Springer Protocols

Yung-I Lee
Edward Chee-Tak Yeung *Editors*

Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols

Humana Press

SPRINGER PROTOCOLS HANDBOOKS

For further volumes:
http://www.springer.com/series/8623

Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols

Edited by

Yung-I Lee

Biology Department, National Museum of Natural Science, Taichung, Taiwan, Republic of China

Edward Chee-Tak Yeung

Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

Humana Press

Editors
Yung-I Lee
Biology Department
National Museum of Natural Science
Taichung, Taiwan, Republic of China

Edward Chee-Tak Yeung
Department of Biological Sciences
University of Calgary
Calgary, Alberta, Canada

ISSN 1949-2448 ISSN 1949-2456 (electronic)
Springer Protocols Handbooks
ISBN 978-1-4939-7770-3 ISBN 978-1-4939-7771-0 (eBook)
https://doi.org/10.1007/978-1-4939-7771-0

Library of Congress Control Number: 2018936747

Printed on acid-free paper

This Humana press imprint is published by the registered company Springer Science+Business Media, LLC part of Springer Nature.
The registered company address is: 233 Spring Street, New York, NY 10013, U.S.A.

Preface

The orchid family is one of the largest families of flowering plants known for their beauty and economic importance. Orchids are especially vital to the horticulture and florist industries. In addition, the potential of using orchids as a source for the pharmaceutical and fragrance industries is currently being explored, resulting in a steady increase in scholarly publications related to orchid biology research. When looking at the literature available, it is surprising to find that there is no comprehensive integration of key areas of research that are important to both scientists and commercial growers alike.

The main purpose of this publication is (1) to provide key practical areas of research (i.e. germination, micropropagation, traditional and current techniques related to plant improvement) and (2) to document methods that ensure survival of plants from laboratories to greenhouses. The topics highlighted in this work is by no means complete but is meant to draw attention to the many techniques available that can be beneficial to one's work on orchid research and development. We hope that this publication can promote cross-talks between scientists and growers. Both groups have different knowledge bases and when combined will ensure successful growth of orchids in their natural habitats or commercial greenhouses. Laymen that are interested in orchid growing will also benefit from having this handy scientific reference.

In this work, we emphasize both the theoretical understanding of methods and practical details. A proper theoretical understanding is essential to the success of a protocol. Hence several overview chapters have been included amongst the protocol chapters. We first emphasize propagation methods using seeds and related techniques that are important to plant conservation and improvement (Part I). Successes in asymbiotic and symbiotic seed germination are keys to orchid conservation and their propagation. Part II summarizes micropropagation methods, common media, and newer methods of micropropagation such as the bioreactor culture procedures. This is followed by a special technique section (Part III) focusing on techniques related to the manipulation of explants in an in vitro environment. Some cell biological methods and transformation techniques are included in Part IV. Plant improvements through transformation are now common and can also be applied to orchid species with some successes; however, transformation protocols can apply only to a limited number of orchid species. The methods and protocols detailed serve to encourage further improvements in this area of research. Successes in a laboratory setting do not guarantee plant survival and propagation in greenhouses and in the natural environment. Hence, in Part V, we focus on greenhouse propagation techniques that are essential to the survival of plants generated from a laboratory setting. A final part is to showcase recent successes on orchid propagation by documenting sample publications and how to present orchids in an artistic fashion for one's enjoyment.

We would like to thank Mr. Colin Chan for his help in editing the figures and graphics and to Professor C.C. Chinnappa for proofreading manuscripts. Finally, we are grateful to all authors for their contributions to this book and their patience and cooperation during the course of preparation and editing.

Taichung, Taiwan, Republic of China — *Yung-I Lee*
Calgary, Alberta, Canada — *Edward Chee-Tak Yeung*

Contents

Contributors

J. Arditti · *University of California, Irvine, CA, USA*

Raffaella Balestrini · *Istituto per la Protezione Sostenibile delle Piante, Consiglio Nazionale delle Ricerche (CNR), Turin, Italy*

Ming-Tsair Chan · *Agricultural Biotechnology Research Center, Academia Sinica, Tainan, Taiwan, Republic of China*

Colin Chan · *Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada*

Chen Chang · *Department of Horticulture, National Chung Hsing University, Taichung City, Taiwan, Republic of China*

Chin-An Chang · *Chaoyang University of Technology, Taichung, Taiwan, Republic of China*

Wen-Huei Chen · *Orchid Research and Development Center, National Cheng Kung University, Tainan, Taiwan, Republic of China*

Shu-Pei Chen · *Applied Zoology Division, Taiwan Agricultural Research Institute, Taichung, Taiwan, Republic of China*

Dong Poh Chin · *Center for Environment, Health and Field Sciences, Chiba University, Kashiwa, Chiba, Japan*

Yi-Tien Chiu · *Department of Horticulture, National Chung Hsing University, Taichung City, Taiwan, Republic of China*

Hua-Ting Chuang · *Department of Horticultural Science, National Chiayi University, Chiayi City, Taiwan, Republic of China*

Laura L. Corey · *Department of Biology, Illinois College, Jacksonville, IL, USA*

Ceci C. Custódio · *Agronomy College, Universidade do Oeste Paulista (UNOESTE), Rodovia Raposa Tavares, Presidente Prudente, SP, Brazil*

Valeria Fochi · *Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università degli Studi di Torino, Turin, Italy*

Shun-Xing Guo · *Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, People's Republic of China*

Lawrence D. Harder · *Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada*

Indra S. Harry · *Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada*

Silvério T. Hosomi · *College of Biological Sciences, Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, SP, Brazil*

Ting-Fang Hsieh · *Floriculture Research Center, Taiwan Agricultural Research Institute, GuKeng, Yunlin, Taiwan, Republic of China*

Shan-Te Hsu · *Department of Horticultural Science, National Chiayi University, Chiayi City, Taiwan, Republic of China*

Kuang-Liang Huang · *Department of Horticultural Science, National Chiayi University, Chiayi City, Taiwan, Republic of China*

Yoon-Sun Huh · *Chungbuk Agricultural Research and Extension Services, Cheongju-si, Chungbuk, Republic of Korea*
Syoichi Ichihashi · *Department of Biology, Aichi University of Education, Kariya, Aichi, Japan*
Steven D. Johnson · *School of Life Sciences, University of KwaZulu-Natal, Scottsville, Pietermaritzburg, South Africa*
Juntaro Kato · *Department of Biology, Aichi University of Education, Kariya, Aichi, Japan*
Yung-I Lee · *Biology Department, National Museum of Natural Science, Taichung, Taiwan, Republic of China*
Yuan-Yuan Li · *Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, People's Republic of China*
Chia-Wen Li · *Department of Life Science, Fu-Jen Catholic University, New Taipei City, Taiwan, Republic of China*
Yu-Hsin Liao · *Department of Horticultural Science, National Chiayi University, Chiayi City, Taiwan, Republic of China*
Alessandro Lopa · *Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università degli Studi di Torino, Turin, Italy*
Nelson B. Machado-Neto · *Agronomy College, Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, SP, Brazil*
Timothy R. Marks · *Comparative Seed Biology, Comparative Plant and Fungal Biology, Royal Botanic Gardens Kew, Ardingly, West Sussex, UK*
Masahiro Mii · *Center for Environment, Health and Field Sciences, Chiba University, Kashiwa, Chiba, Japan*
Hosakatte Niranjana Murthy · *Department of Botany, Karnatak University, Dharwad, India*
Duong Tan Nhut · *Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology, Dalat City, Vietnam*
Kee-Yoeup Paek · *Department of Horticultural Science, Chungbuk National University, Cheongju-si, Chungbuk, Republic of Korea; Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju-si, Chungbuk, Republic of Korea*
Jungmin Park · *Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada*
So-Young Park · *Department of Horticultural Science, Chungbuk National University, Cheongju-si, Chungbuk, Republic of Korea; Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju-si, Chungbuk, Republic of Korea*
Mendel Perkins · *Department of Botany, The University of British Columbia, Vancouver, BC, Canada*
Silvia Perotto · *Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università degli Studi di Torino, Turin, Italy; Istituto per la Protezione Sostenibile delle Piante, Consiglio Nazionale delle Ricerche (CNR), Turin, Italy*
Hugh W. Pritchard · *Comparative Seed Biology, Comparative Plant and Fungal Biology, Royal Botanic Gardens Kew, Ardingly, West Sussex, UK*
Philip T. Seaton · *Comparative Seed Biology, Comparative Plant and Fungal Biology, Royal Botanic Gardens Kew, Ardingly, West Sussex, UK*

Rong-Show Shen · *Department of Horticultural Science, National Chiayi University, Chiayi City, Taiwan, Republic of China*
Patrick I. Siu · *Willoughby, NSW, Australia*
Corrine Stahl · *Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada*
Jiunn-Feng Su · *Plant Pathology Division, Taiwan Agricultural Research Institute, Taichung, Taiwan, Republic of China*
Michio Tanaka · *Department of Horticultural Science, Kagawa University, Takamatsu, Kagawa, Japan*
Ching-Yan Tang · *Department of Life Sciences, National University of Kaohsiung, Kaohsiung, Taiwan, Republic of China*
Hoang Thanh Tung · *Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology, Dalat City, Vietnam*
Wagner A. Vendrame · *Department of Environmental Horticulture, University of Florida, Tropical Research and Education Center, Homestead, FL, USA*
Hui Wang · *Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, People's Republic of China*
Tim W. Yam · *Singapore Botanic Gardens, National Parks Board, Singapore, Singapore*
Edward C. Yeung · *Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada*
Lawrence W. Zettler · *Department of Biology, Illinois College, Jacksonville, IL, USA*

Part I

Orchid Seeds in Plant Propagation and Conservation

Chapter 1

Understanding Seed and Protocorm Development in Orchids

Edward C. Yeung, Yuan-Yuan Li, and Yung-I Lee

Abstract

In this overview, the development of orchid seed and protocorm is summarized. Although the structural organization of seed and protocorm appears simple, information is presented indicating that they have developmental programs similar to and as complex as other flowering plants. The varied suspensor morphologies, the presence of cuticular material covering the surface of the embryo, and the delicate seed coat structure ensure embryo survival, albeit unusual. The embryo is programmed to form a protocorm. The protocorm cells are destined to form a shoot apical meristem at the apical (chalazal) end and to house the symbiont at the basal (micropylar) end of a protocorm. Changes in protocorms during asymbiotic and symbiotic seed germination are discussed.

Key words Embryo, Suspensor, Symbiotic seed germination, Asymbiotic seed germination, Endosperm, Seed storage proteins and lipids, Phytohormones, Mycorrhizal fungi, Seed coat, Carapace, Protocorm, Shoot apical meristem

1 Introduction

The small size and specialized structural features of orchid embryos and seeds and the need to form protocorms prior to plantlets formation have captured the imagination of plant biologists. As a result, many publications including monographs and reviews [e.g., 1–15] have been published, discussing different aspects of orchid embryology, seed germination, and protocorm development. Moreover, when studying orchid seed biology, it is important that we have a broad appreciation of seed development in flowering plants. Recent studies on plant reproduction provide a vast amount of information detailing the molecular and genetic regulations in embryo and seed development [e.g., 16, 17]. All this information can provide insights and methodologies to further our studies on orchids.

In addition to theoretical interests, the applied aspect of asymbiotic and symbiotic seed germination is a key approach used in orchid

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_1,

propagation and conservation. Furthermore, micropropagation of protocorms from asymbiotically germinated seeds and manipulation of protocorms in transformation studies are additional techniques that are important to plant improvements and commercial applications. Successes on orchid seed germination, micropropagation, and transformation studies are summarized in recent reviews [18–21].

In order to further our goals in orchid propagation and conservation and improving current methods, it is essential that we have a good understanding on orchid seed development and changes in protocorm during germination. A detailed perspective on orchid seed and protocorm development has been published recently, emphasizing that although orchid embryos are small, they have complex developmental programs similar to other flowering plants and the protocorms are designed to house their symbiont with the primary goal to form a functional shoot apical meristem [22]. Hence, in this chapter, an abridged account on orchid seed biology is presented and emphasis is placed on changes to the protocorm during asymbiotic and symbiotic seed germination. Other chapters in this book provide current information and protocols on selected topics of seed germination and propagation. Readers are urged to consult these and other publications for references.

2 The Orchid Seeds

2.1 The Embryo

In orchids, after a successful pollination and fertilization event, numerous seeds develop within a single capsule. Seeds are very small and embryos have no obvious histodifferentiation into distinct tissues, such as the root and shoot apical meristem, primary meristems, and organ, i.e., cotyledon. An extensive review by Yam et al. [12] documents embryo features from various groups of orchids and this information will not be repeated here; instead, essential developmental characteristics are featured below.

2.1.1 Suspensor

In flowering plants, the zygote usually divides unequally, giving rise to a larger basal cell and a smaller terminal cell. The basal cell gives rise to a short-lived embryonic organ, known as the suspensor, and the smaller terminal cell divides and gives rise to the embryo proper [23]. One of the most notable features in orchid embryos is the varied morphologies of the suspensor found in different groups of orchids [1]. The number of suspensor cells when present tends to be limited, ranging from 1 to over 30 [10]. Depending on the species, the suspensor can appear as a single enlarged cell or as a multicellular structure with varied forms [1] (Fig. 1). However, it is important to note that not all orchid species have a distinct suspensor. For example, many species in the genus *Spiranthes* are considered to be suspensorless, as the basal cell fails to enlarge and divide further and resembles an embryo proper cell [1, 10, 12] (Fig. 1a). Although the structure of the suspensor appears simple, such as the single suspensor cell of the nun orchid, *Phaius tankervilleae*, dramatic changes in

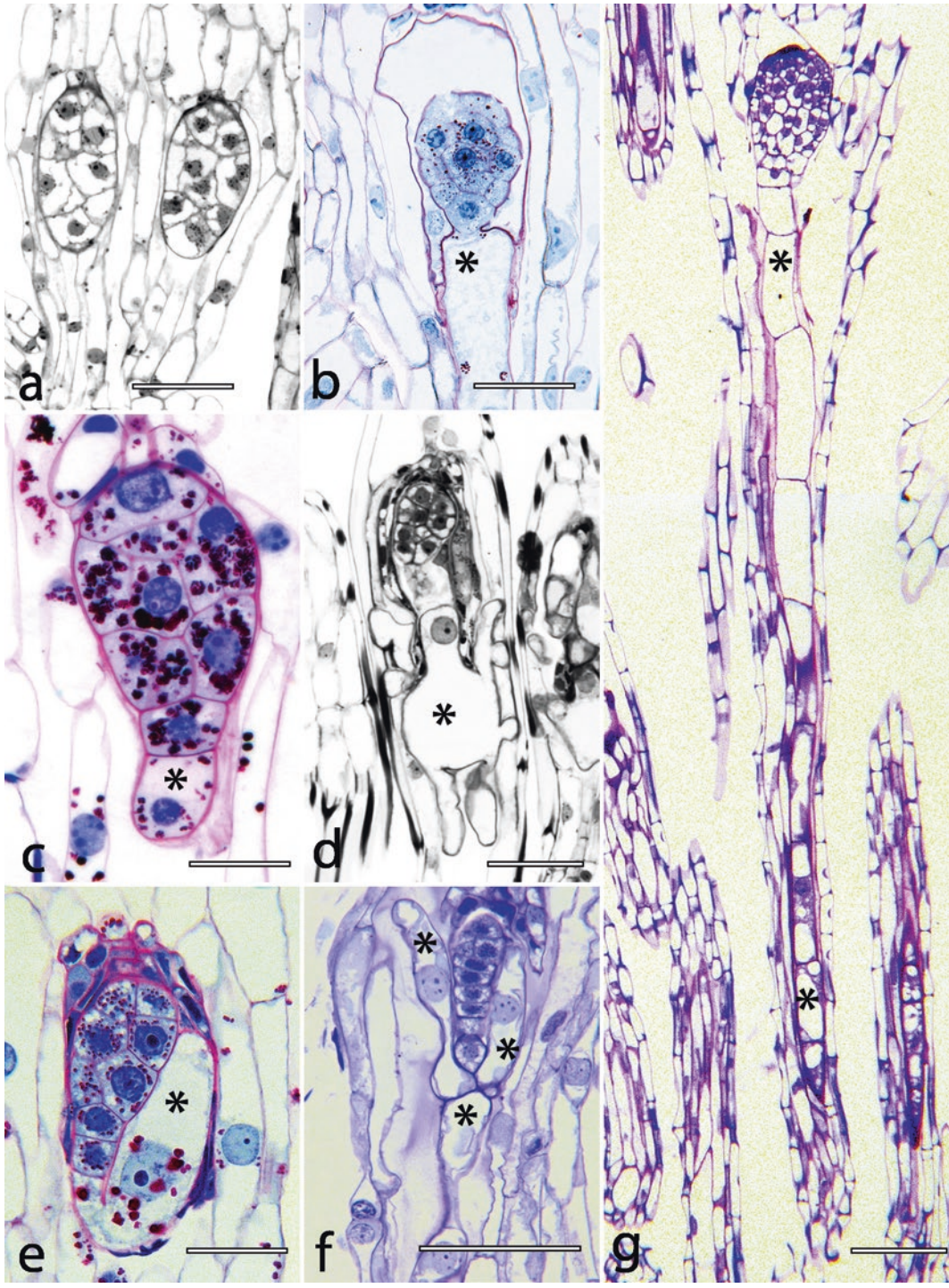


Fig. 1 Varied suspensor morphologies. (**a**) *Spiranthes sinensis* embryos without a suspensor. Scale bar = 60 μm. (**b**) A *Phaius tankervilleae* embryo showing a single, enlarged suspensor cell. Scale bar = 80 μm. (**c**) A *Cypripedium debile* embryo with a single, small suspensor cell. Scale bar = 60 μm. (**d**) A *Bletilla formosana* embryo showing filamentous extensions originating from an enlarged basal suspensor cell. Scale bar = 120 μm. (**e**) The suspensor of *Calypso bulbosa* embryo consists of an enlarged cell and a 4-celled filamentous region. The basal suspensor cell extends toward the embryo proper resulting in the formation of a U-shaped embryo. Scale bar = 60 μm. (**f**) *Cymbidium sinense* embryo showing multidirectional growth of suspensor cells. Scale bar = 200 μm. (**g**) An *Epidendrum ibaguense* embryo showing the long multicellular suspensor. Scale bar = 120 μm

size and shape can occur during its course of development (Fig. 1b). The morphogenetic changes involve coordinated rearrangement in cell cytoskeleton [24]. Changes in the microtubules have also been found during the course of suspensor and proembryo formation in *Cymbidium sinense* [25]. At present, few detailed cellular and ultrastructural studies of orchid embryos are available in the literature. Additional studies are needed in order to obtain insight into how the varied suspensor forms are generated. It is likely that the cytoskeletal elements, i.e., microtubules and actin filaments, are involved in the morphogenesis of suspensors.

The varied morphologies of the suspensor and the haustoria-like behavior indicate that they can play a role in nutrient uptake. In *Paphiopedilum delenatii*, when the suspensor cells are examined using an electron microscope, the cells have a transfer cell morphology with wall ingrowths present [26]. The increase in surface area through the formation of convoluted wall ingrowths enables an increased capacity for nutrient uptake. It is generally accepted that cells with wall ingrowths are highly specialized [27] and function in short distance transport [28, 29].

Based on histochemical staining using toluidine blue O [30], lipidic polymers, i.e., cutin and suberin, and phenolic substances, such as lignin, are absent from the suspensor cell wall. In the absence of these deposits, the primary wall of the suspensor cells remains "porous" and permits the transfer of nutrients from the maternal seed coat to the suspensor. For species without any obvious structural specialization in the suspensor, such as the nun orchid, the plasmolysis and tracer uptake studies by Lee and Yeung [31] clearly demonstrated that the suspensor has unique physiological properties. It was determined that the osmotic potential of the suspensor cell in nun orchid is more negative than surrounding cells. The differences in osmotic potentials can direct a preferential flow of water into the suspensor. When introducing a fluorescent tracer 6-carboxyfluorescein diacetate (CFD) to developing seeds, the fluorescence signal carboxyfluorescein, a hydrolyzed product of CFD, is first detected in the suspensor cytoplasm prior to its appearance in the embryo proper [31]. These results clearly indicate that the suspensor is the uptake site for the developing embryo.

Not all orchid species have a distinct suspensor [3, 10]. Although the nutrient uptake pathway by the suspensorless embryo is not known, it is logical to speculate that the entire surface of the embryo proper must be able to absorb nutrients from surrounding maternal tissues. In *Cyrtosia javanica*, the seed coat is multilayered and remains intact until seed maturation [32]. Dense cytoplasmic accessory and antipodal cells are present, locating at the chalazal end of developing seeds [32]. It is interesting to note that although a cuticle is present, it has a patchy appearance and it is initially absent from the wall of the embryo at the chalazal end [32]. The pattern of cuticle deposition suggests that the entire surface of the

embryo proper can function in nutrient absorption, especially at the chalazal end. In order to determine how nutrient enters into the embryo in the absence of a suspensor, it is necessary to study the microenvironment surrounding the developing embryo.

2.1.2 Embryo Proper

In a majority of flowering plants, the embryo proper undergoes distinct phases of development. After establishing the apical-basal polar axis, histodifferentiation of major tissues takes place, followed by storage product deposition, preparation of germination programs, and final changes leading to developmental arrest [33, 34]. The histodifferentiation phase in flowering plants is responsible for laying down the future body plan of the sporophyte, ready for germination. Distinct primary meristems, i.e., protoderm, ground meristem, and procambium, and the root and shoot apical meristems are clearly delineated at the end of the histodifferentiation phase. Upon germination, a seedling forms quickly due to the activities of the "pre-formed" apical meristems. Orchid embryo is minute in size, with no obvious tissues and organ. Casual examination would lead to the conclusion that a histodifferentiation phase does not exist in orchid embryos.

Not having an easily recognizable tissue pattern an orchid embryo does not necessarily suggest that it is less specialized when compared to other flowering plants. The following observation indicates that histodifferentiation has indeed occurred during early embryogenesis in orchid. In examining the structural organization of the embryo proper, a gradient of cell size is often present with smaller cells located at the future shoot pole (Fig. 2a). The gradient indicates that physiological differences exist among embryo cells in an apical-basal manner. This is demonstrated by the fact that upon germination, the smaller cells at the apical (chalazal) end are destined to form the meristematic zone and the large cells at the basal (micropylar) end will enlarge and destined to house the symbiont. In orchids, the surface layer of the embryo is specialized, taking on an epidermal cell characteristic. A surface cuticle layer has been reported in other flowering plant embryos [35, 36] and similar cuticular depositions can also be found in orchids (Fig. 2b). In *Phalaenopsis amabilis* [37] and *Cyrtosia javanica* [32], a cuticle is present but it takes on a patchy appearance. In *Cymbidium sinense*, the surface cuticle layer is very distinct covering the entire surface of the embryo proper [38]. The ability to synthesize cuticular materials is an epidermal cell characteristic, a specialized feature of a protoderm. Finally, the structural organization of an orchid embryo mirrors the structure of a protocorm at the time of germination. In orchid, immature seeds can germinate asymbiotically to form protocorms, once they reach approximately half way into seed maturation prior to storage product synthesis. The ability to germinate precociously indicates that the protocorm body plan

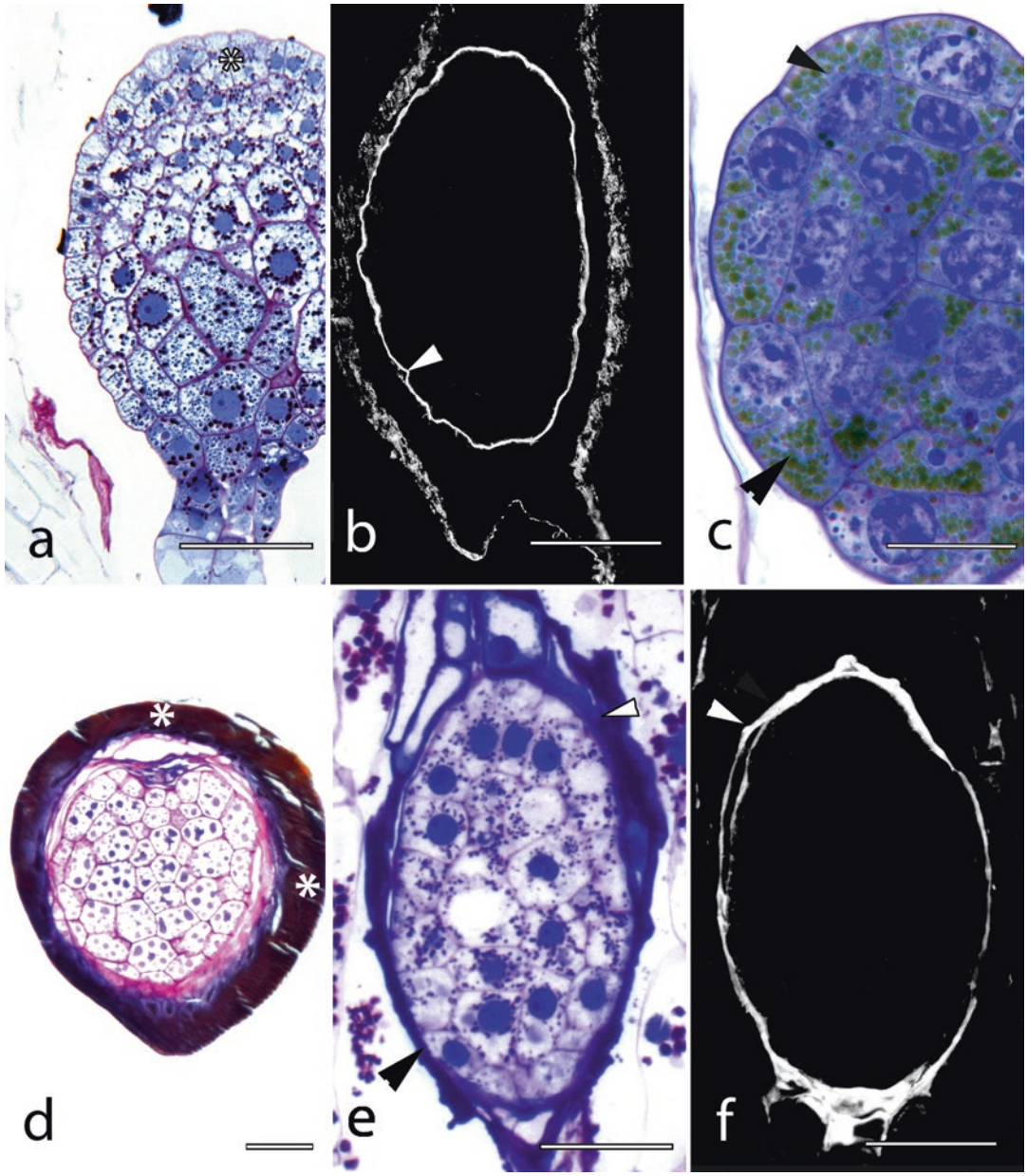


Fig. 2 Some structural characteristics of embryo proper. (**a**) Embryo proper of *Epidendrum ibaguense* showing a gradient of cell size with small cells occupying the future shoot pole (*). Scale bar = 100 μm. (**b**) The embryo proper of *Cyrtosia javanica* showing the presence of a distinct cuticle (arrowhead) on its surface. Scale bar = 100 μm. (**c**) The nun orchid, *Phaius tankervilleae,* at the time of maturation. The embryo cells have an abundant lipid deposits (arrowhead). Scale bar = 40 μm. (**d**) A section of *Vanilla planifolia* seed, showing the unusual thick seed coat (*). Scale bar = 0.3 mm. (**e**) A *Cypripedium plectrochilum* seed showing a thick layer of carapace (arrowhead) that wraps around the embryo. Scale bar = 100 μm. (**f**) Phenolic deposits can be present in the carapace and these deposits are readily detected using a fluorescence microscope. Scale bar = 100 μm

has already established. Precocious germination is only possible if histodifferentiation has occurred.

One of the distinct features in a majority of orchid embryos is the absence of a cotyledon. However, Nishimura [39] reported that a rudimentary cotyledon-like protrusion can be found in seven genera. Except for *Bletilla striata*, the rudimentary protrusion has little function during seed germination. The true nature of cotyledon-like structure in orchids awaits future studies during shoot apical meristem formation [22]. The absence of a cotyledon may represent a unique evolutionary *trend* within Orchidaceae [7].

Orchid embryos are small in size with fewer cells when compared with other flowering plant embryos. This can be a result of cells having a long cell cycle time. Also, the limited number of cells produced may be due to early cessation of mitotic activities. Mitotic activities are sensitive to water deficit [40]. The lack of an endosperm may result in having a lower water holding capacity for the seed, allowing for a more rapid change in water content. This can lead to an early termination of mitotic activity. In dicot seeds, such as beans and canola, the endosperm still fills with liquid when the embryos completed their histodifferentiation phase. Mitotic activities then continue until the early maturation stage of development when the embryo completely fills the endosperm cavity. Future studies in the regulation of mitotic behavior and cell cycle events can provide insight into the growth of orchid embryos.

The histodifferentiation phase in flowering plants is followed by a storage product synthesis and deposition phase. Similar events also occur within orchid embryos. Storage products accumulate rapidly soon after the cessation of mitotic activity, with starch appears first within the cytoplasm (Fig. 1c, e) followed by deposition of storage lipids and proteins (Fig. 2c). Starch granules may or may not be present in mature embryo depending on the species [37, 38, 41]. Abscisic acid (ABA) plays a key role in storage product biosynthesis as shown in *Brassica* microspore-derived embryos [42, 43]. Since rapid accumulation of ABA also occurs during orchid embryo development [44–46], ABA may have a similar role to play in orchids.

In flowering plants, one of the key events at the time of embryo maturation is to acquire desiccation tolerance and prepare for developmental arrest. In *Phalaenopsis amobilis*, desiccation tolerance is established at the last stages of seed maturation [47]. The late embryogenesis abundant (LEA) proteins are shown to accumulate in *Dendrobium officinale* in preparation for seed maturation drying [48]. It is well established that LEA proteins are synthesized at the time of seed maturation in order to cope with water deficit and resistance to drought [49]. The findings in orchid, albeit limited, indicate that orchids use similar strategies in

preparing for seed maturation and developmental arrest as other flowering plants.

The presence of stored mRNA in mature seeds is a well-documented phenomenon in flowering plants. The early work by Dure [50, 51] clearly demonstrates the presence of stored mRNAs formed during late embryogeny, which are essential to successful germination. Proteomic analyses in rice seeds indicate that 20 proteins are upregulated even in the presence of a transcription inhibitor, actinomycin D, demonstrating that the stored mRNAs are translated during germination [52]. In *Spathoglottis plicata*, poly(A)-RNAs are present in all mature embryo cells as determined by the ^{3}H-poly-(U) in situ hybridization method [53]. These stored templates are most likely utilized during the first few days of germination as there is a decrease in mRNA abundance in the embryo [53]. More importantly, the work by Raghavan and Goh [53] demonstrated indirectly the presence of stored mRNAs as in other flowering plants. Future proteomic analyses will confirm the presence of stored mRNAs in mature orchid embryos and provide insight into their role during seed germination.

Because of the small size of the embryo and without obvious structural differentiation into tissues and meristems, one generally concludes that orchid embryos are simple, primitive, and not organized. The above discussion indicates the opposite. When looking at the overall pattern of embryo development and the predetermined cell fates of protocorm cells (see discussion below), orchid embryo has a complex developmental pattern. We still have a lot to learn about the inner workings of orchid embryos during their development. As developmental mechanisms are under genetic control, current genome-wide transcriptome and expression profile analyses, e.g., [54–57], will provide a better insight into the process of seed germination and protocorm development in orchids.

2.2 Endosperm

It is well known that an endosperm is absent from mature orchid seeds. During fertilization, although one of the gamete nuclei enters the central cell, it does not appear to fuse with the polar nuclei forming a single primary endosperm nucleus. In *Epidendrum scutella*, the polar nuclei have begun to fuse before fertilization; however, fusion is not complete [58]. Instead, a varied number of nuclei can be seen. This generates the confusion as to whether the nuclei present within the endosperm cell are products of mitotic divisions of the primary endosperm nucleus or simply representing the unfused nuclei in the primary endosperm cell after fertilization. Based on a majority of publications, it is generally agreed that there is no fusion of nuclei within the central cell and the nuclei soon degenerate within the primary endosperm cell as the embryo begins to develop. In *Vanilla planifolia*, Swamy [59] reported that rounds of divisions occur prior to endosperm degeneration; how-

ever, a recent study by Kodahl et al. [60] indicates that such a process did not occur. Clements [10] doubts that fusion of nuclei occurs within the central cell after fertilization. Furthermore, if some chalazal nuclei persist and "release" into the endosperm cavity, this can also contribute additional nuclei within the cavity [61], contributing to the confusion of nuclei number within the primary endosperm cell. With a vast number of species in Orchidaceae, it is not surprising to see exceptions to the general rule. In order to be sure that the increase in number of nuclei is indeed due to divisions of the primary endosperm nucleus, a clear demonstration of mitotic events such as mitotic figures is needed after fertilization.

The absence of a functional endosperm after fertilization is likely decided during ovule development. Ovule development is complex, and proper specifications of various embryo sac components are essential to their function at the time of fertilization and early stages of embryo development [62]. As discussed by Yeung [22], the reduction in the number of nuclei within an embryo sac, i.e., the "striking" phenomenon, may be an early indication of central cell defects. In addition, modification to one of the male gametes at the time of fertilization can also prevent endosperm formation. In *Epidendrum scutella*, the chromatin in the sperm nucleus appears less dense in the central cell than in the pollen tube [58], suggesting possible changes to the male gamete at the time of fertilization. Further investigations of sperm cells and ovules at the time of fertilization will provide additional insight into the possible cause of endosperm failure in orchid.

In flowering plants, the failure in endosperm formation usually leads to embryo abortion [63]. The fact that orchid embryo continues to develop and mature in the absence of an endosperm indicates that orchids have adopted a different embryo development program. Since orchid embryo can develop without the presence of an endosperm, this removes one of the potential barriers for hybrid failure [64]. Future molecular biology studies such as that discussed by Lopez-Villalobos et al. [62] will provide useful clues as to why endosperm fails to develop in orchid.

2.3 The Seed Coat

Comprehensive information on orchid seed morphology and seed coat structure is available in the literature. The physical and morphological characteristics of orchid seeds are detailed in a review by Arditti and Ghani [65]. Dressler provided a summary of seed structures in his monograph [8], and the developmental features of seed coats are discussed by Molvray and Chase [66].

Orchid seed has a thin seed coat. It is developed mainly from the outer integument of the ovule after fertilization and no vascular tissue present. The inner integument often degenerates. For a majority of orchid seeds, even though the seed coat is thin, the addition of secondary walls, phenolic substances, and cuticular materials offer additional protection to

the embryo within. However, exceptions are noted in the thickness of the seed coat. In the subfamily Vanilloideae, a number of species, e.g., *Vanilla planifolia* [59, 67] (Fig. 2d) and *Cyrtosia javanica* [32], have multiple layers of seed coats and the outermost layer is heavily lignified.

The inner integument of the ovule usually fails to develop much further after fertilization; the cells eventually lose their content and collapse around the embryo proper forming a thin layer, termed as "carapace" [9, 12, 68] (Fig. 2e, f). The thickness of the carapace varies. Synthesis and deposition of phenolic compounds can occur prior to cell collapsing, offering further protection to the embryo [69]. These compounds can be detected easily using a fluorescence microscope (Fig. 2f). In *Dactylorhiza majalis*, a carapace is readily detected and it wraps tightly around the embryo [9]. Although the carapace offers additional protection to the embryo, it can be one of the major causes in inhibiting germination of mature seeds. The low percentage of mature seed germination in *Cephalanthera falcata* is attributed to the accumulation of substances, such as lignin, in the inner integument as the possible cause of germination inhibition [69].

2.4 Phytohormone Studies

In recent years, we have a better appreciation concerning endogenous phytohormones, especially abscisic acid (ABA) [44–46] during seed development. ABA plays many roles in plant growth and development and it is well documented that it is a negative regulator for seed germination [70]. During orchid seed development, ABA level increases mid-way into seed maturation [44–46]. When the ABA level is suppressed by the addition of fluridone, an ABA biosynthesis inhibitor, the percentage of seed germination increases [46]. The success in asymbiotic germination using immature seeds is mostly due to the low levels of ABA at the end of the histodifferentiation phase.

Information on other phytohormones during seed development is limited. Auxin and ethylene have been shown to play essential role during pollination and early stages in fruit and ovule development [71–74]. In a recent study, expression analysis of fertilization and early embryogenesis-associated gene in *Phalaenopsis* indicates that auxin and ethylene can play a role at these critical stages of seed formation [57]. After seed germination, auxin has been shown to regulate first leaf development and protocorm morphogenesis in *Spathoglottis plicata* [75]. In *Epidendrum ibaguense*, cytokinin and gibberellin levels are high and remain high at the time of seed maturation [76]. This differs from other plants in which the levels of these phytohormones decrease at the time of seed maturation. The high endogenous levels of cytokinin and gibberellin may be the reason why growth regulators are usually not needed for seed germination [76]. Although at present only few studies are available in the literature, the information at hand sug-

gests that phytohormones have important roles to play in seed and protocorm development.

3 Seed Germination

The success of asymbiotic seed germination of orchids as demonstrated by Knudson [77, 78] leads to a proliferation of germination and micropropagation studies. In addition, improvements in the isolation and identification of orchid mycorrhizal fungi and culture techniques generate numerous studies on symbiotic seed germination both in vitro and in situ. Both approaches are equally important to orchid propagation and conservation.

Many endogenous and exogenous factors can influence seed germination and subsequent plantlet development [9, 79 also see discussion below]. Chapter 5 presents information on nutrient media and discusses endogenous factors such as plant growth regulators and seed coat structures in seed germination. It is important to note that for in vitro symbiotic and asymbiotic seed germination, culture conditions such as the quality and quantity of lights and temperature can also have important roles to play [e.g., 80–84]. For in situ symbiotic germination, many additional factors are essential to the success of the process. The presence of compatible mycorrhizal fungi and environment factors play determining role in the success of seed germination. For further information, see [85–88]. The following sections focus on in vitro seed germination, with emphasis on protocorm development.

3.1 Asymbiotic Seed Germination of Terrestrial Orchids

Asymbiotic seed germination of terrestrial orchids from temperate regions is often more complicated as compared to epiphytic orchids from tropical areas. Seeds of some temperate terrestrial orchids require a stratification treatment to release from dormancy [89]. On the contrary, seeds of tropical orchids, e.g., *Phalaenopsis*, *Dendrobium*, and *Oncidium* species, germinate readily in the presence of suitable nutrients.

The causes of poor in vitro germination in terrestrial orchids may be related to the impermeability of seed coat [69, 90]. In species, such as *Vanilla planifolia* [59, 67] and *Cyrtosia javanica* [32], the outermost layers of the seed coat are heavily lignified and become impermeable to water and nutrient for germination. The formation of a carapace as discussed earlier can serve as an additional physical barrier to asymbiotic seed germination, especially with additional deposits of phenolic compounds [69]. Furthermore, the induction of physiological dormancy through the accumulation of some inhibitory substances, such as phenolics in *Cymbidium goeringii* [91] and ABA in *Epipactis helleborine* [44], *Calanthe tricarinata* [15] and *Cypripedium formosanum* [46], as seeds approach maturity, can have a negative influence on germination. For cultur-

ing mature seeds of terrestrial orchids, different methods can be applied to improve their germination [92]. The removal of phenolic substances can be achieved by treating with calcium hypochlorite solution and Tween 20 added as the wetting agent (*see* Van Waes and Debergh [93]). In addition, the use of sonication [94] can also be beneficial as this treatment could loosen the carapace enhancing germination. Prechilling [95–98], scarification treatments [45, 99], and liquid suspension culture [97] are useful methods. These treatments may not only scarify the hard seed coat to increase the permeability of seeds but can also reduce the levels of endogenous inhibitors. An understanding of mature seed structure enables us to determine whether a pretreatment step is necessary for seed germination. The duration of treatment needs to be optimized, in order to obtain the desired effect. Moreover, the aforementioned features may be essential to seed survival and symbiotic germination of mature seeds in their natural habitat.

In order to minimize the negative effects of mature seed coat structures and secondary chemicals present, culturing of immature seeds, also known as "green capsule culture," is preferred. This method usually results in a higher germination percentage than mature seeds [92, 93]. This is due to the fact that immature seeds have not yet acquired a hydrophobic seed coat, and inhibitors such as the ABA level are low; consequently, seeds can overcome dormancy after plating on a suitable culture medium. For immature seed culture, the optimum timing of seed harvest is crucial to maximum germination, and this needs to be determined experimentally for each individual species. In *Cypripedium formosanum*, seeds collected from 90 to 105 days after pollination (DAP) are most suitable for in vitro culture [90], and in *Cypripedium macranthos*, seeds collected at 42 DAP give the best germination percentage [100]. In order to determine the window of optimum timing for immature seed culture of a species of interest, be sure to have plenty of developing capsules available.

3.2 Asymbiotic Seed Germination of Epiphytic Orchids

In epiphytic orchids (most orchid genera in the ornamental market), e.g., *Cattleya*, *Dendrobium*, *Oncidium,* and *Phalaenopsis*, the conditions required for seed germination are simpler when compared to temperate terrestrial orchids. There are several notable differences in mature seeds between epiphytic and terrestrial orchids. First, seeds of epiphytic orchids usually do not have a dormancy period after harvest [101]. Second, the optimum time of germination in epiphytic orchids is when the seeds are approaching maturity. Third, in epiphytic orchids, based on histochemical staining, hydrophobic materials, such as lignin and cuticular materials, are not as apparent as those found in terrestrial orchids [102]. In fact, the culture of immature seeds of epiphytic orchids usually results in a lower germination percentage than the culture of

mature seeds [103–105]. For these reasons, mature seeds of epiphytic orchids should be used for asymbiotic germination.

During mature seed collection, if the capsule has split open, it will be necessary to surface sterilize the seeds collected from the capsule. Since most epiphytic orchids do not have a heavily thickened seed coat, their mature seeds cannot tolerate the harsh treatment of surface sterilization [95]. A lower concentration of disinfectant is preferred.

3.3 Changes in Protocorms Associated with Asymbiotic Seed Germination

Upon placement on appropriate culture conditions, the embryo within the seed coat will first enlarge, transforms into a protocorm and resumes mitotic activities, especially at the future shoot pole. The protocorms may turn green, forming a shoot at the apical (chalazal) end, and followed by plantlet formation with both a shoot and roots. Storage products, i.e., storage protein and lipid bodies, are mobilized in the presence of sucrose in the medium [106–108]. The seed storage products are replaced by starch granules in the cytoplasm [106]. The addition of metabolizable sugars provides the added energy necessary to kick start the growth of the protocorm. In the absence of added sucrose in the medium, germination slows or fails. Glyoxysomes which are needed to mobilize lipid reserves fail to form [41, 107]. Harrison [41] suggested that the slow utilization of reserve "could permit the orchid seedling to survive for a longer period until an appropriate endophytic infection is established."

Protocorm formation is a characteristic feature of orchid seed germination. This unique structure has attracted a lot of discussion. Different interpretations as to the nature of protocorm can be found in the literature. It is considered to be equivalent to a hypocotyl [see 13], and a structure that represents an extension of the proembryonic stage of development [105]. Judging from the unique properties and functions of protocorms, Yeung [22] suggested that the protocorm body plan is established during embryo development at the time of histodifferentiation and protocorm cells are preprogrammed with distinct cell fates. This is supported by the fact that the cells at the apical end always give rise to a meristematic zone and the larger parenchymatous cells at the basal end of a protocorm are destined to house the symbiont.

Structural descriptions of protocorm formation can be found in the literature. Leroux et al. [105] detailed protocorm development in *Cypripedium acaule,* using light and scanning electron microscopy. Seeds begin to swell upon placement on a suitable germination medium and transform into protocorms. Mitotic activity begins and this activity is confined mainly to the future shoot pole. Although DNA synthesis occurs in cells at the basal end of the protocorm, these cells failed to divide, resulting in the formation of polyploid cells. A range of 2C to 32C DNA content has been reported [108–111]. The cells at the apical end of the

protocorm become meristematic and are smaller in size when compared to the basal cells. Within the meristematic layers, some cells will differentiate and become shoot apical meristem (SAM) initials, eventually giving rise to a SAM. Once a shoot becomes functional, the protocorm begins to grow in size and turns into a plantlet. Depending on the species, the protocorm can degenerate or remain as part of the plantlet. The plantlets can take on varied forms. Adventitious roots will eventually form near the SAM [6, 11].

Although successes in asymbiotic germination are often recorded, it is noted that asymbiotic germinated plantlets are less vigorous when compared to symbiotic germinated plantlets [112]. In *Paphiopedilum villosum*, asymbiotic germination proves to be difficult and germinated seeds eventually become arrested [113]. In examining seed germination media used, many are relatively simple (*see* Chaps. 5 and 6). Initial media for germination may be adequate; however, for continual development, a more complex medium may be required. Mycorrhizal fungi, besides providing nutrients for general metabolic purposes, are also known to produce a variety of growth regulators [114, 115] that may aid in further development of protocorms and plantlets. If this is the case, a better design of nutrient media and further optimization of growing conditions are essential [e.g., 116, 117].

3.4 Symbiotic Seed Germination: Changes in Protocorms Associated with Symbiotic Seed Germination

In recent years, emphases have been given to the identification of mycorrhizal fungi and symbiotic seed germination with the ultimate goal to understand mycorrhizal fungi-plant interactions in the natural environment. For literature on the study of mycorrhizal fungi, readers are referred to recent publications [e.g., 118–123]. Detailed structural events on symbiotic establishment between compatible mycorrhizal fungi and orchid seeds are readily available in the literature [e.g., 118, 124–127]. In this overview, some current information is highlighted. It is important to note that a majority of studies on symbiotic germination at present are carried out in a laboratory setting. The results obtained from in vitro studies may differ from field investigations. For example, when comparing in vitro vs. field studies, selectivity toward fungal symbionts differs for the same species [118, 128]. Careful comparative studies are needed in order to understand the many challenges encountered by orchids, in order to achieve successes on symbiotic germination in their natural habitat [88].

Under appropriate conditions, the embryo will begin to swell within the seed. If a compatible fungus is present, hyphae will usually penetrate the seed through the micropyle and enter the embryo through the degenerated suspensor as the seed germinates [*see* 129]. Penetration through epidermal cells and rhizoids has also been noted [9, 124, 129]. In *Dactylorhiza majalis*, in order for hyphae colonization and subsequent protocorm development,

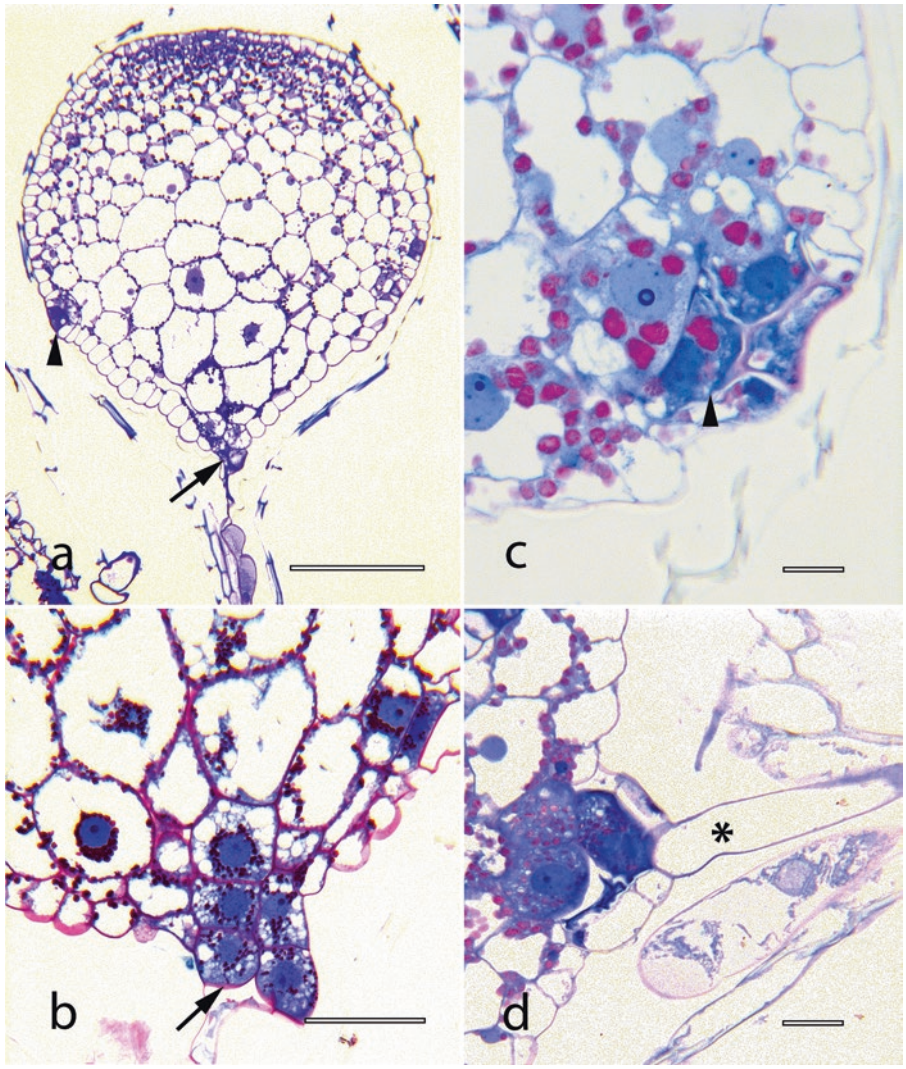


Fig. 3 Some structural features of *Epidendrum ibaguense* protocorm. (**a**) A developing protocorm showing a clear gradient of cell size. The smaller cells are located at the future shoot pole giving rise to the shoot apical meristem. The cells near the suspensor end (arrow) are large with enlarged nuclei. The suspensor has degenerated. In addition, some epidermal cells at the basal end of the protocorm become more cytoplasmic in appearance (arrowhead). Scale bar = 250 μm. (**b**) The embryo proper cells at the site of suspensor degeneration have dense cytoplasm (arrow). Scale bar = 50 μm. (**c**) A cell cluster destined to form rhizoids has dense cytoplasm with distinct starch deposits. Scale bar = 50 μm. (**d**) The surface cells of the cluster differentiate and give rise to rhizoids. Scale bar = 50 μm

hyphae have to enter through epidermal hairs and not through the degenerated suspensor [124]. It is interesting to note that in the study of *Epidendrum ibaguense* protocorm development in vitro, the protocorm cells have a dense cytoplasm at the site of the degenerated suspensor (Fig. 3a, b) and where future rhizoids (Fig. 3c, d) are going to form. Stoutamire [130] indicates that the trichomes

properly function in the formation of mycorrhizal association. The cytological features as shown in Fig. 3 support the notion that specific protocorm cells can have a function to play in attracting and/or allowing an easy access of fungal hyphae.

In *Caladenia tentaculata*, a UV autofluorescing substance is produced by the embryo and it gradually recedes toward the suspensor region [129]. This substance may regulate the establishment of mycorrhizal fungi and limit the site of entry at the suspensor end of the embryo [129]. Since infection occurs through the basal end of the protocorm, the meristematic cell layers are well protected.

After the initial colonization by a mycorrhizal fungus, storage proteins and lipids are rapidly mobilized, and starch granules start to accumulate, especially in the basal protocorm cells [126]. Pelotons soon form within infected cells at the basal end of the protocorm. Their formation indicates success of mycorrhizal establishment. Subsequent digestions of peloton by protocorm cells make nutrients available to protocorm cells. Nutrient transfer between mycorrhizal fungi and protocorm cells is an active area of research. The digestion of pelotons by host cells certainly provides a steady source of nutrient supplies for the protocorm. For mycotrophic orchids, there is little doubt that nutrients have to come from the mycorrhizal fungi. A recent study demonstrates a bidirectional nutrient transfer that can occur between orchid and its symbiont [131, 132]. Future studies using combined techniques as demonstrated by Fochi et al. [131] will provide additional insights and possible mechanisms into nutrient transfer between them.

When considering together the pattern of protocorm development and the process of mycorrhiza establishment, protocorm is a very unusual structure with clear mandates in housing a symbiont for nutrient and to form a shoot apical meristem. Moreover, the protocorm-fungi interaction appears to be a delicate one; changes in nutrient levels and environmental factors can shift from having a stable symbiosis to fungal parasitism leading to death of protocorm [118, 133]. In order to ensure a higher probability of plantlets surviving in their natural environment, the method of reintroductions of well-grown plants back to their native habitats is a useful strategy in conservation [134–136].

3.5 Protocorm and the Shoot Apical Meristem

Irrespective of the type of seed germination, i.e., asymbiotic or symbiotic, the first indication of potential success in germination is the formation of a shoot apical meristem (SAM). The formation of a SAM can be regarded as the primary task of the protocorm. Meristems make the plant [137]. Without meristem formation, there can be no further development into a plantlet. Once a SAM is formed, tissues and leaf primordia are initiated, giving rise to a

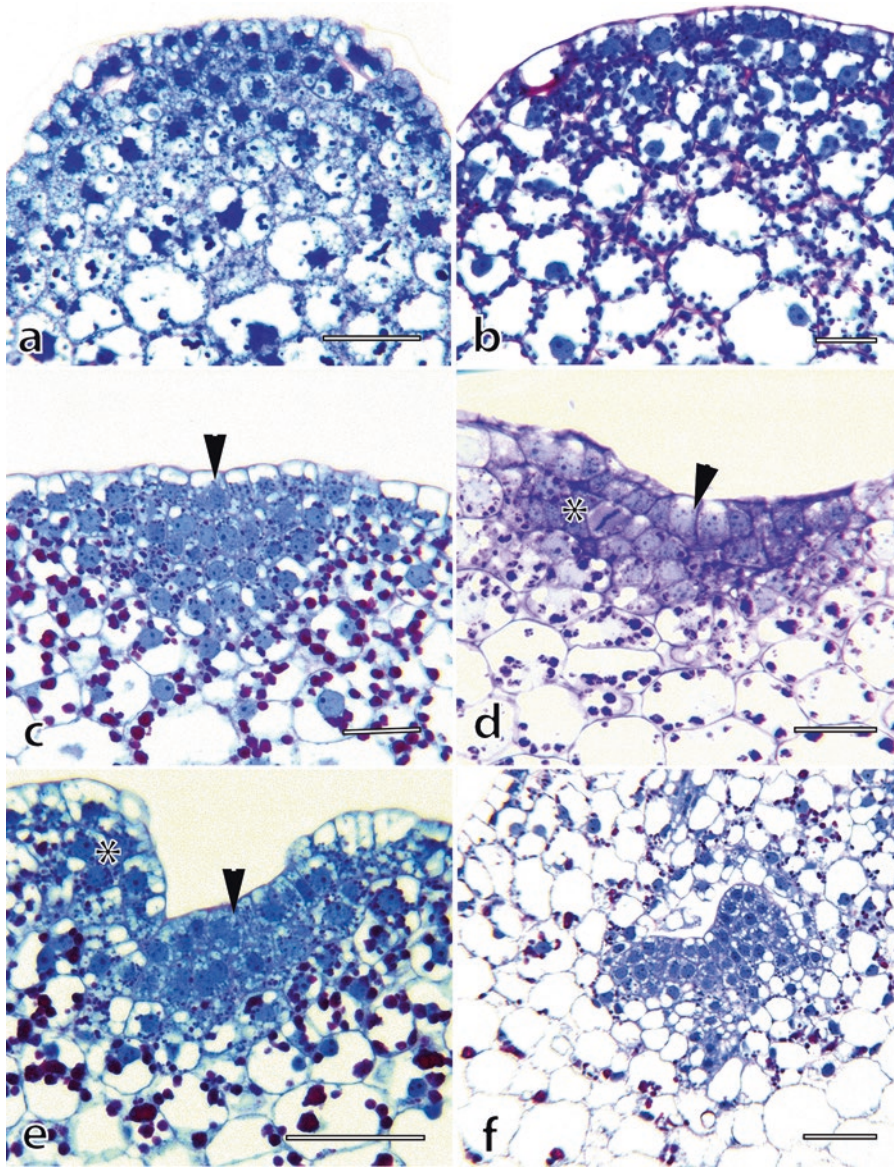


Fig. 4 Shoot apical meristem in *Epidendrum ibaguense* protocorm. (**a**) Abundant storage products are present at the time of seed germination. Scale bar = 60 μm. (**b**) Approximately 1 week after placing seeds on germination medium, embryos enlarge and develop into protocorms. Cells become highly vacuolated. Plastids become abundant with starch granules within protocorm cells. Protocorm cells at the future shoot pole, albeit smaller, have similar cytological features. Scale bar = 45 μm. (**c**) Soon after, future shoot meristem cells (arrowhead) can be identified as they are smaller in size and with a large nucleus to cytoplasm ratio. Starch granules are less abundant. Scale bar = 50 μm. (**d**) The SAM increases in size and starch granules remain less abundant when compared to the rest of the protocorm cells (arrowhead). In the peripheral region of the SAM, the cells start to divide marking the formation of a leaf primordium (*). Scale bar = 50 μm. (**e**) Continual mitotic activities and expansion of peripheral meristem cells result in the formation of the first leaf primordium (*); this results in the formation of a "dimple" with the SAM cells (arrowhead) located at the depression. Scale bar = 45 μm. (**f**) The SAM continues to grow in size, giving rise to additional leaf primordia at a regular interval. Scale bar = 70 μm

shoot. Subsequent adventitious root formation will result in a functional plant.

A SAM is initiated from the meristematic layer at the apical end of a protocorm [22]. Figure 4 depicts a sequence of events in SAM formation in *Epidendrum ibaguense* during asymbiotic seed germination. At the time of placing seeds on germination medium (1/2 MS medium with 2% sucrose), the embryo cells have a dense deposition of food reserves (Fig. 4a). Soon after, the reserve products are utilized and the protocorm cells become vacuolated and chloroplasts with starch granules are abundant within the cell cytoplasm (Fig. 4b). The first indication of a SAM formation is that cells at the future shoot pole become more tightly packed together and starch granules become less abundant when compared to neighboring cells (Fig. 4c). Distinct SAM initials appear within the meristematic zone (Fig. 4d). The appearance delineates a small depression clearly indicating the position of a SAM [21]. A leaf primordium initiates from the peripheral region of the SAM (Fig. 4e). A mature SAM eventually takes on a dome-shape initiating leaf primordium at a regular interval (Fig. 4f). Although information on shoot development is available [*see* 14, 22, 106], the molecular events in SAM initial specification and subsequent elaboration into a mature SAM are not known. Future studies, especially molecular genetics studies similar to the study of SAM in Arabidopsis, will provide new insight into meristem formation in orchid protocorm. In orchid, during the histodifferentiation phase, the SAM initiation program is suppressed or negatively regulated and is only turned on during protocorm formation. Future genome-wide transcriptome and expression profile analysis will provide much needed information on genes involved in SAM formation.

4 Future Perspectives

Orchid embryos are small and they have developmental programs similar to and as complex as other flowering plants. The primary goal of embryogeny is to form a protocorm. The protocorm is a unique structure responsible for the formation of a SAM at the apical end and to house the symbiont at the basal end. Successful establishment with an appropriate symbiont will lead to plantlet formation.

With the information at hand, we have a good general understanding of the asymbiotic and symbiotic germination process. The use of seed pretreatment protocols allows for a higher percentage in asymbiotic seed germination [*see* Chap. 5]. The concurrent studies in the biology of mycorrhizal fungi have allowed better successes in symbiotic seed germination. Many chapters in this current volume contain updated information and serve to demonstrate

current successes in asymbiotic and symbiotic seed germination and other propagation methods.

Moreover, we still need additional information on the physiology, biochemistry, and cell and molecular biology of seed and protocorm development. In recent years, genomics and proteomics data have been readily available [138–142]. Using combined approaches and designing experiments targeting specific questions, it will be possible to generate protocols essential to the propagation and conservation of orchids.

We hope this overview can provoke additional studies in orchid seed biology. In order to celebrate our successes in asymbiotic and symbiotic seed germination, we need to have a true understanding of all processes associated with seed development and germination via protocorms.

References

1. Swamy BGL (1949) Embryological studies in the Orchidaceae. II. Embryology. Amer Midl Nat 41:202–232
2. Wirth M, Withner CL (1959) Embryology and development in the Orchidaceae. In: Withner CL (ed) The orchids. Ronald Press Co., New York, pp 155–188
3. Dodson CH, Gillespie RJ (1967) The biology of the orchids. The Mid-America Orchid Congress, Inc., Nashville
4. Poddubnaya-Arnoldi VA (1967) Comparative embryology of the Orchidaceae. Phytomorphology 17:312–320
5. Withner CL (1974) The orchids: scientific studies. Wiley, New York
6. Veyret Y (1974) Development of the embryo and the young seedling stages of orchids. In: Withner CL (ed) The orchids: scientific studies. Wiley, New York, pp 223–265
7. Arditti J (1992) Fundamentals of orchid biology. Wiley, New York
8. Dressler RL (1993) Phylogeny and classification of the orchid family. Cambridge University Press, Cambridge
9. Rasmassen H (1995) Terrestrial orchids—from seed to mycotrophic plant. Cambridge University Press, Cambridge
10. Clements MA (1999) Embryology. In: Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN (eds) Genera Orchidacearum, General introduction, Apostasioideae, Cypripedioideae, vol 1. Oxford University Press, Oxford, pp 38–58
11. Vinogradova TN, Andronova EV (2002) Development of orchid seeds and seedlings. In: Kull T, Arditti J (eds) Orchid biology: reviews and perspectives, VIII. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 167–234
12. Yam TW, Yeung EC, Ye X-L, Zee S-Y, Arditti J (2002) Embryology- seeds. In: Arditti J, Kull T (eds) Orchid biology: reviews and perspectives VIII. Kluwer, Dordrecht, The Netherlands, pp 287–384
13. Batygina TB, Bragina EA, Vasilyeva VE (2003) The reproductive system and germination in orchids. Acta Biol Cracov Ser Bot 45:21–34
14. Andronova EV (2006) Embryogenesis in Orchidaceae. In: Batygina TB (ed) Embryology of flowering plants, Seed, vol 2. Science Pub., New Hampshire, pp 355–359
15. Lee Y-I, Yeung EC, Chung MC (2007b) Embryo development of orchids. World Scientific Publishing Co. Pte. Ltd., Singapore
16. Sabelli PA, Larkins BA (eds) (2015) Advances in seed biology. Frontiers Media, Lausanne. https://doi.org/10.3389/978-2-88919-675-3
17. Belmonte MF, Kirbridge RC, Stone SL, Pelletier JM, Bui AQ, Yeung EC, Hashimoto M, Fei J, Harade CM, Munoz MD, Le BH, Drews GN, Brady SM, Goldberg RB, Harada JJ (2013) Comprehensive developmental profiles of gene activity in regions and subregions of the *Arabidopsis* seeds. PNAS 110:E435–E444
18. Teixeira da Silva JA (2013) Orchids: advances in tissue culture, genetics, phytochemistry and transgenic biotechnology. Floricult Ornament Biotech 7:1–52
19. Chugh S, Guha S, Rao IU (2009) Micropropagation of orchids: a review on the

potential of different explants. Sci Hortic 122:507–520

20. Hossain MM, Kant R, Van PT, Winarto B, Zeng S-J, Teixeira da Silva JA (2013) The application of biotechnology to orchids. Crit Rev Plant Sci 32:69–139
21. Teixeira da Silva JA (2003) The role of thin cell layers in regeneration and transformation in orchids. Plant Cell Tissue Organ Cult 113:149–161
22. Yeung EC (2017) A perspective on orchid seed and protocorm development. Bot Stud 58:33
23. Yeung EC, Meinke DW (1993) Embryogenesis in angiosperms: development of the suspensor. Plant Cell 5:1371–1381
24. Ye XL, Zee SY, Yeung EC (1997) Suspensor development in the nun orchid, *Phaius tankervilliae*. Int J Plant Sci 158:704–712
25. Huang BQ, Ye XL, Yeung EC, Zee SY (1998) Embryology of *Cymbidium sinense*: the microtubule organization of early embryos. Ann Bot 81:741–750
26. Lee Y-I, Yeung EC, Lee N, Chung MC (2006) Embryo development in the lady's slipper orchid, *Paphiopedilum delenatii* with emphases on the ultrastructure of the suspensor. Ann Bot 98:1311–1319
27. Kawashima T, Goldberg RB (2010) The suspensor: not just suspending the embryo. Trends Plant Sci 15:23–30
28. Offler CE, McCurdy DW, Patrick JW, Talbot MJ (2003) Transfer cells: cells specialized for a special purpose. Annu Rev Plant Biol 54:431–454
29. Thompson RD, Hueros G, Becker HA, Maitz M (2001) Development and functions of seed transfer cells. Plant Sci 160:775–783
30. O'Brien TP, Feder N, McCully ME (1964) Poly-chromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373
31. Lee Y-I, Yeung EC (2010a) The osmotic property and fluorescent tracer movement of developing orchid embryos of *Phaius tankervilliae* (Aiton) BI. Sex Plant Reprod 23:337–341
32. Yang CK, Lee YI (2014) The seed development of a mycoheterotrophic orchid, Cyrtosia javanica Blume. Bot Stud 55:44
33. Bewley JD, Black M (1994) Seeds: physiology of development and germination. Plenum Press, New York
34. Goldberg RB, de Paiva G, Yadegari R (1994) Plant embryogenesis: zygote to seed. Science 266:605–614
35. Rodkiewicz B, Fyk B, Szczuka E (1994) Chlorophyll and cutin in early embryogenesis in *Capsella*, *Arabidopsis*, and *Stellaria* investigated by fluorescence microscopy. Sex Plant Reprod 7:287–289
36. Lackie S, Yeung EC (1996) Zygotic embryo development in *Daucus carota*. Can J Bot 74:990–998
37. Lee Y-I, Yeung EC, Lee N, Chung MC (2008a) Embryology of *Phalaenopsis amabilis* var. *formosa* : embryo development. Bot Studies 49:139–146
38. Yeung EC, Zee SY, Ye XL (1996) Embryology of *Cymbidium sinense*: embryo development. Ann Bot 78:105–110
39. Nishimura G (1991) Comparative morphology of cotyledonous orchid seedlings. Lindleyana 6:140–146
40. Setter TL, Flannigan BA (2001) Water deficit inhibits cell division and expression of transcripts involved in cell proliferation and endoreduplication in maize endosperm. J Exp Bot 52:1401–1140
41. Harrison CR (1977) Ultrastructural and histochemical changes during the germination of *Cattleya aurantiaca* (Orchidaceae). Bot Gaz 138:41–45
42. Taylor DC, Weber N, Underhill EW, Pomeroy MK, Keller WA, Scowcroft WR, Wilen RW, Moloney MM, Holbrook LA (1990) Storage-protein regulation and lipid accumulation in microspore embryos of *Brassica napus* L. Planta 181:18–26
43. Zou JT, Abrams GD, Barton DL, Taylor DC, Pomeroy MK, Abrams SR (1995) Induction of lipid and oleosin biosynthesis by (+)-abscisic acid and its metabolites in microspore-derived embryos of *Brassica napus* L. cv. Reston. Biological responses in the presence of 8-hydroxy abscisic acid. Plant Physiol 108:563–571
44. Van der Kinderen G (1987) Abscisic acid in terrestrial orchid seeds: a possible impact on their germination. Lindleyana 2:84–87
45. Lee YI, Lu CF, Chung MC, Yeung EC, Lee N (2007a) Changes in endogenous abscisic acid levels and asymbiotic seed germination of a terrestrial orchid, *Calanthe tricarinata* Lindl. J Am Soc Hortic Sci 132:246–252
46. Lee Y-I, Chung M-C, Yeung EC, Lee N (2015) Dynamic distribution and the role of abscisic acid during seed development of a lady's slipper orchid, *Cypripedium formosanum*. Ann Bot 116:403–411
47. Schwallier RS, Bhoopalan V, Blackman S (2011) The influence of seed maturation on desiccation tolerance in *Phalaenopsis amobilis* hybrids. Sci Hortic 128:136–140
48. Ling H, Zeng X, Guo S (2016) Functional insights into the late embryogenesis abundant

(LEA) protein family from *Dendrobium officinale* (Orchidaceae) using an *Escherichia coli* system. Sci Rep 6:39693

49. Battaglia M, Olvera-Carrillo Y, Garciarrubio A, Campos F, Covarrubias AA (2008) The enigmatic LEA proteins and other hydrophilins. Plant Physiol 148:6–24
50. Dure LS III (1979) Role of stored messenger RNA in late embryo development and germination. In: Rubenstein I, Phillips RL, Green CE, Gengenbach BG (eds) The plant seed. Academic Press, New York, pp 113–127
51. Dure L (1985) Embryogenesis and gene expression during seed formation. Oxford Surv Plant Mol Cell Biol 2:179–197
52. Sano N, Permana H, Kumada R, Shinozaki Y, Tanabata T, Yamada T, Hiraswa T, Kanekatsu M (2002) Proteomic analysis of embryonic proteins synthesized from long-lived mRNAs during germination of rice seeds. Plant Cell Physiol 53:687–698
53. Raghavan V, Goh CJ (1994) DNA synthesis and mRNA accumulation during germination of embryos of the orchid *Spathoglottis plicata*. Protoplasma 183:137–147
54. Su CL, Chao YT, Alex Chang YC, Chen WC, Chen CY, Lee AY, Hwa KT, Shih MC (2011) *De novo* assembly of expressed transcripts and global analysis of the *Phalaenopsis aphrodite* transcriptome. Plant Cell Physiol 52:1501–1514
55. Lin HY, Chen JC, Wei MJ, Lien YC, Li HH, Ko SS, Liu ZH, Fang SC (2014) Genome-wide annotation, expression profiling, and protein interaction studies of the core cell-cycle genes in *Phalaenopsis aphrodite*. Plant Mol Biol 84:203–226
56. Fang SC, Chen JC, Wei MJ (2016) Protocorms and protocorm-like bodies are molecularly distinct from zygotic embryonic tissues in *Phalaenopsis aphrodite*. Plant Physiol 171:2682–2700
57. Chen JC, Wei MJ, Fang SC (2016) Expression analysis of fertilization/early embryogenesis-associated genes in *Phalaenopsis* orchids. Plant Signal Behav 11(10):e1237331
58. Cocucci AE, Jensen WA (1969) Orchid embryology: the mature megagametophyte of *Epidendrum scutella* following fertilization. Amer J Bot 56:629–640
59. Swamy BGL (1947) On the life-history of *Vanilla planifolia*. Bot Gaz 108:449–459
60. Kodahl N, Johansen BB, Rasmussen FN (2015) The embryo sac of *Vanilla imperialis* (Orchidaceae) is six-nucleate, and double fertilization and formation of endosperm are not observed. J Linn Soc Bot 177:202–213
61. Arekal GD, Karanth KA (1981) The embryology of *Epipogium roseum* (Orchidaceae). Plant Syst Evol 138:1–7
62. Lopez-Villalobos A, Lopez-Quiroz AA, Yeung EC (2016) Asymmetric cell division in the zygote of flowering plants: the continuing polarized event of embryo sac development. In: Rose RJ (ed) Molecular cell biology of the growth and differentiation of plant cells. CRC Press, Boca Raton, pp 257–284
63. Vijayaraghavan MR, Prabhakar K (1984) The endosperm. In: Johri BM (ed) Embryology of angiosperms. Springer, Berlin, pp 319–376
64. van der Pilj L, Dodson CH (1966) Orchid flowers: their pollination and evolution. University of Miami Press, Coral Gables, FL
65. Arditti J, Ghani AKA (2000) Numerical and physical properties of orchid seeds and their biological implications. New Phytol 145:367–421
66. Molvray M, Chase MW (1999) Seed morphology. In: Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN (eds) Genera Orchidacearum, General introduction, Apostasioideae, Cypripedioideae, vol 1. Oxford University Press, New York, pp 59–66
67. Nishimura G, Yukawa T (2010) Dark material accumulation and sclerotization during seed coat formation in *Vanilla planifolia* Jacks: Ex Andrews (Orchidaceae). Bull Natl Mus Nat Sci Ser B 36:33–37
68. Veyret Y (1969) La structure des semences des Orchidaceae et leur aptitude a la germination in-vitro en cultures pures. Travaux du Laboratoire de (La Jaysinia), Museum Nationale D'Histroire Naturelle. Paris Fascicle 3:89–98
69. Yamazaki J, Miyoshi K (2006) *In vitro* asymbiotic germination of immature seed and formation of protocorm by *Cephalanthera falcate* (Orchidaceae). Ann Bot 98:1197–1206
70. Kucera B, Cohn MA, Leubner-Metzger G (2005) Plant hormone interactions during seed dormancy release and germination. Seed Sci Res 15:281–307
71. Zhang XS, O'Neill SD (1993) Ovary and gametophyte are coordinately regulated by auxin and ethylene following pollination. Plant Cell 5:403–418
72. O'Neill SD (1997) Pollination regulation of flower development. Annu Rev Plant Physiol Plant Mol Biol 48:547–574
73. Tsai WC, Hsiao YY, Pan ZJ, Kuoh CS, Chen WH, Chen H (2008) The role of ethylene in orchid ovule development. Plant Sci 175:98–105
74. Novak SD, Luna LJ, Gamage RN (2014) Role of auxin in orchid development. Plant Signal Behav 9(10):e972277

75. Novak SD, Whitehouse GA (2013) Auxin regulates first leaf development and promotes the formation of protocorm trichomes and rhizome-like structures in developing seedlings of *Spathoglottis plicata* (Orchidaceae). AoB Plants 5:pls053
76. Taylor JS, Blackman SJ, Yeung EC (1982) Hormonal and structural aspects of fruit development in the orchid, *Epidendrum*. J Exp Bot 33:495–505
77. Knudson L (1922) Non-symbiotic germination of orchid seeds. Bot Gaz 73:1–25
78. Knudson L (1946) A nutrient for germination of orchid seeds. Amer Orchid Soc Bull 15:214–217
79. Arditti J (1967) Factors affecting the germination of orchid seeds. Bot Rev 33:1–97
80. Kauth PJ, Dutra D, Johnson TR, Stewart SL, Kane ME, Vendrame W (2008) Techniques and applications of in vitro orchid seed germination. In: Teixeira da Silva JA (ed) Floriculture, ornamental and plant biotechnology: advances and topical issues, vol V, 1st edn. Global Science Books, Ltd., Isleworth, UK, pp 375–391
81. Zettler LW, McInnis TM Jr (1994) Light enhancement of symbiotic seed germination and development of an endangered terrestrial orchid (*Platanthera integrilabia*). Plant Sci 102:133–138
82. Yoder JA, Zettler LW, Stewart SL (2000) Water requirements of terrestrial and epiphytic orchid seeds and seedlings, and evidence for water uptake by means of mycotrophy. Plant Sci 156:145–150
83. Baque MA, Shin YK, Elshmari T, Lee EJ, Paek KY (2011) Effect of light quality, sucrose and coconut water concentration on the micropropagation of *Calanthe* hybrids ('Bukduseong' × 'Hyesung' and 'Chunkwang' × Hyesung'). Aust J Crop Sci 5:1247–1254
84. Nikabadi S, Bunn E, Stevens J, Newman B, Turner SR, Dixon KW (2014) Germination responses of four native terrestrial orchids from south-west Western Australia to temperature and light treatments. Plant Cell Tissue Organ Cult 118:559–569
85. Bonnardeaux Y, Brundrett M, Batty AL, Dixon KW, Koch J, Sivasithamparam K (2007) Diversity of mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships and alien invasions. Mycol Res 111:51–61
86. Swarts ND, Sinclair EA, Francis A, Dixon KW (2010) Ecological specialization in mycorrhizal symbiosis leads to rarity in an endangered orchid. Mol Ecol 19:3226–3242
87. McCormick MK, Lee Taylor D, Juhaszova K, Burnett RKJ, Whigham DF, O'Neill JP (2012) Limitations on orchid recruitment: not a simple picture. Mol Ecol 21:1511–1523
88. Rasmussen HN, Dixon KW, Jěrěáková J, Tesitelová T (2015) Germination and seedling establishment in orchids: a complex of requirements. Ann Bot 116:391–402
89. Stoutamire WP (1974) Terrestrial orchid seedlings. In: Withner CL (ed) The orchids: scientific studies. Wiley, New York, pp 101–108
90. Lee YI, Lee N, Yeung EC, Chung MC (2005) Embryo development of *Cypripedium formosanum* in relation to seed germination *in vitro*. J Am Soc Hortic Sci 130:747–753
91. Kako S (1976) In: Torigata H (ed) Study on the germination of seeds of *Cymbidium goeringii*, Seed formation and sterile culture of orchids. Seibundoshinkosha, Tokyo, pp 174–237. (in Japanese)
92. Lee YI (2011) *In vitro* culture and germination of terrestrial Asian orchid seeds. In: Thorpe TA, Yeung EC (eds) Plant embryo culture: methods and protocols. Humana Press, New York, pp 53–62
93. Van Waes JM, Debergh PC (1986) *In vitro* germination of some Western European orchids. Physiol Plant 67:253–261
94. Miyoshi K, Mii M (1988) Ultrasonic treatment for enhancing seed germination of terrestrial orchid, *Calanthe discolor*, in asymbiotic culture. Sci Hortic 35:127–130
95. Masanori T, Tomita M (1997) Effect of culture media and cold treatment on germination in asymbiotic culture of *Cypripedium macranthos* and *Cypripedium japonicum*. Lindleyana 12:208–213
96. Miyoshi K, Mii M (1998) Stimulatory effects of sodium and calcium hypochlorite, prechilling and cytokinins on the germination of *Cypripedium macranthos* seed *in vitro*. Physiol Plant 102:481–448
97. Chu CC, Mudge KW (1994) Effects of prechilling and liquid suspension culture on seed germination of the yellow lady's slipper orchid (*Cypripedium calceolus* var. pubescens). Lindleyana 9:153–159
98. DePauw MA, Remphrey WR (1993) *In vitro* germination of three *Cypripedium* species in relation to time of seed collection, media, and cold treatment. Can J Bot 71:879–885
99. St. Arnaud M, Lauzer D, Barabe D (1992) *In vitro* germination and early growth of seedlings of *Cypripedium acaule* (Orchidaceae). Lindleyana 7:22–27

100. Zhang Y, Lee Y-I, Deng L, Zhao S (2013) Asymbiotic germination of immature seeds and the seedling development of *Cypripedium macranthos* Sw., an endangered lady's slipper orchid. Sci Hortic 164:130–136
101. Lee Y-I, Yeung EC, Lee N, Chung MC (2008b) Embryology of *Phalaenopsis amabilis* var. formosa: embryo development. Bot Stud 49:139–146
102. Hsu RCC, Lee YI (2012) Seed development of *Cypripedium debile* Rchb. f. in relation to asymbiotic germination. HortSci 47: 1495–1498
103. Lee Y-I, Yeung EC (2010b) Embryo development and *in vitro* seed germination of *Bulbophyllum fascinator*. Acta Hortic 878:243–250
104. Suzuki RM, Moreira VC, Pescador R, Ferreira WM (2012) Asymbiotic seed germination and *in vitro* seedling development of the threatened orchid *Hoffmannseggella cinnabarina*. In Vitro Cell Dev Biol Plant 48:500–511
105. Mweetwa AM, Welbaum GE, Tay D (2008) Effects of development, temperature, and calcium hypochlorite treatment on *in vitro* germinability of *Phalaenopsis* seeds. Sci Hortic 117:257–262
106. Leroux GL, Barabe D, Vieh J (1997) Morphogenesis of the protocorm of *Cypripedium acaule* (Orchidaceae). Plant Syst Evol 205:53–72
107. Manning JC, van Staden J (1987) The development and mobilization of seed reserves in some African orchids. Aust J Bot 35:343–353
108. Alvarez MR (1968) Quantitative changes in nuclear DNA accompanying postgermination embryonic development in *Vanda* (Orchidaceae). Am J Bot 55:1036–1041
109. Lim WL, Loh CS (2003) Endopolyploidy in *Vanda* Miss Joaquim (Orchidaceae). New Phytol 159:279–287
110. Chen WH, Tang CY, Kao YL (2009) Ploidy doubling by *in vitro* culture of excised protocorms or protocorm-like bodies in *Phalaenopsis* species. Plant Cell Tissue Organ Cult 98:229–238
111. Chen WH, Kao YL, Tang CY, Lean GT (2011) Endopolyploidy in *Phalaenopsis* orchids and its application in polyploid breeding. In: Chen W-H, Chen H-H (eds) Orchid biotechnology II. World Scientific Publishing Co. Ltd, Singapore, pp 25–48
112. Johnson TR, Stewart SL, Dutra D, Kane ME, Richardson L (2007) Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)—preliminary evidence for the symbiotic culture advantage. Plant Cell Tissue Organ Cult 90:313–323
113. Khamchatra N, Dixon KW, Tantiwiwat S, Piapukiew J (2016) Symbiotic seed germination of an endangered epiphytic slipper orchid, *Paphiopedilum villosum* (Lindl.) Stein. from Thailand. S Afri J Bot 104:76–81
114. Liu H, Luo Y, Liu H (2010) Studies of mycorrhizal fungi of Chinese orchids and their role in orchid conservation in China—a review. Bot Rev 76:241–262
115. Chutima R, Lumyong S (2012) Production of indole-3-acetic acid by Thai native orchid-associated fungi. Symbiosis 56:35–44
116. Dohling S, Kumaria S, Tandom P (2008) Optimization of nutrient requirements for asymbiotic seed germination of *Dendrobium longicornu* Lindl. and *D. formosum* Roxb. Proc Indian Nat Sci Acad 74:167–171
117. Stewart SL, Kane ME (2006) Asymbiotic seed germination and in vitro seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell Tissue Organ Cult 86: 147–158
118. Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, New York
119. Dearnaley JDW (2007) Further advances in orchid mycorrhizal research. Mycorrhiza 17:475–486
120. Dearnaley J, Perotto S, Selosse M-A (2016) Structure and development of orchid mycorrhizas. In: Martin F (ed) Molecular mycorrhizal symbiosis. Wiley, New York, pp 63–86
121. Currah RS, Zettler LW, Hambleton S, Richardson KA (1997) Fungi from orchid mycorrhizae. In: Arditti J, Pridgeon AM (eds) Orchid biology: reviews and perspectives, vol VII. Kluwer Academic Publishers, Dordrecht, pp 117–170
122. Rasmussen HN (2002) Recent developments in the study of orchid mycorrhiza. Plant Soil 244:149–163
123. Rasmussen HN, Rasmussen FN (2009) Orchid mycorrhiza: implications of a mycophagous life style. Oikos 118:334–345
124. Rasmussen HN (1990) Cell differentiation and mycorrhizal infection in *Dactylorhiza majalis* (Rchb. f.) Hunt & Summerh. (Orchidaceae) during germination *in vitro*. New Phytol 116:137–143
125. Peterson RL, Massicotte HB, Melville LHF, Phillips F (2004) Mycorrhizas: anatomy and cell biology. NRC Research Press, Ottawa
126. Richardson KA, Peterson RL, Currah RS (1992) Seed reserves and early symbiotic pro-

tocorm development of *Platanthera hyperborea* (Orchidaceae). Can J Bot 70:291–300

127. Uetake Y, Kobayashi K, Ogoshi A (1992) Ultrastructural changes during the symbiotic development of *Spiranthes sinensis* (Orchidaceae) protocorms associated with binucleate *Rhizoctonia anastomosis* group C. Mycol Res 96:199–209
128. Masuhara G, Katsuya K (1994) *In situ* and *in vitro* specificity between *Rhizoctonia* spp. and *Spiranthes sinensis* (Persoon) Ames. var. amoena (M. Bieberstein) Hara (Orchidaceae). New Phytol 127:711–718
129. Wright M, Guest D, Cross R (2005) Development of mycorrhiza association in *Caladenia tentaculata*. Selbyana 26:114–124
130. Stoutamire WP (1983) Early growth in North American terrestrial orchid seedlings. In: Plaxton EH (ed) North American terrestrial orchids symposium II, proceedings and lectures. Michigan Orchid Society, Livonia, pp 14–24
131. Fochi V, Chitarra W, Kohler A, Voyron S, Singan VR, Lindquist EA, Barry KW, Girlanda M, Grigoriev IV, Martin F et al (2017) Fungal and plant gene expression in the *Tulasnella calospora–Serapias vomeracea* symbiosis provides clues about N pathways in orchid mycorrhizas. New Phytol 213:365–379
132. Dearnaley JDW, Cameron DD (2016) Nitrogen transport in the orchid mycorrhizal symbiosis—further evidence for a mutualistic association. New Phytol 213:10–12
133. Beyrle HF, Smith SE, Peterson RL, Franco CMM (1995) Colonization of *Orchis morio* protocorms by a mycorrhizal fungus: effects of nitrogen nutrition and glyphosate in modifying the responses. Can J Bot 73:1128–1140
134. Stewart SL (2008) Orchid reintroduction in the United States: a mini-review. N Am Native Orchid J 14:54–59
135. Zeng SJ, Wu KL, Teixeira da Silva JA, Zhang JX, Chen ZL, Xia NH, Duan J (2012) Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. Sci Hortic 138:198–209
136. Reiter N, Whitfield J, Pokkard G, Bedggood W, Argall M, Dixon K, Davis B, Swarts N (2016) Orchid re-introductions: an evaluation of success and ecological considerations using key comparative studies from Australia. Plant Ecol 217:81–95
137. Sussex IM (1989) Developmental programming of the shoot meristem. Cell 56:225–229
138. Su CL, Chao YT, Yen SH, Chen CY, Chen WC, Chang YC, Shih MC (2013) Orchidstra: an integrated orchid functional genomics database. Plant Cell Physiol 54:1–11
139. Zhao M-M, Zhang G, Zhang D-W, Hsiao Y-Y, Guo S-X (2013) ESTs analysis reveals putative genes involved in symbiotic seed germination in *Dendrobium officinale*. PLoS One 8:e72705
140. Valadares R, Perotto S, Santos E, Lambais M (2014) Proteome changes in *Oncidium sphacelatum* (Orchidaceae) at different trophic stages of symbiotic germination. Mycorrhiza 24:349–360
141. Cai J, Liu X, Vanneste K, Proost S, Tsai W-C, Liu KW et al (2015) The genome sequence of the orchid *Phalaenopsis equestris*. Nat Genet 47:65–72
142. Chin CF (2016) The proteome of orchids. Agri. Proteomics 1:117–125

Chapter 2

Orchid Mycorrhizal Fungi: Isolation and Identification Techniques

Lawrence W. Zettler and Laura L. Corey

Abstract

In light of the importance of mycorrhizal fungi to the orchid life cycle from an evolutionary and ecological context, it is essential that conservationists adopt protocols to isolate, identify, and safeguard important fungal strains in this age of extinction. The purpose of this chapter is to summarize some of the methods used by researchers worldwide to isolate and identify peloton-forming fungi in the *Rhizoctonia* complex from temperate terrestrials and tropical epiphytes alike. The chapter begins with a summary of preparatory background information leading up to fungal isolation, including terminology, how to spot and identifying small seedlings in the field, how to avoid "senile populations," collection permits, and viable transport of material. The second part of the chapter focuses on fungal isolation techniques, namely field collecting, peloton assessment, surface sterilization, and isolation from roots and protocorms. Maintenance of fungi through subculturing and storage is also discussed. The chapter concludes with methods aimed at provisional and precise fungal identification using cultural characteristics and modern molecular techniques, respectively, with emphasis on the Tulasnellaceae, Ceratobasidiaceae, and Sebacinaceae.

Key words Seedlings, Field collection, Tulasnellaceae, Ceratobasidiaceae, Sebacinaceae, Epiphytic and terrestrial orchids

1 Introduction

Orchids constitute the most diverse family of angiosperms with upwards of 25,000+ species [1], representing 8–10% of all vascular plant species worldwide [2, 3]. Newest evidence suggests that the first orchids arose ca. 112 million years ago, with the major lineages diverging about 90 million years ago, and a shift to an epiphytic lifestyle ca. 35 million years ago [3]. Five of these lineages persist to this day, three of which (apostasioids, cypripedioids, vanilloids) are represented by relatively few species [4]. The driving force behind the rapid diversification of this family is, at least in part, attributed to their close association with mycorrhizal fungi [5]. Unlike most plants, orchids exploit their mycorrhizal fungi as an organic carbon source to meet their early

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_2,

germination needs, and this cleaver adaptation appears to have contributed to the family's global success. All orchids have two features shared in common—a mycotrophic protocorm stage early in the life cycle [6], and tissues/roots harboring coils of intracellular hyphae or pelotons [7] that are then digested, releasing organic carbon [8] and water [9] to the orchid. Understanding how orchids interact with these fungi in the natural setting, particularly at the germination site, is now the subject of intensive study, and holds considerable promise for conservation.

All subfamilies of Orchidaceae, including those first to have evolved, typically form associations with basidiomycetes in the ubiquitous *Rhizoctonia* complex (e.g., *Ceratobasidium*, *Thanatephorus*, *Tulasnella*), implying that this close relationship was a "founding event" [6]. As to why orchids have largely targeted this particular group of fungi is perplexing, but recent studies have revealed that some orchids display a high level of strain-to-species specificity (e.g., [10–12]), suggesting that young seedlings have the ability to distinguish between closely related fungal strains [6]. Considering that fungi within the same genus or family are known to differ in their trophic strategies [13, 14], orchids may have exploited this narrow group of fungi by selecting specific strains that best meet their nutritional needs in a given habitat. By living as heterotrophs for extended periods, orchid seedlings have provided the family with new habitats and evolutionary pathways that would otherwise not be available [15]. This trophic versatility helps to explain why these plants have such a high degree of ecological adaptability for which they are known. Identifying the peloton-forming fungi, therefore, should enable future researchers to pinpoint the ultimate source of organic carbon that orchid seedling stages require [6], and this process is considerably enhanced by modern molecular techniques currently underway.

In light of the importance of mycorrhizal fungi to the orchid life cycle from an evolutionary and ecological context, it is essential that conservationists worldwide adopt protocols to isolate, identify, and safeguard important fungal strains in this age of extinction [16]. Throughout the world, orchids are threatened by many factors including human overpopulation, logging, mining, ecological degradation imposed by climate change, and exploitation [4]. To reduce pressure exerted by the latter, artificial propagation of commercially desirable species would likely diminish the appetite for poaching orchids from the wild (e.g., *Dendrophylax lindenii* in Florida). Using fungi for this purpose (=symbiotic seed germination, Fig. 1) is now a practical alternative, even for tropical epiphytes routinely cultivated on asymbiotic media (e.g., [17, 18]).

The purpose of this chapter is to summarize the methods adopted by researchers worldwide aimed at isolating and identifying peloton-forming fungi in the *Rhizoctonia* complex in tissues of photosynthetic orchids for conservation and horticultural purposes. As Rasmussen [15] aptly pointed out, "*Orchid endophytes are*

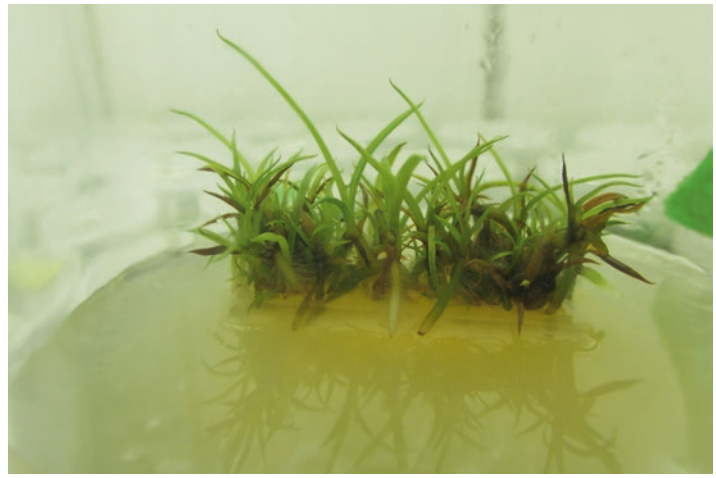


Fig. 1 Seedlings of a terrestrial orchid from Madagascar (*Tylostigma* sp.) grown on an oat-based medium in vitro using a mycorrhizal fungus. Symbiotic germination has practical merit for conservation and horticulture alike for growing epiphytes as well as terrestrials from seed

difficult to trace in soil and difficult to identify as potential symbionts unless they are actually extracted from orchid tissue, and this may become increasingly difficult as orchid populations decline in size." Accordingly, this chapter was written with current and future researchers in mind faced with the burden of securing orchid mycorrhizal fungi from natural areas undergoing rapid and irreversible change. The knowledge they acquire, and the fungi they ultimately secure, will no doubt be valued by future generations. The isolation methods described herein should have a broad application, encompassing orchids in different growth stages (protocorms, seedlings, mature plants), different climes (temperate, subtropical, tropical), and different substrata (terrestrial, epiphytic, lithophytic). In addition, we describe the techniques for provisional identification of mycorrhizal candidates using traditional low-cost means (e.g., cultural characteristics, light microscopy), followed by modern molecular approaches (e.g., ITS sequencing).

2 Discussion of Technical Issues and Concerns

To effectively isolate and identify orchid fungi, one must be armed with the knowledge of *when* and *where* to look for these organisms, both in the natural habitat and in living tissues. This knowledge begins with understanding basic terminology. Existing literature is full of terminology that is often confusing when describing fungal associations of orchids, and some of the major terms are addressed here for the sake of clarity.

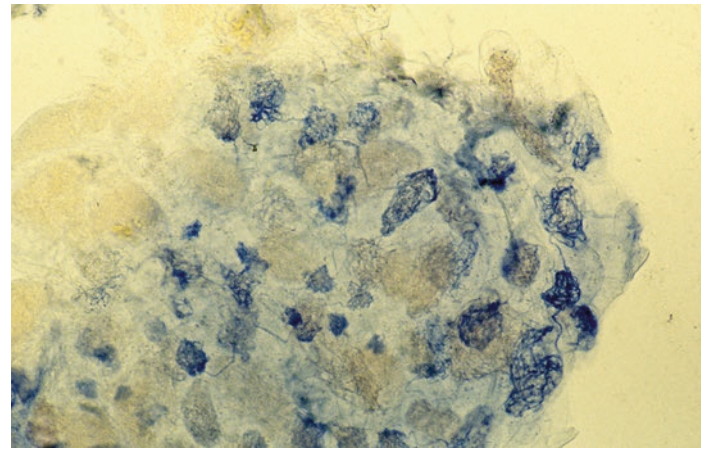


Fig. 2 Pelotons—coils of fungal hyphae—are easily observed in living orchid tissues under light microscopy after staining (e.g., trypan blue). This image shows numerous pelotons within a protocorm of *Encyclia tampensis*—an epiphytic orchid from Florida

2.1 Terminology

"Orchid mycorrhiza" generally refers to the actual plant-fungus relationship, where a mixture of different groups of fungi may occur within the roots. Some of these fungi may provide the orchid with organic carbon as well as nutrients and water, but the extent of this transfer is often unclear. Thus, mycorrhizal fungi are thought to impart some physiological benefit to the orchid. Some of these fungi are transitional, meaning that they do not persist in tissues as the orchid matures, whereas others do. Some of the fungi that facilitate germination remain as "permanent residents" as the orchid matures; others may trigger germination, then be replaced by other types of fungi. The fungi that persist in an intimate manner, usually in the root cortex, are generally referred to as "mycobionts." Most orchid mycobionts display a colonization pattern where fungal hyphae form dense coils (pelotons) within host plant cells (Fig. 2).

These pelotons are often digested as a carbon source (mycotrophy) in a controlled manner, depending on the nutritional needs of the orchid at any given time. The number of pelotons in an orchid root may vary from individual to individual, with seasonality thought to play an important role. For example, roots of tropical epiphytic orchids may harbor more pelotons during the onset of the rainy season compared to the dry season [19]. For temperate terrestrials, peloton formation may coincide with the onset of the growing season. In contrast to mycobionts, "orchid endophytes" constitute a broader term for the fungi that occur in living tissues but have no known role, positive or negative. Fungi isolated from pelotons should technically be regarded as endophytes—not orchid mycorrhizal fungi—until they have been shown experimentally to benefit the orchid (e.g., facilitate germination in vitro). Currah et al. [20] applies the term "endophyte" to fungi that are associated with the cortex and outer layer (epidermis, velamen) of the root.

2.2 Spotting Small Seedlings in the Field

Young orchid seedlings, before they initiate leaves, are regarded as protocorms and are entirely mycotrophic or largely so. Orchid protocorms typically range in size from 0.5 mm to >1 cm, depending on the species, but they often have a universal shape consisting of a rounded or ovoid lower base terminated by a growing tip or shoot that will eventually give rise to leaves in photosynthetic species. Their diminutive size stems from the fact that they arise from tiny, "dust-like" seeds. Although finding young orchid seedlings in situ is challenging because of their small size, examining substrates in close proximity to mature orchids is often a good place to look. Small seedlings of terrestrials and epiphytes can often be found directly beneath a mature orchid that flowered the year before (Figs. 3, 4, 5 and 6). Presumably, some seeds released from capsules above are thought to trickle down to the substrate below where seedlings are spawned by a favorable mycelial mat. Some orchids like *Tipularia discolor* in North America colonize rotting hardwood logs in the forest floor by exploiting wood-rotting fungi [21]. Protocorms of this species can be observed just beneath a thin layer of woody debris on the upper portion of the log (Fig. 7). For epiphytic orchids, ascending the tree canopy poses a formidable challenge (and risk) to those seeking protocorms on arboreal substrates. Nevertheless, close inspection of mosses and/or lichens on branches will often reveal protocorms and tiny seedlings alike, and exposed rocks covered in algae often are good places to look as well (Fig. 8). Terrestrial orchids occupying open sunny areas (e.g., bogs, fens) are sometimes detected in patches of low spots where water accumulates. For forest-dwelling species, woody debris (e.g., rotting logs) or areas near disturbed sites (e.g., adjacent logging roads) should be closely inspected. Seedlings of lithophytic orchids can sometimes be

Fig. 3 A young *Liparis* seedling in the act of elongating its first leaf when unearthed from the base of a mature orchid of the same species in a forest in Madagascar. The protocorm's conical shape is evident at the tip of the ball point pen

Fig. 4 Young orchid seedlings are rooted in a moss mat adjacent to a colony of a mature terrestrial orchid, *Cynorkis gibbosa*, in Madagascar. The young seedlings are presumably of the same species and were easy to spot emerging from the moss

Fig. 5 This very young orchid seedling, shown on the thumb nail of the collector, was discovered in moist soil in contact with mature orchid roots (*Tylostigma* sp.) in Madagascar

found among cracks and crevices on rock surfaces, and especially in pockets where detritus accumulates. Epiphytic orchid seedlings may often be found in close association with mosses, but lichens should also be explored as potential associates. Small seedlings

Fig. 6 A young Ghost Orchid (*Dendrophylax lindenii*) seedling clinging to corrugated bark of its host tree in western Cuba. The pink pin to the right was affixed to the bark for tracking purposes in a long-term monitoring study

Fig. 7 Two seedlings of *Tipularia discolor* (circled) growing within a rotting hardwood log in Maryland, USA. This species utilizes wood-rotting mycorrhizal fungi to facilitate seedling development

have been observed beneath the thallus of foliose lichens on trees in both Ecuador and Madagascar, for example (Figs. 9 and 10). Using a scalpel or spatula to gently lift the moss/lichen layer will often reveal healthy orchid roots of small seedlings, and occasion-

Fig. 8 An advanced-stage protocorm of an epiphytic orchid (arrow) growing on the face of a moist rock coated with algae in the Andean cloud forest near Cuenca, Ecuador

Fig. 9 An advanced-stage protocorm of an epiphytic orchid (green in color) that was discovered beneath the shallow bark of host tree in the Central Highlands of Madagascar, resting within the palm of a hand

Fig. 10 A young seedling, likely a *Bulbophyllum* sp., growing in association with foliose lichens on a host tree in Madagascar. The tip of a ball point pen to the left provides a glimpse of the seedling's small size

ally even protocorms. When seedlings are discovered, the microsite should be photographed, and detailed notes should be taken with respect to plant associates, shade/sunlight availability, orientation/position on bark (for epiphytes), and other factors (e.g., soil pH, nutrient composition).

2.3 Identifying Small Seedlings to Taxonomic Rank

Although often difficult to locate under natural conditions, orchid protocorms are frequently targeted by researchers because they are the growth stage most likely to harbor the endophyte(s) that initiated the germination process. Thus, acquiring fungi from protocorms or small seedlings, especially those from pelotons contained therein, makes it more likely that the resulting fungus will be effective at germinating seeds in vitro using symbiotic techniques. In addition, some of these fungi may be highly effective at germinating seeds of unrelated orchid taxa, not just the donor species (e.g., [19]). One drawback to this method, however, is that it is almost impossible to know what species (or genus, for that matter) the seedling came from based on visual cues alone. Accordingly, molecular techniques are now employed to resolve this issue by matching the DNA from orchid seedlings with the known sequences of chloroplast DNA region *trn*L-F to that of an identified plant (*see* [22]). Thus, some of the orchid tissues collected in the field should be detached and retained (e.g., shoot region) when possible.

Another technique used for obtaining small seedling stages involves the use of seed packets (described in current text, Chap. 3). Mature seeds of a known orchid species are added to nylon (plankton) mesh packets, followed by their deployment in or on natural substrates. This technique, initially devised by Rasmussen and

Whigham [23], allows for the "capture" of minute orchid protocorms as a means to study natural germination processes and to recover endophytes that may have triggered seed germination. Briefly, water and fungal hyphae from the surrounding substrate (soil, mosses) infiltrate the mesh and make physical contact with the seeds triggering germination to the protocorm stage. Protocorms and the mycobionts they contain are thus easily "captured" upon retrieval and transported back to the laboratory for fungal isolation. Depending on the species, protocorms may be captured in this manner in as little as 1 year after sowing (e.g., *Peristylus holochila*; [24]) to as many as 4.5 years (e.g., *Cypripedium calceolus*; [25]). During the 20+ years that seed packets have been used and modified throughout the world (e.g., [26–32]), this technique has yielded a wealth of information on early life history stages of orchids and the fungi that are involved.

2.4 Avoiding Senile Populations

To more effectively isolate the full breadth of fungi that orchids require in nature, our mindset must conform to a tiny cryptic world where moisture and nutrients abound. It is too easy to target roots of a large epiphytic orchid on a tree limb, for example, as a potential source of mycorrhizal fungi when doing so could yield mostly strains that had little to do with meeting that plant's early nutritional needs. Furthermore, just because an orchid population consists of mature, healthy-appearing plants does not necessarily mean that the population is suitable for seedling recruitment. In fact, long-lived orchids in a given area may simply be persisting year after year, releasing seeds in vain if they are not dispersed to distant areas more suited to seedling establishment. Such "senile populations" are characterized as lacking the key components (e.g., specific pollinators, mycorrhizal fungi) necessary for seedling recruitment [7], representing a serious, often overlooked problem for the conservationist. This is especially true if seed packets (baits) are deployed containing seed of critically rare species that then have little or no chance of germinating. Specialists who begin exploring orchid habitats should therefore be alert to this possibility, and be diligent about discovering seedling stages before orchid tissues are collected for study.

2.5 Permits and Transport

Before any orchid material is collected, proper permits must be secured – all of them. This may require international, federal, and/or state permits, and a letter documenting landowner's permission. For research carried out abroad, the legal collection and international transport of orchid material requires a Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) permit. Once this document is secured, there are often several other permits that are then needed before orchid samples may be transported. For example, most countries are obligated to inspect outgoing material for potential pests (e.g., mites, slugs, scale insects), leading to a phytosanitary certificate. If material is

deemed "pest-free," samples are usually then cleared to enter outbound aircraft provided they are housed in shatter-proof containers. These samples will also be exposed to radiation at security checkpoints, but this has little or no apparent negative effect on the viability of fungi and seed embryos. If given the option of transporting samples in carry-on baggage or in check-in luggage, the former is preferable to ensure that the samples remain at ambient temperature throughout the duration of the flight. This also minimizes the risk of losing precious samples due to improper handling by the airline(s).

Upon arrival, researchers should be prepared to present all export permits (CITES, phytosanitation certificate) as well as import permits. In the United States, for example, orchid material (seed, capsules, leaves) may be legally imported by means of a USDA PPQ-587 permit that allows importation of plants and plant products. For orchid root samples, however, a USDA PPQ-526 permit is required because the US Government considers all *Rhizoctonia*-like fungi to be noxious pests given their close affinities to well-known plant pathogens. Moreover, arrangements must be made well in advance to meet inspectors in designated airports (e.g., Atlanta Hartsfield International Airport, ATL), and root samples must be accompanied by clearly marked labels. Once the samples are cleared to enter by customs officials, many countries require the samples to remain sealed until arrival in a designated, certified laboratory. In the United States, for example, all root samples containing *Rhizoctonia*-like fungi of foreign origin must remain in a USDA Level 2 Quarantine Facility under lock and key. All living orchid fungi must remain in this facility at all times, and be destroyed by autoclaving immediately upon exiting. However, orchid fungi in pure culture may be shipped to other researchers outside of the United States for further work or storage via cryopreservation provided the samples are thoroughly sealed in shatterproof containers.

3 Fungal Isolation

3.1 Field Collection

Orchid samples collected for fungal isolation generally consist of actively growing roots excised from established plants in populations that harbor spontaneous seedlings. While it is desirable to collect material from seedling stages, mature orchids are also known to harbor fungi that are effective at facilitating germination in vitro for terrestrials and epiphytes alike (e.g., [18, 33]). Whenever possible, however, protocorms or small seedlings should be targeted for collection, and this usually involves sampling the entire plant. Doing so serves two purposes – it allows for thorough fungal sampling of all pelotons, and also permits molecular identification of the orchid material itself using the shoot region/leaves that are subsequently

Fig. 11 Yellow-orange pigmented areas (various spots) on roots of terrestrial orchids often harbor undigested pelotons in mature plants as well as seedlings. Pictured is a *Spiranthes* species from Illinois, USA, collected during the growing season (mid-summer)

detached and analyzed. Even when the identity of the mature orchid is known, it is considered good practice to collect a leaf piece to accompany roots that are sampled.

When detaching roots in the field, a sterile scalpel is generally used, and the entire length of the root should be collected. The scalpel is then disinfested with rubbing alcohol or a bleach solution between sampling to reduce the likelihood of cross-contamination and/or disease transmission. For many temperate terrestrials (e.g., *Spiranthes*, *Platanthera*), pelotons are often present in lateral branch roots especially in brownish [34] or yellow-orange patches (Fig. 11) along the root length [15, 35–38]. These fleshy roots sometimes are associated with a faint musky odor, and this phenomenon appears to be widespread, having been detected from *Tylostigma* in Madagascar, to *Platanthera* in North America, for example. For some terrestrials in Australia (e.g., *Caladenia* spp., Pterostylidinae), pelotons are often found in the inflated stem-collar region subtending the basal leaf [36, 39], far removed from tubers. Pelotons in some epiphytic orchids are reported to be scarce [40], but they are likely to be present in roots that physically contact the substrate at the root/surface interface [41, 42].

In mature roots of epiphytes in south Florida, pelotons are typically observed in the second centimeter region beyond the root tip (Fig. 12; [43]). Benzing [41], however, found the opposite to be true, i.e., that older roots tended to bear heaviest infections, but it was unclear if the pelotons he observed were viable or undergoing digestion (lysis). Once detached, each individual root is placed into a presterilized glass or plastic vial with a screw cap, and vials are then transported back to the laboratory in darkness

Fig. 12 Roots of actively growing epiphytes that are in close contact with a substrate are known to yield pelotons suitable for fungal isolation. The region of the root 2 cm beyond the growing tip, where the velamen layer begins to form (circle), is often highest in peloton density. Pictured is the root tip of the Ribbon Orchid (*Campylocentrum pachyrrhizum*), a leafless epiphyte from south Florida known to harbor strains of *Ceratobasidium*

within an insulated container (e.g., cooler). Ideally, root samples should be chilled (4 °C) as quickly as possible, usually within 24–48 h following collection. It remains unknown how long pelotons can remain viable in root samples after they are detached, but fungi have been successfully isolated from roots collected 3 weeks prior [22], and many of these strains retained their effectiveness at facilitating seed germination in vitro [19]. Suárez et al. [42], however, reported that hyphae in orchid tissues lose viability even after one night of storage in the laboratory regardless of whether or not the samples are chilled. As mentioned previously, sampling should take place during different times of the year to account for possible changes in the mycoflora possibly triggered by seasonal differences in moisture and/or temperature.

Below is a summary of some basic tenets to keep in mind leading up to fungal isolation in the laboratory:

- *Target younger orchids.* Pelotons in younger orchids (protocorms, seedlings) are more likely to yield the fungal strains that facilitated germination and development, as some mature orchids are known to "switch" fungal relationships over time [7, 44, 45].

- *In older plants, know where to look*. As the root system enlarges in size and differentiates, it becomes more difficult to pinpoint the location of peloton clusters. For terrestrials, pelotons are more prevalent in lateral branch roots or collar regions [36], not starch-filled tubers [37]. For epiphytes, pelotons are often found in roots that physically contact the substrate [46], not aerial roots [41, 42].
- *Isolate from younger roots of mature orchids*. Younger roots are more likely to harbor viable pelotons because hyphal colonization and proliferation would be actively taking place as the orchid root elongates. In contrast, older roots may be actively engaged in mycotrophy which would result in damaged (nonviable) pelotons.
- *Seasonality matters*. Active peloton formation is assumed to be closely tied to temperature and moisture. For temperate terrestrials, the onset of the growing season is thought to be optimal for peloton isolation. For orchids in tropical regions, peloton isolations may be best attempted during the rainy season which would be more conducive to fungal activity.
- *Not all pelotons are composed of fungi that will grow in culture*. Many pelotons—even seemingly viable ones (e.g., those with distinctive intact hyphae under magnification)—will not yield actively growing mycelia in culture for various reasons. Some of the peloton-forming fungi, for example, may be unculturable, whereas others may have succumbed to early process of digestion (lysis). Pelotons that show even the slightest degree of degradation should be discarded ([47] cited in 15).
- *Not all pelotons will yield Rhizoctonia-like fungi*. There are a number of cases worldwide where pelotons have yielded a mixture of fungal taxa, not just those assignable to the *Rhizoctonia* complex (*Ceratobasidium*, *Tulasnella*, and *Sebacina*), for example (see review by [20]).
- *A single peloton may be composed of a mixture of different fungi*. Thus, hyphal tips should be excised from pelotons and subcultured, not the entire peloton itself.

3.2 Peloton Assessment

After collecting roots in the field, many researchers opt to fix, stain, squash, and examine via light microscopy, root regions for evidence of pelotons before beginning fungal isolation attempts (e.g., [38, 42, 48–50]). This serves two purposes: (1) it helps to pinpoint the location of viable pelotons in cortical regions along the root length, and (2) it provides some quantitative assessment of peloton number (density), and their state of degradation (lysis). The former ensures that limited resources (time, supplies) are not wasted on roots that lack suitable fungal material, whereas the latter provides a baseline for ecological studies seeking information on orchid mycorrhizal associations in situ. Matsuda et al. [51], for example, examined root pieces of two

Cephalanthera species and concluded that mycorrhization was significantly different among individuals. They also found that the spatial distribution of mycorrhizas in both orchids was most frequent at the root apex. Fixing and staining, however, results in destruction of potential fungi that might otherwise be isolated. A general rule is to fix and stain 1 cm segments from no more than 1/4th of the total roots collected.

3.3 Surface Sterilization

The literature describing isolation of endophytic fungi from orchid mycorrhizas is consistent in one respect—all typically involve the removal of particles from roots followed by surface sterilization. Many researchers cut roots into segments and then proceed with surface sterilization (e.g., [38, 49]), whereas others surface-sterilize prior (e.g., [17]). The process begins by rinsing intact roots under running tap or DI water, followed by gentle scrubbing (usually by hand) to remove soil or other particles. Some researchers then cut roots into segments and surface-sterilize the epidermis, whereas others prefer to leave the roots intact. Roots/root segments are then placed in presterilized containers (e.g., empty Petri plates) and surface sterilized using cleansing agents. These agents have consisted of sodium (NaOCl) or calcium hypochlorite $Ca(ClO)_2$ bleach [52], ethanol [53], and mercuric chloride [54–56], sometimes coupled with a wetting agent such as dish detergent or tween [20]. The duration time that the roots are exposed to the chemical agents varies, and this factor should be carefully considered given that roots are likely to absorb the chemicals which could result in the death of endophytic fungi [36]. For epiphytic orchids, roots have been soaked as long as 10 min in 20% household bleach (5.25% NaOCl; [42]), to as little as 1 min [57]. Mahendran et al. [56] soaked roots of *Aerides crispum* in 0.05% mercuric chloride for 2 min, followed by 3–7 rinses in sterile DI water. Some researchers expose roots/root pieces to two different chemical agents separately, followed by a DI water rinse. Pereira et al. [58], for example, immersed roots of several epiphytes for 1 min in 70% ethanol, followed by immersion in 2% NaOCl for 5 min, whereas Otero et al. [59] exposed roots of *Ionopsis utricularioides* to 70% ethanol (1 min), 2.5% NaOCl (0.5 min), followed by a 1 min rinse in 70% ethanol. In other cases, the mixture of two chemical agents into a single wash, followed by sterile DI water rinse(s), has been practiced. Nontachaiyapoom et al. [46], for example, used 3% (*v*/*v*) H_2O_2 and 70% (*v*/*v*) ethanol for 10 min, followed by three water rinses. Zettler [37] recommend a mixture of 5 mL absolute ethanol, 5 mL 5.25% NaOCl, and 90 mL DI water; however, use of ethanol (as well as acetone, butanone, isopropyl alcohol, among others) and sodium hypochlorite (NaOCl) in combination is known to produce chloroform as a side reaction (C.F. Spencer, pers. com.) which is cytotoxic. Accordingly, the use of a single chemical agent throughout the surface-sterilization process may be

optimal, and this would also be applicable to protocols aimed at orchid seed surface sterilization. For researchers that opt for household bleach (5.25% NaOCl) as the primary chemical agent, we recommend using a freshly prepared solution, given that beach is known to have a short (<1 year) "shelf life."

3.4 Fungal Isolation from Root Pieces

Unlike fungi forming mycorrhizas in other plants, orchid endophytes can be readily cultured [8], as most are not fastidious about their preference for organic media [20]. A quick and often effective method for recovering a broad range of orchid endophytes is to simply cut thin slices of roots with a sterile scalpel, and place the slices onto the surface of an agar-based growing medium such as corn meal agar (CMA), potato dextrose agar or PDA [46], or phytone yeast extract agar (PYE), among others [20]. This method has been used with success in early and recent studies alike (e.g., [40, 42, 50, 54, 56, 59, 60–62]) and might be especially useful in cases where orchid roots are unusually thin (e.g., *Harrisella porrecta*). The advantage of using root slices is that more fungi from the rhizosphere are potentially cultured, including those external to the root cortex that would be in closer proximity to the soil/bark substrate [20]. A disadvantage, however, is that there is no way to know if the emerging fungi originated from pelotons, or if the fungi entered the root as parasitic invaders [20] or benign saprophytes.

3.5 Fungal Isolation from Pelotons in Cortical Cells and Protocorms

In recent years, the most widely practiced method for isolating orchid endophytes has been to target the cortical pelotons themselves. This process simply involves the removal of a portion of the cortex with a sterile scalpel followed by maceration in a drop of sterile water within the confines of an empty petri dish ([20, 48]; Fig. 13). For thicker roots of terrestrials, the epidermis may be scraped off and discarded prior to maceration [37] which serves to reduce the level of surface contaminants [36], whereas for epiphytes the velamen layer is sometimes discarded in a similar manner (e.g., [42, 46, 63]). For diminutive protocorms, the entire seedling may be immersed in a drop of water and macerated without removal of surface tissues (e.g., [24, 64, 65]). After maceration in the water droplet, lukewarm nutrient agar is poured over the droplet, gently swirled to disperse the peloton/cortical cell matrix, and then allowed to cool and solidify (e.g., [22, 49, 66, 67]). In this manner, pelotons suspended in the agar are spaced far enough apart to facilitate removal of hyphal tip transfers using a sterile scalpel.

Another, more refined, isolation technique involves teasing out pelotons with sterile mounting needles [15, 36, 68]. Although effective, this technique may be rather cumbersome for beginners. Once pelotons are teased out, either with a needle or maceration by scalpel, they may be transferred by Pasteur pipette to new droplets of sterile water which serves as a rinse [20, 69]. The number of transfers (rinses) varies, but several rinses are recommended to

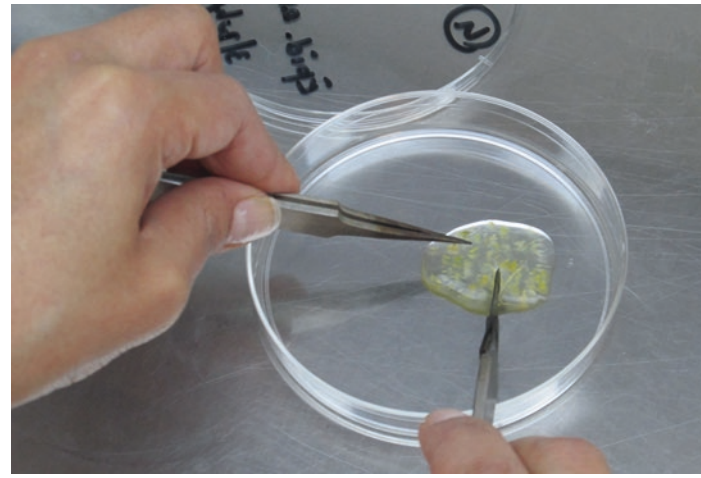


Fig. 13 A 1 cm segment of an epiphytic orchid's root is macerated within 5 mL of sterile DI water within a petri plate. The combination of chopping with a scalpel, and pinching with a forceps, releases cortical pelotons into the water droplet. The addition of molten agar (FIM) followed by gentle swirling, permits pelotons to be spaced far enough apart for fungal isolation once the agar cools and solidifies

reduce the number of contaminants that may be present in the sample. After final rinsing, the pelotons are then transferred to a clean Petri plate and immersed in lukewarm nutrient agar, or pipetted onto the surface of solidified agar. These peloton transfers may be deposited at the outer edge of each Petri plate to provide space for the emerging hyphae to grow. Ramsey and Dixon [36] nicely illustrate this procedure showing up to 12 peloton transfers per plate in such a manner. A slightly modified procedure by Zhu et al. [38] involves transferring teased pelotons into 2 mL Eppendorf tubes followed by 4–24 h incubation at ambient temperature. This procedure allows for living pelotons to be distinguished from nonviable pelotons. Those that yield actively growing hyphae are selected and transferred to 1 cm^2 PDA disks which are then placed on agar in separate plates and allowed to resume growth. The method appears to be especially useful for recovering the slow-growing fungal strains that might otherwise be overlooked or out-competed by faster-growing opportunistic strains.

The media used for facilitating hyphal emergence from pelotons is generally carbohydrate-rich. Currah et al. [20] recommended corn meal agar (CMA), whereas modified Melin-Norkrans agar or MMN [70] has also been frequently reported with good results (e.g., [33, 58, 71, 72]). Zettler et al. [24] utilized both MMN and malt extract agar to isolate endophytes of *Platanthera holochila* in Hawaii, but lowered the pH of the media to 5.0 prior to autoclaving to parallel the acidity of the soils on the archipelago. Shimura and Koda [73] utilized five different kinds of media in an attempt to isolate and maintain orchid endophytes of *Cypripedium*

macranthos: acid water agar medium (AWA), chloramphenicol water agar medium (CWA), sucrose agar medium (PSA), oatmeal agar medium (OMA1), and green pea agar medium (GPA), with CWA and OMA1 provided best results. The high carbohydrate load in some of these media (e.g., MMN = 10 g sucrose/L) has raised concerns that more opportunistic, faster-growing endophytes (e.g., *Ceratobasidium*) might be favored at the exclusion of slower-growing, more important strains (e.g., *Sebacina*). Using media high in carbohydrates also permits rapid growth of aggressive fungal contaminants (e.g., *Pestalotia*, *Trichoderma*) that have the potential to outgrow all orchid endophytes in culture in a short period of time leading to intermingled hyphae. Moreover, CMA and MMN also impart a yellowish tint to the agar making it slightly more difficult to observe fungal hyphae within the plate. Fungal isolation medium or FIM [66], however, has a lower carbohydrate load (2.5 g sucrose/L), and results in tint-free media. Yokoya et al. [22] used FIM to successfully isolate a broad spectrum of endophytes from epiphytic, terrestrial, and lithophytic orchids in Madagascar, and this media appears to be gaining in popularity elsewhere (e.g., [17, 57, 74]).

To reduce bacterial contamination making it easier to isolate emerging fungal hyphae in pure culture, many researchers add antibiotics to media, either before or after autoclaving depending on the antibiotic chosen. Among these include streptomycin sulfate [75], 100 mg/L tetracycline [20], 50 μg/mL oxytetracycline [46]; 50 mg/L novobiocin sodium salt [67], 50 mg/L chloromycetin [62], and 50 μg/mL penicillin [63], with streptomycin being frequently reported (e.g., [22, 38]). Some researchers have had success without using antibiotics (e.g., [33, 42, 57, 58, 65, 71, 76]), and this may, in fact, be preferable given that some bacteria may actually benefit the orchid mycorrhizal association [36]. Moreover, Currah et al. [20] reported that some orchid endophytes (e.g., *Moniliopsis* spp.) are vulnerable to tetracycline and will die in culture unless quickly subcultured to media lacking antibiotics. New research is also being carried out involving endosymbiotic bacteria within orchid mycorrhizal fungi and how they may affect orchid seed germination (M. McCormick, pers. com.). If these studies confirm that endosymbiotic bacteria impart a benefit to orchid seed germination and/or endophyte longevity in storage, the use of antibiotics in fungal isolation procedures will need to be re-evaluated.

3.6 Subculturing and Storage

After plating on agar, pelotons will normally yield actively growing hyphae 24–48 h after incubation at ambient temperature with or without dark incubation. These hyphae can be best viewed using a binocular dissection microscope with a glass stage allowing for a light source to be beamed upward from below into the agar. With gentle manipulation of the light source, the contrast of

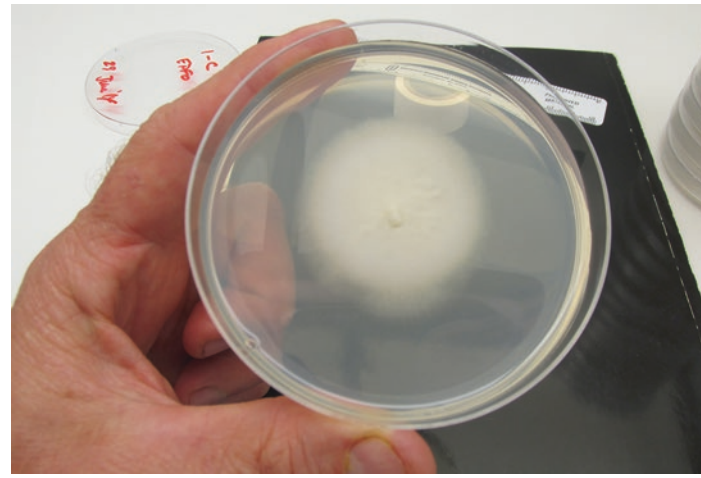


Fig. 14 A newly isolated endophyte (*Tulasnella* spp.) in pure culture acquired from a hyphal tip from a peloton previously suspended in FIM

hyphae will appear to "glow" against the backdrop of the agar making it possible to distinguish single hyphal tips of pelotons from other contaminating fungi. This time frame also permits bacterial colonies to grow and become visible, usually appearing as "disks" suspended in the agar. The use of a sterile scalpel with a narrow pointed end is generally sufficient for excising single hyphal tips and is also useful for cutting around and between potential contaminants. Before excising a hyphal tip of interest, it is a good idea to view the entire depth of the agar from the surface all the way to the bottom of the plate, as some contaminants will remain hidden from view when out of focus. Once the tip is excised, it should be placed onto the surface of agar in a new plate that lacks antibiotics, with potato dextrose agar (PDA) serving this purpose well [20]. PDA is not only a good medium for culturing a wide range of fungi, it also permits fungal colonies to grow and differentiate within and on the surface of the agar in a manner that allows for provisional identification of mycorrhizal candidates. Many different strains of *Rhizoctonia*-like fungi yield lighter-colored mycelia on the surface of agar soon after they are subcultured (Fig. 14), and this characteristic feature is often helpful for initial screening purposes.

Once an orchid endophyte has been isolated in pure culture and selected for further work (e.g., symbiotic germination trials, molecular identification), keeping the culture alive and free of lab contaminants is crucial. Orchid endophytes can be maintained on standard agar-based media for a number of years at ambient temperature and in refrigeration (4 °C) through repeated subculturing. In this respect, orchid endophytes are "user friendly" compared to mycorrhizal fungi associated with non-orchids. However, there

is prevailing concern that some orchid endophytes lose their mycorrhizal capacity (e.g., ability to germinate seeds in vitro) when maintained in the laboratory over a period of years. Alexander and Hadley [77], for example, reported a decline in mycorrhizal capacity for cultures stored at 4 °C on both malt agar and PDA after 2 years, with subculturing at 2 month intervals. Moreover, Currah et al. [20] warned that fungi from tropical orchids do not survive well in cold storage, and advocated that duplicates be maintained at ambient temperature as a back-up. Some researchers have opted to safeguard important *Rhizoctonia*-like strains in culture collections (e.g., UAMH in Canada) through cryopreservation, but it remains unknown if this procedure is effective at conserving mycorrhizal capacity. At least one mycorrhizal fungus (*Tulasnella calospora* 266, UAMH 9824) with a well-established track record for germinating orchid seeds in vitro spanning a number of taxa [78] retained its effectiveness after several years of cryopreservation in Canada (L. Zettler, unpub. Data), but more studies of this kind are warranted. Strains of *Ceratobasidium* may be more problematic to work with considering that they have a shorter life expectancy in cool (4 °C) storage compared to strains of *Tulasnella* [79]. Moreover, some *Ceratobasidium* strains at UAMH have proved difficult to revive from cryopreservation compared to other orchid endophytes (L. Sigler, pers. com.). Resolving the issues related to viability and mycorrhizal capacity of orchid mycorrhizal fungi will be crucial in this age of extinction.

3.7 Summary

There are now several effective techniques available for the successful isolation of peloton-forming fungi from orchid roots and protocorms spanning the globe, but maintaining these fungi in viable condition remains a formidable challenge. For the sake of orchid conservation as a whole, it is the duty of each lab and research team to devise new, more effective ways to isolate and safeguard orchid endophytes, and to share this information in publications, workshops, and collaborations. The recent (2012–2016) collaboration between the Royal Botanic Gardens, Kew, and Illinois College to recover orchid endophytes in Madagascar [80] nicely illustrates this concept. For example, upon three separate collecting trips in Madagascar, both teams split root samples and protocorms and proceeded to isolate orchid endophytes separately in their respective labs, using slightly different techniques. This joint effort resulted in the recovery of a wide range of fungi that were collectively assembled, many of which were isolated in one lab but not the other. Some of these isolates have been shown to be highly effective at facilitating seed germination in vitro [19], and are now stored in cryopreservation.

In the 26 years that the senior author has been isolating orchid endophytes from protocorms, seedlings, and mature plants of temperate terrestrials and tropical epiphytes alike, there

is no single technique that has been effective for any single orchid or sample. Roots of the rare, North American terrestrial *Isotria medeoloides*, for example, have failed to yield orchid endophytes despite many attempts. However, the most effective technique—so far—has been to add a 0.5–1.0 cm root piece to a 5 mL drop of sterile DI water, maceration by scalpel, and immersion in 20 mL fungal isolation medium (FIM) with streptomycin sulfate. This technique has worked for terrestrials as well as tropical epiphytes without the need to remove the epidermis and/or velamen layer after surface sterilization. As crude as this technique may be, it has been generally reliable at recovering a wide range of fungi in the *Rhizoctonia* complex.

4 Fungal Identification

4.1 Background

The first record of a mycorrhizal fungus in an orchid dates back to 1824 from observations made by the German naturalist, Heinrich Link, but the role of the fungus remained ambiguous until the early 1900s after Nöel Bernard experimentally demonstrated a connection between filamentous fungi and seed germination [81]. Attempts to identify these fungi were then carried out mostly through the use of light microscopy. Over the years, it soon became clear that the majority of orchid endophytes were assignable to the club fungi (basidiomycetes), in particular members of the *Rhizoctonia* complex, with few exceptions [82, 83]. This complex consists of a heterogeneous assemblage of mostly root-dwelling saprophytes or parasites that share similar morphological traits, namely (1) white/cream to chestnut brown colony color, (2) hyphal branching at right angles, (3) a constriction of the hyphal branch at the point of origin, with a septum close to the main hypha, and (4) shorter hyphae that frequently branch forming monilioid cells that may develop into dense aggregations (sclerotia) over time [15, 20, 84, 85]. For many years, identifying fungi within the *Rhizoctonia* complex was problematic because only rarely do isolates in pure culture yield reproductive mycelia (teleomorphic stages). As a result, specialists resorted to using several cumbersome techniques including cytomorphological characterization [85], enzymatic assays [86], observing nuclear number in young hyphae assisted by DAPI staining [85], characterization of septal ultrastructure by transmission electron microscopy, and inducing hyphae to fuse leading to anastomosis groupings [85]. With these characters, traditional mycological classification could be executed with more confidence. Moore [87] went so far as to split the *Rhizoctonia* complex into new anamorphic genera based on conservative cytological and ultrastructural characteristics [20]. For example, cultures bearing binucleate hyphae with perforate parenthesomes, and teleomorphs assignable *Ceratobasidium* where

placed into the genus *Ceratorhiza*. Binucleate strains with imperforate parenthesomes and teleomorphs in *Tulasnella* or *Sebacina* were classified as *Epulorhiza* [20]. Multinucleate strains having perforate parenthesomes, and teleomorphs assignable to *Thanatephorus* or *Waitea* were reclassified as *Moniliopsis*. Moore's system soon led to the naming of new anamorphic species (e.g., *Epulorhiza epiphytica*; [72]), augmenting the well-established teleomorphic nomenclature. Designating two scientific names for one fungus, however, imparted an element of confusion that persists to this day. The use of anamorphic classification has since been abandoned in favor of the teleomorphic binomial—a practice that coincided with advances in molecular biology that revolutionized fungal identification.

4.2 Modern Approaches to Identification

During the past 20 years, the modern molecular techniques that have greatly facilitated fungal identification, both internal and external to the *Rhizoctonia* complex, have included PCR, RAPDs, and RFLPs acquired from rDNA. Sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA is currently the preferred method for *Rhizoctonia*-like fungi [88]. As a direct result, we now know that this fungus complex encompasses distinct clades of orchid mycobionts that are genetically divergent from one another [89]. We also know that many specific strains within these clades are worldwide in distribution. Molecular techniques have also benefitted conservation efforts by providing a means for determining if some orchid species might be common or rare by analyzing their fungal diversity. Orchids that utilize a broad spectrum of fungi, for example, have been termed "generalists," whereas those that target specific groups of fungi are known as specialists [16].

4.3 Geographical Patterns of Rhizoctonia-Like Fungi

Most of what we know about the identity of orchid endophytes has been based on fungi isolated from mature terrestrial orchids inhabiting temperate regions (Europe, Australia, North America). During the past decade, a surge in new information has surfaced from tropical regions (e.g., Ecuador, Thailand, China, Madagascar) describing fungi from epiphytic orchids, as well as early seedling stages, augmenting the earlier literature (*see* [7]). Collectively, orchids as a group appear to associate primarily with *Rhizoctonia*-like fungi in two orders—the Cantharellales and Sebacinales [90, 91], and within these orders members of the Ceratobasidiaceae/Tulasnellaceae, and Sebacinaceae, respectively. Moreover, young and mature orchids alike harbor *Rhizoctonia*-like fungi, but the pattern of diversity within this fungal complex may change over time as the orchid matures, depending on the species [7] and/or the individual plant. In other words, some orchids appear to retain their germination mycobionts in a lifelong association, whereas others may "switch" or replace their mycoflora with a more diverse assemblage as they mature.

Among the *Rhizoctonia*-like fungi, the three genera isolated with regularity worldwide have been *Ceratobasidium*, *Tulasnella*,

and *Sebacina*. Of these, the genus *Tulasnella* is probably the most frequently reported endophyte genus recovered from Orchidaceae worldwide, with *T. calospora* (Bourdier) Juel (=*Epulorhiza repens*) being the most prevalent, occurring among roots and seedlings alike [15], and from terrestrials and epiphytes alike [7]. *Ceratobasidium* is also ubiquitous and seems to be an especially common associate in certain groups of orchids and in certain regions. For example, both prairie fringed orchids in North America (*Platanthera leucophaea* and *P. praeclara*) appear to rely primarily on *Ceratobasidium* fungi to complete their life cycles [31, 92]. In south Florida, *Ceratobasidum* appears to be closely tied to the Ghost Orchid, *Dendrophylax lindenii* [17]. Members of the Ceratobasidiaceae have also been shown to facilitate germination in a number of orchid subfamilies, in particular the Apostasioideae, Vanilloideae, Epidendroideae, Orchidoideae [7, 93], and Vandeae [6, 94]. In the Brazilian Atlantic rain forest, both *Ceratobasidium* (=*Ceratorhiza*) and *Tulasnella* (=*Epulorhiza*) were documented from seven neotropical species by Pereira et al. [58]. In the Andes Mountains of Ecuador, however, *Tulasnella* seems to be most prevalent in cloud forest epiphytes based on studies by Suarez et al. [42]. Although the genus *Sebacina* has—so far—been isolated less frequently, it too is a known orchid mycorrhizal associate. For example, one particular strain of *Sebacina* isolated from a *Polystachya concreta* seedling in Madagascar was highly effective at inducing rapid in vitro seedling development of the terrestrial, *Cynorkis purpurea*, outperforming the *Tulasnella* and *Ceratobasidium* isolates tested [19].

Among nonphotosynthetic orchids, other types of basidiomycetes are also involved including *Russula* spp., *Thelephora* spp., and *Sebacina* spp., many of which connect to woody plants in ectomycorrhizal associations [95]. Ascomycetes and imperfect fungi (e.g., molds) are also known to associate with orchids, but their physiological role(s) have yet to be determined [20]. For example, anamorphs assignable to *Fusarium*—a large and variable genus parasitic on higher plants and/or saprophytic [96]—have been isolated from pelotons with regularity from epiphytic orchids in Madagascar as well as Florida (L. Zettler, pers. obser.), but its physiological role remains unknown. As interesting as this new information may be, we still know relatively little about the whereabouts of the *Rhizoctonia*-like fungi with respect to orchids and their global distribution, and even less about other types of fungi that appear to be connected to the association.

4.4 Provisional Identification of Rhizoctonia-Like Fungi: A Practical First Step

The modern-day specialist seeking to identify orchid endophytes in the *Rhizoctonia* complex now has access to powerful new techniques that may be applied to pure cultures as well as orchid tissues (roots, protocorms). For some workers, provisional identification alone may be the only practical alternative until cultures can be screened for their symbiotic potential and identified further by

molecular means. Moreover, the use of molecular techniques alone does not provide sufficient insight into what physiological role(s) these fungi may play unless specific strains are isolated in pure culture and tested for germination efficiency [97]. Accordingly, this section has been written as a practical first step toward identifying typical *Rhizoctonia*-like fungi in pure culture for multitude of purposes. Although we choose to focus on this fungal complex because its members are ubiquitous and represent a crucial biotic component of the orchid life cycle, we would like to emphasize that other groups of fungi (e.g., ascomycetes, molds) may also serve important roles, and should not be discounted in the broader context. Currah et al. [20] provide a detailed synopsis of the fungal diversity associated with orchids, including perfect stages (basidia, basidiospores) of the *Rhizoctonia* complex, as well as ascomycetes and many different types (genera) of imperfect fungi (e.g., molds) associated with the roots and mycorrhizas of orchids. Though somewhat dated, their fungal descriptions, and their procedures for provisional identification, remain very useful.

4.5 Cultural Characteristics of the Cantharellales and Sebacinales

Members of both basidiomycete orders are easily isolated from orchid tissues. Hyphal tips excised from actively growing pelotons on FIM (discussed previously) are typically subcultured to 9 cm diameter Petri dishes containing a sugar-rich medium such as potato dextrose agar or PDA. This medium affords the fungus with sufficient nutrients to then differentiate into a colony that yields morphological characteristics easily distinguished with the unaided eye, namely color and texture. Within a week of growth at ambient temperature, some cultures will also begin to produce morphological structures that can be viewed under light microscopy (e.g., sclerotia, monilioid cells), further aiding in provisional identification. Currah et al. [20] categorize orchid endophytes into three main groups at this early stage consisting of colonies that are (1) black to olive; (2) white, cream, or tan, and (3) brightly colored, i.e., red, green, pink, or orange. Isolates that are lighter in color and are tinted yellow, tan, or chestnut brown are often basidiomycetes (e.g., *Rhizoctonia*-like fungi) especially if they lack spores (e.g., conidia). Below is a general summary of the four major groups of *Rhizoctonia*-like fungi isolated from orchids following descriptions by Currah et al. [20] unless otherwise noted.

4.5.1 Order Cantharellales

Family Tulasnellaceae

Genus *Tulasnella* [anamorphs formerly *Epulorhiza*]

Colonies typically white, cream colored, or slightly yellowish on PDA (Fig. 15), with mostly surface or submerged mycelium and a uniform, sometimes waxy texture. Growth rates generally slow (<0.1 mm/h) at 25 °C on PDA. Hyphae are narrow (2–4 μm broad), binucleate, and septa with imperforate parenthesomes.

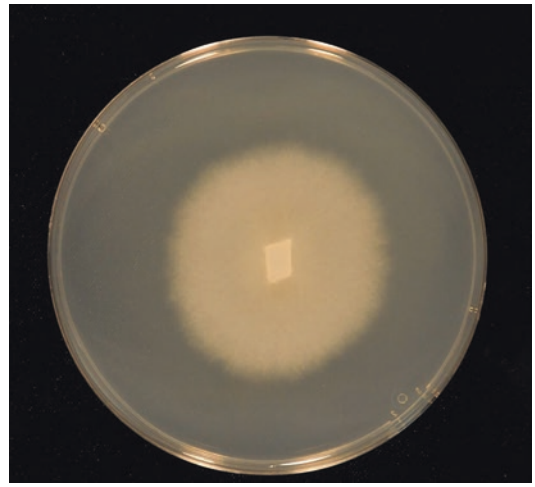


Fig. 15 *Tulasnella calospora* growing within a 9 cm diameter petri plate on potato dextrose agar (PDA) <30 days after it was subcultured. The colony's smooth surface, coupled with the light (creamy) coloration is often indicative of the genus. This strain originated from the roots of the terrestrial orchid, *Spiranthes brevilabris*, from Florida. Photo courtesy of Dr. Mike Kane, University of Florida

Monilioid cells elongate, elliptical or spherical (9–18 μm), often in short branched, or unbranched chains, occasionally developing into loose aggregates of sclerotia. Hyphae typically lack clamp connections, and perfect states rarely observed. Consistently negative for polyphenol oxidase [86].

This genus is largely regarded as the universal mycorrhizal associate of Orchidaceae worldwide, with numerous reports in the literature (*see* [7]). When orchid seeds are inoculated with members of this genus in vitro on oat-based media, rarely does the mycelium overgrow seeds unlike many members of Ceratobasidiaceae. Pure cultures are easily maintained at ambient temperature and 4 °C with regular subculturing, and most strains remain viable after cryopreservation. *Tulasnella calospora* (Bourdier) Juel (=*Epulorhiza repens*) is widely regarded as the most common mycorrhizal associate of photosynthetic orchids. One strain of *T. calospora* (UAMH 9824), for example, is known to germinate a wide range of orchid taxa in vitro [78].

Family Ceratobasidiaceae

Genus *Ceratobasidium* [anamorphs formerly *Ceratorhiza*]

Colonies cream colored to yellowish orange (Fig. 16), rapid growth rates (0.2–0.5 mm/h on PDA at 25 °C), and abundant aerial mycelia, sometimes exhibiting concentric rings or zones. Hyphae broad (4–7 μm), branching at wide angles, binucleate, and septa with perforate parenthesomes. Monilioid cells barrel-shaped, broadly attached, large (15–25 × 7–11 μm), forming

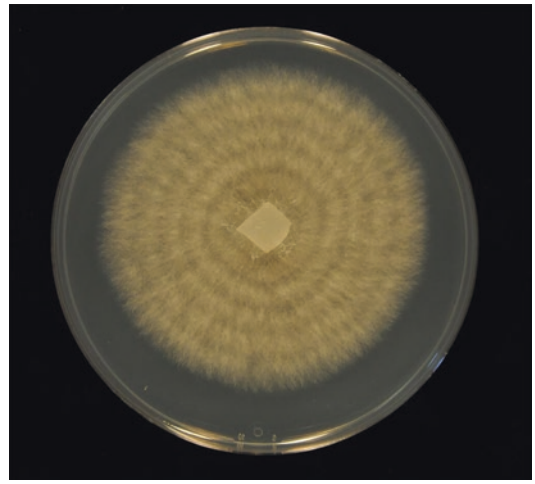


Fig. 16 *Ceratobasidium* sp. growing within a 9 cm diameter petri plate on potato dextrose agar (PDA) 7 days after it was subcultured. The colony's ragged surface, abundant aerial mycelium, and cream-tan coloration are typical of the genus. Many strains of *Ceratobasidium* also yield concentric growth rings evident in this image. This strain was isolated from the roots of the rare Ghost Orchid, *Dendrophylax lindenii*, from south Florida. Photo courtesy of Dr. Mike Kane, University of Florida

abundant sclerotia giving colonies a granular appearance. Basidia known to occasionally form on vegetative hyphae. Generally test positive for polyphenol oxidase [86].

This genus is well known for species that are pathogens of turf grasses and cereal crops [98, 99]. Yukawa et al. [100] proposed that fungi in the Ceratobasidiaceae are linked to orchids in the Vandae tribe, and especially in the Angraecinae subtribe (e.g., *D. lindenii*). This hypothesis is supported by Chomicki et al. [101] and Hoang et al. [17]. *Ceratobasidium cornigerum* (=*Ceratorhiza goodyera-repentis*)—regarded as the most common species isolated from terrestrials [20]. Many strains of *Ceratobasidium* known to become "unruly" (i.e., overgrow seeds) when used in symbiotic germination experiments, even on media low in simple sugars.

Family Ceratobasidiaceae

Genus *Thanatephorus*

Colonies tan to brownish, with dense mycelium, both aerial and submerged on agar surface. Mycelium may be highly variable among strains. Rapid growth rates (0.2–0.6 mm/h on PDA at 25 °C). Hyphae broad (8–11 μm), multinucleate with perforate parenthesomes and prominent dolipore septa. Cultures often produce prolific number of sizable (25–30 μm) monilioid cells, generally globose and in dichotomously branched chains. Forms numerous sclerotia, often coalescing into a crust-like texture on aged (>30 days old) cultures. May yield perfect states (basidia/

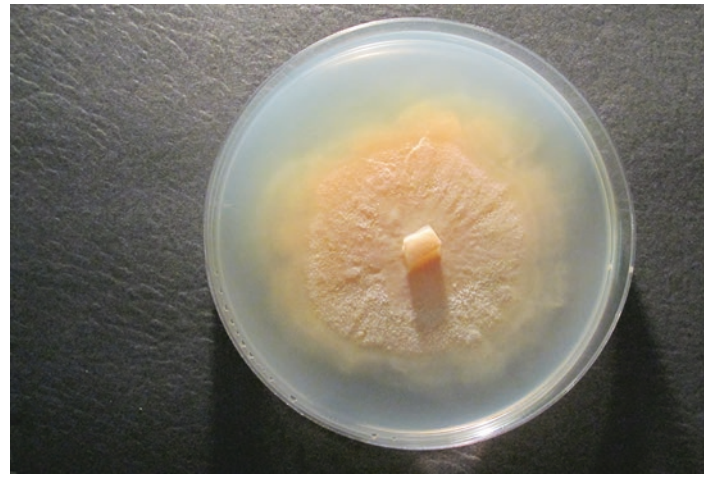


Fig. 17 *Sebacina* sp. growing within a 9 cm diameter petri plate on potato dextrose agar (PDA) >30 days after it was subcultured. The colony's submerged mycelium, coupled with creamy orange coloration, is evident in this image. This strain originated from roots of an epiphytic orchid in Madagascar

basidiospores) under laboratory conditions with culture manipulation in well-known studies by Warcup and Talbot [68, 102–104].

This genus includes the well-known plant pathogen, *Thanatephorus cucumeris* [=*Moniliopsis/Rhizoctonia solani*], occasionally isolated from orchid roots (e.g., [61]). Known orchid mycorrhizal associate under natural and artificial (lab) conditions.

4.5.2 Order Sebacinales

Family Sebacinaceae

Genus *Sebacina*

Members of this genus are generally slow-growing (<0.1 mm/h on PDA at 25 C) and have dense, mostly surface mycelium. Colonies often cream-colored initially, then turning orange with age (>30 days; Fig. 17). Hyphae hyaline, thin-walled, and narrow (<5 μm wide). Monilioid cells are spherical or subglobose, thin-walled and hyaline, usually <15 μm in diameter, often forming closely knit groups or clusters. Anamorphs once placed in *Epulorhiza* [20].

A diverse, cosmopolitan genus known for forming ectomycorrhizal associations with non-orchids including temperate deciduous trees [105], and members of the Ericaceae [106], as well as endomycorrhizal associates of orchids [22, 76, 107], including achlorophyllous taxa [13]. As a group, members of this order are known to be present in a variety of habitats [13]. Rafter et al. [19] demonstrated that this genus has the potential to facilitate successful associations with orchids spanning different life forms in Madagascar. Strains tentatively identified as *Sebacina* have been isolated with regularity from epiphytic orchids in south Florida, including *Encyclia tampensis* and *Prosthechea cochleata* (L. Zettler, unpub. data).

4.6 Molecular Identification of Orchid Mycorrhizal Fungi

The current standard procedure for molecular identification of mycorrhizal symbionts is polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA [108], followed by DNA sequencing using the Sanger method. The resulting sequences can be compared against known sequences using the Basic Local Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (NCBI) web portal [109].

Molecular identification of orchid mycorrhizal fungi generally requires isolation of DNA from agar plates or liquid culture, rather than directly from orchid roots. DNA isolation can be carried out by one of several methods; the most common are the cetyltrimethylammonium bromide (CTAB) method [110], or a commercial kit such as the Omega Fungal E.Z.N.A (Omega Bio-tek, Norcross, GA) or Qiagen DNeasy (Qiagen, Valencia, CA). While CTAB results in DNA of higher purity, the commercial kits are rapid and convenient, do not produce hazardous waste, and produce DNA of sufficient quality for most PCR protocols to be successful.

Following isolation, the fungal DNA is amplified using the PCR with primers specific to the ribosomal DNA internal transcribed spacer region. The internal transcribed spacer region includes the 5.8S ribosomal RNA (rRNA) gene as well as two transcribed spacers between the 18S rRNA gene and the 5.8S rRNA gene, and the 5.8S rRNA gene and the 28S rRNA gene. These spacers are transcribed from the ribosomal DNA, but are then removed from the ribosomal RNAs during maturation. Because the spacers are not used in the final ribosomal structure, they are not subject to strong selection against mutation. In contrast, the ribosomal RNA sequences themselves are subject to very strong selection since there is no possibility of silent mutation (in contrast to a protein-coding region). The ITS region is particularly useful for fungal identification as bacteria lack a 5.8S rRNA gene. Although most fungal isolation procedures involve sterilization of the roots prior to culture, bacterial contamination could be a confounding factor. Additionally, endobacteria are not removed by surface sterilization. Therefore, the use of a eukaryotic-specific region simplifies the identification of mycorrhizal fungi.

The original ITS primers have been improved and optimized for detection of mycorrhizae from particular clades such as *Tulasnella* [111] and *Ceratobasidium* [63]. Based on morphology and growth habit in culture, researchers can select the most likely primer pairs or begin with nonspecific primer pairs and work toward more specialized primers.

Looking ahead, next-generation sequencing holds much promise for the elucidation of entire fungal and orchid genomes. Similarly, procedures such as RNA-seq and other methods of transcriptome analysis can be used to explore the changes in gene regulatory networks prompted by the symbiotic relationship. However, these technologies are currently out of reach for most and remain impractical for routine identification of mycorrhizal isolates.

Acknowledgments

We kindly thank Michael E. Kane (University of Florida) for providing images of *Tulasnella* and *Ceratobasidium* in culture. The long-standing technical support of Andrew L. Stice (Illinois College) is much appreciated. We also thank our collaborators at the Royal Botanic Gardens, Kew (Viswambharan Sarasan, Kazutomo Yokoya, Jonathan Kendon), for the opportunity to collect samples in Madagascar that significantly enhanced this chapter. This chapter would not have been written without the expertise and guidance provided by Hanne N. Rasmussen (University of Copenhagen) for which we are grateful.

References

1. Dressler RL (2005) How many orchid species? Selbyana 26:155–158
2. Scotland RW, Wortley AH (2003) How many species of seed plants are there? Taxon 52:101–104
3. Stokstad E (2015) Orchid's dazzling diversity explained. Science 349:914
4. Cribb P (2011) Orchids in a changing climate. Lankesteriana 11:233–238
5. Waterman RJ, Bidartondo MI (2008) Deception above, deception below: linking pollination and mycorrhizal biology of orchids. J Exp Biol 59:1085–1096
6. Rasmussen HN, Rasmussen FN (2014) Seedling mycorrhiza: a discussion of origin and evolution of Orchidaceae. Bot J Linn Soc 175:313–327
7. Rasmussen HN, Dixon KW, Jersáková J, Těšitelová T (2015) Germination and seedling establishment in orchids: a complex of requirements. Ann Bot 116:391–402
8. Peterson RL, Massicotte HB, Melville LH (2004) Mycorrhizas: anatomy and cell biology. NRC Research Press, Ottawa
9. Yoder JA, Zettler LW, Stewart SL (2000) Water requirements of terrestrial and epiphytic orchid seeds and seedlings, and evidence for water update by means of mycotrophy. Plant Sci 156:145–150
10. McCormick MK, Whigham DF, Sloan D, O'Malley K, Hodkinson B (2006) Orchid-fungus fidelity: a marriage meant to last? Ecology 87:903–911
11. Girlanda M, Segreto R, Cafasso D, Liebel HT, Rodda M, Ercole E, Cozzolini S, Gebauer G, Perotto S (2011) Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations. Am J Bot 98:1148–1163
12. Zettler LW, Hofer CJ (1998) Propagation of the little club-spur orchid [*Platanthera clavellata*] by symbiotic seed germination, and its ecological implications. Environ Exp Bot 39:189–195
13. Weiß M, Sýkorová Z, Garnica S (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. PLoS One 6:e16793
14. Veldre V, Abarenkov K, Bahram M et al (2013) Evolution of nutritional modes of Ceratobasidiaceae (Cantharellales, Basidiomycota) as revealed from publicly available ITS sequences. Fungal Ecol 6:256–268
15. Rasmussen HN (1995) Terrestrial orchids from seed to mycotrophic plant. Cambridge University Press, Cambridge
16. Swarts ND, Dixon KW (2009) Terrestrial orchid conservation in the age of extinction. Ann Bot 104:543–556
17. Hoang NH, Kane ME, Radcliffe EN, Zettler LW, Richardson LW (2017) Comparative seed germination and seedling development of the Ghost Orchid, *Dendrophylax lindenii* (Orchidaceae), and molecular identification of its mycorrhizal fungus from south Florida. Ann Bot 119:379–393
18. Zettler LW, Poulter SB, McDonald KI, Stewart SL (2007) Conservation-driven propagation of an epiphytic orchid (*Epidendrum nocturnum*) with a mycorrhizal fungus. HortSci 42:135–139
19. Rafter M, Yokoya K, Schofield EJ, Zettler LW, Sarasan V (2016) Non-specific symbiotic germination of *Cynorkis purpurea* (Thouars) Kraezl., a habitat-specific terrestrial orchid from the Central Highlands of Madagascar. Mycorrhiza 26:541–552

20. Currah RS, Zelmer CD, Hambleton S, Richardson KA (1997) Fungi from orchid mycorrhizas. In: Arditti J, Pridgeon AM (eds) Orchid biology: reviews and perspectives, vol VII. Springer, Dordrecht, pp 117–170
21. Rasmussen HN, Whigham DF (1998) Importance of woody debris in seed germination of *Tipularia discolor* (Orchidaceae). Am J Bot 85:829–834
22. Yokoya K, Zettler LW, Kendon JP, Bidartondo M, Stice AL, Skarha S, Corey LL, Knight A, Sarasan V (2015) Preliminary findings on identification of mycorrhizal fungi from diverse orchids in the Central Highlands of Madagascar. Mycorrhiza 25:611–625
23. Rasmussen HN, Whigham DF (1993) Seed ecology of dust seeds *in situ*: a new study technique and its application in terrestrial orchids. Am J Bot 80:1374–1378
24. Zettler LW, Wood EM, Johnson LJAN, Kirk AK, Perlman SP (2011) Seed propagation and re-introduction of the U.S. Federally endangered Hawaiian endemic, *Platanthera holochila* [Hbd.] Krzl. (Orchidaceae). Eur J Environ Sci 1:80–94
25. Rasmussen HN, Pedersen HÆ (2011) *Cypripedium calceolus* germination *in situ*: seed longevity, and dormancy breakage by long incubation and cold winters. Eur J Environ Sci 1:69–70
26. Masuhara G, Katsuya K (1994) *In situ* and *in vitro* specificity between *Rhizoctonia* app. and *Spiranthes sinensis* (Persoon) Ames var. *amoena* (M. Bieberstein) Hara (Orchidaceae). New Phytol 127:711–718
27. Van der Kinderen G (1995) A method for the study of field germinated seeds of terrestrial orchids. Lindleyana 10:68–73
28. Zelmer CD, Cuthbertson L, Currah RS (1996) Fungi associated with terrestrial orchid mycorrhizas, seeds and protocorms. Mycoscience 37:439–446
29. Batty AL, Dixon KW, Brundrett M, Sivasithamparam K (2001) Constraints to symbiotic germination of terrestrial orchid seed in a Mediterranean bushland. New Phytol 152:511–520
30. Chen J, Wang H, Guo SX (2012) Isolation and identification of endophytic and mycorrhizal fungi from seeds and roots of *Dendrobium* (Orchidaceae). Mycorrhiza 22:297–307
31. Zettler LW, Piskin KA (2011) Mycorrhizal fungi from protocorms, seedlings, and mature plants of the eastern prairie fringed orchid, *Platanthera leucophaea*: a comprehensive list to augment conservation. Am Midl Nat 166:29–39
32. Zettler LW, Corey LL, Ross AY, Moller-Jacobs L (2011) Protocorms of an epiphytic orchid (*Epidendrum amphistomum* A. Richard) recovered *in situ*, and subsequent identification of associated mycorrhizal fungi using molecular markers. Eur J Environ Sci 1:108–114
33. Stewart SL, Zettler LW, Minso J, Brown PM (2003) Symbiotic germination and reintroduction of *Spiranthes brevilabris* Lindley, and endangered orchid native to Florida. Selbyana 24:64–70
34. Nomura N, Ogura-Tsuita Y, Gale SW, Maeda A, Umata H, Hosaka K, Yukawa T (2013) The rare terrestrial orchid *Nervilia nipponica* consistently associates with a single group of novel mycobionts. J Plant Res 126:613–623
35. Anderson AB (1991) Symbiotic and asymbiotic germination and growth of *Spiranthes magnicamporum* (Orchidaceae). Lindleyana 6:183–186
36. Ramsey MM, Dixon KW (2003) Propagation science, recovery and translocation of terrestrial orchids. In: Dixon KW, Barrett RL, Cribb PJ (eds) Orchid conservation. Natural History Publications [Borneo], Kota Kinabalu, Sabah, pp 259–288
37. Zettler LW (1997) Terrestrial orchid conservation by symbiotic seed germination: techniques and perspectives. Selbyana 18:188–194
38. Zhu GS, Yu ZN, Gui Y, Liu ZY (2008) A novel technique for isolating orchid mycorrhizal fungi. Fungal Divers 33:123–137
39. Otero JT, Thrall PH, Clements M, Burdon JJ, Miller JY (2011) Codiversification of orchids (Pterostylidinae) and their associated mycorrhizal fungi. Aust J Bot 59:480–497
40. Otero JT, Ackerman JD, Bayman P (2004) Differences in mycorrhizal preferences between two tropical orchids. Mol Ecol 13:2393–2404
41. Benzing DH (1982) Mycorrhizal infections of epiphytic orchids in southern Florida. Am Orchid Soc Bull 51:618–622
42. Suárez JP, Weiß M, Abele A, Garnica S, Oberwinkler F, Kottke I (2006) Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in the Andean cloud forest. Mycol Res 110:1257–1270
43. Radcliffe EN, Bishop KM, Corey LL, Zettler LW (2015) Mycorrhizal fungi from mature epiphytic orchids and seedlings native to south Florida, and a technique for pinpointing pelotons in roots. Trans Illinois State Academy Sci [107th Meeting], Supplement to Volume 108, 49
44. McCormick MK, Whigham DF, O'Neill J (2004) Mycorrhizal diversity in photosynthetic terrestrial orchids. New Phytol 163:425–438
45. Jacquemyn H, Merckx V, Brys R, Tyteca D, BPA C, Honnay O, Lievens B (2011) Analysis

of a network architecture reveals phylogenetic constraints on mycorrhizal specificity in the genus *Orchis* [Orchidaceae]. New Phytol 192:518–528
46. Nontachaiyapoom S, Sasirat S, Manoch L (2010) Isolation and identification of *Rhizoctonia*-like fungi from roots of three orchid genera, *Paphiopedilum*, *Dendrobium*, and *Cymbidium*, collected in Chiang Rai and Chiang Mai provinces of Thailand. Mycorrhiza 20:459–471
47. Burgeff H (1909) Die Wurzelpilze der Orchideen, ihre Kultur und ihre Leben in der Pflanze. Gustav Fischer, Jena
48. Currah RS, Sigler L, Hambleton S (1987) New records and new taxa of fungi from the mycorrhizae of terrestrial orchids of Alberta. Can J Bot 65:2473–2482
49. Currah RS, Smreciu EA, Hambleton S (1990) Mycorrhizae and mycorrhizal fungi of boreal species of *Platanthera* and *Coeloglossum* (Orchidaceae). Can J Bot 68:1171–1181
50. Hossain MM, Kant R, Van PT, Winarto B, Zeng S, Teixeira da Silva JA (2013) The application of biotechnology to orchids. Crit Rev Plant Sci 32:69–139
51. Matsuda Y, Amiya A, Ito S (2009) Colonization patterns of mycorrhizal fungi associated with two rare orchids, *Cephalanthera falcata* and *C. erecta*. Ecol Res 24:1023–1031
52. Harvais G (1974) Notes on the biology of some native orchids of Thunder Bay, their endophytes and symbionts. Can J Bot 52:451–460
53. Nieuwdorp PJ (1972) Some observations with light and electron microscope on the endotrophic mycorrhiza of orchids. Acta Bot Neerl 21:128–144
54. Smith SE (1966) Physiology and ecology of orchid mycorrhizal fungi with reference to seedling nutrition. New Phytol 65:488–499
55. Harvais G, Hadley G (1967) The relation between host and endophyte in orchid mycorrhiza. New Phytol 66:205–215
56. Mahendran G, Muniappan V, Ashwini M, Muthukumar T, Narmatha Bai V (2013) Asymbiotic seed germination of *Cymbidium bicolor* Lindl. [Orchidaceae] and the influence of mycorrhizal fungus on seedling development. Acta Physiol Plant 35:829–840
57. Aggarwal S, Nirmala C, Beri S, Rastogi S, Adholeya A (2012) *In vitro* symbiotic seed germination and molecular characterization of associated endophytic fungi in a commercially important and endangered Indian orchid *Vanda coerulea* Griff. ex Lindl. Euro. J Environ Sci 2:33–42
58. Pereira OL, Kasuya MCM, Borges AC, de Araújo EF (2005) Morphological and molecular characterization of mycorrhizal fungi isolated from neotropical orchids in Brazil. Can J Bot 83:54–65
59. Otero JT, Flanagan NS, Herre EA, Ackerman JD, Bayman P (2007) Widespread mycorrhizal specificity correlates to mycorrhizal function in the neotropical, epiphytic orchid *Ionopsis utricularioides* (Orchidaceae). Am J Bot 94:1944–1950
60. Curtis JT (1939) The relation of specificity of orchid mycorrhizal fungi to the problem of symbiosis. Am J Bot 26:390–399
61. Richardson KA, Currah RS, Hambleton S (1993) Basidiomycetous endophytes from the roots of neotropical epiphytic Orchidaceae. Lindleyana 8:127–137
62. Yuan Z, Chen Y, Yang Y (2009) Diverse non-mycorrhizal fungal endophytes inhabiting an epiphytic, medicinal orchid (*Dendrobium nobile*): estimation and characterization. World J Microbiol Biotechnol 25:295–303
63. Porras-Alfaro A, Bayman P (2007) Mycorrhizal fungi of *Vanilla*: diversity, specificity and effects on seed germination and plant growth. Mycologia 99:510–525
64. Keel BG, Zettler LW, Kaplan BA (2011) Seed germination of *Habenaria repens* (Orchidaceae) *in situ* beyond its range, and its potential for assisted migration imposed by climate change. Castanea 76:43–54
65. Zi XM, Sheng CL, Goodale UM, Shao SC, Gao JY (2014) *In situ* seed baiting to isolate germination-enhancing fungi from an epiphytic orchid, *Dendrobium aphyllum* (Orchidaceae). Mycorrhiza 24:487–499
66. Clements MA, Muir H, Cribb PJ (1986) A preliminary report on the symbiotic germination of European terrestrial orchids. Kew Bull 41:437–445
67. Stewart SL, Kane ME (2007) Symbiotic seed germination and evidence for *in vitro* mycobiont specificity in *Spiranthes brevilabris* (Orchidaceae) and its implications for species-level conservation. In Vitro Cell Dev Biol 43:178–186
68. Warcup JH, Talbot PHH (1967) Perfect states of some Rhizoctonias associated with orchids. New Phytol 66:631–641
69. Rasmussen HN, Andersen TF, Johansen B (1990) Temperature sensitivity of *in vitro* germination and seedling development of *Dactylorhiza majalis* (Orchidaceae) with and without a mycorrhizal fungus. Plant Cell Environ 13:171–177
70. Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

71. Currah RS, Zettler LW, McInnis TM Jr (1997) *Epulorhiza inquilina* sp. nov. from *Platanthera* (Orchidaceae) and a key to *Epulorhiza* species. Mycotaxon 61:335–342
72. Pereira OL, Rollemberg CL, Borges AC, Matsuoka K, Kasuya MCM (2003) Epulorhiza epiphytica sp. nov. isolated from mycorrhizal roots of epiphytic orchids in Brazil. Mycoscience 44:153–155
73. Shimura H, Koda Y (2005) Enhanced symbiotic seed germination of *Cypripedium macranthos* var. *rebunense* following inoculation after cold treatment. Physiol Plant 123:281–287
74. Davis BJ, Phillips RD, Wright M, Linde CC, Dixon KW (2015) Continent-wide distribution in mycorrhizal fungi: implications for the biogeography of specialized orchids. Ann Bot 116:413–421
75. Clements MA, Ellyard RK (1979) The symbiotic germination of Australian terrestrial orchids. Am Orchid Soc Bull 48:810–816
76. Wang H, Fang H, Wang Y, Duan L, Guo S (2011) *In situ* seed baiting techniques in *Dendrobium officinale* Kimuraet Migo and *Dendrobium nobile* Lindl.: the endangered Chinese endemic *Dendrobium* [Orchidaceae]. World J Microbiol Biotechnol 27:2051–2059
77. Alexander C, Hadley G (1984) Variation in symbiotic activity of *Rhizoctonia* isolates from *Goodyera repens* mycorrhizas. Trans Br Mycol Soc 97:391–400
78. Massey EE, Zettler LW (2007) An expanded role for in vitro symbiotic seed germination as a conservation tool: two case studies in North America [*Platanthera leucophaea* and *Epidendrum nocturnum*]. Proceedings 3rd International Orchid Conservation Congress. Lankesteriana 7:303–308
79. Furness AN, Fortney J, Zettler LW (2011) Viability assessment of orchid mycorrhizal fungi in prolonged cool [4-6 C] storage to benefit conservation. Transactions Illinois State Academy Sciences [Volume 104, Supplement 82]
80. Sarasan V (2014) Mycorrhizal research supports orchid conservation in Madagascar. Kew Science blog, 29 July 2014. http://www.kew.org/discover/blogs/kew-science
81. Arditti J (1966) Orchids. Sci Am 214:70–78
82. Dearnaley JDW (2007) Further advances in orchid mycorrhizal research. Mycorrhiza 17:475–486
83. Rasmussen HN (2002) Recent developments in the study of orchid mycorrhiza. Plant Soil 244:149–163
84. Roberts P (1999) *Rhizoctonia*-forming fungi: a taxonomic guide. Royal Botanic Gardens, Kew
85. Sneh B, Burpee L, Ogoshi A (1991) Identification of *Rhizoctonia* species. Amer Phytopathological Society, St. Paul
86. Zelmer CD (1994) Interactions between northern terrestrial orchids and fungi in nature. M.Sc. Thesis, University of Alberta, Canada
87. Moore RT (1987) The genera of *Rhizoctonia*-like fungi: *Ascorhizoctonia*, *Ceratorhiza*, gen. nov., *Epulorhiza*, gen. nov., *Moniliopsis* and *Rhizoctonia*. Mycotaxon 29:91–99
88. Otero JT, Mosquea AT, Flanagan NS (2013) Tropical orchid mycorrhizae: potential applications in orchid conservation, commercialization, and beyond. Lankesteriana 13:57–63
89. Taylor DL, Bruns TD, Leake JR, Read DJ (2002) Mycorrhizal specificity and function in myco-heterotrophic plants. In: van der Heijden MGA, Sanders I (eds) Mycorrhizal ecology. Springer, Berlin, pp 375–414
90. Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Proc R Soc Lond B Biol Sci 271:1799–1806
91. Selosse MA, Faccio A, Scappaticci G, Bonfante P (2004) Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microb Ecol 47:416–426
92. Sharma J, Zettler LW, Van Sambeek JW, Ellerseck M, Starbuck CJ (2003) Symbiotic seed germination and mycorrhizae of federally-threatened *Platanthera praeclara* (Orchidaceae). Am Midl Nat 149:79–95
93. Dressler RL (1993) Phylogeny and classification of the orchid family. Dioscorides Press, Portland
94. Irawati I (2009) Self and cross inoculation of *Papilionanthe hookeriana* and *Taeniophyllum obtusum* orchid mycorrhiza. Bull Kebun Raya 12:11–18
95. Smith SE, Read DA (2008) Mycorrhizal symbiosis, 3rd edn. Academic, Sydney
96. Barnett HL, Hunter BB (1998) Illustrated genera of imperfect fungi, 4th edn. The American Phytopathological Society, St. Paul
97. Zettler LW, Corey LL, Jacks A, Gruender L, Lopez A (2013) *Tulasnella irregularis* (Basidiomycota: Tulasnellaceae) from roots of *Encyclia tampensis* in south Florida, and confirmation of its mycorrhizal significance through symbiotic seed germination. Lankesteriana 13:119–128

98. Kataria HR, Hoffman GM (1988) A critical review of plant pathogenic species of *Ceratobasidium* Rogers. J Plant Dis Protect 95:81–107
99. Oniki M, Ogoshi A, Araki T (1986) *Ceratobasidium setariae*, *C. cornigerum*, *C. graminearum*, the teleomorphs of the pathogenic binucleate *Rhizoctonia* fungi from graminaeceous plants. Trans Mycol Soc Jpn 27:147–158
100. Yukawa T, Ogura-Tsujita Y, Shefferson RP, Yokoyama J (2009) Mycorrhizal diversity in Apostasia (Orchidaceae) indicates the origin and evolution of orchid mycorrhiza. Am J Bot 96:1997–2009
101. Chomicki G, Bidel LPR, Jay-Allemand C (2014) Exodermis structure controls fungal invasion in the leafless epiphytic orchid *Dendrophylax lindenii* (Lindl.) Benth. ex Rolfe. Flora Morphol Distrib Funct Ecol Plants 209:88–94
102. Warcup JH, PHH T (1966) Perfect states of some Rhizoctonias. Trans Br Mycol Soc 49:427–435
103. Warcup JH, Talbot PHH (1971) Perfect states of Rhizoctonias associated with orchids II. New Phytol 70:35–40
104. Warcup JH, Talbot PHH (1980) Perfect states of Rhizoctonias associated with orchids III. New Phytol 86:267–272
105. Selosse MA, Wei M, Jany JL, Tiller A (2002) Communities and populations of sebacinoid Basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) L.C.M. Rich. and neighbouring tree ectomycorrhizae. Mol Ecol 11:1831–1844
106. Selosse MA, Setaro S, Glatard F, Urcelay C, Weiß M (2007) Sebacinales are common mycorrhizal associates of Ericaceae. New Phytol 174:864–878
107. Chen J, Wang H, Liu SS, Li YY, Guo SX (2014) Ultrastructure of symbiotic germination of the orchid *Dendrobium officinale* with its mycobiont, *Sebacina* sp. Aust J Bot 62:229–234
108. Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for Basidiomycetes application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118
109. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
110. Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci U S A 81:8014–8018
111. Taylor DL, McCormick MK (2008) Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. New Phytol 177:1020–1033

Chapter 3

Protocols for Establishing Fungi-Protocorm Culture

Yuan-Yuan Li, Hui Wang, and Shun-Xing Guo

Abstract

Establishing fungi-protocorm culture requires the isolation, identification, and in vitro germination test of symbiotic fungi. In situ seed baiting is a useful technique to attract and isolate seed germination-promoting fungi. In this chapter, a protocol for establishing protocorm-fungi culture using a seed baiting technique is detailed. This technique enables the identification of potential mycorrhizal fungi specifically at the time of seed germination and during protocorm development.

Key words Seed baiting, Symbiotic germination, Protocorm, Mycorrhizal fungi, Orchid

1 Introduction

Orchids have associations with mycorrhizal fungi as sources of energy and nutrients through the action of mycotrophy to support seed germination, protocorm development, and seedling growth [1, 2]. Colonization of orchid seeds by appropriate fungal partner is therefore a prerequisite for the successful regeneration cycle in their natural habitat [3]. Most of our knowledge of orchid seed-mycorrhizal fungi associations is based on in vitro studies using fungal isolates taken from roots of mature orchid plants [4–7]. Identification of specific symbionts at early stages of seed germination and protocorm development is difficult. It is not until the introduction of the seed baiting technique by Rasmussen and Whigham [8] that identification of specific symbionts then becomes possible. The seed baiting technique enables the isolation from symbiotic protocorm species-specific and/or germination stage-specific mycorrhizal fungi, resulting in better successes in symbiotic seed germination [9]. This technique has been successfully used by many researchers [10–15] in the identification of mycorrhizal fungi and early events associated with the symbiotic process.

The in situ baiting studies use packets of orchid seeds buried in natural habitats of the adult orchid plants during the growing season to acquire mycorrhizal fungi capable of supporting germination.

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_3,

The seed baiting technique developed by Rasmussen and Whigham [8] has resulted in a large number of in situ germination studies, mostly focusing on terrestrial orchids [16]. For epiphytic orchids, the use of plastic slide mounts is difficult as the mounts are physically rigid and cannot be easily adapted to a small buried location. A modified seed baiting technique developed by Zi et al. [9] and Cruz-Higareda et al. [17], by affixing seed packets to tree bark, is more suitable for most epiphytic orchids. In this chapter, we described a protocol in establishing protocorm-mycorrhizal fungi cultures using the seed baiting technique which sees potential applications in a wide range of orchid species, both terrestrial and epiphytic orchids.

2 Materials

2.1 Laboratory Equipment and Supplies

1. Laminar flow cabinet.
2. Artificial climate growth incubator.
3. Dissecting stereomicroscope.
4. General laboratory supplies such as dissecting tools, scissors, razor blades, scalpel, fine-tipped tweezers, forceps, beakers, flasks, measuring cylinder, plates, slides, and coverslips.
5. Nylon cloth with a pore size of 45 μm (Bo Tong Xing Ye Biotech, Beijing, China).

2.2 Media and Chemicals

1. Potato dextrose agar (PDA) medium: boil 200 g of peeled and diced potatoes in 1 L of water for 20 min and filter through a fine mesh. Add 20 g dextrose and 12 g agar to the filtrate, and dilute with water to 1 L. Adjust the pH to 5.6 before autoclaving at 101.33 kPa and 121 °C for 20 min.
2. Oat meal agar (OMA) medium: boil 4 g oat flakes in 1 L of water for 10 min and filter through a fine mesh. Add 10 g agar to the filtrate, and dilute with water to 1 L. Adjust the pH to 5.2 before autoclaving at 101.33 kPa and 121 °C for 20 min.
3. Common laboratory chemicals such as hydrochloric acid, sodium hydroxide, ethanol, sodium hypochlorite solution.
4. Sterile, autoclaved water.
5. Antibiotics, i.e., streptomycin and penicillin.

2.3 Preparation of the Seed Baiting Packet

The seed baiting packet (8 cm × 4 cm) (**Note 1**) is made of nylon cloth with holes large enough to allow fungal spores to enter but not large enough to allow the seeds to escape. Fold a 8 × 8 cm nylon cloth in half. Seal the folded nylon cloth on two of the three sides and separate into two equal parts by sewing with a needle and thread. Place orchid seeds into the nylon cloth packet (**Note 2**), and seal the packet.

2.4 Equipment and Materials Associated with Molecular Biology Studies

1. PCR equipment.
2. ABI 3730 automated sequencer (Applied Biosystems, USA).
3. E.Z.N.A. Fungal DNA Kit® (Omega Bio-Tek, Doraville, GA, USA).
4. Watson's PCR purification kit (Watson, China).
5. BigDye Terminator sequencing kit (Applied Biosystems, USA).

3 Methods

3.1 Orchid Seeds Baiting In Situ

1. Collect orchid seeds from mature capsules that are about to release seeds. Examine seeds under a dissecting microscope to ensure the presence of mature embryos.
2. Place approximately 500–1000 orchid seeds (depending on the total number of seeds available) in each seed packet (**Note 3**). For terrestrial orchids, position seed packets under the humus and moss (approximate depth 4–6 cm), along transect lines, near the roots of the adult orchids (Fig. 1a). Map the location of each packet in the plot before insertion (Fig. 1b). For epiphytic orchids, affix seed packets onto tree bark or rocks, near adult orchid roots (Fig. 2a). In this process, apply a thin layer of moss on the surface of the packet in order to retain moisture and allow for light penetration.
3. Harvest seed packets after 12–36 months to coincide with the growing season (**Note 4**). Gently recover seed packets from the buried locations. Be sure to keep packets moist between sheets of sterilized wet moss, store in a cool storage box or a mini-refrigerator (0–4 °C), and transport to the laboratory for examination as soon as possible (**Note 5**). Rinse the packets

Fig. 1 (**a**) Seed packets baiting of terrestrial orchids. Several seed packets under the humus and moss. (**b**) Position and placement of seed packets (arrows)

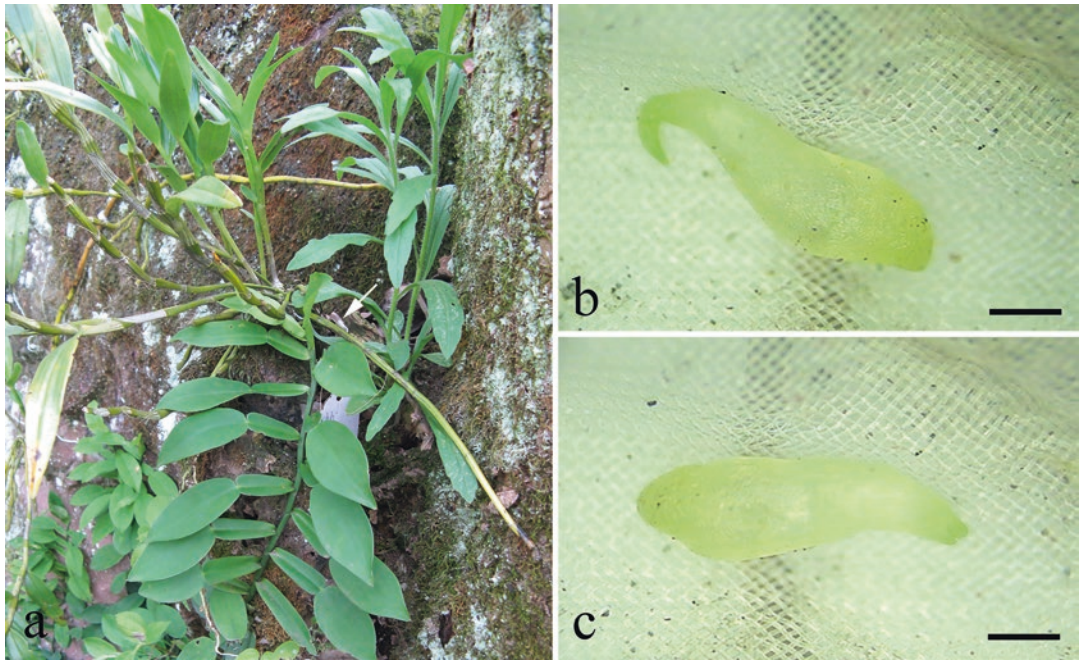


Fig. 2 Seed packets baiting of epiphytic orchids. (**a**) Position and placement of seed packets (arrow), near roots. (**b**, **c**) Protocorms recovered from seed packets. Scale bar = 1 mm

gently with tap water and cut open with a mini-scissors under a dissecting microscope. Score germination and seedling growth and development on a scale of 0–5 according to Stewart et al. [18]. Select protocorms (mycorrhizal seedlings) using a fine-tipped tweezers for isolating endophytic fungi (**Note 6**) (Fig. 2b, c).

3.2 Fungal Isolation

1. Wash the surface of mycorrhizal protocorms gently with sterile distilled water three times to remove surface debris. Sterilize the protocorm surface with 1% sodium hypochlorite solution for 1–3 min (according to the size of protocorms), and finally rinse with sterile distilled water three to four times.
2. Tease apart the sterilized protocorms using a sterile blade to release intracellular hyphal coils (pelotons), and place in PDA medium containing 100 μg/mL streptomycin and 100 μg/mL penicillin (**Note 7**).
3. Incubate the dish at 25.0 ± 0.5 °C in the dark, and examine daily. Once the hyphae emerge from the edge of the broken protocorms, excise the hyphae using a dissecting needle and transfer to fresh PDA medium with antibiotics. Incubate Petri plates at 25.0 ± 0.5 °C in the dark. Subculture fungal colonies from actively growing isolates onto fresh PDA medium without antibiotics to be purified (see Subheading 3.4 and **Note 8**).

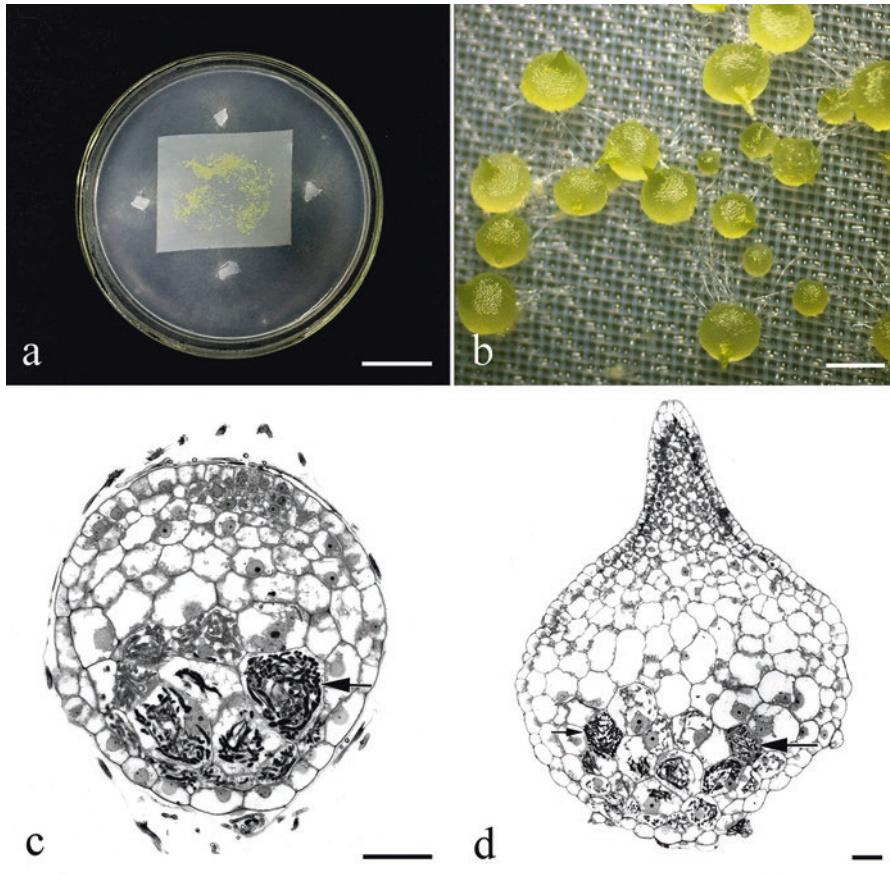


Fig. 3 In vitro symbiotic seed germination of *Dendrobium officinale*. (**a**) Seeds of *D. officinale* inoculated with mycorrhizal fungi. Scale bar = 2 cm. (**b**) Protocorms after 3 weeks of inoculation. Scale bar = 2 mm. (**c**) A stage 2 enlarged protocorm with rupture seed coat. The pelotons (arrow) accumulate in the basal end of the protocorm. Scale bar = 50 μm. (**d**) A stage 3 protocorm with the first crest. The pelotons (arrows) are located within basal protocorm cells. Scale bar = 50 μm

3.3 In Vitro Symbiotic Seed Germination

1. Co-inoculate mycorrhizal fungi and orchid seeds in a 9 cm Petri dish. Sow surface sterilized seeds onto a square of autoclaved nylon cloth (4 cm × 4 cm) and position on the surface of OMA medium. Place four pieces (0.5 cm^3) of medium with mycelia near the nylon cloth with seeds (**Note 9**) (Fig. 3a, b). Plates without fungus serve as controls. Seal the plates with Parafilm.
2. Maintain the Petri plates in an artificial climate incubator at 25.0 ± 2.0 °C. Select light or dark culture conditions according to orchid species (**Note 10**). Assess seed germination and protocorm development regularly using a dissecting stereomicroscope. Score germination and seedling growth and development on a scale of 0–5 [18].

3.4 Identification of Germination-Promoting Fungus

1. Incubate germination-promoting fungus on fresh PDA in darkness at 25.0 ± 0.5 °C for 10 days (according to the growth rate). Isolate actively growing mycelia from culture plates.
2. Extract genomic DNA using the E.Z.N.A.® Fungal DNA Kit (Omega Bio-Tek, Doraville, GA, USA) according to the manufacturer's protocol. Amplify internal transcribed spacer (ITS) regions and the 5.8S and large subunit rRNA (nrLSU) with primer pairs ITS1 and ITS4 and primer pairs ITS1-OF and ITS4-OF [19, 20], respectively (**Note 11**). Perform PCR amplification and sequencing in aqueous volumes of 25 μL. The reactions contain 12.5 μL of 2× Master PCR Mix [0.1 U Taq Polymerase/μL, 500 μM dNTP each, 20 mM Tris–HCl, 100 mM KCl, and 3 mM $MgCl_2$ (TIANGEN Biotech, Beijing, China)], 0.5 μL of each primer (10 μM), 1.0 μL of template DNA (50 ng/μL), and 10.5 μL of ddH_2O. The cycling parameters are as follows: an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 7 min. Prepare negative controls (without DNA template) for every group of amplifications. Purify the PCR products using the Watson's PCR purification kit (Watson, China). Perform sequencing with a BigDye Terminator sequencing kit (Applied Biosystems, USA), and analyze with an ABI 3730 automated sequencer (Applied Biosystems, USA).
3. Perform sequence-based identifications using BLAST with FASTA algorithms, using the Genbank database of fungal nucleotide sequences for reference. Taxa that have more than 99% ITS sequence similarity are identified as reference taxa species; taxa that have less than 99% but more than 95% ITS sequence similarity with previously described species are identified at the genus level [21].

4 Concluding Remarks

This chapter introduces a protocol for establishing fungi-protocorm cultures. The seed baiting technique provides an effective tool to detect and isolate potential mycorrhizal fungi that aid in symbiotic seed germination. Acquiring and identifying appropriate candidates is the first step toward our understanding of the germination process. It is difficult to study symbiotic germination of orchid seeds under natural conditions. Establishing protocorm-mycorrhizal fungi culture under laboratory conditions provides a stable experimental system to investigate the mechanism of symbiotic seed germination.

5 Notes

1. One can vary the size of the baiting package. Although the suggested size is 4 × 4 cm, the small size may create difficulties in making the packages. In this case, the baiting packet (4 × 8 cm) may be a better option. In addition, one baiting packet (4 × 8 cm) can be used for two species, as it can have two separate compartments. This enables the study of an additional species in a small experimental plot, if needed.
2. A sponge or moss can be used to maintain moisture, but sawdust is not an appropriate choice as seeds in packets with sawdust showed low germination rates when compared to packets without sawdust (unpublished observation). Orchid seeds are dispersed on the surface of the sponge [17] or moss in the packet.
3. Since orchid seeds are known to have low germination percentages and the retrieval rates of seed packets under natural conditions may be low, especially for terrestrial orchids, a large number of seed packets (50 or more in each location) should be used.
4. In situ baits are usually subject to prevailing climatic conditions in the natural habitats. Hence, the seed baiting packets should be placed in the natural habitats at the start of the growing season to allow sufficient time for seed germination. Harvest time differs according to the habitats of various orchid species. For some terrestrial orchids, such as *Cypripedium*, seed packets could be buried and exposed to the environment for 4–10 years before harvesting.
5. Recovered seed packets can be stored in the fridge (4 °C) for 7–10 days in the laboratory. They must be kept moist between sheets of wet paper towel.
6. The healthy mycorrhizal protocorms at stages 2 and 3 are preferred. At these stages, a large amount of hyphae accumulates in the basal end of the protocorms, and mycelium growth is very active ([22] and unpublished data) (Fig. 3c, d).
7. Because of their small size, it is difficult to tease apart protocorms at stages 1 and 2 using a sterile blade to release pelotons. Hence, the following methods can be adopted: After surface sterilization, transfer the protocorms to a drop of sterile distilled water in a sterile Petri dish. Pelotons are teased from cortical cells into water using a dissecting needle. Remove protocorm tissue and pour PDA with antibiotics into the Petri dish. When hyphal growth from single coil is observed, transfer the hyphae onto fresh PDA medium without antibiotics.

8. Fungal colonies from actively growing isolates are subcultured by excising the hyphal tips. Sometimes, this step needs to be repeated three to four times until a purified strain is obtained.
9. For some aggressive mycorrhizal fungi, such as *Ceratobasidium* sp., only one 0.5 cm^3 piece of fungal inoculum is used on the dish. A large amount of actively growing fungal hyphae can kill the orchid seeds. OMA medium can be replaced by water agar medium to slow the growth of the fungus.
10. In general, epiphytic orchid seeds are maintained in the light with different photoperiods, i.e., 12/12, 14/10, and 16/8 light/dark cycles, while terrestrial orchid seeds are usually maintained in total darkness. In view of the differences in response to light and dark conditions during symbiotic seed germination, the light requirement for symbiotic germination should be individually tested for different species [9].
11. The majority of orchid mycorrhiza fungi belong to the anamorphic form–genus *Rhizoctonia* which contains three taxa: *Ceratobasidiaceae*, *Sebacinaceae*, and *Tulasnellaceae* [20]. We recommend the use of common primer pairs ITS1/ITS4 and ITS1-OF/ITS4-OF for initial characterization of the mycorrhiza fungi. Meanwhile, some new fungal-selective primers can be applied in identification of *Rhizoctonia*-like fungi, such as ITS4-Tul for *Tulasnella*, SSU1318-Tom, and LSU-Tom4 for Thelephoraceae [20].

References

1. Rasmussen HN (1995) Terrestrial orchids: from seed to mycotrophic plant. Cambridge University Press, Cambridge
2. Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic Press, London, U.K.
3. Stewart SL, Kane ME (2007) Symbiotic seed germination and evidence for in vitro mycobiont specificity in *Spiranthes brevilabris* (Orchidaceae) and its implications for species-level conservation. In Vitro Cell Dev Biol Plant 43:178–186
4. Stewart SL, Zettler LW (2002) Symbiotic germination of three semi-aquatic rein orchids (*Habenaria repens*, *H. quinquiseta*, *H. macroceratitis*) from Florida. Aquat Bot 72:25–35
5. Massey EE, Zettler LW (2007) An expanded role for *in vitro* symbiotic seed germination as a conservation tool: two case studies in North America (*Platanthera leucophaea* and *Epidendrum nocturnum*). Lankesteriana 7:303–308
6. Swarts ND, Dixon KW (2009) Terrestrial orchid conservation in the age of extinction. Ann Bot 104:543–556
7. Chen J, Wang H, Guo SX (2012) Isolation and identification of endophytic and mycorrhizal fungi from seeds and roots of *Dendrobium* (Orchidaceae). Mycorrhiza 22:297–307
8. Rasmussen HN, Whigham D (1993) Seed ecology of dust seeds *in situ*: a new study technique and its application in terrestrial orchids. Am J Bot 80:1374–1378
9. Zi XM, Sheng CL, Goodale UM, Shao SC, Gao JY (2014) *In situ* seed baiting to isolate germination-enhancing fungi for an epiphytic orchid, *Dendrobium aphyllum* (Orchidaceae). Mycorrhiza 24:487–499
10. Masuhara G, Katsuya K (1994) *In situ* and *in vitro* specificity between *Rhizoctonia* spp. & *Spiranthes sinensis* (Persoon.) Ames. Var. amoena (M. Beiberstein) Hara (Orchidaceae). New Phytol 127:711–718
11. Rasmussen HN, Whigham DF (1998) The underground phase: a special challenge in studies of terrestrial orchid populations. Bot J Linn Soc 126:49–64
12. McKendrick SL, Leake JR, Taylor DL, Read DJ (2000) Symbiotic germination and

development of myco-heterotrophic plants in nature: ontogeny of *Corallorhiza trifida* and characterization of its mycorrhizal fungi. New Phytol 145:523–537

13. Brundrett MC, Scade A, Batty AL, Dixon KW, Sivasithamparam K (2003) Development of *in situ* and *ex si*tu seed baiting techniques to detect mycorrhizal fungi from terrestrial orchid habitats. Mycol Res 107:1210–1220
14. Zettler LW, Piskin KA (2011) Mycorrhizal fungi from protocorms, seedlings and mature plants of the eastern prairie fringed orchid, *Platanthera leucophaea* (Nutt.) Lindley: a comprehensive list to augment conservation. Am Midl Nat 166:29–39
15. Wang H, Fang HY, Wang YQ, Duan LS, Guo SX (2013) In situ seed baiting techniques in *Dendrobium officinale* Kimura et Migo and *Dendrobium nobile* Lindl.: the endangered Chinese endemic *Dendrobium* (Orchidaceae). World J Microbiol Biotechnol 27:2051–2059
16. Rasmussen HN (2002) Recent developments in the study of orchid mycorrhiza. Plant Soil 244:149–163
17. Cruz-Higareda JB, Luna-Rosales BS, Barba-Alvarez A (2015) A novel seed baiting technique for the epiphytic orchid *Rhynchostele cervantesii*, a means to acquire mycorrhizal fungi from protocorms. Lankesteriana 15:67–76
18. Stewart SL, Zettler LW, Minso J, Brown PM (2003) Symbiotic germination and reintroduction of *Spiranthes brevilabris* Lindley, an endangered orchid native to Florida. Selbyana 24:64–70
19. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322
20. Taylor DL, McCormick MK (2007) Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. New Phytol 177:1020–1033
21. Sánchez MS, Bills GF, Zabalgogeazcoa I (2008) Diversity and structure of the fungal endophytic assemblages from two sympatric coastal grasses. Fungal Divers 33:87–100
22. Chen J, Wang H, Liu SS, Li YY, Guo SX (2013) Ultrastructure of symbiotic germination of the orchid *Dendrobium officinale* with its mycobiont, *Sebacina* sp. Aust J Bot 62:229–234

Chapter 4

Orchid Seed and Pollen: A Toolkit for Long-Term Storage, Viability Assessment and Conservation

Philip T. Seaton, Silvério T. Hosomi, Ceci C. Custódio, Timothy R. Marks, Nelson B. Machado-Neto, and Hugh W. Pritchard

Abstract

The development of ex situ preservation techniques for seed and pollen provides a vital addition to orchid conservation using in situ and living collection approaches. The ability to both store and later efficiently distribute germplasm for reintroductions and maintenance of genetic diversity provides a powerful tool that can usefully operate beyond the confines of habitat loss and climate change. Currently a wealth of data and experience exists in both professional and amateur fields alike that requires effective global networking and sustainable resourcing to ensure that all practitioners of orchid conservation benefit. In this chapter we have summarised the current state of knowledge concerning the practice of both orchid seed and pollen storage, emphasising some of the problems that may be encountered. We also describe how current research shows that dry seeds, and potentially pollen, of many species have the capacity to survive in storage for a number of decades, if not longer, at low and cryogenic temperatures. Although within the plant kingdom orchid seeds may still be described as being short-lived, we highlight new techniques for storage and assessment of viability and germination that are continually being developed and applied more broadly to a wider range of species, to improve longevity and enhance measurement techniques. We emphasise throughout the need for more comparative data acquisition, information and interpretation of the variation in responses across the family, so as to help inform the global community of how best to handle orchid germplasm.

Key words Seed banks, Pollen, Germination, Longevity, Tetrazolium test, Desiccation

1 Introduction

Banking germplasm (seeds, pollen, somatic embryos, etc.) is a key component of an integrated strategy for the preservation of orchids around the world, whether for conservation or horticulture. Habitats continue to decline. Not only are they prone to natural disasters such as hurricanes or volcanic eruptions but, as human populations continue to grow, the pressure on land use for towns and cities and new land for agriculture is relentless [1]. Forests and other habitats continue to disappear, and wild germplasm

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_4,

continues to be transferred to private collectors [2]. A global network of orchid seed banks [3] has the potential to provide an insurance policy against loss of species and, together with living collections, both amateur and professional, contribute to the reintroduction of species into suitable habitats at some future date (*see* **Note 1**). Whilst such action was first advocated as long ago as 1984 [4], progress in the intervening 30+ years has been erratic, often due to a lack of continuous funding. Overall detailed information is still only available for a limited number of species. Perhaps 5% of an estimated 25,000 species globally [5] are held in seed banks. Encouragingly, recent investigations have confirmed that, if handled appropriately, it may also be possible to store orchid pollen due to a high level of desiccation tolerance in some species, raising the possibility of pollen banking and exchange between institutes and countries, thereby widening the genetic base of living collections [6].

Collecting seed from wild populations can be challenging and requires a considerable investment in both time and money. Ideally seed should be collected immediately prior to capsule dehiscence, and therefore a number of visits by collectors to the site(s) in question to collect seed at the optimum moment may be needed. Due to the distances involved and/or availability of staff, repeated (possibly annual) visits to collect fresh material may not be practical (and indeed the source material may no longer be available). Once established, living collections are an attractive and practical alternative, or addition. They can provide easy access to the material and the opportunity to hand-pollinate and monitor seed capsules on a daily basis as they approach the point of dehiscence.

Despite the continued (and growing) threats to species survival, there is good reason to be optimistic that stored seeds and material from living collections, of both terrestrial and epiphytic species, can be used for successful reintroduction into suitable habitats (e.g. see Seaton [7]; Ferreira et al. [8]; Yam [9]; Yam et al. [10]). Seeds and plants should be maintained in duplicate collections in different locations. Such collections require long-term commitment to their maintenance if they are to make a significant contribution to conservation (*see* **Note 2**). Living collections can be at risk from a combination of factors, including neglect, poor horticulture and problems arising from pests and diseases. Where orchids are being grown in cold climates, the potential for heating failure is of constant concern, as is failure of cooling systems in hot climates. There is a strong case to be made for maintaining living collections in their countries of origin, where orchids are often grown in shade houses in a series of different environments (e.g. at different altitudes), each reflecting the provenance of the species in question. Effective ex situ conservation also requires an integrated approach both within and between institutes that ensures good communication between horticultural and laboratory personnel (*see* **Note 3**).

There are currently a number of seed banking networks throughout the world: for example, Orchid Seed Science and Sustainable Use (OSSSU; osssu.org) is a global network of orchid seed scientists actively promoting conservation coordinated by the Royal Botanic Gardens, Kew, UK; the North American Orchid Conservation Center (NAOCC) focuses on North American species; and the Orchid Seed Bank Challenge in Australia aims to store seed representative of all native orchids from the southwest Australian global biodiversity hotspot [5]. For orchids and other plant families, Kew's Millennium Seed Bank (MSB) provides 'state-of-the-art' backup for in-country seed banks (*see* **Note 4**). The question that most frequently arises when discussing orchid seed preservation is, "How long will orchid seeds remain viable in storage?" The answer will depend upon the species and storage conditions. Seeds of many orchid species are likely to be short-lived, and others will be longer-lived [11]. Lewis Knudson successfully germinated seeds of a range of species after storage in an office drawer for about 20 years, but then again, many species didn't germinate after that time [12]. Air-dried seeds of *Encyclia phoenicea* that were collected by one of the authors in Cuba and kept in a refrigerator at around 5 °C still showed significant (>90%) levels of viability and germination after 11 years of storage (Seaton, unpublished data). Dried seeds of some *Cattleya* species with 3% moisture content (MC) and committed to storage at −18 °C at Universidade do Oeste Paulista (UNOESTE), Brazil, as part of the Darwin Initiative OSSSU project remained highly viable after almost 8 years. Unfortunately in many studies, figures are not available for the initial percentage germination, and yet such information is essential if you want to ascertain the potential longevity of any seed lot [13]. If the initial percentage germination was low, for example, the seed lot is likely to lose its ability to germinate sooner than if it was high.

Seed should be tested for viability and germination as soon as possible after its collection. In this chapter the term 'viability' refers to the seed's positive response to a vital stain, in this case 2,3,5 triphenyl tetrazolium chloride (TZ). Testing for germination and/or viability at regular intervals is highly desirable as it enables monitoring of changes in viability and provides an indication as to when accessions should be renewed either by making fresh collections from the field or by carrying out controlled pollinations in living collections to generate fresh material (*see* **Notes 5** and **6**). This can also be used to generate valuable data on the long-term survival of individual species [14–16]. Accessions with a low percentage of viable seeds can still be valuable, especially where the species in question is of high conservation importance.

An estimate for how long stored seeds will survive can be made using a comparative longevity test, where seeds are artificially aged under higher relative humidity (RH) and temperature. For example,

seeds can be rehydrated at 47% RH in an open container over a non-saturated solution of lithium chloride (LiCl 370 g L^{-1}) at 20 °C for 10 days and then moved to ageing conditions of 60% RH (LiCl 300 g L^{-1}) at 40 °C. Samples of seeds are periodically removed for asymbiotic germination on a suitable medium over time [17]. Preferably, such accelerated ageing should be performed using a range of conditions [11, 18]. Germination can be plotted against time in storage, and the seed survival data interpreted using probit analysis to fit viability equations, from which an estimate of time required to reduce viability to 50% of its original value can be made for the conditions used [19]. Results achieved with 16 species from Australia and elsewhere around the world indicate seeds tolerate drying to 15–20% RH, but accelerated ageing tests suggest that they are relatively short-lived compared to non-orchid species [11, 18]. Other empirical evidence would suggest that this is not the case for all orchid species, emphasising the need for generating more data and better predictive modelling of longevity for orchids, including at conventional seed banking conditions [20]. Variation in the ability of orchid pollen to be stored is likely to mirror the situation in seed storage, and further data covering a wider range of species from diverse habitats is required. For example, high germination in pollen derived from pollinia with MC as low as 5% has been reported [21] or stored for up to 6 years following equilibration at 33% RH [6], whilst other authors suggest that orchid pollen may be recalcitrant [22].

2 Species Identification

Correct identification of each species, either in the field or in ex situ collections, is vital. The World Checklist of Monocotyledons (http://apps.kew.org/wcsp/monocots) provides an up-to-date list of accepted names. Ideally a corresponding herbarium specimen should be lodged within an appropriate institutional herbarium for future reference. Where this is not possible, photographs or drawings should be made of the flower (front and side views) and the whole plant. Data should be collected regarding the geolocation of plants collected in the field (distinct populations may, at some future date, prove to be separate species). Information about habitat (e.g. soil pH, if it is a terrestrial species, and associated vegetation) can facilitate cultivation of plants grown from seed in living collections that may become a source of material for future reintroductions. Accession numbers and collection records should be linked to a database. All field collections must have prior approval by the appropriate authorities in the country (*see* **Note 7**).

2.1 Procedure for Species Identification

1. Check whether a herbarium specimen has been placed in an appropriate institution.
2. Check collection records.
3. Link pollen and seed parents to the herbarium specimen and collection records.
4. Where no herbarium specimen exists, whenever possible, a pressed specimen should be made to include flowers and a leaf as a minimum and lodged in an appropriate institution herbarium
5. Fleshy flowers (that cannot be pressed successfully) can be preserved in spirit (70% v/v ethanol).
6. As a bare minimum, a photographic record (or drawings) should be made of the flower (front and side views) and the whole plant.
7. Where seed is harvested in the field, photographs should also be taken of the habitat, and field data recorded.

3 Pollination for Seed Production

Because orchid flowers have a structure that differs radically from those in other plant families, there is often a requirement to train operatives how to pollinate orchids, both in living collections and in the field. Orchid pollen is aggregated into clumps that are termed pollinia. Pollinia in turn are usually associated with other structures that enable the transfer and adhesion to their respective pollinators (often insects). Pollinia plus the associated structures are termed *pollinaria*. Slipper orchids (*Cypripedium*, *Paphiopedilum*, *Phragmipedium* and *Selenipedium*) are rather different, as their pollinia can be found as sticky masses in either side of the column. The following procedure is designed to increase the chances of obtaining good quality seed collections, i.e. collections that contain a high proportion of 'full' seeds (i.e. seeds that contain embryos).

3.1 Procedure for Pollinating Plants

1. Preferably mature plants should be used as 'pod parents'. Healthy, well-grown plants produce larger capsules. The mother plant should contain sufficient storage reserves to guarantee seed and capsule filling.
2. Outcrossing between different clones is always preferable. Pollination should be between two individuals, each of a distinct genetic make-up.
3. Where only a single plant of a rare species is available, self-pollination may be the only option (but see Sect. 5 below,

pollen and seed exchange). Some species, however, may be self-incompatible [23, 24].

4. Information regarding timing of pollen maturation on the parent plant or how long the pollen remains viable in situ is limited to a few species (e.g. [6]). Flowers of some species remain open for a single day (e.g. some species of *Sobralia* and *Vanilla*), whilst flowers of other species remain open for 2 months or longer (e.g. some *Dendrobium*); some may take several days to open fully (e.g. *Laelia anceps*). Our recommendation is that freshly opened flowers (once they have opened fully) are more likely to be successful than older blooms [6].
5. Orchids vary considerably in the number of flowers they produce at any one time. Some species produce single blooms; others produce large, branching, inflorescences of many tens of flowers. If more than one flower is produced in an inflorescence, then a number of flowers should be pollinated (*see* **Note 8**). If plants possess a large number of small flowers per inflorescence (e.g. some *Oncidium* species), as a guide, we suggest that up to 10% of flowers should be pollinated.
6. Not all pollinations will be successful, so that we recommend pollinating an excess of flowers at the outset where possible. The number of capsules can be gradually reduced, with time, to enhance seed and capsule filling.
7. A plastic or a metallic tag should be attached to the inflorescence of the pod parent with the pollination date (*see* Fig. 1).
8. The information should be transferred to a notebook or a spreadsheet to calculate the maturation time (time between the date of pollination and capsule dehiscence), including how many flowers were pollinated and how they were pollinated (by hand or naturally occurring pollinators).
9. The flowers above the last flower to be pollinated should be removed to enable sufficient plant resources to be available to aid seed filling (*see* Fig. 1).
10. To maximise the number of full seeds per capsule, a full pollen load (i.e. all of the available pollen from one individual flower) should be applied to the stigma of the seed parent.

4 Pollen Storage

When the two proposed parent plants are not in flower at the same time, it may be necessary to store the pollen of the proposed donor for some time until the recipient plant is in flower. The storage protocol for pollen is essentially the same as for seed (see seed storage Sect. 7), and involves storing entire pollinaria at reduced temperature and low RH (*see* **Note 9**). As with seed, the longevity of orchid pollen generally improves with lower temperatures and

Fig. 1 Inflorescences of *Dactylorhiza fuchsii* tagged for identification and excess flowers removed to allow maximum resource allocation to developing seed capsules. Orange arrow: indicates removal of a pollinated flower. White arrow: metallic foil tag with pollination date. Black arrow: top of the inflorescence has been removed

MC, so that potentially cryopreservation may be the best option for long-term preservation, although refrigerator and freezer temperatures can be used where available facilities dictate. Pollen of *Dactylorhiza fuchsii* has been shown to survive for 12 months in liquid nitrogen [25], and pollen of *Disa uniflora* showed ~100% germination after 120 days (unpublished data). Of particular importance is the ability of stored pollen to give rise to functional seeds. Examples of this have been reported in *Dendrobium* hybrids, stored in liquid nitrogen for 24 h [21], and in *D. fuchsii* stored for 6 years at −20 °C [6].

4.1 Procedure for Pollen Storage

Whole pollinaria should be collected from mature flowers of individual plants and placed in suitable sealable containers and their MC brought into equilibrium with the RH, for example, generated over a saturated solution of calcium chloride (~33%) in a desiccator for 3 days (*see* **Note 10**).

4.2 Pollen Germination

Germination is assessed by sowing pollinia on agar-gelled (1% w/v) medium containing 1% (w/v) sucrose and 0.1% (w/v) boric acid, at pH 5.6, and placed in the dark at ~25 °C for 48 h [25]. Counting is performed by suspending the pollinia in a deionized water droplet on a microscope slide and teasing individual pollen

grains apart. Germination is considered to have occurred when the length of the pollen tube is greater than the diameter of the pollen grain (*see* **Note 11**).

5 Pollen and Seed Exchange

There have been a number of proposals to set up orchid pollen exchange schemes (e.g. Lyon Botanical Garden). Single examples of rare and endangered plants representing different genotypes (i.e. the plants are different clones) may be found in botanic gardens and other collections around the globe. Pollen exchange between collections has the potential to enhance living collections by widening their genetic base through cross-pollination, thereby providing additional security for species in ex situ collections. Seed produced in this way should be divided equally between the two participating institutes. Both pollen and seed should be sent using express mail to minimise the impact of any potential adverse environmental conditions during transit. Relevant treaties, such as the Convention on Biological Diversity (CBD) and Convention on International Trade in Endangered Species (CITES) plus in-country regulations, should be adhered to at all times [26]. All orchid species are listed under either Appendix I or Appendix II of CITES (http://www.cites.org).

For both the donor and recipient, it is vital to record data to verify the plants involved and to document the developmental stages of the process. This will establish a time frame for improving future pollinations and plant management. Upon receipt, the pollen should be allowed to equilibrate to ambient conditions of temperature and RH before pollinations are conducted. Plants should be monitored on a regular basis in order not to miss the flowering periods of both the pollen and pod parents and the timing of dehiscence of the seed capsule(s). Daily observation is strongly recommended as the capsule approaches dehiscence.

5.1 Guidelines for Pollen and Seed Exchange

1. Identify species in the living collection of each of the participating institutes.
2. Determine the flowering time of both the pod (the recipient) and pollen (donor) parents.
3. Record the accession number of the pod parent in the recipient garden's database.
4. Record the accession number of the pollen parent in the donor garden's database.
5. Exchange the above information.
6. When the donor plant flowers, remove the pollinaria and record both the date of harvest and the number of pollinaria harvested.

7. Place the pollinaria in suitable sealable containers, and bring their MC into equilibrium with the RH (~33%) generated over a saturated solution of calcium chloride in a desiccator for 3 days.
8. Send the pollinaria to the recipient institute.
9. Record the date of pollination of the pod parent.
10. Record date when the capsule(s) are harvested.

5.2 Procedure for Sending Seed and Pollen Through the Post

1. Seed and pollinia should first be cleaned and ideally dried, as described.
2. To ensure seeds and pollinia are transported dry and are not liable to crushing, they must be placed in glass vessels with seals to prevent rehydration. These can then be securely packaged, using bubble wrap, and posted (*see* **Note 12**).

6 Harvesting Seed

Harvesting from the wild should only proceed with prior approval (preferably in writing) from the appropriate authorities. Although there are exceptions (e.g. *Vanilla* species), orchid seed normally is wind-dispersed. Generally speaking, seed capsules change colour from green to yellow or brown towards the end of their development, but this is not always the case. Seed capsule ripening times vary enormously [27]; *Epidendrum radicans* takes around 51 days, for example, whereas *Cymbidium* capsules may take more than a year [28]. However, this information should be treated as a guideline only. The rate of ripening of a seed capsule will vary according to environmental conditions, greenhouse (shade house) culture, the genetic make-up of the parent plant, etc. Judging the maturity of seed capsules of individual species can be a matter of experience. As the seed capsule approaches dehiscence, regular, daily observation is recommended to guard against potential seed loss.

6.1 Procedure for Seed Collection and Handling

1. Capsules should be collected at, or immediately prior to, dehiscence to ensure a good quantity of mature seeds are harvested (*see* **Note 13**).
2. Record date of seed collection and whether the capsule had begun to split (dehisce).
3. Photograph the capsule at the time of harvest, if possible.
4. Calculate and record time of pollination to maturity of the seed capsule, so that this can be related to seed quality.
5. Capsules can be shaken to release seeds through linear cracks in the capsule wall, or the ends trimmed to aid release of the seeds. A toothpick can be used to liberate the seeds that are attached to the placental tissues, or the capsules, if small, may

be shaken into a small paper envelope. Some capsules are thin-walled (many temperate terrestrial species), and capsule tissue can be peeled away.

6. Capsules that have not yet split, and have been removed from the parent plant, can be allowed to dehisce 'naturally' in a thin paper envelope in a cool, dry room.
7. Seed must be cleaned by separating it from any debris (a potential source of contamination) using forceps and a sieve (*see* **Note 14**).
8. Seeds should be collected on paper and dried at low RH (see below) for a maximum of 7 days, then sieved to remove any remaining debris.

6.2 Ex Situ Collections

Living collections can form a key component in any orchid conservation project. Although wild-collected seed lots are always welcome (*see* **Note 15**), making field collections, particularly in tropical countries, can be difficult, time-consuming and expensive. The advantages of having access to a living collection are many, be it amateur or professional. Amateur growers often have more time to devote to their collections and are an under-utilised resource, as they often have a wealth of expertise in the plants they grow. Ideally, ex situ collections will consist of a number of individuals that reflect the genetic variability of wild populations. Growing of populations of plants, rather than single representatives of each species, conserves and demonstrates the genetic variation within a species and is to be encouraged (*see* Fig. 2). Cross-pollination can ensure problems of self-pollination, or depression of seed quality through the crossing of identical clones does not occur [29–31]. Controlled pollinations can then form a key component of orchid conservation.

6.3 Number of Seeds per Capsule

Because orchid seeds are tiny, it is possible to store a large number in a small volume. Capsules of some orchids may contain very large numbers of seeds. At one extreme, a capsule of *Grammatophyllum speciosum* can contain in excess of 2 million seeds (Marks, unpublished data), whereas a capsule of *Listera cordata* contains around 400 seeds: more typically a seed capsule will contain thousands of seeds (see table in Arditti, [32]).

6.4 Counting the Number of Seeds per Capsule

1. Weigh the total amount of harvested seeds from each capsule separately.
2. A small subsample of seed can then be weighed using an analytical balance with four (preferably more) decimal places, where available.
3. Count the number of seeds in the subsample. This can be done directly using a binocular dissecting microscope or by taking a digital image and later counting on a computer screen.

Fig. 2 Flowers of *Laelia anceps* illustrating the genetic variation that may be found in one species

4. Use this information to calculate the numbers of seeds in each of the individual capsules and consequently in the whole collection.
5. Although seeds can be mixed together for each accession/population, it is often preferable to keep the contents of seed capsules from different plants as separate accessions, as genetic or cultural differences may lead to differences in seed quality.

7 Postharvest Treatment of Seed

Each individual seed collection should be given its own unique accession number that follows it throughout storage and testing for germination and/or viability. Separate collections of the same species should be given individual accession numbers.

Besides being dependent upon the species, seed longevity in storage also depends upon the initial quality of the seed. This can be affected by factors both in the mother plant and in how the seeds have been handled postharvest. Ideally the seed will have been harvested just as the seed capsule has begun to split. Many growers sow immature seed (so-called green pod). This has the advantage of eliminating some of the problems of contamination when sowing and gives a quicker germination result, but unfortunately immature seed, when dried, might have a shorter lifespan, and such material should probably be avoided as the basis of a conservation collection (*see* **Note 17**). Drying of orchid seed to an appropriate seed MC is key to maintaining its longevity. This equilibrium moisture content (eMC) will vary between species but is likely to be between 3 and 5% (i.e. in equilibrium with 15–20% RH). High RH treatments will result in high MCs, with implications for storage potential.

Seed can be dried over saturated solutions of either calcium chloride or lithium chloride [11], both of which give known RH. A saturated solution of calcium chloride produces a 30% RH at 20 °C, whilst a saturated solution of lithium chloride produces 12.4% RH at 20 °C [17, 33]. Higher RHs may be achieved using specific solution concentrations [17]. Where neither calcium chloride nor lithium chloride is available, using oven-dried rice remains an easy, practical method for drying orchid seed, particularly for amateur growers, and can provide a RH equivalent to that of calcium chloride (unpublished data).

7.1 Procedure for Drying Seed

This can be done using air-tight vessels (e.g. Kilner jars) in several ways depending upon the level of technology available (*see* **Notes 10** and **17**).

1. The MC of seeds can be adjusted to <5% w/w by placing seeds in a thin layer in a dish or a thin paper envelope within a desiccator over a solution of lithium chloride solution (*see* **Note 18** and Fig. 3a) (147 g in 200 mL, which equates to 15%RH) solution at 20 °C for no more than 7 days (*see* Table 1).
2. Alternatively this can also be done using a saturated solution of calcium chloride (add $CaCl_2 \cdot 2H_2O$ in excess of 15 g 100 mL^{-1}) at 20 °C.
3. Or a simpler method can employ using dried and cooled rice (*see* **Notes 19** and **20** and Fig. 3b). A drawback of this method is that the final RH is unknown, and variable compared to methods 1 and 2. This can be addressed to some extent by including a small sachet of silica orange as an indicator, which will turn green above c.25% RH (*see* **Note 21**), whereupon the dried rice should be replaced with freshly dried material (*see* **Note 19**).

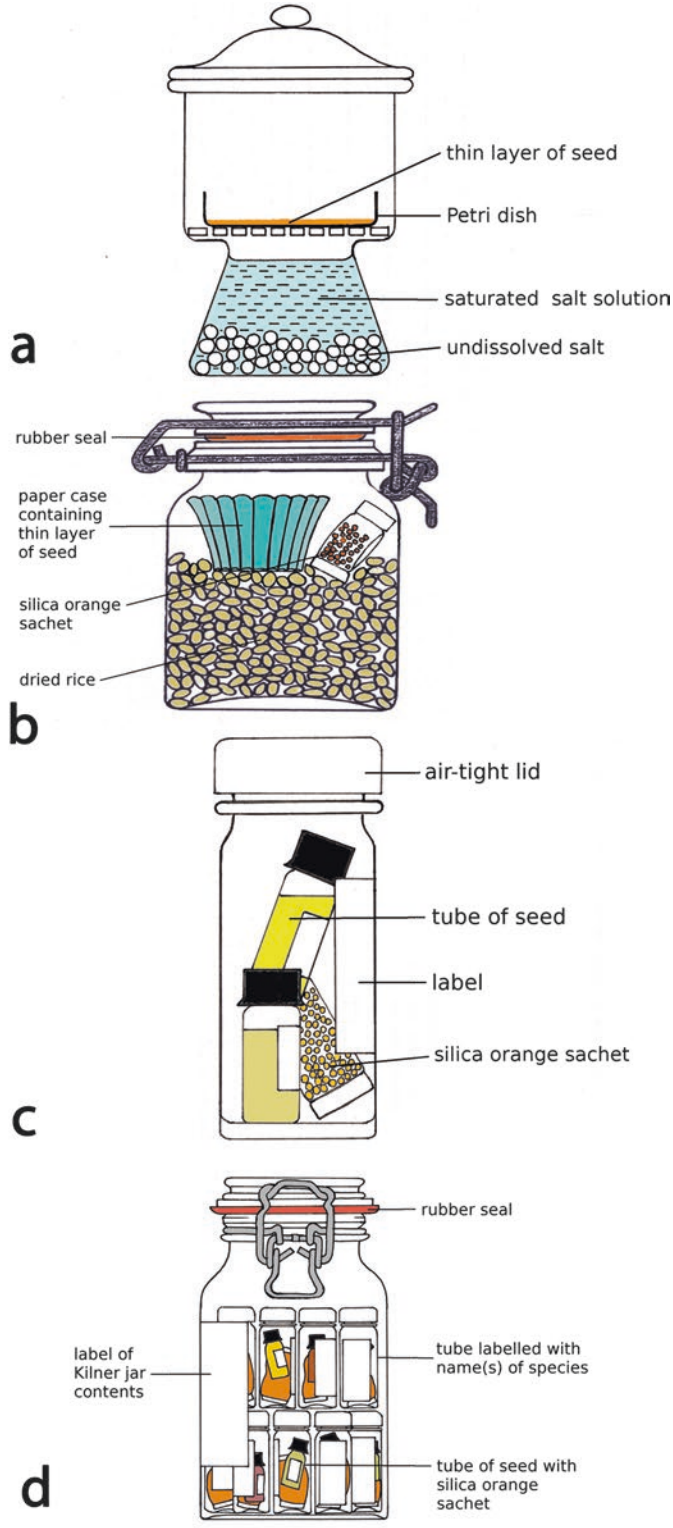


Fig. 3 Illustrations of orchid seed drying and storage equipment. (**a**) Seed being dried in a thin layer over a saturated salt solution. (**b**) Seed being dried in a paper case over dried rice. The silica orange sachet acts as an indicator of relative humidity. (**c**) A 2 mL tubes of seed stored within a 10 mL 'universal' tube bottle. The silica orange sachet acts as an indicator of relative humidity. (**d**) Large Kilner jar providing a double seal for tubes of seed of a range of species

Table 1
Preparation of solutions of lithium chloride to produce specific relative humidity conditions (taken from Technical Information Sheet_09, Kate Gold and Fiona Hay, Royal Botanic Gardens, Kew)

Weight of LiCl (g)	RH (%) generated at 20 °C
174	11
147	15
128	20
104	30
88	40
74	50
60	60
50	70
34	80
26	85
20	90
8	95

Once prepared, the solutions should be allowed to equilibrate in sealed containers for a minimum of 24 h, ideally at a constant temperature of 20 ± 0.5 °C

4. After drying, seed is transferred to hermetically sealed tubes for long-term storage (*see* **Notes 22**, **23** *and* **24** and Fig 3c, d).

7.2 Storage Temperature

Successful orchid seed storage has been reported over a range of temperatures, such as 5, −20, −80 and −196 °C, with tropical, temperate, epiphytic and terrestrial species [34] (*see* **Note 25**). Nevertheless there are indications that the longevity of some species may be compromised at −20 °C but survive better at both lower and higher temperatures (e.g. *Cattleya aurantiaca* [see 35] and other species [11, 19]). It is the ambition of the OSSSU network [36] to generate more data so that recommendations can be developed for many species [34, 37].

7.3 Germination Testing

Seed should be sown on an appropriate artificial medium. Percentage germination often varies with the medium chosen [38, 39]. Most tropical species will germinate on a wide range of media, although only a limited number are commonly used (*see* **Notes 27** and **28**).

Although observation of freshly collected seed under a microscope will give some idea of its viability, i.e. seeds containing plump embryos are likely to have the potential to germinate, this

cannot be used as a substitute for a germination test (*see* **Notes 28–30**). This is particularly true for seeds that have been stored for some time, where dead seeds may have the same appearance as living seeds. Germination, rather than being an event, is a process. Initially the embryo imbibes water and begins to swell. This will take place whether the seed is alive or dead. If the seed is viable, the embryo will continue to grow until it disrupts the testa, eventually bursting free and forming a protocorm. Most, if not all, tropical epiphytes, such as *Cattleya* and *Vanda*, germinate in the light, and the protocorm soon begins to synthesise chlorophyll and turn green. Thus they can be said to have germinated when the testa has split and the protocorm is green in colour. Terrestrial orchids are usually germinated in the dark, and before the formation of creamy white protocorms, the testa splits and rootlike rhizoids can form. Such morphological changes can be used to judge when germination has taken place and may vary between species. Deciding when germination has taken place requires accurate description and measurement over time. We recommend that investigators should record their definition of germination, either as a drawing or a photograph. The time for seed to germinate on a particular medium can vary enormously between species. For example, germination in *Maxillaria acutifolia* and *M. valenzuelana* reached 72% and 74%, respectively, after 28 days on ½B5 [40] medium, but this was 10 and 72% after the same time on ½MS [41] medium (unpublished data).

Choose the medium that gives optimum germination and record and use for future sowings. Although dilutions of ionically stronger media have been used [41], nutrient levels, especially inorganic nitrogen, are generally low [42]. Inorganic nitrogen is sometimes replaced by amino acids [43, 44]. Media are generally prepared with sucrose as the major carbohydrate and are sterilised by autoclaving prior to use. European terrestrial orchids can be more demanding in terms of their nutritional requirements. When sowing seed with the intention of measuring percentage germination (of full seeds), it is beneficial to sow the seed on a medium that does not contain activated charcoal, such as Knudson C [42], as its inclusion can make visualisation difficult.

7.4 Surface Disinfection

Seeds can be disinfected using a variety of methods [45, 46] using either sodium hypochlorite (often 5% solution of household bleach) or 5 g L^{-1} sodium dichloroisocyanurate [47] plus surfactants for about 20 minutes and washing in sterile water at least twice to remove the disinfectant. Effective disinfection times can vary between seed batches and species.

7.5 Estimating Percentage Germination

When counting percentage germination, it should be made clear that you are counting percentage germination of 'full' seeds, i.e. seeds that contain embryos. Empty testae should be disregarded (*see* Fig. 4). Small embryos or seeds that are otherwise abnormal in appearance compared with other 'normal' seeds should also be rejected, but the proportions of each morphological 'type' should be recorded. Not all seeds germinate at once, and percentage germination will increase over time to eventually reach a maximum. Thus it may be necessary to count the numbers of seeds germinating until that maximum is reached and record the time taken to reach maximum germination on the medium being used. To assess whether the germination test has released the full genetic potential of the seed lot or whether some viable seeds remain that have not germinated under these conditions, a TZ test should be used (see below).

Seed germination can be followed at weekly intervals by counting the germinated fields in a Petri or Falcon dish (90 × 16 mm or 60 × 15 mm) either using a binocular microscope (×20 magnification) or by taking pictures of marked fields taken sequentially using a digital camera attached manually to the eyepiece of a stereomicroscope (*see* Fig. 5). Germinated embryos can be more easily counted on a computer screen. As the images in the latter instance are of the same field the fate of individual seeds can followed as the number of germinated seeds increases. Weekly counting should continue until further germination ceases (*see* **Note 27**).

Calculation of the rate of germination (GVI—germination velocity index, following Maguire [48]) can provide additional information that may indicate changes in seed physiology. It can be calculated as follows:

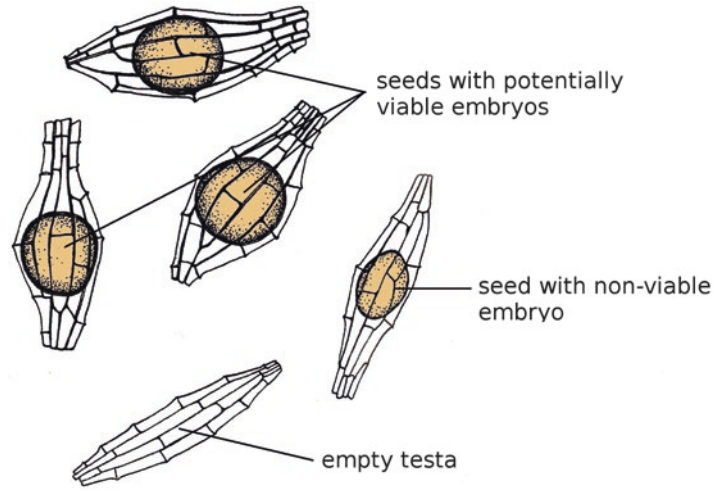


Fig. 4 When counting germination, only seeds with potentially viable embryos should be included

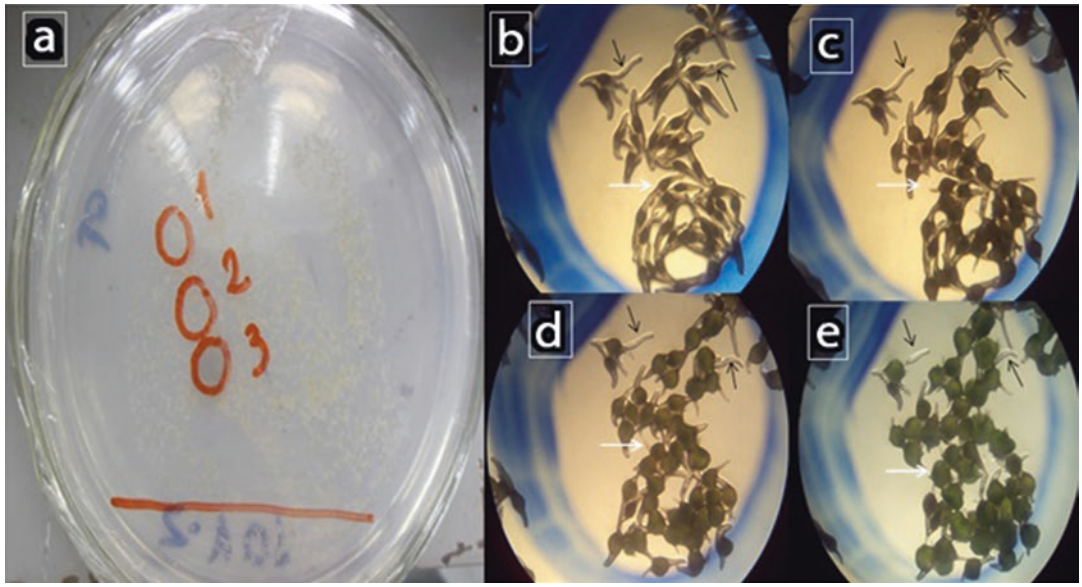


Fig. 5 Seed germination can be followed by pictures taken sequentially in marked fields over a number of weeks, using a digital camera manually attached to the eyepiece of a stereomicroscope. (**a**) Petri dish with seeds and marked fields, (**b**) 1, (**c**) 2, (**d**) 3 and (**e**) 4 weeks after sowing. Black arrows, empty seeds; white arrows, germinating embryos

$$GVI = \frac{G1}{N1} + \frac{G2}{N2} + L + \frac{Gn}{Nn}$$

where $G1$, $G2$ and Gn are germinated seeds in each counted period until n. N is the number of days after sowing in each evaluation (7, 14, 21, etc., until no changes are noted).

Measurements of germination should be done in at least three Petri dishes, with at least 100 seeds counted over the whole dish, or in a prescribed area (to achieve a random sample) if a large number of seeds have been plated. This can easily be done using a grid held under the Petri dish. Seeds can either be counted real-time or from digital images taken under a microscope. In addition to making counting and comparisons easier, the use of digital images allows the use of appropriate software and the saving of a permanent record in the seed collection database (*see* Fig. 5).

7.6 Viability Staining

With limited time, and a large number of accessions, collection managers can be faced with the problem that germination testing can be both labour-intensive and time-consuming, and it is beneficial if a more rapid technique can be used instead. The tetrazolium (TZ) test [49, 50] has the potential to fulfil this role. TZ relies upon the presence of an active dehydrogenase in the embryo, which reduces the TZ chemical stain from colourless 1,3,5-triphenyl tetrazolium chloride to red 1,3,5-triphenyl formazan. The technique

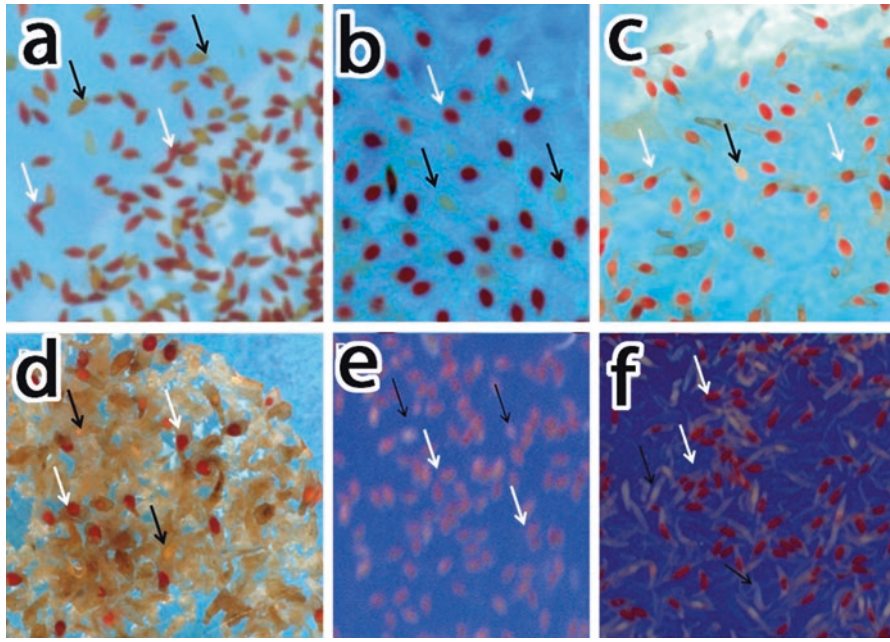


Fig. 6 Seeds coloured with tetrazolium of species with different life forms. (**a**) *Aerides odorata*, epiphytic species from tropical moist forest; (**b**) *Grammatophyllum speciosum*, epiphytic species from tropical moist forest; (**c**) *Dactylorhiza fuchsii*, tuberous geophyte from temperate grassland and open woodland; (**d**) *Disa uniflora*, tuberous geophyte found growing alongside perrenially wet streams; (**e**) *Vanda curvifolia*, epiphytic chamaephyte from tropical moist forest; (**f**) *Cattleya intermedia*, epiphyte with pseudobulbs, from the Atlantic Rainforest

is relatively quick and easy to carry out. After soaking in 10% sucrose for 24 h at room temperature, the seed is washed twice with clean water and incubated for a further 24 h at 30–40 °C (*see* **Note 31**) in 1% tetrazolium (TZ) solution in the dark. When viewed under the microscope, viable embryos will be stained through bright to dark red, depending on the species. Non-viable embryos remain colourless. However, with some species it is more difficult to distinguish between viable and non-viable seeds, and embryos may be pink or salmon-coloured. In some cases there will be a mixture of the two (*see* Fig. 6).

Images of the results can be taken using either a flatbed scanner or a camera with a macro function. Viewing against either a sky blue or a royal blue background improves contrast and aids the visualisation of unstained embryos. The images from either can then be uploaded onto a computer with suitable software, making counting seeds much easier and allowing a permanent record to be held for future reference, especially to compare colour rendition between species. As with the germination test, only full seeds should be counted.

This technique works well for species with pale-coloured seeds and those with thin testae but requires modification for those

seeds with a thick testa or a pigmented carapace. For example, after TZ staining the dark testa of *Vanda curvifolia* can be bleached to reveal the underlying stained embryos by 5 min exposure to NaOCl (5%). Alternatively the thick testa of *Dactylorhiza fuchsii* can be scarified by 2½ min exposure to NaOCl (0.5%) prior to TZ staining, which results in a close correlation between percentage viability and germination scores [51]. As the presence of NaOCl can cause further bleaching, and may interfere with the TZ reaction, seeds should be washed twice in deionised water after its use (*see* **Note 32**). With some modification, such techniques are likely to be effective on other species with problematic morphology such as *Cypripedium*.

TZ provides an estimate of viability and can have a good correlation with germination [50, 51], although problems such as dormancy or germination media inadequacy may confound germination levels achieved. In terms of ease of application, TZ can give a fast and accurate response, but reports of failures in the technique [52, 53] indicate that the technique may require modification with some species. The tetrazolium test following preconditioning is unaffected by seed dormancy, as the respiratory enzymes are still present, and the sucrose treatment enhances their activity.

7.7 Tetrazolium Solutions

Tetrazolium may be prepared as either a non-buffered or, preferably, a buffered solution in either deionised or distilled water and stored in a brown glass bottle, or in a clear glass flask wrapped in aluminium foil, to exclude light and kept at 4 °C for up to 3 months. Alternatively, suitably sized aliquots can be frozen until required.

To maintain a pH of 6.5–7.5 for optimal activity, tetrazolium salt should be dissolved in a phosphate buffer. To prepare 1 L: dissolve 3.63 g potassium dihydrogen orthophosphate (KH_2PO_4) in 400 mL water and dissolve 7.13 g disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) in 600 mL water. Mix together, and add tetrazolium salt at the required concentration (e.g. for a 1% w/v solution, dissolve 1 g tetrazolium salt in 100 mL phosphate buffer).

7.8 Procedure for Viability Testing

The aim is to take a sample that adequately represents the genetic diversity of the species in question. The size of the sample will depend to some extent on the size of the capsule and the seeds and the amount of seed available. A larger capsule like *Cattleya labiata* may contain almost a gram of seeds. In contrast a *Dactylorhiza fuchsii* capsule may contain a 10–20 mg of seed but has multiple capsules per inflorescence. Adjustments may need to be made for seed size, but 10 mg of seed is usually adequate for the test [49, 50] (*see* **Note 33**).

1. Place seeds in a 1.5 mL microtube.
2. Add 1.0 mL 10% sucrose solution and leave at room temperature or 25 °C for 24 h in the light.
3. Draw off solution using a Pasteur pipette or a micropipette, leaving the seed in the bottom of the tube.
4. Add 1 mL of 1% tetrazolium solution, and place in a water bath in the dark at 40 °C (*see* **Note 30**) for 24 h.
5. Remove tetrazolium solution.
6. Wash the seeds twice in distilled or deionised water to remove excess of TZ solution. Seeds may be kept in the refrigerator at 5 °C for at least 1 week before being analysed.
7. Resuspend seed in a small amount of water.
8. Place a few drops of the seed suspension on a microscope slide.
9. Place the slide on a flatbed scanner, and scan at a high resolution (e.g. 3600 dpi or above) placing a clear plastic rule at the side as a scale. The inner face of the scanner cover should be covered with a sheet of either sky blue or royal blue paper for increased contrast. Some small chocks (like a pencil or a crayon) may be put on each side of the scanner to avoid contact between the solution containing the seeds and the cover.
10. The slide can be scanned at 3600 dpi and the image viewed on a computer screen.
11. As an alternative to using a scanner, slides can be photographed in bright light against a light blue background with a grid (e.g. blue graph paper). Semi-permanent slides can be made by placing a cover slip over the seed suspension and sealing with nail varnish.

8 Notes

1. Seeds are commonly stored alone but can be combined with appropriate species of symbiotic fungi necessary for growth in situ. This has been demonstrated by encapsulating seeds of either *Anacamptis morio* or *Dactylorhiza fuchsii* and hyphae of the basidiomycete fungus *Ceratobasidium cornigerum* in calcium alginate beads [54]. These were soaked in 0.75 M sucrose and dried. Both hyphal growth and seed germination were unaffected by storage in liquid nitrogen for 30 days, whilst survival was reduced when stored at −20 and −70 °C. This approach was also effective for the Australian species *Pterostylis saxicola* and *Diuris arenaria* that survived equally well at −18 and −196 °C [55]. Encapsulation and drying are more commonly used for the storage of protocorm-like bodies raised adventitiously from other vegetative plant parts (e.g. Saiprasad

and Polisetty [56]) and are covered more extensively elsewhere in this volume.

2. Dead seeds that have been retained in a seed bank may be of value as a source of useful material for DNA phylogeny studies [57].
3. It is difficult to overstate the importance of regular communication between members of any team, who may represent distinct areas of expertise. Taxonomists may be involved in identification of the species in question; field operatives may be responsible for collecting and cleaning the seed if wild-collected material is being employed or greenhouse staff if employing living collections. Laboratory technicians will be responsible for carrying out germination testing and storing of the seed.
4. A series of technical information sheets can be accessed from the Royal Botanic Gardens, Kew, website. These can be consulted for additional information where required. The methodologies can be equally applied to pollen as well as to seeds. http://www.kew.org/science/collections/seed-collection/millennium-seed-bank-resources
5. Although practical considerations, in availability of staff resources, for example, may limit the amount of testing that is practical, every effort should be made to guard against the loss of potentially valuable genetic material.
6. Protocorms generated through germination testing can be transferred to flasks and grown as a source of genetic material that can either replace plants lost or enhance living collections.
7. Not only is it important to obtain permission from the relevant authorities within the country in question when harvesting seed, but CITES and CBD regulations should be consulted for the latest information when transferring genetic material between countries, and it may be necessary to sign a legally binding material transfer agreement. Seed obtained from illegally collected plants does not become legal, for example.
8. Where appropriate an excess of flowers should be pollinated as some seed capsules may spontaneously abort after developing for some time, for reasons unknown. The number of capsules should gradually be reduced over time so that a maximum quantity of plant resource is available for development of the remaining capsule(s).
9. The desiccation tolerance of orchid pollen has been tested in very few species and is likely to show variation across the family, requiring further assessment of a broader range of species representing different habitats.

10. Adjusting the MC of both seeds and pollen usually means drying them to below ambient RH. Both can be dried in paper cups (one of the authors uses small cupcake cases). After drying they must be kept in air-tight vessels otherwise their MC will increase and lead to loss of viability. Co-storage with a RH sensitive indicator, such as silica orange sachets, can show changes in conditions. Sub-zero temperatures produce a very dry atmosphere, so that 'leaky vessels' may also allow overdrying of contents if inadequately sealed.
11. Pollen grains can occur as single units or in clumps (e.g. tetrads) in pollinia. These need to be carefully teased apart in water to observe individual pollen grain germination, but without damaging emergent pollen tubes.
12. One of the authors regularly receives seed in paper packets (rather than the recommended hermetically sealed glass tubes). Although less than ideal, in this instance the donor should be encouraged to make packets that are secure (i.e. do not allow the seed to leak out of the packet) and to avoid using adhesive tape—to which any stray seeds will stick.
13. Although in extreme circumstances, bagging of capsules is an option in the last stages of capsule ripening, this cannot be recommended: plastic bags will allow water condensation and ethylene accumulation, which may lead to early fruit ripening and seed loss, and paper bags will become wet/dry repeatedly and may be rotten by the time the capsule matures, whilst fabric bags will remain wet for longer than desired allowing the growth of contaminants.
14. Seed can be separated from any debris (a potential source of contamination) using a variety of sieves (one of the authors uses a tea strainer). Transferring seed to small storage tubes can be challenging. Seeds are best handled on a piece of stiff, folded, paper. The crease facilitates pouring into the tubes. As a general rule, mature dry seed will flow easily when poured, as opposed to seed with a higher MC that may tend to form clumps, with some seed adhering to the sides of any glass vessels. In the latter instance, the seed should be dried to a suitable MC as soon as possible. Besides making the seed easier to handle (and also potentially increasing its lifespan in storage), prompt drying of seed may reduce the incidence of future contamination problems when the seed is sown.
15. When collecting seed in the field, as a rough guide, no more than 20% of the available seed should be collected on any one day. Record the location of the population in case at some future date separate populations may become regarded as being separate species.

16. Many growers sow immature seed (so-called green pod). This has the advantage of eliminating some of the problems of contamination when sowing and gives a quicker result. Unfortunately, these wetter seeds are intolerant of drying and have considerably reduced lifespan in storage. Collection of immature seeds should be avoided as their higher MC would make them intolerant to drying and to freezing. A similar response to that described for *Digitalis purpurea* when successful drying and storage are dependent upon the seeds, acquiring mass maturity may be likely [58].
17. At the Millennium Seed Bank (MSB), upon receipt seed is stored in a dry room operating at 15% RH and 15 °C for 7 days before sealing in air-tight glass bottles or cryovials with a rubber or silicone ring in the cap and either stored, respectively, at −20 °C or in the gaseous phase over liquid nitrogen. Seeds can also be usefully stored in folded and stapled paper packets that are convenient for subsequent sterilisation for sowing and should be stored in heat-sealed tri-laminate foil bags [59]. These can be used over the whole range of storage temperatures.
18. Saturated salt solutions of particular RH can be maintained by ensuring the physical presence of crystals. Non-saturated solutions also take up water from the atmosphere, and the user should be aware that their equilibrium relative humidity (eRH) gradually increases over time.
19. Seed can be dried in a thin layer over dried rice for a few days before putting the seed in hermetically sealed tubes and placing them in a refrigerator at 5 °C. The rice should be dried prior to use by placing it in a thin layer on a baking tray and placing it in an oven (conventional or fan) at approximately 100 °C for a few hours. It is ready when it appears to be slightly toasted and should then be placed in an air-tight vessel such as a Kilner jar to cool before use. The only drawback of using dried rice is that we don't know the final RH within the drying jar, and therefore the seed MC can vary with time. Rice is hygroscopic and, with repeated use, will absorb moisture from the atmosphere, and the RH within the desiccator will gradually increase. This can be overcome to some extent by including a small sachet of silica orange as an indicator [60]. If the silica beads begin to turn green, then the dried rice must be either replaced with a new batch or regenerated by redrying as above in an oven. As there is some uncertainty about how many times the dried rice can be regenerated, we would generally recommend using fresh batches of dried rice.
20. Although commonly used as a desiccant, we have reservations about using silica gel, as when freshly regenerated it can produce a very low RH and subsequently overdry seeds and pol-

len. Also, it will absorb moisture from the atmosphere, and unless used with an indicator or is monitored, the resulting eRH will be unknown. Removal of too much water leads to low and potentially damaging MC that can reduce viability or longevity of both pollen [25] and seeds [35].

21. Silica orange sachets contain the indicator methyl violet that changes in colour from orange to green at around 20–25% RH. Blue silica gel granules (that change in colour from blue to pink as RH increases) can no longer be recommended as the indicator in this case is cobalt chloride, which is toxic to humans. It should be emphasised that the silica orange sachets are being used primarily as an indicator, and not as a desiccant.
22. Seeds and pollen are hydroscopic, so will absorb moisture from the ambient air if left in the open, thus it is important that the seed should be stored in an air-tight or hermetically sealed glass vessel. Likewise, sub-zero temperatures produce a very dry atmosphere, so that 'leaky vessels' may also allow overdrying of contents if inadequately sealed. Plastic containers are not suitable, as the seed clings to the walls of the vessel.
23. A number of suitable containers are available for different seed volumes. The aim is to leave as little air space as possible, as the seed will equilibrate with any air left within the container. Large seed accessions will clearly require a larger container than a small accession. A 25 mL 'universal' tube may be suitable for collections of *Cymbidium* seed, for example, whereas a seed collection from a single capsule of a species such as *Restrepia* will only require a tube with a volume of 2 mL, or less (*see* Fig. 3c). One tube can be placed within another vessel for added security, the outer vessel containing a sachet of silica orange as an indicator so that, even if the seal in the seed container leaks, the seed can be seen to be in a dry atmosphere. We have found that Kilner jars (a preserving jar with a rubber seal (*see* Fig. 3d)) are an excellent choice, the rubber seal providing a 'perfect' seal. The rubber seals should be replaced at 10-year intervals to ensure that they are not losing their integrity.
24. Glass vials with silicon seals are suitable for storage in the fridge (5 °C) or freezer (−20 °C) and cryovials if storing in liquid nitrogen vapour (c. −180 °C). Vials should be placed in larger vessels (Kilner jars, cryoboxes or racks) to maintain ease of access to collections under all conditions.
25. Although some facilities, such as the Millennium Seed Bank, possess walk-in cold rooms at −20 °C, because orchid seed is so small, and large numbers of seeds can be stored in a small volume, a small domestic freezer has sufficient capacity to store seeds representing hundreds or thousands of species.
26. We recommend avoiding the use of media for asymbiotic germination that contains activated charcoal when using a con-

ventional binocular microscope with bottom illumination, as it can make the seeds difficult to see, especially if using a grid system under the dish to aid counting. However, incorporating charcoal into the medium can be sometimes be advantageous in making the seeds and protocorms more visible where powerful top or side illumination is employed.

27. Initial levels of germination may be affected by dormancy in some species. For *Epipactis palustris* germination could be improved by combining scarification of the testa with calcium hypochlorite, heat treatment at 27 °C for several weeks, followed by cold stratification for 8–12 weeks at 4–8 °C, and subsequent germination at 20 °C [61].
28. Counting seeds for estimating percentage germination requires some care. Because orchid seeds are tiny, it can be tempting to sow a large number of seeds. If, however, too many seeds are sown, they can either clump together or obscure one another when expanding during germination, or the sheer numbers can be difficult to count.
29. Measuring percentage germination of seeds in a Petri dish or measuring the percentage of seeds with red embryos for the TZ test can be both difficult and time-consuming. There are a number of possible approaches to resolving this problem, and the authors are constantly examining different possibilities and are particularly interested in finding inexpensive solutions. It is, for example, often possible to take a satisfactory photograph down a microscope using a mobile phone camera, although the size of the visual field will be limited. It is generally easier, and less tiring for the operator, to count percentage germination or viability on a computer screen. Inexpensive digital microscopes are becoming widely available and can be connected to a laptop computer. Petri dishes of germinating seeds can be viewed directly, an image captured, and the germinating and non-germinating seeds counted at some later date. Seeds stained with TZ that are suspended in a water droplet can be placed on a microscope slide and the slide placed on a white tile or a piece of paper of the desired background colour that achieves maximum contrast and photographed. A black background will often reveal unstained seeds that are not apparent when viewed against a white background.
30. To aid counting images can be imported into Photoshop or its equivalent using Open Source software, and a grid superimposed to aid counting. A minimum of 100 seeds should be scored, as either germinating or not germinating (empty seed should be disregarded), or for a TZ test having a red embryo or a colourless embryo. If all of the seeds in the image are not going to be scored, squares should be selected using random numbers. Seeds touching the bottom and the left line are

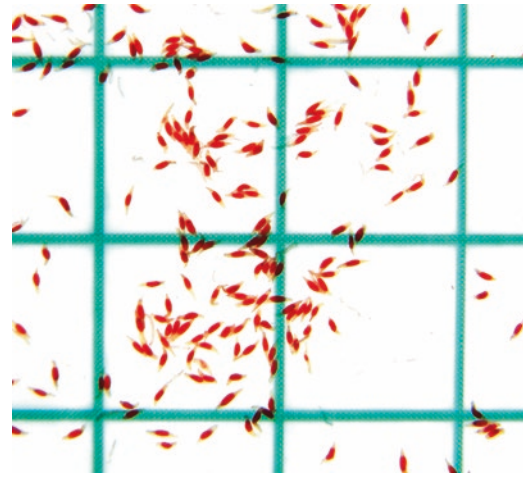


Fig. 7 A grid superimposed over a sample of seeds of *Encyclia caicensis* stained with TZ. Any seed that is touching the left hand or the bottom margin of a square is counted as being inside that square; any seed touching either the right hand or top margin is counted as being inside the adjacent square

counted as being in the square; those touching the top line and the right hand line are outside the square (*see* Fig. 7).

31. Temperatures of both 30 and 40 °C have been reported as effective for the TZ test with both tropical and temperate species. With the species so far tested, innate seed physiology has not been compromised by the higher temperature, although attention may need to be paid to cool temperate species.
32. NaOCl can persist in seeds and may interfere with germination or the reactions involved in the TZ test. To prevent this, seeds should be thoroughly washed in deionized water immediately after NaOCl treatments.
33. The number of seeds in 1 mg varies by as much as a factor of ten: for example, a 1 mg aliquot contains approximately 1100 *Anguloa clowesii* seeds but 177 seeds of *Dendrobium antennatum* (see table in Arditti [32]).

References

1. Koopowitz H (2001) Orchids and their conservation. Timber Press, Oregon
2. Yam TW, Arditti J, Cameron KM (2009) "The orchids have been a splendid sport"—an alternative look at Charles Darwin's contribution to orchid biology. Am J Bot 96:2128–2154
3. Global Strategy for Plant Conservation (2011–2020) https://www.cbd.int/gspc/targets.shtmL
4. Greatwood J (1984) Extract from the proceedings of the International Orchid Commission, March 1984. Am Orchid Soc Bull 53:737
5. Swarts ND, Dixon KW (2009) Perspectives on orchid conservation in botanic gardens. Trends Plant Sci 14:590–598
6. Marks TR, Seaton PT, Pritchard HW (2014) Desiccation tolerance, longevity and seed-siring ability of entomophilous pollen from UK native orchid species. Ann Bot 114:561–569

7. Seaton PT (2009) Orchids in peril. Orchid Rev 117:82–84
8. Ferreira D, Richards M, Seaton PT (2012) Saving Florida's Cigar Orchid. Orchid Rev 120:158–161
9. Yam TW (2013) Native Orchids of Singapore—diversity, identification and conservation. National Parks Board, Singapore Botanic Gardens, Singapore
10. Yam TW, Tay F, Ang P, Soh W (2013) Conservation reintroduction and rediscovery of the native orchids of Singapore—new perspectives on survivorship ecology. In Elliot J, Kurzweil HF, O'Byrne P, Tan KW, Van der Schans AS, Wong SM, Yam TW (Eds) Proceedings of the 20th World Orchid Conference, Singapore, 13–20 November 2011, pp 155–163
11. Pritchard HW, Poynter ALC, Seaton PT (1999) Inter-specific variation in orchid seed longevity in relation to ultra-drying and cryopreservation. Lindleyana 14:92–101
12. Knudson L (1954) Storage and viability of orchid seed. Am Orchid Soc Bull 22:260–260
13. Pritchard HW, Seaton PT (1993) Orchid seed storage: historical perspective, current status, and future prospects for long-term conservation. Selbyana 14:89–104
14. Hay F (2004) The seed viability equations: the normal distribution and probits. Millennium Seed Bank Project Kew. http://data.kew.org/sid/viability/SeedViabilityEquationsFHDec04.pdf. Accessed 9 Sept 2016
15. Hay FR, Mead A, Bloomberg M (2014) Modelling seed germination in response to continuous variables: use and limitations of probit analysis and alternative approaches. Seed Sci Res 24:165–186
16. Newton R, Hay F, Probert R (2014) Protocol for comparative seed longevity testing. Technical Information Sheet_01. Millennium Seed Bank Partnership: Ardingly
17. Hay FR, Adams J, Manger K, Probert R (2008) The use of nonsaturated lithium chloride solutions for experimental control of seed water content. Seed Sci Technol 36:737–746
18. Hay FR, Merritt DJ, Soanes JA, Dixon KW (2010) Comparative longevity of Australian orchid (Orchidaceae) seeds under experimental and low temperature storage conditions. Bot J Linn Soc 164:26–41
19. Ellis RH, Roberts EH (1980) Improved equations for the prediction of seed longevity. Ann Bot 45:13–30
20. Pritchard HW, Dickie JB (2003) Predicting seed longevity: the use and abuse of seed viability equations. In: Smith RD et al (eds) Seed conservation: turning science into practice. Royal Botanic Gardens, Kew, pp 653–721
21. Vendrame WA, Carvalho VS, Dias JMM, Maguire I (2008) Pollination of *Dendrobium* hybrids using cryopreserved pollen. Hortscience 43:264–267
22. Franchi GG, Piotto B, Nepi M, Baskin CC, Baskin JM, Pacini E (2011) Pollen and seed desiccation tolerance in relation to degree of developmental arrest, dispersal, and survival. J Exp Bot 62:5267–5281
23. Johansen BO (1990) Incompatibility in *Dendrobium* (Orchidaceae). Bot J Linn Soc 103:165–196
24. Millner HJ, McCrea AR, Baldwin TC (2015) An investigation of self-incompatibility within the genus *Restrepia*. Am J Bot 102:487–494
25. Pritchard HW, Prendergast FG (1989) Factors influencing the germination and storage characteristics of orchid pollen. In: Pritchard HW (ed) Modern methods in orchid conservation: the role of physiology, ecology and management. Cambridge University Press, Cambridge, pp 1–16
26. Cheyne P (2003) Access and benefit-sharing agreements. In: Smith RD, Dickie JB, Linington SH, Pritchard HW, Probert RJ (eds) Seed conservation turning science into practice. The Royal Botanic Gardens, Kew, pp 5–26
27. Yam TW, Ghani AKA, Ichihashi S, Thame A, Rao AN, Avadhani PN, Nair H, Hew CS, Arditti J, Tatarenko IV (2007) Time from pollination to fruit ripening, seed maturation and germination. In: Cameron KM, Arditti J, Kull T (eds) Orchid biology: reviews and perspectives, IX. Botanical Garden Press, New York, pp 433–506
28. Arditti J (1992) Fundamentals of orchid biology. Wiley, New York, p 539
29. Ferdy JB, Loriot S, Sandmeier M, Lefranc M, Raquin C (2001) Inbreeding depression in a rare deceptive orchid. Can J Bot 79:1181–1188
30. Cozzolino S, Widmer A (2005) Orchid diversity: an evolutionary consequence of deception? Trends Ecol Evol 20:487–494
31. Sletvold N, Grindeland JM, Zu P, Ågren J (2012) Strong inbreeding depression and local outbreeding depression in the rewarding orchid *Gymnadenia conopsea*. Conserv Genet 13:1305–1315
32. Arditti J (1992) Fundamentals of orchid biology. Wiley, New York, pp 303–304
33. Wexler A, Hasegawa S (1954) Relative humidity-temperature relationships of some saturated salt solutions in the temperature range 0 to 50 °C. J Res Nat Bureau of Standards 53, No. 1, Research paper 2512

34. Popova E, Kim HH, Saxena PK et al (2016) Frozen beauty: the cryobiotechnology of orchid diversity. Biotechnol Adv 34:380–403
35. Seaton PT, Hailes NSJ (1989) Effect of temperature and moisture content on the viability of *Cattleya aurantiaca* seed. Modern methods in orchid conservation: the role of physiology, ecology and management. Cambridge University Press, Cambridge, pp 17–29
36. Seaton PT, Pritchard HW (2008) Life in the freezer. Orchids 67:762–773
37. Popova EV, Han SH, Moltchanova E et al (2013) Systematic overestimation of Salicaceae seed survival using radicle emergence in response to drying and storage: implications for *ex situ* seed banking. Acta Physiol Plant 35:3015–3025
38. Arditti J (1982) In: Arditti J (ed) Appendix: Orchid seed germination and seedling culture—a manual in orchid biology—reviews and perspectives II. Cornell University Press, Ithaca, NY, pp 242–370
39. Nadarajan J, Wood S, Marks TR, Seaton PT, Pritchard HW (2011) Nutritional requirements for in vitro seed germination of 12 terrestrial, lithophytic and epiphytic orchids. J Trop For Sci 23:204–212
40. Gamborg OL, Mille RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 1113–1127
41. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15: 473–497
42. Knudson L (1946) A new nutrient solution for the germination of orchid seeds. Bot Gaz 73:1–25
43. Van Waes JM, Debergh PC (1986) *In vitro* germination of some Western European orchids. Physiol Plant 67:253–261
44. Malmgren S (1996) Orchid propagation: theory and practice. In: Allen C (ed) North American native orchids: propagation and production. North American Native Terrestrial Orchid Conference, Germantown, Maryland, pp 63–71
45. Seaton PT, Ramsay M (2005) Growing orchids from seed. Royal Botanic Gardens, Kew
46. Seaton PT, Ramsay M (2009) Cultivo de orquídeas por semillas. Royal Botanic Gardens, Kew
47. Machado-Neto NB, Custódio CC (2005) A medium for non-commercial sowing of orchid seed. Selbyana 26:316–317
48. Maguire JD (1962) Speed of germination aid in selection and evaluation of seedling emergence and vigor. Crop Sci 2:176–177
49. Hosomi ST, Santos RB, Custodio CC, Seaton PT, Marks TR, Machado-Neto NB (2011) Preconditioning *Cattleya* seeds to improve the efficacy of the tetrazolium test for viability. Seed Sci Technol 39:178–189
50. Hosomi ST, Custodio CC, Seaton PT, Marks TR, Machado-Neto NB (2012) Improved assessment of viability and germination of *Cattleya* (Orchidaceae) seeds following storage. In Vitro Cell Dev Biol Plant 48:127–136
51. Custódio CC, Marks TR, Pritchard HW, Hosomi ST, Machado-Neto NB (2016) Improved tetrazolium viability testing in orchid seeds with a thick carapace (*Dactylorhiza fuchsii*) or dark seed coat (*Vanda curvifolia*). Seed Sci Technol 44:1–12
52. Lallana VH, Garcia LF (2013) Pre-treatments effect in *Trichocentrum jonesianum* seeds viability test. Invest Agrar 15:120–132
53. Soares JS, Rosa YBCJ, Tatara MB, Sorgato JC, Lemes CSR (2014) Viability identification of orchid seeds by the tetrazolium test. Semina Ciênc Agrár 35:2275–2284
54. Wood CB, Pritchard HW, Miller AP (2000) Simultaneous preservation of orchid seed and fungal symbionts using encapsulation-dehydration is dependent on moisture content and storage temperature. CryoLetters 21:125–136
55. Sommerville KD, Siemon JP, Wood CB, Offord CA (2008) Simultaneous encapsulation of seed and mycorrhizal fungi for long-term storage and propagation of terrestrial orchids. Aust J Bot 56:609–615
56. Saiprasad GVS, Polisetty R (2003) Propagation of three orchid genera use in encapsulated protocorm-like bodies. In Vitro Cell Dev Biol Plant 39:42–48
57. Walters C, Reilley AA, Reeves PA, Baszcak J, Richards CM (2006) The utility of aged seeds in DNA banks. Seed Sci Res 16:169–178
58. Hay FR, Probert RJ (1995) Seed maturity and the effects of different drying conditions on desiccation tolerance and seed longevity in Foxglove (*Digitalis purpurea* L.) Ann Bot 76:639–647
59. Gold K, Manger K (2014) Selecting containers for long-term seed storage. Technical Information Sheet_06. http://www.kew.org/sites/default/files/06-Containers%20web.pdf
60. Sutcliffe V, Adams J (2014) Low-cost monitors of seed moisture status. Technical Information Sheet_07. http://www.kew.org/sites/default/files/07-Low-cost%20moisture%20monitors%20web.pdf
61. Rasmussen HN (1992) Seed dormancy patterns in *Epipactis palustris* (Orchidaceae): requirements for germination and establishment of mycorrhizal. Physiol Plant 86:161–167

Part II

Orchid Micropropagation

Chapter 5

Orchid Seed Germination and Micropropagation I: Background Information and Related Protocols

Edward C. Yeung, Jungmin Park, and Indra S. Harry

Abstract

Seed germination and micropropagation of orchids are key approaches to orchid conservation and commercial production. The general concepts and methodologies to orchid asymbiotic and symbiotic seed germination and regeneration are clearly established in the literature. This chapter discusses the general techniques and common culture media used in in vitro studies. Also, frequently used additives and related protocols on sterilization techniques and viability staining of seeds are documented.

Key words Asymbiotic seed germination, Symbiotic seed germination, Culture media, Media additives, Sterilization protocols, Viability staining

1 Introduction

Tissue culture of plant cells, tissues, and organs has a long history. Accounts regarding the history of plant tissue culture can be found in reviews by Krikorian and Berquam [1], Gautheret [2], Bhojwani and Razdan [3], Gamborg [4], Thorpe [5], Dodds and Roberts [6], Trigiano and Gray [7], and Vasil [8]. The key historical event related to modern day plant tissue culture and biotechnology occurred in 1902 when the German plant physiologist Gottlieb Haberlandt reported his experiments on the culture of plant cells in his address to the German Academy of Science [1]. He provided the first well-documented case of plant cell culture using the Knop's salt solution in hanging drop cultures [1]. Although the outcome did not show the results he expected, Haberlandt predicted that it would be possible to obtain embryos from somatic cells and that embryo sac fluid may be able to induce cell divisions in culture cells. His experiments and predictions clearly established the concept of totipotency of plant cells and drew attention and provoked others to follow. Hence, Haberlandt is regarded as the father of plant tissue culture. Haberlandt also demonstrated a new experimental approach, i.e.,

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_5,

in vitro culture, to study the nature of plant cells. The new approach allows the study of the behavior of isolated cells/tissues/organs away from the influence of the whole organism. One can also test the effects of various additives on in vitro cultured cells.

One of the key factors in the success of an in vitro study is that explants require a proper nutrient milieu in order to maintain and promote growth processes. Culture media used in the late nineteenth and early twentieth centuries were relatively simple. Many were initially based on the recipes by Knop's and Pfeffer's inorganic nutrient solutions [9]. The early successes were mainly due to the use of explants with existing apical meristems or meristematic tissues such as seed, root, and shoot tips. Since meristematic tissues, especially apical meristems, are self-perpetuating structures, they can be maintained and proliferated in simple media containing inorganic nutrients, vitamins, and an energy and carbon source such as sucrose. The addition of organic additives, e.g., vitamins, coconut water, and amino acids from casein hydrolysates, further improved the success of in vitro cultures. Although reports on successful regeneration were noted in 1939 as exemplified by the work of White and others (*see* [10]), the identification of a cytokinin, kinetin, in 1955 by Miller et al. [11] and the demonstration of the importance of auxin-cytokinin ratio in shoot and root regeneration by Skoog and Miller [12], together with the formulation of a "high" salt medium by Murashige and Skoog in 1962 [13], proved to be important milestones for plant regeneration studies. Shoots and roots are readily regenerated from callus masses. Since then, successes in plant micropropagation have been reported in diverse plant species.

The study of orchid propagation via seed germination has a long history and has been an undertaking for orchid enthusiasts and growers. The history of orchid seed germination is detailed by Yam et al. [14, 15]. At the turn of the twentieth century, the studies by Bernard provided valuable information on the role of mycorrhiza in orchid seed germination [14]. His work in part led to the success in asymbiotic seed germination studies reported by Knudson in 1922. Based on the work of Bernard, Knudson suspected that the mycorrhizal fungi may be supplying certain sugars that are beneficial to seed germination. He tested the hypothesis by adding sucrose to the medium and achieved success in establishing in vitro asymbiotic germination of *Cattleya* seeds [16, 17]. His studies demonstrated the usefulness of sucrose as a nutrient source in culture. Knudson's studies promoted subsequent studies of asymbiotic seed germination of many orchids. The Knudson C medium published in 1946 [18] is still used today. Recent studies on seed germination and plantlet development were listed in an extensive review by Teixeira da Silva [19] and Kauth et al. [20] who discussed current issues on orchid seed germination and related techniques (*see* also Chap. 27 for recent references).

Micropropagation of orchids began to take shape in the mid-twentieth century similar to other flowering plants. A detailed account on the history of orchid micropropagation studies is provided by Yam and Arditti [15]. Chapter 7 serves as an overview on micropropagation studies and methods. In their reviews, Teixeira da Silva [19] and Chugh et al. [21] documented earlier successes in micropropagation studies, and Chap. 27 provides updated published reports on this topic. Also a newly published comprehensive text on orchid micropropagation by Yam and Arditti [22] contains valuable source of information for orchid micropropagation.

The aim of this chapter is to provide information about the common media used in orchid seed germination and micropropagation studies. We also draw attention to a new approach in designing and optimizing one's own medium. Furthermore, when compared to tissue culture media used for other flowering plants, different additives, such as coconut water, banana and potato extracts, and charcoal, are often included in orchid culture media. Some of these additives have positive effects on protocorm and plantlet development. The addition of chitosan to orchid cultures has attracted attention in recent years. Hence, in this chapter, information on the commonly used additives in orchid seed germination and micropropagation are also presented. Related techniques, for seed sterilization methods and viability staining, are also included as they are useful for seed germination and in vitro culture studies. The companion chapter, Chap. 6, provides detailed information on the composition of commonly used media for orchid in vitro studies and recent publications on chitosan and other additives used.

For an in-depth discussion of medium components, the complexity of interactions among various factors, such as pH during media preparation and related techniques in plant tissue culture, and for further details, readers are urged to consult texts, such as those by Bhojwani and Razdan [3], de Fossard [23], George et al. [24], Trigiano and Gray [7], and Vasil and Thorpe [25]. Novices and students learning tissue culture should consult books by Gamborg and Phillips [26], Evans et al. [27], and Smith [28].

2 Practical Issues Regarding Media Preparation

Successes in orchid seed germination and micropropagation depend on many factors, such as the choice of explants, media used, handling techniques, equipment, and culture conditions, including light quality and quantity and temperature, as well as growth facilities. Media preparation certainly plays an important role in the success of the experiments, and this is also the primary focus of this chapter. The information presented below serves as reminders and highlights useful information in media preparation.

2.1 Optimizing Medium for Specific Applications

When embarking on an in vitro culture project, the simplest approach in selecting a medium is to search for suitable protocols for closely related species. If such media are available, one can test media for suitability, and if warranted, the selected medium can be further optimized. For seed germination and micropropagation studies, media used for orchid species can be found in [19, 21, 22] and Chap. 27. Media composition and related information are documented in Chap. 6 and [22].

Investigators can certainly design their own medium for a particular species and explant of interest. This will generate optimum and desirable growth responses, and at the same time, one can gain a better theoretical understanding of how the selected explant responds to the nutrient components of the medium. The Murashige and Skoog [13] medium is the most common medium used in plant tissue culture. The MS formulation was based on careful chemical analyses of tobacco cells, and it took 5 years to standardize a protocol [13]. The composition is based on a one-factor-at-a-time (OFAT) methodology in which medium components are tested one at a time, until the growth of the callus is optimized. This is a common approach used in developing medium formulation such as the Gamborg B5 medium for suspension cultures of soybean root cells [29] and the Vacin and Went [30] formulation for epiphytic orchids. The main drawback of the OFAT approach is that it cannot identify interactions among nutrient components and suitable nutrient proportion, e.g., N/P/K ratio in a medium that affect desirable growth and development features [31]. This is also a time-consuming and tedious approach.

Instead of using the OFAT approach, Ichihashi and colleague [32–35] published a series of papers using the "triangle" method by Hamner [36, 37] to study effects of variation in concentration of selected groups of cations or anions in *Bletilla striata* seed germination and plantlet development. This approach enabled the selection of optimal ion concentrations for a specific stage of seedling development. In the last few years, another approach, i.e., the response surface methodology (RSM), has been used to optimize media for improved growth of explants. The focus is to study the effects of MS mineral nutrients and to determine the most critical salts and/or their combination for improved plant growth responses. Using this systematic and modeling approach, MS medium components can be grouped into various factors. For example, Poothing and Reed [38] used five groups, namely, NH_4NO_3, KNO_3, Mesos, micronutrients, and Fe-EDTA, for optimizing the growth requirements of micropropagated raspberries. Since many orchid species are economically important, it is prudent to design appropriate media for germination and micropropagation. For recent publications utilizing the RSM, one can consult the following publications by Halloran and Adelbert [31], Poothong and Reed [38, 39], Reed et al. [40], and Wada et al. [41].

2.2 Medium Preparation

Once a medium formulation is known, one can prepare one's own or purchase pre-mixed media. When preparing one's own medium, be sure to use quality water and chemicals. It is important to double check the chemical formula before use, as the same chemical compound can have different molecular weights, due to differences in hydration state (*see* **Note 1**). In preparing a medium, although it is easy to weigh chemicals using gram per liter as listed in media, it is important to appreciate the chemicals used based on molar concentrations [23]. Molarity is defined as the number of moles of a substance contained in 1 L of solution. One mole of each compound in 1 L of solution has the same number of molecules. Hence, when expressed in molarity, one can easily determine the quantity of compounds or ions present within and between media [23] (see Chap. 6). For example, the amount of ammonium and nitrate ions or their ratio can have profound effects on protocorm development and bioactive product accumulation [42]. By knowing the molarity of compounds and ions used, one can recognize the importance of a compound or an ion in a cell culture system.

For ease of routine media preparation, stock solutions are prepared [5, 26]. Macroelements are usually prepared as 10× stock, and microelements are usually prepared as 100× stock and kept at 4 °C. Organics are prepared as 1000× stock and aliquot into 1 or 10 mL portions and kept frozen at −20 °C. In order to avoid precipitation of macro- and microelements, separate stock solutions such as calcium salts should be prepared and added to the mix (*see* **Note 2**). Iron is an important micronutrient. Since iron salts tend to precipitate gradually from media, chelated forms are used [43]. The sodium ferric salt of ethylenediaminetetraacetic acid [FeEDTA] is the most common form used in plant tissue culture. This iron stock solution can be prepared by mixing equimolar solutions of ferrous sulfate and sodium EDTA followed by autoclaving to generate a stable soluble complex [26] (*see* **Note 3**). The sodium ferric salt of EDTA and the other chelated ion salt, i.e., Sequestrene 330 Fe iron chelate, are also commercially available [43].

Plant growth regulators play important roles in in vitro morphogenesis, such as shoot and root regeneration. Stock solutions are usually prepared for the ease of media preparation. It is important to realize that solutions prepared for different growth regulators have different shelf life; prolonged storage and use of growth regulator stock solutions are not recommended except for synthetic auxins, such as 2,4-D and a majority of cytokinins. Hart et al. [44] studied the stability of adenine-based cytokinins, i.e., trans-zeatin, 2iP, kinetin, benzyladenine, and m-topolin, and found that they are stable. For a 1.0 mg/L in 0.05 N KOH stock solution, there was no significant degradation even after autoclaving for 30 min [44]. Other growth regulators, i.e., gibberellins, abscisic acid, and indoleacetic acid, should be filter-sterilized and

added to warm autoclaved media before dispensing. In general, when preparing growth regulator stock solutions, we recommend that the stock solutions be filter-sterilized and stored in sterile test tubes at 4 °C, with the date of preparation clearly written on the container (*see* **Note 4**).

Sucrose is the most commonly used carbohydrate and serves as source of energy and carbon for culture explants. For theoretical studies, purified laboratory grade sucrose should be used in order to avoid variability in results. The growth of banana plantlets was significantly better when laboratory grade sucrose was used [45]. Commercial table cane sugar can be used depending on the nature of the study. The cost of table sugar is certainly lower than the purified form and is a low-cost alternative (*see* **Note 5**).

Furthermore, it is also important to note that sterilization by autoclaving media causes hydrolysis of sucrose [46], resulting in glucose and fructose formation. The hydrolysis is pH dependent [47]. It also appears that iron-EDTA catalyzes the breakdown of sucrose during the autoclaving process [48]. Moreover, the inclusion of activated charcoal in media catalyzes sucrose hydrolysis [*see* Subheading 4.4]. Besides sucrose, other carbohydrates can also play a role in orchid seed germination [48–51]. More recently, Johnson and Kane have provided useful insights into the effects of light, nutrient, and carbohydrates on seed germination [52, 53].

Prior to sterilization of media, the pH of the media needs to be adjusted to a range between 5 and 6, using a pH meter. The acidity of a medium can influence ion uptake by the explants [43]. Hence, improper use and calibration of a pH meter can lead to serious errors in pH measurements. It is imperative that the pH meter is in good working condition. Prior to performing pH measurements, the meter should be checked using standard pH reference solutions ensuring the electrode and the meter are recording accurate measurements. To adjust the pH, a 0.2 N HCl and/or NaOH solution is used (*see* **Note 6**).

As discussed in Chap. 7, different gelling agents are available with agar and gellan gum being the most common. Gellan gum is marketed under trade names such as Phytagel [Sigma] and Gelrite [Merck]. Agar is a complex polysaccharide extracted and purified from red algae. It is known to contain different types and amount of impurities and can have batch-to-batch variations, even from the same supplier. It is imperative that one obtains purified agar from a reliable source. Gellam gum is a good alternative to agar. The polysaccharides are obtained from the bacterium *Pseudomonas elodea*, and gel strength is related to the concentration of cations, i.e., Mg^{2+} and Ca^{2+}, in the medium. It is a colorless gel, thus allowing for easy examinations of explants. The selected gelling agent contributes to the complexity of a culture medium [*see* 43].

In order to reduce the cost of in vitro culture, other matrixes, such as coir fibers, can be used [54]. A direct sowing of seeds non-axenically using peat moss pre-inoculated with orchid mycorrhizal

fungus resulted in ex vitro symbiotic seed germination of *Spathoglottis plicata* [55].

For common culture media such as MS, ready-made preparations are available commercially. Although the use of these products saves time, care should be taken when using these products. Since commercial media are normally in a powder form and the recommended storage is 4 °C, it is imperative that the media be allowed to warm up to room temperature before opening the cap in order to avoid condensation. Powder within the medium will begin to react with one another when moisture becomes available. Hence, it is a good practice to use up the contents as soon as possible (*see* **Note 7**).

2.3 Media Sterilization

Sterilization of media is a must prior to in vitro cultures. Different sterilization techniques are available depending on the nature of the culture. For small batches of liquid media, especially with heat-sensitive compounds, filter sterilization method can be used. Different sizes of sterilization units with 0.22 μm membrane are commercially available. Autoclaving, using pressurized heat, is the most common method. In general, the liquid is autoclaved at 121 °C for no longer than 20 min. For proper sterilization, an appropriate size container should be used in conjunction with a certain volume of medium. This is to avoid "boiling" over and loss of medium during autoclaving. The container should have about 40% head space in order to avoid boiling over. We routinely autoclave 1 L of medium in a 2 L wide-bottom flask. When autoclaving 2 L or more, the gelling agent should be melted prior to autoclaving to ensure proper homogeneous sterilization of components. Also magnetic stirring bars can be autoclaved with the media to enable mixing of the components or additives before pouring.

Prolong autoclaving can have a negative effect on medium components, such as sucrose. If there is a significant change in the color of the medium before and after autoclaving, this indicates possible heat alteration to some components. One needs to have a proper understanding of the heat sensitivity of the chemicals used. Some adjustment to the autoclaving procedure may be necessary. Depending on the components of a medium, the pH of a medium can vary before and after autoclaving [43]. It is likely that autoclaving can induce additional changes, which can have positive or negative effects on culture explants.

2.4 Selecting Proper Culture Vessels and Their Storage

A great variety of culture vessels are available commercially. The selection is based on the size of the explants and the duration and nature of the experiments. For small explants, such as seeds and small tissue pieces, Petri dishes are commonly used. Proper selection of containers saves physical space needed to maintain the cultures in the culture room. Since containers are manufactured using different materials, such as glass, polystyrene, polypropylene, and polycarbonate, they have different clarities, and this can affect the growth of explants.

Media should be used soon after preparation, as certain components, such as growth substances, can deteriorate overtime. In humid environments with a potentially higher contamination rate, it is advisable to allow the media to rest for a few days to ensure that there is no unwanted microbial growth. If longer-term storage of media is needed, the culture vessels, usually Petri dishes, should be stored at 4 °C in darkness to avoid pH changes [43]. In addition, condensation is often present during the storage of Petri plates; therefore, they should be carefully checked for microbial growth before use.

Plates can be adequately sealed with Parafilm®, which allows for gas exchange. Several commercial, presterilized containers come with lids containing 0.2 μM PTFE (Teflon) filters, which allow for gas exchange. Containers should be selected based on clarity, permeability, explant/seedling size and whether or not they can be re-autoclaved. Overall, containers should be cost effective for the species being propagated.

2.5 General Safety Concerns

In any laboratory, proper safety procedures need to be established [see recommendations by Gamborg and Phillips [26]]. For medium preparation, a proper understanding on the nature of the chemicals and how to handle these chemicals is a must. Material safety data sheet information for chemicals used should be readily available to all laboratory personnel. It is imperative to understand how to operate an autoclave and take necessary precautions when handling hot liquids.

Clean benches, like laminar airflow cabinets, are often used in medium preparation and the handling of explants. If UV light is present within a clean bench, one has to ensure that it is turned off before using it. If open flames, such as alcohol lamps or a Bunsen burner, are used within a clean bench for sterilization of tools or surfaces of culture vessels, etc., extra care is needed. Since 95% ethanol is often used in conjunction with tool sterilization, the solution can catch fire if due care is not exercised. Extra care should be taken to avoid spillage of ethanol and having the solution too close to the flame. Furthermore, a fire extinguisher or appropriate chemicals, such as baking soda, have to be available in case a fire erupts due to alcohol spills during the course of an operation. Improper "flaming" of tools after dipping in ethanol can result in burning one's fingers. Hence, if possible, a glass bead heat sterilizer can be used instead of an open flame (*see* **Note 8**).

When handling pathogenic material, especially virus-infected explants, proper handling of explant is essential in order to avoid contaminations and spread of pathogens. Tools, such as dissecting knives, etc., must be properly disinfected in order to avoid cross infection and the spread of pathogens (*see* **Note 9**). Pathogenic material needs to be discarded properly by placing them in autoclavable bags followed by autoclaving. After completion of the

experiment, the work area has to be properly disinfected. Similarly, all transgenic materials need to be autoclaved prior to their disposal to avoid unnecessary escape of transgenic materials to the natural environment.

3 Culture Media and Related Protocols

3.1 Media for Asymbiotic Seed Germination

Different culture media have been formulated over the years. Knudson's success in obtaining seed germination as early as 1922 encouraged others to follow. For asymbiotic seed germination, the current trend involves the use of developing seeds, approximately 1/2 to 2/3 into seed maturation and culture on a relatively simple medium with sucrose and without growth regulators. As discussed in Chap. 1, midway into seed development, endogenous inhibitor level is low, and the protective seed coat has not fully matured. These conditions favor seed germination as long as proper nutrients, especially sucrose, are present. One added advantage for immature seed culture is that a vigorous seed sterilization procedure is usually not necessary as the internal cavity of the capsule is usually free of other microorganisms. One potential negative aspect of culturing immature seeds from green capsules is that seeds are at different stages of maturity. Since fertilization is asynchronous, differences in seed maturity within the same capsule can contribute to variability of results.

For mature seeds, especially terrestrial species, asymbiotic seed germination is more difficult. This can be due to the presence of endogenous inhibitors, such as abscisic acid [56–58], and the mature seed coat with a carapace can act as a physical barrier [59], slowing the germination process. To remedy this situation, different seed pretreatments, such as sodium hypochlorite solution treatment [*see* Subheading 3.5.1], have been reported that can enhance seed germination. To encourage seed germination, exogenous plant growth regulators and additives, such as coconut water, can be added. Not all seeds can be readily germinated asymbiotically, and an obligate requirement for proper mycorrhizal establishment may be needed.

The commonly used medium formulations for seed germination are listed in Chap. 6. Knudson's C formulation [18] is still used for seed germination studies to date. Vicin and Went [30] realized poor pH stability of Knudson's medium, and they devised a formulation with a more stable pH for orchid cultures. Their medium is still popular among orchidologists interested in seed germination. Although the MS medium [13] was designed for the growth of tobacco callus, it is the most common medium used for seed germination and micropropagation studies of orchids, albeit the medium is often diluted for different species studied. Since then other medium formulations have been published each with

specific objective aiming at seed germination and early development of the seedlings. Some of the more notable studies are listed below, and Chap. 6 details media compositions of the commonly used basal media. In 1957 Thomale [60] and subsequently, Fast [61] developed media for *Paphiopedilum* species. The medium devised by Lindemann et al. [62] was intended for meristem culture of *Cattleya*. Mitra et al. [63] developed a medium for the study of protocorm formation. Harvais [64, 65] and Malmgren [66, 67] devised media focusing on the optimization of asymbiotic propagation of *Cypripedium* species. Ichihashi and Yamshita [32] an Ichihashi [33–35] optimized media for *Bletilla* seed germination. Van Waes and Debergh [68] developed a medium for in vitro germination of some Western European orchids. The above media often serve as basal media for other reported studies. Besides the media indicated above, many more media are available in the literature for in vitro studies. Teixeira de Silva [19] detailed the literature on orchid seed germination with information on media used until 2005. Samples of recent publications showing successes in asymbiotic seed germination of immature and mature seeds are shown in Chap. 27.

A special note is needed for the fertilizer Hyponex medium first reported by Kano [69] (also *see* Chap. 6). Media containing Hyponex are commonly used by Asian investigators. The Hyponex medium is prepared using Hyponex fertilizer powder with a N-P-K ratio of 6.5-6-19. This product is manufactured by Hyponex Japan and is not readily available elsewhere, especially in North America. However, other Hyponex formulations are commercially available elsewhere and have been used for seed germination and plantlet development studies (for additional references, see Winarto et al. [70]).The Hyponex medium is simple to prepare. To prepare 1 L of medium, depending on the Hyponex formulation, 1.0–3.0 g of fertilizer is used, together with 25–30 g sugar and 7.0–8.0 g agar, pH 5.7. Additives such as coconut water, potato and banana extracts, and activated charcoal which are often added as micronutrients are not present/listed in the Hyponex formula.

3.2 Microbiological Media Agar Used in Fungal Isolation and Symbiotic Seed Germination

The process of in vitro symbiotic seed germination requires the isolation and identification of mycorrhizal fungi of interest. The method detailed by Warcup and Talbot [71] provides the basic technique in the isolation of mycorrhizal fungi associated with orchids. The use of the seed baiting technique [72, 73] enables the identification of specific compatible mycorrhizal fungi that are important to seed germination. For additional information and methodologies, readers can consult Chaps. 2 and 3.

Once a fungus is isolated, it can grow on selected media. Different fungi prefer different carbon sources. Although the cellulose agar medium was used earlier for the study of symbiotic germination of orchid seeds [74, 75], the oatmeal and potato media

appear to be more commonly used for symbiotic seed germination. Commercial preparations for various fungal culture media are available from different sources, and additional nutrients are often added to the basal medium. The protocols for the more commonly used media are detailed below.

3.2.1 The Oatmeal Agar (Warcup [76])

Blend oatmeal flakes 2.5–3.0 g in 200 mL of water and bring it to 1 L of water without sieving, and add 10–12 g/L agar; autoclave the medium, and dispense into plates.

3.2.2 The Potato Dextrose Agar Medium

The potato extract is first prepared by boiling 200 g of washed slices of potato in approximately 1 L of water for 30 min. The soupy solution is decanted and filtered through cheese cloth. Dextrose 20 g/L and agar 15 g/L are then added. The volume is adjusted to 1 L followed by autoclaving. Instead of using fresh potato, potato starch 4 g/L can be used.

3.2.3 Fungal Isolation Medium and Associated Modified Oats Medium for Symbiotic Seed Germination (Clement et al. [77])

Fungal isolates from different sources are cultured on the "fungal isolating medium" consisting of 0.5 g/L $Ca(NO_3)_2{\cdot}4H_2O$, 0.2 g/L KH_2PO_4, 0.1 g/L KCl, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L yeast extract, 5 g/L sucrose, and 10.0 g/L agar.

With the establishment of fungal isolates, orchid seeds are then prepared for symbiotic germination. Seeds are surface sterilized for 3–10 min in 3–5% sodium hypochlorite solution containing 0.01% of a wetting agent. Seeds are then washed onto sterile Whatman filter paper using a sterile vacuum filtration unit. The paper squares with seeds are then placed onto "modified oats medium" for germination, and selected fungal isolate is inoculated at the edge of the medium. The modified oats medium is composed of 0.2 g/L $Ca(NO_3)_2{\cdot}4H_2O$; 0.2 g/L KH_2PO_4; 0.1 g/L each of KCl, $MgSO_4{\cdot}7H_2O$, and yeast extract; 2 g/L sucrose; 10 g/L agar; and 3.5 g/L powdered rolled oats.

3.3 Media for Micropropagation Using Protocorm and Other Explants

Although seed germination is important to orchid propagation and conservation, in order to conserve and propagate genotypes with desirable characteristics, micropropagation from individual plant parts is necessary. For orchid micropropagation studies, the MS formulation is the most common medium used. Other media with broader applications for monocots and dicots have been devised and used in the study of orchid micropropagation. These include the Schenk and Hildebrandt medium [78], the B5 medium [26], and DKW medium [79]. These basal media are detailed in Chap. 6. Recent literature on orchid micropropagation can be found in [19, 21] and in Chap. 27.

In contrast to a majority of seed germination studies, different plant growth regulators are added to culture media to promote regeneration. 1-Naphthaleneacetic acid and 6-benzylaminopurine (BA) appear to be the most common growth regulators used in

regeneration studies. In recent years, meta-topolin, an aromatic natural cytokinin, has shown positive effects with orchid and other species micropropagation [*see* 80]. This growth substance can be used instead of BA.

3.4 Commercial Media for Multiplication, Maintenance, and Replating of Orchid Plantlets

After plantlet formation, commercial media formulations are available for the maintenance, replating, and multiplication of plantlets. Companies, such as Sigma, PhytoTechnology Laboratories, and Duchefa Biochemie, manufacture their own trademark media for researchers and consumers. The compositions of media are clearly documented on their websites. In general, additives, such as banana powder, activated charcoal, and peptone, are present. These media are buffered using 2-[*N*-morpholino] ethanesulfonic acid (MES), which provides a stable pH for long-term cultures. Investigators can examine the composition of commercial media and determine the suitability of these media for their own work.

3.5 Protocol for Decontamination of Seeds and Other Explants

3.5.1 Seed Sterilization

Proper sterilization of seeds is an important step toward the success of seed germination and micropropagation studies. The amount of time required and the concentration of disinfectants used depend on the sensitivity of the tissues toward the disinfectants and how difficult it is to remove the foreign organisms. The most common disinfectant solutions are sodium hypochlorite (0.5–5%), calcium hypochlorite (9–10%), and commercial bleach solutions (10–20%) (*see* **Note 10**). This can be augmented by a short treatment in solutions of ethanol [70–90%], hydrogen peroxide (10%), and mercuric chloride only when necessary (0.1–0.2%) (*see* **Note 11**).

For immature and mature seed germination studies, if maturing capsules are still intact and have not yet split open, capsules can be sterilized using a strong 20–30% commercial bleach solution with a drop of wetting agent, i.e., Tween 20, ensuring proper wetting of surfaces for decontamination. The capsules can be stirred gently in the bleach solution for 15 min, rinsed in sterile water, then dipped in 70% ethanol for another minute, and washed with several changes of sterile water. Open the capsule in a clean bench, and transfer seeds to appropriate media.

For mature seeds from dehisced capsules, the seeds can be processed using small sterile filter paper packets [81] or placed in nylon bags. The packets with seeds are placed directly into a sterilizing solution such as 5–10% bleach solution with a drop of surfactant, i.e., Tween 20, stirred gently for about 10 min. Inside a laminar flow bench, the paper packets are then washed several times with sterile distilled water before opening the packets and transferring seeds onto appropriate medium [81]. As an alternative, the seeds can be placed directly into a hypochlorite/bleach solution and stirred gently. Inside a clean bench, the seeds are poured into a sterile filter unit with funnel and flask connecting to a vacuuming device. The bleach is removed with a mild suction (vacuum), and the seeds are then washed

with several changes of sterile water and placed onto appropriate medium [77]. Seeds can also be decontaminated using the syringe method as detailed by Ponert et al. [82].

For seeds that are difficult to disinfect, such as those from the genus *Disa*, Thompson et al. [83] designed the following regime for seed disinfection. The seeds are first sprayed with 70% EtOH, followed by a 20 min treatment of 1.75% NaOCl +1% Tween 20. This is immediately followed by a 10 min treatment of 1% Benlate®. The seeds are then rinsed 3× with sterile dH_2O. More recently, Jevsnik and Luthar [84] introduced a new protocol for seed decontamination. Seeds are disinfected in a solution of dichloroisocyanuric acid, sodium salt, 16.6 g/L with a drop of Tween 20 (Sigma) for 8 min in a 1.5 mL micro-centrifuge tube. The disinfectant and subsequent sterile water washes are removed by centrifugation.

3.5.2 Sterilization of Different Plant Parts

Surface disinfectants of explants from other plant parts are performed similar to that detailed above. In addition to the use of commercial bleach or sodium hypochlorite solution, for difficult materials, other disinfectants, such as ethanol, hydrogen peroxide, and/or mercuric chloride solution, can be used in conjunction with a hypochlorite treatment. Preliminary testing of disinfection procedures may be necessary. If possible, a slightly larger explant is excised from the plant for initial disinfection treatment, and the damaged cut edges can be removed at the time of culture.

3.6 Seed Germination Enhancement Techniques

Different treatments of seeds have been reported to enhance germination [85–92]. Besides serving as disinfectants, sodium and calcium hypochlorite can also enhance seed germination. A range of concentration from 0.2 to 5% can be used. It is believed that these solutions can help to scarify the surface of the seed, improving permeability of nutrients, and allow leaching of inhibitors [81, 85, 86]. After treatment, seeds usually become lighter in color indicating the removal of substances from the seed coat. For germinating seeds from green capsules, although the sodium hypochlorite disinfection treatment is often not necessary, immature seeds benefit from such treatments, and they have been shown to enhance seed germination [87].

Since hypochlorite is an alkaline solution, other alkaline solutions, i.e., sodium hydroxide solution (1 N), have been found to be effective in enhancing seed germination [*see* 81, 83]. Sodium hydroxide treatment may extract endogenous inhibitors, such as abscisic acid, from seeds [87].

Sonication treatments can be useful in plant tissue culture [88]. Once the treatment time is optimized, sonication of orchid seeds enhances seed germination, especially for terrestrial orchids, such as *Calanthe discolor* [89] and *Paphiopedilum armeniacum* [90]. Sonication can loosen the embryo from the tightly sealed seed coat. Also, seeds with carapace will benefit from this treatment.

Difficult to germinate species often benefit from cold treatments [59, 91]. Low-temperature treatment has been reported to improve seed germination. In a recent study, a short treatment of *Phalaenopsis* seeds with liquid nitrogen did not have adverse effects on germination [91]. *Hadrolaelia grandis* seeds stored at −80 °C can still germinate after 36 months of storage [92]. Several weeks of cold-moist stratification at 5 °C improved in vitro seed germination and seedling development in *Platanthera chapmanii* [93]. Cold treatment/storage of seeds prior to fungal inoculation can benefit symbiotic seed germination [94, 95]. Besides the leaching of inhibitors, the cold treatments may cause physiological changes within seeds, preparing them for germination, especially in establishing the mycorrhizal association. Further physiological and biochemical studies are needed in order to device better protocols for seed germination.

3.7 Test of Seed Viability

Since orchid seeds are small, prior to embarking on an experiment, seed viability needs to be assessed. The most common procedure is the tetrazolium chloride staining method introduced by Lakon [*see* 96]. Triphenyl tetrazolium chloride [TTC] is a redox indicator indicating cellular respiration activities. If cells are still alive, TCC will be reduced enzymatically to red 1,3,5-triphenylformzan, which is readily detected. As an alternative, the fluorescence staining of viable cells using fluorescein diacetate can be used [97] [also *see* Chap. 4]. In the presence of esterases, the colorless fluorescein diacetate molecule is hydrolyzed, generating fluorescein that fluoresces bright yellow under UV excitation.

3.7.1 TTC Test [98]

Moist seeds are stained using a 1% 2,3,5-triphenyltetrazolium chloride [TCC] solution. The pH of the solution is adjusted to 6.5 with 1 N NaOH. The seeds are incubated in darkness for 24 h at 30 °C. Viable embryos stain pink to red.

3.7.2 Fluorescein Diacetate Test [97]

A stock fluorescein diacetate [FDA] solution is prepared by dissolving 5 mg of FDA in 1 mL acetone. This stock solution should be stored at −20 °C. A staining solution is prepared just prior to use by diluting the stock solution with water or medium to a concentration of 10 μg/mL. The seeds are stained for various periods of time and examined with a fluorescence microscope. Embryos with a bright yellow florescence are regarded to be positively stained.

4 Common Additives

In orchid culture media, one or more of the following additives are often included, i.e., coconut water, banana powder, potato extract, casein hydrolysate, and charcoal. Chitosan has recently

received some attention as it appears to have a positive effect on micropropagation of orchids. Other less common additives, such as pineapple juice and tomato water, have also been used. Selected publications are documented in Chap. 6 indicating the promotive effects of additives in some experimental systems. In this section, the common additives used in orchid cultures are discussed. It is important to point out that the exact compositions of many additives used are not clear. The source of the additives and the methods of preparation also vary. Hence, their modes of actions are not known; they contribute little to our theoretical understanding of orchid seed germination and micropropagation.

4.1 Coconut Water [CW]

Coconut water refers to the liquid endosperm of the fruit [47]. CW has been a common organic additive to tissue culture medium for many decades. Its reported effects as a natural growth promoter allows for reduced addition of PGRs, which can sometimes cause somaclonal variation [99]. Common benefits of adding CW to tissue culture medium include promotion of germination and cell division. The first reported use of coconut water as an organic additive in tissue culture was published in 1941 by van Overbeek et al. [100], where *Datura stramonium* proembryos were transplanted onto media containing coconut milk. Due to its positive effects, coconut water has proven to be a useful additive in different experimental systems [47]. Analyses of CW content have been reported by different investigators and are summarized by Thorpe et al. [47] and more recently by Yong et al. [101]. CW is a rich source of amino acids, inorganic elements, organic acids, sugars, vitamins, and growth substances.

The suggested mode of action for CW is due mainly in part to the presence of cytokinins. Phytohormones that are present in CW include auxin, gibberellins, cytokinins, and abscisic acid. Cytokinins have many functions in plant development, such as plant cell division, formation of shoot meristems, seed germination, morphogenesis, and root development. The most abundant cytokinin in CW is trans-zeatin riboside and is commonly used to induce plantlet regeneration from callus [47].

Coconut water can be purchased commercially, or one can prepare it if fresh, good quality coconut is obtained in local markets [*see* 47]. Unripe nuts are preferred.

CW can be drained from young nuts by drilling holes through the “eyes” of the coconut. Each nut yields approximately 100 mL of liquid. If necessary, the liquid can be de-proteined by heating the liquid at 80–100 °C for about 10 min. After coarse filtration through filter papers, aliquots of 100 mL portions can be filter-sterilized and frozen until used. Coconut water is normally used at a concentration of 5–20% [v/v] (*see* **Note 12**).

4.2 Banana Extract

The use of banana homogenate in orchid seedling culture can be dated back to 1950 [*see* 102]. A detailed composition of banana pulp is documented by Arditti [103]. In addition to supplying carbohydrates and ions, various phytohormones, i.e., IAA, GAs, and cytokinins, are present [103]. Banana extract promotes seed germination and development of *Dendrobium lituiflorum* [104], and positive effects are noted for the multiplication and development of protocorm from a *Dendrobium* orchid [105] and other species.

In preparing the banana extract, a known weight of banana is mashed into a paste and added to the medium before autoclaving. Banana powder is available commercially, and approximately 30 g of powder is used per liter of medium.

4.3 Potato Extract

Potato is a rich source of nutrient. Nutritional analyses indicate that potato contains starch, sugars, proteins, amino acids, vitamins, and minerals, in the range of 1.7–2.1 g per 100 g of fresh weight of boiled potato [106]. Potato extract has been shown to have positive promotive effects on orchid growth [43].

In preparing the potato extract, unsprouted potatoes should be used in order to avoid formation of additional glucose from starch and the generation of toxic glycoalkaloids [107]. Approximately 30–100 g of potato is used per liter of medium [86].

4.4 Activated Charcoal

AC is an additive that has been used regularly in different micropropagation systems [43].The possible modes of actions have been discussed in several reviews [108–110] and in Chap. 7. It is generally known that AC can adsorb different organic and inorganic compounds. Secondary metabolites from plant tissues can also be removed, reducing their harmful effects on plant growth. The addition of AC increases sucrose hydrolysis to glucose and fructose [109]. Although the general effects are known, precise changes within the culture medium and how these changes affect growth and development of explants are difficult to determine. The positive effects of AC addition on orchid seed germination and micropropagation have been summarized by Thomas [110]. A wide range of AC concentrations have been used in different experiment systems. Paek and Yeung [111] used 0.01 g/L AC to enhance rhizome production and fresh weight gain in *Cymbidium forrestii*. In *Zygostates grandiflora*, 3 g/L AC has a positive effect on seed germination and seedling development [112]. For commercial replating and propagation maintenance media, 2 g/L of AC is used. In general, the addition of AC appears to be more beneficial in promoting seedling development after germination. AC is purchased commercially from various suppliers (*see* **Note 13**).

4.5 Casein Hydrolysate, Peptone, and Tryptone

These products are good natural sources of amino acids, peptides, and proteins for cell cultures. Some micronutrients and vitamins are also present [47]. Hydrolysates can be prepared

using enzymatic digestion or acid hydrolysis of different natural products, such as milk products, plant and animal tissues, and microbial cultures. Various hydrolysates are available from different commercial sources.

Using hydrolysates in in vitro orchid culture has resulted in mixed recommendations [113]. It is known that the response of plant tissues to a culture medium in an in vitro system depends on the nitrate to ammonium ratio [43]. The added organic nitrogen may shift the ratio resulting in either a positive or a negative response. A range of concentrations from 0.1 g to 2.0 g/L is used for different species. For commercial media used for replating, maintenance, and propagation as indicated in Sect. 3.4, 2 g/L of hydrolysate is present.

4.6 Chitosan

Chitin is a polymer present in crustaceans and fungi, and it is the second most abundant polymer on earth, only after cellulose. Chitosan is the deacetylated form of chitin, and it has been available commercially for several decades [114]. Chitosan is known for its benefits in agricultural applications and reported to stimulate plant growth, have antifungal properties, increase phytochemical production, and decrease transpiration rates. Positive benefits of chitosan applications on plant growth and in vitro cultures can be found in recent reviews [114, 115]. Specific summaries on the effects of chitosan on orchid growth are also available in the literature [116, 117]. Recent publications on the effects of chitosan in orchid seed germination and micropropagation are listed in Chap. 6.

Although positive effects of chitosan application are known, the exact mechanism(s) on how it promotes plant growth is still not understood. Hadwiger [114] discussed how plants response to chitosan application. It appears that chitosan serves as an elicitor in plant defense, enhancing the stress responses by plants. It is not clear as to how these defense and stress responses translate into positive effects on seed germination and plantlet development in orchids. It is interesting to note that other flowering plants, somatic embryo production can be induced by different stress treatments [118]. In a recent study, the partially depolymerized products of chitosan, chitooligomers, can affect the growth of wheat seedlings under salt stress with the activation of antioxidant enzyme activities [119]. Future studies will yield better insight in chitosan action in plants.

The standard protocol for preparation of chitosan is outlined in a publication by Limpanavech et al. [120]. Crab shells (600 g) are first demineralized by soaking in 10 L of 1.5 M HCl for 24 h; the acid solution is replaced every 8 h with a freshly prepared solution. The demineralized crab shells are then deproteinized by placing them in 10 L of 1.5 M NaOH for 24 h; the hydroxide solution is also replaced every 8 h. These steps yielded a chitin product, which was then deacetylated by soaking in 50% (w/w) NaOH for 48 h with a fresh change of hydroxide solution after 24 h of initial treatment.

The resulting product is washed in distilled water. Molecular weight of chitosan is determined using gel permeation chromatography. A procedure for the production of high-quality chitin and chitosan from shrimp shells can be found in [121] (*see* **Note 14**).

4.7 Other Additives

Besides the additives discussed above, other additives, such as pineapple and tomato juices and extracts from taro, green kidney bean pod, soybean seedling, and rice and wheat seed, have been used and reported to promote seed germination and seedling growth [122, 123]. Recently, zinc sulfate was reported to have a positive effect on *Ocimum* regeneration [124].

5 Conclusion

We are now approaching the centennial of Knudson's discovery on asymbiotic seed germination. The general concepts and approaches to asymbiotic and symbiotic seed germination and tissue cultures are clearly established in the literature. Successes are reported regularly in various publications. The third edition of Micropropagation of Orchids by Yam and Arditti [22] clearly demonstrates progress made in past decades.

In recent years, through improvements in the identification and culture of mycorrhizal fungi and through the use of current cell biology and molecular genetic techniques, the process of symbiotic seed germination is better understood. The utilization of developing seeds and seed treatment techniques greatly improve successes in asymbiotic seed germination. In order to further improve conservation efforts through seed germination, a better theoretical understanding of seed development and subsequent germination process leading to the formation of protocorm is needed. What causes early developmental arrest of embryo development? What physiological changes are associated with asymbiotic and symbiotic seed germination and protocorm development? Are there key media components that regulate seed germination and protocorm morphogenesis? There are still many unanswered questions. The use of combined approaches, i.e., cellular, physiological, biochemical, and molecular methods in the study of seed development and germination, will guarantee development of improved protocols for asymbiotic and symbiotic seed germination and successes in the conservation efforts.

6 Notes

1. Always double check media tables for correctness before using the printed information on a routine basis. Be sure to recalculate the amounts needed if different chemical formula is used based on mg/L calculation.

2. In preparing medium, always add an appropriate amount of water to the container before adding stock solutions. If stock solutions are added together first, the high concentration of ions can cause precipitate formation.
3. According to Gamborg and Phillips [26], to prepare a 10× iron-EDTA stock solution, dissolve separately 5.57 g of $FeSO_4 \cdot 7H_2O$ and 7.45 g of Na_2EDTA each in 350 mL of water. Combine both solutions, and bring it to 1 L with water. Autoclave the solution in order to complete the chelation reaction; the solution should be golden yellow in color after autoclaving.
4. Many commercial companies provide practical information in their website. For example, Sigma provides recommended procedures for solubilization and storage of plant growth regulators in their website, i.e., http://www.sigmaaldrich.com/technical-documents/protocols/biology/growth-regulators.html.
5. If sugar is purchased from local food stores, it is advisable to use fresh batches of sugar in media preparation in order to avoid potential deterioration due to oxidation of impurities upon prolong storage.
6. The NaOH solution should be replaced with fresh solution from time to time, as it can absorb carbon dioxide from the air. This can result in generating unwanted sodium carbonate, which can cause precipitate formation with other components in the medium.
7. If granulation and clumping appear on the surface of the powder medium or there is a color change, the medium should be discarded. This is caused by moisture absorption by medium components. It is advisable to use powder medium soon after purchase.
8. Heat sterilization is the most common practice for sterilizing tools and glass culture vessels when performing sterile protocols using a laminar flow hood. Although the Bunsen burner is a very effective tool, one has to determine the suitability of having this type of burner in a laminar flow hood. If a Bunsen burner is preferred, one should purchase an appropriate size burner with a foot switch in order to minimize open flame during operation. As an alternative, an electric heat sterilizer can be used instead. If necessary, one can supplement the electric heat sterilizer with a small alcohol lamp.
9. When handling or dissecting pathological specimens, it is advisable to have several sets of dissecting tools ready to be used. This will prevent cross contamination of dissected specimens. The used tools should be submerged in a 20% bleach solution right after use to avoid the spreading of pathogens. Furthermore, the clean bench needs to be carefully disinfected after use.

10. Household bleach such as Chlorox is commonly used as disinfectant. Be sure to close the cap securely in order to maintain its potency. The date when it is first opened should be noted. The bleach solution should be replaced regularly.
11. Mercuric chloride is a highly toxic compound and should be handled with extra care. It is slightly volatile at room temperature. Be sure to read safety data sheets before use. Standard operation procedures should be followed for storage, weighing, mixing, usage, and disposal.
12. Coconut water is commercially available from companies, such as Sigma (product number C5915) and PhytoTechnology Laboratories (product number C195). It is sold as sterile solution ready to use. Product information is available on their website.
13. There are different types of activated charcoal available based on different sources and size of the granules. Activated charcoal suitable for plant cell culture is available from different sources, such as Sigma and PhytoTechnology Laboratories.
14. Chitosan is available from different manufacturers as it has broad applications in agriculture. Sigma/Aldrich provides different forms/molecular weights in their catalogue. Information on the production of fungal chitosan can be found in [125] and is available for purchase from Kittolife Co. Ltd., Korea, as indicated in [116].

References

1. Krikorian AD, Berquam DL (1969) Plant cell and tissue cultures: the role of Haberlandt. Bot Rev 35:59–84
2. Gautheret RJ (1983) Plant tissue culture: a history. Bot Mag Tokyo 96:393–401
3. Bhojwani SS, Razdan MK (1983) Plant tissue culture: theory and practice. Elsevier, New York
4. Gamborg GL (2002) Plant tissue culture and biotechnology milestones. In Vitro Cell Dev Biol Plant 38:84–92
5. Thorpe TA (2006) History of plant tissue culture. Methods Mol Biol 318:9–32
6. Dodds JH, Roberts LW (1995) Experiments in plant tissue culture. Cambridge University Press, Cambridge
7. Trigiano RN, Gray DJ (2005) Plant development and biotechnology. CRC Press, Boca Raton
8. Vasil IK (2008) A history of plant biotechnology: from the cell theory of Schleiden and Schwann to biotech crops. Plant Cell Rep 27:1423–1440
9. White PR (1932) Plant tissue cultures. Annu Rev Biochem 11:615–628
10. Thorpe TA (1994) Morphogenesis and regeneration. In: Vasil IK, Thorpe TA (eds) Plant cell and tissue culture. Kluwer, Dordrecht, pp 17–36
11. Miller CO, Skoog F, Von Saltza MH, Strong FM (1955) Kinetin, a cell division factor from deoxyribonucleic acid. J Am Chem Soc 77:1392
12. Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp Soc Exp Biol 11:118–131
13. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
14. Yam TW, Nair H, Hew CS, Arditti J (2002) Orchid seeds and their germination: an historical account. In: Kull T, Arditti J (eds) Orchid biology: review and perspectives, VIII. Kluwer, The Netherlands, pp 387–504

15. Yam TW, Arditti J (2009) History of orchid propagation: a mirror of the history of biotechnology. Plant Biotechnol Rep 3:1–56
16. Knudson L (1922) Nonsymbiotic germination of orchid seeds. Bot Gaz 73:1–25
17. Arditti J (1990) Lewis Knudson (1994–1958): his science, his times, and his legacy. Lindleyana 5:1–79
18. Knudson L (1946) A nutrient for germination of orchid seeds. Am Orchid Soc Bull 15:214–217
19. Teixeira da Silva JAT (2013) Orchids: advances in tissue culture, genetics, phytochemistry and transgenic biotechnology. Floricult Ornament Biotechnol 7:1–52
20. Kauth PJ, Dutra D, Johnson TR, Stewart SL, Kane ME, Vendrame W (2008) Techniques and applications of in vitro orchid seed germination. In: Teixeira da Silva JA (ed) Floriculture, ornamental and plant biotechnology: advances and topical issues, 1st ed, vol V. Global Science Books, Ltd., Isleworth, pp 375–391
21. Chugh S, Guha S, Rao IU (2009) Micropropagation of orchids: a review on the potential of different explants. Sci Hortic 122:507–520
22. Yam TW, Arditti J (2017) Micropropagation of orchids, 3rd edn. Blackwell, Cambridge
23. de Fossard RA (1976) Tissue culture for plant propagators. The University of New England Printery, Armidale
24. George EF, Hall MA, De Klerk G-J (2008) Plant propagation by tissue culture, 3rd edn. Springer, Dordrecht
25. Vasil IK, Thorpe TA (eds) (1994) Plant cell and tissue culture. Kluwer, Dordrecht
26. Gamborg OL, Philips GC (1995) Plant cell, tissue and organ culture—fundamental methods. Springer, Berlin
27. Evans DE, Coleman JOD, Kearns A (2003) Plant cell culture. Bios Scientific Publishers, London
28. Smith RH (2013) Plant tissue culture: technique and experiments, 3rd edn. Elsevier, Amsterdam
29. Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158
30. Vacin F, Went FW (1949) Some pH changes in nutrient solutions. Bot Gaz 110:605–613
31. Halloran SM, Adelberg J (2011) A macronutrient optimization platform for micropropagation and acclimatization: using turmeric (*Curcuma longa* L.) as a model plant. In Vitro Cell Dev Biol Plant 47:257–273
32. Ichihashi S, Yamashita M (1977) Studies on the media for orchid seed germination. I. The effects of balances inside each cation and anion group for the germination and seedling development of *Bletilla striata* seeds. J Jpn Soc Hort Sci 45:407–413
33. Ichihashi S (1978) Studies on the media for orchid seed germination. II. The effects of anionic and cationic combinations relevant to seeding populations and culture periods on the growth of *Bletilla striata* seedlings. J Jpn Soc Hort Sci 46:521–529
34. Ichihashi S (1979) Studies on the media for orchid seed germination. III. The effects of total ionic concentration, cation/anion ratio, NH_4^+/NO_3^- ratio, and minor elements on the growth of *Bletilla striata*. J Jpn Soc Hort Sci 47:524–536
35. Ichihashi S (1979) Studies on the media for orchid seed germination. J Jpn Soc Hort Sci 48:345–352
36. Hamner CL (1940) Growth responses of Biloxi soybeans to variation in relative concentrations of phosphate and nitrate in the nutrient solution. Bot Gaz 101:637–649
37. Hamner KC, Lyon CB, Hamner CL (1942) Effect of mineral nutrition on the ascorbic-acid content of the tomato. Bot Gaz 103:586–616
38. Poothong S, Reed BM (2015) Increased $CaCl_2$, $MgSO_4$, and KH_2PO_4 improve the growth of micropropagated red raspberries. In Vitro Cell Dev Biol Plant 51:648–658
39. Poothong S, Reed BM (2016) Optimizing shoot culture media for *Rubus* germplasm: the effects of NH_4^+, NO_3^-, and total nitrogen. In Vitro Cell Dev Biol Plant 52:265–275
40. Reed BM, Wada S, DeNoma J, Niedz RP (2013) Mineral nutrition influences physiological responses of pear in vitro. In Vitro Cell Dev Biol Plant 49:699–709
41. Wada S, Niedz RP, DeNoma J, Reed BM (2013) Meso components ($CaCl_2$, $MgSO_4$, KH_2PO_4) are critical for improving pear micropropagation. In Vitro Cell Dev Biol Plant 49:356–365
42. Cui H-Y, Murthy HN, Moh SH, Cui Y-Y, Paek K-Y (2015) Establishment of protocorm suspension cultures of *Dendrobium candidum* for the production of bioactive compounds. Hort Environ Biotechnol 56:114–122
43. George EF, De Klerk G-J (2008) The components of plant tissue culture media I: macro- and micro-nutrients. In: George EF, Hall MA, De Klerk G-J (eds) Plant propagation by tissue culture, 3rd edn. Springer, Dordrecht, pp 65–114

44. Hart DS, Keightley A, Sappington D, Nguyen PTM, Chritton C, Seckinger GR, Torres KC (2016) Stability of adenine-based cytokinins in aqueous solution. In Vitro Cell Dev Biol Plant 52:1–9
45. Placide R, Clement U, Fracoise U, Védaste A (2012) Comparative study of effects of table sugar, laboratory grade sucrose and mannitol on growth of banana plantlets under in vitro conditions. Rwanda J Ser E Agric Sci 28:76–83
46. Ball E (1953) Hydrolysis of sucrose by autoclaving media, a neglected aspect in the technique of culture of plant tissues. Bull Torrey Bot Club 80:409–411
47. Thorpe TA, Stasolla C, Yeung EC, De Klerk G-J, George EF (2008) The components of plant tissue culture media II: organic additions. In: George EF, Hall MA, De Klerk G-J (eds) Plant propagation by tissue culture, 3rd edn. Springer, Dordrecht
48. Schenk NS, Hsiao K-C, Bornman CH (1991) Avoidance of precipitation and carbohydrate breakdown in autoclaved plant tissue culture media. Plant Cell Rep 10:115–119
49. Ernst R, Arditti J (1990) Carbohydrate physiology of orchid seedlings III. Hydrolysis of maltooligosaccharides by *Phalaenopsis* (Orchidaceae) seedlings. Am J Bot 77:188–195
50. Ernst R, Arditti J, Healey PL (1971) Carbohydrate physiology of orchid seedlings II. Hydrolysis and effects of oligosaccharides. Am J Bot 58:827–835
51. Stewart SL, Kane ME (2010) Effect of carbohydrate source on the in vitro asymbiotic seed germination of terrestrial orchid *Habenaria macroceratitis*. J Plant Nutr 33:1–11
52. Johnson TR, Kane ME, Perez HE (2011) Examining the interaction of light, nutrients and carbohydrates on seed germination and early seedling development of *Bletia purpurea* (Orchidaceae). Plant Growth Regul 63:89–99
53. Johnson TR, Kane ME (2013) Differential germination and developmental responses of *Bletia purpurea* (Orchidaceae) to mannitol and sorbitol in the presence of sucrose and fructose. J Plant Nutr 36:702–716
54. Aggarwal S, Nirmala C (2012) Utilization of coir fibers as an eco-friendly substitute for costly gelling agents for *in vitro* orchid germination. Sci Hortic 133:89–92
55. Aewsakul N, Maneesorn D, Serivichyaswat P, Taluengjit A, Nontachaiyapoom S (2013) *Ex vitro* symbiotic seed germination of *Spathoglottis plicata* Blume on common orchid cultivation substrates. Sci Hortic 160:238–242
56. Van der Kinderen G (1987) Abscisic acid in terrestrial orchid seeds: a possible impact on their germination. Lindleyana 2:84–87
57. Lee YI, Lee N, Yeung EC, Chung M-C (2005) Embryo development of *Cypripedium formosanum* in relation to seed germination in vitro. J Am Soc Hort Sci 130:752–753
58. Lee YI, Lu C-F, Chung M-C, Yeung EC (2007) Developmental changes in endogenous abscisic acid concentrations and asymbiotic seed germination of a terrestrial Orchid, *Calanthe tricarinata* Lindl. J Am Soc Hort Sci 132:246–252
59. Rasmussen HN (1995) Terrestrial orchids: from seed to mycotrophic plant. Cambridge University Press, Cambridge
60. Thomale H (1957) Die Orchideen, 2nd edn. Verlag Eugen Ulmer, Stuttgart, pp 89–90
61. Fast G (1971) Versuchezur Anzucht von Paphiopedilumaus Samen. Orchidee 22:189–192
62. Lindermann EGP, Gunckel JE, Davidson OW (1970) Meristem culture of *Cattleya*. Am Orchid Soc Bull 39:100–127
63. Mitra GC, Prasad RN, Roychowdhury A (1976) Inorganic salts and differentiation of protocorms in seed callus of an orchid and correlated changes in its free amino acid content. Indian J Exp Biol 14:350–351
64. Harvais G (1973) Growth requirements and development of *Cypripedium reginae* in axenic culture. Can J Bot 51:327–332
65. Harvais G (1982) An improved culture medium for growing the orchid *Cypripedium reginae* axenically. Can J Bot 60:2547–2555
66. Malmgren S (1992) Large-scale asymbiotic propagation of *Cypripedium calceolus*—plant physiology from a surgeon's point of view. Micropropag News 1:59–64
67. Malmgren S (1996) Orchid propagation. Theory and practice. In: Allen C (ed) North American Native Terrestrial Orchids. Propagation and production. Proceedings of the North American Native Terrestrial Orchid Conference, pp 63–71
68. van Waes JM, Debergh PC (1986) *In vitro* germination of some Western European orchids. Physiol Plant 67:253–261
69. Kano K (1965) Studies on the media for orchid seed germination. Mem Fac Agric Kagawa Univ 20:1–68
70. Winarto B, Teixeira da Silva JAT (2015) Use of coconut water and fertilizer for in vitro proliferation and plantlet production of *Dendrobium* 'Gradita 31'. In Vitro Cell Dev Biol Plant 51:303–314

71. Warcup JH, Talbot PHB (1967) Perfect states of *Rhizoctonias* associated with orchids. New Phytol 66:631–641
72. Zi XM, Sheng CL, Goodale UM, Gao JY (2014) *In situ* seed baiting to isolate germination-enhancing fungi for an epiphytic orchid, *Dendrobium aphyllum* Orchid. Mycorrhiza 24:487–499
73. Khamchatra N, Dixon K, Chayamarit K, Apisitwanich S, Tantiwiwart S (2016) Using *in situ* seed baiting technique to isolate and identify endophytic and mycorrhizal fungi from seeds of a threatened epiphytic orchid, *Dendrobium friedericksianum* Rchb.f. (Orchidaceae). Agric Nat Resour 50:8–13
74. Hadley G (1969) Cellulose as a carbon source for orchid mycorrhiza. New Phytol 68: 933–939
75. Warcup JH (1973) Symbiotic germination of some Australian terrestrial orchids. New Phytol 72:387–392
76. Warcup JH (1981) The mycorrhizal relationships of Australian orchids. New Phytol 87: 371–381
77. Clements MA, Muir H, Cribb PJ (1985) A preliminary report on the symbiotic germination of European terrestrial orchids. Kew Bull 41:437–445
78. Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and plant growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50: 199–204
79. Driver J, Rodrigues R, Kuniyuki AH (1984) *In vitro* propagation of paradox walnut rootstock. HortSci 19:507–709
80. Bhattacharyya P, Kumar V, Van Staden J (2017) Assessment of genetic stability amongst micropropagated *Ansellia africana*, a vulnerable medicinal orchid species of Africa using SCoT markers. S Afr J Bot 108: 294–302
81. Butcher D, Marlow SA (1989) Asymbiotic germination of epiphytic and terrestrial orchids. In: Pritchard HW (ed) Modern methods in orchid conservation: the role of physiology, ecology and management. Cambridge University Press, Cambridge, pp 31–38
82. Ponert J, Vosolsobe S, Kmecova K, Lipavska H (2011) European orchid cultivation—from seed to mature plant. Eur J Environ Sci 1: 95–107
83. Thompson DL, Edwards TJ, van Staden J (2006) Evaluating asymbiotic seed culture method and establishing *Disa* (Orchidaceae) germinability *in vitro*: relationships, requirements and first-time reports. Plant Growth Regul 49:269–284
84. Jevšnik T, Luthar Z (2015) Successful disinfection protocol for orchid seeds and influence of gelling agent on germination and growth. Acta Agric Solv 105:95–102
85. Richardson KA, Peterson RL, Currah RS (1992) Seed reserves and early symbiotic protocorm development of *Platanthera hyperborean* (Orchidaceae). Can J Bot 70: 291–300
86. Lee YI (2011) *In vitro* culture and germination of terrestrial Asian orchid seeds. Methods Mol Biol 710:53–62
87. Bae K-H, Ko MS, Lee MH, Kim NY, Song JM, Song G (2013) Effects of NaOCl treatment on in vitro germination of seeds of a rare endemic plant, *Oreorchis coreana* Finet. J Plant Biotechnol 40:43–48
88. Gaba V, Kathiravan K, Amutha S, Singer S, Xiaodi X, Ananthakrishnan G (2008) The uses of ultrasound in plant tissue culture. In: Gupta D, Ibaraki Y (eds) Plant tissue culture engineering. Springer, New York, pp 417–426
89. Miyoshi K, Mii M (1988) Ultrasonic treatment for enhancing seed germination of terrestrial orchid, *Calanthe discolor*, in asymbiotic culture. Sci Hortic 35:127–130
90. Zhang Y-Y, Wu K-L, Zhang J-X, Deng R-F, Duan J, Teixeira da Silva JAT, Huang W-C, Zeng S-J (2015) Embryo development in association with asymbiotic seed germination in vitro of *Paphiopedilum armeniacum* S.C. Chen et F.Y. Liu. Sci Rep 5:16356
91. Mweetwa AM, Welbaum GE, Tay D (2008) Effects of development, temperature, and calcium hypochlorite treatment on *in vitr*o germinability of *Phalaenopsis* seeds. Sci Hortic 117:257–262
92. Vudala SM, LLF R (2017) Seed storage and asymbiotic germination of *Hadrolaelia grandis* (Orchidaceae). S Afr J Bot 108:1–7
93. Poff KE, Sharma J, Richards M (2016) Cold-moist stratification improves germination in a temperature terrestrial orchid. Castanea 81: 292–301
94. Zettler LW, McInnis TM Jr (1993) Symbiotic seed germination and development of *Spiranthes cernua* and *Goodyera pubescens* (Orchidaceae: Spiranthoideae). Lindleyana 8:155–162
95. Shimura H, Koda Y (2005) Enhanced symbiotic seed germination of *Cypripedium macranthos* var. *rebunense* following inoculation after cold treatment. Physiol Plant 123: 281–287

96. Bennett N, Loomis WE (1949) Tetrazolium chloride as a test reagent for freezing injury of seed corn. Plant Physiol 24:162–174
97. Pritchard HW (1985) Determination of orchid seed viability using fluorescein diacetate. Plant Cell Environ 8:727–730
98. Vujanovic V, St-Arnaud M, Barabe TG (2000) Viability testing of orchid seed and the promotion of colouration and germination. Ann Bot 86:79–86
99. Ng C-Y, Saleh N (2010) In vitro propagation of *Paphiopedilum* orchid through formation of protocorm-like bodies. Plant Cell Tissue Organ Cult 105:193–202
100. Van Overbeek J, Conklin ME, Blakeslee AF (1941) Factors in coconut milk essential for growth and development of very young *Datura* embryos. Science 94:350–351
101. Yong JWH, Ge L, Ng YF, Tan SN (2009) The chemical composition and biological properties of coconut (*Cocos nucifera* L.) water. Molecules 14:5144–5164
102. Arditti J (1967) Factors affecting the germination of orchid seeds. Bot Rev 33:1–97
103. Arditti J (2009) Micropropagation of orchid, 2nd edn. Blackwell Publishing, Malden
104. Vyas S, Guha S, Bhattacharya M, Rao U (2009) Rapid regeneration of plants of *Dendrobium lituiflorum* Lindl. (Orchidaceae) by using banana extract. Sci Hortic 121:32–37
105. Islam MO, Islam MS, Saleh MA (2015) Effect of banana extract on growth and development of protocorm like bodies in *Dendrobium* sp. Orchid Agriculturists 13: 101–108
106. Storey M (2007) The harvested crop. In: Vreugdenhil D (ed) Potato biology and biotechnology: advances and perspectives. Elsevier, Amsterdam, pp 441–470
107. Gnasekaran P, Rathinam X, Sinniah UR (2010) A study on the use of organic additives on the protocorm-like bodies (plbs) growth of *Phalaenopsis violacea* orchid. J Phytology 2:29–33
108. Yam TW, Ernst RE, Arditti J, Nair H, Weatherhead MA (1990) Charcoal in orchid seed germination and tissue culture media: a review. Lindleyana 5:256–265
109. Pan MJ, van Staden J (1998) The use of charcoal in *in vitro* culture—a review. Plant Growth Regul 26:155–163
110. Thomas TD (2008) The role of activated charcoal in plant tissue culture. Biotechnol Adv 26:618–631
111. Paek KY, Yeung EC (1991) The effects of 1-naphthaleneacetic acid and N6-benzyladenine on the growth of *Cymbidium forrestii* rhizomes in vitro. Plant Cell Tissue Organ Cult 24: 65–71
112. Pacek-Bieniek A, Dyduch-Sieminska RM (2010) Influence of activated charcoal on seed germination and seedling development by the asymbiotic method in *Zygostates grandiflora* (Lindl.) Mansf. (Orchidaceae). Folia Hortic 22:45–50
113. Gupta A (2016) Asymbiotic seed germination in orchids: role of organic additives. Int Adv Res J Sci Eng Technol 3:143–147
114. Hadwiger LA (2013) Multiple effects of chitosan on plant systems: solid science or hype. Plant Sci 208:42–49
115. Pichyangkura R, Chadchawan S (2015) Biostimulant activity of chitosan in horticulture. Sci Hortic 196:49–65
116. Nge KL, New N, Chandrkrachang S, Stevens WF (2006) Chitosan as a growth stimulator in orchid tissue culture. Plant Sci 170:1185–1190
117. Uthairatanakij A, Teixeira da Silva J, Obsuwan K (2007) Chitosan for improving orchid production and quality. Orchid Sci Biotechnol 1:1–5
118. Yeung EC (1995) Structural and developmental patterns in somatic embryogenesis. In: Thorpe TA (ed) *In Vitro* embryogenesis in plants. Kluwer Academic Publishers, Dordrecht, pp 205–248
119. Zhang X, Li K, Liu S, Xing R, Yu H, Chen X, Qin Y, Li P (2017) Relationship between the degree of polymerization of chitooligomers and their activity affecting the growth of wheat seedlings under salt stress. J Agric Food Chem 65:501–509
120. Limpanavech P, Chaiyasuta S, Vongpromek R, Pichyangkura R, Khunwasi C, Chadchawan S, Lotrakul P, Bunjongrat R, Chaidee A, Bangyeekhun T (2008) Chitosan effects on floral production, gene expression, and anatomical changes in the *Dendrobium* orchid. Sci Hortic 116:65–72
121. Toan NV, Ng CH, Aye KN, Trang TS (2006) Production of high-quality chitin and chitosan from preconditioned shrimp shells. J Chem Technol Biotechnol 81: 1113–1118
122. Ichihashi S, Islam MO (1999) Effects of complex organic additives on callus growth in three orchid genera, *Phalaenopsis*, *Doritaenopsis*, and *Neofinetia*. J Jpn Soc Hortic Sci 68:269–274

123. Islam MO, Matsui S, Ichihashi S (2000) Effects of complex organic additives on seed germination and carotenoid content in *Cattleya* seedlings. Lindleyana 15:81–88
124. Verma SK, Sahin G, Das AK, Gurel E (2016) *In vitro* plant regeneration of *Ocimum basilicum* L. is accelerated by zinc sulphate. In Vitro Cell Dev Biol Plant 52:20–27
125. New N, Stevens WF (2002) Production of fungal chitosan by solid substrate fermentation followed by enzymatic extraction. Biotechnol Lett 24:131–134

Chapter 6

Orchid Seed Germination and Micropropagation II: Media Information and Composition

Jungmin Park and Edward C. Yeung

Abstract

This chapter serves as a summary of various media constituents for orchid seed germination and micropropagation. The fifteen commonly used media are arranged into individual tables listing compounds in both mg/L and molar concentration. A comparative table of mineral salt content is also included. Recent publications on the beneficial effects of organic additives such as chitosan, coconut water, banana, and potato are listed to serve as a quick reference source on the subject.

Key words Culture media, Seed germination, Micropropagation, Molarity, Organic additives, Chitosan, Coconut water, Banana powder, Potato extract

1 Introduction

Media in the context of this chapter can be defined as a solution consisting of inorganic macro- and microelements, vitamins, sucrose, organic additives, and agar (or other gelling agents such as Gelrite®) for tissue culture.

Different formulations have been used for orchid seed germination and in vitro studies. With the numerous species in Orchidaceae, not one medium will be a proper fit for all orchid species. Media formulations are commonly modified to suit the needs of a species and the different types of explants used in an experiment. Hence, it is important to perform prior research to choose the appropriate medium, as certain concentrations of macro- and microelements can prove to be toxic to certain tissues [1]. A suggestion for researchers prior to choosing media or determining what modifications should be performed would be to refer to *Micropropagation of Orchids* [1], which contains tables of complete media composition for each orchid species. Recent reviews [2, 3] and Chap. 27 also list recent publications on orchid seed germination and micropropagation studies with information on the basal media used.

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_6,

In the literature, inaccuracies and errors are often noted in reporting basal medium formulations [4, 5]. Owen and Miller [5] drew attention to this problem and reported corrected inorganic components of eight common plant tissue culture basal media. With many culture media available to date, it is imperative that investigators carefully check media formulations prior to their regular use. This chapter is a companion to Chap. 5, and it is intended to serve as a detailed reference source for the commonly used media in orchid seed germination and micropropagation studies (Tables 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, and 16). For many of these culture media, prepared mixes can be purchased from companies specializing in plant tissue culture. It is important to note that for commercial preparations, modifications to the original formulations can be found. We draw attention to these modifications in accompanying notes detailing changes from the original publication, if there are any. A list of commercial websites is provided enabling quick access to information and products of interest (Table 17).

A common feature in orchid in vitro studies is that organic additives such as coconut water are often added to basal media (*see* Chaps. 5 and 7). Tables 18 and 19 contain selected recent publications demonstrating the usefulness of some of the additives in orchid seed germination and micropropagation studies.

2 Media Tables

Tables 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 list the compounds used to prepare each medium with the amount in both weight per volume (mg/L) and molar concentration (mM for macronutrients, μM for micronutrients). The compositions listed are the exact formulations presented in the original publication. Table 16 details molar concentrations (mM) of individual ions; the information enables researchers to determine and compare the concentration of individual ions among the common media used in in vitro culture studies. The importance of including mole values in media tables is emphasized by de Fossard [6]. Additional media used in orchid in vitro studies are listed in the monograph by Rasmussen [7].

It is important to note that many of the original formulations were published decades ago and could be somewhat outdated, e.g., the use of iron salts. For commercial preparations, it is commonly seen that some components are replaced or modified in compliance with recent findings or ease of access. These modifications are stated in the Notes section that accompanies the URLs to commercial sites.

Table 1
Gamborg B_5 medium [17] (Note 1)

Macroelements	mg/L	mM
KNO_3	2500	24.7
$NaH_2PO_4 \cdot H_2O$	150	1.1
$CaCl_2 \cdot 2H_2O$	150	1.02
$(NH_4)_2SO_4$	134	1.0
$MgSO_4 \cdot 7H_2O$	250	1.0
[a]Fe	28	0.05
Microelements	**mg/L**	**μM**
$MnSO_4 \cdot H_2O$	10	59.17
H_3BO_3	3	48.52
$ZnSO_4 \cdot 7H_2O$	2	6.95
KI	0.75	4.52
$Na_2MoO_4 \cdot 2H_2O$	0.25	1.03
$CoCl_2 \cdot 6H_2O$	0.025	0.11
$CuSO_4$	0.025	0.10
Organic	**mg/L**	**μM**
m-Inositol	100	555.1
Thiamine HCl	10	29.7
Nicotinic acid	1	8.12
Pyridoxine HCl	1	4.86
Sucrose	20,000	58.43 mM
2,4-D	2	–

[a]Sequestrene 330 Fe from Geigy Agricultural Chemicals, Saw Mill River Road, Ardsley, New York

Table 2
DKW/Juglans medium [18] (Note 2)

Macroelements	mg/L	mM
NH_4NO_3	1416	17.7
K_2SO_4[a]	1559	8.95
$Ca(NO_3)_2 \cdot 4H_2O$	1960	8.3
$MgSO_4 \cdot 7H_2O$	739.4	3
KH_2PO_4	260	1.9
$CaCl_2 \cdot 2H_2O$	147	1

(continued)

Table 2
(continued)

Macroelements	**mg/L**	**mM**
$FeSO_4 \cdot 7H_2O$	33.4	0.12
Na_2EDTA	44.7	0.12
Microelements	**mg/L**	**µM**
$MnSO_4 \cdot H_2O$	33.8	200
$Zn(NO_3)_2 \cdot 6H_2O$	17	57.2
H_3BO_3	4.8	77.6
$Na_2MoO_4 \cdot 2H_2O$	0.39	1.6
$CuSO_4 \cdot 5H_2O$	0.25	1
Organic	**mg/L**	**µM**
Myo-inositol	100	555
Glycine	2	26.6
Thiamine HCl	2	5.9
Nicotinic acid	1	8.1
Sucrose	30,000	87.64 mM
Gelrite	2000	–

[a]The original publication lists 8.95 mM of KH_2SO_4 which is a compound that does not exist; in cross-checking with media formulations from several commercial websites (Himedia Labs, Duchefa Biochemie, and Phytotechnology Laboratories), it was found to be an error and K_2SO_4 is used

Table 3
Fast medium (see Rasmussen [7]) (Note 3)

Macroelements	**mg/L**	**mM**
KCl	167	2.24
$(NH_4)(NO_3)$	167	2.086
KH_2PO_4	83	0.61
$MgSO_4 \cdot 7H_2O$	83	0.337
$Ca(NO_3)_2 \cdot 4H_2O$	83	0.351
FeNa-EDTA	17	0.046
Microelements	**mg/L**	**µM**
H_3BO_3	1	16.173
$MnSO_4 \cdot 4H_2O$	0.1	0.45
$ZnSO_4 \cdot 7H_2O$	1	3.48

(continued)

Table 3
(continued)

Macroelements	**mg/L**	**mM**
$NiCl_2 \cdot 6H_2O$	0.03	0.126
$AlCl_3$	0.03	0.22
$CuSO_4 \cdot 5H_2O$	0.03	0.12
KI	0.01	0.06
Organic	**mg/L**	**μM**
Peptone	1670	–
Fresh Brewer's yeast	8300	–
Nicotinic acid	0.1	0.812
Biotin	0.01	0.0409
Fructose	5000	27.75 mM
Sucrose	11,670	34.1 mM
Agar	8300	–

Table 4
Harvais medium [19] (Note 4)

Macroelements	**mg/L**	**mM**
$(NH_4)(NO_3)$	400	5
KNO_3	200	1.98
$Ca(NO_3)_2 \cdot 4H_2O$	400	1.69
KH_2PO_4	200	1.47
KCl	100	1.34
$MgSO_4 \cdot 7H_2O$	200	0.81
Microelements	**mg/L**	**μM**
Ammonium ferric citrate	25	95.43
$MnSO_4 \cdot 4H_2O$	2.03	9.10
H_3BO_3	0.5	8.09
$CuSO_4 \cdot 5H_2O$	0.5	2.00
$ZnSO_4 \cdot 7H_2O$	0.5	1.74
KI	0.1	0.60
$Co(NO_3)_2 \cdot 6H_2O$	0.025	0.085
$Na_2MoO_4 \cdot 2H_2O$	0.02	0.083

Table 5
Heller medium (see [1]) (Note 5)

Macroelements	mg/L	mM
KCl	750	10.06
$NaNO_3$	600	7.06
$MgSO_4 \cdot 7H_2O$	250	1.01
$NaH_2PO_4 \cdot H_2O$	125	0.91
$CaCl_2 \cdot 2H_2O$	75	0.51
Microelements	**mg/L**	**μM**
H_3BO_3	1	16.2
$FeCl_3 \cdot 6H_2O$	1	3.7
$ZnSO_4 \cdot 7H_2O$	1	3.48
$MnSO_4 \cdot 4H_2O$	0.1	0.45
$AlCl_3 \cdot 6H_2O$	0.03	0.22
$NiCl_2 \cdot 6H_2O$	0.03	0.13
$CuSO_4 \cdot 5H_2O$	0.03	0.12
KI	0.01	0.06

Adapted from *Micropropagation of Orchids* (p. 143) [1]

Table 6
Knudson C medium (see Vacin and Went [20]) (Note 6)

Macroelements	mg/L	mM
$Ca(NO_3)_2 \cdot 4H_2O$	1000	6.09
$(NH_4)_2SO_4$	500	3.78
KH_2PO_4	250	1.84
$MgSO_4 \cdot 7H_2O$	250	1.01
$FeSO_4 \cdot 7H_2O$	25	0.09
$MnSO_4 \cdot 4H_2O$	7.5	0.03
Organic	**mg/L**	**mM**
Sucrose	20,000	58.42
Agar	18 g	–

Table 7
Lindemann orchid medium [21] (Note 7)

Macroelements	**mg/L**	**mM**
KCl	1050	14.08
$(NH_4)_2SO_4$	1000	7.57
$Ca(NO_3)_2$	500	2.16
KH_2PO_4	135	0.99
$MgSO_4$	120	0.49
Microelements	**mg/L**	**μM**
$FeC_6H_5O_7 \cdot 3H_2O$	5.4	17.96
H_3BO_3	1.014	16.34
$AlCl_3$	0.031	2.32
$ZnSO_4$	0.565	1.98
KI	0.099	0.60
$MnSO_4$	0.068	0.30
$NiCl_2$	0.017	0.13
$CuSO_4$	0.019	0.08
Organic	**mg/L**	**μM**
Cytidylic acid	161.6	500
Guanylic acid	181.6	500
Myo-inositol	18.016	100
Glutamic acid	14.7	100
Asparagine	13.2	100
Folic acid	4.414	10
Nicotinic acid	1.231	10
Thiamine HCl	0.338	1.0
Ca-D-pantothenate	0.476	1.0
Pyridoxine HCl	0.205	1.0
Biotin	0.024	0.1
NAA	0.06–0.33 opt.	–
Kinetin	0.05 opt.	–
Gibberellic acid	0.2–1.2 opt.	–
Coconut water[a]	15% v/v	–

[a]Or casein hydrolysate (100 mg/L)

Table 8
Malmgren orchid medium (Malmgren [22] as prepared by PhytoTechnology Laboratories) (Note 8)

Macroelements	mg/L	mM
$MgSO_4$	97.69	0.81
KH_2PO_4	75	0.43
$Ca_3(PO_4)_2$	75	0.24
$FeSO_4 \cdot 7H_2O$	27.8	0.1
$Na_2EDTA \cdot 2H_2O$	37.26	0.1
$MnSO_4 \cdot H_2O$	1.54	0.01
Organic	**mg/L**	**μM**
Myo-inositol	100	555.1
Nicotinic acid	5	40.6
Thiamine HCl	10	29.6
Glycine	2	26.6
Pyridoxine HCl	5	24.3
Folic acid	0.5	1.1
D-Biotin	0.05	0.2
Enzymatic hydrolysate casein	400	–
Activated charcoal	1000	–
Pineapple powder	20,000	–
Agar	7000	–

Table 9
Mitra orchid medium [23] (Note 9)

Macroelements	mg/L	mM
KNO_3	180	1.78
NaH_2PO_4	150	1.25
$MgSO_4 \cdot 7H_2O$	250	1.01
$Ca(NO_3)_2 \cdot 4H_2O$	200	0.85
$(NH_4)_2SO_4$	100	0.76
$FeSO_4 \cdot 7H_2O$	27.8	0.1
$Na_2EDTA \cdot 2H_2O$	37.3	0.1
Microelements	**mg/L**	**μM**

(continued)

Table 9
(continued)

Macroelements	mg/L	mM
H_3BO_3	0.6	9.70
$MnCl_2 \cdot 4H_2O$	0.4	2.02
$Na_2MoO_4 \cdot 2H_2O$	0.05	0.21
$CuSO_4 \cdot 5H_2O$	0.05	0.20
KI	0.03	0.18
$ZnSO_4 \cdot 7H_2O$	0.05	0.17
$Co(NO_3)_2 \cdot 6H_2O$	0.05	0.17
Organic	**mg/L**	**μM**
Nicotinic acid	1.25	10.2
Pyridoxine HCl	0.3	1.46
Thiamine HCl	0.3	0.89
Folic acid	0.3	0.68
Biotin	0.05	0.20
Riboflavin	0.05	0.13
Sucrose	20,000	58.42 mM
Agar	7000	–

Table 10
Murashige and Skoog medium [16] (Note 10)

Macroelements	mg/L	mM
NH_4NO_3	1650	20.61
KNO_3	1900	18.79
$CaCl_2 \cdot 2H_2O$	440	2.99
$MgSO_4 \cdot 7H_2O$	370	1.5
KH_2PO_4	170	1.25
Na_2EDTA	37.3	0.1
$FeSO_4 \cdot 7H_2O$	27.8	0.1
Microelements	**mg/L**	**μM**
$MnSO_4 \cdot 4H_2O$	22.3	100
H_3BO_3	6.2	100
$ZnSO_4 \cdot 4H_2O$	8.6	29.90
KI	0.83	5.00

(continued)

Table 10
(continued)

Macroelements	mg/L	mM
$Na_2MoO_4 \cdot 2H_2O$	0.25	1.03
$CoCl_2 \cdot 6H_2O$	0.025	0.11
$CuSO_4 \cdot 5H_2O$	0.025	0.10
Organic	**mg/L**	**μM**
Myo-inositol	100	555.1
Glycine	2	26.60
Nicotinic acid	0.5	4.06
Pyridoxine HCl	0.5	2.43
Thiamine HCl	0.1	0.30
Indole acetic acid	1~30	–
Kinetin	0.04~10	–
Sucrose	30,000	87.64 mM
Agar	10,000	–

Table 11
New Dogashima medium [24] (Note 11)

Macroelements	mg/L	mM
$(NH_4)(NO_3)$	480	6.0
KH_2PO_4	550	3.76
KCl	150	2.0
$Ca(NO_3)_2 \cdot 4H_2O$	470	2.0
KNO_3	200	2.0
$MgSO_4 \cdot 7H_2O$	250	1.0
Microelements	**mg/L**	**μM**
Fe-EDTA	21	60.9
$MnSO_4 \cdot 4H_2O$	3	13.5
H_3BO_3	0.5	8.09
$ZnSO_4 \cdot 7H_2O$	0.5	1.74
$CoCl_2 \cdot 6H_2O$	0.025	0.11
$Na_2MoO_4 \cdot 2H_2O$	0.025	0.10
$CuSO_4 \cdot 5H_2O$	0.025	0.10

(continued)

Table 11
(continued)

Macroelements	mg/L	mM
Conc. H_2SO_4	0.5 mL	–
Organic	**mg/L**	**μM**
Myo-inositol	100	555.1
L-Cysteine	1	8.3
Niacin	1	8.1
Adenine	1	7.4
Pyridoxine HCl	1	4.9
Thiamine HCl	1	3.0
Calcium pantothenate	1	2.1
D-Biotin, cryst.	0.1	0.4
Sucrose	20,000	58.42 mM
Agar	8000	–

Table 12
Schenk and Hildebrandt medium [25] (Note 12)

Macroelements	mg/L	mM
KNO_3	2500	24.73
$NH_4H_2PO_4$	300	2.61
$MgSO_4 \cdot 7H_2O$	400	1.62
$CaCl_2 \cdot 2H_2O$	200	1.36
Microelements	**mg/L**	**μM**
H_3BO_3	5	80.8
$FeSO_4 \cdot 7H_2O$	15	53.9
Na_2EDTA	20	53.7
$MnSO_4 \cdot H_2O$	10	59.2
KI	1	6.02
$ZnSO_4 \cdot 7H_2O$	1	3.48
$CuSO_4 \cdot 5H_2O$	0.2	0.80
$CoCl_2 \cdot 6H_2O$	0.1	0.42
$Na_2MoO_4 \cdot 2H_2O$	0.1	0.41

(continued)

Table 12
(continued)

Macroelements	mg/L	mM
Organic	**mg/L**	**μM**
Myo-inositol	1000	5551
Nicotinic acid	5	40.61
Thiamine HCl	5	14.83
Pyridoxine HCl	0.5	2.43
pCPA	2	8.00
Kinetin	0.1	0.47
Sucrose	30,000	87.64 mM
Agar	6000	–

Table 13
Thomale-GD medium [14] (Note 13)

Macroelements	mg/L	mM
$(NH_4)(NO_3)$	370	4.62
KNO_3	400	3.96
KH_2PO_4	300	2.20
$(NH_4)_2SO_4$	60	0.45
$MgNO_3{\cdot}6H_2O$	110	0.41
$FeSO_4{\cdot}7H_2O$	20	0.1
Organic	**mg/L**	**μM**
Wuchsstoff 66_f	10	–
Nicotinic acid amide	1	8.20
Glucose	10,000	550.8 mM
Fructose	10,000	550.8 mM

Table 14
Vacin and Went medium [20] (Note 14)

Macroelements	mg/L	mM
KNO_3	525	5.19
$(NH_4)_2SO_4$	500	3.78
KH_2PO_4	250	1.84
$MgSO_4{\cdot}7H_2O$	250	1.01

(continued)

Table 14
(continued)

Macroelements	**mg/L**	**mM**
$Ca_3(PO_4)_2$	200	0.64
Microelements	**mg/L**	**µM**
$Fe_2(C_4H_4O_6)_3{\cdot}2H_2O$	28	47.3
$MnSO_4{\cdot}4H_2O$	7.5	33.6
Organic	**mg/L**	**mM**
Sucrose	20,000	58.43
Agar	16,000	–

Table 15
Van Waes and Debergh medium [26] (Note 15)

Macroelements	**mg/L**	**mM**
$(NH_4)(NO_3)$	370	4.62
KNO_3	400	3.95
KH_2PO_4	27.85	2.2
$(NH_4)_2SO_4$	300	0.45
$MgSO_4{\cdot}7H_2O$	110	0.41
Microelements	**mg/L**	**µM**
H_3BO_3	10	161.7
Na_2EDTA	37.3	100
$FeSO_4{\cdot}7H_2O$	27.8	100
$MnSO_4{\cdot}4H_2O$	25	112
$ZnSO_4{\cdot}7H_2O$	10	34.8
$Na_2MoO_4{\cdot}2H_2O$	0.25	1.03
$CuSO_4{\cdot}5H_2O$	0.025	0.1
Organic	**mg/L**	**µM**
Myo-inositol	100	555.1
Nicotinic acid	5	40.6
Pyridoxine HCl	0.5	2.43
Thiamine HCl	0.5	1.48
Biotin	0.5	3.5
Folic acid	0.5	1.13
Glycine	2	26.6
Sucrose	20,000	58.42 mM
Agar	6000	–

Table 16
Comparative table of mineral salt content for orchid germination and micropropagation media (Note 16)

	Media														
Constituents	**B_5**	**DKW**	**Fast**	**HV**	**Heller**	**KC**	**LM**	**MM**	**Mitra**	**MS**	**NDM**	**SH**	**T-GD**	**VW**	**VD**
Macronutrients (mM)															
Ammonium	2.03	17.7	2.09	5.0	–	7.57	15.14	–	1.51	20.61	6.0	2.61	5.52	7.57	5.52
Calcium	1.02	9.3	0.35	1.7	0.51	4.23	2.12	0.73	0.85	3	2.0	1.36	–	1.93	–
Chlorine	2.04	2	2.24	1.34	11.1	–	14.08	–	0.004	6	2.01	2.72	–	–	–
Magnesium	1.01	3	0.34	0.81	1.01	1.01	0.49	0.81	1.01	1.5	1.01	1.62	0.41	1.01	0.41
Nitrate	24.7	34.4	2.79	10.36	7.06	8.47	2.12	–	3.47	39.4	9.97	24.7	8.99	5.19	8.57
Potassium	24.7	10.85	2.85	4.80	10.06	1.84	15.07	0.55	1.78	20.0	8.03	24.7	6.16	7.03	6.15
Phosphate	1.08	1.9	0.61	1.47	0.91	1.84	0.99	1.0	1.25	1.25	4.04	2.61	2.2	3.13	2.2
Sulfate	2.08	12.3	0.34	0.81	1.02	4.92	8.10	0.92	1.87	1.73	1.03	1.70	0.55	4.92	1.07
Sodium	1.09	0.24	0.046	0.00016	7.97	–	–	–	1.45	0.2	0.0002	0.1	0.2	–	0.2
Micronutrients (μM)															
Boron	48.5	77.6	16.17	8.09	16.17	–	16.4	–	9.7	100	8.09	80.9	–	–	161.7
Cobalt	0.1	–	–	0.085	–	–	–	–	0.17	0.1	0.11	0.42	–	–	0.11
Copper	0.15	1	0.12	2.00	0.12	–	0.1	–	0.2	0.1	0.1	0.80	–	–	0.1
Iron	50	120	46.32	95.4	3.7	89.9	18.0	100	100	100	60.86	54	100	94.6	100
Iodine	4.5	–	0.06	0.60	0.06	–	0.6	–	0.18	5	–	6.02	–	–	–
Manganese	59.2	200	0.45	9.1	0.45	33.6	37.63	9.11	2.02	100	13.45	59.2	–	33.6	112

Molybdenum	1.03	1.6	–	0.08	–	–	–	–	0.207	1.0	0.1	0.413	–	–	1.03
Nickel	–	–	0.13	–	0.12	–	0.24	–	–	–	–	–	–	–	–
Aluminum	–	–	0.22	–	0.22	–	–	–	–	–	–	–	–	–	–
Zinc	7.0	57.2	3.48	1.74	3.48	–	3.5	–	0.174	36	1.74	3.48	–	–	34.78
Total *N* (mM)	25.7	52.1	4.88	15.36	7.06	16.0	17.26	–	5	60.0	15.97	27.31	14.51	12.76	14.09
NH_4^+; NO_3^-	0.08	0.51	0.75	0.48	–	0.89	7.14	–	0.44	0.5	0.60	0.11	0.61	1.46	0.64

Abbreviations: *B5* Gamborg B5 medium, *DKW* DKW/Juglans medium, *Fast* fast medium, *HV* Harvais medium, *Heller* Heller medium, *KC* Knudson C medium, *LM* Lindemann Orchid medium, *MM* Malmgren Orchid medium, *Mitra* Mitra Orchid medium, *MS* Murashige and Skoog medium, *NDM* New Dogashima medium, *SH* Schenk and Hildebrandt medium, *T-GD* Thomale-GD medium, *VW* Vacin and Went medium, *VD* Van Waes and Debergh medium

2.1 The Hyponex Medium

The Hyponex medium was used by Kano [8] with the intention to devise a simple and easy to prepare medium for orchid seed germination. When compared to the Knudson C medium, a 3% Hyponex medium resulted in better growth of germinating seeds of *Dendrobium* and *Laeliocattleya* hybrids [8]. Simple media were proposed for four genera of orchids, i.e., *Dendrobium*, *Cattleya* group, *Cymbidium*, and *Paphiopedilum* [8]. The same formulations were published as a note in the American Orchid Society Bulletin [9]. Since its publication, the Hyponex medium has become popular among the Asian investigators. The earlier Hyponex medium is prepared using Hyponex fertilizer powder [N-P-K: 6.5:6:19]. Moreover, this product is manufactured by Hyponex Japan and is not readily available elsewhere, especially in North America. Nowadays, other Hyponex formulations with different N-P-K ratio are used for seed germination and plantlet development studies (for additional references, see Winarto et al. [10]). Current formulations are primarily a mixture of N-P-K (6.5:6:19) and N-P-K (20:20:20) and used at a concentration of 3 g/L (e.g., see Chap. 8).

The Hyponex medium is simple to prepare. To prepare 1 L of medium, depending on the Hyponex formulation, usually 3.0 g of fertilizer is used, together with 25–30 g sucrose and 7.0–8.0 g agar, pH 5.7. Because the medium is relatively simple and the microelements are not known, additives such as coconut water, potato and banana extracts, and activated charcoal are often added. In Kano's studies, the addition of tomato and apple juices showed positive effects on the growth of *Dendrobium* hybrid plantlets [8].

2.2 Common Additives

It is interesting to note that in in vitro culture of orchids, organic additives especially coconut water are often added to the basal medium. Positive effects of organic additives are often reported to improve germination and/or plantlet growth. Many of these additives such as coconut water and banana powder are readily available from various commercial sources. Information on the common additives used for orchid in vitro cultures can be found in reviews [2, 3] and in Chaps. 5 and 27. Table 17 showcases recent reports on the beneficial effects of common additives such as coconut water and banana and potato extracts on seed germination and micropropagation. Although many publications have reported findings of optimal concentrations and/or volumes of the said organic additives, their mode of action in a culture system is not clear.

In recent years, chitosan has been shown to promote growth of orchid explants (see Chap. 5). Table 18 provides selected recent literature on this additive for a quick reference. Readers should consult original publications for additional information.

Table 17
A sample of recent literature on the use of organic additives in orchid in vitro cultures

Orchid species	Stage	Medium	Reference
Bulbophyllum dhaninivatii Seidenf.	Shoot culture	VW (Vacin and Went) medium supplemented with 50, 100, and 150 mL/L coconut water, 25 and 50 g/L potato extract, and 25 and 50 g/L banana homogenate	[27]
Cypripedium macranthos Sw.	Asymbiotic seed germination and seedling development	¼ MS (Murashige and Skoog medium) with 0, 50, 100, and 200 mL/L coconut water; 0, 50, 100, and 200 mL/L maple sap; 0, 50, 100, and 200 mL/L birch sap; 0, 15, 30, and 60 g/L banana powder; and 0, 1, 2, and 4 g/L peptone	[28]
Cypripedium macranthos Sw.	Asymbiotic seed germination and seedling development	Harvais medium supplemented with 50 and 100 mL/L coconut water; 25 and 50 g/L banana homogenate; or 25 and 50 g/L potato homogenate	[29]
Dendrobium 'Gradita 31'	PLB growth and proliferation	½ MS supplemented with 0, 7.5, 15, and 30% (v/v) coconut water	[10]
Dendrobium aqueum	In vitro regeneration from protocorms	½ MS with cytokinins at 1, 3, 5, 7, and 10 mg/L; banana powder and coconut water at 1%, 3%, 5%, 7%, and 10%; and auxins at 1, 3, 5, 7, and 10 mg/L	[30]
Dendrobium candidum	Protocorm suspension culture	MS medium with 1, 3, 5, 7, and 9% banana homogenate; 1, 3, 5, 7, and 9% coconut extract; 1, 3, 5, 7, 9% potato extract	[31]
Dendrobium lituiflorum Lindl.	Germination and seedling growth	MS or KC (Knudson C medium) supplemented with 0, 1, 2.5, 5, 10, and 20% banana extract	[32]
Dendrobium sp. var. Sonia	PLB development	½ MS supplemented with banana extract at 12.5, 25, 50, 100, and 200 mL/L	[33]
Phalaenopsis violacea	PLB growth	MS medium supplemented with 10, 20, 30% of banana cultivars extracts, papaya extract, tomato extract, and coconut water	[34]
Vanda helvola Blume	Seed germination and seedling development	KC medium supplemented with 10, 15, and 20% (v/v) tomato juice or coconut water and 0.1, 0.2, and 0.3% (w/v) peptone or yeast extract	[35]
Vanda Kasem's Delight	PLB growth	VW medium with potato, papaya, and tomato organic extracts at concentrations of 0, 5, 10, 20, and 30%	[36]
Vanda roxburgii	Germination and seedling growth	Hyponex medium with 0, 25, 50, 100, and 200 mL/L of potato extract	[37]
Zygostates grandiflora (Lindl.) Mansf.	Seed germination and seedling development	MS with indoleacetic acid and benzylaminopurine, with 0, 1, and 3 charcoal and on a medium that contained 1.0 and 3.0 g/L activated charcoal	[38]

Table 18
Recent publications demonstrating positive effects of chitosan in orchid in vitro cultures

Orchid species	Stage	Medium	Reference
Hybrid *Cymbidium* Twilight Moon 'Day Light'	PLB formation	Teixeira *Cymbidium* medium supplemented with 0.1, 1, 10, and 50 mg/L commercial chitosan, non-commercial chitosan, and hyaluronic acid	[39]
Dendrobium 'AW 179'	Plantlet growth	Modified VW medium supplemented with 0, 50, 250, or 1250 mg/L chitosan	[40]
Dendrobium 'Eiskul'	PLB multiplication	VW medium modified with 37.7 mg/L of Na_2EDTA and 27.8 mg/L of $FeSO_4.7H_2O$, with the addition of 15%v/v coconut water. 70%, 80%, and 90% oligomeric and polymeric chitosan were used at concentrations of 10, 20, 40, and 80 mg/L	[41]
Dendrobium 'White Pancy Lip'	Seedling growth	Modified VW medium supplemented with 10, 20, 40, and 60 mg/L chitosan	[42]
Dendrobium bigibbum var. *compactum* and *Dendrobium formosum*	Seed germination and protocorm growth	Modified VW medium supplemented with polymer and oligomer form of chitosan, each with deacetylation degree of 70. 80, 90% at five different concentrations (0, 10, 20, 40, or 80 mg/L)	[43]
Dendrobium mannii and *Dendrobium mirbelianum*	PLBs	Modified MS supplemented with 1.5 mg/L of 2- isopentenyl adenine, 0.1 mg/L IAA, 4 mg/L calcium panthotenate, 10% (v/v) coconut water, and 0, 1, 2, 3, and 4 mg/L of chitosan	[44]
Dendrobium phalaenopsis	Growth of meristem explants into protocorm-like bodies	VW medium with 15% coconut water with 15 ppm of 10, 100, and oligomer 1 kDa molecular weight chitosan	[45]
Dendrobium sp.	PLBs	VW medium with 15% coconut water, and chitosan at 0, 5, 10, 15, 20, and 25 ppm	[46]
Phalaenopsis gigantea	PLBs	NDM (New Dogashima medium) and VW medium with chitosan at 0, 5, 10, 15, 20, and 25 mg/L and TDZ at concentrations of 0, 0.1, and 0.5 mg/L	[47]
Phalaenopsis gigantea	PLBs	NDM and VW medium with 20% coconut water and 0, 5, 10, 15, 20, and 25 mg/L of chitosan	[48]
Rhynchostylis gigantea	Protocorm growth	Modified VW medium supplemented with chitosan at 0, 10, 20, 40, and 60 mg/L	[49]

3 Commercial Suppliers

Table 19 lists a sample of commercial companies that carry a wide range of supplies use in orchid cultures. Prior to purchasing media and supplies from a commercial supplier, it is important to study the information data sheet and other relevant information to determine the suitability of the products in one's own studies.

Table 19
Website information on a sample of suppliers in plant tissue culture

Supplier	Media available for purchase
Bio World https://www.bio-world.com	Gamborg B5 medium Knudson C Orchid medium, Morel Modification Mitra Maintenance/Replate medium DKW/Juglans medium, w/ vitamins Malmgren Terrestrial Orchid medium, w/ pineapple powder Lindemann Orchid medium Schenk and Hildebrandt medium Heller medium Fast Terrestrial Orchid medium
Caisson Labs http://www.caissonlabs.com	Murashige and Skoog with vitamins. Without glycine. Vacin and Went Orchid Basal Salts DKW Basal Salts
Dephyte https://dephyte.com/	Knudson C medium Morel-mod. NDM | New Dogashima medium
Duchefa Biochemie https://www.duchefa-biochemie.com	Murashige and Skoog medium Gamborg B5 medium Knudson C orchid medium Vacin and Went medium Heller medium DKW/Juglans medium Lindemann Orchid medium Schenk and Hildebrandt Basal Salt medium
Fisher Scientific https://www.fishersci.ca	MP Biomedicals Murashige and Skoog Media
Henduan Bio tech http://hengduanbiotech.com/	GD-Thomale original medium, complete
Himedia Labs http://himedialabs.com	Gamborg B5 medium PT016 w/ $CaCl_2$, vitamins and sucrose; w/o IAA, kinetin and agar Mitra Orchid medium Vacin and Went medium DKW / Juglans medium Malmgren Modified Terrestrial Orchid medium Lindemann Orchid medium Schenk and Hildebrandt medium Heller medium w/ macroelements and microelements; w/o vitamins, sucrose, and agar

(continued)

Table 19
(continued)

Supplier	Media available for purchase
MP Bio http://www.mpbio.com	Murashige and Skoog basal medium Vacin and Went Modified Basal Salt medium, Mother Flasking medium I Malmgren's Modified Terrestrial Orchid medium
Phytotechnology Laboratories https://phytotechlab.com	Murashige and Skoog Basal medium with vitamins Knudson C Orchid medium Mitra Replate/Maintenance medium Vacin and Went Orchid medium with sucrose DKW medium with vitamins Fast Orchid medium Malmgren Orchid medium Lindemann Orchid Basal medium Schenk and Hildebrandt Modified Basal medium w/ vitamins and sucrose
Plant Media http://www.plantmedia.com	Murashige and Skoog (MS) medium, w/ vitamins Knudson C Orchid medium, Morel Modification Mitra Maintenance/ Replate medium DKW Basal medium, w/ 30 g/L sucrose Fast Terrestrial Orchid medium Malmgren Terrestrial Orchid medium, w/ pineapple powder Schenk and Hildebrandt medium
Sigma-Aldrich http://www.sigmaaldrich.com	Murashige and Skoog Basal medium Gamborg's B-5 Basal medium with minimal organics Knudson C Modified Orchid medium Schenk and Hildebrandt Basal Salt Mixture
Thomas Scientific http://www.thomassci.com/	Gamborg B5 medium Vacin and Went medium
VWR https://us.vwr.com	Murashige/Skoog Multiplication medium

4 Notes

1. Sequestrene 330 Fe from Geigy Agricultural Chemicals, Saw Mill River Road, Ardsley, New York, was used for the original formulation. Supplier Duchefa Biochemie uses 36.70 mg/L (100 μM) of FeNaEDTA instead of Sequestrene 330 Fe. Sequestrene 330 Fe is replaced with 37.3 mg/L Na_2EDTA and 27.80 mg/L $FeSO_4 \cdot 7H_2O$ in the formulation from Himedia Labs. According to Arditti [1], the chelated form of iron is preferred.
2. Media available from Himedia Labs use anhydrous calcium nitrate, magnesium sulfate, and calcium chloride and no sucrose and agar. Duchefa Biochemie uses FeNaEDTA instead

of a mixture of Na_2EDTA and $FeSO_4 \cdot 7H_2O$, and has no vitamins, agar, and sucrose included. The formulation from PhytoTechnology Laboratories includes 0.005 mg/L of $NiSO_4 \cdot 6H_2O$, which is not present in the original formulation. This modification follows McGranahan et al. [11].

3. The Fast medium formulation is published in the appendix section of Rasmussen's monograph [7]. The medium calls for the addition of 0.8 mL/L of Heller's trace elements, but because the method of preparation of the stock solution is not stated, Heller's microelements composition from [1] is listed instead.
4. This medium is unavailable as a prepared mix from commercial suppliers.
5. As shown in [1], Heller's medium is often modified with addition of organic components, vitamins, and plant growth regulators. Upon observations of several modified Heller's medium, the macro- and microelements are consistent, and only the organic additives are modified. The table shown here is Heller's medium without any organic additives, but with just macro- and micronutrients and is assumed to be the original formulation. This medium is available from suppliers with the same composition of macro- and micro- elements, albeit sometimes with the use of different hydrates of compounds. Himedia Labs uses equivalent molar concentrations of magnesium sulfate and anhydrous monobasic sodium phosphate. Duchefa Biochemie also uses equivalent molar concentrations of magnesium sulfate, calcium chloride, and sodium phosphate monobasic anhydrous forms.
6. The original formulation of the Knudson C medium is listed in Table 6, which is published in Vacin and Went [20]. Moreover, the formulation provided by suppliers often includes the Morel modification (Table C-6 in [1]). The modifications are as follows: increase $(NH_4)_2SO_4$ from 500 to 1000 mg and reduce $Ca(NO_3)_2 \cdot 4H_2O$ from 1000 to 500 mg. Two compounds not originally present, NH_4NO_3 and KCl, are added, at 250 mg/L each.
7. The original publication calls for either coconut water 15% v/v or 100 mg/L of casein hydrolysate as an organic additive.
8. The composition listed in the table is based on the formulation provided by PhytoTechnology Laboratories (Phytotech: https://phytotechlab.com/media/documents/ProdInfo/M551-Info.pdf). This formulation from PhytoTechnology Laboratories is used in several publications, including [12, 13]. The original recipe by Malmgren published in [26] lists an approximate range of macroelements and are as follows: $MgSO_4 \cdot 7H_2O$ 50–100 mg/L, KH_2PO_4 50–100 mg/L, $Ca_3(PO_4)_2$ 50–100 mg/L, sucrose 10–20 g (10 g in sowing

medium, 15–20 g in growing medium), activated charcoal 0.5 g, 10–25 mL of pineapple juice (with pH of 5.5–6), 300 mg of Vamin (amino acids), 0.1–0.3 amp of Soluvit (vitamins), and 6 g of agar in 1000 mL of tap water. The pH of this medium will be at 5.5–6, as opposed to 3.75–4.75 for the medium prepared by PhytoTechnology Laboratories.

9. The original recipe calls for 3 mL of $Na_2FeEDTA$. However, according to [1], $Na_2FeEDTA$ may not be easily accessed and thus can be substituted with 37.3 mg/L of Na_2EDTA and 27.8 mg/L of $FeSO_4{\cdot}7H_2O$.
10. For the iron source, 5 mL/L of a stock solution mix of 7.45 g Na_2EDTA and 5.57 g $FeSO_4{\cdot}7H_2O$ in 1 L of H_2O was used. Supplier Duchefa Biochemie uses 36.70 mg/L (100 μM) of FeNaEDTA instead of a mixture of Na_2EDTA and $FeSO_4{\cdot}7H_2O$. Edamin (1 g/L) is listed as an optional organic additive. Often, this medium is reduced in salt concentration by a factor of 2 or 4 according to the needs of the plant.
11. This medium mixture is available for purchase from Dephyte; the formula uses anhydrous calcium nitrate ($Ca(NO_3)_2$) and magnesium sulfate, manganese sulfate monohydrate instead of tetrahydrate, and does not include concentrated sulfuric acid. It includes biotin, but no folic acid.
12. Duchefa Biochemie uses anhydrous magnesium sulfate and calcium chloride; FeNaEDTA instead of $FeSO_4$ + Na_2EDTA; this supplier also does not include kinetin, sucrose, and agar in the formulation. PhytoTechnology Laboratories uses anhydrous calcium chloride and magnesium sulfate and 10 g/L of sucrose and does not include kinetin and agar. Himedia labs uses anhydrous magnesium sulfate and doesn't include kinetin, sucrose, and agar.
13. Medium mixture is available for purchase from Hengduan biotech; the supplier uses 10 mg/L of Nicotinic acid instead of 1 mg of nicotinic acid amine. Wuchsstoff 66_f is replaced with 0.1 mg/L of 1-Naphthaleneacetic acid (NAA) as it is no longer commercially available.
14. The media formulas provided by suppliers often substitute ferric tartrate for other sources of iron such as ferric sulfate. PhytoTechnology and Caisson Labs use equivalent iron molar concentration of ferric sulfate heptahydrate. Duchefa Biochemie and Himedia Labs use manganese sulfate monohydrate instead of dihydrate, anhydrous magnesium sulfate instead of heptahydrate, and anhydrous ferric tartrate instead of dihydrate. The medium prepared by Phytotechnology Laboratories includes 0.4 mg/L of Thiamine HCl, which is absent in the original formulation.
15. The original publication used a modified macroelement solution based on the media composition described in Thomale

[14], microelement and vitamin solution according to Nitsch and Nitsch [15], and Fe-EDTA solution according to Murashige and Skoog [16].

16. The calculations for the individual ion and compound concentrations were performed in the same manner as presented in de Fossard [6].

References

1. Arditti J (2008) Micropropagation of orchids, 2nd edn. Blackwell Publishing, Oxford
2. Chugh S, Guha S, Rao IU (2009) Micropropagation of orchids: a review on the potential of different explants. Sci Hortic 122:507–520
3. Teixeira da Silva JA (2013) Orchids: advances in tissue culture, genetics, phytochemistry and transgenic biotechnology. Floricult Ornament Biotechnol 7:1–52
4. Dodds JH, Roberts LW (1995) Experiments in plant tissue culture. Cambridge University Press, Cambridge
5. Owen HR, Miller R (1992) An examination and correction of plant tissue culture basal medium formulations. Plant Cell Tissue Org Cult 28:147–150
6. de Fossard RA (1976) Tissue culture for plant propagators. Armidale, NSW, Dept. of Botany, University of New England
7. Rasmussen HN (1995) Terrestrial orchids: from seed to mycotrophic plant. Cambridge University Press, Cambridge
8. Kano K (1965) Studies on the media for orchid seed germination. Mem Fac Agric Kagawa Univ 20:1–68
9. Tsukamoto Y, Kano K, Katsuura T (1963) Instant media for orchid seed germination. Am Orchid Soc Bull 32:354–355
10. Winarto B, Teixeira da Silva JAT (2015) Use of coconut water and fertilizer for *in vitro* proliferation and plantlet production of *Dendrobium* 'Gradita 31'. In Vitro Cell Dev Biol Plant 51:303–314
11. Mcgranahan GH, Driver JA, Tulecke W (1987) Tissue culture of *Juglans*. In: Bonga JM, Durzan DJ (eds) Cell and tissue culture in forestry. Martinus Nijhoff Publishers, Boston, pp 261–271
12. Stewart SL, Kane ME (2006) Asymbiotic seed germination and in vitro seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell Tissue Organ Cult 86:147–158
13. Kauth PJ, Dutra D, Johnson TR, Stewart SL, Kane ME, Vendrame W (2008) Techniques and applications of *in vitro* orchid seed germination. In: Teixeira da Silva J (ed) Floriculture, ornamental and plant biotechnology: advances and topical issues. Global Science Books, Middlesex, pp 375–391
14. Thomale H (1954) Die Orchideen. Verlag Eugen Ulmer, Stuttgart
15. Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87
16. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497
17. Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspensions cultures of soybean root cells. Exp Cell Res 50:151–158
18. Driver JA, Kuniyuki AH (1984) *In vitro* propagation of paradox walnut rootstock. Hortic Sci 19:507–509
19. Harvais G (1982) An improved culture medium for growing the orchid *Cypripedium reginae* axenically. Can J Bot 60:2547–2555
20. Vacin EF, Went FW (1949) Some pH changes in nutrient solutions. Bot Gaz 110:605–613
21. Lindemann EGP, Gunckel JE, Davidson OW (1970) Meristem culture of *Cattleya*. Am Orchid Soc Bull 39:1002–1004
22. Malmgren S (1996) Orchid propagation: theory and practice. In: Allen C (ed) Proceedings of the North American terrestrial orchids: propagation and production conference. Washington, National Arboretum, pp 63–72
23. Mitra GC, Prasad RN, Choudhury AR (1976) Inorganic correlated salts and differentiation of protocorms in seed of an orchid (*Dendrobium fimbriatum*) and correlated changes in its free amino acid context. Indian J Exp Biol 14:350–351
24. Tokuhara K, Mii M (1993) Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. Plant Cell Rep 13:7–11
25. Schenk RV, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50:199–204

26. Van Waes JM, Deberg PC (1986) *In vitro* germination of some Western European orchids. Physiol Plant 67:253–261
27. Kongbangkerd A, Watthana S, Srimuang KO (2016) Influence of organic supplements on growth and development of *in vitro* shoots of *Bulbophyllum dhaninivatii* Seidenf. Appl Mech Mater 855:42–46
28. Huh YS, Lee JK, Nam SY, Paek KY, Suh GU (2016) Improvement of asymbiotic seed germination and seedling development of *Cypripedium macranthos* Sw. with organic additives. J Plant Biotechnol 43:138–145
29. Zhang Y, Lee Y-I, Deng L, Zhao S (2013) Asymbiotic germination of immature seeds and the seedling development of *Cypripediu macranthos* Sw., an endangered lady's slipper orchid. Sci Hortic 164:130–136
30. Parthibhan S, Rao MV, Kumar TS (2015) *In vitro* regeneration from protocorms in *Dendrobium aqueum* Lindley—an imperiled orchid. J Genet Eng Biotechnol 13:227–233
31. Cui HY, Murthy HN, Moh SH, Cui YY, Paek KY (2015) Establishment of protocorm suspension cultures of *Dendrobium candidum* for the production of bioactive compounds. Hortic Environ Biotechnol 56:114–122
32. Vyas S, Guha S, Bhattacharya M, Rao IU (2009) Rapid regeneration of plants of *Dendrobium lituiflorum* Lindl. (Orchidaceae) by using banana extract. Sci Hortic 121:32–37
33. Islam MO, Islam MS, Saleh MA (2016) Effect of banana extract on growth and development of protocorm like bodies in *Dendrobium* sp. orchid. Agriculturists 13:101
34. Gnasekaran P, Rathinam X, Sinniah UR, Subramaniam S (2010) A study on the use of organic additives on the protocorm-like bodies (PLBS) growth of *Phalaenopsis violacea* orchid. J Phytology 2:29–33
35. David D, Jawan R, Marbawi H, Gansau JA (2015) Organic additives improves the *in vitro* growth of native orchid *Vanda helvola* Blume. Not Sci Biol 7:192–197
36. Gnasekaran P, Poobathy R, Mahmood M, Samian MR, Subramaniam S (2012) Effects of complex organic additives on improving the growth of PLBs of *Vanda* Kasem's Delight. Aust J Crop Sci 6:1245–1248
37. Islam M, Akter M, Prodhan A (2012) Effect of potato extract on *in vitro* seed germination and seedling growth of local *Vanda roxburgii* orchid. J Bangladesh Agric Univ 9:211–215
38. Pacek-Bieniek A, Dyduch-Siemińska M, Rudaś M (2010) Influence of activated charcoal on seed germination and seedling development by the asymbiotic method in *Zygostates grandiflora* (Lindl.) Mansf. (*Orchidaceae*). Folia Hortic 22:45–50
39. JATD S, Uthairatanakij A, Obsuwan K, Shimasaki K, Tanaka M (2013) Elicitors (Chitosan and Hyaluronic Acid) affect protocorm-like body formation in hybrid *Cymbidium*. Floricult Ornament Biotechnol 7:77–81
40. Tantasawat P, Wannajindaporn A, Chantawaree C, Wangpunga C, Poomsom K, Sorntip A (2010) Chitosan stimulates growth of micropropagated *Dendrobium* plantlets. Acta Hortic 878:205–212
41. Pornpienpakdee P, Singhasurasak R, Chaiyasap P, Pichyangkura R, Bunjongrat R, Chadchawan S, Limpanavech P (2010) Improving the micropropagation efficiency of hybrid *Dendrobium* orchids with chitosan. Sci Hortic 124:490–499
42. Obsuwan K, Sawangsri K, Yoodee S, Uthairatanakij A (2010) Effects of chitosan concentration on *in vitro* growth of *Dendrobium* hybrid seedlings. Acta Hortic 878:289–294
43. Kananont N, Pichyangkura R, Chanprame S, Chadchawana S, Limpanavech P (2010) Chitosan specificity for the *in vitro* seed germination of two *Dendrobium* orchids (Asparagales: Orchidaceae). Sci Hortic 124:239–247
44. Rahmah S, Wiendi NMA, Suwarno WB, Krisantini K (2015) Proliferation of Protocorm-like bodies of *Dendrobium mannii* and *Dendrobium mirbelianum* in chitosan-containing media in vitro. J Trop Crop Sci 2:22–28
45. Nge KL, Nwe N, Chandrkrachang S, Stevens WF (2006) Chitosan and as a growth stimulator in orchid tissue culture. Plant Sci 170: 1185–1190
46. Restanto DP, Santoso B, Kriswanto B, Supardjono S (2016) The application of chitosan for Protocorm like bodies (PLB) induction of orchid (*Dendrobium* sp.) *in vitro*. Agric Agric Sci Proc 9:462–468
47. Samarfard S, Kadir MA, Kadzimin SB, Saud HM, Ravanfar SA, Danaee M (2014) *In vitro* propagation and detection of somaclonal variation in *Phalaenopsis gigantea* as affected by chitosan and thidiazuron combinations. Hortic Sci 49:82–88
48. Samarfard S, Kadir MA, Kadzimin SB, Ravabfar S, Saud HM (2013) Genetic stability of *in vitro* multiplied *Phalaenopsis gigantea* protocorm-like bodies as affected by chitosan. Not Bot Horti Agrobo 41:177–183
49. Obsuwan K, Yoodee S, Uthairatanakij A (2010) Application of chitosan on *in vitro* growth of *Rhynchostylis gigantea* protocorms and seedlings. Acta Hortic 878:283–228

Chapter 7

Orchid Micropropagation: An Overview of Approaches and Methodologies

Tim W. Yam and J. Arditti

Abstract

Micropropagation can be used as means of such ex situ conservation. Sometimes, when there is only one plant left in the natural habitat, the only way to bulk up the population is by micropropagation. One of Singapore's critically endangered species, *Robiquetia spathulata*, has been successfully cloned and reintroduced using this method. However, plantlets produced by cloning are genetically homogenous and thus are less suitable for conservation and repopulation than genetically diverse seedlings. This chapter provides an overview on various types of explants used in orchid micropropagation. General methodologies in the handling and culturing of explants are discussed.

Key words Micropropagation, Culture conditions, Culture media, Explant isolation, Medium additives, Plant growth regulators, Sterilization procedures

1 Introduction

Why do we use plants raised by micropropagation for conservation? Plantlets raised from micropropagation are genetically identical; these cloned plants lack genetic diversity and therefore are not ideal for conservation work. However, when a plant is critically endangered and sometimes there are only one or a few plants left in nature, micropropagation may be the only means of increasing their number.

Micropropagation was first proposed in 1968 and defined as an aseptic procedure for the asexual production of plantlets from organs, tissues, and cells bypassing the sexual process or other means of asexual propagation [1, 2].

Shih Wei Loo and Ernest A. Ball [3–5] cultured the first shoot tips and meristems (in asparagus and *Tropaeolum majus* L. and *Lupinus albus* L.) in 1945 and 1946, independently of each other. *Dahlia* plants were freed of spotted wilt virus through shoot-tip cuttings in 1948. A year later, Dr. Gavino Rotor [6] cultured

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_7,

Phalaenopsis flower stalk sections in the first ever propagation of an orchid through the method now known as tissue culture or micropropagation. Hans Thomale (1919–2002), a German nurseryman, was the first to culture an orchid shoot tip and published his work in 1956 and 1957 [7, 8]. George Morel published his report on the micropropagation of orchids in 1960 [9]; unfortunately it was devoid of detailed information about techniques and procedures. The first publication of orchid shoot-tip culture containing all relevant information (the accepted practice among scientists) was by the late Professor Donald E. Wimber (1930–1997) in 1963 [10]. Those interested in a detailed history of orchid micropropagation should refer to Arditti and Krikorian [11].

This chapter provides an overview on orchid micropropagation emphasizing some practical issues in the handling and culturing of explants. For detailed information and discussion, readers are referred to Arditti [12–14], Arditti and Ernst [15], and the recently published monograph by Yam and Arditti [16]. Chapter 5 provides companion information on in vitro orchid seed germination.

2 Explants

As already mentioned, the first orchid explants to be cultured were *Phalaenopsis* flower stalk segments and shoot tips. Other explants from different parts of the plant body have been successfully cultured from both mature plants and seedlings. However, when explants are taken from seedlings, it is more difficult to selectively propagate outstanding cultivars because their quality of the original plant is not known. However, it is possible to rapidly increase the number of plants using the available seedlings. Taking explants from plants which were produced from an explant should be avoided because excessive proliferation, especially through the use of high levels of growth regulators, can cause undesirable mutations [17, 18]; for a review, see [19–22]. Explants can be removed before or after the donor plant has been surface sterilized. When the explants are taken before sterilization of the donor plant, they must be surface sterilized following their removal.

2.1 Root Cultures

The first paper which suggested that orchid root cultures should be done appeared in Beechey [23]. At about the same time, students from Joseph Arditti's laboratory attempted to culture the root tips of *Epidendrum* using a modified medium originally developed for the culture of wheat root tips [24]. The roots grew in length only and after 2 years lost their chlorophyll [25]. In 1976, *Phalaenopsis* roots were cultured successfully [26]. Since then, roots of many other orchids have been cultured successfully

and used to produce plantlets: *Catasetum* [27–30], *Cattleya* [31], *Cymbidium* [32], *Cypripedium yatabeanum* [33], *Cyrtopodium* [34], *Doritaenopsis* [35, 36], *Rhynchostylis* [37, 38], and other orchids [39]. Root culture seems to be a viable method of orchid micropropagation.

2.2 Rhizome and Tuber

Professor H. Torikata at the University of Nagoya in Japan successfully cultured rhizome tips [40]; for a review, see Rao [41]. Other orchids propagated from rhizome explants are *Cymbidium aloifolium* [42], *Cymbidium ensifolium* [43–45], *Cymbidium kanran* Dougiu [43], *Cymbidium goeringii* [46], *Cymbidium kanran* Namkuk [46], *Cymbidium kanran* Toja [43], *Cymbidium kanran* Jeju, *Cymbidium goeringii* [43], *Cymbidium niveo-marginatum* [46], *Cymbidium sinense* [45], *Geodorum densiflorum* [47], and other orchids. The first report of tuber explant culture is that of *Pachystoma senile* [48].

2.3 Leaf Cultures

The advantage of leaf-tip cultures is that removal of explants does not endanger the donor plant. It has been observed that juvenile leaves on protocorms have a tendency to produce protocorm-like bodies; this led to the development of micropropagation methods through culture of leaf bases [49]. The first well-documented report that leaves can produce protocorm-like bodies was made in cultures derived from *Cymbidium* shoot tips [10]. In the early 1970s, students of Ernest Ball and Joseph Arditti propagated leaf tips of *Epidendrum* and *Laeliocattleya* successfully [50–56]. One of the most important criteria for successful leaf-tip culture is that the leaves must be immature; otherwise they would lose their ability to form callus and cease to develop when placed in culture. Other orchid species that have been successfully cloned from leaf tips include *Acampe praemorsa* [57], *Aerides maculosum* [58], *Aranda* [59–62], *Ascocenda* [60, 61], *Cattleya* [60, 61], *Cymbidium* [31, 32, 63], *Dendrobium* [63], *Laeliocattleya* [64], *Mokara* [65], *Oncidium* [66, 67], *Papilionanthe teres* [68], *Phalaenopsis* [69–73], *Renantanda* [74, 75], *Renanthera imschootiana* [76, 77], *Rhynchostylis retusa* [78], terrestrial species [79], *Vanda* [69], *Vanda coerulea* [80], *Vanda cristata* [81], *Vanda teres* [82], and other orchids [17, 83]. For reviews, see [11–16, 42, 84–89].

2.4 Stems

Sympodial orchids have multiple stems, and there are many nodes on each stem. These stem nodes provide excellent source of explants for orchid micropropagation. Stem culture of *Arundina* was first mentioned in Bertsch [90]. But a detailed research paper on stem disk culture of *Arundina* was only published by Mitra [91]. Nodes of *Dendrobium* were cultured in 1973 [92–95]. The stem of other orchids has also been cultured (for reviews see [11–16, 42, 84–89]).

2.5 Flower Buds, Flowers, Floral Segments, and Reproductive Organs

Flower cultures also have the advantage in that removal of explants does not endanger the donor plant. The ovaries of the first orchid flower segments were cultured by Professor I. Ito at the Kyoto Prefectural University in Japan [96, 97]. Professor Ito was also the first to culture immature *Dendrobium* seeds [98]. Immature seed cultures of other orchids have also been reported: *Vanilla* [99], *Phalaenopsis* [100], *Dendrobium* [101], *Vanda* [102], and *Paphiopedilum* [103]; for reviews see [13, 41, 84, 88, 104]. Although this is not a usual method of micropropagation, the contents of ovaries are scraped onto a culture medium, and some plantlets can form from ovary tissue and/or cells [16].

Intuwong and Sagawa [105] reported that they successfully cultured young flower buds of *Ascofinetia*, *Neostylis*, and *Vascostylis*. Subsequently, those of *Cymbidium* [106, 107], *Phalaenopsis*, *Phragmipedium* [108], and other orchids were cultured (for reviews see [11–16, 42, 84–89]).

2.6 Inflorescences

Dr. Gavino Rotor was the first researcher who cultured *Phalaenopsis* flower stalk nodes. Subsequently, inflorescence explants of other orchids have also been cultured: *Aranda* [109], *Dendrobium* [110], *Doritaenopsis* [111, 112], *Mokara* [65], *Oncidium* [113], and *Phalaenopsis* [71, 114–125]; for reviews, see [11–16, 42, 86–91].

2.7 Plant Diseases and Meristems

It is known more than 60 years ago that healthy clones of plants can be obtained from stem tips or root cuttings from unhealthy plants (see North [126], Krikorian [1] for literature citations). Although stem-tip culture is able to produce a larger number of pathogen-free plants in a relatively short period of time ([127–131]; Morel and Muller [129]; Gautheret [130, 131]), apical meristems are not necessarily free of virus infection. This has led to considerable difficulties in freeing many clones and cultivars of viruses [132]. *Odontoglossum* ring spot (ORSV) and *Cymbidium* mosaic (CyMV) viruses have been eradicated through chemotherapy in vitro. An eradication method using Virazole was developed nearly two decades ago [133]. To ensure the cloned plants are virus free, these plantlets should be assayed for virus. Virus-free plantlets should be planted in potting mix in a greenhouse. Infected plantlets should be destroyed.

3 Culture Media

Several basic media are used for orchid micropropagation (Knudson C [134], LS [135], MPC [136], MS [137], and VC [138] are the main ones), but each of them is usually modified to suit a particular species or hybrid. Each of these media contains macroelements, microelements, iron chelate, and supplements such as:

1. Plant growth regulators, e.g., auxins [e.g., indoleacetic acid (IAA), indolebutyric acid (IBA), indolepropionic acid (IPA), naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D)] and cytokinins [e.g., benzyladenine (BA) which is also known as benzylaminopurine (BAP), isopentenyl adenosine (IPA), and zeatin]
2. Vitamins (mostly niacin, pyridoxine, and thiamine, but also biotin, folic acid, pantothenic acid, and others)
3. Complex additives (e.g., banana homogenate, casein hydrolysate, coconut water, fish emulsion, peptone, and yeast extract)
4. A darkening agent (vegetable charcoal)
5. A solidifier (e.g., agar or Phytagel)

Media must be prepared carefully and in accordance with instructions or experience. All media must be sterilized by autoclaving. Depending on the medium and procedure, some heat-sensitive components may have to be added after sterilization.

3.1 Macroelements

Macroelements are chemicals added in relatively large amounts. They include calcium (Ca), magnesium (Mg), nitrogen (N), phosphorus (P), potassium (K), and sulfur (S). Depending on the medium, several salts may be used to supply each mineral [e.g., potassium can be provided as KNO_3, KH_2PO_4, K_2HPO_4, and KCl among others; nitrogen may be added as KNO_3, NH_4NO_3, $Ca(NO_3)_2$, $(NH_4)_2SO_4$, other salts, and/or urea (see **Note 1**)]. The combinations of salts in each medium are designed to provide an appropriate balance of nutrients in proper concentrations and meet the demands or preferences of the explants or tissues being cultured. The sugar content [139] and pH [140] of the media affect the uptake of these macroelements.

Stock solutions of macroelements (except those containing nitrogen) can be stored at room temperature. Nitrogen-containing stock solutions should be kept in a refrigerator or freezer. Stock solutions should be stored in containers that are capped tightly to prevent evaporation. All macroelement salts can be sterilized by autoclaving.

3.2 Microelements

Different culture media require varying amounts of microelements. It is very important to keep in mind that many microelements can be toxic at higher concentrations. Like macroelements, all microelement salts are heat stable and can be autoclaved.

When ethylenediaminetetraacetic acid [free acid (EDTA, MW 292.25), disodium salt (Na_2EDTA, MW 336.02), and disodium dihydrate ($Na_2EDTA{\cdot}2H_2O$, MW 272.24)] became available as chelating agents, they found widespread use in culture media including, of course, the well-known Murashige-Skoog (MS)

medium [137]. The amounts used in MS are 37.3 mg Na_2EDTA L^{-1} and 27.8 mg $FeSO_4.7H_2O$ L^{-1}. However, many iron salts are not sufficiently soluble.

3.3 Plant Growth Regulators

3.3.1 Auxins and Antiauxin

The most commonly used auxins in orchid tissue culture media are the naturally occurring auxin, indoleacetic acid (IAA), and the synthetic naphthaleneacetic acid (NAA), indolebutyric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D). The effects of one auxin on a certain species may be different from those of other auxins and may differ with the orchid. The effects of different concentrations of the same auxin may differ in respect to one species and may not be the same with another orchid. Auxins should never be substituted, and their concentrations should never be changed without prior tests.

Most auxins are not destroyed by autoclaving at 110–120 °C for 50–60 min especially if the pH is non-acidic. However, autoclaving at low pH and in the presence of other factors may destroy IAA [141]. In many instances, orchid explants and tissues grow and develop well on media that are autoclaved following the addition of auxin. Illumination provided by cool white fluorescent tubes causes the degradation of both IAA and IBA in both liquid and solid media. IBA is more stable than IAA under these conditions. Charcoal can absorb up to 97% of IAA and IBA in MS medium [142].

Some media contain the antiauxin trans-cinnamic acid (tCA) to break bud dormancy. Heat sterilization of tCA is not advisable without prior determination that this will not affect its usefulness.

3.3.2 Cytokinins

The synthetic kinetin (6-furfuryl aminopurine), benzyladenine (N6-benzylaminopurine, N6-benzyladenine, BA, BAP), dimethylaminopurine (DMAP), thidiazuron (TDZ), and the naturally occurring zeatin are used most commonly in orchid culture media. The cytokinins and concentrations used are based on empirical findings, and unnecessary changes should be avoided. The effects of different cytokinins and their concentrations differ like those of auxins. Experiments with aqueous solutions of kinetin, zeatin, and isopentenyladenosine have shown that they are not broken down when autoclaved for 1 h at 120 °C [143]. In general, cytokinins should be treated like auxins in respect to sterilization.

Thidiazuron (N-phenyl-N′-1,2,3-thiadiazol-5-yl-urea, TDZ) is a relatively recent addition to the list of cytokinins which are used in orchid micropropagation [144–146]. It was first used for micropropagation of orchids, specifically *Phalaenopsis* [147]. TDZ is soluble in dimethyl sulfoxide (DMSO), N,N-dimethylformamide (DMF), 0.1 or 0.01 N KOH or NaOH, 0.5 N HCl, and 50% or more ethanol [148]. It can be autoclaved, but autoclaving may cause some loss of activity. Therefore, some investigators prefer to filter sterilize it. It can also be dissolved in 70–95% ethanol and added to media after autoclaving.

3.3.3 Gibberellins and Anti-Gibberellin

Gibberellins are seldomly used in culture media for orchids. When used, GA_3 is the most common form. Autoclaving reduces gibberellin activity by more than 90% [149]. Therefore, gibberellins must be cold sterilized through filtration or by dissolving them in ethanol. The anti-gibberellin ancymidol has been used in an orchid medium [150]. It should be dissolved in 50–95% ethanol and added to media after autoclaving.

3.4 Other Additives

3.4.1 Amino Acids

The most commonly used amino acid in orchid culture media is glycine [137]. Other amino acids are also used in some media. The effects of autoclaving on amino acids may vary, and it is best to follow the original procedures in each case. If in doubt, both autoclaving and cold sterilization (filtration or dissolving in ethanol) should be tested before deciding which sterilization method to use.

3.4.2 Polyol

The only polyol used in orchid culture media is myoinositol because it is part of the MS medium [137]. The function of inositol was not clear for a long time. More recently it has been suggested that inositol is a component of cellular signalling molecules [151]. Inositol is usually sterilized by autoclaving.

3.4.3 Polyamines

All plants contain polyamines. They play roles in and affect cell division, embryogenesis, flower development, fruit ripening, root induction, and tuber formation. Putrescine, spermidine, and spermine are the most common polyamines in plants. Some have been used in tissue culture including orchid micropropagation to promote adventitious root initiation, shoot formation, and somatic embryogenesis.

3.4.4 Vitamins

Niacin (nicotinic acid), pyridoxine (vitamin B6), and thiamine (vitamin B1) are most commonly added to orchid culture media as part of several media including the MS medium. Biotin, folic acid, and pantothenic acid (as calcium pantothenate) are also used in some media. Media that contain vitamins are routinely heat-sterilized without any ill effects. In practical applications, it is best to just follow instructions.

3.4.5 Charcoal

Activated carbon or charcoal is characterized by an extremely large area/weight ratio (up to 2000 $m^2\ g^{-1}$) and is used for the adsorption of substances. Dr. Peter Werkmeister was the first to use charcoal to darken orchid culture media [152–154]; for historical accounts, see [11, 89]. He did it to study the effects of dark media on root growth and development.

Activated vegetable charcoal (2 mg L^{-1}) improved seedling growth of the terrestrial *Paphiopedilum* [155] and the epiphytic *Phalaenopsis* [156, 157] orchids. These observations from experiments based on Werkmeister's work [155–157] led to the development of practical charcoal-containing media, which gained widespread and rapid acceptance. Apart from media for seedlings,

charcoal is now added to many tissue culture media for orchids and other plants (for a few examples, see [11, 158–169]; for reviews see [11, 169]).

The beneficial effects of charcoal in culture media are attributed to [1] darkening which simulated soil conditions [170] and [2] adsorption of unidentified morphogenetically active [171], toxic [164], or harmful substances [167, 172, 173].

However, charcoal also has the capacity to adsorb hormones and vitamins and thereby inhibit growth [166, 167, 174–176]. When the pH of a medium was first adjusted to 5.8, after the addition of charcoal but before autoclaving, the pH changed to 5.4 (Sigma-neutralized charcoal), 5.9 (Sigma-acid-washed charcoal), or 5.4 [Merck No. 2186, acid-washed NuChar, and SN-neutralized NuChar [171]).

Given these findings, charcoal should be added with caution to media that contain additives which may be adsorbed, especially if they are required by the tissues. On the other hand, if explants, tissues, plantlets, and seedlings grow well on a medium that contains both charcoal and the additives it adsorbs, there is no reason to make changes (see **Note 2**).

3.4.6 Banana

Banana homogenate enhances the growth of orchid seedlings. In micropropagation, banana homogenate is added to media for plantlet development. It can be sterilized by autoclaving. For reviews see Withner [104, 177] and Arditti [178].

3.4.7 Coconut Water

Coconut water (CW) is the clear liquid endosperm of coconut seeds. As an additive to culture media, CW has beneficial effects on some orchid explants. CW can also increase proliferation without causing mutations. Because of these effects, CW is incorporated in several media (for reviews see 15, 179). The most commonly used and recommended CW is that from green (unripe) nuts. CW from ripe nuts can also be used. CW can be autoclaved. The reasons for the beneficial effects of CW are not clear. Plant hormones and related substances may be reasons for these effects.

3.4.8 Hydrolysates and Autolysates

Hydrolysates of peptone, tryptone, yeast extract, and chitosan (from deacetylated exoskeleton of crustaceans) are used in some orchid micropropagation media.

3.4.9 Sugars

Sucrose ($C_{12}H_{22}O_{11}$, MW 342.30) is most commonly used in orchid micropropagation. If necessary glucose and/or fructose can be substituted for sucrose in special tissue culture media, it is necessary to keep in mind that due to the different molecular weights of sucrose relative to glucose and fructose, the amount has to be adjusted.

3.4.10 Anti-Contaminants

For conservation work, very often plant materials have to be collected from the natural habitat. In some instances, it may not be possible to adequately surface-sterilize tissues or seeds, or a valuable culture may become contaminated. When this happens, the incor-

poration of bactericides or fungicides in the culture medium may save the cultures by either eliminating the contaminant(s) or at least keeping it/them in check until seedlings or plantlets are large enough to be moved to community pots. These formulations cannot be used routinely because they inhibit the development of the plants but are useful in special cases (see **Note 3**). Medium is prepared as usual for tropical orchids through the step of dissolving the agar. The anti-contaminants are dissolved or suspended and mixed.

Amphotericin B (92.7% pure, 10 ppm), nystatin (4020 units mg^{-1}, 25 ppm), and Sodium Omadine (90% pure, 5 ppm) when used singly delayed the development of *Cymbidium* shoot-tip explants but had no other deleterious effects [180]. Penicillin G (1595 units mg^{-1}, 100 ppm) did not affect callus growth but inhibited plantlet formation. Benomyl (50% pure, 50 ppm), Dowicide (97% pure, 5 ppm), gentamicin (from a sterile ampoule, 50 ppm), quintozene (PCNB; 99% pure, 100 ppm), and vancomycin (97.8% pure, 50 ppm) were inhibitory. All combinations of these compounds inhibited callus formation, growth, and plantlet development [180]. Plant Preservative Mixture™ (PPM) was tested for its ability to control contamination in seed and seedling cultures of *Vanda sanderiana* seedlings. At 4 mL L^{-1}, PPM eliminated contamination [181].

These anti-contaminants have not been tested widely and may be unsuitable for some explants. It is advisable to test each anti-contaminant with a few cultures prior to large-scale use. Care should be exercised in any attempts to use them. Some anti-contaminants may delay the development of explants, while others may inhibit plantlet formation. Except in rare and unusual cases, anti-contaminant should not be used for orchid micropropagation. More details about the use of anti-contaminants in orchid micropropagation can be found in Yam and Arditti [16].

3.5 Solidifiers

Agar was the major solidifier used for orchid micropropagation media. More recently gellan gum sold under a number of brand names (Gelrite® and Phytagel™) is now being used in many formulations.

3.5.1 Agar

For experimental purposes, it is necessary to use reagent grade agar. Technical grade or kitchen quality agar may contain additives, which could interfere with cultures; these should only be used for practical micropropagation after tests and with caution.

A common procedure is to add the agar slowly with stirring to the medium at room temperature and bring the mixture to a gentle boil. After the agar has dissolved, the medium is autoclaved before or after dispensing it into culture vessels. Another method is to dispense the agar into a cold solution, disperse it thoroughly, and dissolve it by autoclaving. Depending on the agar, gelling temperature can vary from 25 to 30 °C.

3.5.2 Gellan Gum

Gellan gum is an agar substitute consisting of glucuronic acid, rhamnose, and glucose produced through fermentation by *Pseudomonas elodea*. Gellan gum produces a high-strength, colorless, and clear gel which gels at 27–31 ° C. The most commonly used concentrations for plant tissue culture are 1.2–1.5 to 2–2.5 g L^{-1}. Gellan gum, Phytagel, and Gelrite require the presence of divalent cations for gelling. Most orchid culture media contain enough calcium and magnesium to make gelling possible. If more dilute media are used, higher concentrations of Phytagel or Gelrite may be required. Gellan gums (Phytagel, Gelrite) must be added to media at *room temperature* with rapid stirring to avoid the formation of lumps. Two methods can be used for the preparation of gellan gum-containing media depending on whether a medium is to be dispensed before or after autoclaving.

Method 1: Medium will be dispensed after autoclaving.

Step 1. Prepare medium under room temperature.

Step 2. Add gellan gum slowly while stirring the medium continuously until gellan gum is completely and thoroughly dispersed.

Step 3. Sterilize medium.

Step 4. Add other additional components after autoclaving if any. Do it with great care since the medium is hot after autoclaving.

Step 5. Dispense medium while it is still liquid.

Step 6. Allow medium to cool and solidify.

Method 2: Medium is to be dispensed before autoclaving.

Step 1. Prepare medium under room temperature.

Step 2. Add the gellan gum slowly while stirring the medium continuously until gellan gum is completely and thoroughly dispersed.

Step 3. Heat the medium to a gentle boil until the solidifier is completely dissolved. Remove medium from heat source, continue to stir medium for a few minutes, and make sure gellan gum is completely dissolved.

Step 4. Dispense medium into culture vessels.

Step 5. Autoclave medium.

Step 6. Allow medium to cool and solidify. Should a medium tend to form a precipitate, swirl or stir every vessel to disperse whatever precipitates. Handle the hot media with great care.

Step 7. For the preparation of certain specific media, procedures are available in Yam and Arditti [16].

3.5.3 Agargel

Agargel is a product which combines the positive attributes of agar and Phytagel. Depending on the required strength of a gel, Agargel should be used at 2.5–5 g L^{-1}.

3.5.4 Transfergel

Transfergel is intended to be used as a carrier gel for microcuttings and somatic embryos. Transfergel is added to media at 1.5–2% (w/v). It hydrates and thickens immediately on being added to media. Therefore, it requires considerable and continuous stirring to ensure complete mixing and to get it to dissolve completely. Sigma-Aldrich recommends that it should be autoclaved in a container four times its volume (e.g., 500 mL in a 2 L flask) to avoid potential boilover.

3.5.5 Alginic Acid

Alginate [182] is a polysaccharide which can be used to prepare beads which can contain cell, protoplasts, tissues, protocorm-like bodies, and artificial seeds. Beads of alginate should be prepared accordance with the instructions for each procedure.

3.6 pH of a Medium

The pH of a culture medium indicates its alkalinity or acidity (i.e., hydrogen ion concentration). The pH of a medium is important because it affects the availability of nutrients, and it has a direct effect on the life processes of cells. The pH of culture media should be indicated in each method, determined experimentally, or adjusted to 4.8–6.0. It is best to set the pH to the levels recommended by the original investigators. To measure the pH of a medium accurately, it is best to use a pH meter. When the pH of a medium is above the desired value, it is too alkaline and must be adjusted down with a few drops of a 0.1 N acid (hydrochloric, nitric, phosphoric, or sulfuric) solution. Should the pH be lower than required, the medium is too acid and must be adjusted up with a 0.1 N solution of a base (alkali) such as ammonium hydroxide, potassium hydroxide, or sodium hydroxide.

4 Some Practical Issues

4.1 Stock Solution Preparation

If you have to prepare culture media regularly, it is better to prepare stock solutions of most media components (see **Note 4**). These solutions are concentrated solutions of 10, 100, or even 1000 times. Stock solutions save work and increase accuracy because it is easier to measure large or small volumes of solution accurately than it is to weigh solids.

Each stock solution must be properly labeled with the following: name of compound, formula of compound, concentration, amount to use per liter of culture medium, date of preparation, and name of the person who made the solution. Stock solutions containing nitrogen (e.g., nitrate, ammonia, and urea) tend to become contaminated on standing. Many researchers prefer to

weigh the solid chemicals every time. For microelements, all components should be combined into one stock solution. If stock solutions are prepared, they must be kept in a refrigerator or frozen between uses.

Stock solutions of hormones and vitamins should be in 70 or 95% ethanol. For some compounds, it may be necessary to add a few drops of sodium or potassium hydroxide or hydrochloric, sulfuric, nitric, or acetic acid to increase the solubility. To prevent contamination, such stock solutions should be stored in a freezer. Plant growth regulators, vitamins, and amino acids may not be stable for prolonged periods. It is best, therefore, to prepare only small volumes (10–15 mL) of stock solutions. Stock solutions of organic substances should be stored in a freezer or refrigerator.

4.1.1 Preparation of the Medium

A 1 L culture media for micropropagation can be prepared by the following procedure.

1. Add the correct amount of macroelement stock solutions to 250 mL of distilled water.
2. Dispense the appropriate amount of microelement stock solution.
3. Add inositol (if it is included in the medium).
4. Incorporate all the complex additives (some media may not require such additives).
5. Add distilled water to bring the total volume to approximately 900 mL.
6. Adjust the pH.
7. Add sugar (sugar may also be added before pH adjustment).
8. Adjust the total volume of culture medium to 1 L with distilled water. Transfer medium to an Erlenmeyer flask or bottle with a capacity twice the total volume of the solution being sterilized (e.g., 1 L of medium should be sterilized in a 2 L flask).
9. Add solidifier for solid media.
10. Sterilize the medium in an autoclave. A flask may be adequately covered for autoclaving by aluminum foil or inverting a beaker over the neck.
11. Sterilize empty culture vessels (either before the medium is sterilized or at the same time).
12. Get ready all the other heat-sensitive additives. Combine appropriate volumes of all plant growth regulators, vitamin, amino acid, and any other necessary stock solutions (usually not exceeding 5–6 mL). Keep it in a 5–10 mL sterile Erlenmeyer flasks or similar container. Place the stoppered container in the working area, and sterilize its external surfaces by spraying with 70% ethanol or household bleach as described before.

13. Remove the sterilized medium from the autoclave, and put it inside a laminar flow cabinet.
14. While the medium is still hot (and therefore still liquid if it contains agar), pour the contents of the container described in item 12 into the medium.

4.2 Sterilization of Media

To avoid contamination, culture media, tools, working space, and tissues must be sterilized, and work has to be carried out under aseptic conditions to ensure success. Different methods can be used to ensure sterility.

4.2.1 Heat Sterilization Using an Autoclave

Spores of microorganisms in liquid or solid culture media must be eliminated and sterilized by autoclaving under high temperature and pressure (usually 121 °C and 1.2×10^5 Pa which can also be expressed as 121 °C and 1 atm, or 15–20 psi, or 10.5 kg 6.5 cm^{-2}).

Inorganic components (macro- and microelements), sugars, agar, and some complex organic additives (coconut water, casein hydrolysate, peptone, yeast extract, banana homogenate, etc.) can be heat-sterilized.

Culture media should be autoclaved at 121 °C for 15 min at that temperature. Sterilization time depends on the volume per vessel which is being sterilized; guidelines can be found in the following website: (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Plant_Biotechnology/Tissue_Culture_Protocols/Media_Sterilization.html).

4.2.2 Filter Sterilization

Certain media components (e.g., certain plant growth regulators) are sensitive to high temperature and cannot be heat-sterilized. Solutions containing these chemicals may be sterilized by passing them through sterilizing filters (e.g., Millipore brand filter) which permit the passage of liquids but not particles larger than 0.22 or 0.45 μm and thereby retain all contaminants. Some sterilizing filters can be used for a number of solvents; others are suitable only for water. Therefore, it is important to read all instructions carefully (see **Note 5**).

4.3 Explant Excision and Sterilization

4.3.1 Tissue Explant Excision

Shoot tips or meristems are the primary explants for the establishment of orchid micropropagation. For most orchid species, meristems are located at the tips of shoots or buds and protected by sheathing petioles, leaves, or scales. These protective structures maintain the sterility of the meristem and protect the shoots from harmful sterilants. Apical meristems of *Paphiopedilum* do not have such protection and can be damaged during surface sterilization. This is one reason why it is difficult to clone *Paphiopedilum*. In vitro grown seedlings are easier to culture because they do not require surface sterilization.

The process of meristem or shoot-tip excision from stems, buds, or other organs requires several important, sensitive, and critical steps.

1. Remove the entire plant or growth from the soil or potting mix or excise part of a plant (new shoot from a sympodial orchid, apical part or top cut from a monopodial plant).
2. Wash and scrub the plant (or the excised parts) with a soft bristle toothbrush, a mild household detergent, and running water. Avoid long exposures to high concentrations of detergents because they may damage plant tissues [183, 184].
3. Excision of explants must be carried carefully:
   (a) Surface sterilization with sodium hypochlorite solution of about 2.4–2.6% is suitable. Instructions for surface sterilization are given in Sects. 4.3.2 and 4.3.3.
   (b) Remove the mature leaves, scales, dead tissues, debris, roots, and other parts using a sharp scalpel or razor blade. Do this carefully to prevent damage to the internal tissues and/or the part to be excised and cultured.
   (c) When excising a meristem, the lower portion of the organ (e.g., a stem) should be removed so that the exposed cut edge is perpendicular to the axis.
   (d) Dissection of shoot tips requires careful removal of leaf primordia under a dissecting microscope. The meristem should be excised on a cube of subjacent tissue. The younger leaf primordia surrounding the shoot tip are closely associated with the meristem. These primordia are more difficult to excise without damaging the apical dome. Damage to the meristem can be avoided by excising primordia under a dissecting microscope. The dissecting microscope must be wiped with water, followed by a mild detergent and 70% ethanol to eliminate contaminants.
   (e) Once all the young primordia have been removed, the meristem can be excised on a small cube of subjacent tissue (usually not larger than 1 cm^3, and not smaller than 0.5 cm^3) and lifted on a scalpel.
   (f) Lateral buds can also be removed during the dissection process prior to excision of the terminal meristem or shoot tip. In most orchids, lateral buds are clearly visible in the axils of leaves and appear as glistening raised domes (usually 0.5–1 cm in diameter), similar in appearance to the apical meristem. As each leaf is dissected, lateral buds along with subjacent tissue can be excised.
   (g) Secure the top portion of the explant by mounting the flat cut edge on a Styrofoam block or a cork and affixing it with long pins inserted at an angle.

Mounting in this fashion stabilizes the organ and allows for (1) easy surface sterilization by inverting the mounted organ into a sterilizing solution and (2) micro-excision of the part to be cultured (shoot tip or any other tissue explant).

Root tips can also be excised for tissue culture. Plantlets have been raised from roots, but this method is not as common as using shoot tips. Since roots of many orchids have mycorrhiza which may make surface sterilization and decontamination of the root difficult or even impossible, it is advisable to use excise root tips from aerial roots that have not been in contact with soil, potting mix, benches, and bark surface that could lead to penetration of a fungus.

Seedling raised from seeds can also be propagated by tissue culture. However, cloned plants that originate from seedlings lack genetic diversity and therefore are not ideal for conservation work. Explants from seedlings growing in vitro can also be very useful when the intent is to test a medium or a procedure while eliminating the possible effects of surface sterilization.

Thin layers from leaves, nodes, shoot tips, stems, and protocorms have been used for regeneration and transformation of orchids (for a review, see 185).

The nature of the dissection (e.g., longitudinal vs. cross sections through an organ) and the placement, density, and size of explants may contribute to successful micropropagation. Therefore, it is important to follow published reports precisely.

4.3.2 Hypochlorite Solutions

Commercial household bleaches such as Clorox and Purex are excellent sterilants. They contain between 4.75 and 6% sodium hypochlorite. Undiluted bleaches can be used to wash tools, working areas, and the outside of glasswares. If used to sterilize plant tissues, these bleaches should be diluted according to instructions in specific procedures. A few drops of wetting agent such as Tween 20 should be added to enhance the wetting properties of the sterilants. Even a household liquid detergent can be used for practical purposes.

Calcium hypochlorite can also be used to surface-sterilize tissues and seeds. A saturated solution of calcium hypochlorite is prepared by dissolving 10 g calcium hypochlorite in 140 mL water (7 g/100 mL); the solution is stirred vigorously before it is allowed to stand for 3–5 min. The solution is stirred again and allowed to stand until the precipitate has settled. The solution is filtered through filter paper. The clear, yellowish liquid must be used for sterilization within 12 h.

Ethanol and isopropanol can be used to sterilize tools, surfaces of culture flasks, and working areas. These alcohols in 70% aqueous solutions can be used. Methanol is toxic and should be avoided.

4.3.3 Surface Sterilization of Explants

The first step in the process is usually a thorough washing with water and mild household detergent. This is followed by a rinse with distilled water and the sterilization procedure. Calcium hypochlorite and sodium hypochlorite solution with appropriate dilutions, both with the addition of a few drops of mild household liquid detergent and 70% ethyl or 95% alcohol, are the most commonly used sterilants.

Alcohol is usually utilized only as a momentary dip. Either of the hypochlorite solutions are used for a more prolonged soaking of the donor organs or explants. Sensitivity of tissues to these sterilants may vary. Therefore published procedures should be followed carefully, and great care must be taken when using a new sterilant-tissue combination. After the sterilization, tissues must be washed with sterile distilled water.

The general recommended procedures are as follows:

1. Remove dirt and soil from explants by gently washing and scrubbing.
2. Dip explants in either 70 or 95% ethanol solution for 2–3 s.
3. Immerse explants (5–20 min) in a diluted sodium hypochlorite (household bleach) or calcium hypochlorite solution (follow procedures for concentrations and time (see **Note 7**).
4. Remove sterilant from the tissues with several sterile water washings in a laminar flow cabinet.
5. For sections of plant material, they are usually sterilized by soaking in calcium hypochlorite for 5–20 min. The sterilant is then removed by washing with sterile distilled water.

4.4 Culture Vessels

Erlenmeyer flasks, test tubes, and a variety of specially designed plastic and glass containers can be used for orchid micropropagation. However, clear glass containers, plastic containers, polyethylene and polypropylene bags [186], and disposable film vessels [187–189] have also been used. Containers with very wide necks which are not specially designed for tissue culture (e.g., jars) should be used with caution because cultures in such vessels are easily contaminated; a cap with a cotton-filled vent must be screwed on tightly and withstand autoclaving. The adhesive should also be capable of bonding the tube to the cap (i.e., be able to bond two different materials) and leave no cracks.

Presterilized culture vessels which are specially designed for tissue culture can be used as they come out of the packages. However, it is important to make sure that these vessels are opened under sterile conditions. Containers that were used previously for any purpose, including tissue culture, must be thoroughly washed and dried before use (see **Note 8**). Glass culture flasks can be sterilized by burning. Natural gas burner is good because it produces a clean and high-temperature flame. An alcohol

flame (methylated spirits or denatured ethanol can be used as they are more economical) can be used. Metal tools should be dipped in alcohol (methyl, ethyl, or isopropyl) before flaming by an alcohol flame.

Containers must be fitted with a cover before being filled with medium (except for designed culture vessels which have their own covers). The cover can be a rubber stopper with one or two holes stuffed with non-adsorbent cotton. The cover can be variable depending on the size of the culture vessel. Once prepared, stoppers with cotton-filled holes can be used repeatedly. The stoppers must be washed, but the cotton must be dried completely or replaced before the next use. The cotton must always be kept clean. Some covers are plastic with a built-in filter, allowing for gas exchange and preventing entry of microorganisms. These covers are excellent but expensive.

Both rubber stoppers and cotton buns should be covered with aluminum foil. Paper can be used if foil is not available; it should be secured with a rubber band or wire below the neck. In high-humidity areas (if the cultures are not kept in air-conditioned room), moisture may condense on the cotton bun or plugs below the aluminum foil, allowing fungi to grow and contaminate cultures. In such areas, paper rather than aluminum foil should be used. Some researchers have also used aluminum foil only as covers. When plastic films (such as cling film) are used to cover cotton buns or stoppers, make sure that they are not tied too tightly which may prevent gas exchange and/or accumulate water condensate; this in turn may cause contamination of cultures.

It is important that there is sufficient gas exchange because (1) ethylene produced by tissue culture materials can inhibit growth and (2) oxygen and carbon dioxide may need to be replenished. Cotton plugs and filters allow diffusion of gases but prevent entry of contaminants.

4.5 Culture Conditions

Culture conditions affect the initiation of callus from explants, growth of callus masses, protocorm-like bodies (PLBs), and plantlets. Proper cultural condition is one of the most important factors in determining the success of orchid micropropagation. Most explants can be cultured at 22–25 °C, under 12–24 h photoperiods provided by a variety of light sources (see Light Sources later). Some explants, species, or hybrids may have special requirements for photoperiods and light intensity. These can only be learned from experiments or published methods.

4.5.1 Temperature

Most explants can be cultured at 22–25 °C. However some explants, species, or hybrids may have special requirements for temperature. These can only be learned from experiments or published methods. When adjusting the temperature for tissue culture, it is important to follow instructions carefully.

4.5.2 Agitation

Agitation of liquid media helps to improve gaseous exchange, enhance contact between tissues and liquid, and influence growth and development. Agitation may be gyro-rotatory (wrist action), oscillatory (back and forth), or rotatory (rotating on a wheel with its axis parallel or at an angle to the ground). Shakers for these purposes can be purchased from scientific equipment companies.

Speed of agitation affects the growth and differentiation of explants. It is important to follow the recommendation employed in the original research paper. Normally, rotatory shakers should be set at 1–3 rpm, oscillating ones should move back and forth 60 times a minute, and gyro-rotatory units should shake approximately 30–40 times per minute. These can be adjusted if necessary.

4.5.3 Illumination

Light duration, its presence or absence, intensity, wavelength, and source candescence are of great importance in the micropropagation of orchids. Most orchid explants require some illumination. However, certain explants, such as some vandaceous orchids, should be kept under subdued light or in the dark, during the initial stages of culture. It is important to follow the light conditions of the original research paper. If in doubt, experiments should be carried out by using explants from similar but more common plants. Some explants should be placed in the dark for varying periods of time to find out if the explants would benefit from the exclusion of light. Plantlets require light to grow properly; therefore, explants must be transferred to the light eventually. When explants are transferred from darkness to light, it should be done gradually.

Except for explants that must be kept in the dark, orchid tissue cultures require illumination which can range from a few hours a day to a 24-h illumination. For rare species that have not been cloned before, photoperiods must be determined experimentally. When established procedures are employed, it is important to follow the light duration of the original research paper.

The choice of light source for orchid tissue culture depends on many factors such as availability, cost, and coverage. An inexpensive light system can consist of two cool white tubes mounted on a standard fluorescent light fixture. The addition of two 25–50-W incandescent bulbs between the fluorescent tubes will improve the light spectrum to which the plants will be subjected. A combination of one cool white and one warm white tube may produce somewhat better illumination, especially if they are combined with incandescent bulbs. The area illuminated by these lights should be roughly equal to (or slightly larger than) that of the fixture itself, which is usually mounted 45–50 cm above the plants.

If only two cool white tubes are used, the light intensity provided at plant level by such a fixture should be between 110 and 130 ft-c (ca. 3.81 W m^{-2} or 17.14 μmol m^{-2} s^{-1} PAR) or 1100–1300 lx. Four tubes may provide 250–320 ft-c (2500–3200 lx,

or ca. 9.52 W m^{-2} PAR or 42.86 μmol m^{-2} s^{-1} PAR), whereas new ones can be expected to produce 350–420 ft-c (3500–4200 lx, or ca. 12.70 W m^{-2} PAR or 57.14 μmol m^{-1} s^{-1} PAR). The addition of incandescent bulbs will increase the illumination levels and broaden the spectrum. The emission spectra of plant growth tubes contain wavelengths appropriate for plant growth and should be considered, especially if their prices are reasonable. Combinations of plant growth and other fluorescent tubes and/or incandescent bulb can also produce good results.

LED lights reduce the use of energy, and they are more lasting. Recently they have become more readily available and affordable; all these factors make LED lights increasingly attractive as sources of illumination for orchid tissue culture.

4.5.4 Carbon Dioxide

Carbon dioxide (CO_2) is the source of carbon for photosynthesis, but the low atmospheric levels can be a limiting factor. It has been shown that elevated levels of CO_2 can bring about increased growth by orchid plantlets and seedlings [190–194]. Researchers have yet to design a simple and/or inexpensive system for CO_2 enrichment for orchid micropropagation. This is probably why CO_2 enrichment is not a common practice [16].

4.5.5 Work Area

Many of the micropropagation procedures should be carried out under aseptic conditions. This is especially important when mixing heat-sterilized solutions with components in 70–95% ethanol stock solutions, pouring medium into culture vessels, and excising explants and placing them in culture. Such conditions can be obtained in several ways.

The laminar flow hood (or laminar flow cabinet) is the best and most efficient means of assuring sterility in the working area. Air coming into these hoods is driven through filters that remove all particles such as fungal spore and bacteria. The sterile air is blown gently across the working area toward the operator, and this generally prevents contamination of cultures. Tools and work surfaces must still be sterilized, even when used inside the laminar flow hoods. All unsterile tools and glasswares must be wiped with 70% alcohol before placing them inside the laminar flow hood, to prevent the introduction of contaminants.

The surfaces inside the hood must be sterilized before the hood is used. This can be done by spraying the inside of the hood with 70% ethanol prior to use. Some laminar flow hoods are installed with germicidal ultraviolet (UV) lamp; the working surface inside of the hood should be irradiated with the UV lamp before each use or while it is not being used. The front opening of the hood should be curtained off with a plastic curtain so as to prevent the entry of dust into the hood. The plastic curtain also provides protection of workers because UV light can cause severe damage to eyes. UV light must be turned off, while people are

working inside the hood. The work space inside these hoods is large enough for efficient movements. There is also space inside for tools, a gas or alcohol burner for heat-sterilizing tools, aluminum foil and culture glassware, and microscopes.

5 Conclusion

Although orchid micropropagation has been around for more than 60 years, the existing tissue culture and clonal propagation methods still need to be improved. So look out for new publications with improved methods of cloning orchids.

As conservation becomes more and more important, new methods will be developed for genera and species which have not been cultured as yet either because they are less popular in the mass market or there are difficulties in cloning them (e.g., *Cypripedium* and *Phragmipedium*). Recently, more researchers have developed new methods for cloning these new genera such as *Cypripedium* [34], *Habenaria* [195], *Nervilia* [196], and others (see [16]). It is likely that future methods will be developed for other recalcitrant and/or less popular orchids.

Recently, there are exciting developments in computerized flow systems for orchid tissue culture, robotization, bioreactors, flow systems, computerization, and automation [197–206]. More of such new and improved methods will be developed in the future.

6 Notes

1. Certain chemicals may not be available in some countries, it is sometimes necessary to make substitutions and modifications in recipes. If this becomes necessary, the changes should be made by experienced workers with great caution because what may appear to be small and insignificant changes can introduce major modifications.
2. If darkening of a medium is necessary and charcoal cannot be used, 2 g/L of graphite [207, 208] can be employed instead. Graphite should be used only in special cases, as, for example, when the intent is to darken a medium with an agent which does not have the adsorptive capacity of charcoal.
3. Some of the anti-contaminants are antibiotics which can affect humans and animals and should be used with care.
4. Distilled water must be used as a solvent in the preparation of culture media. When this is not available, deionized water may be used. Rainwater collected in a glass or plastic container, in areas where the atmosphere is not polluted (e.g., no acid rain),

can also be employed. Tap and well water can be used for practical purposes after testing.

5. Sterilization of heat-sensitive substances can be done by dissolving them in stock solution in 70 or 95% ethanol which is an excellent sterilant. The addition of up to 5–6 mL of 70% ethanol per liter of medium does not have a deleterious effect on cultures. Methanol, methylated spirits, and denatured ethanol should not be used for this purpose.
6. If used previously for any purpose, the containers must be washed thoroughly with soap and running water. They must be rinsed several times with distilled water after the wash. Containers which are used for tissue culture must have covers which allow for air exchange and at the same time prevent entry of contaminants.
7. Calcium and sodium hypochlorite are the most commonly used surface sterilants in micropropagation. Hydrogen peroxide (H_2O_2) is used occasionally (discussed online in plant-tc@lists.umn.edu) to save contaminated flasks; however, this method is very laborious, and it may be used with very small amount of very special plant materials.
8. It is important that all glassware (culture vessels, volumetric flasks or cylinders, beakers, test tubes, etc.) be cleaned thoroughly and rinsed with distilled water prior to initial use. Disposable presterilized culture vessels are widely available and relatively inexpensive at present. They do not require washing.

References

1. Krikorian AD (1982) Cloning higher plants from aseptically cultured tissues and cells. Biol Rev 57:151–218
2. Hartman HT, Kester DE (1983) Plant propagation, principles and practices, 4th edn. Prentice-Hall, Englewood Cliffs
3. Loo SW (1945) Cultivation of excised stem tips of intact plants under aseptic conditions. Ph.D. Diss., California Institute of Technology, Pasadena
4. Loo SW (1945) Cultivation of excised stem tips of *Asparagus* in vitro. Am J Bot 32:13–17
5. Ball EA (1946) Development in sterile culture of stem tips and subjacent regions of *Tropaeolum majus* L., and of *Lupinus albus* L. Am J Bot 33:301–318
6. Rotor G Jr (1949) A method for vegetative propagation of *Phalaenopsis* species and hybrids. Am Orchid Soc Bull 18:738–739
7. Thomale H (1956) Neue Wege der Orchideenvernehrung. Die Orchidee 7:104–105
8. Thomale H (1957) Die Orchideen, 2nd edn. Verlag Eugen Ulmer, Stuttgart
9. Morel G (1960) Producing virus-free cymbidiums. Am Orchid Soc Bull 29:495–497
10. Wimber DE (1963) Clonal multiplication of cymbidiums through tissue culture of the shoot meristem. Am Orchid Soc Bull 32:105–107
11. Arditti J, Krikorian AD (1996) Orchid micropropagation: the path from laboratory to commercialization and an account of several unappreciated investigators. Bot J Linn Soc 122:183–241
12. Arditti J (1977) Clonal propagation of orchids by means of leaf cultures in vitro: a short review. Orchid Rev (England) 85:102–103
13. Arditti J (1977) Clonal propagation of orchids by means of tissue culture—a manual. In: Arditti J (ed) Orchid biology: reviews and perspectives, vol I. Cornell University Press, Ithaca, pp 203–293

14. Arditti J (2008) Micropropagation of orchids, 2nd edn. Blackwell Publishing, Oxford
15. Arditti J, Ernst R (1993) Micropropagation of orchids, 1st edn. Wiley, New York
16. Yam TW, Arditti J (2017) Micropropagation of orchids, 3rd edn. Wiley, Oxford
17. Vajrabhaya T, Vajrabhaya M (1976) The study of various organs of orchids *in vitro*. I. Stems, leaves and buds. Rep Sci Res. Fac Sci Chulalongkorn Univ 1:105–115
18. Vajrabhaya T, Vajrabhaya M (1976) The study on growth of organs of orchids *in vitro* (nutrition). Chulalongkorn Res J 3:109–127
19. Vajrabhaya T (1977) Variations in clonal propagation. In: Arditti J (ed) Orchid biology: reviews and perspectives, vol 1. Cornell University Press, Ithaca, pp 176–201
20. Sahavacharin O (1980) Mutation in the tissue culture of orchids. In: Sukshom Kashemsanta MR (ed) Proc. 9th World Orchid Conf., Bangkok, 1978, pp 223–225
21. Rentoul JN (1981) Mericloning and colour pigmentation. Aust Orchid Rev 46:249–252
22. Teo CKH (1981) Developing better plants through tissue culture. Malayan Orchid Rev (Singapore) 15:25–27
23. Beechey C (1970) Propagation of orchids from aerial roots? Am Orchid Soc Bull 41:1085–1088
24. Ojima K, Fujiwara A (1962) Studies on the growth promoting substance of the excised wheat root. III. Effects of tryptophan and some related substances. Tohoku J Agric Res 13:69–98
25. Churchill M-E, Ball EA, Arditti J (1972) Tissue culture of orchids. II. Methods for root tips. Am Orchid Soc Bull 41:726–730
26. Tanaka M, Senda Y, Hasegawa A (1976) Plantlet formation by root-tip culture in *Phalaenopsis*. Am Orchid Soc Bull 45:1022–1024
27. Kerbauy GB (1984) Regeneration of protocorm-like bodies through *in vitro* culture of root tips of *Catasetum* (Orchidaceae). Z Pflanzenphysiol 113:287–291
28. Colli S, Kerbauy G (1993) Direct root tip conversion of *Catasetum* into protocorm-like bodies. Effects of auxin and cytokinin. Plant Cell Tissue Organ Cult 33:39–44
29. APA V, Kerbauy G, Figueiredo-Ribeiro RCL (1998) Changes in soluble carbohydrates and starch partitioning during vegetative bud formation from root tips of *Catasetum fimbriatum* (Orchidaceae). Plant Cell Tissue Organ Cult 54:105–111
30. Peres LEP, Amar S, Kerbauy GB, Slatino A, Zaffari GR, Mercier H (1999) Effects of auxin, cytokinin and ethylene treatments on the endogenous ethylene and auxin-to-cytokinin ratio related to direct root tip conversion of *Catasetum fimbriatum* Lindl. (Orchidaceae) into buds. J Plant Physiol 155:551–555
31. Kerbauy GB (1991) *In vitro* conversion of *Cattleya* root tip cells into protocorm-like bodies. J Plant Physiol 138:248–251
32. Pindel A, Miczycski K (1996) Regeneration of *Cymbidium* orchids from leaf and root explants. Folia Holtic 8:95–105
33. Jo S, Nishimura N, Okazawa Y (2001) Micropropagation of *Cypripedium yatabeanum* through root tip culture *in vitro*. In: S. Ichihashi (ed) Proc. of APOC7 (2001), Nagoya, Japan. Secretariat of 7th APOC, 1 Imahori, Ushida, Chiryu, Aichi 472-0007, Japan, pp 58–59. http://village.infoweb.ne.jp/~fwkb0570/index-e.htm
34. Sanchez ML (1988) Micropropagation of *Cyrtopodium* (Orchidaceae) through root-tip culture. Lindleyana 3:93–96
35. Tsukazaki H, Mii M, Tokuhara K, Ishikawa K (2000) Cryopreservation of *Doritaenopsis* suspension culture by vitrification. Plant Cell Rep 19:1160–1164
36. Park SY, Ichihashi S, Paek KY (2001) Micropropagation of *Doritaenopsis* hybrids through root-tip cultures. In: Ichihashi S, Nagata H (eds) Proc. 7th Asia Pacific Orchid Conf. CD available from Secretariat APOC 7, 1 Imohori, Ushida, Chiryu, Aichi 472–0007, Japan, pp 200–201
37. Sood A, Vij SP (1986) *In vitro* root segment culture of *Rhynchostylis retusa* Bl. In: Vij SP (ed) Biology, conservation, and culture of orchids. Published for the Orchid Society of India by the Affiliated East-West Press, New Delhi, pp 463–468
38. Vij SP, Pathak P, Sharma M (1987) On the regeneration of *Rhynchostylis retusa* root segments. J Orchid Soc India 1:71–74
39. Vij SP (1993) Regeneration response of orchid roots: a study *in vitro*. J Orchid Soc India 7:61–72
40. Ueda H, Torikata H (1972) Effects of light and culture medium on adventitious root formation by *Cymbidium* in aseptic culture. Am Orchid Soc Bull 41:322–327
41. Rao AN (1977) Tissue culture in the orchid industry. In: Reinert J, Bajaj YPS (eds) Plant cell, tissue, and organ culture. Springer, New York, pp 207–248, pp 44–69, 207–248
42. Nayak NR, Chand PK, Rath SP, Patnaik SN (1998) Influence of some plant growth regulators on the growth and organogenesis of *Cymbidium aloifolium* (L.) Sw. Seed-derived rhizomes *in vitro*. In Vitro Cell Dev BiolPlant 34:185–188
43. Paek KY, Yoo GJ, Park MR, Hisajima S (1993) Effects of sucrose and changes of free sugar content during organogenesis through

rhizome culture of temperate *Cymbidium*. Proc Nagoya Orchid Show 93:55–63
44. Chang C, Chang WC (1998) Plant regeneration from callus culture of *Cymbidium ensifolium* Var. misericors. Plant Cell Rep 17:251–255
45. Chang C, Chang WC (2000) Effect of thidiazuron on bud development of *Cymbidium sinense* Willd *in vitro*. Plant Growth Regul 30:171–175
46. Paek KY, Kozai T (1998) Micropropagation of temperate *Cymbidium* via rhizome culture. HortTechnology 8:283–287
47. Sheelavantmath SS, Murthy HN, Pyati AN, Kumar HGA, Ravishankar BV (2000) *In vitro* propagation of the endangered orchid, *Geodorum densiflorum* (Lam.) Schltr. through rhizome section culture. Plant Cell Tissue Organ Cult 60:151–154
48. Vij SP, Sood A, Sharma A, Shekhar N (1983) *In vitro* tuber culture of *Pachystoma senile*—a ground growing orchid. Trop Plant Sci Res 1:211–213
49. Champagnat M, Morel G, Mounetou B (1970) La multiplication végétative des *Cattleya* a partir do jeunes feuiles cultivées aseptiquement in vitro. Ann Sci Nat Bot Biol Veg Ser 12(11):97–114
50. Arditti J, Ball EA, Churchill ME (1971) Propagacion clonal de orquideas utilizando apices de hojas. Orquidea (Mexico) 2: 290–300
51. Ball EA, Arditti J, Churchill ME (1971) Clonal propagation of orchids from leaf tips. Orchid Rev 79:281–288
52. Churchill ME, Arditti J, Ball EA (1971) Clonal propagation of orchids from leaf tips. Am Orchid Soc Bull 42:109–113
53. Churchill ME, Ball EA, Arditti J (1971) Production of orchids from seedling leaf tips. Orchid Digest 34:71–273
54. Churchill ME, Flick BH, Ball EA, Arditti J (1971b) Vermehrung von Orchideen durch Blattspitzen. Orchidee 22:147–151
55. Churchill ME, Arditti J, Ball EA (1972) Propagao clonal de orquideas a partir de apices de folha. Biol Soc Campineira Orquideas 2:23–28
56. Churchill ME, Ball EA, Arditti J (1973) Tissue culture of orchids. I. Methods for leaf tips. New Phytol 72:161–166
57. Nayak NR, Patnaik S, Rath SP (1997) Direct shoot regeneration from foliar explants of an epiphytic orchid, *Acampe praemorsa* (Roxb.) Blatter and McCann. Plant Cell Rep 16:583–586
58. Murthy HN, Pyati AH (2001) Micropropagation of *Aerides maculosum* Lindl. (Orchidaceae). In Vitro Cell Dev Biol Plant 37:223–226
59. Loh CS, Rao AN, Goh CJ (1975) Clonal propagation from leaves in the orchid *Aranda*. J Singapore Natl Acad Sci 4:97–99
60. Fu FML (1978) Clonal propagation of *Aranda*, *Ascocenda*, and *Cattleya* by leaf tissue culture. Gard Bull (Singapore) 31:132–138
61. Fu FML (1979) Studies on the tissue culture of orchids. 2: Clonal propagation of *Aranda*, *Ascocenda*, and *Cattleya* by leaf tissue culture. Orchid Rev 87:343–346
62. Manorama P, Rao AN, Goh CJ, Loh CS (1986) Leaf callus development in *Aranda* and *Dendrobium*. In: Rao AN (ed) Proc Fifth ASEAN Orchid Cong, 1984, Singapore, pp 102–109
63. Gopalan G, Krishnan PN, Seeni S (1992) Levels of starch and α-amylase during protocorm like-body formation in foliar explants of a *Cymbidium* hybrid. Indian J Exp Biol 30:796–800
64. Matos M, De García E (1991) Plant regeneration from young leaves of *Laeliocattleya* John Cunningham. Phyton 52:119–124
65. Abdul Ghani AK, Haris H (1992) Plantlet formation from young leaves of *Mokara*. Lindleyana 7:11–12
66. Chen JT, Chang C, Chang WC (1999) Direct somatic embryogenesis on leaf explants of *Oncidium* Gower Ramsey and subsequent plant regeneration. Plant Cell Rep 19: 143–149
67. Chen JT, Chang WC (2001) Effects of auxins and cytokinins on direct somatic embryogenesis on leaf explants of *Oncidium* 'Gower Ramsey'. Plant Growth Regul 34:229–232
68. Pathak P, Vij SP (2001) *In vitro* regeneration of *Papilionanthe teres* (Roxb.) Schltr.: utility of foliar explants. In: Ichihashi S, Nagata H (eds) Proc. of APOC7, Nagoya, Japan. Secretariat of APOC 7, 1 Imohori, Ushida, Chiryu, Aichi 472-0007, Japan, pp 226–227
69. Tanaka M, Hasegawa A, Goi M (1974) Studies on the clonal propagation of monopodial orchids by tissue culture. I. Formation of protocorm-like bodies from leaf tissue in *Phalaenopsis* and *Vanda in vitro*. J Jpn Soc Horti Sci (cited in Tanaka et al., 1976)
70. Tanaka M, Sakanishi Y (1977) Clonal propagation of *Phalaenopsis* by leaf tissue culture. Am Orchid Soc Bull 46:733–737
71. Tanaka M (1992) Micropropagation of *Phalaenopsis* spp. In: Baja YPS (ed) Biotechnology in agriculture and forestry. Springer, Berlin, pp 246–268
72. Park SY, Paek KY (1999) Occurrence of abnormal plantlets and their morphological characteristics as affected by clones and culture periods in clonally propagated *Phalaenopsis* by leaf culture. J Korean Soc Hortic Sci 40: 731–734

73. Park SY, Murthy HN, Paek KY (2002) Rapid propagation of *Phalaenopsis* from floral-derived leaves. In Vitro Cell Dev Biol Plant 38:168–172
74. Goh CJ, Tan H (1982) Clonal propagation from leaf explants in *Renantanda* orchid hybrid. Orchid Rev 90:295–296
75. Abdul Ghani AK, Haris H, Bin Haji Ujang N (1992) Production of *Renantanda* plantlets from shoot tips *in vitro*. Lindleyana 7:3–6
76. Seeni S, Latha PG (1992) Foliar regeneration of the endangered red *Vanda*, *Renanthera imschootiana* Rolfe (Orchidaceae). Plant Cell Tissue Organ Cult 29:167–172
77. Fukui H, Li YH, Shiraki H (2001) Plant regeneration from shoot tip and leaf and histomorphological observation of callus in *Vanda coerulea*. In: Ichihashi S, Nagata H (eds) Proc. 7th Asia Pacific Orchid Conf. (Apoc7, Aichi-Nagoya 2001). Compact Disk published by Secretariat of APOC7, 1 Imohori, Ushida, Chiryu, Aichi 472-0007, Japan, pp 193–195
78. Vij SP, Sood A, Plaha KK (1984) Propagation of *Rhynchostylis retusa* Bl (Orchidaceae) by direct organogenesis from leaf segment cultures. Bot Gaz 145:210–214
79. Allenberg H (1976) Notizen zur Keimung, Meristemkultur and Regeneration von Erdorchideen. Die Orchidee 27:28–31
80. Seeni S, Latha PG (2000) *In vitro* multiplication and ecorehabilitation of the endangered blue *Vanda*. Plant Cell Tissue Organ Cult 61:1–8
81. Sharma V, Vij SP (1997) Effect of $CuSO_4{\cdot}5H_2O$ on *in vitro* regenerative capacity of foliar explants excised from mature *Vanda cristata* Lindl. plants. Phytomorphology 47:203–208
82. Niraula R, Rajbhandary SB (1988) Regeneration of plants from leaf explants in orchid *Vanda teres* Lind. In: Prasad BN, Ghimire GPS, Agrawal V (eds) Role of biotechnology in agriculture: first regional conference. Kathmandu, Nepal. International Science Publisher, New York, pp 103–107
83. Chaturvedi HC, Sharma AK (1986) Mericloning of orchids through culture of tips of leaves and roots. In: Vij SP (ed) Biology, conservation, and culture of orchids. Published for the Orchid Society of India by the Affiliated East-West Press, New Delhi, pp 469–472
84. Zimmer K (1978) Die labormässige vegetative Vermehrung der Orchideen. In: Briger FG, Maatsch R, Senghas K (eds) Rudolf Schlechter's Orchideen, vol 3, 3rd edn. Paul Parey, Berlin, pp 158–159
85. Zimmer K (1978b) Zur vegetativen Vermehrung von Orchideen. Dtsch. Gartenbauwissenschaft 34:330–334
86. Fast G (1979) Klonvermehrung von *Phragmipedium* Sedenii and *Phalaenopsis* hybr. aus Bliitenknospen. Die Orchidee 30:241–244
87. Arditti J, Goh CJ (1981) Tissue culture of orchids. In: Lawler LJ, Kerr R (eds) Proc. Orchid Symp.13th Bot. Congr., Orchid Society of New South Wales, Sydney, Australia, pp 65–72
88. Czerevczenko TM, Kushnir GP (1986) Orhidei v kulture. Naukova Dumka, Kiev
89. Yam TW, Arditti J (2009) History of orchid propagation: a mirror of the history of biotechnology. Plant Biotechnol Rep 3:1–56
90. Bertsch W (1966) A new frontier: Orchid propagation by meristem tissue culture. In: de Garmo LR (ed) Proc. 5th World Orchid Conf., Long Beach, California, 1965, pp 225–229
91. Mitra GC (1971) Studies of seeds, shoot-tips and stem-disks of an orchid grown in aseptic culture. Indian J Exp Biol 9:79–85
92. Arditti J, Mosich SK, Ball EA (1973) *Dendrobium* node cultures: a new means of clonal propagation. Aust Orchid Rev 38:175–179
93. Mosich SK, Ball EA, Arditti J (1973) Propagation clonal de *Dendrobium* por mediodel cultivo de nodos. Orquidea (Mexico) 3:244–260
94. Mosich SK, Ball EA, Arditti J (1974) Clonal propagation of orchids by means of node cultures. Am Orchid Soc Bull 43:1005–1061
95. Mosich SK, Ball EA, Flick BH, Arditti J (1974) Klonvermehrung von *Dendrobium* durch die Kultivierung von Stammnodien. Orchidee 25:129–134
96. Ito I (1960) Culture of orchid seedlings by way of completing the growth of cut flowers. Jpn Orchid Soc Bull 6:4–7
97. Ito I (1961) In vitro culture of ovary and seed in orchids. Kyoto, Olericulture Lab., Fac Agric Kyoto Prefectural University
98. Ito I (1955) Germination of seeds from immature pod and subsequent growth of seedlings in *Dendrobium nobile* Lndl. Sci Rep Saikyo Univ Agric 7:35–42
99. Withner CL (1955) Ovule culture and growth of *Vanilla* seedlings. Am Orchid Soc Bull 24:380–382
100. Ayers J (1960) Embryo culture *of Phalaenopsis* seedlings. Am Orchid Soc Bull 29:518–519
101. Niimoto DH, Sagawa Y (1961) Ovule development in *Dendrobium*. Am Orchid Soc Bull 30:813–819
102. Rao AN, Avadhani PN (1964) Some aspects of vitro culture of *Vanda* seeds. In: Yeoh BC (ed) Proceedings of the 4th World Orchid Conference, Singapore 1963. Straits Times Press (M), Singapore, pp 194–202
103. Ernst R (1982) Paphiopedilum. In: Arditti J (ed) Orchid biology: reviews and per-

spectives, vol II. Cornell University Press, New York, pp 350–353
104. Withner CL (1959b) Orchid culture media and nutrient solutions. In: Withner CL (ed) The orchids, a scientific survey. Ronald Press, New York, pp 589–599
105. Intuwong O, Sagawa Y (1973) Clonal propagation of Sarcanthinae orchids by aseptic culture of inflorescences. Am Orchid Soc Bull 42:209–215
106. Kim KW, Kako S (1984) Studies on clonal propagation in the *Cymbidium* floral organ culture in vitro. J Korean Soc Hortic Soc 25:65–71
107. Shimasaki K, Uemoto S (1991) Rhizome induction and plantlet regeneration of *Cymbidium goeringii* from flower bud culture *in vitro*. Plant Cell Tissue Organ Cult 25:45–52
108. Fast G (1980) Vermehrung and Anzucht. In: Fast G (ed) Orchideenkultur. Eugen, Stuttgart, pp 201–283
109. Goh CJ, Wong PF (1990) Micropropagation of the monopodial orchid hybrid *Aranda* 'Deborah' using inflorescence explants. Sci Hortic 44:315–321
110. Singh H, Sagawa Y (1972) Vegetative propagation of *Dendrobium* by flower stalk cuttings. Hawaii Orchid J 1:19. (pagination in each issue of this volume is separate)
111. Tokuhara K, Mii M (1993) Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. Plant Cell Rep 13:7–11
112. Yamazaki J, Takahashi Y, Tsuyuki Y, Kohara H, Kataoka K (1997) The difference of a shoot and PLB formation ability of the axillary bud and shoot tip culture in *Doritaenopsis* and *Phalaenopsis*. Bull Fac Agric Tamagawa Univ 37:49–61. (in Japanese with an English summary)
113. Chen JT, Chang WC (2000) Efficient plant regeneration through somatic embryogenesis from callus cultures of *Oncidium* (Orchidaceae). Plant Sci 160:87–93
114. Ichihashi S (1992) Micropropagation of *Phalaenopsis* through the culture of lateral buds from young flower stalks. Lindleyana 7:208–215
115. Ichihashi S (1992) Micropropagation of *Phalaenopsis* through the culture of lateral buds from young flower stalks. In: Ichihashi S, Nagata H (eds) Proc. Nagoya Int. Orchid Show '92. The Organizing Committee of the Nagoya International Orchid Show '92, Secretariat of NIOS'92 11-1 Kawaseyama, Igaya-cho, Kariya-shi, Aichi-ken 448, Japan, pp 103–109
116. Tokuhara K, Mii M (1998) Somaclonal variations in flower and inflorescence axis in micropropagated plants through flower stalk bud culture of *Phalaenopsis* and *Doritaenopsis*. Plant Biotechnol 15:23–28
117. Tokuhara K, Mii M (2001) Induction of embryogenic callus and cell suspension culture from shoot tips excised from flower stalk buds of *Phalaenopsis* (Orchidaceae). In Vitro Cell Dev Biol Plant 37:457–461
118. Chen Y, Piluek C (1995) Effects of thidiazuron and N^6-benzylaminopurine on shoot regeneration of *Phalaenopsis*. Plant Growth Regul 16:99–101
119. Duan JX, Yazawa S (1995) Induction of precocious flowering and seed formation of x*Doriella* Tiny (*Doritis pulcherrima* x *Kingiella philippinensis*) *in vitro* and *in vivo*. Acta Hortic 397:103–110
120. Duan JX, Yazawa S (1995) Floral induction and development in *Phalaenopsis in vitro*. Plant Cell Tissue Organ Cult 43:71–74
121. Ichihashi S, Hiraiwa H (1996) Effects of solidifier, coconut water, and carbohydrate source on growth of embryogenic callus in *Phalaenopsis*, and allied genera. J Orchid Soc India 10:81–88
122. Jiménez VM, Guevara E (1996) Propagacion *in vitro* de *Phalaenopsis* (Orchidaceae) mediante el cultivo de secciones de ejes florales despues de la senescencia de las flores. Agr Costar 20:75–79
123. Park YS, Kakuta S, Kani A, Okabe M (1996) Efficient propagation of protocorm-like bodies of *Phalaenopsis* in liquid medium. Plant Cell Tissue Organ Cult 45:79–85
124. Islam MO, Ichihashi S, Matsui S (1998) Control of growth and development of protocorm like body derived from callus by carbon sources in *Phalaenopsis*. Plant. Biotechnology 15:183–187
125. Ichihashi S, Tsuzuki M, Aswath CR (2000) Studies on micropropagation of *Phalaenopsis* alliance. Bull Aichi Univ Educ 49:51–56
126. North C (1953) Experiments with root cuttings of Brussel sprouts. Ann Appl Biol 40:250–261
127. Morel G, Martin S (1955a) Guérison de pommes de terre atteintes de maladies à virus. C R Acad Agric Fr 119:877–878
128. Morel G, Martin S (1955b) Guérison de plantes atteintes de maladies à virus par culture de méristèmes apicaux. Report of the XIV International Horticulture Congress, The Hague, Netherlands, vol 1. Veenman & Zonern, Wageningen, pp 303–310
129. Morel G, Muller JF (1964) Le culture in vitro du méristemè apical de la pomme de terre. C R Acad Sci Paris 258:5250–5252
130. Gautheret RJ (1983) Plant tissue culture: a history. Bot Mag Tokyo 96:393–410
131. Gautheret RJ (1985) History of plant tissue and cell culture: a personal account. In: Vasil

IK (ed) Cell culture and somatic cell genetics, vol 2. Academic, Orlando, pp 1–59
132. Kassanis B (1957) The use of tissue cultures to produce virus-free clones from infected potato varieties. Ann Appl Biol 45:422–427
133. Albouy J, Flouzat C, Kusiak C, Tronchet M (1988) Eradication of orchid viruses by chemotherapy from *in-vitro* cultures of *Cymbidium*. Acta Hortic 234:413–420
134. Knudson L (1946) A new nutrient solution for the germination of orchid seed. Am Orchid Soc Bull 15:214–217
135. Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127
136. Mitra GC, Prasad RN, Roychowdhury A (1976) Inorganic salts and differentiation of protocorms in seed-callus of an orchid and correlated changes in its free amino acid content. Indian J Exp Biol 14:350–351
137. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
138. Vacin E, Went FW (1949) Some pH changes in nutrient solutions. Bot Gaz 110:605–613
139. Hew CS, Lim LY (1989) Mineral uptake by orchid plantlets grown on an agar medium. Soilless Cult 25:23–34
140. Lee YK, Hew CS, Loh CS (1987) Uptake of ammonium and nitrate in callus tissue cultures of orchid *Aranda* Noorah Alsagoff. Singapore J Primary Ind 15:37–41
141. Posthumus AC (1971) Auxins. In: van Bragt J, Mossel DAA, Pierik RLM, Veldstra H (eds) Effects of sterilization on components in nutrient media. H. Veenman & NV Zonen, Wageningen, pp 125–128
142. Nissen SJ, Sutter EG (1990) Stability of IAA and IBA in nutrient medium to several tissue culture procedures. Hortscience 25:800–802
143. Dekhuijzen HM (1971) Sterilization of cytokinins. In: van Bragt J, Mossel DAA, Pierik RLM, Veldstra H (eds) Effects of sterilization on components in nutrient media. H. Veenman & NV Zonen, Wageningen, pp 129–132
144. Mok MC, Mok DWS, Armstrong DJ, Shudo K, Isogai Y, Okamoto T (1982) Cytokinin activity of N-phenyl-*N′*-1,2,3-thiadiazol-5-ylurea (Thidiazuron). Phytochemistry 21:1509–1511
145. Mok MC, Mok DWS, Turner JE, Mujer CV (1987) Biological and biochemical effects of cytokinin-active phenylurea derivatives in tissue culture systems. Hortscience 22:1194–1196
146. Mok MC, Mok DWS (1985) The metabolism of (^{14}C)-thidiazuron in callus tissues of *Phaseolus lunatus*. Physiol Plant 65:427–432
147. Ernst R (1994) Effect of thidiazuron on *in vitro* propagation of *Phalaenopsis* and *Doritaenopsis* (Orchidaceae). Plant Cell Tissue Organ Cult 39:273–275
148. Huetterman CA, Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ Cult 33:105–119
149. van Bragt J, Pierik RLM (1971) The effect of autoclaving on the gibberellin activity of aqueous solutions containing gibberellin A_3. In: van Bragt J, Mossel DAA, Pierik RLM, Veldstra H (eds) Effects of sterilization on components in nutrient media. H. Veenman & NV Zonen, Wageningen, pp 133–137
150. Chen JT, Chang WC (2003) Effects of GA_3 ancymidol, cycocel and paclobutrazol on direct somatic embryogenesis of *Oncidium in vitro*. Plant Cell Tissue Organ Cult 72:105–108
151. Dotzauer D, Wolfenstetter S, Eibert D, Schneider S, Dietrich P, Sauer N (2010) Novel PSI domains in plant and animal H^+-inositor symporters. Traffic 11:767–781
152. Werkmeister P (1970b) Die Steuerung von Vermehrung (Proliferation) and Wachstum in der Meristemkultur von *Cymbidium* und die Verwendung eines Kohlen-Nahrmediums. Orchidee 21:126–131
153. Werkmeister P (1970a) Über die Lichtinduktion der geotropen Orientirung von Luft—und Bodenwurzeln in Gewebekulturen von *Cymbidium*. Ber Deutsch Bot Ges 83:19–26
154. Werkmeister P (1971) Light induction of geotropism and the control of proliferation and growth of *Cymbidium* in tissue culture. Bot Gaz 132:346–350
155. Ernst R (1974) The use of activated charcoal in asymbiotic seedling culture of *Paphiopedilum*. Am Orchid Soc Bull 43:35–38
156. Ernst R (1975) Studies on asymbiotic culture of orchids. Am Orchid Soc Bull 44:12–18
157. Ernst R (1976) Charcoal or glasswool in asymbiotic culture of orchids. In: Senghas K (ed) Proceedings of the 8th World Orchid Conference, Frankfurt, German Orchid Society, pp 379–383
158. Reuveni Q, and Lillien-Kipnis H (1971) Studies on the *in vitro* culture of date palm (*Phoenix dactylifera*) tissue and organs. Pamphlet No. 145. Volcani Inst Agric Res, Rehovoth, Israel
159. Nakamura A, Itagaki R (1973) Anther culture in *Nicotiana* and the characteristics of the haploid plants. Jpn J Breed 23:71–78
160. Anagnostakis SL (1974) Haploid plants from anthers of tobacco—enhancement with charcoal. Planta 115:281–283
161. Irikura Y (1975) Induction of haploid plants in tuber bearing species and interspecific hybrids of *Solanum*. Potato Res 18:133–140

162. Irikura Y (1975) Cytogenetic studies on the haploid plants of tuber bearing *Solanum* species. 1. Induction of haploid plants of tuber bearing solanums. Res Bull No. 112 Hokkaido Natl Agric Exp Stn, Japan
163. Bajaj YPS, Reinert J, Heberle E (1976) Factors enhancing in vitro production of haploid plants in anthers and isolated microspores. In: Gautheret RJ (ed) A la memoire de Georges Morel. Masson et Cie, Paris, pp 47–58
164. Wang PJ, Huang LC (1976) Beneficial effects of activated charcoal on plant tissue and organ cultures. In Vitro Cell Dev Biol Plant 12:260–262
165. Wernicke W, Kohlenbach HW (1976) Investigations in liquid culture medium as a means of anther culture in *Nicotiana*. Z Pflanzenphysiol 79:189–198
166. Weatherhead MA, Burdon J, Henshaw GG (1978) Some effects of charcoal as an additive to plant tissue culture media. Z Pflanzenphysiol 89:141–147
167. Weatherhead MA, Burdon J, Henshaw GG (1979) Effects of activated charcoal as an additive to plant tissue culture media: Part 2. Z Pflanzenphysiol 94:399–405
168. Wann SR, Veazey RL, Kahammer J (1997) Activated charcoal does not catalyze sucrose hydrolysis in tissue culture media during autoclaving. Plant Cell Tissue Organ Cult 50:221–224
169. Yam TW, Ernst R, Arditti J, Nair H, Weatherhead MA (1990) Charcoal in orchid seed and tissue culture media: a review. Lindleyana 5:256–265
170. Proskauer J, Berman R (1970) Agar culture medium modified to approximate soil conditions. Nature 227:1161
171. Klein B, Bopp M (1971) Effect of activated charcoal in agar on the culture of lower plants. Nature (Lond) 230:474
172. Pan MJ, van Staden J (1998) The use of charcoal in *in vitro* culture—a review. Plant Growth Regul 26:155–163
173. Thomas TD (2008) The role of activated charcoal in plant tissue culture. Biotechnol Adv 26:618–631
174. Fridborg G, Eriksson T (1975) Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiol Plant 34:306–308
175. Constantin MJ, Henke RR, Mansur MA (1977) Effects of activated charcoal on callus growth and shoot organogenesis in tobacco. In Vitro Cell Dev Biol Plant 13:293–296
176. Fridborg G, Pedersen M, Landstrom LE, Eriksson T (1978) The effects of activated charcoal on tissue cultures: adsorption of metabolites inhibiting morphogenesis. Physiol Plant 43:104–106
177. Withner CL (1974) Developments in orchid physiology. In: Wither CL (ed) The orchids, scientific studies. Wiley, New York, pp 129–168
178. Arditti J (1968) Germination and growth of orchids on banana fruit tissue and some of its extracts. Am Orchid Soc Bull 37:112–116
179. Ernst R (1967) Effect of select organic nutrient additives on growth in vitro of *Phalaenopsis* seedlings. Am Orchid Soc Bull 36:386–394
180. Brown DM, Groom CL, Cvitanik M, Brown M, Cooper JL, Arditti J (1982) Effects of fungicides and bactericides on orchid seed germination and shoot tip cultures *in vitro*. Plant Cell Tissue Organ Cult 1:165–180
181. Bautista NR, Quilang AB, Taylan GB, Madera RF, Pulma CC (2002) Effect of plant preservative mixture™ (PPM) on contamination rate and growth of *Vanda sanderiana* Reichb. F seedlings (Orchidaceae) in vitro. In: Proc World Conf Science Technology: harnessing science and technology to meet the global challenge. Association for the Advancement of Science, Manila Philippine, 13–15 September 2001, pp 121–126
182. Cameron SI (2008) Plant tissue culture gelling agents and supports: history, development and function. In: Teixeira da da Silva JA (ed) Floriculture and plant biotechnology, vol 18. Global Science Boo, Isleworth, pp 171–191
183. Ernst R, Arditti J, Healey PL (1971) Biological effects of surfactants. I. Influence on the growth of orchid seedlings. New Phytol 70:456–475
184. Healey PL, Ernst R, Arditti J (1971) Biological effects of surfactants. II. Influence on the ultrastructure of orchid seedlings. New Phytol 70:477–482
185. Teixeira da Silva JA (2013) The role of thin cell layers in regeneration and transformation in orchids. Plant Cell Tissue Organ Cult 113:149–161
186. Lee SM, Lam-Chan LT (1995) Study on the use of transparent polybags to culture orchid plantlets *in vitro*. Singapore J Primary Ind 23:53–54
187. Tanaka M, Jinno K, Goi M, Higashiura T (1988) The use of disposable fluorocarbon polymer film culture vessel in micropropagation. Acta Hortic 230:73–80
188. Tanaka M (1991) Disposable film culture vessels. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 17. Springer, Berlin, pp 212–228
189. Tanaka M (1991) Micropropagation of orchids by using a novel film culture vessel. In: Kimura T, Ichihashi S, Nagata H (eds) Proc. Nagoya Int. Orchid Show. Frontier Orchid Co., Ltd., Aichi-ken, pp 97–99

190. Hew CS, Hin SE, Yong JWH, Goul SS, Tanaka M (1995) *In vitro* CO_2 enrichment of CAM orchid plantlets. J Hortic Sci 70:721–736
191. Lootens P, Heursel J (1998) Irradiance, temperature, and carbon dioxide enrichment affect photosynthesis in *Phalaenopsis* hybrids. Hortscience 33:1183–1185
192. Mitra A, Dey S, Sawarkar SK (1998) Photoautotrophic *in vitro* multiplication of the orchid *Dendrobium* under CO_2 enrichment. Biol Plant 41:145–148
193. Tanaka M, Takamura T, Watanabe H, Endo M, Yanagi T, Okamoto K (1998) *In vitro* growth of *Cymbidium* plantlets cultured under superbright red and blue light-emitting diodes (LEDs). J Hortic Sci Biotechnol 73:39–44
194. Gouk SS, He J, Hew CS (1999) Changes in photosynthetic capability and carbohydrate production in an epiphytic CAM orchid plantlet exposed to super-elevated CO_2. Environ Exp Bot 41:219–230
195. Latha PG (1999) Micropropagation and *in vitro* flower induction in *Habenaria crinifera* Lindl. J Orchid Soc India 13:47–53
196. Latha PG (1999) *In vitro* culture of the regionally endangered medicinal orchid, Nervilia aragoana. Gaud J Orchid Soc India 13:59–64
197. Koch L (1974) Untersuchungen zur vegetativen Vermehrung bei Phalaenopsis in vitro. Diss., Technical Univ., Hannover, Germany
198. Koch L (1974) Erbgleiche Vermehrung von *Phalaenopsis in vitro*. Gartenwelt 74:482–484
199. Koch L (1974) Neues Verfahren zur Vermehrung von Orchideen-Protocormen. Erwerbsgartner 19:734–735
200. Kuhn L (1981) The Eric Young micropropagation centre. Am Orchid Soc Bull 50:47–49
201. Kuhn L (1981) New products for orchid propagators. Am Orchid Soc Bull 50:1465–1468
202. Tisserat B, Vandercook CE (1985) Development of an automated plant culture system. Plant Cell Tissue Organ Cult 5:107–117
203. Tisserat B, Vandercook CE (1986) Computerized long-term tissue culture for orchids. Am Orchid Soc Bull 55:35–42
204. Hew CS, Chia TF, Lee YK, Loh CS (1987) The need for a flow orchid tissue culture system. Malaysian Orchid Rev 21:30–34
205. Okamoto T (1996) Robotization of orchid protocorm transplanting in tissue culture. JARQ (Japan International Research Center for Agricultural Sciences) 30:213–220
206. Paek KY, Hahn EJ, Son SH (2001) Application of bioreactor for large-scale micropropagation systems of plants. In Vitro Cell Dev Biol Plant 37:149–157
207. Thurston KC, Spencer SJ, Arditti J (1979) Phytotoxicity of fungicides and bactericides in orchid culture media. Am J Bot 66:825–835
208. Thurston KC, Spencer SJ, Arditti J (1980) Incorporation of fungicides and bactericides in orchid seed and seedling culture media. In: Kashemsata MRS (ed.), Proc. 9th World Orchid Conf., Bangkok, 1978, pp 179–190

Chapter 8

Common Protocols in Orchid Micropropagation

So-Young Park, Yoon-Sun Huh, and Kee-Yoeup Paek

Abstract

Orchids are popular plants because of the wide range of colors, shapes, sizes, and fragrances of their flowers. The demand for orchids as cut flowers and pot plants is ever increasing in the market. The survival and subsequent germination of orchid seeds are comparatively low in the wild. Thus, to meet the demand of the horticultural industry, the in vitro germination of orchid seeds needs to be performed. Although tropical orchids can germinate on relatively simple media, the temperate orchids might require complex media or the presence of a symbiotic fungus. This chapter focuses on two common micropropagation methods using seed and protocorm-like body (PLB) cultures, respectively. For seed culture, the micropropagation of *Cypripedium macranthos* is detailed. Regeneration of orchids can also be achieved using protocorms or PLBs from vegetative explants. PLBs are excellent explants for clonal propagation, artificial seed development, and genetic engineering because they are organized and easily regenerable somatic embryos that propagate rapidly. Here, the development of PLBs from leaf sections and root tips of *Phalaenopsis* orchids is also described.

Key words Orchids, Seed culture, Protocorm, Protocorm-like bodies, Leaf thin sections, Root tip culture, *Cypripedium macranthos*, *Phalaenopsis*

1 Introduction

Orchids are commercially important plants cultivated as cut flowers and pot plants around the world [1, 2]. They are popular primarily because of their exotic values, such as spectacular variations in colors, sizes, intriguing shapes, fragrances, and long shelf life of their flowers [3]. Growing orchids is an international business covering nearly 8% of the world floriculture trade [4].

Orchids produce numerous minute seeds, which have a low propagation rate in nature because ovules are not present or poorly developed at the time of anthesis, and endosperms, which contain nutrient reserves for germination, are absent from mature seeds. Thus, alternative techniques for mass production of orchids are required to meet their ever-increasing demand in the market. Commercial production of plants through micropropagation techniques has several advantages over the traditional methods of

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_8,

propagation through seeds, cuttings, graftings, etc. [5]. In the last few decades, the achievements of modern biotechnology have revolutionized plant propagation and this effect is obvious in many sectors. Plant tissue culture technology is one of the major components of biotechnology, especially employed for the conservation, multiplication, and improvement of crops. A single explant can be multiplied, generating a large number of plants in a relatively short period of time and space under controlled conditions, irrespective of the season and weather [6]. Apart from their use as a research tool, plant tissue culture techniques have gained major industrial importance in the area of plant propagation, disease elimination, plant improvement, and secondary metabolite production in recent years. Since a number of orchids are also known to be of medicinal importance, this is another reason that plant tissue culture technique is being utilized by industries for the commercial production of useful secondary metabolites [7].

Asymbiotic germination is a useful and common technique for the micropropagation of orchids. However, the in vitro germination of terrestrial orchids, such as *Cypripedium*, is difficult when compared to the epiphytic orchids. The mature seeds of terrestrial orchids commonly contain a rigid and hard seed coat with high hydrophobicity. Additionally, certain inhibitory substances, such as abscisic acid, accumulate in mature seeds, resulting in their low germination rate [8]. Four genera (*Cypripedium*, *Paphiopedilum*, *Phragmipedium*, and *Selenipedium*) of the subfamily Cypripedioideae are considered to be more difficult to culture in vitro; particularly, *Cypripedium* is considered to be the most difficult [9]. Since orchid seeds germinate only symbiotically following a fungal infection, seed germination methods utilizing fungi have been employed for a long time. Since Knudson's discovery [10, 11] that orchid seeds could be germinated on a relatively simple mineral and sugar-containing medium, asymbiotic procedures gained a wide acceptance and are now a key approach used in the propagation and conservation of orchids ([12, 13], see Chap. 4).

The clonal propagation of highly heterozygous varieties of orchids became possible on a large scale following the work of Morel [14, 15], who showed that in vitro culture of shoot tips of *Cymbidium* and other orchid genera might lead to the formation of structures similar to seedling protocorms. A protocorm-like body (PLB) is regarded as orchid somatic embryo [16], with one end destined to form a shoot. With further manipulation of medium components and thin section of PLBs, additional secondary PLBs can be produced [17]. By repeated multiplication of these PLBs, large tissue stocks of any one clone can be obtained within a relatively short period of time [18]. If the culture is left undisturbed, many plantlets with normal shoots and roots can be regenerated from the PLBs. Somatic tissue cultures, such as shoot

meristem, leaf, root tip, and flower stalk, in orchids usually result in either the direct formation of PLBs from the explant or the indirect formation through the formation of a callus [19]; this is considered as either direct or indirect embryogenesis [20].

We have developed seed culture techniques for the large-scale production of *Cypripedium macranthos* plantlets [21, 22] and a PLB culture technique for the large-scale production of plantlets in *Phalaenopsis* orchids [23–25]. These well-established reproducible culture techniques are presented in this chapter. The media, equipment, supplies, and other requirements mentioned in this chapter are readily available in commercial tissue culture laboratories. During media preparation, it is important that all measurements are performed accurately and organic supplements prepared specifically as mentioned herein.

2 Materials

2.1 Seed Culture

1. Plant material: Collect seed capsules (75 days after cross-pollination) from *C. macranthos* plants that have been cultivated in the field or greenhouses (Fig. 1).
2. Surface sterilization of seed capsules: 2–3% of sodium hypochlorite solution 50 mL, sterilized water 500 mL, sterilized bottle.
3. Seed germination medium: Quarter-strength MS medium [26] supplemented with 10 g/L sucrose, 10% (v/v) coconut, and 0.7% (w/v) agar, adjusted to pH 5.8.
4. Rhizome and bud development medium: ½-strength inorganic salts of MS medium with 30 g/L sucrose, 10% (v/v) coconut water, and solidified with 7.5 g/L agar.
5. Aseptic dissection: Blade (No. 10, 11), forceps, autoclaved plates.

Fig. 1 Flowers (**a**) and a 75-day-old seed pod (**b**) of *Cypripedium macranthos* Sw. in the field

6. Washing solution for rhizomes: 0.05% (w/v) Benlate solution.
7. Potting mixture for seedlings: Perlite, Seramis clay granulate, pumice gravel, and kanumatsuchi (1:1:1:1).
8. In vitro culture: Petri dish (diameter 9 cm × height 1.5 cm).
9. Styrofoam boxes.
10. Plastic box (Φ120 × H105 mm).

2.2 Protocorm-Like Body (PLB) Culture

1. Plant material: For induction of PLBs from leaf thin sections using in vitro-grown plantlets derived from nodal buds of flowering stalks, floral stalks with 3–5 open flowers should be used (see Subheading 3.2.1). For PLB formation from root-tip culture, root tips from the in vitro-grown plantlets derived from floral stalks can be used (see Subheading 3.2.2).
2. Flower stalk culture medium: VW or Hyponex medium (see Tables 1 and 2), 2% (w/v) sucrose, 20% (v/v) coconut water, and 1% Bacto agar.
3. Protocorm multiplication (PM) medium (see Table 2): Hyponex (N:P:K = 6.5:6:19, 1 g/L), Hyponex (N:P:K = 20:20:20, 1 g/L), 2 g/L peptone, coconut water 10% (v/v), unsprouted potato homogenate 30 g/L, activated charcoal 0.05%, and 0.8% agar adjusted to pH 5.5.
4. Leaf thin-section culture medium: Half-strength MS medium supplemented with 2.0 mg/L thidiazuron (TDZ) or 10.0 mg/L BA, 10.0 mg/L adenine sulfate, 2% (w/v) sucrose, 20% (v/v) coconut water, and 0.23% Gelrite. Adjust the pH of the medium to 5.7 before adding Gelrite.
5. Root tip culture medium: Half-strength MS medium, 1.0 mg/L TDZ, 20% (v/v) coconut water, 10.0 mg/L adenine sulfate, and 0.23% (w/v) Gelrite.
6. Coconut water: Drain the liquid endosperm of coconut from the ripe nuts, filter through two layers of cheese cloth or coffee filter paper and either used immediately or frozen in deep freezer (−70 °C).
7. Unsprouted potato homogenate: After peeling off the skin, homogenize 30–100 g of potatoes with 200 mL water in a blender for 30 s. The liquid extract is mixed immediately with other medium components. Potatoes that have not had long storage show the best results. The homogenate obtained from sprouted potatoes can cause medium browning after culture.
8. Activated charcoal: Add finely divided activated charcoal to the medium. It is recommended to use vegetable charcoal because it has a much higher percentage (95–99%) of active charcoal (Sigma) than the charcoal obtained from other sources.

Table 1
Composition of the modified MS, VW, and Knudson media

Component	MS (mg/L)	VW (mg/L)	Knudson (mg/L)
Macronutrients			
NH_4NO_3	825	–	–
$(NH4)_2SO_4$	–	500	500
$Ca_3(PO_4)_2$	–	200	–
$Ca(NO_3)_2{\cdot}4H_2O$	–	–	1000
$CaCl_2{\cdot}H_2O$	220	–	–
$MgSO_4{\cdot}7H_2O$	185	250	250
KNO_3	950	525	–
KH_2PO_4	85	250	250
Micronutrients			
Na_2EDTA	18.65	–	25
$FeSO_4{\cdot}7H_2O$	13.9	–	–
$Fe_2(C_4H_4O_6)_3{\cdot}2H_2O$	–	28	–
H_3BO_3	3.1	–	–
$CoCl_2{\cdot}6H_2O$	0.0125	–	–
$CuSO_4{\cdot}5H_2O$	0.0125	–	–
$MnSO_4{\cdot}4H_2O$	11.15	7.5	–
KI	0.145	–	–
$Na_2MoO_4{\cdot}2H_2O$	0.125	–	–
$ZnSO_4{\cdot}4H_2O$	4.3	–	–
Organics			
Glycine	2	–	–
Myoinositol	100	–	100
Nicotinic acid	0.5	–	1
Pyridoxine	0.5	–	–
Thiamine HCl	0.1	–	1
Adenine sulfate	10	–	–

9. Sterilization solutions: 3% Sodium hypochlorite solution with two drops of Tween 20®/100 mL for vegetative explants.
10. Erlenmeyer flasks: 125 and 250 mL.
11. Plastic petri dishes: Diameter 9 cm × height 1.5 cm.

Table 2
Composition of the modified Hyponex media

Component	Seed germination	Protocorm multiplication (PM)	First transplanting (1st TP)	Second transplanting (2nd TP)
Hyponex (g/L)				
N:P:K = 6.5:6:19	3.0	1.0	1.0	1.0
N:P:K = 20:20:20	–	1.0	1.0	1.0
Adenine sulfate (mg/L)	5.0	–	–	–
Peptone (g/L)	–	2.0	2.0	3.0
Coconut water (%)	20	10.0	10.0	–
Potato or banana homogenate (g/L)	–	30–100	–	–
Activated charcoal (%)	0.05	0.05	0.05	0.05

3 Methods

3.1 Seed Culture of Cypripedium macranthos

1. During blooming season (from April to June), cross-pollinate the flowers of *C. macranthos* manually by transferring pollinia onto the stigma of another flower (**Note 1**). Harvest seed capsules of *C. macranthos* at 60–70 days after cross-pollination (Fig. 1). Seeds collected during this time are still premature; however, this is the best stage for obtaining maximum germination. The seeds harvested before or after this time exhibit poor germination (*see* **Note 2**).
2. Wash the green undehisced capsules thoroughly under tap water followed by wiping with 70% EtOH. Dip the seed capsules in 70% EtOH for a few seconds. Subsequently, immerse the capsules into 3% sodium hypochlorite solution for 15 min and rinse three times with sterile distilled water to complete the surface sterilization step (*see* **Note 3**).
3. Sterilize the capsule surface by open flame for a second before opening the capsule on a clean bench. To remove immature seeds, cut the green capsules with a sterile scalpel and scrape out the seeds with a sterile spatula.
4. Sow the surface-sterilized seeds onto the germination medium. For medium preparation, if coconut water is not available, then 10% (v/v) birch and maple sap can be used instead (Table 3). Culture the plated seeds in darkness at 23 ± 2 °C for 3 months.
5. Transfer germinated protocorms on the 1/2 MS medium containing 3% (w/v) sucrose. At the rhizome development stage, the nutrient content and sucrose concentration of basal medium are important for proper rhizome development (**Note 4**).

Table 3
Comparison of nutrient constituents in coconut water, birch, and maple sap added in the culture medium

Nutrient	Constituents	Coconut water (100 g)	Birch sap (100 g)	Maple sap (100 g)
Sugars (g)	Total	2.5	2.3	2.8
Sugar alcohols (mg)	Mannitol	0.1	–	–
	Sorbitol	1.4	–	–
Inorganic ions (mg)	Ca	24.2	58.6	63.4
	Fe	0.3	0.1	0.6
	Mg	25.3	11.3	14.5
	P	20.4	6.4	2.7
	K	242.1	120.4	204.2
	Mn	0.1	1.1	3.3
	Cu	0.04	0.03	0.7
	Na	36.5	5.3	10.4
Vitamins (mg)	Thiamin (V_{B1})	0.03	–	0.01
	Riboflavin (V_{B2})	0.05	–	0.01
	Niacin (V_{B3})	0.08	–	0.03
	Pantothenic acid (V_{B5})	0.04	–	0.03
	Pyridoxine(V_{B6})	0.03	–	0.002
	Myoinositol	0.01	–	–
	Ascorbic acid (V_C)	2.4	0.3	0.9
Organic acids (mg)	Malic	289.3	359.1	141.7
	Citric	23.7	6.8	15.0
	Succinic	8.4	11.5	12.2
Phytohormones (nM)	IAA	25.6	–	–
	Trans-ZR	10.2	–	–
	GA	16.7	–	–
	ABA	8.5	–	–

6. After 2 months of rhizome culture, two to three roots have developed from the rhizome. Transfer well-developed rhizomes onto fresh medium. At this stage, the optimal inoculum density is 4 g/L medium.
7. Harvest seedlings with well-developed rhizomes from the medium and rinse with running water to remove the gelled-agar medium, and subsequently immerse in a 0.05% benlate

solution for 5 min to prevent fungal development. Remove excessive water from the rhizomes. Plant the young seedlings comprising rhizomes and new buds in styrofoam boxes containing soil substrate mixtures (**Note 5**). Keep these styrofoam boxes in the dark at 4 °C for 3 months (**Note 6**).

8. After 3 months of chilling treatment, transplant seedlings into a plastic box (Φ120 × H105 mm) containing a mixed granular substrate comprising equal amounts of perlite, Seramis clay granulate, pumice gravel, and kanumatsuchi, and then transfer to a greenhouse. Maintain the plants at a temperature of 23 ± 2 °C and shade them using a nylon netting to provide 40–50% (approximately 40 μmol/m^2/s PPFD) of the full sunlight, and water once every week regularly (**Note 7**).
9. One month after transferring to a greenhouse, when plants have successfully acclimatized in ex vitro conditions, leaves begin to develop from the rhizome, followed by the complete development of plantlets after 3 months. This is the best time to apply fertilizer (**Note 8**). Apply solid fertilization OsmoCote (14-14-14, Scotts Co., Australia) at 5 g/L of potting volume. OsmoCote, a slow-release fertilizer, is applied at the end of March and June.

3.2 PLB Culture

3.2.1 PLB Culture from Flower Stalks

1. The floral stalks, which have 3–5 open flowers with buds in their node, are a good material for inducing adventitious shoots (Fig. 2).
2. Cut the stalks into sections with one lateral bud in the center of a 3–4-cm-long stalk. Immerse the nodal sections into 3% sodium hypochlorite solution containing one drop of Tween 20 for 10 min and then rinse thrice with sterilized water. Excise the bleached ends of the sections before placing the bases of the flower stalk sections in the medium.
3. Place trimmed flower stalk sections in the VW medium (Table 1) with 2% (w/v) sucrose, 20% (v/v) coconut water, and 1% (w/v) agar. Addition of 3.0 mg/L 6-benzylaminopurine (BAP) or 1.0 mg/L TDZ in the medium stimulates shoot development.
4. Place the cultures in the culture room at 26–28 °C under 16-h photoperiod at the light intensity of 30 μmol/m^2/s PPFD.
5. Shoots with two or three leaves generally appear within 1–2 months of culture, depending on the species and genotypes. Leaves from flower stalk culture are a suitable material for making thin leaf segments. Cut five to seven 1 mm segments transversely using a surgical blade from the proximal (basal) portion of the youngest leaf from each plantlet (*see* **Note 9**).

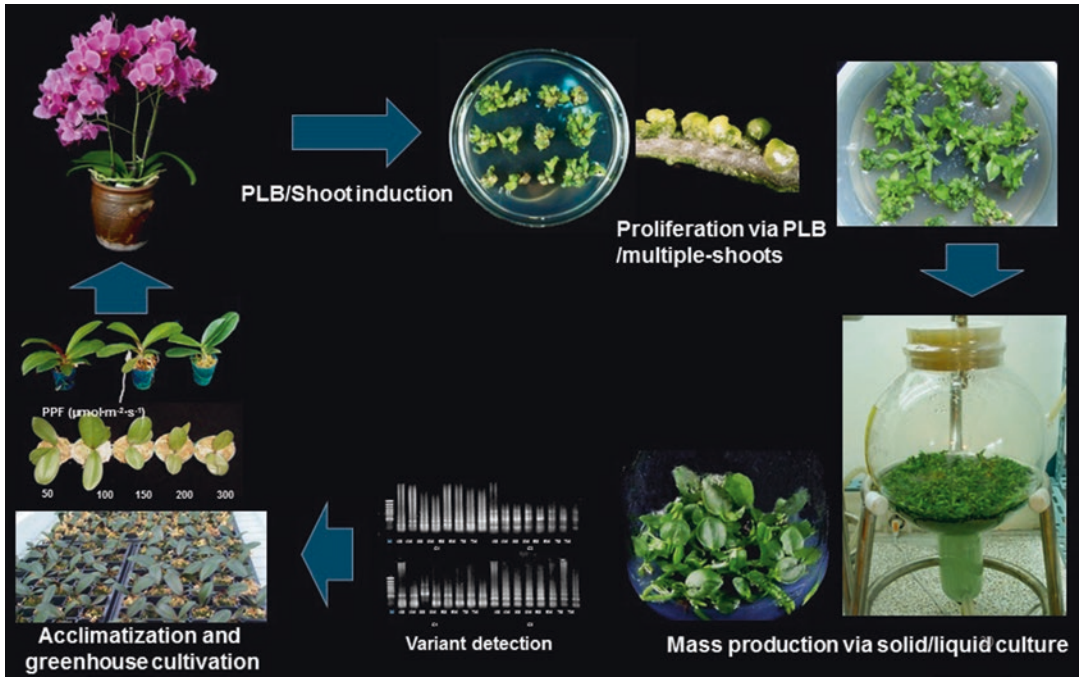


Fig. 2 Vegetative propagation of elite *Phalaenopsis* via protocorm-like body (PLB) multiplication

6. Soak the thin-sectioned segments in the half-strength MS liquid medium for 2 h.
7. Place the soaked sections (~40) with their cut sides down in Petri dishes (10 cm in diameter) containing 30 mL of the leaf thin-section culture medium.
8. Incubate cultures for 1 week at 27 °C in the dark. Thereafter, transfer the cultures to a tissue culture room at 25 °C under a 16-h photoperiod at the light intensity of 20 μmol/m^2/s PPFD and maintain for 6 weeks (*see* **Note 10**).

3.2.2 PLB Formation from Root Tip Culture

1. Root tips (less than 0.5 cm long) dissected from in vitro plantlets derived from flower stalk culture (see Subheading 3.2) can be used for PLB formation.
2. Place the roots in the root tip culture medium with their cut sides down. In terms of cytokinins, TDZ is more effective than BAP or zeatin for inducing formation of PLBs from root tips.
3. Place approximately 20 root tips in a plastic Petri dish (10 cm diameter) containing 25 mL of medium under the same culture conditions as in leaf thin-section culture.
4. Two to six PLBs can be obtained from a root tip after 8 weeks of culture.

3.3 Proliferation of PLBs

1. Select well-growing greenish globular protocorms for proliferation.
2. Remove small shoots developed on top of protocorms and transfer to the fresh PM medium (Table 2) at 4-week intervals. For more rapid proliferation, divide protocorms into 2–4 pieces longitudinally. One piece of protocorm produces 10–20 protocorms after 4 weeks in culture.
3. Discard small yellowish protocorms and separate protocorm clumps into single protocorms before subculture. Subculture protocorms well in time to prevent the formation of old and/or deteriorated protocorms, which give rise to abnormal shoots and growth retardation both in vitro and ex vitro (**Note 11**).
4. Place 20 pieces of protocorms in one disposable Petri dish (10 cm in diameter) containing 25 mL of the PM medium. Maintain the cultures at 25 °C for 4 weeks with a 16-h photoperiod under 30 μmol/m^2/s PPFD in light intensity.

3.4 Subculture and Multiplication of PLBs

1. For subculture and further proliferation of PLBs, their upper portions should be used as explants for proliferation to reduce the occurrence of variation, and their lower portions should be discarded (*see* **Note 12**). The sucrose-free VW medium with 20% coconut water or PM medium can be used for the multiplication of PLBs.
2. Discard all abnormal PLBs during subculture. Generally, 4-week intervals are advisable (**Note 13**).
3. Cultures in liquid medium respond better when agitated on a horizontal gyratory (100 rpm) or a vertical wheel-type (2–3 rpm) shaker.

3.5 Transplantation and Acclimatization of Seedlings

1. Once the seedlings develop the first leaf, transplant them onto the first transplanting (TP) medium. After 2–3 months of the first transfer, transplant seedlings with two more leaves and roots onto the second TP medium and culture for approximately 3 months (Table 2). Addition of 5% (v/v) coconut water into the medium is favorable for the growth of seedlings.
2. Acclimatization conditions are important to maximize the survival rate and to stimulate the vigorous growth of seedlings. After 6 months of in vitro culture, seedlings might develop 4–5 leaves and 3–4 roots. Wash away the medium adhering to the roots in tap water. It is important to remove agar from the roots because the agar-trapping sucrose and other organic compounds in roots cause disease and infection.
3. The seedlings are put into a potting mix and hardened in a greenhouse setting. The most common method of hardening small batches of seedlings is to place them in benches after

planting them in pots or plugs covered with clear plastic lids or with plastic domes made of thin film (*see* **Note 14**). Seedlings kept in a greenhouse after their removal from flasks must be shaded up to 90% from direct light for 7–10 weeks. Direct light can be increased gradually, up to 70% for photosynthesis by the seedlings. The most suitable temperature range for the acclimatization of seedlings is 20–25 °C under high humidity.

4 Concluding Remarks

In vitro propagation of orchids using seeds and PLB cultures can get complicated as cultural requirements can vary depending on the species, genus, or genotype. Therefore, apart from having a thorough knowledge regarding the techniques, it is imperative to establish an efficient protocol for a particular orchid.

5 Notes

1. When flowers are in bloom, they should be cross-pollinated for a better germination percentage. The cross-pollinated seeds show a germination rate of 60%, whereas the self-pollinated seeds show approximately 30% germination in 60–70-day-old seed capsules (Table 4).
2. The best time for harvesting seed capsule is 75 days after pollination (DAP) as they have the late globular embryos (Fig. 3). This stage shows maximum seed germination rate as well as protocorm formation.

Table 4
Effect of pollination type and seed maturity on the germination and protocorm formation after 4 months of culture in agar-gelled culture of *Cypripedium macranthos* Sw

Pollination type	Days after pollination (DAP)	Germination (%)	Protocorm formation (%)
Self-pollination	45	16.5 d[A]	21.7 d
	60	37.2 b	42.4 b
	75	39.3 b	46.0 b
	90	20.2 cd	23.1 d
Cross-pollination	45	17.4 d	24.7 d
	60	62.3 a	68.5 a
	75	64.0 a	71.2 a
	90	25.4 c	34.1 c

[A]Mean separation by Duncan's multiple range test at $P \leq 0.05$

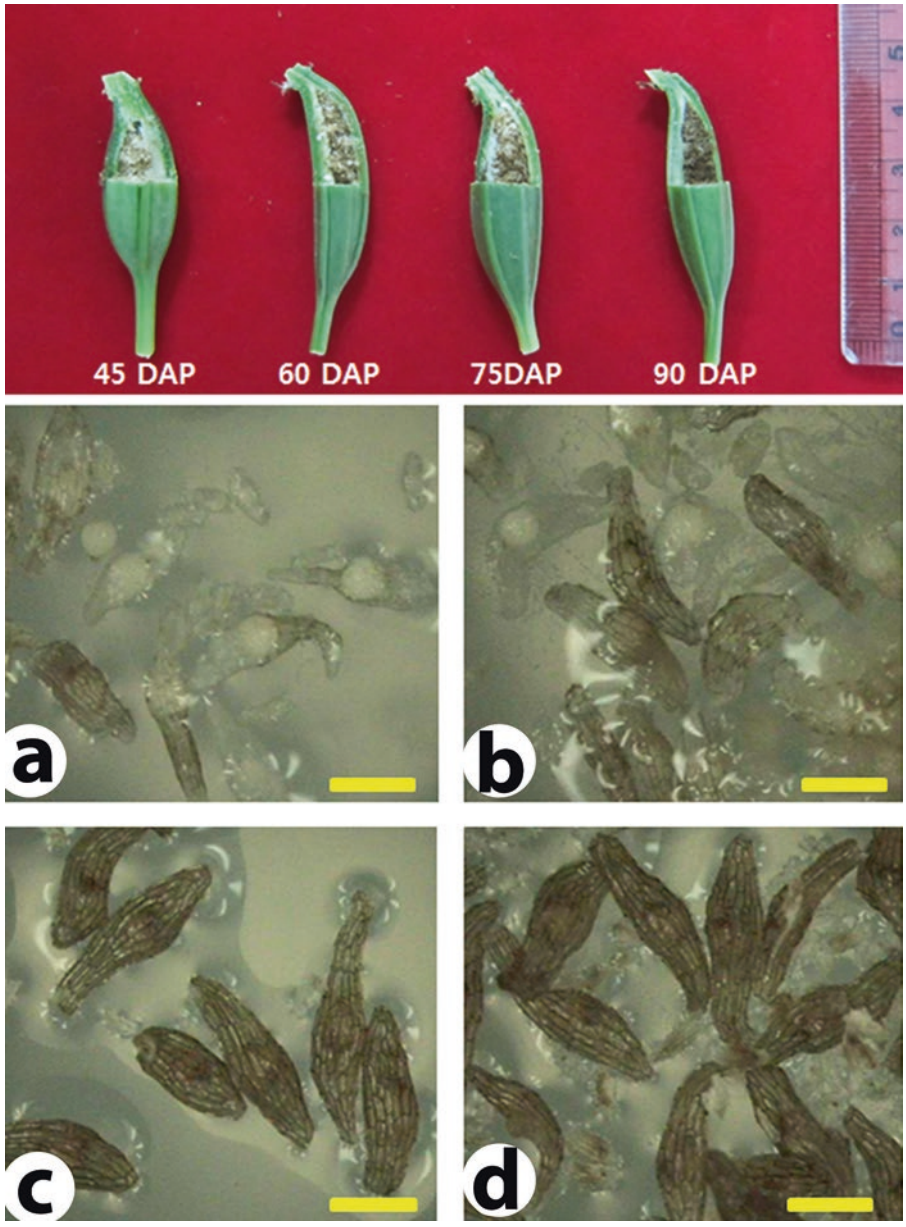


Fig. 3 Comparison of seed morphology after sowing, depending on days after cross-pollination of *Cypripedium macranthos* Sw. (**a**) At 45 days after pollination (DAP), seeds are yellowish white and moist, and early globular or globular embryos can be observed. (**b**) At 60 DAP, the seeds begin to turn light brown. (**c**) At 75 DAP, late globular embryos and seed desiccation are observed. (**d**) At 90 DAP, the seeds turn brown and have an impermeable inner integument to forming a tight layer or carapace enclosing the globular embryo. Scale bar = 0.5 mm

3. Two methods are used for seed culture sterilization: surface sterilization of seed capsules and direct sterilization of seeds from the dehisced pods. The former method provides better results during germination.
4. The nutrient content and sucrose concentration of the basal medium are important for proper rhizome development. If the

medium strength is increased over onefold of MS, then the color of protocorms starts to turn brown, and when it is increased over twofold, the death rate of protocorms is also increased. The rhizomes differentiated on the medium with 1% sucrose show the highest fresh and dry weight and rhizome number as well as the lowest death rate.

5. Growing substrates: The growth characteristics of seedlings potted in a mixed granular substrate as well as vermiculite and perlite mixture are superior to those seedlings potted in other soil media containing rich organic matter. The survival rate of these seedlings shows a significant increase to more than 70%. These inorganic soil media containing granules show beneficial effects because of their high porosity contributing to aeration and drainage.
6. Prechilling treatment: A prechilling treatment for at least 3 months is essential for the successful acclimatization of young seedlings as it increases their survival by breaking the physiological dormancy. Appropriate chilling treatment is beneficial for stimulating the dormant buds of *C. macranthos* to initiate their growth and enlargement during ex vitro acclimatization and subsequent cultivation.
7. Light: The light intensity of 40 μmol m^{-2} s^{-1} promotes the photosynthetic capacity and induces healthy and vigorous growth of young seedlings. Excessive irradiance might result in chlorophyll destruction and decrease of chlorophyll contents.
8. Fertilization: The application of 5 g OsmoCote, a slow-release fertilizer, encourages young seedlings to grow vibrantly and generates buds that will develop into new shoots in the next spring. Constant low levels of nutrients supplied by the slow-release fertilizer might provide plants with stable mineral nutrition for extended periods. This helps to minimize nutrient leaching, reduce plant damage, and improve the overall fertilizer-use efficiency.
9. Formation of PLBs and callus-like bodies is more sensitive to ethylene released from the thin leaf sections than thick leaf segments (over 5 mm). Changes in ethylene concentration in the culture vessel during the culture period are seen to be closely related to the percentage of PLB-forming explants.
10. In thin leaf section culture, wounding caused by the excision process plays an important role in PLB production. Polyphenolics are released into the medium immediately from the cut side of explants; subsequently, they oxidize and cause browning of the medium. Finally, overaccumulation of these compounds causes the explants to die. Therefore, it is desirable to subculture the explants once or twice (at 1–2-week intervals) in fresh media at an early stage of culture to increase the survival and formation of PLBs.

11. The explants should not be subcultured for more than 1 year to reduce the number of off-type plantlets. Production of less than 30,000 plantlets per capsule (a standard for *Phalaenopsis*) is advisable for commercial purposes.
12. Endoreduplication varies according to the tissue types, ages, and parts in one tissue. When PLBs are used as explants, the high proliferation rate often results in somaclonal variation, especially tetraploidy, which tends toward high levels of endoreduplication. Shoot apical meristem in the upper part of PLBs having low degree of endoreduplication is, therefore, more suitable for stable in vitro culture as compared to that in the lower part of PLBs.
13. It is important not to damage protocorm clumps during the separation and transfer of proliferated protocorm clumps. Damaged protocorms or seedlings are one of the sources of the growth-inhibiting phenolic compounds, which turn the medium brown.
14. It is necessary to distinguish the normal and off-type PLBs to reduce the frequency of somaclonal variation after transplanting plantlets to the greenhouse. The off-type PLBs are categorized into two types: one type is translucent and turgid, and the other is small and branched. Both these types are difficult to develop into plantlets, and the latter has the characteristics of new PLBs differentiating from the surface.

References

1. Chang C, Chang WC (2000) Micropropagation of *Cymbidium ensifolium* var. *Misericors* through callus-derived rhizome. In Vitro Cell Dev Biol 36:517–520
2. Kabir MI, Mortuza MG, Islam MO (2012) Morphological features growth and development of *Dendrobium* sp. orchid as influenced by nutrient spray. J Environ Sci Nat Resour 5:309–318
3. Chugh S, Guha S, Rao IU (2009) Micropropagation of orchids: a review on the potential of different explants. Sci Hortic 122:507–520
4. Gnasekaran P, Mahmood M, Subramaniam S (2016) Ultrastructure study of Vanda Kasem's Delight orchid's protocorm-like body. Hort Brasileira 34:333–339
5. Garcia-Gonzales R, Quiroz K, Carrasco B, Caligari P (2010) Plant tissue culture: current status, opportunities and challenges. Cien Inv Agr 37:5–30
6. Akin-Idowu PE, Ibitoye DO, Ademoyegun OT (2009) Tissue culture as a plant production technique for horticultural crops. Afr J Biotechnol 8:3782–3788
7. Paek KY, Chakrabarty D, Hahn EJ (2005) Application of bioreactor systems for large scale production of horticultural and medicinal plants. Plant Cell Tissue Organ Cult 81:287–300
8. Lee YI (2007) The asymbiotic seed germination of six *Paphiopedilum* species in relation to the time of seed collection and seed pretreatment. In: ISHS Acta Horticulturae 755: International Conference on Quality Management in Supply Chains of Ornamentals. Acta Hortic 755:50
9. Arditti J (2008) Micropropagation of orchids, 2nd edn. Blackwell Publishing Ltd., Hoboken, p 544
10. Knudson L (1922) Non-symbiotic germination of orchid seeds. Bot Gaz 73:1–25
11. Knudson L (1946) A new nutrient solution for the germination of orchid seed. Am Orchid Soc Bull 14:214–217

12. Arditti J, Clements MA, Fast G, Hadley G, Nishimura G, Ernst R (1982) Orchid seed germination and seedling culture—a manual. In: Arditti J (ed) Orchid biology—reviews and perspectives, II. Cornell University Press, Ithaca, pp 245–370
13. Ernst R (1975) Studies in asymbiotic culture of orchids. Am Orchid Soc Bull 44:12–18
14. Morel G (1960) Producing virus-free cymbidiums. Am Orchid Soc Bull 29:495–497
15. Morel G (1964) Clonal propagation of orchids by meristem culture. Cymbidium Soc News 20:3–11
16. Lee YI, Hsu ST, Yeung EC (2013) Orchid protocorm-like bodies are somatic embryos. Am J Bot 100:2121–2131
17. Teixeira da Silva JA (2013) The role of thin cell layers in regeneration and transformation in orchids. Plant Cell Tissue Organ Cult 113:149–161
18. Yam TW, Arditti J (2009) History of orchid propagation: a mirror of the history of biotechnology. Plant Biotechnol Rep 3:1–56
19. Ichihashi S (1997) Research on micropropagation of *Cymbidium*, nobile-type *Dendrobium*, and *Phalaenopsis*. In: Arditti J, Pridgeon AM (eds) Orchid biology: reviews and perspective, VII. Kluwer Academic Publishers, Dordrecht, pp 285–310
20. George EF (1996) Plant propagation by tissue culture. Exegetics Limited, Edington
21. Huh YS (2016) Establishment of *in vitro* proliferation and cultural practice of Endangered *Cypripedium macranthos* Sw. through asymbiotic seed culture, Unpublished doctoral thesis. Chungbuk National University, Chungbuk, Republic of Korea
22. Huh YS, Lee JK, Nam SY, Paek KY, Suh GU (2016) Improvement of asymbiotic seed germination and seedling development of *Cypripedium macranthos* Sw. with organic additives. J Plant Biotechnol 43:138–145
23. Park SY, Murthy HN, Paek KY (2003) Protocorm-like body induction and subsequent plant regeneration from root tip culture of *Doritaenopsis*. Plant Sci 164:919–923
24. Park SY, Murthy HN, Paek KY (2002) Rapid propagation of *Phalaenopsis* from floral stalk derived leaves. In Vitro Cell Dev Biol Plant 38:168–172
25. Park SY, Yeung EC, Chakrabarty D, Paek KY (2002) An efficient direct induction of protocorm-like bodies from leaf subepidermal cells of *Doritaenopsis* hybrid using thin-section culture. Plant Cell Rep 21:46–51
26. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Chapter 9

Micropropagation of Orchids by Using Bioreactor Technology

Hosakatte Niranjana Murthy, Kee-Yoeup Paek, and So-Young Park

Abstract

Orchids comprise an important group of flowering plants that produce a wide and distinct variety of flowers with characteristic shapes, size, colors, and fragrance. In horticulture industry, orchids are used as cut flowers and as potted plants. Many of the orchids have also seen medicinal use as part of traditional Chinese medicine. To meet the extensive demand for their attractive and showy flowers in the commercial market, tissue culture methods have been developed in many ornamental orchids for their large-scale multiplication. Recently, the bioreactor systems such as airlift, bubble, and temporary immersion bioreactors have been established for large-scale propagation of protocorm-like bodies (PLBs), shoots, and plantlets of various orchids. This chapter describes the bioreactor culture methods for micropropagation of *Anoectochilus formosanus* and *Dendrobium candidum*. Methods of estimation of total phenolics, flavonoids, polysaccharides, and antioxidant activities of biomass produced from *Dendrobium candidum* are also discussed.

Key words *Anoectochilus formosanus*, Bioreactor culture, *Dendrobium candidum*, Flavonoids, Phenolics, Polysaccharides, Protocorm-like bodies

1 Introduction

Orchids are a highly fascinating group of flowering plants with a wide variety of long-lasting flowers. A tremendous diversity in flowers with respect to size and shape exists and they can even mimic a variety of life forms including bees, moths, birds, and several other animal forms [1]. Diversity also exists in flower color ranging from greenish, white, yellow to intense red, blue, and brown. Flower coloration mimics the female insect to lure the male insect pollinator [2]. Many species are highly fragrant due to scents produced by the flowers to attract pollinators [3] and for setting the fruits [4]. As orchids are the rich sources of valuable secondary metabolites including vanillin [5], various orchids are used as herbal medicine to cure a wide range of aliments [6].

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_9,

Conventional propagation of orchids utilizes vegetative propagules such as kiekies and back-bulbs. Shoot division is a slow process and is not conducive for large-scale plant propagation. Asymbiotic seed germination is another method for large-scale propagation of orchids; however, the plants raised by this method are highly heterozygous [7]. Therefore, breeding for traits and creating many individual plants with the same traits are more difficult. Therefore, micropropagation, a method which creates many individuals with the same genetic makeup, is used. Micropropagation using various explants such as shoot tip culture, leaf segment culture, inflorescence and flower bud culture, rhizome segment culture, root segment culture, and thin cell layers of different explants has been followed [8, 9]. However, current micropropagation methods are time consuming and expensive as use of semisolid/gelled medium involves periodic transfer of plant material to fresh medium because of the exhaustion of the nutrients in the medium due to rapid growth of cultures [10]. Therefore, liquid-based culture methods have been developed to reduce the production cost of the plantlets and such cultures, allowing automation of procedures [11]. Recently, large-scale cultures such as airlift column bioreactors, airlift balloon bioreactors, and temporary immersion cultures have been developed for the propagation of orchids which are highly effective for propagation of orchids to meet the demand at commercial market [12, 13]. Bioreactor systems ease the control of physical parameters namely medium pH, temperature, and gaseous environment which promotes the growth of healthy plantlets. The use of bioreactors for micropropagation of orchids not only helps in scaling up of production but also decreases the cost of production. Moreover, the plant biomass produced in the bioreactors is useful for extraction of valuable secondary metabolites of pharmaceutical importance [14–18].

Anoectochilus formosanus is popularly known as "jewel orchid" because of its attractive foliar venation (Fig. 1). It is a valuable

Fig. 1 *Anoectochilus formosanus* showing its inflorescence

medicinal plant because of its diverse pharmacological effects such as liver protection, cancer prevention, antidiabetic properties, and cardiovascular protection [19]. This plant has been used in the preparation of herbal tea in Taiwan and other Asian countries [20]. *Dendrobium candidum* is another ornamental orchid which is native of China, Japan, Korea, and the Himalayas; it has been used in traditional Chinese medicine as tonic. Phenoloics, flavonoids, alkaloids, coumarins, terpenes, and polysaccharides are the active chemical components of *Dendrobium* [21]. We have developed bioreactor technologies [13–18] for the large-scale production of plantlets of *Anoectochilus formosanus* and protocorm-like bodies (PLBs) of *Dendrobium candidum* with the objective of utilization of the biomass thus produced as raw material by the pharmaceutical and nutraceutical industry. Shoot tip and/or nodal explants of *Anoectochilus formosanus* were used as initial explants and on the Hyponex medium supplemented with benzyladenine (BA) the explants differentiated into shoot buds within 30 days of culture without any intervening callus or protocorm-like body formation [22]. However, PLBs were used as initial explants for large-scale biomass production in bioreactors containing Murashige and Skoog medium [MS, 23] supplemented with 0.5 mg/L naphthalene acetic acid (NAA), 2.5% (w/v) sucrose, 150 mg/L NaH_2PO_4, and 1% banana homogenate. The PLBs were involved in the formation of secondary PLBs; further multiplication generated mass of multiple PLBs. The bioreactor culture methods which were used for large-scale propagation of *Anoectochilus formosanus* plantlets and PLB biomass of *Dendrobium candidum* are presented in this chapter. Further, standardized methods for quantification of bioactive compounds such as phenolics, flavonoids, and polysaccharides which were present in PLB biomass are also presented here.

2 Materials

2.1 Shoot Proliferation of Anoectochilus formosanus in Bioreactor Cultures

1. Shoot cuttings (10–15 mm in length) of *Anoectochilus formosanus*.
2. Murashige and Skoog (MS) [23] medium stocks (MS stocks I, II, III, and IV): Stocks may also be prepared using individual chemicals (Table 1). Store the medium in freezer or cold room at 4 °C (*see* **Note 1**).
3. Hyponex medium is supplied by Hyponex Co. Ltd. Japan. Freeze-dried Hyponex powder (1 g/L 20 N:20P:20 K + 2 g/L 6.5 N:4.5P:19 K) is dissolved in 1 L deionized water and can be stored in freezer or cold room at 4 °C.
4. Benzyladenine (BA) (Duchefa, The Netherlands): Prepare stock solution (100 μM) and store in freezer at −20 °C (*see* **Note 2**).
5. Gelrite (Duchefa Biochemie BV; Haarlem, The Netherlands).

Table 1
Composition of MS medium[a]

Chemical constituents	Concentration (mg/L)	Volume per liter (mL)
Major inorganic nutrients		
NH_4NO_3	33,000	50
KNO_3	38,000	
$CaCl_2{\cdot}2H_2O$	8800	
$MgSO_4{\cdot}7H_2O$	7400	
KH_2PO_4	3400	
Minor inorganic nutrients		
KI	166	5
H_3BO_3	1240	
$MnSO_4{\cdot}4H_2O$	4460	
$ZnSO_4{\cdot}7H_2O$	1720	
$Na_2MoO_4{\cdot}2H_2O$	50	
$CuSO_4{\cdot}5H_2O$	5	
$CoCl_2{\cdot}6H_2O$	5	
Iron source		
$FeSO_4{\cdot}7H_2O$	5560	5
$Na_2EDTA{\cdot}2H_2O$	7460	
Organic supplement		
Inositol	20,000	5
Nicotinic acid	100	
Pyridoxine-HCl	100	
Thiamine HCl	100	
Glycine	400	
Carbon source		
Sucrose	As per the experiment	

[a]After dissolving all the stock solutions in enough deionized water make it up to 1 L, adjust the pH to 6.0 (add 2 g/L gelrite or agar if semisolid medium), autoclave at 125 °C for 25 min

6. Petri dishes (15 mm × 100 mm).
7. 250-mL Erlenmeyer flasks.
8. 3- and 5-L Balloon-type bioreactors (Fig. 2; available with Samsung Scientific Company; Seoul, South Korea) (*see* **Note 3**).
9. Polytetrafluoroethylene air filters: 0.20 μm PTFE membrane filters (Midisart 2000) (Sartorius Inc., Mississauga, Canada).

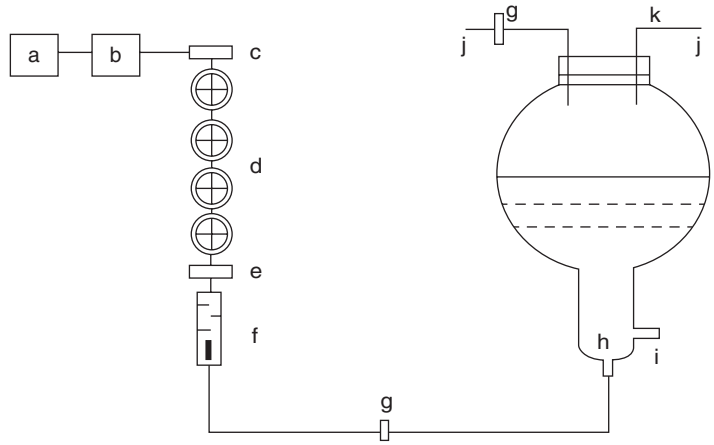


Fig. 2 A schematic diagram of an airlift bioreactor: (**a**) air compressor, (**b**) air reservoir, (**c**) air cooling device, (**d**) air filter system, (**e**) air dryer, (**f**) air flow meter, (**g**) membrane filter, (**h**) glass sparger, (**i**) medium feeding and sampling port, (**j**) vent, (**k**) prefilter

2.2 PLB Proliferation of Dendrobium candidum in Bioreactor Cultures

1. PLBs obtained from shoot tip cultures of *Dendrobium candidum*.
2. Murashige and Skoog (MS) [23] medium stocks (MS stocks I, II, III, and IV): Stocks can be prepared using individual chemicals (Table 1). Store the medium in freezer or cold room at 4 °C (*see* **Note 1**).
3. Benzyladenine (BA) (Duchefa, The Netherlands): Prepare stock solution (100 μM) and store in freezer at −20 °C (*see* **Note 2**).
4. Naphthalene acetic acid (NAA) (Duchefa, The Netherlands): Prepare stock solution (100 μM) and store in freezer at −20 °C (*see* **Note 2**).
5. Banana homogenate (homogenized banana fruits are used).
6. Gelrite (Duchefa Biochemie BV; Haarlem, The Netherlands).
7. Petri dishes (15 mm × 100 mm).
8. 250-mL Erlenmeyer flasks.
9. 3- and 5-L Balloon-type bioreactors (available with Samsung Scientific Company; Seoul, South Korea) (*see* **Note 3**).
10. Polytetrafluoroethylene air filters: 0.20 μm PTFE membrane filters (Midisart 2000) (Sartorius Inc., Mississauga, Canada).

2.3 Estimation of Total Phenols in the PLB Biomass of Dendrobium candidum

1. 2 N Folin-Ciocalteu reagent (Sigma): Store at 4 °C.
2. Gallic acid (Sigma): Generally 1000 ppm stock is prepared and stored at 4 °C in the dark (*see* **Note 4**).
3. 20% Sodium carbonate solution: Prepare a stock solution and store at 4 °C.
4. 80% Methanol, high-performance chromatographic (HPLC) grade.

2.4 Estimation of Total Flavonoids in the PLB Biomass of Dendrobium candidum

1. (+) Catechin (Sigma): Prepare 1000 ppm stock solution and store at 4 °C in the dark.
2. 10% Aluminum chloride solution (Sigma): Store stock solution at 4 °C.
3. 5% Sodium nitrate solution. Store stock solution at 4 °C.

2.5 Estimation of Total Polysaccharides in PLB Biomass of Dendrobium candidum

1. D-Glucuronic acid lactone (Sigma): Prepare 1000 mg mL^{-1} standard solution and store at 4 °C in the dark.
2. 0.125% Carbazole prepared in absolute ethanol: Store stock solution at 4 °C.
3. 5% (v/v) Sulfuric acid.

2.6 Scavenging Effect on 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical (Scavenging Activity of Natural Antioxidants)

1. 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) (*see* **Note 5**).
2. Ultraviolet (UV)-visible spectrophotometer (UV-1650PC, Shimadzu, Japan).

3 Methods

3.1 Induction of Multiple Shoots from Nodal and Leaf Explants of Anoectochilus formosanus

1. Grow *Anoectochilus formosanus* plants under controlled conditions of 16-h photoperiod, 200 μmol m^2 s^{-1}, at 24 ± 2 °C/21 ± 2 °C, day/night. Water plants daily and apply fertilizers once in every 15 days; foliar spray, N:P:K, 10:4:4 (Wuy-il Co., Seoul, Republic of Korea).
2. Collect the healthy nodes and shoots from the plants and wash thoroughly in tap water. Immerse in 95% ethanol for 10 s and surface sterilize in a 20% sodium hypochlorite solution containing few drops of Tween-30 detergent for 10 min (*see* **Note 6**).
3. Rinse 3× in cold, sterile distilled water for 5 min.
4. Prepare nodal explants (1-cm-long segments containing axillary bud) and shoot tips (1–2 mm) under the laminar flow bench and culture individually on a semisolid MS medium. Supplement with 50 g/L sucrose, 5 μM BA, 0.5 g/L activated charcoal, and 0.7% agar, pH 6.0.
5. Incubate the cultures at 24 ± 2 °C for 12 weeks under cool white fluorescent light (40 μmol m^2 s^{-1}) with a 16-h photoperiod. Shoot tip and nodal explants differentiate shoot buds within 4 weeks of culture without any intervening callus or protocorm-like body formation [23]. Subculture the explants to fresh medium at 4-week intervals.

3.2 Multiplication of Shoots of Anoectochilus formosanus in Bioreactors Using Liquid Medium

1. Collect actively growing shoots from the semisolid culture (8 g/L shoot cuttings; 1.0–1.5 cm long, and containing a single node) and culture in a 5 L capacity airlift bioreactors containing 3 L of Hyponex medium (1 g/L 20 N:20P:20 K + 2 g/L 6.5 N:4.5P:19 K) containing 50 g/L sucrose, 5 μM BA, 2.0 g/L peptone, and 0.5 g/L activated charcoal.
2. Maintain the bioreactor cultures at 24 ± 2 °C and 50 μmol m^2 s^{-1} light intensity (cool white fluorescent light), 16-h photoperiod per day. The cultures should be aerated with an airflow of 0.1 vvm (air volume/culture volume per min). Shoots grow profusely and multiply in bioreactor cultures (Fig. 3a) (*see* **Note 7**).

Fig. 3 (**a**) Shoot cultures of *Anoectochilus formosanus* (in 5 L airlift bioreactors). (**b**) Biomass (plantlets harvested) of *Anoectochilus formosanus* produced from bioreactor cultures

Fig. 4 Acclimatized plants of *Anoectochilus formosanus*

3. After 8 weeks of culture, assess the plantlet biomass (Fig. 3b) in terms of number of shoots, shoot length, number of leaves per plantlets, fresh weight, dry weight, and growth ratio.
4. Transfer the plantlets to vermiculite and perlite mixture (1:1) for acclimatization (Fig. 4) (*see* **Note 8**).

3.3 Induction of PLBs from Nodal Explants of Dendrobium candidum

1. Grow *Dendrobium candidum* plants under controlled conditions of 16-h photoperiod, 200 μmol m^2 s^{-1}, at 24 ± 2 °C/21 ± 2 °C, day/night. Water the plants daily and supply fertilizers once in 15 days; foliar spray, N:P:K, 10:4:4 (Wuy-il Co., Seoul, Republic of Korea).
2. Collect healthy stem segments containing node and wash thoroughly in tap water. Immerse in 95% ethanol for 10 s, and surface sterilize in a 20% sodium hypochlorite solution containing few drops of Tween-30 detergent for 10 min (*see* **Note 6**).
3. Rinse 3x in cold, sterile distilled water for 5 min.
4. Prepare nodal explants (1-cm-long segments containing an axillary bud) under the laminar flow bench and culture individually on half-strength semisolid MS medium with 30 g/L sucrose, 0.5 mg/L NAA, 0.5 mg/L BA, 0.5 g/L activated charcoal, and 0.7% agar, pH 6.
5. Incubate the cultures at 24 ± 2 °C for 12 weeks under cool white fluorescent light (40 μmol m^2 s^{-1}) with 16-h photoperiod. The PLBs will give rise to additional secondary PLBs. Approximately 5–8 PLBs will develop on the surface of nodal explants within 2 weeks. Subculture the explants to the fresh medium at 4-week intervals.

3.4 Culture and Multiplication of PLBs of Dendrobium candidum in the Liquid Medium

1. Collect actively proliferating PLBs (0.2–0.3 cm in diameter) from the semisolid cultures, and inoculate 5.6 g PLB inoculum into 250-mL flask containing 70 mL of half-strength MS liquid medium containing 30 g/L sucrose (*see* **Note 9**).
2. Incubate cultures at 24 ± 2 °C, under cool white fluorescent light (40 μmol m^2 s^{-1}) with 16-h photoperiod, and shake at 100 rpm on an orbital shaker. Maintain cultures by regular subculturing at 4-week intervals.
3. PLBs will multiply rapidly in the liquid medium and 8–12 secondary PLBs will develop per PLB explant.

3.5 Multiplication of Protocorm Biomass of Dendrobium candidum in Bioreactors Using Liquid Medium

1. Culture 50 g/L PLBs in a 3 L capacity airlift bioreactor containing 2 L of half-strength MS medium supplemented with 0.5 mg/L NAA, 2.5% (w/v) sucrose, 150 mg/L NaH_2PO_4, and 1% banana homogenate.
2. Maintain the bioreactor cultures at 24 ± 2 °C and 50 μmol m^2 s^{-1} light intensity (cool white fluorescent light), and with a 16-h photoperiod. Aerate the cultures with an airflow of 0.1 vvm (air volume/culture volume per min). Shoots grow profusely and multiply in bioreactor cultures (Fig. 5a) (*see* **Note 7**).
3. After 5 weeks of culture, assess the protocorm biomass (Fig. 5b) for its fresh weight, dry weight, and growth ratio.

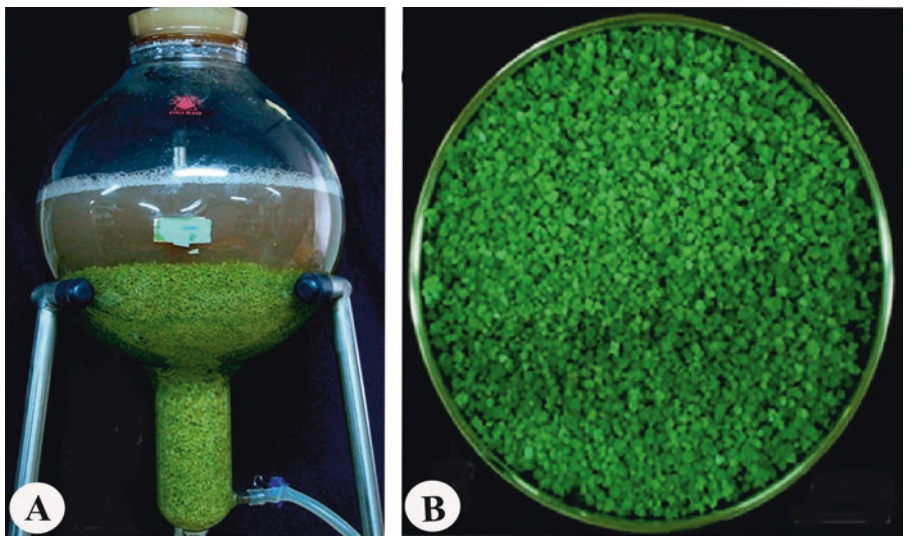


Fig. 5 (**a**) A PLB suspension culture in 3 L capacity airlift bioreactors. (**b**) PLB biomass harvested from bioreactor cultures

3.6 Estimation of Plant Biomass of Anoectochilus formosanus and Dendrobium candidum

1. Separate the plantlets or PLB biomass from the media by passing them through a stainless steel sieve. Determine the plant fresh weight/fresh biomass after rinsing with sterile water and blotting excess surface water.
2. Record the dry weight/dry biomass after drying the plantlets/PLBs at 40 °C in a forced-air oven to a constant weight (*see* **Note 10**).
3. Determine the growth ratio by GR = harvested dry biomass (g) − inoculated dry biomass (g)/inoculated dry biomass (g).

3.7 Preparation of PLB Extract of Dendrobium candidum for Estimation of Carbohydrates, Phenols, and Flavonoids

1. Place 0.5 g ground-dried PLB material into 50 mL polypropylene tube containing 10 mL of 80% ethanol and stir for 15 min.
2. Centrifuge contents at 3000 rpm (11.76 × *g*) in a refrigerated centrifuge for 10 min. Filter the supernatant solution under vacuum into a volumetric flask and save the filtrate.
3. Re-extract the residue as in steps 1 and 2 and make up the final volume to 25 mL.

3.8 Estimation of Total Carbohydrate Contents in the PLBs of Dendrobium candidum

1. The amount of total carbohydrates in the PLB extract can be analyzed spectrophotometrically by using carbazole reagent.
2. Desiccate the methanolic extract of PLBs in an oven at 60 °C. Hydrolyze 0.2 g powdered extract in 5 mL of 5% (v/v) sulfuric acid at 100 °C for 2 h. After acid hydrolysis, make up the volume of the solution to 100 mL using distilled water. Filter the solution using filter paper (Avantec 110 mm, Toyo Rosihi Kaisha Ltd., Japan).
3. Prepare 0.2% carbozole solution using absolute ethanol. Add 0.2 mL of filtrate to 6 mL of sulfuric acid and boil the solution at 100 °C on a water bath. Add 0.2 mL carbazole solution and mix the content vigorously. Prepare standard D-glucuronic acid lactone in the similar manner. Keep the solution for 2 h in darkness at room temperature.
4. Determine the absorbance of solution at 530 nm on a UV-visible spectrophotometer. Compare the measurements to the standard curve of D-glucuronic acid lactone. Express the results as mg per gram dry weight.

3.9 Estimation of Total Phenolics in the PLBs of Dendrobium candidum

1. The amount of total phenolic in the PLB extracts is analyzed spectrophotometrically using Folin-Ciocalteu reagent.
2. Mix 100 μL methanolic extract with 2.5 mL deionized water, and add 0.1 mL 2-N Folin-Ciocalteu reagent. Mix the contents and allow to stand for 6 min.
3. After 6 min, add 0.15 mL 20% sodium carbonate solution. The color develops after incubation at room temperature for 30 min.

4. The absorbance of solutions is detected at 760 nm on spectrophotometer. Compare the measurements with the standard curve of gallic acid. Express the results as mg of gallic acid equivalent per gram of dry protocorms (*see* **Note 11**).

3.10 Estimation of Total Flavonoid Contents in the PLBs of Dendrobium candidum

1. Analyze the amount of total flavonoids in the PLB extract spectrophotometrically by the aluminum chloride method (*see* **Note 12**).
2. Mix a 0.25 mL methanolic root extract and a (+) catechin standard solution with 1.25 mL deionized water. Add 75 μL 5% sodium nitrate solution and allow it to stand for 6 min.
3. After 6 min, add 0.15 mL 10% aluminum chloride solution and allow the mixture to stand for 5 min. Add 0.5 mL 1 M sodium hydroxide and mix the contents well.
4. Measure the absorbance immediately at 510 nm on a spectrophotometer (UV-1650PC, Shimadzu, Japan). The results are expressed as mg of (+) catechin equivalents per gram of PLBs.

3.11 Scavenging Effect on 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Radical (Scavenging Activity of Natural Antioxidants)

1. For the analysis of antioxidants, mix 0.5 mL aliquots of each extract with 300 μL 1 mM methanolic solution of DPPH* in a 4 mL cuvette. Bring it to a total volume of 3.0 mL with methanol. Prepare DPPH* solution fresh daily and store in the dark at 4 °C in a flask covered with aluminum foil.
2. After incubation in the dark at room temperature for 15 min, assay the reaction mixture at 517 nm using a UV-visible spectrophotometer (*see* **Note 13**).
3. In order to eliminate interference with the DPPH* reaction by extracted pigments, assay blanks of the extracts using 300 μL methanol instead of the DPPH* solution. Prepare and assay a DPPH* blank sample, containing 2.7 mL methanol and 300 μL of DPPH* solution daily. Perform all experiments in duplicate and repeat at least twice.
4. Record the percentage of decrease in the absorbance at 517 nm for each concentration; calculate the percentage of quenching of the DPPH* radical on the basis of the observed decrease of the radical. Calculate the inhibition percentage according to the following formula:

   Inhibition percentage = $[(A_{DPPH} - A_{Extr})/A_{DPPH}] \times 100$, where A_{DPPH} is the absorbance value of the DPPH* blank sample and A_{Extr} is evaluated as the difference between the absorbance value of the test solution and that of its blank. Curves showing inhibition percentage/μL of extract are used to find the concentration at which 50% radical scavenging occurred (EC_{50}).

4 Notes

1. The most efficient way of preparing plant culture media is to prepare stock solutions of major inorganic elements, iron source, vitamins, and individual growth regulators. See Table 1 for the preparation of the MS medium. Store the vitamins at −20 °C in small batches. Thaw and mix fully before use. Keep other stock solutions in the refrigerator at 4 °C, and check frequently and discard if precipitation occurs. Inorganic stock solutions should not be stored for more than 1 month. It is advisable to prepare stocks of growth regulators fresh for each batch of medium, as even small changes in concentrations due to precipitation can considerably affect the growth of the cultures. Alternatively, stock solutions of macroelements, microelements, and vitamins are commercially available.
2. Filter sterilized growth regulators such as BA and NAA should be added to the medium after autoclaving and cooling to temperatures lower than 40 °C.
3. Silicon tubes connecting to incoming and outgoing air filters should be clipped tight and the seal should be checked thoroughly before and after autoclaving. Air filters should be of high quality; 0.20 μm PTFE membrane filters should be changed after using 3–4 times.
4. Prepare a stock solution of gallic acid by weighing 1.0050 g gallic acid and dissolve in 1 L deionized distilled water. The addition of 1 mL ethanol will help in the dissolution. Store this stock solution in the amber glass container in a refrigerator and use for fresh working standards.
5. Stock solution (1 mM methanolic solution) of DPPH* should be prepared fresh daily and stored in the dark at 4 °C in a flask covered with aluminum foil.
6. Sometimes explants cultured on the medium are prone to infection and in such cases stringent surface sterilization of explants is needed; use 0.1% mercuric chloride and sterilize explants for 10–15 min. Subsequently wash explants thoroughly with sterilized distilled water and culture on the nutrient medium.
7. PLBs or shoot biomass accumulation in bioreactor cultures is dependent on physical factors, such as culture conditions other than chemical composition of the medium. For example, inoculum density, aeration volume, light/dark, and temperature conditions developed over several series of experiments. Variations in these parameters could severely affect the biomass production as well as accumulation of secondary metabolites in the biomass. Therefore, physical conditions explained in the protocol should be strictly maintained to obtain reproducible results.

8. In vitro-regenerated plantlets of *Anoectochilus formosanus* can be acclimatized using peat-moss or vermiculite and perlite mixture (1:1) and can be reared in growth chambers with 80% relative humidity and at 24 ± 2 °C for 4 weeks under cool white fluorescent light (200 μmol m^2 s^{-1}) with 16-h photoperiod. The plants can then be transferred to greenhouse conditions.
9. During the initiation of the first batch of PLB suspension cultures, PLBs of 2–3 mm in diameter should be cultured in shake flasks. After two to three passages of subcultures PLBs proliferate profusely in liquid cultures. Such PLBs should be used as inoculum for establishing bioreactor cultures.
10. PLBs should be dried at 40 °C in a forced-air oven to moisture content about 10%. Higher temperatures are not used because of the loss of phenolic compounds. After drying, the PLBs are stored at −20 °C, protected from light and humidity until further use.
11. Prepare working standard of 20, 50, 100, 150, and 200 ppm by using 1000 ppm standard gallic acid solution. Careful measurements for all liquid dilution transfer steps should be made to assure accuracy in the results. In addition, time duration for all the reactions should be maintained precisely for samples as well as for standards.
12. The principle of aluminum chloride colorimetric method is as follows: Aluminum chloride forms acid-stable complex with the C-4 keto group and either the C-3 or the C-5 hydroxyl group of flavones and flavonols [24].
13. The DPPH radical has widely been used to evaluate the free radical scavenging activity of natural antioxidants [25]. DPPH is a purple-colored radical that, after being reduced by an antioxidant, turns into yellow product, DPPH* purple + antioxidant → yellow non-radical product.

References

1. Cozzolino S, Widmer A (2005) Orchid diversity: an evolutionary consequence of deception? Trends Ecol Evol 20:487–494
2. Schuter PM, Schiestl FP (2002) Molecular mechanism of floral mimicry in orchids. Trends Plant Sci 13:228–235
3. Kaiser R (1993) The scent of orchids: olfactory and chemical investigations. Elsevier, Amsterdam
4. Neiland MRM, Wilcock CC (1998) Fruit set, nectar reward, and rarity in the Orchidaceae. Am J Bot 78:95–104
5. Walton NJ, Mayer MJ, Narbad A (2003) Molecules of interest: vanillin. Phytochemistry 63:505–515
6. Bulpitt CJ, Li Y, Bulpitt PF, Wang J (2007) The use of orchids in Chinese medicine. J R Soc Med 100:558–563
7. Arditti J, Clements MA, Fast G, Hadley G, Nishimura G, Earnt R (1982) Orchid seed germination and seedling culture—a manual. In: Arditti J (ed) Orchid biology: reviews and perspectives, vol II. Cornell University Press, Ithaca, pp 243–270

8. Chugh S, Guha S, Rao U (2009) Micropropagation of orchids: a review on the potential of different explants. Scientia Hort 122:507–520
9. Yam TW, Arditti J (2009) History of orchid propagation: a mirror of the history of biotechnology. Plant Biotechnol Rep 3:1–56
10. Maene L, Debergh P (1985) Liquid medium additions to established tissue cultures to improve elongation and rooting *in vivo*. Plant Cell Tiss Org Cult 5:23–33
11. Aitken-Christie J (1991) Automation. In: Debergh PC, Zimmerman RJ (eds) Micropropagation: technology and application. Kluwer Academic Publishers, Dordrecht, pp 363–388
12. Park SY, Murthy HN, Paek KY (2000) Mass multiplication of protocorm-like bodies using bioreactor system and subsequent plant regeneration in *Palaenopsis*. Plant Cell Tiss Org Cult 63:67–72
13. Wu ZH, Chakrabarty D, Hahn EJ, Paek KY (2007) Micropropagation of an endangered jewel orchid (*Anoectochiuls formosanus*) using bioreactor system. Hortic Environ Biotechnol 48:376–380
14. Cui HY, Murthy HN, Moh SH, Cui YY, Paek KY (2015) Establishment of protocorms suspension cultures of *Dendrobium candidum* for production of bioactive compounds. Hortic Environ Biotechnol 56: 114–122
15. Cui HY, Murthy HN, Moh SH, Cui Y, Lee EJ, Paek KY (2014) Protocorm culture of *Dendrobium candidum* in balloon type bubble bioreactors. Biochem Eng J 88:26–29
16. Cui HY, Murthy HN, Moh SH, Cui YY, Lee EJ, Paek KY (2014) Production of biomass and bioactive compounds in protocorm cultures of *Dendrobium candidum* Wall ex Lindl. using balloon type bubble bioreactors. Ind Crop Prod 53:28–33
17. Cui HY, Murthy HN, Moh SH, Cui YY, Lee EJ, Paek KY (2014) Comparison of conventional and ultrasound assisted methods for extraction of nutraceutical compounds form *Dendrobium candidium*. CyTA—J Food 12:355–359
18. Yoon YJ, Murthy HN, Hahn EJ, Paek KY (2007) Biomass production of *Anoectochilus formosanus* Hayata in a bioreactor system. J Plant Biol 50:573–576
19. Kan WS (1986) Pharmaceutical botany, 7th edn. National Research Institute of Chinese Medicine, Taiwan
20. Wang SY, Kuo YH, Chang HN, Kang PL, Tsay HS, Lin KF, Yang NS, Shyur LF (2002) Profiling and characterization antioxidant activities in *Anoectochilus formosanus* Hayata. J Agric Food Chem 50:1859–1865
21. Ng TB, Liu J, Wong JH, Ye X, SCW S, Tong Y, Zhang KY (2012) Review of research on *Dendrobium*, a prized folk medicine. Appl Microbiol Biotechnol 93:1795–1803
22. Ket NV, Hahn EJ, Park SY, Chakrabarty D, Paek KY (2004) Micropropagation of an endangered orchid *Anoectochilus formosanus*. Biol Plant 48:339–344
23. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
24. Mabry TJ, Markham KR, Thomas MB (1970) The systematic identification of flavonoids. Springer, New York
25. Brand-Williams W, Cuvelier ME, Berset C (1995) Use of free radical method to evaluate antioxidant activity. Lebensm-Wiss Technol 28:25–30

Chapter 10

Enhanced Growth and Development of *Cymbidium* and *Phalaenopsis* Plantlets Cultured In Vitro Under Light-Emitting Diodes

Duong Tan Nhut, Hoang Thanh Tung, and Michio Tanaka

Abstract

The usefulness of the light-emitting diodes (LEDs) in the growth of orchid plantlets is presented in this chapter. The construction of the Culture Pack-Rockwool (CP-RW) system and the LED Pack is described. When *Phalaenopsis* and *Cymbidium* plantlets were cultured using the CP-RW system under different blue to red LED ratios, the growth of plantlets was enhanced than those under plant growth fluorescent lamps (PGF). The positive effects of LED irradiation on plant growth indicate that LEDs will be a major light source for use in a wide range of micropropagation systems.

Key words *Cymbidium*, Light-emitting diodes, *Phalaenopsis*, Photosynthetic rate, Culture Pack-Rockwool system, Plant growth fluorescent lamps

1 Introduction

The use of LEDs as a radiation source for plants has attracted considerable interest and attention in recent years because of their vast potential for commercial applications [1, 2]. The "total" LED irradiation system has several advantages over the existing lighting systems used in tissue culture for the following reasons: (a) the peak emissions of the blue (B) and red (R) LEDs coincide closely with the absorption peaks of chlorophylls and the reported wavelengths of maximum photosynthetic efficiency [3]; (b) LEDs have a long lifespan, making them economically and environmentally viable; (c) LEDs generate very little heat, thereby minimizing the need for an extensive cooling system in the plant growth facilities; (d) the low energy consumed by LEDs makes commercial clonal propagation more cost effective; and (e) the LED system is compact, requiring less physical space. In addition, LEDs have features which are far better than the commonly used radiation sources, namely, fluorescent, metal halide, high-pressure sodium,

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_10,

and incandescent lights. LEDs have wavelength specificity and narrow bandwidths, allowing control over the wavelength given to plants. These features combined may very well solidify the use of LEDs in plant propagation for many generations to come.

LEDs have been used in many areas of photobiological research such as chlorophyll synthesis [4], photosynthesis [5], and morphogenesis [6]. A number of plant species have also been reported to grow successfully under LEDs. These include seedlings of pepper, cucumber, wheat, and spinach [6–10], potato plantlets cultured in vitro [3], and *Rehmannia glutinosa* plantlets cultured in vitro with or without sucrose and ventilation [11]. However, in earlier studies, the plants were grown under red LEDs supplemented with blue or far-red radiation derived from broad-spectrum metal halide or blue fluorescent light sources because there were no blue LEDs with high photon levels.

Moreover, a total LED plant irradiation system requires LEDs that emit high photon levels in the blue and red spectral regions. Earlier blue LEDs emitted radiation of low photon levels and were also expensive [7]. Nichia Chemical Co. Ltd. in Japan succeeded in developing superbright blue LEDs [12, 13]. This invention paved the way for the development of a total LED plant irradiation system. Recent results on the effects of LEDs on plant growth in controlled environment are summarized in a review by Agarwal and Gupta [2].

Although orchids can be propagated from seeds, *Cymbidium* cultivars prefer to be propagated through vegetative means. Several techniques are available, ranging from those requiring proper laboratory facilities to a simple division of plants. Since *Cymbidium* is one of the more popular orchids in cultivation, different tissue culture procedures have been developed. However, the production cost of tissue culture plantlets (mericlones) is still high, and growers always require high-quality plantlets for their pot plants and cut flower production. Using red and blue LEDs applied in combination has been shown to enhance the growth of *Cymbidium* plantlets cultured in the absence or presence of CO_2 enrichment [12]. Callus induction, callus proliferation, and protocorm-like body (PLB) formation of *Cymbidium* orchids were highest when grown under 75% red +25% blue LEDs [14]. The effectiveness of using a total LED irradiation system for the micropropagation of orchids has been proven by the enhanced growth of *Cymbidium* plantlets.

Investigations on clonal propagation of *Phalaenopsis* from leaf tissue were carried out by Tanaka and Sakanishi [15, 16]. Initial experiments were carried out with emerging leaves on mature plants and those taken from seedlings. Leaves of mature plants do not produce PLBs; instead, they develop on leaf explants taken from very young seedlings. Production of PLBs is also reduced with increasing age of the seedlings [17]. Tanaka et al. [16] was successful in inducing PLB formation by using leaf tissue derived from nodal shoot of *Phalaenopsis* flower-stalk sections. This method is

currently used for commercial micropropagation application. LEDs also show positive effects on the micropropagation, vegetative growth, and flower initiation of *Phalaenopsis* orchids [18, 19].

In this chapter, the construction of the Culture Pack-Rockwool (CP-RW) system and the LED Pack is described. The positive effects of LEDs in the production of quality *Cymbidium* and *Phalaenopsis* plantlets are presented as case histories.

2 Materials

2.1 Culture Systems

2.1.1 Culture Pack-Rockwool System

The Culture Pack-Rockwool system (CP-RW) is constructed to house the rockwool and sterile plantlets. The "Culture Pack" is made up of a stainless-steel frame (CP: 7.5 × 7.5 × 10.5 cm; the outside dimension of the frame) with a Neoflon® PFA film cover (PFA film, 25 μm in thickness; Daikin Industries, Japan) [20]. The supporting substrate for the plantlets is rockwool (RW: 16 joined blocks, 4 by 4, of Grodan® Rockwool Multiblock AO 18/30, Grodania A/S, Denmark) (*see* **Note 1**). These are combined to form the "Culture Pack"-Rockwool system (CP-RW) (Fig. 1) [21].

2.1.2 LED Irradiance Devices

LED Pack 3 (Nichia Chemical Co. Ltd., Tokushima, Japan) has an aluminum frame with a dimension of 36 (W) × 38 (H) × 46 cm (D). The frame enables the mounting of LED boards with different ratios of the light spectrum, i.e., blue to red ratios (100% red LED, 90% red +10% blue LED, 80% red +20% blue LED, 70% red +30% blue LED) (Fig. 2) [22]. Each LED Pack 3 can have 1120 LEDs

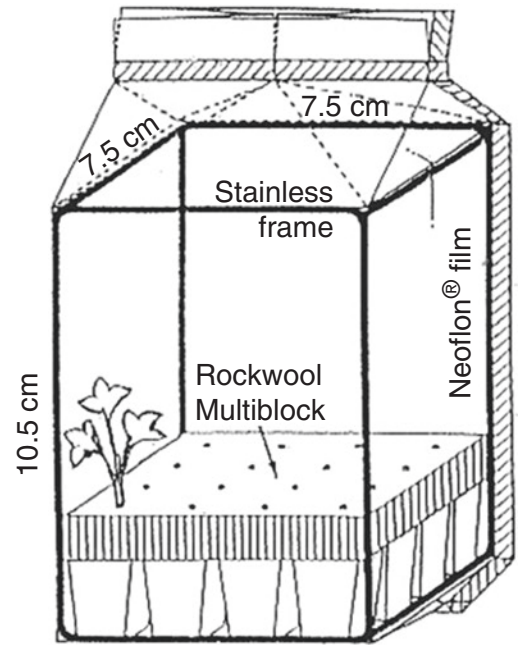


Fig. 1 The "Culture Pack-Rockwool system"

Fig. 2 LED Pack with various blue to red LED ratios

mounted on the board and secured to the top of the frame. The irradiance level measured at the surface of the rockwool is kept constant at 45 μmol m^{-2} s^{-1}. LED Pack 3 can be adjusted to 60 and 75 μmol m^{-2} s^{-1} by a regulator. Electric circuit and DC current supplies are provided on the backside of the LED Pack.

2.2 Plant Materials, Culture Media, and Supplies

1. Use excised shoots of *Cymbidium* Great Katy "Love Me," 3 cm in length with three leaves as explants. The *Cymbidium* plantlets are generated from micropropagated PLBs derived from shoot-tip cultures (*see* [12] and **Note 2**).
2. Use *Phalaenopsis* Gallant Beau "George Vasquez" plantlets 1.5 cm in length, with two leaves and two roots as explants. The *Phalaenopsis* plantlets are generated from flowering stalk culture (*see* **Note 3**).
3. Tissue culture media: (1) For *Cymbidium* cultures, use a modified Vacin and Went medium [23] supplemented with 2 g/L tryptone, 0.1 mg/L naphthaleneacetic acid (NAA), 0.1 mg/L kinetin (K), and Nitsch microelements [24] without sugar. (2) For *Phalaenopsis* cultures, use a modified Kyoto liquid medium (3 g L^{-1} Hyponex) supplemented with 100 mg/L NH_4NO_3, 30 mg/L Fe-EDTA, and Nitsch microelements [24] without sugar.

4. Laboratory equipment and supplies: laminar flow cabinets, Petri dishes, tissue culture chemicals, and supplies.

2.3 Equipment for Determining Photosynthetic Activities

1. A chlorophyll meter (SPAD-502, Minolta Co., Ltd., Japan) for the determination of SPAD (soil plant analysis development) values.
2. A portable photosynthetic system (LI-COR Inc., USA) for determining photosynthetic rates.

3 Methods

3.1 Assembling the Culture Pack-Rockwool System

Sterilize the rockwool using a drying oven (150°C, 1 h), and then place into the steel frames lined with Neoflon® PFA fluorocarbon polymer film (Fig. 1). Autoclave the assembly for 35 min at 121°C. Add 120 mL sterile liquid culture medium into a CP-RW system for the growth of *Cymbidium* and *Phalaenopsis* plantlets. Insert 16 plantlets into the small holes made on each rockwool multiblock. Heat-seal the Neoflon® PFA film to maintain a sterile interior. Perform all transfer operations under aseptic conditions.

3.2 LED Boards for the Growth of Cymbidium and Phalaenopsis Plants

For the *Cymbidium* and *Phalaenopsis* case studies, different types and mixes of LEDs were assembled on LED boards. Three types of LED boards were used, i.e., (1) 176 blue LEDs (material: GaA1As, NLPB 500 = 1 cd, 5 mm in diameter, Nichia Chemical Co. Ltd., Tokushima, Japan), (2) 176 red LEDs (material: GaN, GL5UR3K1 = 3 cd, 5 mm in diameter, Sharp Electric Ltd., Tokyo, Japan), and (3) a combination of 88 red and 88 blue LEDs. Keep the irradiance level measured at the center of each box constant at 45 μmol m^{-2} s^{-1}. Place the LED Packs in the culture room with the CO_2 concentration maintained at 3000 ppm. For comparison, place the culture systems on the shelf under plant growth fluorescent lamps (PGF) (45 μmol m^{-2} s^{-1}, Homo-Lux, National Electric Ltd., Tokyo, Japan) in the same room.

3.3 Data Recording

1. Record the numbers of leaves, plant height, shoot fresh weight, number of roots, and root length and root fresh weight of plantlets after 1–3 months of culture depending on the plant material. Determine fresh weight of shoots (stems and leaves) and roots of the plantlets separately.
2. Determination of dry weight: Dry shoots and roots in an oven at 105 °C for 30 min, and then keep at 60 °C for 48 h to reach a constant dry weight.
3. Chlorophyll measurement: Measure chlorophyll content in the third leaf (from the shoot apex) of the sampled plantlets as single-photon avalanche diode (SPAD) value using a chlorophyll meter (SPAD-502, Minolta Co., Ltd., Japan).

4. Measurement of photosynthetic rate: Determine the photosynthetic rate in plantlets after 3 months of culture using the portable photosynthetic system (LI-COR Inc., USA). Measure the photosynthetic rate in *Cymbidium* plantlets during the day due to its C3 photosynthetic properties. For *Phalaenopsis* plantlets, measure the photosynthetic rate at the end of the night [phase II of the CAM (Crassulacean acid metabolism) cycle].

4 Case History 1: *Cymbidium*

4.1 Effects of Various Blue to Red LED Ratios on the Growth of Plantlets

The effects of various blue to red LED ratios (100% red LED, 90% red +10% blue LED, 80% red +20% blue LED, 70% red +30% blue LED, and PGF) on the growth and SPAD value of *Cymbidium* plantlets are shown in Figs. 3 and 4.

The plantlets elongated under 100% red LED and were weak with a thin stem when compared to plants grown under the other blue to red LED ratios; the leaves also turned yellowish green, while the leaves were dark green in the other treatments (Fig. 3).

The number of leaves and the leaf diameter of plantlets under 100% red LED were the largest (Fig. 4c, e). Both root number and root length under 100% red LED were the lowest, but there were no differences between the values among the other treatments (Fig. 4c, d). The height of plantlets and root length were also recorded. The results indicate that *Cymbidium* plantlets grew well under 80% red +20% blue LED ratio and the results were higher than PGF (Fig. 4a, b). The SPAD values of leaves under 80% red +20% blue LEDs and PGF were higher as compared to the other treatments (Fig. 4f). Even though they have similar values, the

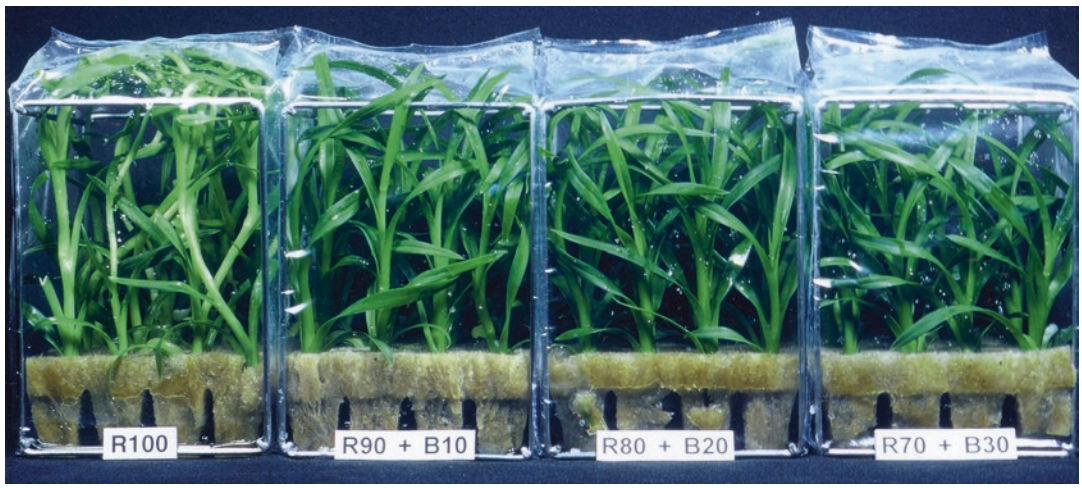


Fig. 3 In vitro growth of *Cymbidium* plantlets cultured in modified Vacin and Went liquid medium under CO_2 enrichment under various blue to red LED ratios. The plantlets under 100% red LED tend to elongate with thin stems

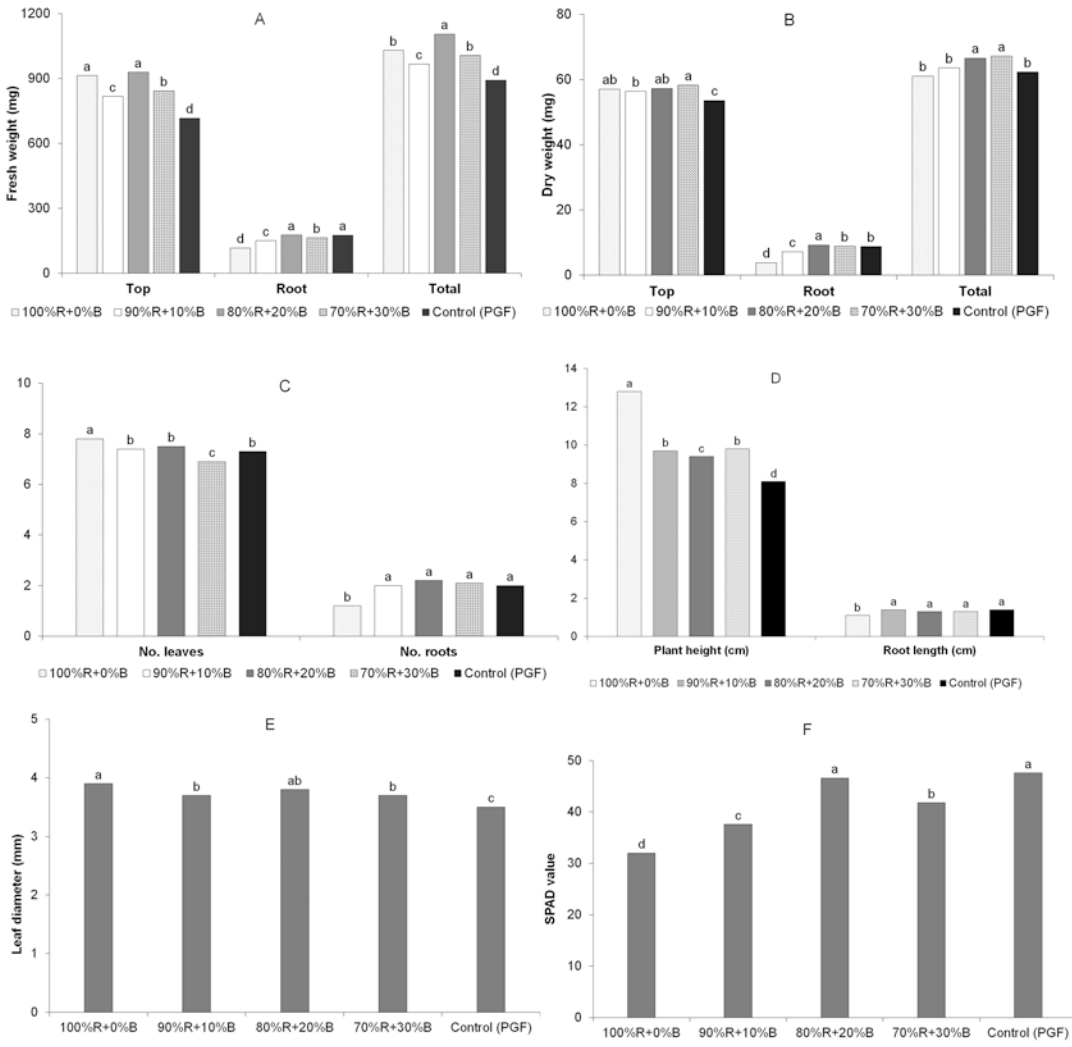


Fig. 4 Effects of various blue to red LED ratios on the growth and SPAD value of *Cymbidium* plantlets cultured in modified Vacin and Went medium under CO_2 enrichment

total fresh and dry weights under 80% red +20% blue LEDs were higher than those under PGF (Fig. 4a, b).

The photosynthetic rate after 3 months of culture is shown in Fig. 5. The photosynthetic rate of plantlets cultured under PGF was lower than those under 90% red +10% blue LED, 80% red +20% blue LED, and 70% red +30% blue LED, with the exception of 100% red LED. The results indicated that the photosynthetic rate under 80% red +20% blue LED was the best. Hence, judging from the overall results, the 80% red +20% blue LED ratio could be recommended for the micropropagation of *Cymbidium*.

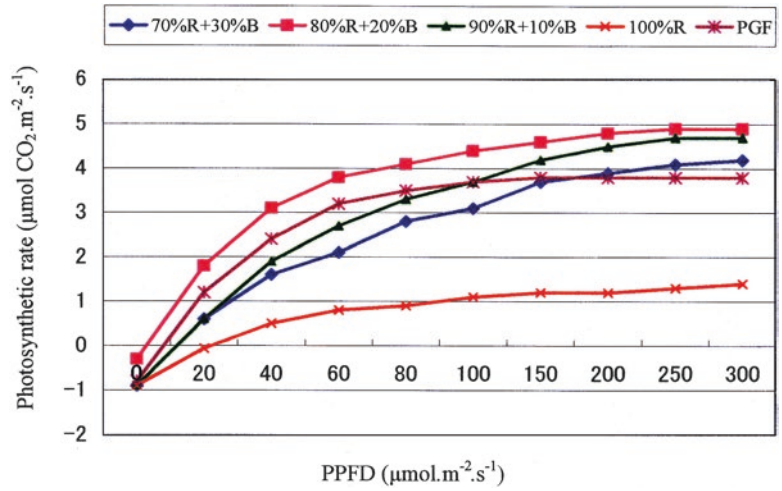


Fig. 5 The photosynthetic rate of *Cymbidium* plantlets cultured in modified Vacin and Went liquid medium under CO_2 enrichment and various blue to red LED ratios. The photosynthetic rate of plantlets cultured under PGF is lower than other LED ratios with the exception of 100% red LED

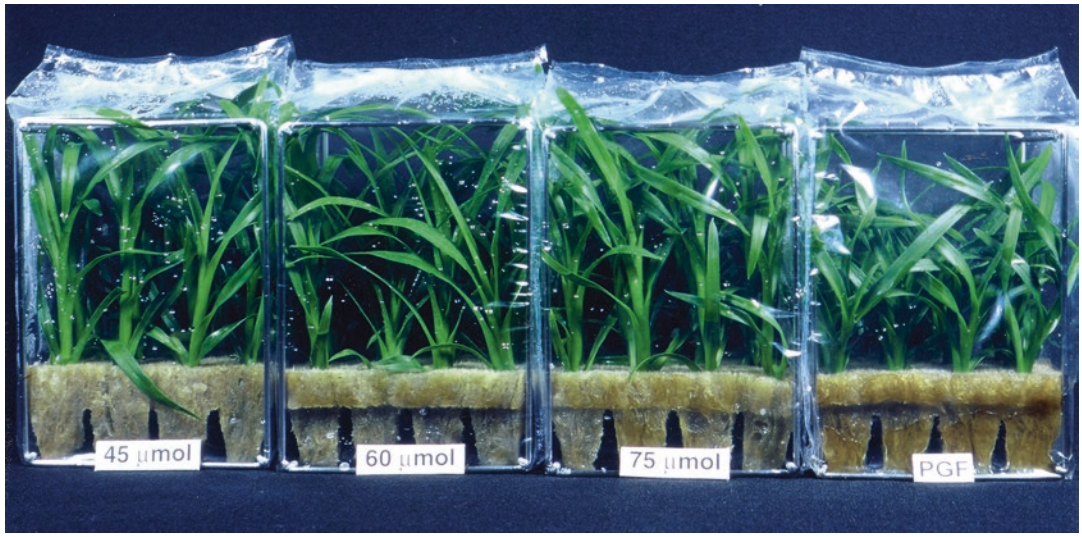


Fig. 6 Using 90% red +10% blue LED as a light source, *Cymbidium* plantlets grow normally under different irradiation levels

4.2 Effects of Different LED Irradiation Levels on the Growth of Plantlets

For investigating the effects of LED irradiation levels on the growth of *Cymbidium* plantlets in the CP-RW system, plants were cultured under 90% red +10% blue LED at 45, 60, or 75 μmol m^{-2} s^{-1} with CO_2 enrichment. Forty-eight plantlets in each treatment were cultured for 90 days. The results are shown in Figs. 6 and 7.

The plantlets were normal in every LED irradiation level (Fig. 6). Fresh and dry weights, plant height, and root length were

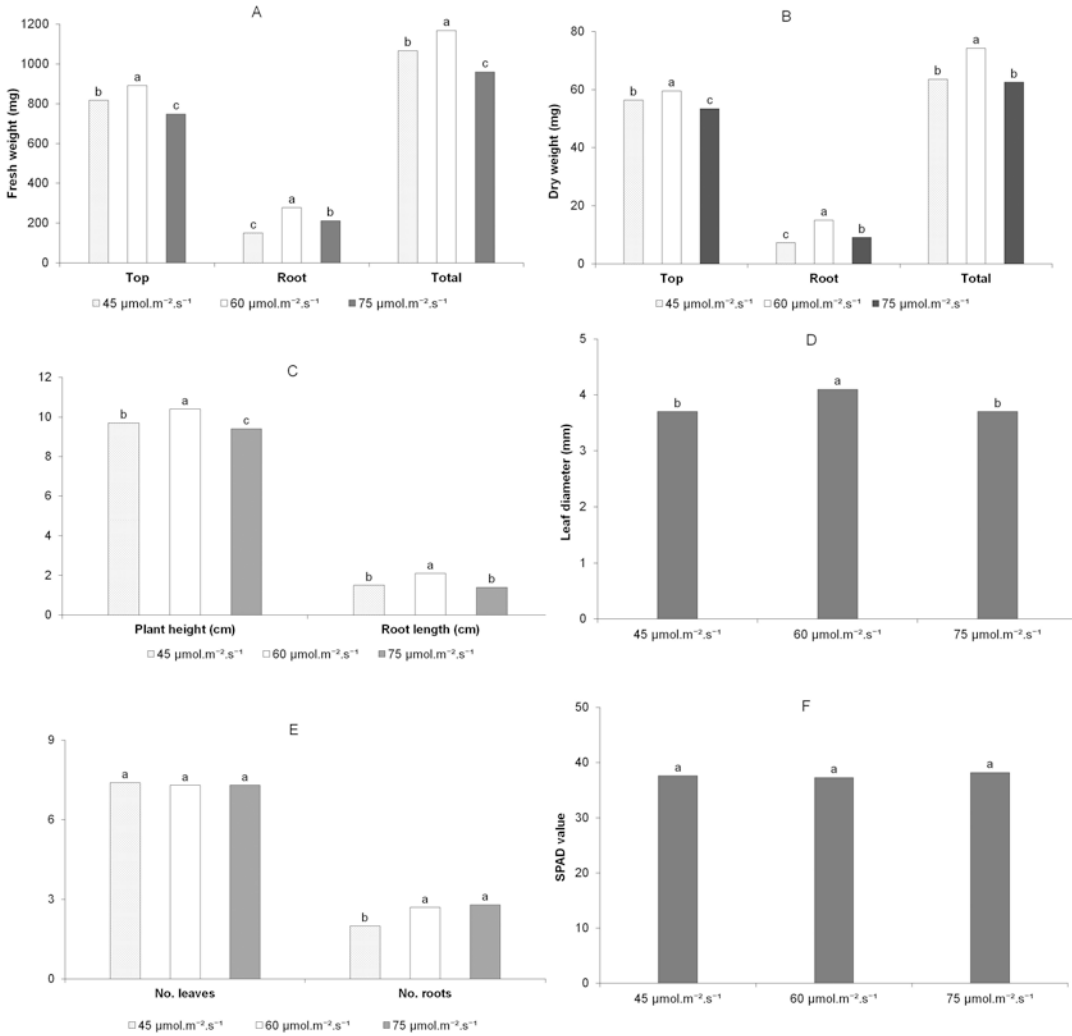


Fig. 7 Quantitative analyses on the effects of different irradiation levels on the growth and SPAD value of *Cymbidium* plantlets indicate that 90% red +10% blue LED as a light source at 60 μmol m^{-2} s^{-1} gives the best results

higher when grown under the light intensity of 60 μmol m^{-2} s^{-1} as compared to those under 45 and 75 μmol m^{-2} s^{-1} (Fig. 7a–c). The lowest shoot fresh weight was recorded under 75 μmol m^{-2} s^{-1}, while the lowest root fresh weight was noted under 45 μmol m^{-2} s^{-1} (Fig. 7a, b). The number of leaves produced was the same, while the average leaf diameter under 60 μmol m^{-2} s^{-1} was larger than that under 45 μmol m^{-2} s^{-1} (Fig. 7d, e). The SPAD value of leaves was equal among treatments (Fig. 7f).

The results indicate that the *Cymbidium* plantlets grew well at 90% red +10% blue LED when irradiation level was increased from 45 to 60 μmol m^{-2} s^{-1}. It is important to note that blue

LEDs are still expensive; therefore, using 90% red +10% blue LED as a light source at 60 μmol m^{-2} s^{-1} instead of 80% red +20% blue LED at 45 μmol m^{-2} s^{-1} is more economical, and this combination is recommended for the micropropagation of *Cymbidium* plantlets.

5 Case History 2: *Phalaenopsis*

5.1 Effects of Various Blue to Red LED Ratios

Effects of various blue to red LED ratios on the growth and SPAD value of *Phalaenopsis* plantlets cultured in the CP-RW system are shown in Figs. 8 and 9.

All plantlets elongated under different ratios of red and blue LEDs. Moreover, the leaf color was slightly yellow, and the leaf shape was abnormal under 100% red LED irradiation (Fig. 8). The plantlets under 90% red +10% blue LED had greener leaves. The growth of plantlets was normal under 80% red +20% blue LED and 70% red +30% blue LED. The leaf color was dark green under 80% red +20% blue LED, and the leaf shape was oval (Fig. 8).

The best fresh weight under LEDs was recorded in the plantlets grown under 80% red +20% blue LED, and the value was higher than those under PGF (Fig. 9a). The highest root fresh weight was obtained under 70% red +30% blue LED (Fig. 9a). The number of leaves and the leaf length of plantlets were almost equal in all treatments. The number of roots cultured under 80% red +20% blue LED, 70% red +30% blue LED, and PGF was slightly higher than that under 100% red LED (Fig. 9c). The roots under 70% red +30%

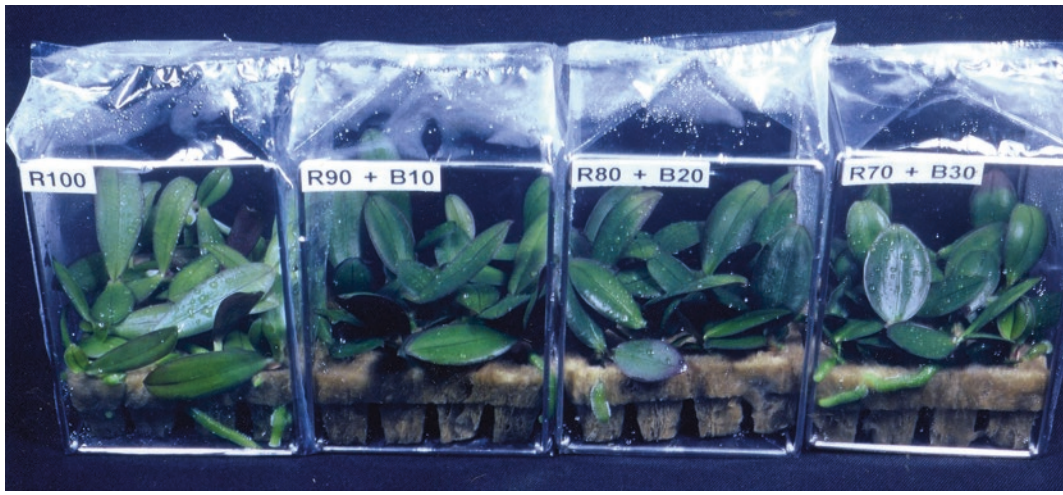


Fig. 8 In vitro growth of *Phalaenopsis* plantlets cultured in modified Kyoto liquid medium under CO_2 enrichment and various blue to red LED ratios. Under red irradiation, the leaves are not as green and show slight yellowing and tend to elongate more when compared to other LED ratios

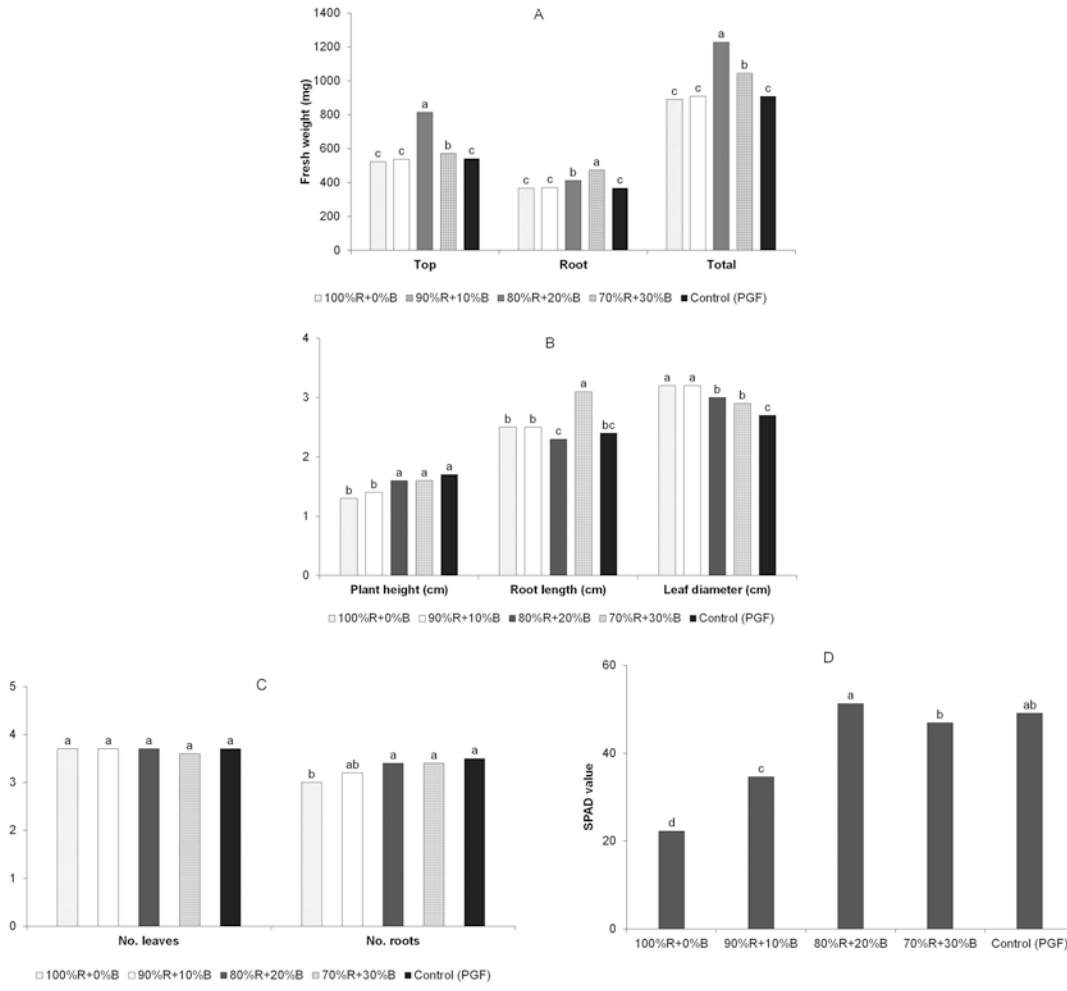


Fig. 9 Quantitative analyses on the effects of various blue to red LED ratios on the growth and SPAD value of *Phalaenopsis* plantlets indicate that plantlets grew well under 80% red +20% blue LED

blue LED were the longest (Fig. 9b). The SPAD value of leaves under 80% red +20% blue LED was the best (Fig. 9d).

The photosynthetic rate is shown in Fig. 10. When plantlets were cultured under 80% red +20% blue LED and PGF for 90 days, the photosynthetic rates were equal.

The overall results indicate that *Phalaenopsis* plantlets grew well under 80% red +20% blue LED and this LED ratio could be recommended for the micropropagation of *Phalaenopsis* plantlets.

5.2 Effect of LED Irradiation Level

For investigation of the effects of LED irradiation level on the growth of *Phalaenopsis* plantlets using the CP-RW system, plants were cultured under 80% red +20% blue LED at 45, 60, or 75 μmol m^{-2} s^{-1} in a CO_2 enrichment environment. Forty-eight plantlets in each treatment were cultured for 90 days.

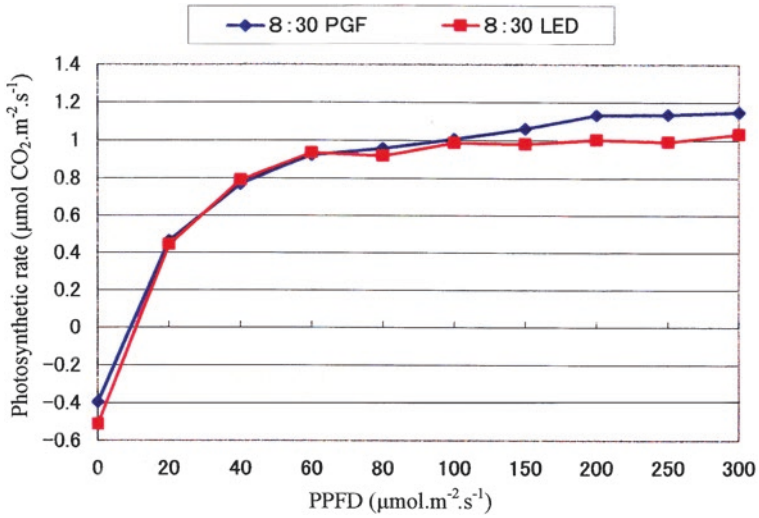


Fig. 10 The photosynthetic rate of *Phalaenopsis* plantlets is similar under 80% red +20% blue LED ratio and PGF

The results are summarized in Fig. 11. The plantlets were vigorous and normal under all LED irradiation levels. The best shoot fresh weight and plant height were observed in the plantlets cultured under 60 μmol m^{-2} s^{-1}, while the highest root fresh weight was obtained under 75 μmol m^{-2} s^{-1} (Fig. 11a, c). Total shoot and root dry weight of plantlets cultured under 60 and 70 μmol m^{-2} s^{-1} was higher than that under 45 μmol m^{-2} s^{-1} (Fig. 11b).

The number of leaves under 45 and 75 μmol m^{-2} s^{-1} LED was higher than that under 60 μmol m^{-2} s^{-1}, while leaf diameter was equal across all treatments (Fig. 11c, d). The number of roots under 60 μmol m^{-2} s^{-1} was greatest, while the root length was the same for all treatments (Fig. 11c, d). The SPAD value of leaves under 75 μmol m^{-2} s^{-1} was the best (Fig. 11e).

From the results, it is clear that the growth of *Phalaenopsis* plantlets was enhanced under 80% red +20% blue LED (60 μmol m^{-2} s^{-1}) as compared to that under 45 μmol m^{-2} s^{-1}. This LED irradiation level could enhance micropropagation of *Phalaenopsis*.

6 Comments

From the data obtained in this study, the use of the total irradiation system by using red and blue LEDs shows positive effects on plant growth and can be applied to the micropropagation of *Cymbidium* and *Phalaenopsis*. The total irradiation system is an effective light source for the photosynthetic process. Similar observation has been reported for *Bletilla* [25] and *Calanthe* [26]. From the results and the attractive features of LEDs and continual improvements in LED designs, e.g., [27], it is reasonable to expect that the total LED irradiation system will be a major light source for a wide range of micropropagation systems in the future.

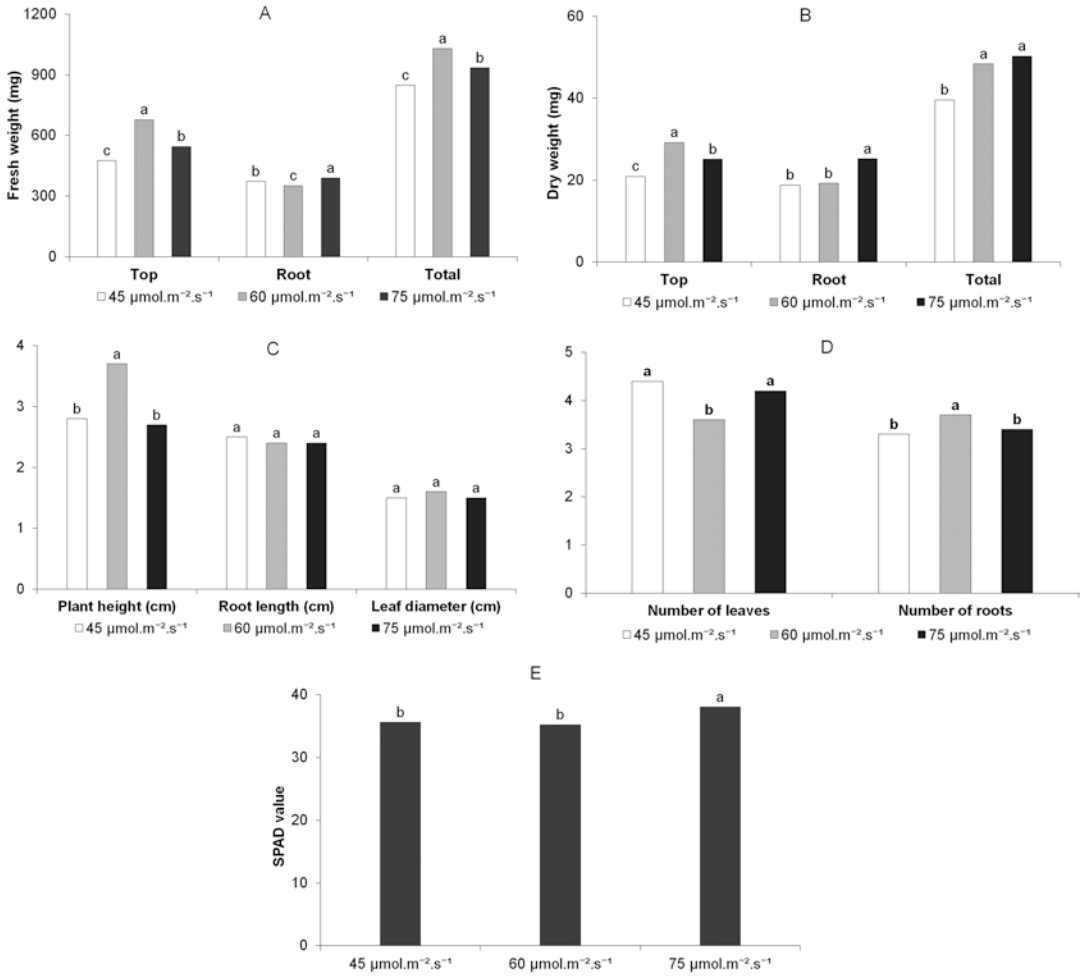


Fig. 11 Under 80% red +20% blue LED, the growth of *Phalaenopsis* plantlets is enhanced at 60 µmol m^{-2} s^{-1} as compared to that under 45 µmol m^{-2} s^{-1}

7 Notes

1. Rockwool is a lightweight hydroponic substrate made by the melting of basaltic rock and is widely used for the commercial production of horticultural crop such as hydroponic tomatoes [28].
2. PLBs were first produced from shoot-tip cultures of *Cymbidium* Sleeping Beauty "Golden Bird." Developing shoots were subsequently harvested from PLBs and used as explants [14].
3. The explants were obtained from 2-month-old PLB-derived plantlets through floral stalk-derived leaves cultured in vitro on modified Kyoto liquid medium (3 g L^{-1} Hyponex,

100 mg L^{-1} NH_4NO_3, 30 mg L^{-1} Fe-EDTA, microelements [23]). A detailed protocol for the rapid propagation of *Phalaenopsis* from floral stalk-derived leaves has been published by Park et al. [29].

Acknowledgments

This chapter was supported by the doctoral thesis "*In vitro* growth and physiological of some horticultural plantlets cultured under red and blue led light-emitting diodes (LEDs)" of Duong Tan Nhut at Kawaga University, Japan, 2002.

References

1. Gupta SD, Jatothu R (2013) Fundamentals and applications of light-emitting diodes (LEDs) in *in vitro* plant growth and morphogenesis. Plant Biotechnol Rep 7:211–220
2. Agarwal A, Gupta SD (2016) Impact of light-emitting diodes (LEDs) and its potential on plant growth and development in controlled-environment plant production system. Curr Biotechnol 5:28–43
3. Miyashita Y, Kitaya Y, Kozai T, Kimura T (1995) Effects of red and far-red light on the growth and morphology of potato plantlets *in vitro*: using light emitting diodes (LEDs) as a light source for micropropagation. Acta Hortic 393:189–194
4. Tripathy BC, Brown CS (1995) Root-shoot interaction in the greening of wheat seedlings grown under red light. Plant Physiol 107: 407–411
5. Tennessen DJ, Singsaas EL, Sharkey TD (1994) Light-emitting diodes as a light source for photosynthesis research. Photosynth Res 39:85–92
6. Hoenecke ME, Bula RJ, Tibbitts TW (1992) Importance of blue photon levels for lettuce seedlings grow under red light-emitting diodes. Hortic Sci 27:427–430
7. Bula RJ, Morrow RC, Tibbitts TW, Ignatius RW, Martin TS, Barta DJ (1991) Light-emitting diodes as a radiation source for plants. Hortic Sci 26:203–205
8. Brown CS, Shuerger AC (1993) Growth of pepper, lettuce and cucumber under light - emitting diodes. Plant Physiol 102:808–813
9. Schuerger AC, Brown CS, Stryjewski EC (1997) Anatomical features of pepper plants (*Capsicum annuum* L.) grown under red light emitting diodes supplemented with blue or far-red light. Ann Bot 79:273–282
10. Yanagi T, Yachi T, Okuda N, Okamotob K (2006) Light quality of continuous illuminating at night to induce floral initiation of *Fragaria chiloensis* L. CHI-24-1. Sci Hortic 109:309–314
11. Hahn EJ, Kozai T, Paek KY (2000) Blue and red light-emitting diodes with or without sucrose and ventilation affects *in vitro* growth of *Rehmannia glutinosa* plantlets. J Plant Biol 43:247–250
12. Tanaka M, Takamura T, Watanabe H, Endo M, Yanagi T (1998) *In vitro* growth of *Cymbidium* plantlets cultured under superbright red and blue light-emitting diodes. J Hortic Sci Biotechnol 73:39–44
13. Nair GB, Dhoble SJ (2015) A perspective perception on the applications of light-emitting diodes. Luminescence 30:1167–1175
14. Huan LVT, Tanaka M (2004) Effect of red and blue light-emitting diodes on callus induction, callus proliferation, and protocorm-like body formation from callus in *Cymbidium* orchid. Environ Control Biol 42:57–64
15. Tanaka M (1977) Clonal propagation of *Phalaenopsis* by leaf tissue culture. Am Orchid Soc Bull 46:733–737
16. Tanaka M (1985) Regenerative capacity of *in vitro* culture leaf segments excised from mature *Phalaenopsis* plant. Bull Osaka Pref Ser B 37:1–4
17. Tanaka M, Hasegawa A, Goi M (1975) Studies on the clonal propagation of monopodial orchids by tissue culture. I Formation of protocorm-like bodies from leaf tissues in *Phalaenopsis* and Vanda. J Jpn Soc Hort Sci 44:47–58

18. Wongnok A, Piluek C, Techasilpitak T (2008) Effects of light emitting diodes on micropropagation of *Phalaenopsis* orchids. Acta Hortic 788:149–156
19. Dewi K, Purwestri YA, Astuti YTM, Natasaputra L, Parmi P (2014) Effects of light quality on vegetative growth and flower initiation in *Phalaenopsis*. Indonesian J Biotechnol 19:33–42
20. Tanaka M (1991) Disposable film culture vessels. In: Bajai YPS (ed) Biotechnology in agriculture and forestry, High-tech and Micropropagation I, vol 17. Springer, Berlin, pp 212–228
21. Tanaka M, Giang DTT, Murakami A (2005) Application of a novel disposable film culture system to photoautotrophic micropropagation of *Eucalyptus uro-grandis* (*Urophylla* × *grandis*). In Vitro Cell Dev Biol Plant 41:173–180
22. Nhut DT (2002) *In vitro* growth and physiological aspects of some horticultural plantlets cultured under red and blue light emitting diodes (LEDs). Doctoral thesis, Kagawa University, Japan
23. Vacin EF, Went FW (1949) Some pH changes in nutrient solutions. Bot Gaz 110:605–613
24. Nitsch C, Nitsch JP (1967) The induction of flowering *in vitro* in stem segments of *Plumbago indica* L. Planta 72:355–370
25. Godo T, Fujikawa K, Guan K, Miyoshi K (2011) Effect of wavelength of LED-light on in vitro asymbiotic germination and seedling growth of *Bletilla ochracea* (Orchidaceae). Plant Biotechnol 28:397–400
26. Bae KH, Oh KH, Kim SY (2015) In vitro seed germination and seedling growth of *Calanthe discolor* Lindl. Plant Breed Seed Sci 71:109–119
27. Chin LY, Chong KK (2012) Study of high power light emitting diodes (LED) lighting system in accelerating the growth rate of *Lactuca sativa* for indoor cultivation. Int J Phys Sci 7:1773–1781
28. Morgan L (1999) Hydroponic lettuce production: a comprehensive, practical and scientific guide to commercial hydroponic lettuce production. Casper Publications, North Manly, NSW
29. Park SY, Murthy H, Paek KY (2002) Rapid propagation of *Phalaenopsis* from floral stalk-derived leaves. In Vitro Cell Dev Biol Plant 38:168–172

Part III

Special Techniques

Chapter 11

Tracking Pollen Fates in Orchid Populations

Steven D. Johnson and Lawrence D. Harder

Abstract

Pollination in most plants is mediated by complex interactions with animal pollinators. The fates of pollen, such as the rates of self-pollination and export to conspecific stigmas, can strongly influence components of reproductive success such as seed production and male siring success. Tracking pollen fates is therefore one of the key aims in plant reproductive biology. This chapter outlines the two main methods (pollen staining and microtagging) that have been developed to track pollen fates in orchids and discusses their advantages and disadvantages. We show that these tracking methods can be used to quantify the efficiency of pollen transfer in orchid populations, the rates of self-pollination and spatial patterns of pollen export. Finally, we outline some of the insights into orchid reproductive biology that have been revealed using these tracking methods.

Key words Geitonogamy, Gene flow, Microtag, Orchid, Orchidaceae, Paternity analysis, Pollen, Pollination, Self-pollination

1 Introduction

Pollination is a key process during seed-plant life cycles, as it is required for sexual reproduction and has a major influence on the genetic structure of populations [1]. Pollination involves both female and male components [2]. The female component, pollen receipt by a plant's stigmas, is relatively easy to measure by counting the proportion of flowers that receive pollen and the amount of pollen received. The main challenge in measuring the female component of pollination success is to measure the quality of pollen received [3]. In particular, the fraction of self-pollen deposited on stigmas plays a critical role in determining the overall fraction of selfed seeds (female selfing rate) of self-compatible plants [4] and can also influence the efficiency of conversion of ovules into seeds and pollen export [5]. The male component of pollination success, dispersal of a plant's pollen to stigmas, is notoriously hard to measure, as it involves tracking the dispersal of pollen to recipient stigmas [6, 7]. Observations of pollinator

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_11,

movements between flowers generally underestimate the spatial extent of pollen dispersal if stigmas remove only a fraction of the pollen on pollinators during individual flower visits (pollen carryover) [8]. In a few species, natural pollen colour or size polymorphisms can be used to track granular pollen [9, 10]; however, methods for quantifying pollen dispersal directly are not yet available for most plant species. Orchids (Orchidaceae) and milkweeds (Apocynaceae: Asclepiadoideae and Secamonoideae) are exceptions, as their pollen is aggregated in pollinia [11] that allow application of labels that can be used to track pollen dispersal. In this chapter we outline the application of these methods in orchid populations to estimate the incidence and intensity of self- and cross-pollination, the spatial extent of cross-pollination, and the overall efficiency of pollen transfer.

1.1 Paternity Analysis Versus Pollen Tracking

The male component of mating success is most commonly measured through paternity analysis based on molecular markers assayed in seeds or seedlings [12, 13]; however, this information conflates pollination outcomes with postpollination attrition during pollen germination, tube growth, ovule fertilization and seed development. For example, molecular markers are uninformative in estimating the amount of pollen that is not removed from anthers or is lost during transfer between flowers [14, 15]. Furthermore, molecular markers do not allow estimation of pollinator-mediated self-pollination in species that are self-incompatible or have early-acting inbreeding depression, even though such pollination can represent significant lost opportunities for pollen export (pollen discounting) [16]. Molecular markers also cannot provide information about the extent to which pollen is dispersed to the stigmas of other species that are not genetically compatible [17]. Thus, unambiguous studies of the pollination process and its influences require direct tracking of pollen dispersal in plant populations. Direct pollen tracking is much cheaper and quicker than paternity analysis based on molecular markers and can be used in large populations, for which paternity assignment using molecular markers can be very difficult due to uncertainties about the uniqueness of genotypes. For orchids, the use of molecular markers in paternity analysis has been particularly challenging because of the difficulty in obtaining sufficient DNA from progeny when seeds are difficult to germinate. However, the technology for germinating orchid seeds has improved considerably, and the first study of paternity analysis in orchids based on DNA extracts from protocorms of two Australian *Chiloglottis* species was recently published [18].

The most commonly used method for estimating pollen dispersal for plants with granular pollen involves dusting coloured dye particles (usually fluorescent paint powders) onto anthers and then tracking the dispersal of these dye particles, usually with the

aid of a UV-emitting light source [8, 19]. However, the dispersal properties of dye particles can differ significantly from that of actual pollen [8, 20]. Furthermore, the number of dye particles applied to anthers cannot be controlled or estimated accurately, so that this method is not useful for quantifying pollen removal and the proportional fates of removed pollen.

The production of pollen of most orchids in pollinia has profound implications for mating patterns, because in most of these species a flower's entire pollen complement can be removed during a single visit. In many orchid species, pollinia are also deposited on stigmas as single units; however, the pollinia of many other orchid species are divided into small subunits (massulate) or are smear-like and thus potentially deposited piecemeal over a sequence of stigmas [21].

The packaging of pollen of most orchids into pollinia offers excellent opportunities for tracking of pollen fates, because pollinia can be stained with unique colours or even labelled with unique tags. Peakall [22] introduced a method for staining pollen in orchids with massulate or mealy pollinia. He injected various histochemicals into anthers of the Australia orchid *Prasophyllum fimbria* and recorded the presence of a smear of stained pollen on recipient stigmas. Johnson et al. [23] later showed that the method could be used for exact tracking of the proportion and number of pollen massulae that are dispersed among flowers. Peakall's [22] method is suitable for tracking pollen in the subfamily Orchidoideae and has also recently been used for slipper orchids in the subfamily Cypripedioideae [24]. Nilsson et al. [25] introduced the use of uniquely numbered microtags for tracking pollen dispersal in a study of the orchid *Aerangis ellisii*, which has 1.1 × 0.9 mm pollinia. The method is suitable for orchids with solid pollinia, which includes the Epidendroideae, the largest subfamily of orchids. Alexandersson [26] used a combination of microtags and pollen staining to track pollinia of the epidendroid orchid *Calypso bulbosa* (Fig. 1a). Flowers of this species produce four pollinia arranged in two pairs, and microtags could be attached only to the front pollinia (Fig. 1b). The rear pollinia were labelled with various colours using a pen paint marker (Edding 751, Ahrensburg, Germany).

2 Technical Comments

The main technical concern about staining or tagging pollen is that it could affect the dispersal properties of the pollen. Peakall [22] showed that staining did not affect the probability of pollinarium removal by pollinators. Similarly, application of microtags did not affect removal of solid pollinaria from flowers of several epidendroid

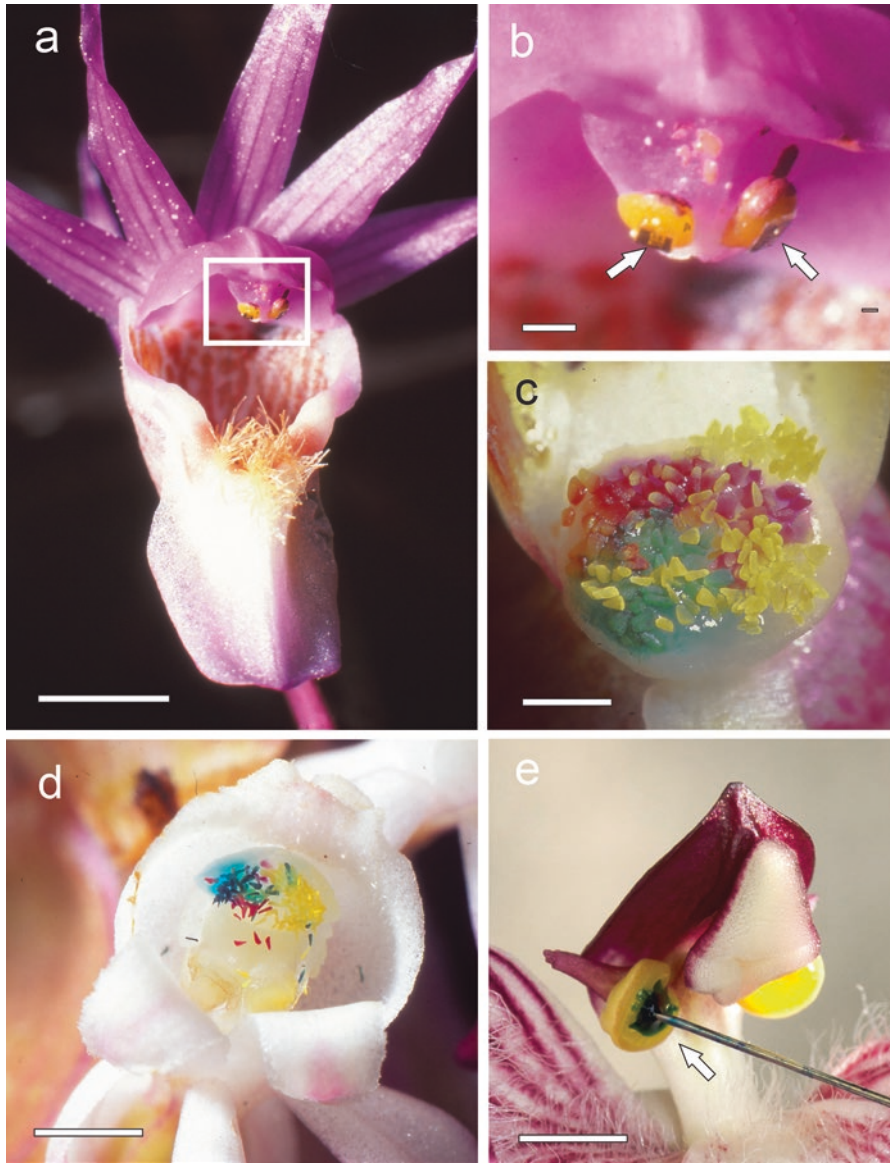


Fig. 1 Labelling of orchid pollen with microtags and colour stains. (**a**) Flower of the epidendroid orchid *Calypso bulbosa*. Scale = 5 mm. (**b**) Close-up of the rostellum of the flower illustrated in a panel showing uniquely numbered microtags attached to each pollinium. Scale = 1 mm. (**c**) Stigma of the orchidoid orchid *Disa cooperi* showing deposition of a combination of unstained yellow pollen massulae and pollen massulae stained with the histochemicals rhodamine pink and fast green. Scale = 1 mm. (**d**) Stigma of the orchidoid orchid *Satyrium longicauda* with unstained pollen massulae and pollen massulae stained with the histochemicals methylene blue, fast green and neutral red. Scale = 2 mm. (**e**) Injection of fast green stain onto a pollinium of *Cypripedium tibeticum*. Scale = 4 mm. Photographs by Steve Johnson (**a–d**) and Lawrence Harder (**e**)

orchids [25, 26]. This is to be expected, because in most orchids the pollinia attach to pollinators via a viscidium that connects to the pollinia via a stipe or caudicle. Careful staining of the pollinia thus does not interfere with the mechanism of attachment to

pollinators. Pollen staining could conceivably affect the internal cohesion of pollinia or the adhesion of pollen to stigmas, but experimental studies have shown that the dispersal properties (e.g. patterns of pollen carryover) of stained massulate pollinia are usually indistinguishable from those of unstained pollen [23, 27] (*see* **Note 1**). Similarly, microtags did not influence the likelihood of deposition of solid pollinaria of *Calypso bulbosa* in cage experiments [26].

3 Materials

3.1 Pollen Production and Transfer Efficiency

1. Clear sticky tape
2. Hand lens (20×) or dissecting microscope
3. Intact pollinaria (c. 20 per species)
4. Methylene blue (10%)
5. Microcentrifuge tubes
6. Centrifuge
7. Xylene
8. Ethanol (70%, 95%, 100%)

3.2 Pollen Staining

1. Histochemical stains diluted in water. Neutral red (1%), rhodamine pink (0.2%), fast green (1%), methylene blue (1%), orange G (10%), trypan red (2%), gentian violet (unknown concentration in a premixed medicinal preparation, Alpha Pharmaceuticals)
2. Hamilton microsyringe (5 or 10 μL) or Pipetman (0–50 μL) automatic pipette
3. Teepol soap
4. Hand lens or dissecting microscope
5. Latex gloves

3.3 Microtags

1. Microfilm (e.g. Kodak Imagelink HQ 361 Sp 615, thickness 0.06 mm) with sequences of unique three-character letter plus number combinations (e.g. A12, B36)
2. Dissecting microscope
3. A fine hair pencil
4. Self-adhesive glue or cyanoacrylate glue (e.g. Conloc Super 528, Gleitmo Technik AB, Sweden)
5. Chloroform
6. Protective paper strip
7. Hand lens (20×) or head-mounted magnifier (e.g. OptiVisor, USA)

4 Methods

4.1 Pollen Production, Removal and Deposition

Studies of pollen production in orchids have generally been based on counts of pollen aggregates (pollinia, massulae), although individual pollen grains have been counted in some cases (*see* below). Pollinia are extracted from fresh flowers by grasping the viscidium with forceps. To quantify the massulae in individual pollinia, we typically dab them repeatedly onto clear sticky tape (placed on a bright blue background with the sticky side facing up) until all massulae have been removed and count the adhering massulae under a dissecting microscope. For population estimates of mean massula production, this process is repeated for 10–30 pollinia. Fresh pollinia can be stored for several weeks at 5 °C.

Lukasiewicz [28] described an effective method for counting individual pollen grains. She placed individual pollinia in microcentrifuge tubes, which were left open for 24 h until the pollinia dried. To dissolve the sporopollenin covering hard pollinia and the elastoviscin interconnections between massulae and tetrads in sectile and mealy pollinia, she added 0.75 mL xylene to each tube. Exposure to xylene varied from 24 h for mealy or sticky pollinia, 48 h for sectile pollinia and 96 h for hard pollinia. This treatment dissociated pollinia into individual grains, except for species with mealy pollinia for which tetrads remained. After xylene treatment, samples were centrifuged at 2938 × *g* for 3 min and the supernatant removed. The precipitate was then washed in 1 mL of 100% ethanol for 15 min and recentrifuged and washed three times before being washed with 95% and then 70% ethanol. Samples can be stored in 70% ethanol until counting, preferably using an electronic particle analyser [29].

Pollen removal and deposition are ideally quantified in recently wilted flowers (at this stage, further removal and deposition cannot take place, and massulae on the stigma are not usually fully absorbed in the stigmatic mucilage). Typically, we collect a single flower from each of 20–200 plants in each population. For orchids with sectile pollinia, the number of massulae deposited on stigmas can be determined using a 20× hand lens or head-mounted magnifier. In older flowers, massulae that have been partially or fully absorbed in stigmatic mucilage are more easily visualized if the entire stigma has been dipped briefly in a 10% solution of methylene blue stain.

4.2 Pollen Staining

The objective of pollen staining is to trace stained pollen from anthers of a donor plant to stigmas on recipient plants. Typically, four different stain colours are used. In principle, more colours could be used, but in practice some similar colours can be difficult to distinguish in the field, particularly once pollen massulae hydrate and swell on the stigma, which tends to lighten the stain colour (*see* **Note 2**) (Fig. 1c, d). Thus, a red stain, for example, may appear

pink within a few hours of pollination. Peakall [22] used a single plant per stain colour during each trial, which makes identification of donor plants unambiguous but also makes it difficult to acquire a large sample of dispersal events, particularly if pollinators visit infrequently. To ensure sufficient sample sizes, we tend to use several donor plants for each stain colour in each trial, but ensure that donor plants with a particular stain colour are separated from each other by a distance greater than the typical distance of pollen dispersal so that each dispersal event can be traced to a likely donor plant. Plants stained with different colours can be located close together.

Stain is injected slowly into the anther sac of intact flowers until the pollinium is covered, being careful that as the stain is absorbed into the pollinium, excess stain does not leak onto the viscidium or other floral structures. Thus, the amount of stain injected into each anther depends on the size of the pollinium. Stain volumes injected into anthers in previous studies ranged from 0.5 μL in *Satyrium longicauda* [30] to 1–2 μL in *Prasophyllum fimbria* [22], *Anacamptis morio* [27], *Disa pulchra* [31] and *Cypripedium candidum* [24] and 4 μL in *Disa cooperi* [23]. Automatic pipettes or Hamilton syringes can be used to inject stain into the anthers. We have obtained the best results using Hamilton syringes with shortened needles (c. 20 mm in length) and blunt tips (Fig. 1e). The biggest problem with these syringes is that pollen massulae and dried stain tend to clog the needles, which can be very difficult to clean. Addition of a small amount of soap to the stain can help to break the surface tension when stain is injected into the anther sac and aids penetration. Latex gloves are worn when injecting stains, as it is almost impossible to avoid some spillage onto fingers that are used to steady the flower during injection or used to hold the vial when withdrawing stain. To avoid spilling vials of stain and minimize contact with fingers, we often place them in a small polystyrene rack. As Hamilton syringes are designed to penetrate gas chromatograph septa, vial openings could be covered with a rubber membrane, which is penetrated by the needle each time the syringe has to be refilled. Covering vials in this way would prevent accidental spillage if the vial tip over. In general, we do not recommend bagging donor plants before staining if the aim is to assess natural patterns of pollen dispersal. Bagging can increase the number of open flowers and nectar availability, both of which can increase self-pollination among flowers (geitonogamy) if individual pollinators visit more flowers per plant [32].

Some time (typically 1–3 days) after staining, stigmas of all open flowers on likely recipient flowers (including the donor for self-pollination) are inspected, and stained pollinia/massulae (Fig. 1c, d) are counted with a 20× hand lens (loupe) or head-mounted magnifier (e.g. Optivisor, USA). Lenses or head-mounted

magnifiers with a built-in light source are very helpful for counting massulae on stigmas that are hidden within the flower. If staining is used only to assess rates of self-pollination, then only the stigmas of stained plants need to be examined for the presence of stain [27, 31–33]. In most cases, researchers will also be interested in the pollen export and the spatial extent of pollen dispersal, which involves examining the stigmas of all potential recipient plants. This can be achieved in populations of up to about 200 plants but is not feasible in populations of thousands of plants.

For best success, preliminary information would be gathered concerning species-specific details of staining methods before implementing an experiment. For massulate species, it is essential to inject stain into several test anthers and, after the stain has dried (c. 30–60 min), to dissect the pollinia and ascertain that all massulae were effectively stained (*see* **Note 3**). Walsh and Michaels [24] left a drop of stain on each pollinium for 2 min and then blotted off the excess with paper towel, repeating the process if staining was considered incomplete. It is also useful to establish how quickly the colour of stained massulae fades after deposition on a stigma. In our experience, waiting more than 3 days after staining before checking stigmas for deposition of stained pollen risks false negatives (i.e. incorrectly concluding that no stained massulae were deposited).

4.3 Microtags

This method involves gluing minute (c. 0.5 mm diameter) unique tags on solid pollinia (Fig. 1a, b) [25]. Sequences of unique three-character letter plus number combinations (e.g. A12, B36) are photographed on microfilm (Kodak Imagelink HQ 361 Sp 615, thickness 0.06 mm) using a special microfilm camera (*see* **Note 4**). Microtags are produced by cutting the microfilm under a dissecting microscope. A fine hair pencil is used to apply minute drops of glue to the microtags. Nilsson et al. [25] dissolved glue from self-adhesive paper tape (Esselte) using chloroform and applied this to the microtags. Microtags were applied to pollinia on the tips of insect pins after first opening the anther sac. For storage, individual tags with glue were placed on the protective paper strip that comes with the self-adhesive paper tape. Alternatively, Alexandersson [26] applied quick setting cyanoacrylate glue ("super glue") to the pollinium using a pine needle and then placed the microtag onto the glue using an insect pin. Nilsson et al. [25] showed that microtags do not interfere with pollen viability.

Given the longevity of solid pollinaria, several days can elapse between removal and deposition, particularly in deceptive orchids, which are visited infrequently. Alexandersson [26] found two instances of single pollinia that were deposited 10 days after they were removed from separate plants. These were deposited on the same flower and caused fruit production, showing that the pollen was still viable.

4.4 Pollination Metrics

Measurements of the production, removal and deposition of unstained pollen and the dispersal of stained pollen enable calculation of various metrics describing pollen fates for orchids [23, 34]. For unstained plants, mean pollen removal failure is measured as the proportion of pollinaria that remain in the anthers of wilted flowers. In addition, pollen transfer efficiency (PTE), the proportion of removed pollen that reaches stigmas in a population, can be estimated as $d_L/r_L m$, where d_L is the average number of pollen units (grains, massulae or pollinia) deposited per flower lifespan, r_L is the average number of pollinaria removed per flower lifespan and m is the number of pollen units per pollinarium. Note that this measure of PTE assumes that pollen disperses only within the population (or that any export out of the population is matched exactly by import of pollen into the population) and includes both self- and cross-pollination.

Counts of stained pollen allow estimation of overall self-pollination and its components that occur among flowers (geitonogamy) (*see* **Note 5**) and within flowers (autogamy), as well as the fraction of cross-pollen import and export (*see* **Note 6**). Self-pollination is identified as pollen on stigmas that is stained the same colour as that applied to a plant's anthers. Thus, total self-pollination during a staining experiment, s_S, is the sum of self-stained pollen units on stigmas of all of a plant's open flowers. Flowers with self-stained pollen can fall into two categories. A total of f_g flowers retain all of their stained pollinia, so that the s_g stained pollen units on their stigmas must have come from other flowers on the same plant (geitonogamy). In contrast, the s_{ag} self-stained pollen units on the stigmas of the f_{ag} flowers from which stained pollinaria were removed could have been deposited either autogamously or geitonogamously. Given these counts, the number of pollen units deposited autogamously can be estimated as $s_a = (f_g s_{ag} - f_{ag} s_g)/f_g$, and the total number deposited geitonogamously is $g = s_g + s_{ag} - s_a$. If all pollinia on sample plants were stained, so that the u unstained pollen on stigmas represents cross-pollen, then the female self-pollination rate is $s/(s + u)$. In contrast, the male self-pollination rate is $s/(s + e)$, where e is the number of stained pollen units exported to other plants. The preceding estimates relate outcomes during a staining experiment, which will typically be briefer than the lives of orchid flowers. Given a count of the total number of removed stained pollinaria, r_S, the amount of removed pollen involved in self-pollination during floral lifespans can be estimated as $s_L = s_S r_L/r_S$.

5 Concluding Remarks

Pollen tracking provides a valuable means of addressing a range of basic and applied questions concerning orchid reproduction. As examples of evolutionary questions, the technique has been

used to show that addition of nectar to flowers of deceptive orchids increases the rate of pollinator-mediated self-pollination [24, 27, 31]. Such consequences of reward versus deceit for pollination quality and quantity have been examined with an extensive survey of pollination outcomes based on staining experiments [34]. Pollen tracking has also been used to confirm the deleterious effect of self-pollination on pollen export [23] and male interference with pollination efficiency [35]. The technique has also been used in a study showing that selection through male pollination success may favour different traits to those favoured through female success [36]. It has also revealed that mating can occur between widely separated individuals and that days, even weeks, can elapse between pollen removal and deposition [26]. Of particular interest, given the dozens of studies that have used pollinarium removal as a proxy for male pollination success, pollen tracking has shown that pollen export is highly stochastic and often linked only weakly with pollen removal [23]. Pollen tracking has also proved very useful for addressing questions about pollinator-mediated reproductive isolation in orchids [17]. With respect to applied questions in conservation biology, pollen tracking has shown that orchids in small populations can be vulnerable to increased rates of pollinator-mediated self-pollination [33].

These are just some of the many applications of a method that is remarkably inexpensive and simple. Indeed, future applications are limited only by the imagination of researchers who employ these methods.

6 Notes

1. Kropf and Renner [37] used dye powders to colour-code pollinia of *Dactylorhiza sambucina* and *Himantoglossum hircinum*. They removed pollinia from the anther without disturbing the viscidia and coated the surface of each pollinium with fluorescent dye powders (Day-Glo colour, USA) and then placed the pollinia back into the anther. High levels of geitonogamy were recorded, but it is not yet known whether dye powder accurately mimics the dispersal properties of pollen massulae.
2. Some stain colours can be difficult to distinguish. Peakall [22] noted this problem for methylene blue and brilliant green after exposure of pollen to rain. He also observed that rain diluted orange G on pollinia. Johnson et al. [23] noted that Bismarck brown was unsuitable for staining massulae of *Disa cooperi* because it was difficult to distinguish massulae stained with this histochemical from unstained massulae that had turned brown on the stigma.

3. Penetration of stain into pollinia is greatly aided by addition of Teepol soap into stain solutions at a dilution of 1:100. If stain does not penetrate all massulae, then it is possible to calculate the number of stained massulae removed from flowers as the product of the total number of massulae produced and the proportion of massulae stained.
4. Microfilm has largely been replaced by digital storage solutions and is no longer commonly used, and microfilm cameras are thus also now harder to obtain. However, many companies continue to offer microfilm services.
5. Comparison of the amount of pollen deposited on emasculated versus non-emasculated inflorescences is an alternative method for assessing overall rates of self-pollination [38, 39]. This method is not recommended when pollinators seek pollen as a reward or use its presence as a signal of nectar availability and may therefore be discouraged from visiting emasculated flowers. However, orchid pollen is very rarely sought as a reward by pollinators due to its packaging in pollinia [11]. Emasculation of single flowers on an inflorescence can provide additional insights into the contribution of within-flower self-pollination to the overall rate of self-pollination [4, 39]. Emasculation has rarely been used to quantify self-pollination in orchids. A study comparing emasculated and intact inflorescences of *Platanthera bifolia* found that geitonogamy accounted for 23–38% of pollen deposited on stigmas in a small population, whereas geitonogamy was not detectable in a larger population [40].
6. The spatial scale of pollen dispersal can be expressed either as a histogram of actual distances between source and recipient plants [23, 25] or as a histogram of the number of plants skipped between source and recipient. Both measures give useful information, but the number of plants skipped between source and recipient is often a more useful indication of the behaviour of pollinators. Pollinators visiting deceptive orchids, for example, often skip several plants between landings, whereas pollinators feeding on rewarding orchids often move between neighbouring plants [34]. These differences in behaviour have implications for the patterns of pollen flow [34].

References

1. Barrett SCH, Harder LD (2017) The ecology of mating and its evolutionary consequences in seed plants. Ann Rev Ecol Evol Syst 48:135–157
2. Willson MF (1979) Sexual selection in plants. Amer Nat 113:777–790
3. Aizen MA, Harder LD (2007) Expanding the limits of the pollen-limitation concept: effects of pollen quantity and quality. Ecology 88:271–281
4. Eckert CG (2000) Contributions of autogamy and geitonogamy to self-fertilization in a mass-flowering, clonal plant. Ecology 81:532–542

5. Barrett SCH (2002) Sexual interference of the floral kind. Heredity 88:154–159
6. Campbell DR (1989) Inflorescence size: test of the male function hypothesis. Amer J Bot 76:730–738
7. Snow AA, Lewis PO (1993) Reproductive traits and male fertility in plants: empirical approaches. Annu Rev Ecol Syst 24:331–351
8. Waser NM, Price MV (1982) A comparison of pollen and fluorescent dye carry-over by natural pollinators of *Ipomopsis aggregata* (Polemoniaceae). Ecology 63:1168–1172
9. Thomson JD, Thomson BA (1989) Dispersal of *Erythronium grandiflorum* pollen by bumblebees: implications for gene flow and reproductive success. Evolution 43:657–661
10. Massinga PH, Johnson SD, Harder LD (2005) Heteromorphic incompatibility and efficiency of pollination in two distylous *Pentanisia* species (Rubiaceae). Ann Bot 95:389–399
11. Harder LD, Johnson SD (2008) Function and evolution of aggregated pollen in angiosperms. Int J Plant Sci 169:59–78
12. Bernasconi G (2004) Seed paternity in flowering plants: an evolutionary perspective. Perspect Plant Ecol Evol Syst 6:149–158
13. Isagi Y, Suyama Y (2011) Single-pollen genotyping. In. Springer, Tokyo
14. Harder LD (2000) Pollen dispersal and the floral diversity of monocotyledons. In: Wilson KL, Morrison DW (eds) Monocots: systematics and evolution. CSIRO Publishing, Melbourne, pp 243–257
15. Harder LD, Routley MB (2006) Pollen and ovule fates and reproductive performance by flowering plants. In: Harder LD, Barrett SCH (eds) Ecology and evolution of flowers. Oxford University Press, Oxford, pp 61–80
16. Harder LD, Wilson WG (1998) A clarification of pollen discounting and its joint effects with inbreeding depression on mating system evolution. Amer Nat 152:684–695
17. Xu SQ, Schluter PM, Scopece G, Breitkopf H, Gross K, Cozzolino S, Schiestl FP (2011) Floral isolation is the main reproductive barrier among closely related sexually deceptive orchids. Evolution 65:2606–2620
18. Whitehead MR, Linde CC, Peakall R (2015) Pollination by sexual deception promotes outcrossing and mate diversity in self-compatible clonal orchids. J Evol Biol 8:1526–1541
19. Linhart YB (1973) Ecological and behavioral determinants of pollen dispersal in hummingbird-pollinated *Heliconia*. Amer Nat 107:511–523
20. Thomson JD, Price MV, Waser NM, Stratton DA (1986) Comparative studies of pollen and fluorescent dye transport by bumble bees visiting *Erythronium grandiflorum*. Oecologia 69:561–566
21. Johnson SD, Edwards TJ (2000) The structure and function of orchid pollinia. Plant Syst Evol 222:243–269
22. Peakall R (1989) A new technique for monitoring pollen flow in orchids. Oecologia 79:361–365
23. Johnson SD, Neal PR, Harder LD (2005) Pollen fates and the limits on male reproductive success in an orchid population. Biol J Linn Soc 86:175–190
24. Walsh RP, Michaels HJ (2017) When it pays to cheat: examining how generalized food deception increases male and female fitness in a terrestrial orchid. PLoS One 12:e0171286
25. Nilsson LA, Rabakonandrianina E, Pettersson B (1992) Exact tracking of pollen transfer and mating in plants. Nature 360:666–668
26. Alexandersson R (1999) Reproductive ecology of the deceptive orchid *Calypso bulbosa*. PhD Dissertation, Umea University
27. Johnson SD, Peter CI, Agren J (2004) The effects of nectar addition on pollen removal and geitonogamy in the non-rewarding orchid *Anacamptis morio*. Proc Royal Soc London Series B-Biol Sci 271:803–809
28. Lukasiewicz MJ (1999) Maternal investment, pollination efficiency and pol1en:ovule ratios in Alberta orchids. MSc thesis. University of Calgary
29. Harder LD (1990) Pollen removal by bumble bees and its implications for pollen dispersal. Ecology 71:1110–1125
30. Jersáková J, Johnson SD (2007) Protandry promotes male pollination success in a moth-pollinated orchid. Funct Ecol 21:496–504
31. Jersáková J, Johnson SD (2006) Lack of floral nectar reduces self-pollination in a fly-pollinated orchid. Oecologia 147:60–68
32. Harder LD, Johnson SD (2005) Adaptive plasticity of floral display size in animal-pollinated plants. Proc Royal Soc B-Biol Sci 272:2651–2657
33. Johnson SD, Torninger E, Agren J (2009) Relationships between population size and pollen fates in a moth-pollinated orchid. Biol Lett 5:282–285
34. Hobbhahn N, Johnson SD, Harder LD (2017) The mating consequences of rewarding vs. deceptive pollination systems: is there a quantity–quality trade-off? Ecol Monogr 87:91–104
35. Duffy KJ, Johnson SD (2014) Male interference with pollination efficiency in a hermaphroditic orchid. J Evol Biol 27:1751–1756

36. Ellis AG, Johnson SD (2010) Gender differences in the effects of floral spur length manipulation on fitness in a hermaphrodite orchid. Int J Plant Sci 171:1010–1019
37. Kropf M, Renner SS (2008) Pollinator-mediated selfing in two deceptive orchids and a review of pollinium tracking studies addressing geitonogamy. Oecologia 155:497–508
38. Eckert C, Samis K, Dart S (2006) Reproductive assurance and the evolution of uniparental reproduction in flowering plants. In: Harder LD, Barrett SCH (eds) The ecology and evolution of flowers. Oxford University Press, Oxford, pp 183–203
39. Lloyd DG (1992) Self- and cross-fertilization in plants. II. The selection of self-fertilization. Int J Plant Sci 153:370–380
40. Maad J, Reinhammar LG (2004) Incidence of geitonogamy differs between two populations in the hawkmoth-pollinated *Platanthera bifolia* (Orchidaceae). Can J Bot 82: 1586–1593

Chapter 12

Cut-Column Pollination Method to Overcome Pollination Barrier in *Phalaenopsis*

Hua-Ting Chuang, Kuang-Liang Huang, and Shan-Te Hsu

Abstract

Hybridization allows the transfer of desirable traits from one cultivar to another with the possibility of selecting superior cultivars from hybrid progenies. However, in *Phalaenopsis*, hybridization barriers are observed frequently in breeding programs. The possible causes of hybridization failure of some *Phalaenopsis* cultivars may include chromosome abnormalities, e.g., aneuploidy and triploidy, and the failure of recognition between the pollen and pistillate parents. To overcome pollination barrier in *Phalaenopsis* cultivars due to the possible failure of recognition between parents, we developed the cut-column method to improve the fertility of some difficult-to-hybridize cultivars, e.g., *Phalaenopsis* Taipei Gold 'STM' and *Phalaenopsis* Sunrise Goldmour 'KHM637'. Successful capsule setting (25–77.8%) with fertile seed formation was achieved from four combinations using *P.* Taipei Gold 'STM' and *P.* Sunrise Goldmour 'KHM637' as the pistillate parents. Hybrid seedlings were obtained from asymbiotic germination cultures. The cut-column pollination method could be applicable to other difficult-to-hybridize *Phalaenopsis* cultivars.

Key words *Phalaenopsis* cultivars, Breeding, Compatibility, Fertility, Hybridization, Cross-pollination, Polyploidy, Pollen-stigma interactions

1 Introduction

The genus *Phalaenopsis* belongs to the orchid family which comprises approximately 60 species native to tropical and subtropical Asia and can be grouped into five subgenera, i.e., *Proboscidioides*, *Aphyllae*, *Parishianae*, *Polychilos*, and *Phalaenopsis* [1]. *Phalaenopsis* has a high horticultural value as potted plants or cut flowers due to its graceful inflorescences and long-lasting flowers. It is also one of the most important floricultural plants in Taiwan. In 2016, its estimated gross export value has reached $128.7 million USD in Taiwan [2]. In the USA, the wholesale value of orchids was estimated at $288 million USD (USDA, 2016), of which more than 80% are *Phalaenopsis*.

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_12,

The ornamental plant market is always looking for new and novel cultivars. Over the past few decades, tens of thousands of *Phalaenopsis* cultivars were bred and selected in Taiwan. Currently, more than 31,000 *Phalaenopsis* hybrids have been registered in RHS (*see* the database of International Orchid Register, Royal Horticultural Society, 2013). For breeding new *Phalaenopsis* cultivars, hybridization allows the transfer of traits of interest from one cultivar to another with the possibility of selecting superior cultivars from hybrid progenies. Due to the complex genetic background in modern *Phalaenopsis* hybrids, failures in producing capsules with fertile seeds are frequently encountered in intergeneric, interspecific, and intraspecific hybrids of *Phalaenopsis*. Incompatibility barriers in interspecific hybridizations occur commonly and could be classified into two types, i.e., pre-zygotic (before fertilization) and post-zygotic (after fertilization) barriers [3]. Moreover, the different degrees of fertility in hybrids may be due to irregular chromosome pairing during meiosis that resulted in the formation of abnormal pollen [4–6]. Besides possible chromosome abnormalities, it is also not clear which type of incompatibility and incongruity may result in the formation of hybridization barrier in *Phalaenopsis*, and whether these barriers are caused by genetic or external factors is not known. Until now, basic information and relevant studies are lacking.

In our previous reports, we investigated the possible causes in hybridization failure in some commercial *Phalaenopsis* cultivars [7, 8]. One of the reasons of failure in producing fertile seedlings may be caused by chromosome abnormalities, e.g., aneuploidy and triploidy. In addition, the hybridization barrier may be caused by the failure of recognition between the pollen and pistillate parents. In most *Phalaenopsis*, the stigma normally closes, and the column becomes swollen after a successful pollination event [9]. From the investigations of hybridization barriers in numerous *Phalaenopsis* cultivars, we observed a phenomenon that some difficult-to-hybridize cultivars have problem with stigma closure after pollination. To overcome this hybridization barrier, we developed a new method using "cut-column pollination" to improve the fertility in a few difficult-to-hybridize *Phalaenopsis* cultivars with the problem of stigma closure after pollination [10, 11]. Here, we describe the possible hybridization barriers in some *Phalaenopsis* cultivars and detail the procedures of the cut-column pollination method to overcome hybridization barriers in *Phalaenopsis*.

2 Lessons Learned from Examining Hybridization Barriers

2.1 Approaches and Methodologies

The procedures for evaluating hybridization barriers in selected *Phalaenopsis* cultivars are illustrated in Fig. 1. Mature plants of selected *Phalaenopsis* cultivars were grown and maintained in a greenhouse at the Horticultural Technology Center, National

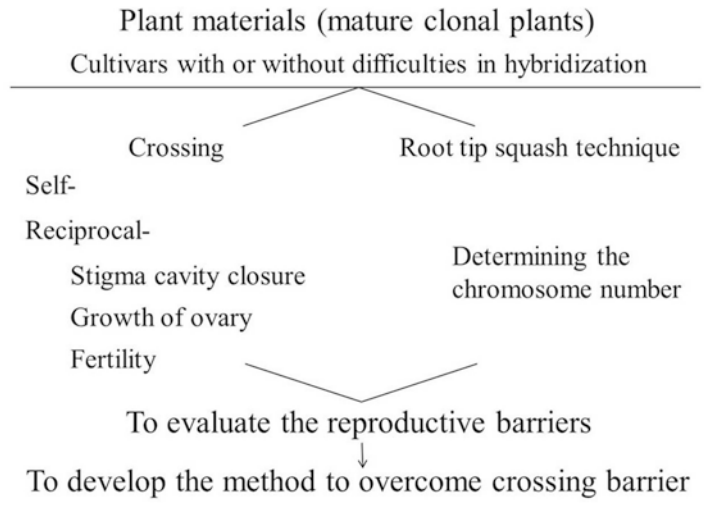


Fig. 1 The flow chart of the cut-column pollination method

Chiayi University, Chiayi, Taiwan, R.O.C. As the first step, we surveyed whether the hybridization barrier occurs among the selected cultivars. The selected *Phalaenopsis* cultivars were self- or cross-pollinated to check their fertilities.

To understand if chromosome abnormalities are the causes of low fertility, we examined the somatic chromosome of the selected cultivars by the root tip squash technique [12]. Previous studies have shown that all *Phalaenopsis* species have the same chromosome number of $2n = 2x = 38$ [12–15]. However, chromosome sets may vary in existing wild or cultivated *Phalaenopsis* species due to artificial hybridization and natural $2n$ gamete formation. The differences in chromosome number may lead to failure of chromosome pairing, which is commonly observed in other crops [13, 16–18].

After pollination, key structural changes were examined regularly, i.e., the occurrence of stigma cavity closure and in vivo pollen germination and tube elongation [19]. The changes in stigma cavity before and after pollination can be studied by careful observation using a stereomicroscope. Pollen germination and tube growth in different crossing combinations can be monitored using the aniline blue staining method [20] (*see* **Note 1**). Finally, seed formation and viability are evaluated by asymbiotic seed germination as detailed by Lin et al. [21] (*see* **Note 2**).

From these pollination experiments, the selected cultivars can be classified into two groups: the difficult-to-hybridize group, including *P.* Taipei Gold 'STM' and *P.* Sunrise Goldmour 'KHM637', *P.* Ever Spring Prince '#1', *P.* Queen Beer 'KHM 159', *P.* Taida Salu 'KHM 101', and *P.* Brother Girl 'Brother', and

the normal-to-hybridize group, including *P.* Brother Irene Irene 'Feng Fong', *P.* Tai Lin Red Angel 'V31', *P.* Sogo Yukidian 'V3', *P. bellina* (previously known as *P. violacea*), and *P. fasciata* (*see* Table 1). When performing the pollination experiments, it was observed that some difficult-to-hybridize cultivars have the problem of stigma closure. Conversely, the stigma of normal-to-hybridize cultivars closed soon after pollination (Fig. 2).

2.2 The Case Studies Using Different Phalaenopsis Cultivars

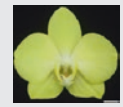
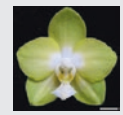
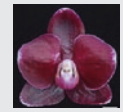
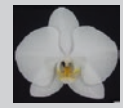
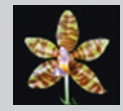
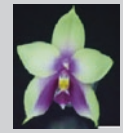
Cytological analyses indicate that two native species, i.e., *P. bellina* and *P. fasciata*, are diploid ($2n = 2x = 38$), five cultivars are triploid ($2n = 3x = 57$), and four cultivars are tetraploid ($2n = 4x = 76$) (Table 1). Among the selected cultivars, the diploid species and tetraploid cultivars are fertile, except for *P.* Sunrise Goldmour 'KHM637', while all triploid cultivars are difficult-to-hybridize. These results indicate that the low fertility of triploid cultivars may be due to the problems of chromosome pairing during meiosis [7, 8, 10, 11]. It is noteworthy that *P.* Sunrise Goldmour 'KHM637', a tetraploid cultivar, should be fertile without the problem of possessing aneuploid gametes.

In *Phalaenopsis*, the stigma usually closes at 48 h after pollination [9, 22, 23]. We found that the absence of stigma closure usually occurred in some difficult-to-hybridize cultivars. Inside the style and ovary, pollen germination and the growth of pollen tube could be observed using aniline blue staining [20]. The absence of pollen tube elongation indicates the occurrence of hybridization barrier. Since the failure of capsule setting is usually observed by the senescence of ovary in one to two weeks after pollination, the observation of stigma closure and pollen tube growth may provide a quick assessment in the presence of a hybridization barrier within one or two days. Therefore, we performed a series of pollination experiments using the normal-to-hybridize and difficult-to-hybridize cultivars as the pollen or pistillate parents to investigate stigma closure and pollen tube elongation after pollination.

In self-pollination experiments, as expected, the five normal-to-hybridize cultivars showed stigma closure and pollen tube elongation and finally produced many seedlings (data not shown). On the contrary, the six difficult-to-hybridize cultivars failed to produce any seedlings [7, 8, 10, 11]. Among the difficult-to-hybridize cultivars, it is interesting to note that the occurrence of stigma closure and pollen tube elongation could be observed in *P.* Taida Salu 'KHM 101' and *P.* Brother Girl 'Brother' after self-pollinations, but those phenomena could not be observed in *P.* Taipei Gold 'STM', *P.* Sunrise Goldmour 'KHM637', *P.* Ever Spring Prince '#1', and *P.* Queen Beer 'KHM 159' (Table 2).

In the cross-pollination experiments, when the six difficult-to-hybridize cultivars were cross-pollinated reciprocally with the normal-to-hybridize cultivars, all capsules failed to develop when

Table 1
The performance of crossing, chromosome number, sources, and flower morphology of *Phalaenopsis* cultivars/species concerned in this chapter

Description	Cultivar/species	Chromosome number	Reference	Sources	Flower morphology[a]
Difficult-to-hybridize	*P.* Taipei Gold 'STM'	$2n = 3x = 57$	[7]	I-Hsin Biotechnology Inc.	
	P. Sunrise Goldmour 'KHM637'	$2n = 4x = 76$	[7]	I-Hsin Biotechnology Inc.	
	P. Ever Spring Prince '#1'	$2n = 3x = 57$	[8]	Taiwan Sugar Corporation	
	P. Queen Beer 'KHM 159'	$2n = 3x = 57$	[8]	I-Hsin Biotechnology Inc.	
	P. Taida Salu 'KHM 101'	$2n = 3x = 57$	[8]	I-Hsin Biotechnology Inc.	
	P. Brother Girl 'Brother'	$2n = 3x = 57$	[8]	Taida Horticultural Co., Ltd.	
Normal-to-hybridize	*P.* Brother Irene Irene 'Feng Fong'	$2n = 4x = 76$	[8]	Star Orchids	
	P. Sogo Yukidian 'V3'	$2n = 4x = 76$	[8]	Taiwan Sugar Corporation	
	P. Tai Lin Red Angel 'V31'	$2n = 4x = 76$	[8]	Star Orchids	
	P. fasciata	$2n = 2x = 38$	[14]	National Chiayi University	
	P. bellina	$2n = 2x = 38$	[13]	National Chiayi University	

[a]Bar = 1 cm

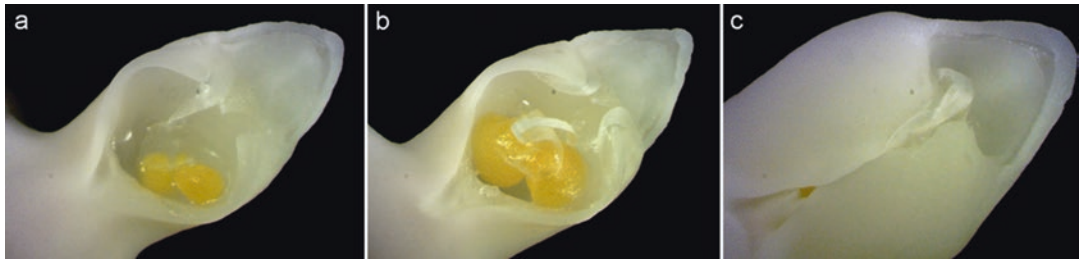


Fig. 2 The stigma closure of ***Phalaenopsis*** Taipei Gold 'STM' and ***P.*** Sogo Yukidian 'V3', one day after pollination. Stigma closure is only observed in ***P.*** Yukidian 'V3' when used as the pistillate parent. (**a**) The self-pollination of ***P.*** Taipei Gold 'STM' (no closure); (**b**) the cross-pollination of ***P.*** Taipei Gold 'STM' (pod parent) and ***P.*** Sogo Yukidian 'V3' (pollen parent) (no closure); (**c**) the cross-pollination of ***P.*** Sogo Yukidian 'V3' (pod parent) and ***P.*** Taipei Gold 'STM' (pollen parent)

Table 2
Stigma closure, pollen tube elongation, and fertility in self-pollination of six difficult-to-hybridize *Phalaenopsis* cultivars

Cultivar	2*n*	Stigma closure	Pollen tube elongation	Offspring
P. Taipei Gold 'STM'	3*x*	No	No	No
P. Sunrise Goldmour 'KHM637'	4*x*	No	No	No
P. Ever Spring Prince '#1'	3*x*	No	No	No
P. Queen Beer 'KHM 159'	3*x*	No	No	No
P. Taida Salu 'KHM 101'	3*x*	Yes	Yes	No
P. Brother Girl 'Brother'	3*x*	Yes	Yes	No

the difficult-to-hybridize cultivars were used as pollen parents (Table 3) [8]. Reciprocally, when the normal-to-hybridize cultivars were used as pollen parents, stigma closure and pollen tube elongation could be observed in *P.* Taida Salu 'KHM 101' and *P.* Ever Spring Prince '#1'; a small number of seedlings could be obtained (Table 4). These results suggest that in addition to the aneuploid gametes, additional unknown factor(s) can also serve as hybridization barrier. From the observation, it appears that if the stigma can close and with successful pollen tube growth, it is still possible to obtain hybrid seedlings from some difficult-to-hybridize cultivars.

2.3 The Cut-Column Pollination Method to Improve the Fertility

In the cross-pollination experiments, it is possible to obtain a few hybrid seedlings from some difficult-to-hybridize *Phalaenopsis* cultivars, i.e., *P.* Taida Salu 'KHM 101' and *P.* Ever Spring Prince '#1', when used as pistillate parents. When cross-pollinated with

Table 3
Stigma closure, pollen tube elongation, and fertility in the cross-pollination of the normal-to-hybridize cultivar, e.g., *P.* Sogo Yukidian 'V3' (as the pistillate parent) with six difficult-to-hybridize *Phalaenopsis* cultivars (as the pollen parents)

Cultivar	2*n*	Stigma closure	Pollen tube elongation	Offspring
P. Taipei Gold 'STM'	3*x*	Yes	Yes	No
P. Sunrise Goldmour 'KHM637'	4*x*	Yes	Yes	No
P. Ever Spring Prince '#1'	3*x*	No	No	No
P. Queen Beer 'KHM 159'	3*x*	No	No	No
P. Taida Salu 'KHM 101'	3*x*	Yes	Yes	No
P. Brother Girl 'Brother'	3*x*	Yes	Yes	No

Table 4
Stigma closure, pollen tube elongation, and fertility in the cross-pollination of six difficult-to-hybridize *Phalaenopsis* cultivars (as the pistillate parents) with the normal-to-hybridize cultivar, e.g., *P.* Sogo Yukidian 'V3' (as the pollen parent)

Cultivar	2*n*	Stigma closure	Pollen tube elongation	Offspring
P. Taipei Gold 'STM'	3*x*	No	No	No
P. Sunrise Goldmour 'KHM637'	4*x*	No	No	No
P. Ever Spring Prince '#1'	3*x*	Yes	Yes	Yes/no
P. Queen Beer 'KHM 159'	3*x*	Yes	Yes	No
P. Taida Salu 'KHM 101'	3*x*	Yes	Yes	Yes
P. Brother Girl 'Brother'	3*x*	Yes	Yes	No

the normal-to-hybridize cultivars, their stigmas closed after 48 h. We further observed successful pollen germination and pollen tube growth in the style of *P.* Taida Salu 'KHM 101' and *P.* Ever Spring Prince '#1' by aniline blue staining (Table 4).

Different techniques such as pollen heating, electrical stimulation, chemical treatment, bud pollination, mentor pollination, style grafting, and cut-style and placental pollination have been developed to overcome pre-fertilization barriers of various plants [24–33]. If the stigma of difficult-to-hybridize *Phalaenopsis* cultivars contains unknown chemicals inhibiting pollen germination or the growth of pollen tube similar to self-incompatible individuals,

Table 5
Effects of cut-column method on capsule setting, fertility, and seedling production in the difficult-to-hybridize cultivars, *Phalaenopsis* Taipei Gold 'STM' and *P.* Sunrise Goldmour 'KHM637', and the pollen parents were selected from the normal-to-hybridize cultivars, e.g., *P.* Sogo Yukidian 'V3', *P. fasciata*, and *P. bellina*

Pollination combination (♀ × ♂)	No. of crossing	Capsule setting (%)	Capsule with fertile seeds (%)	No. of seedlings
P. Taipei Gold 'STM' ×*P.* Sogo Yukidian 'V3'	9	88.9	33.3	51
P. Taipei Gold 'STM' ×*P. fasciata*	4	50.0	25.0	80
P. Sunrise Goldmour 'KHM637' ×*P.* Sogo Yukidian 'V3'	9	77.8	77.8	83
P. Sunrise Goldmour 'KHM637' ×*P. bellina*	4	75.0	75.0	149

The capsule setting was recorded at 60 days after pollination

it may be possible to improve the fertility by removing the column to bypass the barrier.

Thus, we modified the cut-style pollination procedure used in lily [24, 30] and developed the "cut-column pollination method" for overcoming breeding barriers in *Phalaenopsis* [10, 11]. This method has been proven to overcome breeding barriers in *P.* Taipei Gold 'STM' and *P.* Sunrise Goldmour 'KHM637', known to have the problem of stigma closure after pollination (Table 5). In these two difficult-to-hybridize *Phalaenopsis* cultivars, the unknown pre-fertilization barriers in the upper part of the column could be circumvented by the cut-column pollination method. A detailed protocol is described below.

3 Materials

3.1 Plant Material

Phalaenopsis cultivars: Grow and maintain plants in 12–15 cm transparent plastic pots filled with sphagnum moss in the greenhouse equipped with the pad-and-fan cooling system with 32 °C days and 27 °C nights. Fertilize the plants regularly with a complete fertilizer (20–20–20). In order to hasten the *Phalaenopsis* to bloom, place mature plants in a cool room at 25 °C days and 20 °C nights to trigger flower initiation.

3.2 Major Equipment

An epifluorescence microscope, a stereomicroscope, a laminar-flow cabinet, growth room facilities.

3.3 General Laboratory Equipment and Supplies

Forceps, blades, slides and cover glasses, alcohol lamp, paraffin film (BRAND® PARAFILM® M sealing film), and tissue culture supplies for in vitro seed germination.

3.4 Media and Solutions

1. The seed germination medium: 1/4 strength Murashige and Skoog (MS) medium salt [33], supplemented with 0.5 mg/L niacin, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HCl, 20 mg/L glycine, 100 mg/L myo-inositol, 170 mg/L NaH_2PO_4, 1 g/L activated charcoal, 1 g/L peptone, 6 g/L potato powder, 20 g/L sucrose, and 8 g/L agar. Adjust the pH to 5.2 before autoclaving at 121 °C for 15 min.
2. Aniline blue dye for callose staining: 0.1% aniline blue in 50 mM KPO_4 buffer (4.17 mL 1 M K_2HPO_4 + 0.83 mL 1 M KH_2PO_4 + 995 mL H_2O, pH 7.5).
3. Metaphase arresting agent: 2 mM 8-hydroxyquinoline solution.
4. 70% (v/v) ethanol.
5. 1 N HCl.
6. 8 M NaOH.
7. Glycerol.
8. 10% Giemsa staining solution.
9. Farmer's fixative (ethanol/acetic acid = 3:1, v:v).
10. FAA solution (formalin/acetic acid/70% ethanol = 1:1:18, v:v:v).
11. Commercial bleach (5% NaOCl) for surface sterilization.

4 Methods

4.1 Chromosome Staining Procedure

1. Collect actively growing root tips from the plants in the greenhouse near noon time, and subsequently treat with 2 mM 8-hydroxyquinoline solution at 20 °C for 4 h.
2. Wash the root tips with distilled water twice for 10 min, and fix them in Farmer's fixative at 4 °C for 24 h.
3. Wash the root tips with distilled water twice for 10 min, and transfer the root tips to an Eppendorf tube containing 1 N HCl solution at 60 °C for 20–25 min.
4. Wash the root tips with distilled water twice for 10 min, and carefully excised the meristem cells onto a clean slide, and immediately, apply a drop (20 μL) of Farmer's fixative on top of the meristem cells.

5. Squash the meristem cells in the Farmer's fixative on the slide.
6. Dry the slide by heating with an alcohol lamp.
7. Stain the slide with 10% Giemsa solution for 20 min, and air-dry the slide preparation.
8. Observe and count the chromosome number under a Zeiss microscope (Zeiss Axioskop 2).

4.2 Staining of Pollen Tubes

1. Excise columns from the pollinated flowers of each crossing at 7 days after pollination (DAP).
2. Fix the excised columns in the FAA solution immediately at room temperature for 24 h.
3. Soften the excised columns by submerging in the 8 N NaOH solution at 60 °C for 1.5 h.
4. Wash the samples three times in distilled water, and stain with a 0.1% aniline blue solution [20] at 4 °C for 24 h.
5. Transfer a sample onto a slide, and add a drop of glycerin, apply a cover glass, and squash the sample gently.
6. Examine the sample under an epifluorescence microscope (Zeiss Axioskop2, EM: BP 365–390 nm). A pollen grain showing tube length exceeding twice its own diameter is considered to have germinated.

4.3 Cut-Column Pollination Method

1. Select blooming plants as the pistillate or pollen parents at the time of anthesis (*see* **Note 3**), and take them into a clean room for further manipulation (*see* **Note 4**).
2. Gently excise and remove the sepals and petals at the receptacle of the pistillate parent flower, with the column and ovary remain attached to the inflorescence. Remove other unused flowers, e.g., old flowers or buds from the inflorescence if necessary.
3. Cut away the upper part of column using a new and sharp blade (*see* **Note 5**), revealing the lower part of column with the stigma cavity (*see* procedures in Fig. 3).
4. Remove the pollinia from the pollen parent, and place it onto the stigma cavity of the pistillate parent.
5. Gently seal the stigma cavity with a paraffin film, and return the plant to its original culture environment (*see* **Note 6**).
6. The ovary will turn green and become swollen if pollination is successful (Fig. 4a, b).
7. Collect developing capsules at 60 DAP and sow the seeds on the germination medium (Fig. 4c) (*see* **Note 2**).

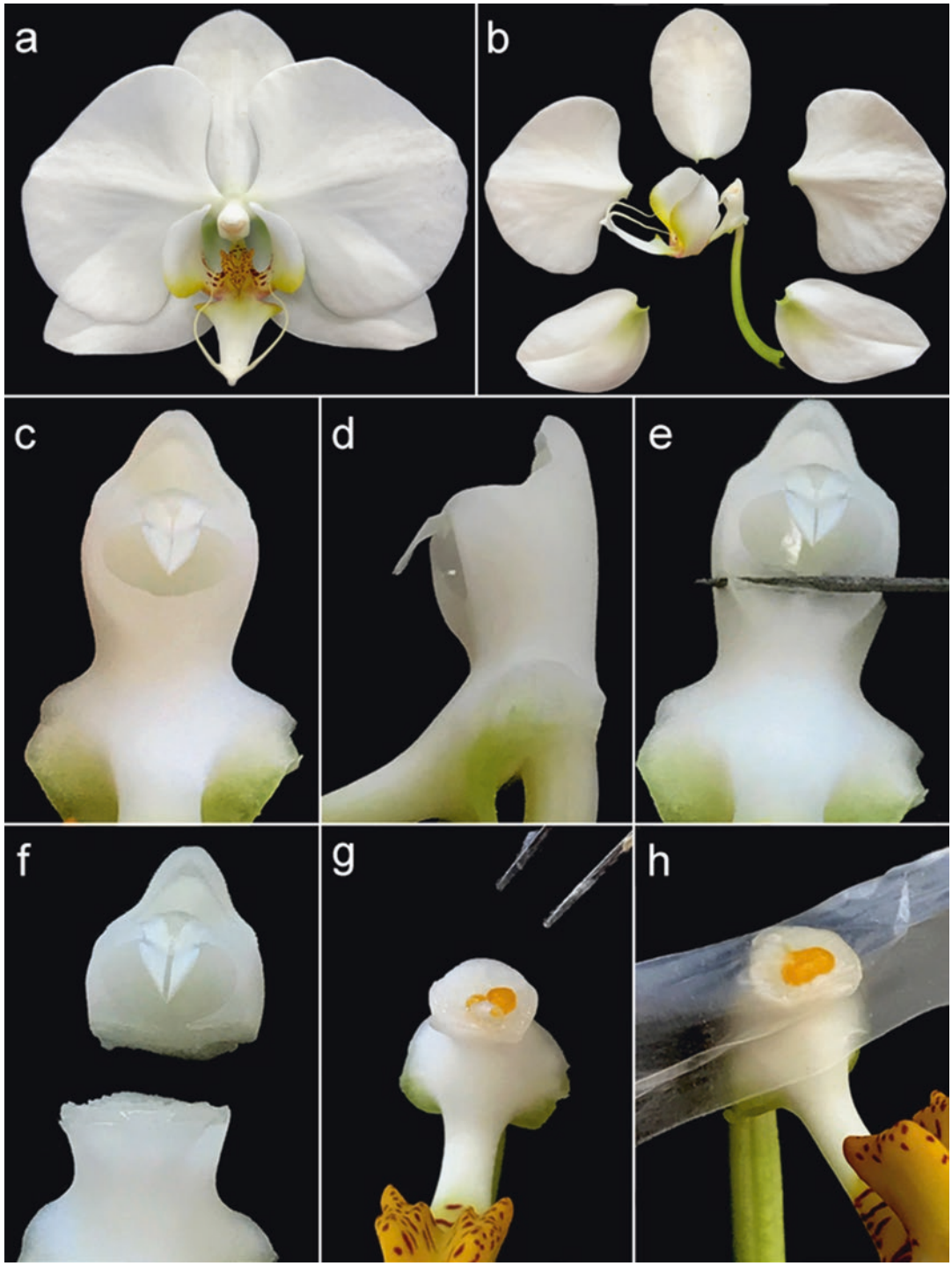


Fig. 3 Schematic illustration of the cut-column pollination method used to overcome breeding barriers. (**a**) An intact flower; (**b**) flower dissected into sepals, petals, and lip with column attached; (**c**) the adaxial view of column; (**d**) the side view of column; (**e**) cut the column with sharp and clean blade; (**f**) the upper part of the column is removed, and the lower part of the column showing the opening of the stigma cavity; (**g**) placing the pollinia from the pollen parent onto the stigma cavity of the pod parent; (**h**) the stigma cavity is sealed immediately with a paraffin film

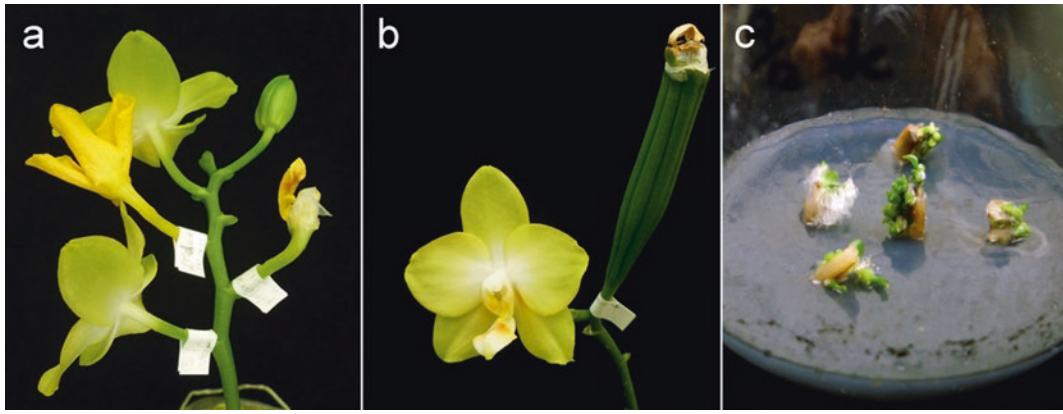


Fig. 4 Fruit setting, development, and seed germination. (**a**) A capsule develops following the cut-column pollination method. The flower wilts at 16 days after pollination without cut column; (**b**) capsules are successfully obtained following the cut-column pollination method; (**c**) seeds germinate under aseptic culture. Scale bars = 1 cm

4.4 Seed Germination Procedure

1. Harvest the capsules at 60 DAP.
2. Surface sterilize the capsules in the 2% NaOCl solution with one drop of Tween 20 for 20 min, and subsequently wash the capsules three times with sterile water.
3. Cut and open the capsules longitudinally on a sterile Petri dish in the laminar-flow cabinet. Remove gently the immature seeds using forceps, and sow them onto the germination medium.
4. Place the cultures under light conditions (photon flux density of 10 μmol m^{-2} s^{-1}, PAR) with a 12-h light/dark photoperiod at 25 ± 2 °C.

5 Comments

Our studies demonstrate the possible hybridization barriers occurring in *Phalaenopsis* cultivars. After pollination, the stigma closure prevents the access of unsuitable pollen and ensures the protection of ovules in *Phalaenopsis*. The wet stigma surface of *Phalaenopsis* flower provides guidance and nutrients, supporting the germination and growth of pollen tubes. The lack of stigma receptivity may result in the failures of stigma closure and pollen tube growth in some difficult-to-hybridize cultivars, e.g., the yellow cultivars (*P.* Taipei Gold 'STM' and *P.* Sunrise Goldmour 'KHM637'). The results suggest a reproductive barrier on the pre-zygotic level, and the cross-compatibility among *Phalaenopsis* cultivars may also be

Fig. 5 Flowers from hybrid plants (**a–d**) derived from crossing using the cut-column method. The pistillate parent is ***Phalaenopsis*** Sunrise Goldmour 'KHM637' with ***P. bellina*** as the pollen parent. Scale bars = 1 cm

influenced by the genetic background of parents. The cut-column pollination method bypasses the pollen-stigma recognition and thus improves the fertility of difficult-to-hybridize cultivars. Successes are illustrated in Fig. 5 (the cross using *P.* Sunrise Goldmour 'KHM637' as the pistillate parent and *P. bellina* as the pollen parent) and Fig. 6 (the cross using *P.* Taipei Gold 'KHM637' as the pistillate parent and *P. fasciata* as the pollen parent). Since pollen-stigma interactions in self- or cross-pollinations involve complex and cellular and molecular interactions, further studies will contribute to understanding of possible hybridization barriers in *Phalaenopsis* and assist in breeding new cultivars.

6 Notes

1. Since the column of most *Phalaenopsis* cultivars is relatively large, the durations of fixation, staining, and softening are longer than other plants. For the observation of pollen tube growth, it is necessary to dissect the column if the column is too thick.

Fig. 6 Flowers from hybrid plants (**a–d**) derived from crossing using the cut-column method. The pistillate parent is ***Phalaenopsis*** Taipei Gold 'KHM637' with ***P. fasciata*** as the pollen parent. Scale bars = 1 cm

2. We usually harvest the green capsules containing immature seeds derived from the cut-column method. Since the developing seeds are delicate, extra care is needed when removing immature seeds from green capsules and sowing them onto the germination medium.
3. The flowers of most *Phalaenopsis* cultivars can last for more than two weeks. For the pollen and pistillate parents, only select the freshly opened flowers for the use.
4. Before performing the cut-column method, disinfect the working area. Clean the workbench using commercial bleach (2% available chlorine), and clean hands by soap and 70% EtOH solution.
5. A clean cut of the column is important. We prefer breakable carbon steel double-edge blades as our cutting knives. Besides, it is necessary to treat the tools, including forceps, blades, and paraffin film, by 70% EtOH solution before use.
6. To avoid the contamination and rot of the cut column, do not spray water on the flowers before cutting, and keep the cut column dry after pollination.

References

1. Christenson EA (2001) *Phalaenopsis*: a monograph. International Phalaenopsis Alliance, Timber Press, Inc., Portland, OR
2. Customs Administration (2017) Trade statistics. Trade statistics search system. http://www.customs.gov.tw/StatisticWeb/News.aspx
3. Rieseberg LH, Carney SE (1998) Plant hybridization. New Phytol 140:599–624
4. Aoyama M, Kojima K, Kobayashi M (1994) Morphology of microspores in *Phalaenopsis* F1 hybrids. Kinki Chugoku Agric Res 88:49–53. [in Japanese]
5. Bolanos-Villegas P, Chin SW, Chen FC (2008) Meiotic chromosome behavior and capsule setting in *Doritaenopsis* hybrids. J Amer Soc Hort Sci 133:107–116
6. Lee YI, Chang FC, Chung MC (2011) Chromosome pairing affinities in interspecific hybrids reflect phylogenetic distances among lady's slipper orchids (*Paphiopedilum*). Ann Bot 108:113–121
7. Chuang HT, Hsu ST, Shen TM (2008) Breeding barriers in yellow *Phalaenopsis* orchids. J Taiwan Soc Hort Sci 54:59–66
8. Hsu ST, Chuang HT, Shen TM (2010) Breeding barriers in red *Phalaenopsis* orchids. Acta Hort 878:145–152
9. Zhang XS, O'Neill SD (1993) Ovary and gametophyte development are coordinately regulated by auxin and ethylene following pollination. Plant Cell 5:403–418
10. Chuang HT, Huang KL, Hsu ST (2013) Using cut-column pollination method to overcome breeding barriers in *Phalaenopsis* Taipei Gold 'STM'. J Taiwan Soc Hort Sci 59:103–111
11. Chuang HT, Huang KL, Shen RS, Miyajima I, Hsu ST (2014) Using cut–column pollination method to overcome crossing barriers in *Phalaenopsis* sunrise Goldmour 'KHM637'. J Fac Agr Kyushu Univ 59:265–271
12. Shindo K, Kamemoto H (1963) Karyotype analysis of some species of *Phalaenopsis*. Cytologia 28:390–398
13. Kao YY, Chang SB, Lin TY, Hsieh CH, Chen YH, Chen WH, Chen CC (2001) Differential accumulation of heterochromatin as a cause for karyotype variation in *Phalaenopsis* orchids. Ann Bot 87:387–395
14. Lin S, Lee HC, Chen WH, Chen CC, Kao YY, Fu YM, Chen YH, Lin TY (2001) Nuclear DNA contents of *Phalaenopsis* sp. and *Doritis pulcherrima*. J Amer Soc Hort Sci 126:195–199
15. Lee HC, Chiou DW, Chen WH, Markhart AH, Chen YH, Lin TY (2004) Dynamics of cell growth and endoreduplication during orchid flower development. Plant Sci 166:659–667
16. Veilleux RE (1985) Diploid and polyploid gametes in crop plants: mechanisms of formation and utilization in plant breeding. Plant Breed Rev 3:253–288
17. Werner JE, Peloquin SJ (1991) Occurrence and mechanisms of 2n egg formation in 2× potato. Genome 34:975–982
18. Lim KB, Shen TM, Barba-Gonzalez R, Ramanna MS, Van Tuyl JM (2004) Occurrence of SDR 2N-gametes in *Lilium* hybrids. Breed Sci 54:13–18
19. Stead AD (1992) Pollination-induced flower senescence: a review. Plant Growth Regul 11:13–20
20. Kho YO, Baër J (1968) Observing pollen tubes by means of fluorescence. Euphytica 17:298–302
21. Lin HJ, Chuang HT, Hsu ST, Shen TM (2008) Effects of developmental temperature and capsule harvest time on the aseptic seed germination in *Phalaenopsis aphrodite* subsp. *formosana*. Plant Nursery 10:59–71
22. Goh CJ, Strauss MS, Arditti J (1982) Flower induction and physiology in orchids. In: Arditti J (ed) Orchid biology: reviews and perspectives, II. Cornell Univ Press, Ithaca, NY, pp 213–241
23. O'Neill SD, Nadeau JA, Zhang XS, Bui AQ, Halevy AH (1993) Interorgan regulation of ethylene biosynthetic genes by pollination. Plant Cell 5:419–432
24. Janson J (1993) Placental pollination in *Lilium longiflorum* Thunb. Plant Sci 90:105–115
25. Janson J, Reinders MC, Van Tuyl JM, Keijzer CJ (1993) Pollen tube growth in *Lilium longiflorum* following different pollination techniques and flower manipulations. Acta Bot Neerl 42:461–472
26. Magdalita PM, Drew RA, Godwin ID, Adkins SW (1998) An efficient interspecific hybridization protocol for *Carica papaya* L.×*C. cauliflora* Jacq. Aust J Exp Agric 38:523–530
27. Ronald WG, Ascher PD (1975) Effects of high temperature treatments on seed yield and self-incompatibility in *Chrysanthemum*. Euphytica 24:317–322
28. Shen TF, Liao CI (1976) Factors affecting the degree of self-incompatibility and the breaking of self-incompatibility in Yehsen cabbage. Sci Dev 4:2298–2305

29. Van Tuyl JM, Straathof TP, Bino RJ, Kwakkenbos AAM (1988) Effect of three pollination methods on embryo development and seedset in intra- and interspecific crosses between seven *Lilium* species. Sex Plant Reprod 1:119–123
30. Van Tuyl JM, Van Diën MP, Van Creij MGM, Van Kleinwee TCM, Franken J, Bino RJ (1991) Application of *in vitro* pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Sci 74:115–126
31. Vervaeke I, Delen R, Wouters J, Deroose R, de Proft MP (2004) Semi *in vivo* pollen tube growth of *Aechmea fasciata*. Plant Cell Tiss Org Cult 76:67–73
32. Visser T (1983) The role of pioneer pollen in compatible and incompatible pollinations of apple and pear. Acta Hortic 139:51–57
33. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Chapter 13

In Vitro Flowering and Breeding of *Erycina pusilla*

Yi-Tien Chiu and Chen Chang

Abstract

Erycina pusilla is a miniature orchid species native to tropical America. The unique characteristics, e.g., a short juvenile stage and its miniature size, make *E. pusilla* a perfect material for producing the ornamental flask product and for the study of flowering physiology and in vitro breeding. In this chapter, we demonstrate the culture steps of in vitro flowering of *E. pusilla*. Using method detailed, it is possible to produce blooming *E. pusilla* plants within 1 year from in vitro seed germination and producing ornamental flasks for commercial sale. This culture protocol can also be used for in vitro breeding to select additional desirable traits for ornamental flask production.

Key words *Erycina pusilla* flowering, Miniature orchids, Breeding, Pollination, Subcultured, In vitro ornamental orchid product

1 Introduction

The initiation of flowering in higher plants involves the switch from vegetative to reproductive stage of growth and is primarily regulated by photoperiod, vernalization, endogenous phytohormones, and autonomous pathways. The technique of in vitro flowering provides an ideal system to study the physiology of flowering because it allows for a better control of factors of interest than the greenhouse or field-grown plants [1]. The application of plant growth regulators, carbohydrate concentrations, and the control of light intensity, photoperiod, and temperature to promote flowering in vitro have been well documented in several plant species [2, 3]. Furthermore, the explants cultured in vitro are usually capable of reducing the juvenile phase and reaching the adult phase earlier, facilitating the breeding progression [4].

Orchids, such as phalaenopsis, oncidiums, and cymbidiums, are important ornamental crops with exotic and colorful flowers [5]. Most orchids require 1–3 years (or even longer) to reach the mature stage for flowering [6]. For example, in phalaenopsis, dendrobiums, and oncidiums, it usually takes 2–3 years from seed germination to blooming. The successful induction of in vitro

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_13,

flowering enables an earlier assessment of floral characteristics such as flower colors. This greatly shortens the schedule required for normal evaluation in greenhouse cultivation [4].

Erycina pusilla (L.) Williams & Chase is a tropical miniature orchid [7] that has been described by Richb. (1863; *see* [8]) as *Oncidium pusillum* and by Dodson and Dressler [8] as *Psygmorchis pusilla*. *E. pusilla* is a noteworthy orchid species because it grows rapidly and reaches maturity near 1 year in natural conditions [3, 9–13]. The unique characteristics, e.g., a short juvenile phase and the miniature size of *E. pusilla*, make it a perfect material for producing the "in vitro bouquets" in commercial markets [4]. In our previous studies, we reported that it is possible to induce *E. pusilla* to bloom in vitro and were successful in completing in vitro pollination, capsule formation, and seed germination [14] (Fig. 1). In other words, we have established an "in vitro breeding system" for *E. pusilla*. In this chapter, we document the procedures of in vitro flowering culture, the production of ornamental bottles, and the case study of an "in vitro breeding system" showcasing *E. pusilla* 'Hsingda Golden' as an in vitro new cultivar.

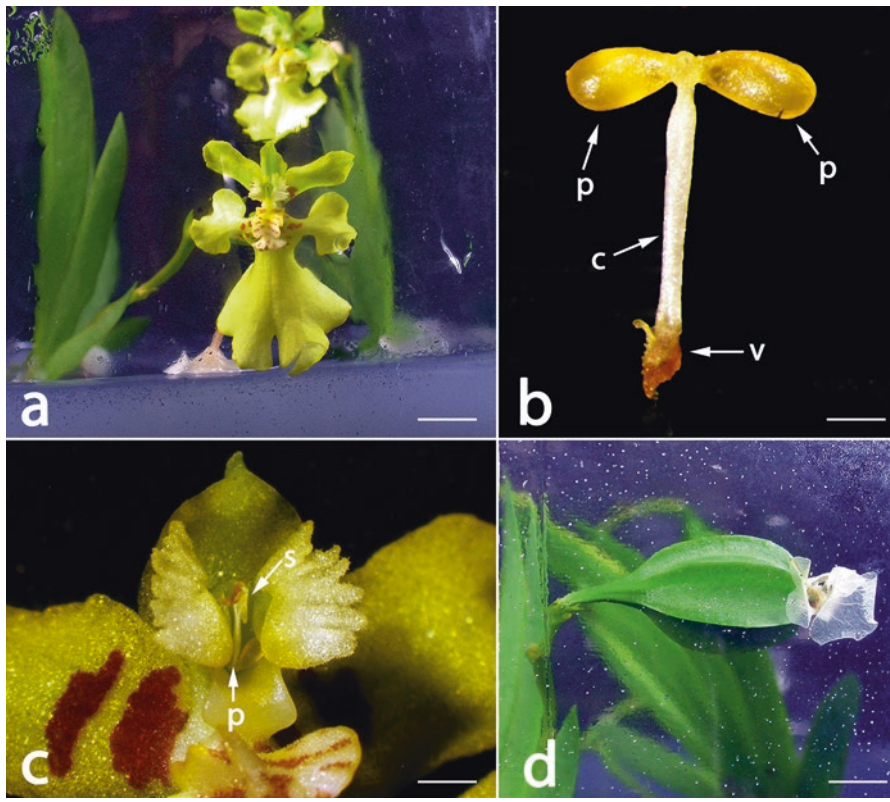


Fig. 1 In vitro pollination and fruit set of *E. pusilla*. (**a**) Flowering plant in vitro (bar = 10 mm); (**b**) pollinarium contains two pollinia, a caudicle, and a viscidium (bar = 0.3 mm). p, pollinia; c, caudicle; v, viscidium; and s, stigma, are indicated; (**c**) hand-pollinated flower (bar = 1 mm); (**d**) fruit set of in vitro plant (bar = 5 mm)

2 Materials

2.1 Plant Materials

The plants *E. pusilla* are maintained in the greenhouse at National Chung Hsing University, Taichung, Taiwan (*see* **Note 1**).

2.2 Equipment and General Laboratory Supplies

1. Laminar flow cabinet
2. Stereomicroscope for pollination
3. Tools, i.e., dissecting needles, forceps, and scalpels
4. The artificial food dye (Everlight Chemical Industrial Co., Taipei, Taiwan)
5. 3 M™ Micropore™ Surgical Tape (3 M, Minnesota, USA)

2.3 Solutions and Culture Media

1. The germination medium: 1/4 strength Murashige and Skoog (MS) medium salt [15], supplemented with (mg/L)—niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (20), *myo*-inositol (100), NaH_2PO_4 (170), activated charcoal (1000), peptone (1000), potato powder (6000), coconut milk (150 mL/L), sucrose (20,000), and agar (8000). Adjust the pH of the medium to 5.2 before autoclaving for 15 min at 121 °C, 1.2 kg cm^{-2}. Dispense 8 mL medium into each test tube (15×20 mm, pyrex no.9820, USA).
2. The subculture medium: 1/2 strength MS salt, supplemented with (mg/L)—niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (20), *myo*-inositol (100), NaH_2PO_4 (170), activated charcoal (1000), peptone (1000), potato powder (6000), coconut milk (150 mL/L), sucrose (20,000), and agar (8000). Adjust the pH of the medium to 5.2 before autoclaving for 20 min at 121 °C, 1.2 kg cm^{-2}. Dispense 100 mL medium into each 600 mL flask.
3. The ornamental flask medium: 1/2 strength MS, supplemented with (mg/L)—niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (20), *myo*-inositol (100), NaH_2PO_4 (170), sucrose (20,000), and Gelrite (4000), artificial dye (2). Adjust the pH of the medium to 5.2 before autoclaving for 15 min at 121 °C, 1.2 kg cm^{-2}.
4. NaOCl 2% solution with one or two drops of Tween 20
5. 70% EtOH solution

3 Methods

3.1 Seed Germination and Growing of Plants In Vitro

1. Select healthy plants in which the flowers have fully opened (*see* **Note 2**) in the greenhouse. Remove two masses of pollinia with the viscidium from the anther by forceps, and transfer them onto the stigma of the same flower.

2. Harvest the capsules approximately 12 weeks after pollination. Within the capsules, the seeds are near maturation.
3. Surface sterilize the capsules in 70% EtOH solution for about 5 s and then in the 2% NaOCl solution with one or two drops of Tween 20 for 20 min, and subsequently wash the capsules three times with sterile water in the laminar flow cabinet.
4. Cut and open the capsules longitudinally on a sterile Petri dish. Gently remove the tiny seeds using fine forceps, and sow them onto the germination medium.
5. Place approximately 100 seeds in each test tube, and place the cultures under light conditions (photon flux density of 6 μmol m^{-2} s^{-1}, PAR) with a 12-h light/dark photoperiod at 25 ± 2 °C.
6. About 1 month after sowing, the seeds have germinated, and developing protocorms with pale green color are visible. About 3 months after sowing, some seedlings have come to less than 1 cm in length. Select and transfer the young seedlings (less than 1 cm high) from the germination medium to the subculture medium (approximately 300 seedlings per 600 mL flask). Place the subcultures under light conditions (photon flux density of 55 μmol m^{-2} s^{-1}, PAR) with a 12-h light/dark photoperiod at 25 ± 2 °C.

3.2 In Vitro Flowering

1. In order to obtain the vigorous seedlings for in vitro flowering culture, subculture seedlings (subculture 1) every 3 months. Place 30 seedlings of 1–1.5 cm in length per 600 mL flask.
2. After 3 months (subculture II), place 25 seedlings of 2.0 cm high per 600 mL flask.
3. After another 3 months (subculture III), place 20 plantlets of 2.4 cm high per 600 mL flask (*see* **Note 3**).
4. After three subcultures, select vigorously grown in vitro plantlets of 4 cm high with flowers for in vitro pollination (Fig. 1a).

3.3 In Vitro Pollination and Capsule Formation

1. For in vitro pollination operation, carefully remove one blooming plant from the flask and place on a sterile Petri dish (*see* **Note 4**). Under a stereomicroscope in the laminar flow cabinet, remove the pollinia from the anther by forceps, and transfer them onto the stigma as described above in the greenhouse plant pollination (Fig. 1b, c) (*see* **Note 5**).
2. Transfer one pollinated plant into a new 600 mL flask with the subculture medium, and place the culture under light conditions in the culture room.

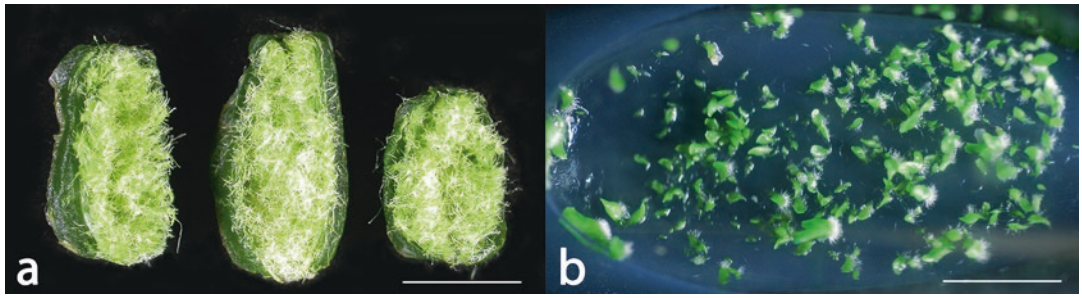


Fig. 2 In vitro seeds and germination of *E. pusilla*. (**a**) Plentiful seeds in the in vitro set fruit (bar = 1 cm); (**b**) in vitro seed germination (bar = 1 cm) after 3 months

3. After 12 weeks of pollination, harvest the capsules (Fig. 1d) and sow the seeds (Fig. 2a, b) as described above. Therefore, the entire procedures of seed germination, growth of seedlings, and flowering can be accomplished and cycled in vitro.

3.4 The Ornamental Flask Product

1. Select vigorous plants from subculture III, and place 20 plantlets of 2.4 cm high per 600 mL flask with the subculture medium.
2. After subculture IV, select one vigorous plant with two blooming flowers or buds from the subcultured materials (*see* **Note 6**), and transfer to the handmade apple-shaped glass or other shaped containers (*see* **Note 7**) with 70 mL ornamental flask medium. Gently put the roots into the colored medium, and adjust the orientation of the plant (*see* **Note 8**).
3. Seal up the apple-shaped glass bottle neck with three layers of 3 M™ Micropore™ Surgical Tape (*see* **Note 9**), and place it under light conditions (photon flux density of 55 μmol m^{-2} s^{-1}, PAR) with a 12-h light/dark photoperiod at 25 ± 2 °C (Fig. 3). The plant of *E. pusilla* will continue to produce flowers more than 6 months under the above conditions.

4 In Vitro Breeding: A Case Study of *Erycina pusilla* 'Hsingda Golden'

1. Since *E. pusilla* is a heterozygous species, there are variations among the seedlings from self- or cross-pollinations. For better performance of ornamental flask products, we aim at selecting the clones with traits, including larger flower size, less brown spots on the sepals and petals, multiple floral spikes and less abortion of floral buds, etc. (*see* **Note 10**).
2. We selected vigorously growing seedlings from the self-pollinated seedlings and labeled each individual carefully for the further observation and evaluation (*see* **Note 11**). From 2009 to 2010, 11 in vitro flowering plants of *E. pusilla* were

Fig. 3 An in vitro ornamental product of *E. pusilla* 'Hsingda Golden'. The opening near the top is concealed using the 3 M™ Micropore™ Surgical Tape

selected and labeled (denoted by PSYP 1–11) from 10,000 seedlings.

3. After evaluation of their traits, two individuals (PSYP 1 and PSYP 5) showed vigorous growth, while PSYP 1 had the characteristics of precocious (short juvenile period) and larger flower size. Therefore, we finally selected PSYP 1 for clonal propagation in vitro, and the plantlets bloomed in vitro in July of 2011 that were all uniform and stable. PSYP 1 has golden color flowers with very light brown spots on the base of lateral petals; the labellum and callus are also distinct from other *E. pusilla* flowers (Fig. 4a, b). Besides, it has rounder flower shape and produces multiple floral spikes at the same time and has a shorter interval between bloomings (Fig.4c, d).
4. We named the clone PSYP 1 as *E. pusilla* 'Hsingda Golden' and have proposed the application of Plant Variety Rights in R.O.C. in September 2013.

5 Concluding Remarks

Here we demonstrate the procedures of in vitro flowering of *E. pusilla* (Fig. 5). Using our protocol, blooming *E. pusilla* plants can be produced within 1 year from seed germination in vitro. We

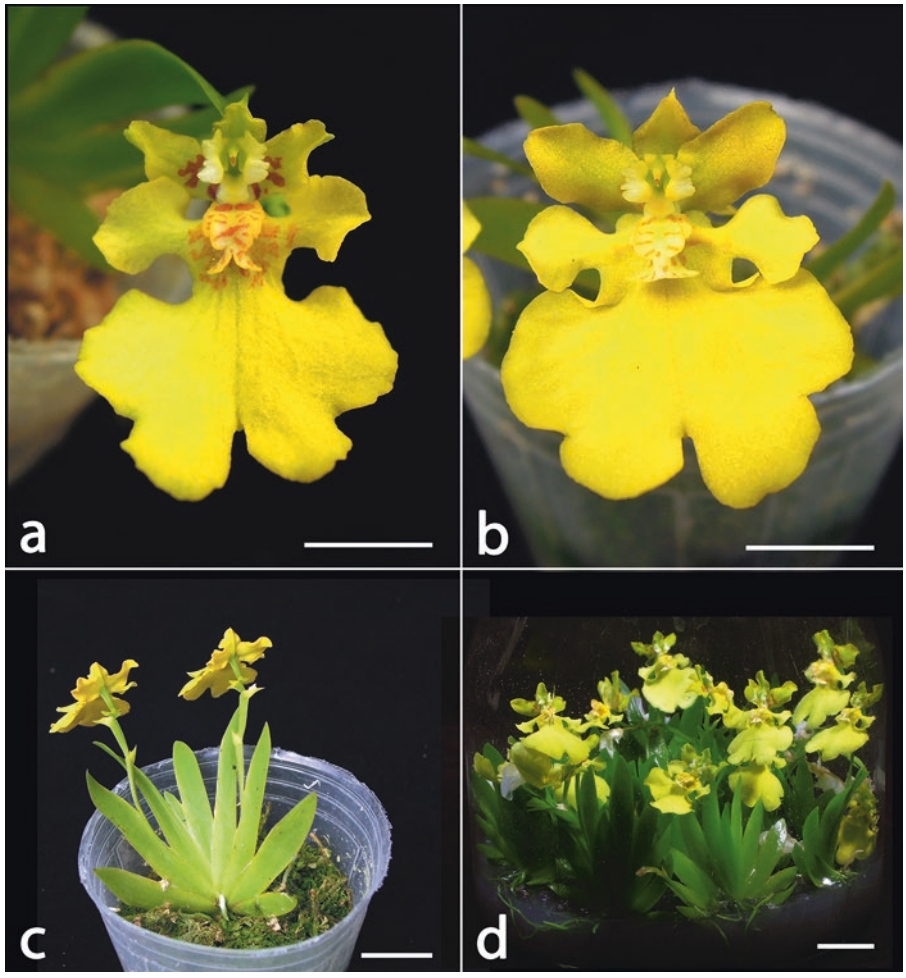


Fig. 4 Plant and flower characteristics of *E. pusilla* 'Hsingda Golden'. (**a**) Flower of *E. pusilla* (bar = 0.5 cm); (**b**) flower of *E. pusilla* 'Hsingda Golden' (bar = 0.5 cm); (**c**) flowering plant of *E. pusilla* 'Hsingda Golden' (bar = 1 cm); (**d**) in vitro flowering of 'Hsingda Golden' (bar = 1 cm)

Fig. 5 The procedure of producing in vitro flowering plantlets of *E. pusilla*

also use this culture system for in vitro breeding to select the desirable traits for the ornamental flask production. Furthermore, we have used this in vitro breeding system to cross *Tolumnia* (a relative genus to *Erycina*) with *E. pusilla* and have created novel hybrids with red orange flower colors (unpublished data) for ornamental flask production.

6 Notes

1. *Erycina pusilla* plants are maintained in the greenhouse under a 70% shading cloth at about 27 °C and 70% humidity at the National Chung Hsing University, Taichung, Taiwan. The plants are grown in a 5 cm diameter plastic pot using sphagnum moss as the potting material. Since it is an epiphytic orchid species, its roots require good aeration. Hence, pots with several drainage holes are used for growing this miniature species. The potting medium must dry out before the next watering. 1 g/L Peters fertilizer (20N-20P_2O_5-20K_2O, The Scotts Company, Ohio) is applied during watering once a month.
2. Newly bloomed flowers guarantee successful pollination and capsule setting. We do not use flowers older than 6 days after anthesis.
3. Without regular subcultures (once every 3 months), seedlings will gradually decay. For the production of ornamental flasks, it usually requires 3–4 times of subcultures.
4. Be careful of contamination during the operation of in vitro pollination in the laminar flow cabinet. The blooming plants with flower spikes are much larger as compared to the protocorms or young seedlings. They can be difficult to handle.
5. Because the flowers are delicate, perform the pollination under a stereomicroscope using a fine pair of sterilized fine forceps. The reddish-brown point above the stigma is the viscidium; by gently pulling out the viscidium, the two yellow masses of pollinia will be visible.
6. Select vigorously grown plants with multiple floral spikes to ensure continual performance of the ornamental flask products. Plants about 15 months after germination exhibiting a size of 4 cm in length seem to be optimal for use in ornamental flask production.
7. The most important consideration in selecting bottles/containers for ornamental flask production is that the bottles/containers are pleasing to the eyes. Certainly clear bottle is essential. In our experience, the narrow bottle is not a good candidate because the flowers will easily touch the inner walls of bottle and wilt quickly. For the apple-shaped container, a hole about 2.5–3 cm is present near the top for inserting the plant. They can be obtained from Ya Song Hot Resistant Glass Co., Ltd. (http://yasong.com.tw). The opening is sealed using the 3 M™ Micropore™ Surgical Tape to prevent contamination. Other suitable culture vessels such as baby food jars can also be used. Different types of containers can be obtained from PhytoTechnology Laboratories (https//phytotechnlab.com).
8. For keeping the ornamental flask medium clear, avoid using activated charcoal or additives such as potato and banana

powders in the culture medium. For a better appreciation of flowers within an ornamental flask, it is important to adjust the orientation of the plant with flowers or buds facing the front of the flask. During the placement of plant into the flask, hold onto the base of the plant at the root-shoot junction; insert the plant carefully into the flask, avoiding injuring to the plant and floral spikes.

9. Blow-dry the water vapor/condensation inside the bottle/container in the laminar flow cabinet before transferring a blooming plant. A hair dryer can be used to aid in the removal of condensation along the walls of the container. Seal the bottle/container with three layers of 3 M™ Micropore™ Surgical Tape, allowing for good ventilation and minimizing contamination.
10. Before selection, knowing what desirable traits to select is essential, such as the beautiful flower color and shape, good blooming habit (multiple floral spikes), long-lasting flowers, easy-to-perform clonal propagation, etc. In our experience, the small flower buds are easily aborted, and this character should be avoided.
11. Subculture each selected individual in one flask, and carefully label the selected clones after clonal propagation. This is important for further observation and evaluation.

Reference

1. Murthy KSR, Kondamudi R, Chalapathi RPV, Pullaiah T (2012) *In vitro* flowering-a review. J Agric Technol 8:1517–1536
2. Chang C, Chang WC (2003) Cytokinins promotion of flowering in *Cymbidium ensifolium* var. misericors *in vitro*. Plant Growth Regul 39:217–221
3. Vaz APA, Càssia RD, Figueiredo-Ribeiro L, Kerbauy GB (2004) Photoperiod and temperature effects on *in vitro* growth and flowering of *P. pusilla*, an epiphytic orchid. Plant Physiol Biochem 42:411–415
4. Teixeira da Silva JA, Kerbauy GB, Zeng S, Chen Z, Duan J (2014) *In vitro* flowering of orchids. Crit Rev Biotechnol 34:56–76
5. Lopez RG, Runkle ES (2005) Environmental physiology of growth and flowering of orchids. HortSci 40:1969–1973
6. Hew CS, Yong JWH (1997) Physiology of tropical orchids in relation to the industry. World Scientific Publishing Co. Pte Ltd, Singapore
7. Williams NH, Chase MW, Fulcher T, Whitten WM (2001) Molecular systematics of the Oncidiinae based on evidence from four DNA sequence regions: expanded circumscriptions of *Cyrtochilum*, *Erycina*, *Otoglossum,* and *Trichocentrum* and a new genus (Orchidaceae). Lindleyana 16:113–139
8. Dodson CH, Dressler RL (1972) Two undescribed genera in the Orchidaceae - Oncidiinae. Phytologia 24:285–292
9. Dodson CH (1957) *Oncidium pusillum* and its allies. Amer Orchid Soc Bull 26:170–172
10. Livingston R b (1962) *Oncidium pusillum*-blooming in flask! Amer Orchid Soc Bulletin 31:1007
11. Vaz APA, Kerbauy GP (2000) Effects of mineral nutrients on *in vitro* growth and flower formation of *Psygmorchis pusilla* (Orchidaceae). Acta Hort 520:149–156
12. Vaz APA, Kerbauy GB (2008) *In vitro* flowering studies in *Psygmorchis pusilla*. Glo Sci 42:427–432
13. Baker ML, Baker CO (2006) Orchid species culture: oncidium/odontoglossum alliance. Timber Press, Portland, OR, p 992
14. Chiu YT, Lin CS, Chang C (2011) *In vitro* fruiting and seed production in *Erycina papilla* (L.) N.H. Williams & M.W. Chase. Prop Ornamental Plants 11:131–136
15. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Chapter 14

Virus Elimination Through Meristem Culture and Rapid Clonal Propagation Using a Temporary Immersion System

Rong-Show Shen and Shan-Te Hsu

Abstract

The generation of virus-free plants is a key requirement in the international trade of orchid seedlings. Shoot apical meristem isolation and its successful culture is one of the methods that can generate virus-free plants. In this chapter, a general protocol for meristem isolation and subsequent micropropagation of meristem explants and regenerated plantlets for *Phalaenopsis* is detailed. Patience, practice, and cleanliness are key elements in shoot apical meristem excision. Information on the temporary immersion system in the mass propagation of virus-free *Phalaenopsis* plantlets is also presented.

Key words Shoot apical meristem, Meristem isolation, Virus, Virus-free plants, Temporary immersion system, *Phalaenopsis*

1 Introduction

Orchids are highly popular international trade products with a notable market that exceeds US$2 billion per year. Known as the Kingdom of *Phalaenopsis*, Taiwan is a global leader in the breeding and cultivation of new orchid cultivars, and new hybrids are produced constantly. Cultivars with high market values are often produced through micropropagation techniques in order to satisfy the commercial demand. However, endogenous viral infections can also be transmitted and multiplied through micropropagation, affecting the growth and flower quality of plants. Virus-infected plants can also lead to trade issues and disputes. Therefore, authentication and verification of virus-free plants have become key requirements in the international trade of orchid seedlings.

Taiwan is the world's largest producer of commercial *Phalaenopsis* seedlings. However, the infection and replication of two orchid viruses, namely *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV), have frequently caused significant losses for both buyers and sellers (Fig. 1). At least twenty-eight viruses have been identified in the orchid family in

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_14,

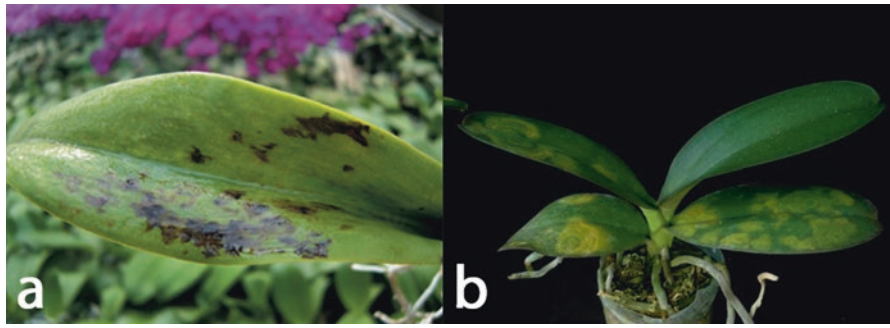


Fig. 1 Symptom of two common viruses in export-oriented commercial Phalaenopsis seedlings. (**a**) *Cymbidium mosaic virus* (CymMV). (**b**) *Odontoglossum ringspot virus* (ORSV)

the literature, of which CymMV and ORSV are distributed worldwide and have resulted in the most severe economic losses. The primary symptoms of CymMV are gangrene stripes or spots that appear on the leaves or flowers, and ORSV-infected plants have mosaic and spotted patterns on leaves and stains on flowers. The co-infection of these two viruses can compound the overall severity of symptoms than do single infection [1]. Hence, producing virus-free seedlings is an absolute must by orchid producers, ensuring the quality and quantity of healthy seedlings. This has become a quintessential assignment for the orchid industry.

The early history of shoot tip culture with the aim to produce healthy clones of plants is summarized by Yam and Arditti [2]. In 1952, Morel and Martin [3] isolated apical meristems from virus-infected dahlias and successfully obtained virus-free plants through in vitro culture, and they also produced virus-free potatoes using the same method [3]. Their successes lead Morel [4] to apply the meristem isolation and culture technique to the *Cymbidium*, for virus elimination [4]. Since then, reports on apical meristem and shoot tip culture appear regularly in the literature (*see* Nehra and Kartha [5] and Panattoni et al. [6]).

At present, feasible methods for producing virus-free plantlets include heat treatment, chemical therapy, meristem culture, heat therapy followed by meristem culture, adventitious shoot formation followed by meristem culture, and grafting of meristems on virus-free root stock [6]. Of these methods, apical meristem culture is the most commonly applied method to produce virus-free healthy plantlets. The lack of direct vascular connections between the shoot apical meristem and the rest of the plant makes it least likely to be infected or have a lower concentration of virus when compared to other parts of the plant [7]. Hence, the apical meristem represents a possible virus-free zone and their culture in vitro provides a feasible method for obtaining virus-free plants [8, 9]. One added advantage of meristem culture is that plants regenerated

from excised meristem tips possess identical hereditary characteristics to those of the parent plant, which reduces the possibility of variation [10] and guarantees the acquisition of stable hereditary traits. For the generation of virus-free plants, Quak [11] indicated the following strategies should be used, i.e., identifying the virus type, determine the type of therapy to be used, further testing of treated plants, and continual testing of propagated cured plants in order to prevent reinfection.

In this chapter, a protocol on virus elimination through meristem culture is detailed for *Phalaenopsis*. The indirect ELISA virus detection method can be found in Chap. 18 and will not be repeated. For additional information on this topic, readers are urged to consult tissue culture texts [9, 12–14] and a recent review by Panattoni et al. [6]. Mass propagation is also a key objective to the success of generating virus-free plants after the eradication of viruses. Hence, the temporary immersion system of micropropagation for *Phalaenopsis* is also described.

2 Shoot Apical Meristem Culture

The shoot apical meristem (SAM) is located at the extreme tip of each growing shoot. It is defined as the region between or above the youngest leaf primordium [15]. Although a SAM can take on various shapes, it usually appears as a small dome and has a size ranges between 40 μm (*Syringa*) and 3300 μm (*Cycas*) [15].

The size of the dissected SAM is important to the success of generating virus-free plants. It is often noted that the smallest explant with just the apical dome "will be the least successful during *in vitro* culture, but will produce the highest proportion of virus-free material" [7]. For example, Kumar et al. [16] used 0.3-mm *Chrysanthemum* meristems as explants to eliminate *Cucumber mosaic virus* and *Tomato aspermy virus* infections. The results of a reverse transcription polymerase chain reaction (RT-PCR) test showed that 65–70% of the specimens were free of virus [16]. Wang and Valkonen [17] adopted 0.5 and 1.0 mm *Ipomoea batatas* meristems to eliminate *Sweet potato chlorotic stunt virus* and *Sweet potato feathery mottle virus*; the RT-PCR test results indicated that both viruses were eliminated. Verbeek et al. [18] selected the meristems of garlic (*Allium sativum* L.) cloves and bulbils to explore the effect of virus eradication, specifically for the *Leek yellow stripe virus*, *Onion yellow dwarf virus*, *Garlic common latent virus*, and *Onion mite-borne latent virus*. Their results showed that the total virus eradication rate was 80% for 0.23-mm meristems from garlic cloves and only 27% for 0.21-mm axillary bud meristems from garlic bulbils. In *Phalaenopsis* Sogo Yukidian, Kang et al. [19] noted that apical dome with a single primordium has the

highest percentage of being virus-free, while in *Doritaenopsis* Queen Beer, meristems with three primordia remain uninfected. Optimal meristem size for meristem culture needs to be determined for each selected species and cultivars. In general, a smaller meristem explant is preferred. The success of using the smallest SAM explants in meristem culture is most likely due to the lack of direct mature vascular tissue connections between the SAM and the rest of the plant.

Viruses enter the plants through damaged leaf surfaces. Once entered, they can be transmitted through mesophyll cells via the symplastic pathway, entering the vascular bundle sheath cells to reach the vascular tissues, and are then transported over long distances via the phloem sieve tubes [20] or the xylem tissues [21, 22]. Once entering the plant body, viruses can also be transported over short distances through phloem parenchyma cells, companion cells, vascular bundle sheath, and mesophyll cells to uninfected leaves, which triggers systemic infection [23, 24]. It is generally noted that fast-growing parts of a plant, i.e., the shoot and root tips, tend to contain no or lower virus contents and are classified as virus-free zones. By contrast, relatively mature or senescent organs of a plant typically have higher virus contents. Several reasons may contribute to this difference. First, because shoot tips lack mature vascular bundles and divide rapidly, this slows the movement of viruses into fast-growing tips. Hull [25] indicated that meristem tips have various growth areas that can range from 100 to 1000 μm, which can be potentially virus-free. Second, viruses cannot establish affinity within meristematic cells of a host, which exhibit high mitotic activity. The ribonucleic acid replication of a virus, which is required for its reproduction, is inhibited by the meristem cells of the host [26]. Third, shoot tips contain a substantial amount of endogenous auxin that can intervene and inhibit the metabolism and multiplication of the viral nucleic acid [26]. Thus, the culturing of meristem-tip has the potential to produce virus-free plants with genotypes and phenotypes identical to those of their parent plant. However, plants can only be verified as virus-free and healthy through the use of the highly sensitive detection technology, such as the ELISA virus detection method (*see* Chap. 18) or through the use of Biochips which are available commercially (Dr. Chip Biotech Inc., Taiwan).

Although small meristem size is preferred for meristem culture, the small explant size can prove to be difficult to culture in vitro. Physical excision and the in vitro environment generate stresses which can have negative effects on the growth of meristem explants after excision. Lim et al. [27] excised 0.1–1.0 mm meristem tips of axillary buds from monopodial Mokara orchids. After 3 months of culture, only 0%–5% of explants survived. Although larger tissues have a higher survival rate, they remained virus-

infected. Similar results were obtained by Loi et al. [28] on culturing 1.0-mm meristem tips of *Dendrobium*. In order to improve the survival and growth of small meristem explants, the in vitro culture conditions and media must be optimized.

Another approach in enhancing the success of generating virus-free plant is to apply a chemotherapy treatment, i.e., the addition of ribavirin, an antiviral compound in conjunction with meristem culture [6, 11, 27]. Co-culturing meristem tips with the broad-spectrum antiviral agent ribavirin can enhance the generation of virus-free plants. However, the antiviral agent can have a negative effect on the growth of explants; the survival rate of explants tends to decrease [27].

In orchids, besides meristem culture, the use of protocorm-like bodies (PLBs) as explants has also generated successes in obtaining virus-free plants [24, 27, 29] (*see* **Note 1**). PLBs regenerate readily from protocorms, other PLBs and PLB sections. The relatively fast regeneration process and rapid growth of PLBs may have prevented viral infection. Furthermore, PLBs differentiate from surface cells of explants [30]; this may reduce the chances of continual infection during regeneration.

Once virus-free plantlets are generated and passed the ELISA/Biochip screening tests, the temporary immersion system can be used for mass propagation of plantlets. This semi-automated culture system provides the most natural environment for in vitro culture of plants (for reviews, *see* Etienne and Berthouly [31], Watt [32], and Georgiev et al. [33]). This method improves plantlet production when compared with conventional method using the semi-solid medium [19].

3 Material

3.1 Equipment and General Laboratory Supplies

1. Laminar flow cabinet
2. Stereomicroscope for meristem isolation
3. Dissecting tools, i.e., dissecting needles, forceps, scalpels (*see* **Note 2**)
4. ELIZA supplies for virus detection (*see* **Note 3**)
5. Multiplex RT-PCR, orchid virus detection kit (Dr. CHIP Co., Taiwan) (*see* **Note 4**)
6. Ventilated Plantima containers (A-Tech Bioscientific Co. Ltd., Taiwan) for use in conjunction with the temporary immersion system (*see* **Note 5**)
7. Basic equipment of the temporary immersion system (TIS) (*see* **Note 6**)
8. Tissue culture related supplies and chemicals

3.2 Solutions and Culture Media

1. NaOCl 1% solution with one or two drops of Tween 20
2. EtOH 70% solution
3. *Phalaenopsis* protocorm-like body (PLB) regeneration medium. The medium is based on the formulation by Shen et al. [34] and is composed of 1/3 Murashige and Skoog medium (MS) [35] with 0.1 mg/L 1-naphthaleneacetic acid (NAA), 2 mg/L 6-benzyladenine purine (BAP), 200 mL/L coconut water, 5 g/L sucrose, and 8 g/L agar. Adjust the pH of the medium to 5.4 ± 0.1 before autoclaving for 25 min at 121 °C, 1.05 kg cm^{-2}.
4. *Phalaenopsis* shoot multiplication medium for use in the temporary immersion system. The liquid culture medium is composed of 1.5 g/L HYPONeX No. 1 (Hyponex Co. USA), 0.1 mg/L NAA, 4 mg/L BAP, 200 mL/L coconut water, and 20 g/L sucrose. Adjust the pH of the medium to 5.4 ± 0.1 before autoclaving for 25 min at 121 °C, 1.05 kg cm^{-2}.
5. *Phalaenopsis* plantlet root initiation medium: 1/3 Murashige and Skoog medium (MS) [35] or 3.0 g/L HYPONeX No. 1 (Hyponex Co.), 0.5 mg/L NAA, 1 mg/L IBA, 50 g/L banana homogenate, 200 mL/L coconut water, and 30 g/L sucrose, and 8 g/L agar. Adjust the pH of the medium to 5.4 ± 0.1 before autoclaving for 25 min at 121 °C, 1.05 kg cm^{-2}.

4 Method

4.1 Explant Preparation Prior to Culture

1. Prescreening of explants for viruses

   Obtain short stem tips from commercial flask plantlets in vitro and/or meristems of inflorescence tips of flowering plants, as well as stem tips from commercial mature seedlings (Fig. 2) [19, 36]. These materials need to be pretested using an enzyme-linked immunosorbent assay (ELISA) to ensure that they are not infected with CymMV, ORSV, or a combination of both viruses. For a detailed procedure, *see* Chap. 18.
2. Structural organization of the shoot apical meristem

   Although this step is not necessary, it is desirable to understand the structural organization of the SAM prior to its excision. This enables the investigator to determine the appropriate size needed in order to enhance the chance of obtaining a meristem free of viruses. Detailed procedures for the paraffin wax embedding method can be found in Stasolla and Yeung [37]. Figure 3 reveals the structural characteristics of a *Phalaenopsis* apical meristem. Leaf primordia are distinct with procambium traces. Mature vascular tissues are absent near the SAM.

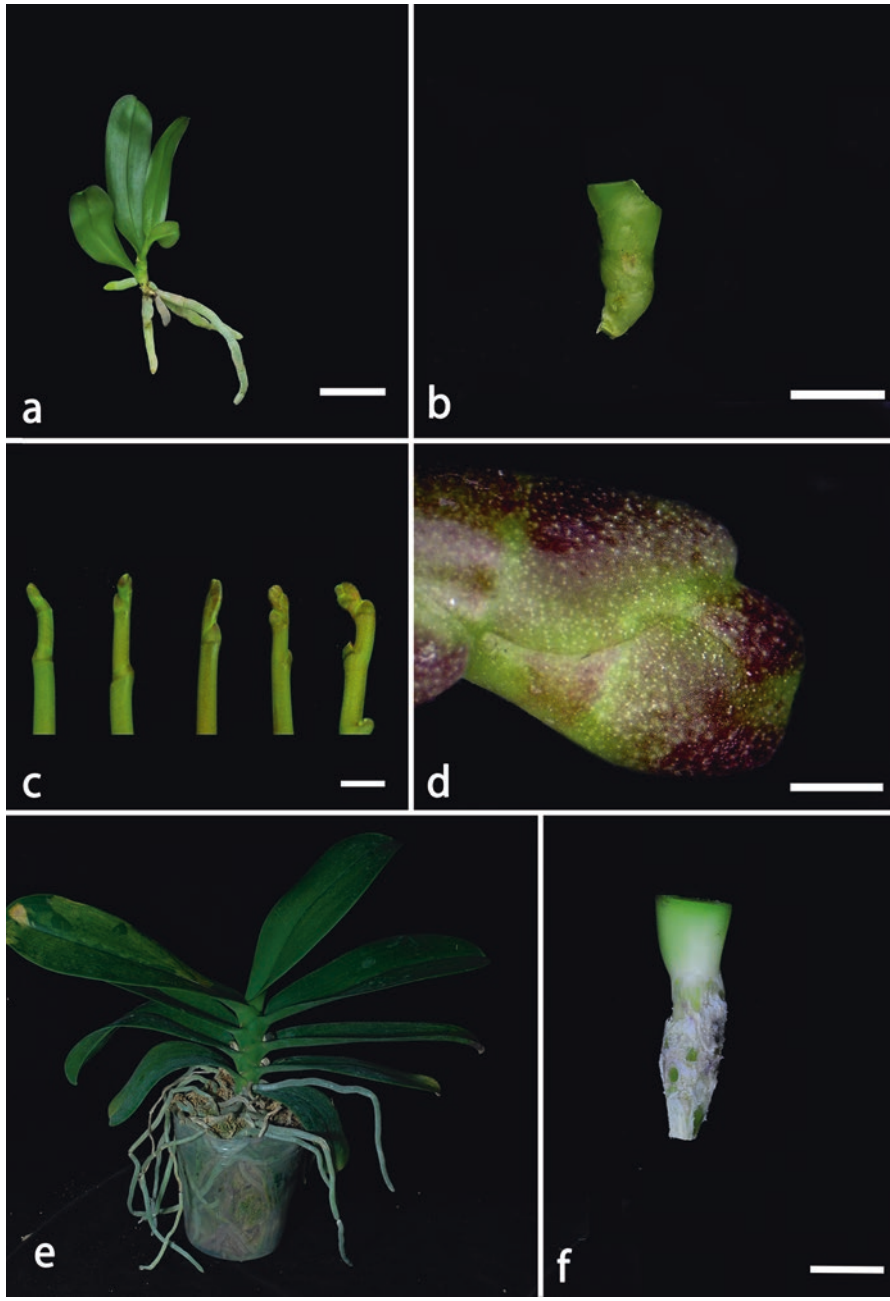


Fig. 2 Preparation of meristem tips from the short stem of plantlets in vitro, inflorescence tips, and short stem mature seedlings in *Phal*. Sogo Yukidian "V3." (**a**) A commercial flask plantlet, Bar = 3 cm. (**b**) Short stem with leaf base after roots and leaves have been removed from plantlets, Bar = 1 cm. (**c**) Inflorescence tip, Bar = 1 cm; (**d**) Enlarged inflorescence tip with approximately ten leaf primordia, Bar = 2 mm. (**e**) A commercial seedling. (**f**) A short stem explant after roots and leaves have been removed from plants, Bar = 1 cm

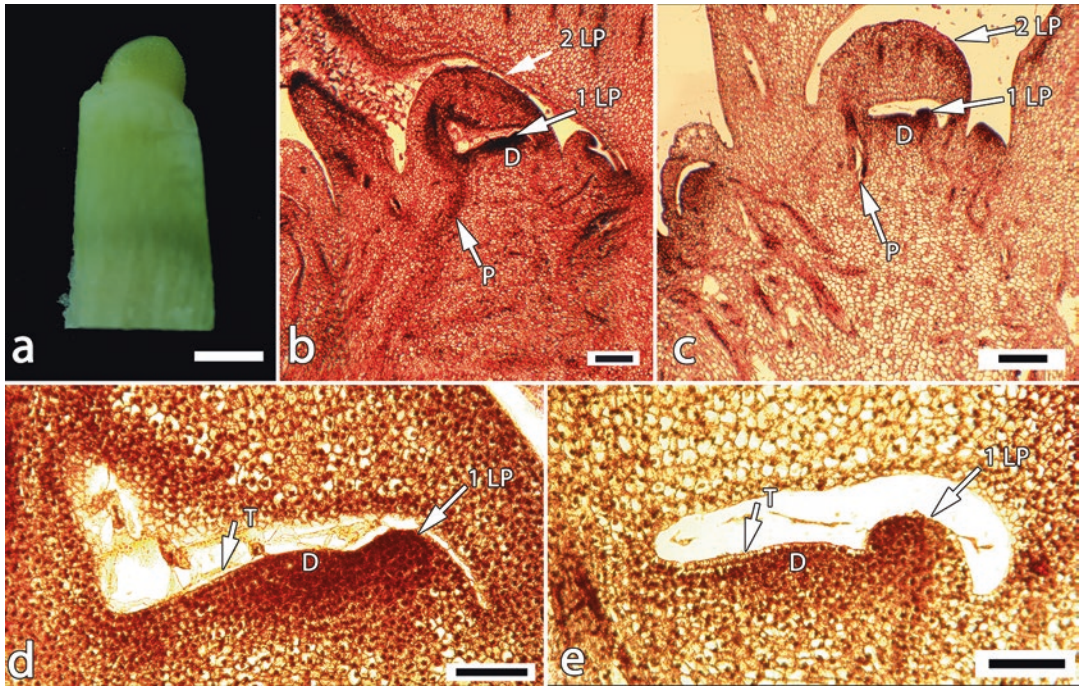


Fig. 3 Histological observation of the meristem tips of commercial mature seedlings in *Phal.* Sogo Yukidian "V3" and *Dtps.* Queen Beer. (**a**) A short stem with a shoot tip of *Phalaenopsis*, Bar = 3 mm. (**b**) *Phalaenopsis* Sogo Yukidian "V3." (**c**) *Doritaenopsis* Queen Beer apical meristems, Bar = 300 μm. (**d**) *P.* Sogo Yukidian "V3" and (**e**) *D.* Queen Beer apical meristems at a higher magnification, showing the slight dome-shaped feature and the dense cytoplasm of meristem cells, Bar = 100 μm. D: dome; P: procambium; LP: leaf primordium; T: tunica

4.2 Sterilizing the Explants

4.2.1 Short Stem Segments from Commercial Flask Plantlets

Work under aseptic conditions using a laminar flow cabinet, remove plantlets from commercial flasks. All stem tips are excised and placed inside a Petri dish and kept for dissection. Utilize the stem tips soon after excision.

4.2.2 Meristem Tips of Inflorescence

Excise approximately 2 cm long of inflorescence tips and place the tips in a 1% NaOCl solution and manually shake for 1 min. Drain the solution and replace by a fresh 1% NaOCl with one to two drops of the wetting agent Tween-20 added. Maintain the meristem tips are in the solution for 8 min via gentle manual shaking. Finally, rinse the tips four times under aseptic condition using sterile water and keep for dissection. Be sure to utilize the meristem tips soon after excision.

4.2.3 Stem Tips from Commercial Mature Seedlings

Remove leaves from selected stem pieces. Rinse the stem tips gently with a mild detergent such as a household dish washing detergent for 2 min. Then place the stem tips in a 1% NaOCl solution and shake manually for 10 min before removing the disinfectant.

Treat the stem tips again with a 0.5% NaOCl solution with one to two drops of Tween-20 added for an additional 15 min via manual shaking. Finally, rinse stem tips four times under aseptic condition using sterile water. Utilize the stem tips soon after excision.

4.3 Meristem Isolation and Culture

Meristem isolation techniques are adopted from Teng et al. [38] for the elimination of systemic pathogens in ginseng micropropagation. Excise the meristem tips from three explant sources under a sterile dissecting microscope housed inside a laminar flow cabinet.

4.3.1 General Dissection Techniques

1. Secure the explant firmly with a pair of forceps at its basal end. Be sure the apical end is clearly visible and focused under a stereomicroscope (*see* **Note 7**).
2. Remove leaf primordium one at a time using a pair of very fine forceps. Follow the phyllotaxis (the arrangement of leaves), peel off cleanly the entire leaf primordium at its base. Gently rotate the explant in order that the next oldest leaf primordium is clearly visible before removing it. Continue to excise as many leaf primordia as possible.
3. Once the apical dome becomes visible, use a sharp knife to remove the apical dome with one or two remaining primordia attached and transfer at once to the culture medium by touching the tip of the knife on the medium surface to dislodge the explant (*see* **Note 8**). Be sure to use a sharp blade to make clean excision in order to avoid unnecessary physical damage to the SAM. A dull knife tends to crush the cells instead of cutting through them (*see* **Note 2**).
4. In dissecting meristems from mature plants and inflorescences from commercial sources, it is imperative to use new sterile dissecting tools in dissecting each specimen in order to avoid potential cross-viral infection (*see* **Note 9**).

4.3.2 Meristem of Stem Segments from Commercial Flask Plantlets

Excise meristem carefully from a stem tip with a single leaf primordium attached. Place the excised meristems onto the Phalaenopsis PLB regeneration medium. Maintain the explants at 25 °C with a 16 h photoperiod and a light intensity of 15 μmol m^{-2} s^{-1}. After 8 weeks of culture, the meristem tips will enlarge and turn green and PLBs will appear after 16 weeks. Excise and subculture the PLBs using fresh PLB regeneration medium for additional 8–10 weeks to allow for plantlet development (Fig. 4).

4.3.3 Meristem Tips of Inflorescence from Commercial Mature Seedlings

Excise flowering meristems with 1–3 leaf primordia from inflorescence tips (Fig. 5a–c) and transfer to the Phalaenopsis PLB regeneration medium. After 8 weeks of culture, calli and PLBs are formed at the meristem tips. Isolate and subculture the PLBs for another 8 weeks in the PLB regeneration medium, following which rooted plantlets with true leaves can be obtained.

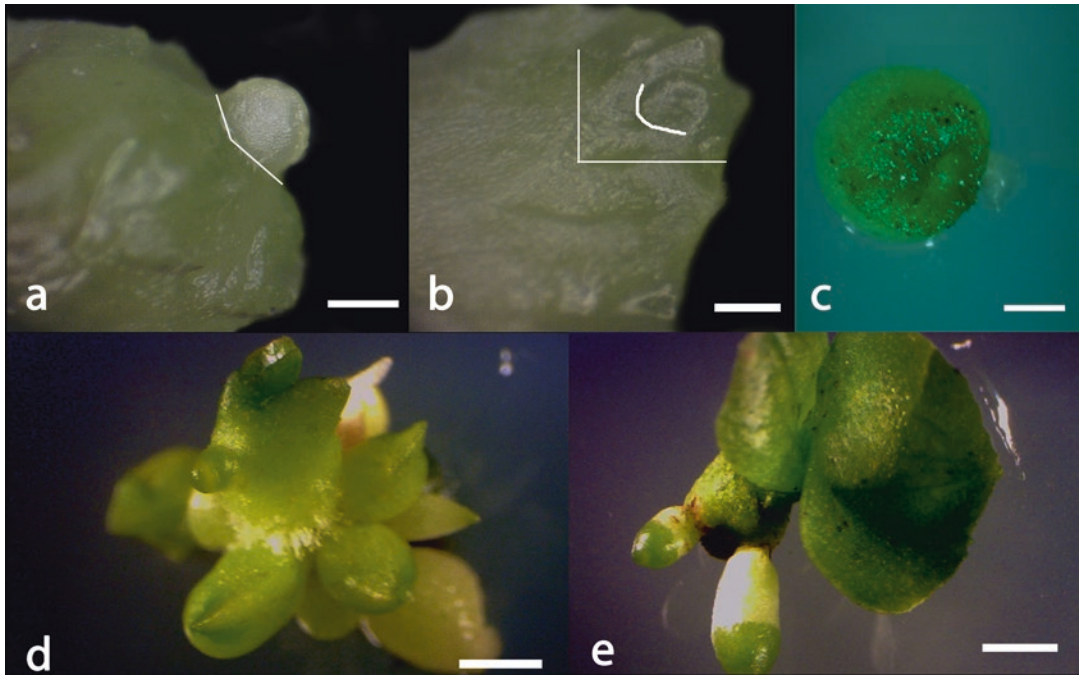


Fig. 4 Isolation of apical meristem and protocorm-like body (PLB) formation from short stem commercial *Phal.* Sogo Yukidian "V3" plantlets in vitro. (**a**) Isolation from the dome with two leaf primordia (*solid line*), Bar = 300 μm. (**b**) Dissected from dome with one primordium (*dotted line*), Bar = 200 μm. (**c**) Swollen and greening of an excised meristem after 8 weeks of culture, Bar = 500 μm. (**d**) PLB formation after 16 weeks of culture, Bar = 3 mm. (**e**) Plantlet regeneration after 8–10 weeks of subculture from PLBs, Bar = 1 cm

4.3.4 Meristem of Short Stems from Commercial Mature Seedlings

Excise apical meristems with one leaf primordium attached from stem tips of mature commercial mature plants (Fig. 5d–f). After 6–8 weeks of culture, the isolated meristems will swell and turn green. Meristem explants will give rise to PLBs after 12–14 weeks of culture. The newly formed PLBs are subsequently excised and subcultured in fresh PLB regeneration medium to form rooted plantlets after another 10–12 weeks.

4.4 Temporary Immersion System for Mass Propagation of Virus-Free Phalaenopsis

This manipulation aims to gain multiple shoots. Selected/coded stock plants tested to be healthy and virus-free are retrieved from flasks under aseptic conditions. Leaves and roots are first removed from stems and placed into ventilated Plantima containers (A-Tech Bioscientific Co. Ltd., Taiwan) for immersion culture (Fig. 6). The conditions for the immersion culture used are detailed by Prommee [39]. Specifically, the short stem segments are immersed in 250 mL of the *Phalaenopsis* shoot multiplication medium at a frequency of once every 4 h for 10 min each time (*see* **Note 7**). The number of axillary shoot buds produced correlates with the number of days in culture. For shoot multiplication, place ten explants per initial Plantima containers. Cut and separate the multiple shoots into

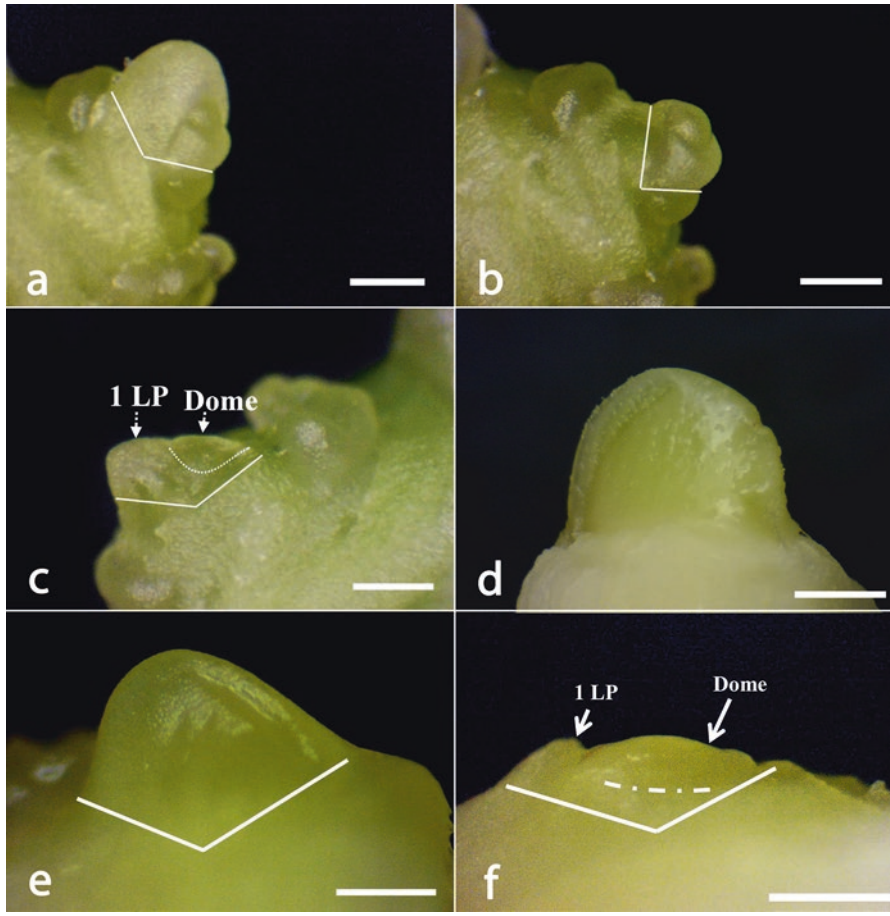


Fig. 5 Dissection and isolation of inflorescence tip and short stem meristems from commercial mature seedlings of *Phal*. Sogo Yukidian "V3." (**a**) Inflorescence tip meristem dissected from a dome with three leaf primordia (*solid line*), Bar = 300 μm. (**b**) Inflorescence tip meristem dissected from a dome with two leaf primordia (*solid line*), Bar = 300 μm. (**c**) Inflorescence tip meristem dissected from a dome (*dotted line*) with one leaf primordium (*solid line*), Bar = 300 μm. (**d**) Short stem meristem dissected from a dome with three leaf primordia, Bar = 1 mm. (**e**) Short stem meristem dissected from a dome with two leaf primordia (*solid line*), Bar = 300 μm. (**f**) Short stem meristem dissected from a dome (*dotted line*) with one leaf primordium (*solid line*), Bar = 300 μm

short stem segments once every month, and change the liquid shoot multiplication medium at the same time.

On average, one single virus-free short stem segment can regenerate 200 plantlets after 5 months of culture (Fig. 7). Once a large number of multiple shoots is obtained, cut and separate the multiple shoots into single shoot and place them onto the Phalaenopsis plantlet root initiation medium. After about 3–4 months of culture, the plantlets with well-developed roots will be ready for deflasking. This method proves to be effective for the mass production of virus-free plants and can serve as a reference for industrial applications (Fig. 8).

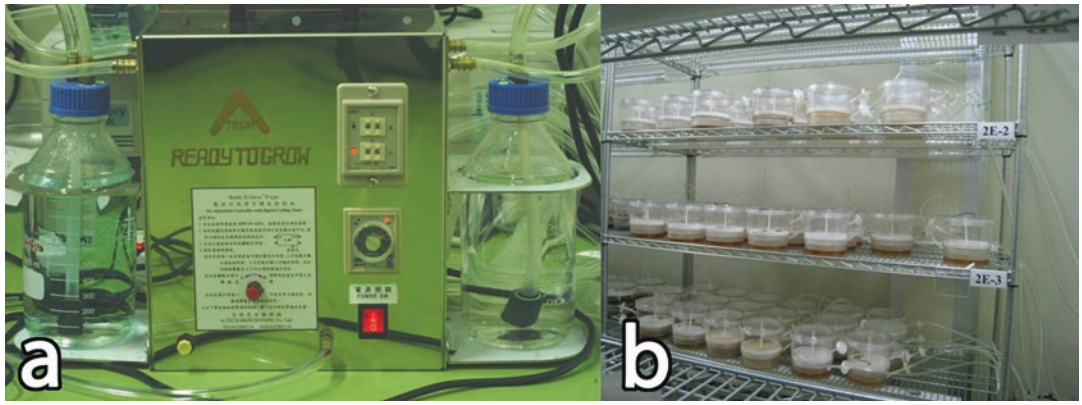


Fig. 6 (**a**) Basic equipment of the temporary immersion system (TIS) and (**b**) ventilated Plantima plant tissue culturing containers

5 Conclusion

Meristem isolation is relatively easy to perform. Since the objective is to isolate potential virus-free SAMs, extra precautions are needed, such as the use of sterilized tools in order to avoid cross-contamination. A minimal size of meristem explants is preferred as this increases the chance of obtaining virus-free plants. Besides issues with physical excision, the ability of the SAM to develop in vitro requires careful selection of culture media and optimization of growth regulators used in promoting the growth and differentiation of the explants. Patience and practice are needed to ensure success in meristem isolation and the production of virus-free plants.

6 Notes

1. Besides apical meristem culture, generation of virus-free *Phalaenopsis* plants can be achieved through seed germination. Clean seedlings can be obtained through aseptically germinated seeds in vitro, as seeds seldom harbor viruses.
2. Fine dissecting tools such as knives, special blades, and forceps can be purchased from suppliers specializing in surgical instruments such as the Fine Science Tools (www.finescience.ca).
3. For details on ELIZA supplies for virus detection and related detection methods, *see* Chap. 18.
4. Orchid virus detection kits can be obtained commercially from companies such as the Dr. Chip Company (www.bio-drchip.com.tw). This is a one-step multiplex RT-PCR system. The latest DR. Orchid-5®Kit can detect five different kinds of viruses simultaneously.

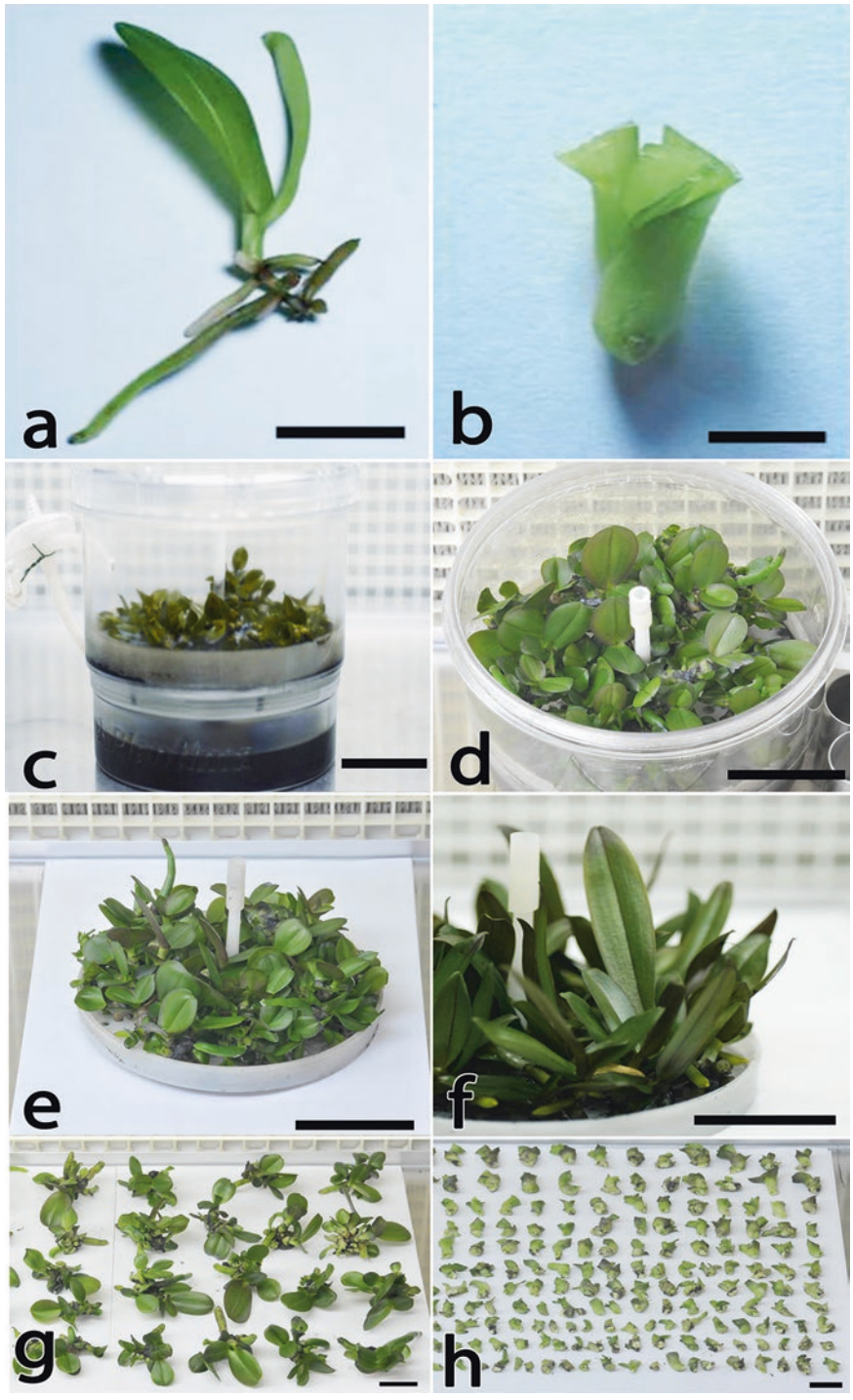


Fig. 7 Mass propagation of virus-free *Phalaenopsis* plantlets via the temporary immersion system (TIS). (**a**) Healthy virus-free donor plantlet, Bar = 1 cm. (**b**) Short stem with most of its leaves and roots removed, Bar = 1 cm. (**c**) TIS device (Plantima), Bar = 5 cm. (**d**, **e**) Growth of shoots after 4 months of subculturing, Bar = 5 cm. (**f**) Young plantlets with elongated leaves and roots after 5 months of culturing, Bar = 5 cm. (**g**, **h**) On average, 200 shoots can be regenerated from a single virus-free shoot of *Phal.* Sogo Yukidian "V3" after 5 months of subculture; (**g**) Bar = 2 cm, (**h**) Bar = 1 cm

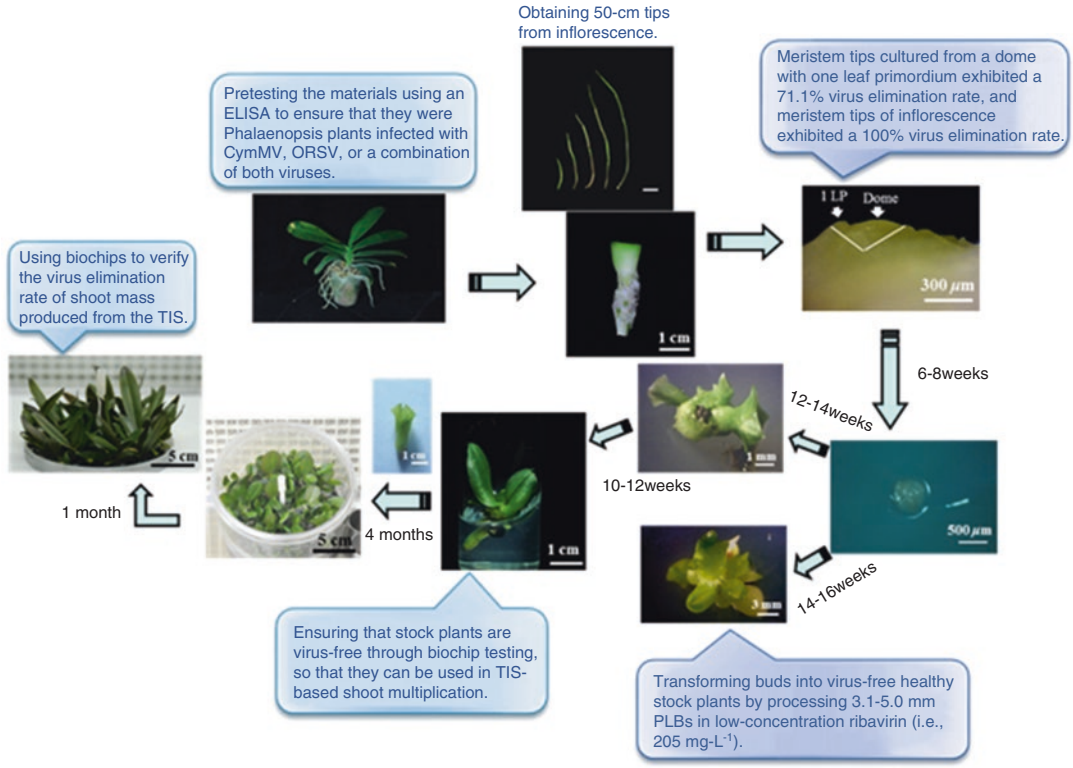


Fig. 8 Virus-free seedlings cultured from *Phalaenopsis* meristem tips and PLBs, and the rapid multiplication performance of explants using the TIS

5. Ventilated Plantima containers are produced by A-Tech Bioscientific Co. Ltd., Taiwan. The container has an upper and lower compartment separated by a perforated platform. The upper compartment serves to house the plantlets while the bottom portion serves as nutrient reservoir.
6. General information and how the system works can be found in the A-Tech Bioscientific Company website (www.atechbios.com.tw). The operation includes four stages: (a) a stand-by static state, (b) the aeration stage using increased gas pressure to force the liquid culture medium to enter the culture zone supplying nutrients to the plantlets, (c) an aeration and immersion stage with continual agitation and oxygenation, and (d) flow back of liquid medium back to the reservoir at the bottom of the culture vessel by gravity.
7. All contaminated materials/tools should be autoclaved irrespective of whether they will be reused or not. After each dissection, tools should be placed into 10% commercial bleach solution and followed by autoclaving before reuse. After each dissection, all working areas and laminar flow cabinet should be decontaminated by cleaning with aseptic cleaner.

8. A shoot apical meristem needs to be excised quickly using shape dissecting knives. During the course of dissection, it is essential that the meristems are kept moist and not dehydrated. At the time of dissection, the explant can be placed on solid nutrient medium and use it as a support. The agar medium provides moisture during the course of dissection, preventing desiccation of explants.
9. Theoretically, it would be nice to remove all the leaf promorida surrounding the apical meristem dome. Since the apical dome tends to be very small, any attempt to remove the last one or two leaf primordia can cause major physical damage to the apical meristem. Thus, the meristem is usually excised together with one or two leaf primordia attached.

References

1. Chang CA (2006) Diagnosis, appraisal, and detection of orchids virus. Symposium of health inspection, quarantine diagnosis, and detective technology of plant disease. Vol V. Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, Executive Yuan. Taiwan; p. 103–118
2. Yam TW, Arditti J (2009) History of orchid propagation: a mirror of the history of biotechnology. Plant Biotechnol Rep 3:1–56
3. Morel G, Martin C (1952) Guerison de dahlias atteints dune maladie a virus. Comptes rendus hebdomadaires des seances de l academie des. Sciences 235:1324–1325
4. Morel G (1960) Producing virus-free *Cymbidiums*. Amer Orchid Soc Bull 29:495–497
5. Nehra NS, Kartha KK (1994) Meristem and shoot tip culture: requirements and applications. In: Vasil IK, Thorpe TA (eds) Plant cell and tissue culture. Kluwer, Dordrecht, pp 37–70
6. Panattoni A, Luvisi A, Triolo E (2013) Review. Elimination of viruses in plants: twenty years of progress. Span J Agric Res 11:173–188
7. Meri K, Hosokawa D (1977) Localization of viruses in apical meristem and production of virus-free plant by means of meristem and tissue culture. Acta Hort 78:389–397
8. Grout BWW (1990) Meristem-tip culture for propagation and virus elimination. In: Hall RD (ed) Plant cell culture protocols. Humana Press, Totowa, NJ, pp 115–125
9. Kane ME (2005) Shoot culture procedures. In: Trigiano RN (ed) Plant development and biotechnology. CRC Press, Boca Raton, FL, pp 145–157
10. Novak FJ, Maskova I (1979) Apical shoot tip culture of tomato. Sci Hortic 10:337–344
11. Quak F (1977) Meristem culture and virus-free plants. In: Reinert J, Bajaj YPS (eds) Applied and fundamental aspects of plant cell, tissue, and organ culture. Springer, Berlin, Heidelberg/New York, pp 598–615
12. Vasil IK, Thorpe TA (eds) (1994) Plant cell and tissue culture. Kluwer, Dordrecht
13. George EF, Hall MA, De Klerk GJ (2007) Plant propagation by tissue culture 3rd edition: volume 1. The background. Exegetic, Basingstoke
14. Trigiano RN, Gray DJ (2000) Plant tissue culture: concepts and laboratory exercises, 2nd edn. CRC Press, Boca Raton, FL
15. Cutter EG (1971) Plant anatomy. Experiment and interpretation. Part 2. Organs. Edward Arnold, London
16. Kumar S, Kan MS, Raj SK, Sharma AK (2009) Elimination of mixed infection of *Cucumber mosaic* and *Tomato aspermy virus* from *Chrysanthemum morifolium* Ramat. cv. Pooja by shoot meristem culture. Sci Hortic 119:108–112
17. Wang Q, Valkonen JPT (2008) Elimination of two viruses which interact synergistically from sweet potato by shoot tip culture and cryotherapy. J Virol Methods 154:135–145
18. Verbeek M, Dijk P, Well PMA (1995) Efficiency of eradication of four viruses from garlic (*Allium sativum*) by meristem-tip culture. Eur J Plant Pathol 101:231–239
19. Kang F-U, Hsu S-T, Shen R-S (2011) Virus elimination through meristem culture and rapid clonal propagation by temporary immer-

sion system in *Phalaenopsis*. J Taiwan Soc Hort Sci 57:207–218

20. Santa Cruz S (1999) Perspective: phloem transport of viruses and macromolecules- what goes in must come out. Trends Microbiol 7:237–241
21. Moreno IM, Thompsont JR, Garcia-Arenal F (2004) Analysis of the systemic colonization of cucumber plants by *Cucumber green mottle mosaic virus*. J Gen Virol 85:749–759
22. Opalka N, Brugidou C, Bonneau C, Nicole M, Beachy RN, Yeager M, Fauquet C (1998) Movement of rice yellow mottle virus between xylem cells through pit membranes. Proc Natl Acad Sci 95:3323–3328
23. Carrington JC, Kasschau KD, Mahajan SK, Schaad MC (1996) Cell-to-cell and long-distance transport of viruses in plants. Plant Cell 8:1669–1681
24. Hsieh SC (2006) Studies on the virus-elimination techniques of green onion 'Lan Yang No. 1'and 'Tao Yuan No. 3'. MS thesis. National Chung Hsing University Taichung, Taiwan
25. Hull R (2002) Mathews' plant virology, 4th edn. Academic Press, Cambridge, MA
26. Pennazio S, Roggero P (1996) Plant hormones and plant virus diseases. The auxins. New Microbiol 19:369–378
27. Lim ST, Wong SM, Goh CJ (1993) Elimination of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* from orchids by meristem culture and thin section culture with chemotherapy. Ann Appl Biol 122:289–297
28. Loi JS, Lee SM, Lam-Chan LT, Fan S, Yiang WH (1991) Eradication of orchid viruses in *Dendrobium* Sonia using virazole. Singapore J Primary Ind 19:16–22
29. Chien K-W, Agrawal DC, Tsay H-S, Chang C-A (2015) Elimination of mixed 'Odontoglossum ring sport' and 'Cymbidium mosaic' viruses from Phalaenopsis hybrid 'V3' through shoot-tip culture and protocorm-like body selection. Crop Prot 67:1–6
30. Park SY, Yeung EC, Chakrabarty D, Paek K (2002) An efficient direct induction of protocorm-like bodies from leaf subepidermal cells of *Doritaenopsis* hybrid using thin-section culture. Plant Cell Rep 21:46–51
31. Etienne H, Berthouly M (2002) Temporary immersion systems in plant micropropagation. Plant Cell Tiss Org Cult 69:215–231
32. Watt MP (2012) The status of temporary immersion system (TIS) technology for plant micropropagation. Afr J Biotechnol 11:14025–14035
33. Georgiev V, Schumann A, Pavlov A, Bley T (2014) Temporary immersion systems in plant biotechnology. Eng Life Sci 14:607–621
34. Shen TM, Shen RS, Chan SI (2004) Protocorm like bodies induction of *Phalaenopsis* hybrids using short-stems derived *in vitro* shoots as explants. APOC 8:293–307
35. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–479
36. Kang FY (2012) Virus elimination through shoot apical meristem, protocorm-like bodies culture and rapid clonal propagation by temporary immersion system in Phalaenopsis. MS thesis. National Chiayi University, Chiayi, Taiwan
37. Stasolla C, Yeung EC (2015) Paraffin and polyester waxes. In: Yeung ECT, Stasolla C, Sumner MJ, Huang BQ (eds) Plant microtechniques and protocols. Springer, New York, pp 45–66
38. Teng WL, Sin T, Teng MC (2002) Explant preparation affects culture initiation and regeneration of *Panax ginseng* and *Panax quinquefolius*. Plant Cell Tiss Org Cult 68:233–239
39. Prommee P (2009) Establishment of regeneration system via axillary bud culture in *Phalaenopsis* by temporary immersion system. MS thesis, Department of Horticulture National Chiayi University, Chiayi, Taiwan, ROC

Chapter 15

Cryopreservation

Wagner A. Vendrame

Abstract

The diversity and beauty of orchids place them among the top flowering plants in the international market. They are commercialized either as cut flowers or potted plants. With over 25,000 estimated known species and over 100,000 hybrids developed, they represent about 10% of all flowering plants. The development of feasible techniques for the conservation of orchid material with low maintenance costs, greater storage time, and good phytosanitary conditions is essential for the conservation of species as well as commercial use of hybrids. Cryopreservation is a technique that involves the conservation of biological material at ultra-low temperatures, generally at −196 °C in liquid nitrogen or at −150 °C in nitrogen's vapor phase. Currently, this is the only feasible technique for the long-term preservation of genetic material from plants that are vegetatively propagated, or with unviable or recalcitrant seeds. Due to the large diversity of orchids, this is a developing and relevant field that requires continued research and development of improved techniques according to the species and/or hybrid. In this chapter, we present simple methods and their applications for the cryopreservation of orchid seeds, protocorms, and pollen.

Key words Conservation, Germplasm storage, Liquid nitrogen, Orchidaceae, Pollinia, Protocorm, Seed, Ultra-low temperature

1 Introduction

Cryopreservation is a process by which live plant cells, tissues, or organs are stored at ultra-low temperatures (−196 °C in liquid nitrogen or −150 °C in nitrogen's vapor phase) over long periods of time with reduced risks of genetic and physiological variations [1, 2]. Cryopreservation allows storage of valuable genotypes while maintaining viability of germplasm at low cost and under stable and safe conditions from diseases or damages caused by the environment [3]. This technique allows the long-term storage of different types of tissues and organs, including shoot tips, somatic and zygotic embryos, whole seeds, pollen, anther, and buds.

There are different types of cryopreservation techniques. Traditional cryopreservation techniques are based on freeze-induced dehydration for cryostorage of undifferentiated cultures

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_15,

and apices of cold-tolerant species [4]. The process includes slow cooling of tissues to a defined prefreezing temperature, which allows supercooling of cells and the external medium, followed by rapid immersion in liquid nitrogen [5]. Because expensive programmable freezers are required for such techniques, operations are usually complex. However, slow freezing can also be performed with a simple domestic or laboratory freezer [6, 7].

More advanced cryopreservation techniques, such as vitrification-based procedures, involve cell dehydration prior to freezing. Samples are exposed to a cryoprotective medium usually called vitrification solution, with or without supplemental air desiccation, followed by rapid cooling. This prevents the formation of intracellular ice formation. The advantages of vitrification procedures include ultra-rapid freezing, which is more adequate for complex organs, such as shoot tips and embryos, which contain different types of cells, and therefore unique requirements for freeze-induced dehydration, and can be used in any tissue culture laboratory [8]. Vitrification systems are simpler to operate because they do not require controlled freezers, prevent intracellular ice formation, and therefore allow broader application, with only minor modifications required for different cell types [7]. This makes the system cost-effective, simple, and feasible for commercial applications. They also allow cryopreservation to be applied to a wide variety of plant species, particularly non-cold hardy tropical plants [9].

Seven vitrification-based procedures have been developed and are described by [10, 11]: (1) Encapsulation–dehydration; (2) Vitrification; (3) Encapsulation–vitrification; (4) Dehydration; (5) Pregrowth; (6) Regrowth–dehydration; and (7) Droplet freezing.

Encapsulation–dehydration is based on the technology developed for encapsulating somatic embryos in calcium alginate beads as synthetic seeds [12]. This technique involves the preculture of explants in liquid medium containing a high sucrose concentration, followed by partial desiccation to low moisture content prior to immersion in liquid nitrogen. This prevents the formation of intracellular ice and can enhance explant survival, particularly for sensitive organs, such as somatic embryos, protocorms, and protocorm-like bodies (PLBs) after cryopreservation [13]. Many species have been cryopreserved using encapsulation–dehydration techniques [2, 14–19]. These include *Centaurium rigualii* [1], alfalfa [20], hop and pelargonium [21], citrus [22], grapevine [23], cocoa [24], *Melia azedarach* [25], gentian [26], and raspberry [27], among others.

Several orchid species and hybrids have also been successfully cryopreserved, including *Bletilla striata* [28–30], *Dendrobium species and hybrids* [31–34], *Oncidium flexuosum* [35], *Phalaenopsis* [36], and *Vanda pumila* [37], among many others. Cryopreservation techniques and protocols have been reported and reviewed for

orchid seeds, pollen, protocorms, meristems, and shoot apices for many different species and hybrids [38, 39].

1.1 Cryopreservation of Seeds

Orchids have very small, dust-like seed, which do not contain endosperm and are produced in large numbers (thousands to millions) within seed capsules. This provides unique possibilities for orchid seed storage and long-term conservation of different orchid species. Orchid seed storage allows preservation of endangered material with little space required, which allows storage of plant material for breeding and genetic improvement programs [40]. Orchid germplasm storage also facilitates distribution of plant material at reduced costs [41]. Conventional storage methods used for seeds in general involve temperatures of −18 °C and humidity reduced to 5%, and orchid seeds can be stored under similar conditions. However, traditional methods are not recommended for long-term orchid seed conservation, as seed viability and longevity are highly variable [41]. In addition, data on viability of orchid seeds after years of storage is limited [42]. The storage conditions for orchid seeds are influenced by the storage method used, the species selected, and the seed lot [38]. Storage of seeds from 30 different orchid species showed that viability was maintained for 3 years when stored at −10 °C, with subsequent decline over 10 years of storage, leading to death of seeds [43, 44]. However, Ito [45] indicated that seeds should be fresh when storing them in ultra-low temperatures (−79 °C), and moderate desiccation should be considered prior to storage for successful recovery.

In early cryopreservation studies, Pritchard et al. [46] showed successful recovery of seeds from ten orchid species after cryopreservation, whereby seeds maintained viability. However, as indicated above, differences in seed lots affect cryopreservation and subsequent seed viability. Seaton and Hailes [47] reported loss of viability in one lot of *Guarianthe aurantiaca* seeds stored for 50 days at −18 °C, while a different lot stored for 400 days under the same conditions showed high viability [48].

Orchid seed cryopreservation techniques have advanced in recent years resulting in more simple and effective protocols for long-term storage of orchid seeds under ultra-low temperatures. The use of the encapsulation–dehydration technique for the cryopreservation of immature seeds of *Cyrtopodium hatschbachii* returned a 64% increase in seed germination and survival of the acclimatized plants after cryopreservation [49]. By evaluating new cryoprotectants for the cryopreservation of seeds of *Dendrobium* hybrids, Galdiano Jr. et al. [33] showed that 1% phloroglucinol added to a previtrification solution (PVS2) returned higher seed germination rates (79%) after cryopreservation compared to 1% Supercool X1000 ®, which showed no effect in seed germination. Vitrification has been shown to be a simple, safe, and cost-effective

method for cryopreservation of orchid seeds. However, somaclonal variation [50] caused by the genetic instability of explants [51] can occur as a result of exposure to excessive low temperatures and certain cryoprotectants. Galdiano Jr. et al. [34] evaluated the genetic stability of cryopreserved seeds from *Dendrobium* hybrids using flow cytometry. Results confirmed the genetic integrity of seedlings recovered after cryopreservation.

1.2 Cryopreservation of Protocorms

Protocols for cryopreservation of orchid protocorms have been developed for species and hybrids. Protocorm cryopreservation allows the long-term storage of germplasm for breeding and conservation programs as they provide a fast means of plant regeneration [28].

Among different factors affecting the recovery of cryopreserved protocorms, the culture media play an important role, as demonstrated by Popova et al. [52]. The authors showed the effect of different culture media on the development of protocorms from cryopreserved *Bratonia* hybrid seeds. Best results were obtained in Morel [53] and MS [54] media.

Similar to seeds, the selection of proper cryoprotectants prior to cryopreservation is essential for the successful recovery of cryopreserved protocorms. Combinations of cryoprotectants have been evaluated for cryopreservation of orchid protocorms, such as for *Dendrobium nobile* [55]. The use of a 2 M glycerol solution (20 min) combined with a vitrification (PVS2) solution with 1% phloroglucinol (10 min) provided the best recovery, with 68% survival of protocorms. The cryopreservation of *Dendrobium* hybrid Den. Swartz 'Dong Yai' protocorms was evaluated using two cryoprotectants, phloroglucinol and Supercool X1000 [33]. In this study, pretreatment of protocorms was evaluated with 0.3 M sucrose for 24 h, followed by exposure to PVS2 solution containing either 1% phloroglucinol or 1% Supercool X1000 for 15 min at 0 °C prior to immersion in liquid nitrogen. Protocorm survival was evaluated after 75 days and germination percentage was either higher or similar to the initial germination for treatments using 1% phloroglucinol alone. Such studies showed the potential of phloroglucinol as an alternative cryoprotectant.

Different cryopreservation techniques have also been evaluated for the cryopreservation of orchid protocorms. Gogoi et al. [56] compared vitrification and encapsulation–vitrification for cryopreservation of protocorms of *Cymbidium eburneum* L. Higher regeneration (66%) was obtained when using the encapsulation–vitrification method. Mata-Rosas and Lastre-Puertos [57] compared four different techniques, including encapsulation–dehydration, encapsulation–vitrification, encapsulation–dehydration–vitrification, and vitrification alone for the cryopreservation of protocorms of *Brassavola nodosa* (L) Lind.

Although protocorm recovery and growth was observed for all techniques, the authors suggest the use of vitrification due to the lower cost and lesser labor.

1.3 Cryopreservation of Pollen

The use of germplasm banks allows the preservation of pollen of rare, threatened and/or endangered species [2, 58–61]. Long-term pollen storage is of importance for the conservation of genetic diversity and for use in plant breeding programs. Pollen storage can also assist in the development of haploid plants through the development of embryos from pollen grains [62]. Cryopreservation of pollen provides a feasible means for storage of large amounts of genetic material [61] as well as studies in fundamental and applied pollen biology [63]. Pritchard et al. [64] suggest the use of cryopreservation for the creation of orchid germplasm banks with seeds and pollen.

Proper pollen storage is important for orchid production and reproduction, enabling crosses between plants that exhibit temporal and spatial separation in their periods of sexual reproduction [65]. Temperatures for pollen storage vary from −20, −4 to +4 °C, and lower temperatures provide greater longevity for pollen, provided that intracellular water content is maintained low [61].

In early studies, Ito [45] indicated that fresh pollen from different orchid species and hybrids could be preserved in ultra-low temperature (−79 °C), combined with moderate desiccation for proper recovery. However, more recently Vendrame et al. [65] reported the cryopreservation of pollen from two *Dendrobium* hybrids using simple and direct storage in liquid nitrogen without the need for pretreatments or cryoprotectants. Cryopreservation effectiveness was evaluated by using pollen that had been cryopreserved in the pollination of flowers of the same hybrids. Although the pollen had been submitted to the different cryopreservation treatments, no significant differences were observed among treatments and controls. All flowers pollinated with cryopreserved pollen from all treatments and controls formed capsules and viable seeds.

2 Materials

2.1 Laboratory Equipment

1. Stereomicroscope, e.g., Leica MZ12.5 stereoscope
2. Fluorescent light
3. Laminar flow hood
4. Cryotank
5. Orbital shakers
6. Drying oven
7. Growth chamber such as the Percival E30B (Percival Scientific, Inc., Perry, IA, USA)

2.2 General Laboratory Supplies

1. Dissection tools, i.e., scalpels, forceps, scissors, and needles
2. Routine laboratory supplies such as flasks, disposable transfer pipette, and Petri plates
3. Microscope slides
4. Syringes
5. Cryovials (2 mL)
6. Culture vessels such as baby food jars, Phytotech P700 culture boxes, and Magenta GA7 boxes
7. Coconut coir (Coco Gro-Brick, OFE International, Miami, FL, USA)
8. Aluminum foil

2.3 Chemicals and Solutions

1. 2,3,5-triphenyl tetrazolium chloride (TTC) for viability testing
2. Fluorescein diacetate (FDA) for viability testing
3. Ethanol (70 and 95%)
4. Sterilizing solution—0.3 and 0.6% sodium hypochlorite solutions
5. Sucrose
6. Agar (Fisher, Chicago)
7. PVS2 [66], consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide (DMSO) in half-strength MS medium [54] with 0.4 M sucrose (pH 5.7).
8. Supercool X-1000 ® (21st Century Medicine, Fontana, CA, USA)
9. Liquid nitrogen (LN)
10. Phloroglucinol (1,3,5-trihydroxybenzene)
11. Murashige and Skoog culture medium
12. Peters Orchid Food (Spectrum Group, St. Louis, MO, USA) consisting of 30% total N, 10% P_2O_5, 10% K_2O, 0.5% Mg, 0.02% B, 0.05% chelated Cu, 0.1% Fe, 0.05% Mn, 0.0005% Mo, and 0.05% Zn.

3 Methods

3.1 Seeds

3.1.1 Seed Selection, Collection, Measurement, and Viability

Seeds can be obtained from either mature or immature seed capsules (Fig. 1a, b). Orchid seed capsules from species or hybrids are usually collected and stored at room temperature (27 ± 2 °C) in a desiccator for 24 h. For purposes of record keeping, capsule size (length × width) is measured using a caliper. Seeds are removed from capsules (Fig. 1c, d) and seed size evaluated with a micrometer by measuring length x width of individual seeds under a

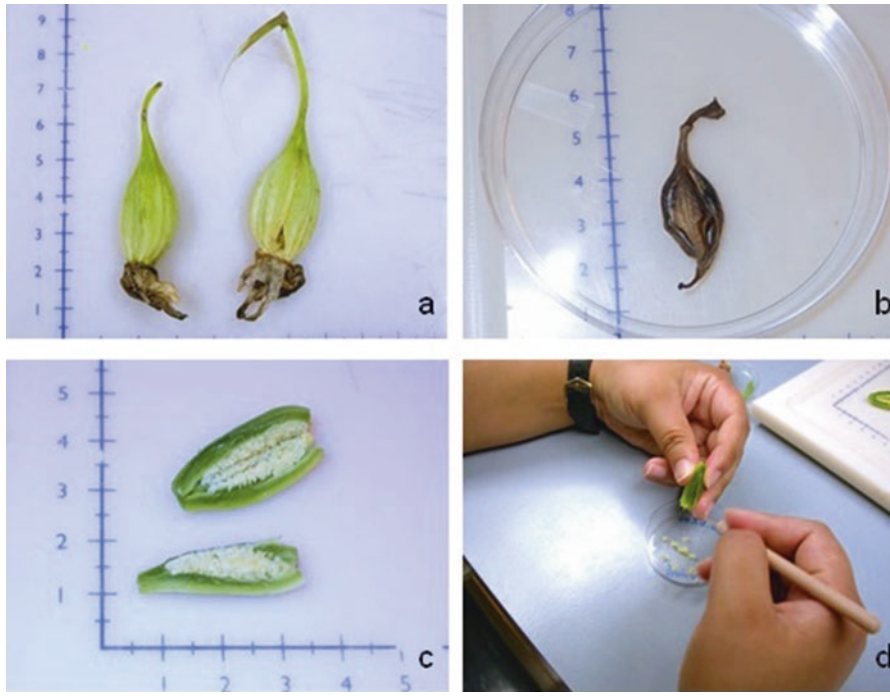


Fig. 1 Immature (**a**) and mature (**b**) seed capsules from orchids. Immature seed capsule open showing orchid seeds inside (**c**). Removal of orchid seeds from seed capsule (**d**)

stereoscope, such as the Leica MZ12.5 stereoscope (Leica Microsystems, Buffalo, NY, USA) at 50× magnification. Capsule weight with and without the seeds is calculated. Seeds are weighted and oven-dried at 103 °C for 17 h to constant weight, and seed moisture content is determined. If mature capsules are open, seeds will need to be disinfected, which will reintroduce moisture. Therefore, final seed moisture content may also have to be determined after seed are disinfected. However, if using intact mature seed capsules or immature seed capsules, which are fully closed, seed disinfection is not necessary and therefore only the initial seed moisture is calculated as described previously. Seed viability can be determined using the fluorescein diacetate (FDA) staining technique [44]. Briefly, orchid seeds are first removed from capsules (Fig. 2a) and immersed in distilled water. Next, seeds are mixed 1:1 (v/v) on a microscope slide with a solution of FDA at 0.5% (w/v) in absolute acetone. Embryos within the seed are observed under UV light for fluorescence, shown as a bright yellow color, thus indicating viability (Fig. 2b). Seed viability can also be assessed using a modified 2,3,5-triphenyl tetrazolium chloride (TTC) test [67]. The TTC viability test consists of exposing seeds to 1% TTC (by volume) at pH 6.5 for 24 h in dark at 27–30 °C. Viable embryos stain red (Fig. 2c, d). If necessary, a seed germination test can also be performed to verify the consistency of the viability tests.

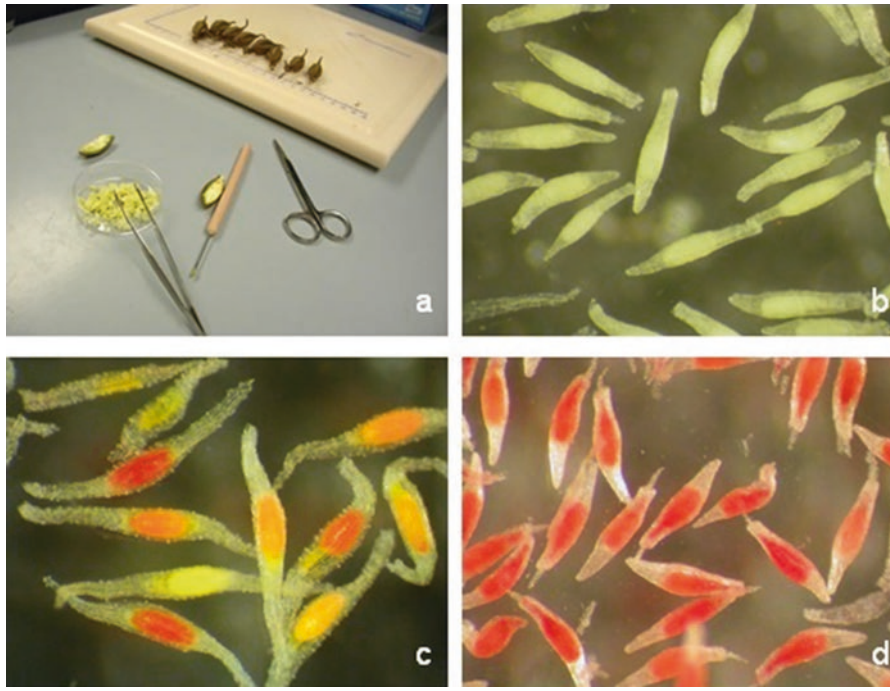


Fig. 2 Seeds removed from capsule prior to viability tests (**a**). Viability test using fluorescein diacetate, showing seeds with viable embryos emitting fluorescence (**b**). Viability test using tetrazolium chloride, showing seeds with various degrees of viability (**c**), and seeds with fully viable embryos stained dark red (**d**)

The number of seeds per capsule can be estimated by weight difference (capsule weight with seeds—capsule weight without seeds) and by calculating the weight of 100 seeds.

3.1.2 Seed Disinfection and Preparation

As indicated above, if mature capsules are open, seeds will need to be disinfected. About 250 mg of seeds from each species or hybrid is placed inside a 100-mL sterile syringe containing 50 mL of 70% ethanol (v/v) and agitated for 1 min (Fig. 3a, b). The ethanol is removed with a plastic disposable transfer pipette and 50 mL of 0.6% sodium hypochlorite is added, followed by agitation for 20 min. After removal of the sodium hypochlorite solution, seeds are rinsed in sterile deionized water twice and transferred to a sterile 150-mL flask. They are left in the flasks overnight at room temperature (27 ± 2 °C). The following day, seeds are distributed in 2-mL cryovials, containing about 1 mL of seed suspension per vial. Because moisture is reintroduced during disinfection, seed moisture content may be determined again after seed are disinfected. Through serial dilutions, 1 mL of solution is calibrated to contain approximately 1000 seeds. Prior to serial dilutions, samples are removed, weighed, and oven-dried as described above to verify the variation in seed moisture content as affected by the seed disinfection technique.

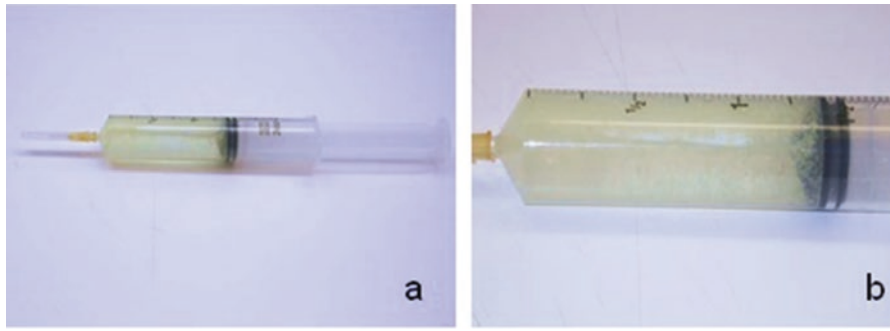


Fig. 3 Orchid seeds are placed inside a syringe for disinfection (**a**). Close-up of syringe containing orchid seeds and disinfection solution (**b**)

3.1.3 Vitrification Procedure and Treatments

Prior to any treatments to be applied, sterile deionized water is removed from each cryovial and 1 mL cryoprotective solution (2 M glycerol and 0.4 M sucrose, pH 5.7) is added. Cryovials are left for 30 min at room temperature (27 ± 2 °C) prior to the addition of a plant vitrification solution (Fig. 4a, b). The plant vitrification solution, designated PVS2 [66], consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide (DMSO) in half-strength MS medium [54] with 0.4 M sucrose (pH 5.7).

The following treatments were evaluated with *Dendrobium* hybrids [40] to determine the best combination of low temperature (precooling) with dehydration treatments prior to cryopreservation for successful recovery and germination of seeds. For cryopreservation, samples in cryovials are placed in racks, which are immersed in liquid nitrogen inside a cryotank (Fig. 4c, d). Therefore, treatments may vary from species to species, or hybrid to hybrid and need to be adjusted accordingly. Treatments consisted of seeds left in PVS2 either at room temperature (27 ± 2 °C) or precooled in ice (0 °C) for 1, 2, 3, 4, or 5 h prior to immersion in liquid nitrogen (LN). Controls consist of seeds placed for germination into Petri dishes containing half-strength MS medium immediately after exposure to—control 1: no PVS2, room temperature, no LN; control 2: 3 h in PVS2, ice temperature, no LN; control 3: addition of PVS2 followed by immediate immersion in LN for 14 days (30-s exposure period to PVS2 prior to LN); control 4: no PVS2, room temperature, LN for 14 days. Control 1 contained 1 mL liquid half-strength MS medium with 58.5 mM sucrose (pH 5.7) replacing PVS2.

Additional treatments could determine the efficiency of cryoprotectants for seed cryopreservation. We have evaluated phloroglucinol and Supercool X-1000 ® as potential cryoprotectants for orchid seeds using the following treatments: control 1 (no PVS2, no phloroglucinol, no Supercool X-1000 ®); control 2 (PVS2 for 60 min); treatment 1 (PVS2 60 min + 1% phloroglucinol);

Fig. 4 Cryovials left at room temperature (27 ± 2 °C) prior to the addition of a plant vitrification solution (**a**, **b**). Cryovials are placed in racks (**c**), which are immersed in liquid nitrogen inside a cryotank (**d**)

treatment 2 (PVS2 60 min + 1% Supercool X-1000 ®); and treatment 3 (PVS2 60 min + 1% phloroglucinol +1% Supercool X-1000 ®). Similar to precooling treatments, they need to be evaluated and adjusted according to each species and/or hybrid.

3.1.4 Post-cryopreservation Recovery and Seed Germination

For controls with no exposure to LN, seeds are placed on germination medium after each control procedure. For controls and treatments submitted to LN, after 14 days cryovials are rapidly re-warmed in a 40 °C water bath for 1–2 min and the PVS2 solution is removed using a plastic disposable transfer pipette. About 1 mL of half-strength liquid MS medium with 1.0 M sucrose is added to each vial and held for 1 h at room temperature, followed by two rinses in 1 mL half-strength liquid MS medium with 58.5 mM sucrose (pH 5.7). Seeds are then placed in Petri dishes

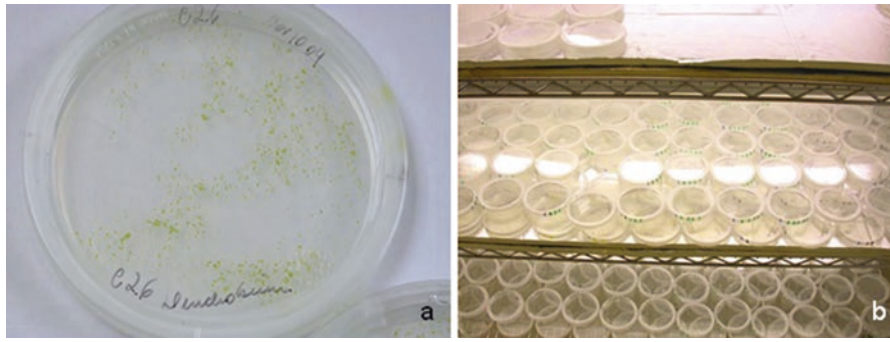


Fig. 5 Orchid seeds placed in Petri dishes containing germination medium (**a**). Petri dishes are maintained under controlled temperature and light conditions (**b**)

containing half-strength MS medium with 58.5 mM sucrose and solidified with 0.6% agar (Fisher, Chicago, IL, USA) (Fig. 5a) under controlled environmental conditions (27 ± 2 °C; 60 μmol m^{-2} s^{-1}; 18/6 light/dark; 2 × 9A Philips® fluorescent bulbs) (Fig. 5b). Plates should be visually monitored on a weekly basis for germination occurrence. Germination percentage is determined by counting the number of germinated seeds under a stereoscope, such as the Leica MZ12.5 stereoscope at 50× magnification. Seed survival is measured by germination.

3.1.5 Seedling Growth and Transplantation

Germinated seedlings should be monitored weekly for shoot and root development (Fig. 6a). As seedlings grow, they may need to be transplanted into either Magenta GA7 boxes (Sigma-Aldrich Co., St. Louis, MO, USA), baby food jars, or Phytotech P700 culture boxes (Phytotechnology Laboratories, Shawnee Mission, KS, USA) containing the same medium as described above for germination, where they are maintained for continued growth and development (Fig. 6b). Light and temperature conditions are the same as for germination; 27 ± 2 °C; 60 μmol m^{-2} s^{-1}; 18/6 light/dark; 2 × 9A Philips® fluorescent bulbs. Once seedlings are fully developed, they can be transplanted to pots (10.16 cm diameter) with 3–4 plants per pot containing coir dust (Coco Gro-Brick, OFE International, Miami, FL, USA) as the substrate. Pots can be maintained in an environmentally controlled incubator, such as the Percival E30B (Percival Scientific, Inc., Perry, IA, USA) at 27 ± 2 °C; 320 μmol m^{-2} s^{-1}; 18/6 light/dark; 6 × 9A Philips® fluorescent bulbs, or they can simply be transferred to a greenhouse for acclimatization and further growth and development (Fig. 6c, d). Pots should be fertilized every other day. We have used a solution of Peters Orchid Food (Spectrum Group, St. Louis, MO, USA) consisting of 30% total N, 10% P_2O_5, 10% K_2O, 0.5% Mg, 0.02% B, 0.05% chelated Cu, 0.1% Fe, 0.05% Mn, 0.0005% Mo, and 0.05% Zn. Plant survival is determined by growth and development of normal seedlings into plants.

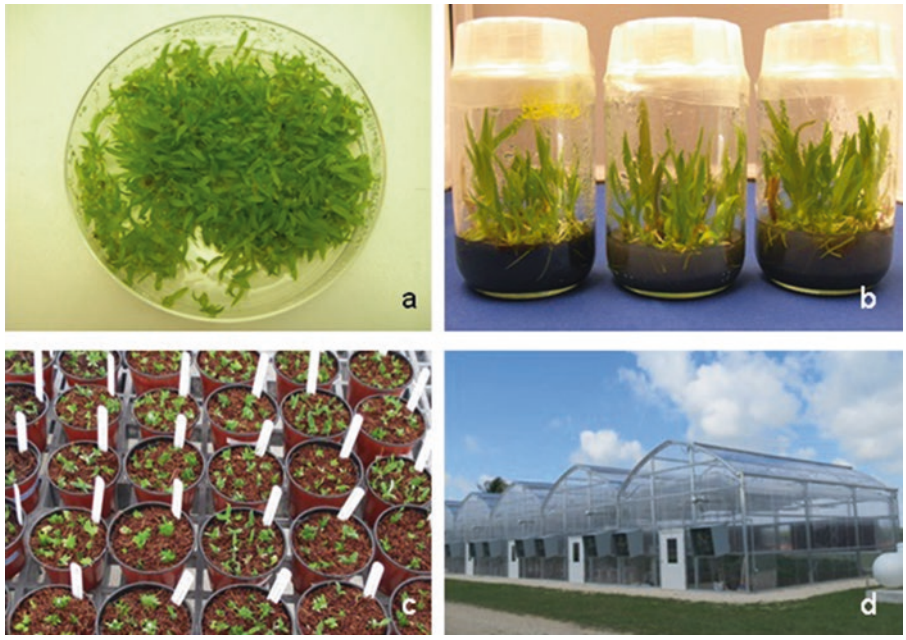


Fig. 6 As orchid seedlings grow and develop (**a**), they can be transferred to larger containers, such a baby food jars (**b**). Once seedlings are fully developed, they are transferred to pots containing growing mix (**c**) and maintained in a greenhouse (**d**)

Fig. 7 Orchid protocorms selected for cryopreservation (**a**). Protocorms are precultured in 125-mL Erlenmeyer flasks containing ½ MS medium for 24 h, placed on an orbital shaker at 110 rpm and 25 °C in the dark (**b**)

3.2 Protocorms

3.2.1 Protocorm Formation and Preparation for Cryopreservation

Mature seed capsules from orchid species or hybrids are collected and surface sterilized in a solution of 3% sodium hypochlorite for 20 min, under a sterile laminar flow hood. Sterile capsules are dissected using a scalpel. Seeds are removed from the capsule and placed for germination in Petri dishes containing half-strength MS medium [54] containing 58.5 mM sucrose (pH 5.7) and solidified with 0.6% agar (Fisher®, Chicago, IL, USA). Petri dishes are maintained under controlled environmental conditions; 27 ± 2 °C; 120 μmol m^{-2} s^{-1}; 16/8 light/dark; 2 × 9A Philips® fluorescent bulbs. Protocorms (1–2 mm diameter) are usually formed within 30–60 days, although this depends on species and/or hybrid (Fig. 7a).

Protocorms are precultured in 125-mL Erlenmeyer flasks containing half-strength MS liquid culture medium supplemented with sucrose. The amount of sucrose needs to be determined and adjusted for each species and/or hybrid, and concentrations commonly used are 0.06, 0.3, 0.6, or 0.9 M sucrose. Flasks are maintained for 24 h on an orbital shaker (110 rpm) at 25 °C in the dark (Fig. 7b). Following preculture, protocorms are treated with 1 mL cryoprotective loading solution at 25 °C for 20 min. The cryoprotective solution consists of 2 M glycerol and 0.4 M sucrose. Because of the higher sensitivity of protocorm tissues to potential toxic components in the PVS2 solution as compared to seeds, exposure times (0, 15, 30, or 45 min) to PVS2 at 0 °C prior to immersion in liquid nitrogen (LN) should be evaluated for each species and/or hybrid.

3.2.2 Cryopreservation Procedure and Treatments

For each treatment, ten protocorms are selected from Petri dishes (Fig. 8a) and placed in 2-mL cryovials containing a different loading solution (2 M glycerol alone; 0.4 M sucrose alone; or 2 M glycerol combined with 0.4 M sucrose, as indicated below) for 20 min at 25 °C, as described by Nishizawa et al. [68]. After that they are transferred to a plant vitrification solution, PVS2 [66], consisting of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide (DMSO) in half-strength MS medium with 0.4 M sucrose (pH 5.7), for 10 min at 25 °C. Treatments consist of protocorms immersed in the different cryopreservation loading and vitrification solutions, respectively, with or without 1% phloroglucinol, prior to immersion in liquid nitrogen. This is performed to evaluate the effectiveness of cryoprotectants for the cryopreservation and successful recovery of protocorms. The following treatments were evaluated for *Dendrobium nobile* [55]:

Control: No glycerol, no PVS2, no phloroglucinol
Treatment 1: 2 M glycerol (20 min) + PVS2 (10 min)
Treatment 2: 2 M glycerol (20 min) + PVS2 with 1% phloroglucinol (10 min)
Treatment 3: 0.4 M sucrose (20 min) + PVS2 (10 min)

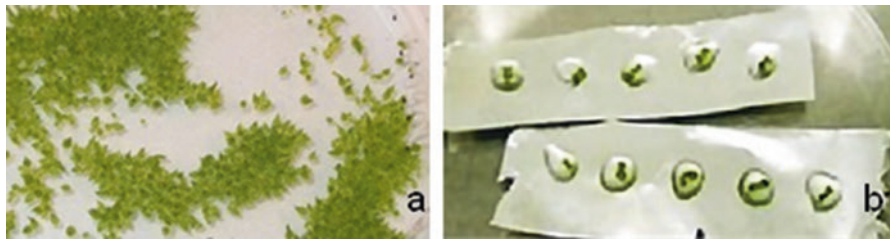


Fig. 8 Protocorms are selected from Petri dishes for cryopreservation (**a**). Droplet-vitrification is an alternative cryopreservation method, whereby protocorms are placed in a sterile aluminum foil strip with droplets of cryoprotectant solution (**b**)

Treatment 4: 0.4 M sucrose (20 min) + PVS2 with 1% phloroglucinol (10 min)
Treatment 5: 2 M glycerol (20 min) + 0.4 M sucrose (20 min) + PVS2 (10 min)
Treatment 6: 2 M glycerol (20 min) + 0.4 M sucrose (20 min) + PVS2 with 1% phloroglucinol (10 min)

Alternatively, protocorms can be cryopreserved using the droplet-vitrification method. In this method, protocorms are dehydrated in 10 drops of 2 μL of PVS2 solution alone or PVS2 solution supplemented with 1% phloroglucinol for 15 min at 0 °C and placed on a sterile aluminum foil strip (7 × 20 mm; width × length) (Fig. 8b). Foil strips are placed into 2-mL cryovials containing LN and immediately transferred to a cryotank containing LN at −196 °C.

3.2.3 Post-cryopreservation Recovery

After cryopreservation, cryovials are removed and rapidly rewarmed in 40 °C water bath for 1.5 min. Cryopreservation solutions are removed from cryovials with a sterile plastic disposable transfer pipette under laminar flow hood. Protocorms are rinsed with half-strength MS medium with 1.2 M sucrose (pH 5.7) and transferred to Petri dishes containing half-strength MS medium containing 58.5 mM sucrose (pH 5.7) solidified with 0.6% agar. Petri dishes with protocorms are maintained in dark for 1 week at 27 ± 2 °C, and then transferred to light under 27 ± 2 °C, 120 μmol m^{-2} s^{-1}; 16/8 light/dark; 2 × 9A Philips® fluorescent bulbs. After approximately 1 month, depending on species and/or hybrid, protocorm recovery and survival can be determined as the percentage of protocorms showing green color and regrowth. The percentage of protocorms forming leaves and roots, and subsequently seedlings, is evaluated after about 40–60 days of culture. Rooted seedlings are transferred to 125-mL culture flasks for further growth and development using the same medium and under the same conditions as described above. After approximately 30 days, depending on species and/or hybrid, fully developed seedlings are transplanted to trays containing coconut coir (Coco Gro-Brick, OFE International, Miami, FL, USA) as the growing mix. Trays are transferred to an incubator, such as the Percival E30B (Percival Scientific, Inc., Perry, IA, USA) under controlled environmental conditions (27 ± 2 °C; 320 μmol m^{-2} s^{-1}; 18/6 light/dark; 6 × 9A Philips® fluorescent bulbs) for acclimatization, or directly to the greenhouse. Trays are fertilized every other day. We have used a solution of Peters Orchid Food (Spectrum Group, St. Louis, MO, USA) consisting of 30% total N, 10% P_2O_5, 10% K_2O, 0.5% Mg, 0.02% B, 0.05% chelated Cu, 0.1% Fe, 0.05% Mn, 0.0005% Mo, and 0.05% Zn. Plant survival is determined by growth and development of seedlings into normal plants.

Fig. 9 Pollinia from Doritaenopsis (**a**), Phalaenopsis (**b**), and Epidendrum (**c**) orchids

3.3 Pollen

3.3.1 Pollen Collection and Preparation for Cryopreservation

Orchid pollen (pollinia) is collected when flowers are completely open. Pollinia can be quite diverse, depending on species and/or hybrids (Fig. 9a–c). After collection, pollen is stored in a desiccator containing silica gel for 24 h. Three samples of four pollinia each, for each species of hybrid, are oven-dried at 103 °C for 17 h to constant weight, and the average initial pollen moisture content is determined before and after desiccation following standard procedures described by the International Seed Testing Association [69].

3.3.2 Cryopreservation Procedure and Treatments

Groups of pollinia from the same flower are transferred to 2-mL cryovials and 1 mL cryoprotective solution (2 M glycerol and 0.4 M sucrose, pH 5.7) is added. Cryovials are left for 30 min at room temperature (27 ± 2 °C) before the addition of a plant vitrification solution. The PVS2 [66] plant vitrification solution consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide (DMSO) in half-strength MS medium [54] supplemented with 0.4 M sucrose (pH 5.7). The high concentration of PVS2 prevents ice crystallization during cryopreservation. Different treatments can be evaluated for optimization of the cryopreservation process and effectiveness of recovery and survival of pollen. However, in a previous study with *Dendrobium* hybrids [65], we determined that no pretreatment, such as precooling or the addition of a cryoprotectant, was necessary for the successful cryopreservation of pollinia. Yet, treatments can be evaluated and adjusted accordingly, if proven necessary and depending on species or hybrid. Here we describe the treatments previously evaluated for Dendrobium hybrids [65] as a reference for future consideration. It is also important to note that most studies involving pollen viability evaluate pollen germination as a measure of success. In our particular study, we evaluated pollen germination by pollinating flowers of the same hybrids with pollinia that had been previously cryopreserved. We also evaluated subsequent seed formation and germination.

Treatments consisted of pollinia left in PVS2 either at room temperature (27 ± 2 °C) or precooled in ice (0 °C) for 1, 2, 3,

or 4 h before immersion in liquid nitrogen (LN) for 48 h. Four controls were established. Control 1 consisted of fresh pollinia collected from one flower and immediately used to pollinate another flower with no subsequent LN. Control 2 consisted of desiccated pollinia maintained in PVS2 for 2 h with no subsequent LN. Controls 3 and 4 consisted of desiccated pollinia placed directly into LN with or without the addition of PVS2, respectively. For control 2 (PVS2, no LN), pollinia were recovered and used to pollinate open flowers of plants from the same hybrids used in the experiment. Pollinated flowers in controls 1 and 2 were emasculated before pollination. Similarly to seeds and protocorms, treatments can be evaluated and adjusted according to the species and/or hybrid.

3.3.3 Post-cryopreservation Assessments

After cryopreservation, pollinia are removed from LN and are rapidly thawed at 40 °C for 3 min. The PVS2 solution is removed from each cryovial using a plastic, disposable transfer pipette. For evaluation of the effectiveness of pollinia cryopreservation in the study previously mentioned [65], approximately 1 mL of a 1.0 M sucrose solution (pH 5.7) was added to each vial and held for 1 h at room temperature followed by two rinses in 1 mL deionized water for both controls 3 and 4. The group of four pollinia from controls 3 and 4 and from each treatment were removed from cryovials and used to pollinate open flowers of plants from the same hybrids used in the experiment (Fig. 10a). All pollinated flowers were emasculated before pollination. Pollinated plants were maintained in a greenhouse for 6 months, and pollen germination was evaluated by the success of the crosses performed for each control or treatment. Fruit (capsule) and viable seed formation was further evaluated (Fig. 10b). Seed samples were removed from capsules, and seed viability was determined using both the 2,3,5-triphenyltetrazolium chloride (TTC) reduction assay [70] and the fluorescein diacetate (FDA) staining technique [44, 71],

Fig. 10 Flowers of *Dendrobium* hybrids selected for pollination using cryopreserved pollen from the same hybrid (**a**). Capsule formation in Dendrobium hybrids after pollination with cryopreserved pollen from the same hybrid (**b**)

as previously described for seeds. Seed samples were germinated in 1-L glass bottles containing half-strength MS medium [54] supplemented with 58.5 mM sucrose and solidified with 0.6% agar (Fisher, Chicago) and maintained at 27 ± 2 °C, 60 μmol m^{-2} s^{-1}, 18/6 light/dark, 2 × 9A Philips fluorescent bulbs (Philips Lighting Co., Somerset, NJ). Seed germination was evaluated for all treatments. Germinated seedlings were monitored weekly for shoot and root development transplanted into Magenta GA7 boxes (Sigma-Aldrich Co., St. Louis, MO) containing the same medium as described for germination. Seedlings were monitored for growth and development and transferred to the greenhouse. Using this protocol, we determined that cryopreservation of *Dendrobium* hybrid pollinia was successful without the need for pretreatments, vitrification solutions, or cryoprotectants. This indicates that the direct immersion of pollinia into liquid nitrogen might be a viable, simple, and fast alternative for cryopreservation of orchid pollen. However, this needs to be assessed for each species and/or hybrid selected.

4 Final Comments

The techniques described above are not the definitive techniques that can be used for all orchid species and/or hybrids. They provide a guideline for the proper development of cryopreservation techniques for orchids. The large diversity of orchids also extends to their seeds, protocorms, and pollen. Therefore, adjustments might be necessary according to the species and/or hybrid selected. Such adjustments may involve seed/protocorm/pollen sterilization procedures, type and concentration of cryoprotectants, the time for precooling (where applicable) prior to cryopreservation, post-cryopreservation recovery procedures, growth and development, environmental conditions and techniques in vitro and in the greenhouse, among other parameters. Adjustments may be also required for the type of containers for cryopreservation of orchids, where seeds and pollen are usually amenable to 2-mL cryovials, while protocorms had better results when using aluminum foil. Therefore, the techniques described in this chapter serve as a general reference for the development of new and improvement of existing methods for the cryopreservation of orchid seeds, protocorms, and pollen.

References

1. Gonzalez-Benito ME, Perez C (1997) Cryopreservation of nodal explants of an endangered plant species (*Centaurium rigualii* Esteve) using the encapsulation–dehydration method. Biodivers Conserv 6:583–590
2. Reed BM (2008) Plant cryopreservation: a practical guide. Springer, New York
3. Harvengt L, Meier-Dinkel A, Dumas E, Collin E (2004) Establishment of a cryopreserved gene bank of European elms. Can J For Res 34:43–55
4. Engelmann F (2004) Plant cryopreservation: progress and prospects. In Vitro Cell Dev Biol Plant 40:427–433

5. Mazur P (1984) Freezing of living cells: mechanisms and applications. Amer J Physiol Cell Physiol 247:125–142
6. Kartha KK, Engelmann F (1994) Cryopreservation and germplasm storage. In: Vasil IK, Thorpe TA (eds) Plant cell and tissue culture. Kluwer, Dordrecht, pp 195–230
7. Engelmann F (1997) In vitro conservation methods. In: Callow J, Ford-Lloyd B, Newbury HJ (eds) Biotechnology and plant genetic resources: conservation and use. CAB Intl., Wallingford, pp 119–161
8. Rao NK (2004) Plant genetic resources: advancing conservation and use through biotechnology. Afr J Biotechnol 3:136–145
9. Hirai D, Sakai A (2003) Simplified cryopreservation of sweet potato [*Ipomoea batatas* (L.) Lam.] by optimizing conditions for osmoprotection. Plant Cell Rep 21:961–966
10. Engelmann F (2000) Importance of cryopreservation for the conservation of plant genetic resources. In: Engelmann F, Takagi H (eds) Cryopreservation of tropical plant germplasm: current research progress and applications. JIRCAS, Tsukuba, pp 8–20
11. Panis B, Piette B, Swennen R (2005) Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all *Musaceae*. Plant Sci 168:45–55
12. Redenbaugh K (1993) Synseeds: applications of synthetic seeds to crop improvement. CRC Press, Boca Raton, FL
13. Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. In Vitro Cell Dev Biol Plant 47:5–16
14. Fabre J, Dereuddre J (1990) Encapsulation-dehydration: a new approach to cryopreservation of *Solanum* shoot-tips. CryoLetters 11:413–426
15. Dereuddre J, Blandin S, Hassen N (1991) Resistance of alginate-coated somatic embryos of carrot (*Daucus carota* L.) to desiccation and freezing in liquid nitrogen: 1. Effects of preculture. CryoLetters 12:125–134
16. Engelmann F, Takagi H (2000) Cryopreservation of tropical plant germplasm-current research progress and applications. JIRCAS, Tsukuba
17. Gonzalez-Arnao MT, Engelmann F (2006) Cryopreservation of plant germplasm using the encapsulation–dehydration technique: review and case study on sugarcane. CryoLetters 27:155–168
18. Sakai A, Engelmann F (2007) Vitrification, encapsulation–vitrification and droplet–vitrification: a review. CryoLetters 28:151–172
19. Engelmann F, Gonzalez-Arnao MT, Wu WJ, Escobar RE (2008) Development of encapsulation–dehydration. In: Reed BM (ed) Plant cryopreservation: a practical guide. Springer, Berlin, pp 59–76
20. Shibli RA, Haagenson DM, Cunningham SM, Berg WK, Volenec JJ (2001) Cryopreservation of alfalfa (*Medicago sativa* L.) cells by encapsulation-dehydration. Plant Cell Rep 20:445–450
21. Dumet D, Grapin A, Bailly C, Dorion N (2002) Revisiting crucial of an encapsulation/desiccation based cryopreservation process: importance of thawing method in the case of pelargonium meristems. Plant Sci 163:1121–1127
22. Gonzalez-Arnao MT, Juaréz J, Navarro L, Duran-Vila N (2003) Cryopreservation of ovules and somatic embryos of citrus using the encapsulation-dehydration technique. CryoLetters 24(2):85–94
23. Wang QC, Mawassi M, Sahar N, Li P, Violeta CT, Gafny R, Sela I, Tanne E, Perl A (2004) Cryopreservation of grapevine (*Vitis* spp.) embryogenic cell suspensions by encapsulation–vitrification. Plant Cell Tiss Org Cult 77:267–275
24. Fang JY, Wetten A, Johnston J (2008) Headspace volatile markers for sensitivity of cocoa (*Theobroma cacao* L.) somatic embryos to cryopreservation. Plant Cell Rep 27:453–461
25. Scocchi A, Faloci M, Medina R, Olmos S, Mroginski L (2004) Plant recovery of cryopreserved apical meristem-tips of *Melia azedarach* L. using encapsulation/dehydration and assessment of their genetic stability. Euphytica 135:29–38
26. Suzuki M, Akihama T, Ishikawa M (2005) Cryopreservation of encapsulated gentian axillary buds following 2 step-preculture with sucrose and desiccation. Plant Cell Tiss Org Cult 83:115–121
27. Wang Q, Laamanen J, Uosukainen M, Valkonen JPT (2005) Cryopreservation of in vitro-grown shoot tips of raspberry (*Rubus idaeus* L.) by encapsulation–vitrification and encapsulation–dehydration. Plant Cell Rep 24:280–288
28. Ishikawa K, Harata K, Mii M, Sakai A, Yoshimatsu K, Shimomura K (1997) Cryopreservation of zygotic embryos of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification. Plant Cell Rep 16:754–757
29. Hirano T, Godo T, Mii M, Ishikawa K (2005) Cryopreservation of immature seeds of *Bletilla striata* by vitrification. Plant Cell Rep 23:534–539

30. Jitsopakul N, Thammasiri K, Ishikawa K (2008) Cryopreservation of *Bletilla striata* mature seeds, 3-day germinating seeds and protocorms by droplet-vitrification. CryoLetters 29:517–526
31. Lurswijidjarus W, Thammasiri K (2004) Cryopreservation of shoot tips of *Dendrobium* Walter Oumae by encapsulation/dehydration. Sci Asia 30:293–299
32. Vendrame WA, Carvalho VS, Maguire I (2007) In vitro propagation and plantlet regeneration from *Doritaenopsis* purple gem 'Ching Hua' flower explants. HortScience 42(5):1256–1258
33. Galdiano RF Jr, Lemos EGM, Faria RT, Vendrame W (2012) Cryopreservation of *Dendrobium* hybrid seeds and protocorms as affected by phloroglucinol and supercool X1000. Sci Hort 148:154–160
34. Galdiano RF Jr, Lemos E, Vendrame W (2014) Seedling development and evaluation of genetic stability of cryopreserved *Dendrobium* hybrid mature seeds. Appl Biochem Biotechnol 172:2521–2529
35. Galdiano RF Jr, Lemos EGM, Vendrame W (2013) Cryopreservation, early seedling development, and genetic stability of *Oncidium flexuosum* Sims. Plant Cell Tiss Org Cult 114:139–148
36. Tsukazaki H, Mii M, Tokuhara K, Ishikawa K (2000) Cryopreservation of *Doritaenopsis* suspension culture by vitrification. Plant Cell Rep 19:1160–1164
37. Na HY, Kondo K (1996) Cryopreservation of tissue-cultured shoot primordia from shoot apices of cultured protocorms in *Vanda pumila* following ABA preculture and desiccation. Plant Sci 118(2):195–201
38. Vendrame W, Faria RT, Sorace M, Sahyun SA (2014) Orchid cryopreservation. Ciênc Agrotec 38(3):213–229
39. Vendrame WA, Khoddamzadeh AA (2017) Orchid biotechnology. Hort Rev 44:173–228
40. Vendrame WA, Carvalho VS, Maguire I, Dias JMM (2007) In vitro germination and plant regeneration of cryopreserved *Dendrobium* hybrid mature seeds. Sci Hort 114:188–193
41. Pritchard HW, Seaton PT (1993) Orchid seed storage: historical perspective, current status, and future prospects for long-term conservation. Selbyana 14:89–104
42. Merritt DJ, Hay FR, Swarts ND, Sommerville KD, Dixon KW (2014) Ex situ conservation and cryopreservation of orchid germplasm. Int J Plant Sci 175:46–58
43. Bowling JC, Thompson PA (1972) On storing orchid seed. Orchid Rev 80:120–121
44. Pritchard HW (1985) Growth and storage of orchid seeds. In: Tan KW (ed.) Proc. eleventh world orchid conference. 11th W.O.C., Miami, pp 290–293
45. Ito I (1965) Ultra-low temperature storage of orchid pollinia and seeds. Jpn Orchid Soc Bull 11:4–15
46. Pritchard HW (1984) Liquid nitrogen preservation of terrestrial and epiphytic orchid seed. CryoLetters 5:295–300
47. Seaton PT, Hailes SJ (1989) Effect of temperature and moisture content on the viability of *Cattleya aurantiaca* seed. In: Pritchard HW (ed) Modern methods in orchid conservation: the role of physiology, ecology and management. Cambridge University Press, Cambridge, pp 17–29
48. Thornhill A, Koopowitz H (1992) Viability of *Disa uniflora* Berg (Orchidaceae) seeds under variable storage conditions: is orchid genebanking possible? Biol Conserv 62:21–27
49. Surenciski MR, Gonzales EA, Terada G, Mroginski LA, Rey HY (2012) Cryopreservation of *Cyrtopodium hatschbachii* Pabst (Orchidaceae) immature seeds by encapsulation-dehydration. Biocell 36:31–36
50. Harding K (2004) Genetic integrity of cryopreserved plant cells: a review. CryoLetters 25:3–22
51. Panis B, Lambardi M (2006) Status of cryopreservation technology in plants (crops and forest trees). In: Ruane J, Sonnino A (eds) The role of biotechnology in exploring and protecting agricultural genetic resources. Food and Agriculture Organization, Rome, pp 61–78
52. Popova EV, Nikishina TV, Kolomeitseva GL, Popov AS (2003) The effect of seed cryopreservation on the development of protocorms by the hybrid orchid *Bratonia*. Russian. J Plant Physiol 50:672–677
53. Morel G (1960) Producing virus free *Cymbidium*. Amer Orchid Soc Bull 29:459–497
54. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
55. Vendrame WA, Faria RT (2011) Phloroglucinol enhances recovery and survival of cryopreserved *Dendrobium nobile* protocorms. Sci Hort 128:131–135
56. Gogoi K, Kumaria S, Tandon P (2012) A comparative study of vitrification and encapsulation-vitrification for cryopreservation of protocorms of *Cymbidium eburneum* L., a threatened and vulnerable orchid of India. CryoLetters 33:443–452
57. Mata-Rosas M, Lastre-Puertos E (2015) Long-term conservation of protocorms of *Brassavola*

nodosa (L) Lind. (Orchidaceae): effect of ABA and a range of cryopreservation techniques. CryoLetters 36(5):289–298

58. Connor KF, Towill LE (1993) Pollen-handling protocol and hydration/dehydration characteristics of pollen for application to long-term storage. Euphytica 68:77–84
59. Grout BWW, Roberts AV (1995) Storage of free pollen, pollen embryos and the zygotic embryos of seed by cryopreservation and freeze drying. In: Grout BWW (ed) Genetic preservation of plant cells in vitro. Springer, Berlin, pp 63–74
60. Sacks EJ, St Clair DA (1996) Cryogenic storage of tomato pollen: effect on fecundity. HortScience 31:447–448
61. Towill LE (2002) Cryopreservation of plant germplasm: introduction and some observations. In: Towill LE, Bajaj YPS (eds) Cryopreservation of plant germplasm II. Springer, Berlin, pp 4–21
62. Pritchard HW, Prendergast FG (1989) Factors influencing the germination and storage characteristics of orchid pollen. In: Pritchard HW (ed) Modern methods in orchid conservation: the role of physiology, ecology and management. Cambridge University Press, Cambridge, pp 1–16
63. Ganeshan S, Rajasekharan PE, Shashikumar S, Decruze W (2008) Cryopreservation of pollen. In: Reed BM (ed) Plant cryopreservation: a practical guide. Springer, Berlin, pp 443–464
64. Pritchard HW, Poynter ALC, Seaton PT (1999) Interspecific variation in orchid seed longevity in relation to ultra-dry storage and cryopreservation. Lindleyana 14(2):92–101
65. Vendrame WA, Carvalho VS, Dias JMM, Maguire I (2008) Pollination of *Dendrobium* hybrids using cryopreserved pollen. HortScience 43:264–267
66. Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Rep 9: 30–33
67. Hosomi ST, Custódio CC, Seaton PT, Marks TR, Machado-Neto NB (2012) Improved assessment of viability and germination of *Cattleya* (*Orchidaceae*) seeds following storage. In Vitro Cell Dev Biol Plant 48:127–136
68. Nishizawa S, Sakai A, Amano Y, Matsuzawa T (1993) Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. Plant Sci 91:67–73
69. International Seed Testing Association (1993) International rules for seed testing 1993. Seed Sci Technol:21
70. Singh F (1999) Differential staining of orchid seeds for viability testing. Amer Orchid Soc Bull 50:416–418
71. Pritchard HW (1985) Determination of orchid seed viability using fluorescein diacetate. Plant Cell Environ 8:727–730

Chapter 16

Haploid Seed Formation via Parthenogenesis in *Bletilla*

Juntaro Kato and Syoichi Ichihashi

Abstract

This chapter provides a protocol to induce haploid plant formation in *Bletilla* via parthenogenesis by the application of 1-naphthaleneacetic acid sodium salt through the stigmatic surface of the column. Although the seed number is low, in vitro seed germination results in the formation of viable seedlings, and the ploidy levels as determined using flow-cytometric analyses indicate the formation of haploid and diploid (doubled haploid) plants. The successful induction of haploid plants through parthenogenesis can be a powerful tool in orchid breeding.

Key words *Bletilla*, Haploids, Stigma, 1-Naphthaleneacetic acid sodium salt, Parthenogenesis, Flow cytometry

1 Introduction

Haploid plant production is a powerful tool in plant breeding. In major crops, when a breeder produces a F_1 hybrid, the usual next step is to breed F_2 segregated populations and then select elite candidates to use for further breeding. However, F_2 individuals have many heterozygous loci, and breeders cannot decide whether the expressed characteristics from elite candidates are due to homozygosity or heterozygosity.

In pure lines, whose genotypes are homozygous at all loci, they are useful for plant breeding. The self-pollinated progenies of pure lines have uniform characteristics identical to the parent. The F_1 plants obtained from crosses among different pure lines will express uniform characteristics with hybrid vigor. To generate pure lines, instead of recurrent self-pollination, haploid production will aid in speeding up the process.

Two types of haploid cells are well known in plants: megaspores within ovaries and microspores in anthers. Hence, to produce haploid plants, one can culture isolated microspores or megaspores or through anther and ovule/ovary cultures. The process is generally known as androgenesis and gynogenesis, respectively [1].

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_16,

Anther culture is more widely used in the production of haploids because anthers are easy to isolate and manipulate experimentally; many defined protocols are available in the literature [1]. Successes have been reported in the generation of haploid *Brassica* species through the use of isolated microspores [2] and horticultural species such as *Cyclamen persicum* through anther culture [3]. There are fewer studies in generating haploids from ovules and female gametophyte [1]. Moreover, successes have been recorded through ovary culture of *Gerbera* [4], ovule culture as in *Gentiana* [5], and pseudo-fertilized ovary culture using X-ray inactivated pollen in *Dianthus* [6]. In barley, the well-known chromosome elimination protocol (the *Hordeum bulbosum* method) enables the generation of haploid barley embryo as the chromosomes of *Hordeum bulbosum* are preferentially eliminated during early embryogeny [7].

In orchid breeding, interspecific and/or intergeneric hybridization is a usual and traditional method in generating hybrids, because many orchids have highly interspecific crossability. Once an excellent interspecific hybrid is obtained, the plant can be propagated through micropropagation and a new cultivar can be established. This new cultivar can be used as a genetic resource for further hybridization. Hence, for most ornamental crops, including orchids, many are bred through arbitrary crossing because elite offsprings are easily propagated by micropropagation in vitro. Although elite hybrids can be propagated vegetatively in vitro, micropropagation protocols can generate somaclonal variations. This is one of the main drawbacks in micropropagation, especially via protocorm-like body (PLBs) formation. Propagation through etiolated shoot cuttings seems to reduce somaclonal variations; however, shoot multiplication speed is slow and the chance of being infected by virus is high.

Seedling plants are often utilized efficiently for mass production of orchids in early stages of commercial production. Moreover, the majority of cultivars are not genetically homozygous; crossing using heterozygous varieties as parents will produce many seedling descendants with different characteristics. To produce uniform seedlings with the same genetic background, production of homozygous plants is required. The use of haploid plants is an excellent alternative method to produce homozygous pure lines through chromosome doubling of haploid plants. After establishing homozygous pure lines, seedling cultivars can be established by inbreeding or by cross combination between different pure lines, generating F_1 cultivars.

Reports on the generation of haploid orchids are few. In this chapter, we detail a method to induce haploid and dihaploid plants from *Bletilla* species and their hybrid, *B.* 'Brigantes', by the naphthaleneacetic acid sodium salt (NAA-Na) treatment. *B. striata* is an exceptionally easy-growing orchid and a very common and

popular garden plant in Japan. However, the color and the shape of flowers are simple and can be further improved. We conducted cross breeding of *Bletilla* species using a yellow flower species, *B. ochracea*, to extend the potential of *Bletilla* as ornamental garden plants. During the breeding program of *Bletilla*, we tested the NAA-Na treatment to induce apomictic seeds [8] and produced haploid plants unexpectedly. Thereafter we started to optimize a protocol on haploid plant production using auxins [9]. This chapter reports a procedure for the generation of haploid plants from *Bletilla* species and their hybrids.

2 Materials

2.1 Plant Materials

Bletilla striata, *B. ochracea*, and their hybrid, *B.* 'Brigantes', are used as experiment materials (*see* **Note 1**).

2.2 Major Laboratory Equipment

Laminar flow cabinet; stereomicroscope (SZ60, Olympus, Japan); light microscope; flow cytometer analysis equipment, e.g., ploidy analyzer type PA (Partec Co.; Germany); incubator; and water bath.

2.3 NAA-Na Solution and Paste

For solution, dissolve 1% 1-naphthaleneacetic acid sodium salt (NAA-Na) in distilled water and store at 4°C. When used as a paste, dissolve by mixing 2% NAA-Na in lanolin paste in a 70 °C hot water bath.

2.4 Seed Germination and Subculture Media

1. Hyponex seed germination medium 6.5–6–19 (N:P:K; Hyponex Japan Co. Ltd.). Dissolve 3 g/L Hyponex and 20 g/L sucrose in water; adjust pH to 5.6 ± 0.1. Using a hot water bath, dissolve 8 g/L agar in the Hyponex medium. Dispense 100 mL medium into flasks and sterilize in an autoclave at 115 °C for 15 min.
2. Subculture medium: Half-strength Murashige and Skoog (MS) medium, adjust pH to 5.6–5.8 [10] supplemented with 2% sucrose and 0.3% gellan gum.

2.5 Staining of Nuclei for Flow Cytometry

DAPI-staining solution: 10 mM Tris-HCl, pH 7.5, containing 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) polyvinylpyrrolidone (PVP 40, Sigma-Aldrich), 0.1% (v/v) Triton X-100, and 2 mg/L 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride.

2.6 Solutions for Chromosome Squash and Staining

1. 8-Hydroxyquinoline (2 mM) or 0.01% (w/v) colchicine solution.
2. Cell wall digestion enzymes: enzyme solution containing 4.0% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Ind. Co, Japan), 1.0% (w/v) Pectolyase Y-23 (Kyowa Chemical Products

Co. Ltd., Osaka, Japan), 0.3% (w/v) Macerozyme R10 (Yakult Pharmaceutical Ind. Co., Japan), and 1 mM EDTA, adjust pH to 4.2 according to the method of Fukui and Iijima [11].

3. Fixation solution—acetic acid: EtOH (1:3; v:v).
4. Giemsa's staining solution, 1% (Merck).

2.7 Other Solutions

Autoclaved sterile distilled water and sodium hypochlorite solution (1% available chlorine concentration) with 1 drop of Tween 20 (Sigma Co.).

2.8 Laboratory Supplies

Scissors, micro-pipets, forceps, beakers, Petri dishes, 50 mL centrifuge tubes with screw cap for tissue sterilization, filter paper (No. 2, 110 mm diameter) sterilized by dry heat at 150°C for 1 h (Advantec Co., Japan), 30 μm nylon mesh, and sealing film, such as Novix-II (butadiene rubber paraffin, Iwaki, Chiba, Japan).

3 Methods

The methods below describe (1) NAA-Na application on stigma, (2) in vitro seed culture, (3) confirmation of ploidy level by flow cytometry, and (4) confirmation of ploidy level by cytological observation.

3.1 NAA-Na Treatment on Stigma

For NAA-Na treatment, first prepare NAA-Na solution or lanolin paste (*see* **Note 2**). Prior to applying NAA-Na aqueous solution or lanolin paste on the stigma, emasculate the flower by removing the pollinia in order to eliminate any possibilities of self- or natural crossing. For emasculation, first remove the anther cap using forceps, exposing the pollinia, and gently remove the pollinia. Carefully excise the lip from each emasculated flower. This will reduce the chance of cross-pollination as bees and gadflies cannot land on the lip when attempting to obtain nectar from flowers. Apply 10 μL NAA-Na solution or inject an equal amount of lanolin paste onto the stigma by a micropipette. The stigma of *Bletilla* flower appears as a small depression on the front side of the column (*see* Fig.1 and **Note 3**).

Fig. 1 Procedure of NAA-Na application on stigma. (**a**) Emasculation. (**b**) Lip removal. (**c**) Apply NAA to stigma

Fig. 2 Differences of hypertrophied ovaries between self-pollination (**a**) and NAA-Na treatment on stigma (**b**)

3.2 Capsule Development and In Vitro Seed Germination

Upon normal pollination, capsules develop and seeds are fully matured after 6 months. After NAA-Na treatment on stigmas, the columns deteriorate and dry quickly. Some capsules will enlarge, albeit slower than the control. Since immature seeds germinate better prior to seed maturation, perform in vitro seed germination studies 4 months after pollination or NAA-Na treatment.

3.2.1 Collecting and Sterilization of Capsules

After 4 months, obtain enlarged capsules from the NAA-Na treatment. The size of the capsules is usually smaller than those obtained by crossing (Fig. 2). Approximately 60% of the ovaries treated with NAA-Na become hypertrophied (Table 1).

Place a single capsule into a 50 mL centrifuge tube containing sodium hypochlorite (1% available chlorine) solution with a drop of Tween 20. Cap the tube, shake the tube gently by hand for 2 min, and allow it to stand for another 15 min (*see* **Notes 4** and **5**).

Decant the sodium hypochlorite solution and rinse the capsule with sterile distilled water twice. Transfer the capsule by forceps onto sterile filter papers wetted with sterilized distilled water inside a laminar flow cabinet.

3.2.2 In Vitro Seed Germination

Cut the capsule along its longitudinal axis carefully using a surgical knife and collect the immature seeds inside by forceps or spatula (*see* **Note 6**). Spread the seeds onto the surface of the Hyponex germination medium (*see* Fig. 3). Seal the culture dishes with polyethylene butadiene rubber paraffin film (Novix-II, Iwaki, Chiba, Japan) and maintain the cultures at 22 ± 1 °C under constant 189.6 ± 21.9 lux illumination of fluorescent lamps (Plantlux, Toshiba, Japan). Examine the cultures for seed germination after 4 weeks using a stereoscopic microscope. Germinated seeds appear green in color (Fig. 4) and are obtained from a few cultivars/strains (Table 2). The number of germinable seeds is small (Table 2).

Table 1
Number of hypertrophied ovaries by treatment of NAA-Na in *Bletilla striata*, *B. ochracea*, and *B.* 'Brigantes'

Species/strains	Ploidy level	No. of flowers treated with NAA-Na	No. of flowers with hypertrophied ovaries
B. striata	2*x*	5	3
B. striata var. *alba*	2*x*	5	3
B. ochracea	2*x*	5	4
B. 'Brigantes'			
H4-3	2*x*	5	5
H5-2	3*x*	5	5
H5-7white	2*x*	5	4
H5-10	2*x*	5	5
H5-11 pale	2*x*	5	5
H5-11 deep	2*x*	5	5
H5-11 pale lip	2*x*	5	4
H5-large petal	3*x*	5	4
H6-4	2*x*	5	5
H6-5	2*x*	5	5
H6-late flowering	3*x*	5	4
No. 4	2*x*	5	4

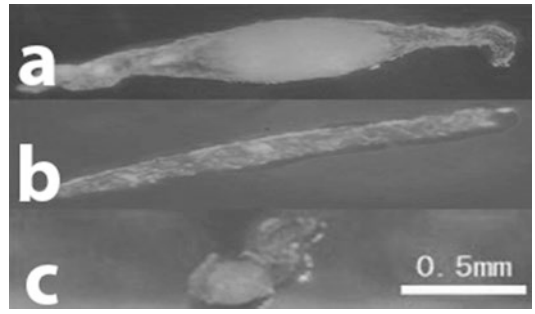


Fig. 3 Differences of seed shapes between self-pollination and NAA treatment on stigma. (**a**) A seed with embryo derived from self-pollination. (**b**) A sterile seed without embryo derived from NAA-Na treatment. (**c**) A fertile seed with embryo derived from NAA-Na treatment

3.2.3 Plantlet Development

After 15 weeks of culture, transplant NAA-Na induced plantlets to half-strength MS subculture medium supplemented with 2% sucrose and 0.3% gellan gum in 25 mm × 118 mm culture tubes,

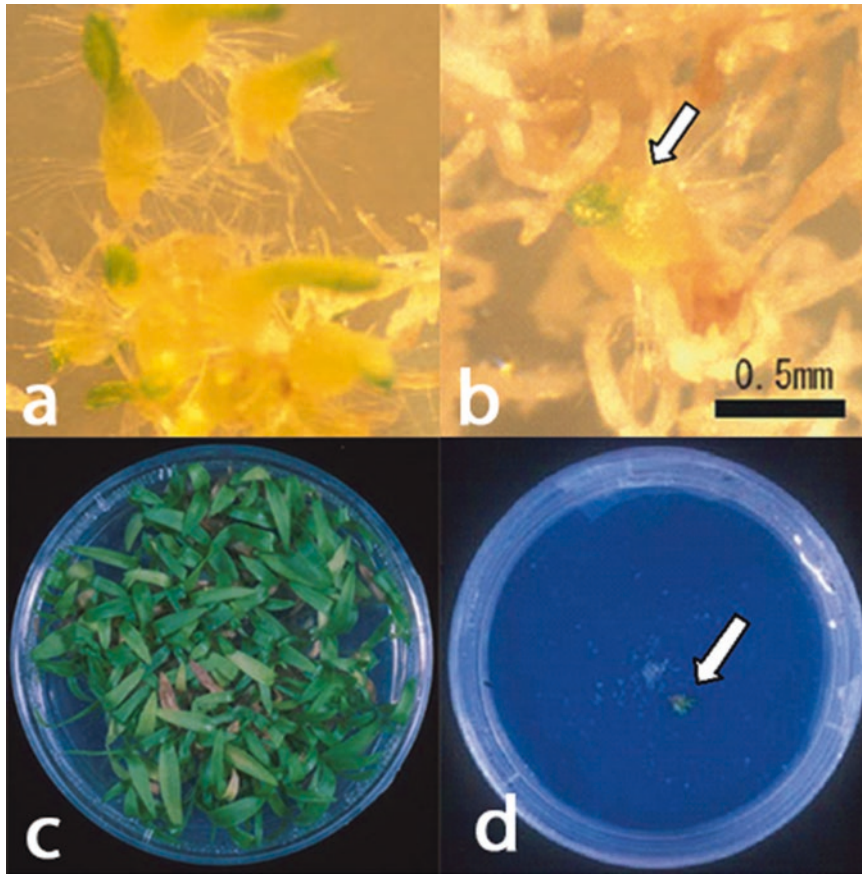


Fig. 4 (**a**, **c**) Germination of self-pollinated seeds. (**b**, **d**) Germination of seeds from the NAA-Na treatment

as plastic Petri dishes will be too small for normal plantlet development (Fig. 4).

Select well-developed rooted plantlets and transfer to pots with sphagnum moss which has been previously soaked in the Hyponex solution.

3.3 Estimation of Ploidy Level by Flow Cytometry (FCM)

When haploids are produced, they should theoretically show the half-value of DNA content of the parent plants. Flow-cytometric analysis is a useful method to determine DNA content and to estimate ploidy level. The following procedures detail our method of flow-cytometric analysis using a PA-type UV detector flow cytometer (Partec Co., Germany).

Excise approximately 0.5 cm^2 leaf segments or a single protocorm onto a plastic Petri dish with 0.2 mL of nuclei extraction solution "CyStain UV Precise P" (Partec Co., Germany), and finely chop the tissues with a surgical knife. Add 1 mL DAPI-staining solution to the tissue mixture in the same plastic dish. After 30 s of incubation, filter the solution with a 30 μm nylon

Table 2
The percentage of seeds with embryos and germinated seeds obtained by NAA-Na treatment in *B. striata*, *B. ochracea*, and *B.* 'Brigantes'

Species and strains	Ploidy level	No. of seeds w(*a*)	No. of seeds with embryo (*b*)	No. of germinable seeds (*c*)	The percentage of seeds with embryos (*b*/*a**100)	The percentage of germinable seeds (*c*/*b**100)
B. striata	2*x*	261	0	0	0.00	0.00
B. striata var. *alba*	2*x*	706	10	0	1.42	0.00
B. ochracea	2*x*	0	0	0	0.00	0.00
B. 'Brigantes'						
H4-3	2*x*	2202	10	0	0.45	0.00
H5-2	3*x*	2376	2	0	0.08	0.00
H5-7white	2*x*	2814	42	0	1.49	0.00
H5-10	2*x*	2376	3	1	0.13	33.33
H5-11 pale	2*x*	7242	17	0	0.23	0.00
H5-11 deep	2*x*	13,890	81	17	0.58	20.99
H5-11 pale lip	2*x*	1770	3	0	0.17	0.00
H5-large petal	3*x*	2252	11	0	0.49	0.00
H6-4	2*x*	3359	22	0	0.65	0.00
H6-5	2*x*	3806	3	0	0.08	0.00
H6-late flowering	3*x*	3201	4	0	0.12	0.00
No. 4	2*x*	3045	5	0	0.16	0.00

mesh to remove tissue residue. Collect the filtrate and measure the DNA content according to the manufacturer's protocol (*see* **Note 7**). Internal standards for determination of DNA content using appropriate plant tissues are needed (*see* **Note 8**). The results are shown in Fig. 5.

Spontaneous chromosome doubling often occurs in haploid plants. Because FCM analysis can be conducted for small plant segments or calli, it is important that FCM analysis should be performed at a younger stage of plantlet development or protocorms soon after germination. While initial plantlets generated from protocorms show a haploid peak, most of all vigorous developing plantlets have at least two major peaks, i.e., haploid and diploid or as diploid and tetraploid.

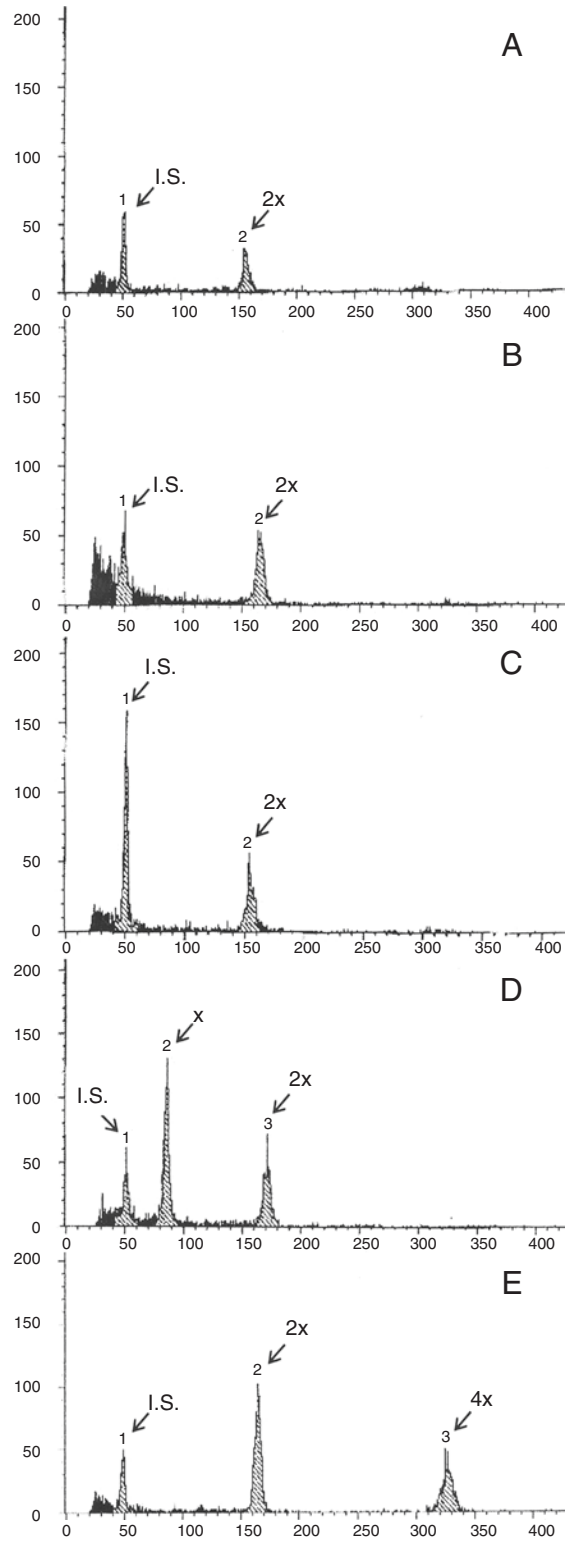


Fig. 5 Flow-cytometric profiles of *B. striata* (**a**), *B. ochracea* (**b**), *B.* 'Brigantes' (**c**), a haploid plant derived from NAA-Na treatment (**d**), and a doubled haploid by natural chromosome doubling (**e**). *IS* internal standard

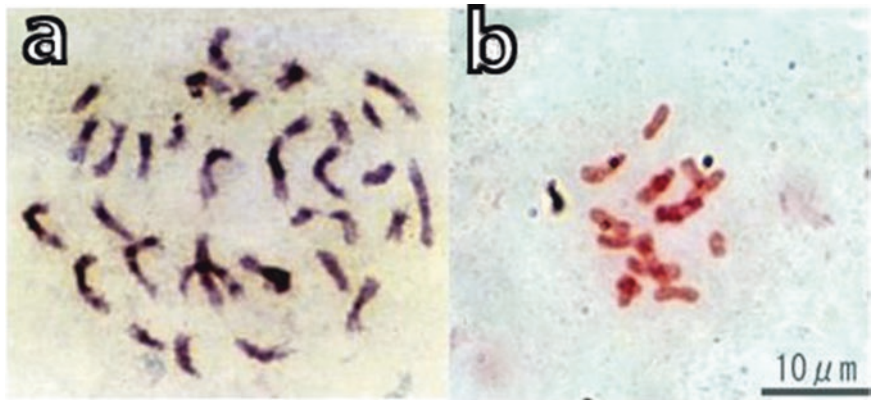


Fig. 6 Somatic chromosome of *B.* 'Brigantes' ((**a**) $2n = 2x = 32$) and haploid plant ((**b**) $2n = x = 16$) induced by the NAA-Na treatment

3.4 Confirmation of Ploidy Level by Somatic Cell Observation

Somatic chromosome count is the ultimate confirmation of ploidy level. Although the squash method is traditionally used, the cell wall often interferes with chromosome examinations. The enzyme maceration method detailed below is an easier alternative, especially for beginners.

Excise growing root tips or protocorms and pretreat them with 2 mM 8-hydroxyquinoline or 0.01% (w/v) colchicine solution for 4 h at 15 °C (*see* **Note 9**). Fix the samples in ethanol-acetic acid [3:1 (v/v)] and keep at 4 °C. Before enzyme maceration, transfer root tips to distilled water and keep at 4 °C overnight before use. Rinse root tips several times with cold distilled water, and then macerate the root tips with the macerating enzyme solution. Treat the tissue for 1 h at 37 °C in a 0.5 mL microtube. After digestion with wall enzymes, rinse the root tips or protocorms carefully with distilled water twice and transfer the tissue onto a glass slide. Add a drop of fixing solution (ethanol-acetic acid [3:1 (v/v)]) on the tissue and macerate the tissue using forceps. With practice, cells will disperse on the slide.

Allow the cells to dry on the slide, and stain the cells with a 1.0% (v/v) Giemsa's solution (Merck, Darmstadt, Germany). Apply a coverglass and examine and count the chromosomes under a light microscope (BX-51, Olympus, Tokyo, Japan) (*see* Fig. 6).

4 General Comments

In this study, the NAA-Na treatment shows positive effects on the generation of haploid plants through parthenogenesis, albeit the number of germinable seeds is small (Table 2). Though immediate column death indicates that the concentration of NAA-Na might be too high, a single application of NAA-Na may not provide a sufficiently strong signal to induce parthenogenesis. A continual

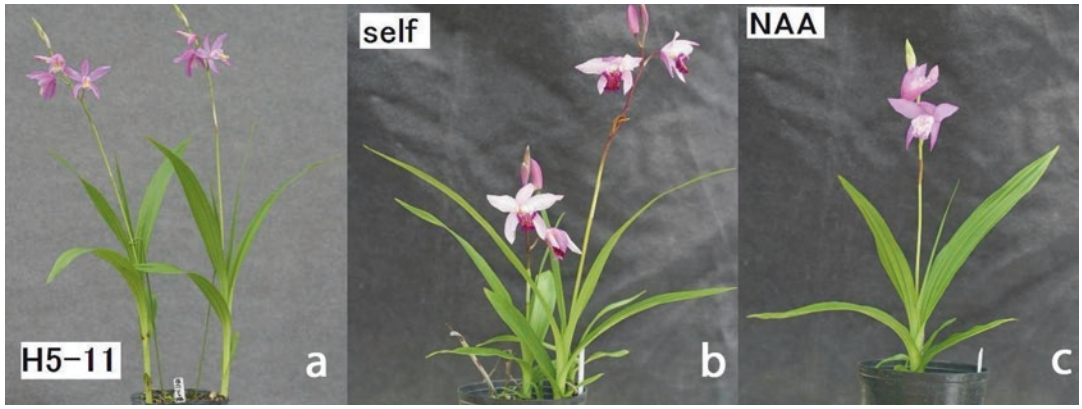


Fig. 7 (**a**) Flower of *Bletilla* 'Brigantes' (H5–11). (**b**) A selfed plant. (**c**) A NAA-Na-induced dihaploid plant

supply of auxin at low concentration may be more beneficial. Judging from Table 2, the genotype selected may also play an important role in the success or failure of haploid parthenogenesis. Additional modifications to the current protocol are needed.

Spontaneous chromosome doubling is common in all haploid plants grown normally. This result suggests that the nature of haploid genome is less stable and the stable diploid genome is preferred. Some doubled haploids induced from the NAA-Na treatment came into bloom (Fig. 7). Although the initial growth of acclimatized plants with chromosome doubling showed slight growth depression, the flower characteristics and fertility are normal (Fig. 7).

5 Notes

1. *Bletilla* 'Brigantes' is a hybrid produced by interspecific hybridization between *B. striata* and *B. ochracea*. *B. striata* is a Japanese garden plant and this species is easy to grow in Japan. *B. ochracea* is a common Chinese *Bletilla* species. However, the optimum growing temperature of this species is lower than that of *B. striata*, and it is also difficult to survive during the Japanese summer season. Moreover, their hybrid, *B.* 'Brigantes', has similar growing characteristics as *B. striata*, and it is easy to grow in the ground or as potted plants in Japan.
2. When preparing the NAA-Na solution using naphthaleneacetic acid (NAA), dissolve NAA in 1 mL 8 M NaOH solution and then dilute with warmed distilled water, and keep at 4 °C.
3. The concentration/volume of NAA-Na used needs to be tested and optimized. For example, when the stigma of *Habenaria radiata* was treated with 0.2% NAA-Na, the region between the stigma and ovary degenerated the next day. In the case of *Bletilla*, the upper part of column also degenerated quickly.

It is important to test the flowers from different species for sensitivity toward NAA concentrations.

4. When the capsules collected have a lot of dust or mold, the capsules must be washed under running water for 30 min before use.
5. Concentration of available chlorine and sterilization time differ from species to species, because the tolerance to chlorine differs.
6. When the capsule is cracked at the time of harvest, dried mature seeds are collected into a pipette, ca. 5 cm long and 1 cm diameter. The pipette can be plugged with cotton and the seeds can be sterilized by filling the pipette with 0.5% sodium hypochlorite solution, allowing it to stand for 5 min followed by discharging the solution by applying a slight pressure at one end. The seeds are repeatedly washed using sterile water. After removing one side of a cotton plug, seeds can be collected by forceps and spread on a medium surface aseptically.
7. Some species have noisy peaks. When noisy peaks are detected, young fresh leaves should be used. Adding 20 mM dithiothreitol (DTT) into the nuclei extraction buffer can sometimes resolve this noisy problem because DTT may be effective in inhibiting some biochemical oxidization processes.
8. The prerequisite for choosing an internal standard is that it must have a smaller DNA content than the haploid *Bletilla*. Nuclei from fresh *B. striata* leaves collected between May and September are used as internal standards.
9. Pretreatment can also be performed with ice water. The glass vial with distilled water and collected roots or protocorms can be placed into an ice box for approximately 20 h. After pretreatment, the distilled water is replaced immediately by the fixing solution.

References

1. Reed SM (2005) Haploid cultures. In: Trigiano RN, Gray DJ (eds) Plant development and biotechnology. CRC Press, Boca Raton, FL
2. Ferrie AMR, Mollers C (2011) Haploids and doubled haploids in *Brassica* spp. for genetic and genomic research. Plant Cell Tiss Org Cult 104:375–386
3. Ishizaka H, Uematsu J (1993) Production of plants from pollen in *Cycalmen persicum* Mill through anther culture. Japan J Breed 43:207–218
4. Miyoshi K, Asakura N (1996) Callus induction, regeneration of haploid plants and chromosome doubling in ovule cultures of pot gerbera (*Gerbera jamesonii*). Plant Cell Rep 16:1–5
5. Doi H, Hoshi N, Yamada E, Yokoi S, Nishihara M, Hikage T, Takahata Y (2013) Efficient haploid and doubled haploid production from unfertilized ovule culture of gentians (*Gentiana* spp.) Breed Sci 63:400–406
6. Sato S, Katoh N, Yoshida H, Hagimori N (2000) Production of doubled haploid plants of carnation (*Dianthus caryophyllus* L.) by pseudofertilized ovule culture. Sci Hortic 83:301–310
7. Devaux P, Kasha KJ (2009) Overview of barley doubled haploid production. In: Touraev A

et al (eds) Advances in haploid production in higher plants. Springer, New York

8. Mori G, Yamaoka K, Imanishi H (1989) Studies on the possibility of inducing apomixes in some orchids. J Japan Soc Hort Sci 58(Suppl 2):536–537. (In Japanese)
9. Ichihashi S, Ito H, Kato J (2013) Breeding of *Bletilla* hybrids and haploid induction via reduced female gamete parthenogenesis by NAA-Na treatment to establish seedling F1 cultivars in orchid. Bull Aichi Univ Educ 62(Natural Sciences):25–33
10. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
11. Fukui K, Iijima K (1991) Somatic chromosome map of rice by imaging methods. Theor Appl Genet 81:589–596

Chapter 17

A Protocol for the Induction of Polyploids in *Phalaenopsis* Orchids by In Vitro Method Without Using Anti-microtubule Agents

Wen-Huei Chen and Ching-Yan Tang

Abstract

Polyploidy plays an important role in the breeding of many crops as well as horticultural plants of the world. It is also important for the variety improvement of *Phalaenopsis* orchids. Most of the commercial orchids are tetraploids. However, most of the wild species of *Phalaenopsis* are diploid. Thus, a barrier to hybridization between these two groups of varieties limits the source of germplasm available for the breeding programs. Hence, a simple technique to scale up the ploidy level of diploid species is needed. Traditionally, chemical induction using anti-microtubule agents such as colchicine or oryzalin is used for polyploid induction. However, there are several disadvantages to these methods, such as complicated procedures, problems of toxicity to plants, and the occurrence of unfavorable chimeras. Search for alternate procedures might give better result to achieve the same objective. Due to the occurrence of endopolyploid cells in the tissues of protocorms and PLBs of the orchid, there is a chance to regenerate polyploid plants from these cells. A protocol for dissecting protocorms or PLBs in tissue culture without using anti-microtubule agents and the use of flow cytometry with 4,6-diamidino-2-phenylindole (DAPI) staining is described in details in this chapter. This is a simple, effective, and reliable technique to produce large numbers of polyploid plants in *Phalaenopsis* orchids. It might have a great impact on new variety development of the orchid in the future.

Key words Chromosome doubling, DAPI, Endopolyploidy, Flow cytometry, In vitro methods, *Phalaenopsis* orchids, Polyploidy induction, Protocorm, Protocorm-like body

1 Introduction

Because of the great diversity in plant types and flower colors, the Moth orchid (*Phalaenopsis* species) is one of the most popular orchids in the world market. The basic chromosome number of this group of orchids is $2n = 2x = 38$. Most of the commercial varieties/hybrids are tetraploids while the wild species are diploid. Although the genus *Phalaenopsis* comprises 63 species [1], only one-fourth of them have been popularly used for the breeding of commercial hybrids. One of the problems is the occurrence of sterility after hybridization between tetraploid varieties and

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_17,

diploid species. Therefore, there is a need to transform diploid species to tetraploid in order to widen the source of germplasm for development of new commercial varieties for the markets.

Polyploidy is important in the breeding of many crops as well as horticultural plants of the world [2]. Chromosome doubling in plants is usually achieved by chemical induction using anti-microtubule agents such as colchicine or oryzalin. Colchicine was used successfully to produce polyploid plants in several species of *Phalaenopsis* [3–5] as well as in other orchids [6]. Although the chemical-induction technique is a workable method for polyploidization, the disadvantages of complicated procedures and the problems of dealing with the toxicity of chemical agents as well as the occurrence of chimeras are unfavorable.

Endopolyploidy, the occurrence of different ploidy levels among cells of an organism, is commonly observed in many plants [7, 8]. It is also a common phenomenon in many species of orchids, such as in *Dendrobium* spp. [9], *Phalaenopsis* spp., *Oncidium varicosum* [10, 11], *Vanda* [12, 13], and *Spathoglottis plicata* [14]. In recent studies, it was observed that endopolyploidy occurred in different tissues of *Phalaenopsis* spp. throughout all stages of plant development including germinating seeds, protocorms, seedlings, and mature plants [11, 15, 16]. Simply speaking, polyploid cells already exist in the tissues of *Phalaenopsis* orchids under the natural environment.

One of the techniques of *Phalaenopsis* orchid micropropagation is to dissect the protocorm-like-bodies (PLBs) horizontally to produce the next cycle of PLBs [17, 18]. One of the problems of this method is the occurrence of somaclonal variation, especially tetraploidy, which probably resulted from endoreduplication of cells from the lower portion of the PLBs [19]. Therefore, precaution should be taken in the process of micropropagation. However, this observation suggests that it is possible to obtain tetraploid plants through similar procedures of tissue culture.

Another factor which determines the feasibility of the procedure is the method of detecting polyploidy. Traditionally, identification of polyploidy was carried out by chromosome counting using microscopy. This method is laborious and requires good skills. Due to the advancement of new technology, flow cytometry has become a popular technique to determine the nuclear DNA content of an organism [20]. This is an efficient, accurate method for genome size determination and ploidy identification. By using this new technology, genome sizes and fluorescence ratios of 50 species of *Phalaenopsis* species were determined by flow cytometer using either propidium iodide (PI) or 4,6-diamidino-2-phenylindole (DAPI) as the fluorochromes [21, 22]. These sets of data are important reference for the breeding programs as well as in the experiments of polyploid induction of *Phalaenopsis* orchids.

With the information on the studies of endopolyploidy and development on the technologies of micropropagation and genome

Table 1
Mean percentages and standard deviations of different ploidy levels (2*x*, 4*x*, 8*x*) in PLBs of clones derived from first and second sectioning cycles of *P. aphrodite* subsp. *formosana* protocorms [23]

Sectioning cycle	Explant for sectioning	Initial ploidy level	No. of clones analyzed	2*x* (%)	4*x* (%)	8*x* (%)	Ploidy doubling (%)[a]
1st cycle	Protocorm	Diploid	22	65.9 ± 20.7	33.5 ± 20.8	0.6 ± 1.5	34.1 ± 20.7
2nd cycle	1st cycle PLB	Diploid	131	48.3 ± 10.7	49.5 ± 9.3	2.1 ± 2.7	51.7 ± 10.7
		Tetraploid	74	0	94.0 ± 7.7	6.0 ± 7.7	6.0 ± 7.7

[a]% ploidy doubling was the sum of the percentage of polyploid higher than the initial ploidy level

size determination, a method using in vitro culture of excised protocorms or PLBs in *Phalaenopsis* species has been developed [23]. By this method, about 33–50% of the next-generation PLBs were tetraploids after the first or second cycle of the sectioned explants (either protocorms or PLBs) of the diploid *P. aphrodite* (Table 1). It was also found that about 10–45% of the next-generation PLBs derived from the cutting of protocorms in seven other *Phalaenopsis* species were polyploids [23]. Tetraploid plants derived from this method grew to maturity like other diploid plants. These results are clear indications of the feasibility of the procedure.

In this chapter, a protocol for the induction of polyploids in *Phalaenopsis* orchids by horizontal sectioning of protocorms or PLBs without using anti-microtubule agents is described. This is a simple and effective method to obtain polyploid plants in high frequency [23, 24]. Polyploid plants produced by this method are highly stable and normal in development, which can be evaluated directly. The procedure for the identification of polyploids by flow cytometry using DAPI is also discussed.

2 Practical Considerations

Protocorm is the small tuber-like body formed by germinating orchid seed. It has the potential to develop into a fully grown orchid plant. Protocorm-like bodies (PLBs) are structures similar to protocorms but are formed by tissue explants and/or callus in vitro [19]. Although protocorms and PLBs do not have the mature embryonic structure like many other plants, they still have certain cellular orientation and polarity that dictate the direction of plant development. Therefore, PLBs are claimed to be somatic embryos [25]. Since higher levels of endopolyploidy and production of polyploid plants in the basal portion (the suspensor end) of PLBs were observed [15, 16, 19], they are used for the induction of polyploids in this protocol. However, the choice of protocorms

or PLBs as the initial materials for polyploid induction depends on the objectives of the experiment because their genetic constitutions are different. A group of protocorms represents a range of genotypes of a population. However, PLBs from the same source is a clone which represents only a single genotype. Therefore, they should be treated differently (*see* Subheadings 4.1.2 and 4.1.3).

Occurrence of crystalline calcium oxalate crystals and endopolyploidy, especially in the mature tissues of *Phalaenopsis* orchids, often causes problems in the process of analysis by flow cytometry [15, 26]. To minimize these problems, young and small pieces of tissues, such as PLBs, young leaves of culture plantlets, or ovary before pollination should be used for the analysis [15, 21].

For identification of ploidy level using flow cytometry, fluorochromes of propidium iodide (PI) or 4′,6-diamidino-2′-phenylindole (DAPI) dihydrochloride can be used. However, due to practical reasons, DAPI is recommended because of the simplicity of its use. For example, staining using DAPI can be done under room temperature. Removal of RNA is not required for DAPI staining.

3 Materials

3.1 Plant Materials

1. For a particular *Phalaenopsis* spp. of interest, prepare an appropriate amount of protocorms or protocorm-like bodies (PLBs) produced by plant tissue culture technique under aseptic condition (*see* **Note 1**). Plant materials to be used for induction of polyploids should have a size of 3–5 mm in length, before the expansion of the first leaf (Fig. 1).
2. PLBs or in vitro plantlets having 3–4 leaves formed after the polyploid induction are used for the evaluation of ploidy levels by flow cytometry (*see* Subheading 4.2).

3.2 Laboratory Equipment and Supplies for Tissue Culture and Regeneration of Plants

1. Laminar flow transfer cabinet with HEPA filter.
2. Autoclave for media and equipment sterilization.
3. Water purification system for media preparation.
4. Growth chamber or growth room equipped with incubation shelves, fluorescent lights, and airconditioner for tissue growth and regeneration.
5. General laboratory equipment such as electronic balance, pH meter, and hot plate/stirrer.
6. General laboratory supplies such as scalpel handle (#3) and blade (#11), beakers, flasks, measuring cylinder, forceps, disposable pipettes, Petri dishes (10 cm diameter), conical flasks (500 mL), spatula, magnetic stirring bars, alcohol lamp or Bunsen burner, Parafilm M® tape, tape for autoclave indicator, aluminum foil, 70% ethanol for sterilization, etc.

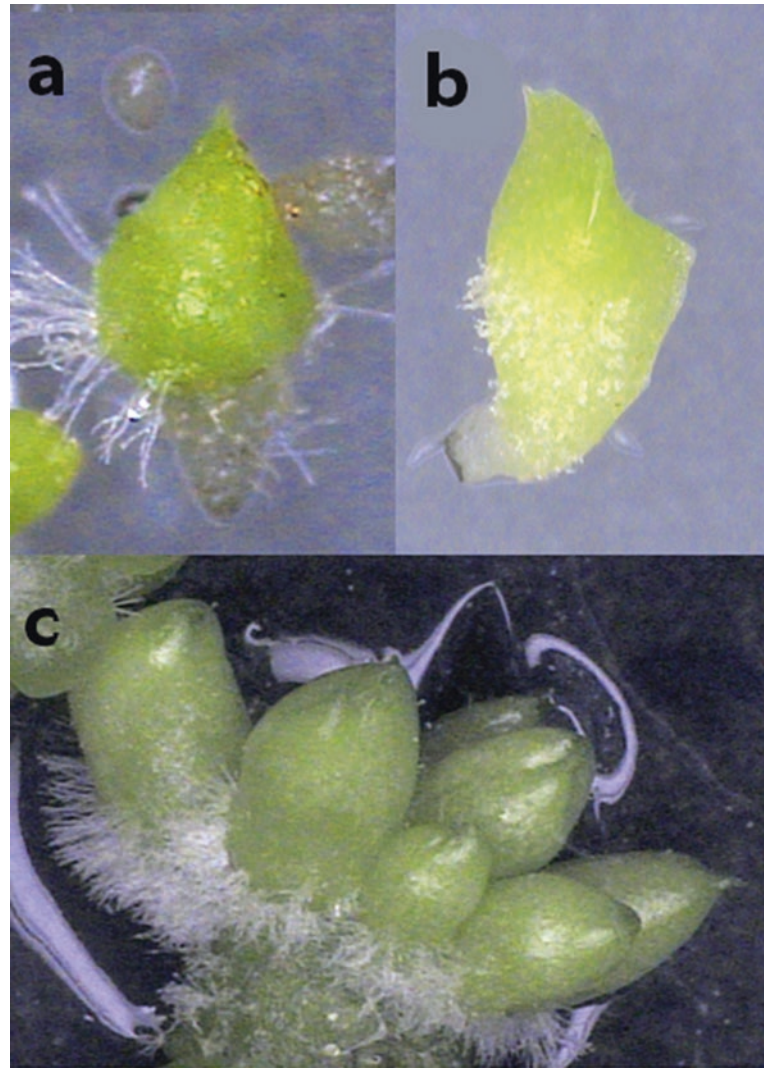


Fig. 1 Protocorms and PLBs of *P. aphrodite*. (**a**) Protocorm; (**b**) PLB; (**c**) PLBs regenerated from the lower portion of PLB after cutting

3.3 Media and Culture Conditions

3.3.1 Medium and Growing Conditions for PLB Induction

1. T2 medium: The induction medium is a semi-solid medium with the following ingredients – 3.5 g L^{-1} Hyponex No. 1 (N-P-K = 7:6:19), 1.0 g L^{-1} tryptone, 0.1 g L^{-1} citric acid, 1 g L^{-1} activated charcoal, 20 g L^{-1} sucrose, 20 g L^{-1} homogenized potato, 25 g L^{-1} homogenized banana, and 7.5 g L^{-1} agar; pH 5.5. Autoclave prepared medium at 121 °C for 20 min before pouring into 10 cm Petri dishes (25 mL per plate) in a laminar flow cabinet for solidification.
2. Growing conditions: Incubate Petri dishes containing the plant materials at 25 ± 2 °C and 16/8 h L/D photoperiod at a light intensity of 10–30 μmol m^{-2} s^{-1} for 2–3 months until the size of PLBs reaches 3–5 mm in length.

3.3.2 Medium and Growing Conditions for Plantlet Regeneration from PLB

1. T2 medium: Same as in Subheading 3.3.1. Subculture PLBs into new T2 medium every 2 months for further development into plantlets. Sealed conical flasks with T2 medium should be used for subculturing large plantlets.
2. Growing conditions: Same as in Subheading 3.3.1 except that the light intensity is increased to 30–40 μmol m^{-2} s^{-1}. Culture PLBs until plantlets having 3–4 or more leaves are formed.

3.3.3 Medium and Growing Conditions for Growing Polyploid Plants in the Growth Room

1. Pots and potting mix: Take mature plantlets from the Petri dishes or conical flasks: wash and transplant into plastic pots of appropriate size filled with good-quality sphagnum moss.
2. Growing conditions: Maintain and grow potted plantlets in the growth room at 27 ± 2 °C and 16/8 h L/D photoperiod with a light intensity of 60–100 μmol m^{-2} s^{-1}. Use standard protocols on irrigation and fertilization according to standard protocols of culture and management of *Phalaenopsis* orchids (*see* **Note 2**).

3.4 Laboratory Equipment and Supplies for Identification of Polyploids by Flow Cytometry

3.4.1 Equipment and Supplies for Flow Cytometry

1. Flow cytometer equipped with a HBO-100 mercury lamp for analysis of UV-excited fluorochromes (Ploidy Analyser PA-I, Partec GmbH, Münster, Germany, or from different suppliers).
2. Use vortex mixer with cup head for mixing nuclei suspensions in sample tubes before flow cytometer measurements.
3. Accessories for cytometer: Sample tubes for flow cytometry (FC tube) and racks, 30 μm disposable filters (CellTrics, Partec, Münster, Germany) and racks, 6 cm flat-bottom glass Petri dishes, sharp double-edged razor blades, hobby knife holder, and 1.2 mL micro-dilution tubes and racks (Fig. 2).
4. Common laboratory supplies such as micro-pipettes, forceps, wash bottles, ice bucket, and aluminum foil.

Fig. 2 Machine and accessories for flow cytometry

3.4.2 Reagent Kit for Flow Cytometry and Chemicals

1. CyStain® UV Precise P Kit (05-5002, Partec, Münster, Germany) consists of (1) an extraction buffer for nuclear extraction and (2) staining buffer with DAPI for the staining of nuclear DNA from plant tissues.
2. Common laboratory chemicals such as 10% Clorox solution and double-distilled water.

4 Methods

4.1 Induction of Polyploid Using PLBs

4.1.1 Dissecting of Protocorms or PLBs for Induction of Polyploids

1. Place a group of protocorms or PLB-derived protocorms with a size of 3–5 mm in length in a sterile Petri dish inside a laminar flow cabinet under aseptic condition. By using the scalpel (#3) with a sharp blade (#11), carefully cut the protocorms or PLBs transversely, approximately at the midpoint to separate it into an upper and a lower portion. The upper portion containing the growing point can be discarded or used for ploidy identification using flow cytometry (*see* Subheading 4.2).
2. Subculture the lower portions of the protocorms or PLBs in a Petri dish (10 cm in diameter) containing solid inducing medium (T2 medium, *see* Subheading 3.3.1), and place the cut surface facing upward so that the next-generation PLBs can develop from the cut surface. Place about 20–30 pieces of cut protocorms or PLBs in a Petri dish and seal with a Parafilm M® stripe.
3. Place the Petri dishes containing the cut protocorms or PLBs on the shelves of a culture room or growth chamber under the condition as indicated in Subheading 3.3.1, and culture for 2–3 months until the next-generation PLBs reach a size of 3–5 mm in length.
4. Separate the next-generation PLBs carefully using dissecting knives for further growth, identification of ploidy level, or further cutting by repeating **steps 1–3**, one or more times if necessary.

4.1.2 Induction of Polyploids from PLBs from a Clone of the Same Genotype

1. Produce a clone of PLBs from the same genotype induced by "flower-stalk culture," and follow by "leaf thin-section culture" under aseptic condition (*see* **Note 1**).
2. Select 20–40 PLBs with proper size from a clone and follow the steps of Subheading 4.1.1 to initiate the process of polyploid induction.
3. Repeat steps of Subheading 4.1.1 one or two more times to produce 30–40 clusters of the next-generation PLBs (*see* **Note 3**).
4. Separate individual PLB and place in T2 medium for regeneration of plantlets as shown in Subheading 3.3.2.

5. Assign a number for identity to each plantlet having 3–4 leaves or above. Take a small piece (about 0.3–0.4 cm^2 in size) of a young leaf from each plantlet under aseptic condition (*see* **Note 4**), and use for ploidy identification (*see* Subheading 4.2).
6. Save the plantlets with doubling of ploidy level (*see* **Notes 5** and **6**) for further growth in culture until they are large enough for transferring to the growth room according to the procedure in Subheading 3.3.3 (*see* **Note 2**).
7. Confirmation of the ploidy level of the mature plants should be carried out (*see* **Note 7**) before using for further evaluation and application. Plantlets without ploidy doubling can be discarded or saved for regeneration as controls for comparison.

4.1.3 Induction of Polyploids from Protocorms of Various Genotypes

1. Select 100 or more protocorms of 3–5 mm in length germinated from seeds of the same species (*see* **Note 8**) and follow the **steps 1–2** of Subheading 4.1.1 to conduct the first cycle of cutting.
2. Assign a line number to each cut protocorms for its identity. After the first cutting, the next-generation PLBs produced from each protocorm should be considered as a clone with its own identity.
3. Cut the next-generation PLBs of each line to produce about 10–20 newly generated PLBs within each line and follow **steps 3–6** in Subheading 4.1.2 to regenerate plantlets for further development and evaluation (*see* **Note 9**).

4.2 Identification of Polyploid Plants by Flow Cytometry

4.2.1 Preparation of Testing Samples

1. Put fresh tissue of a PLB (whole PLB or the upper portion of a PLB with fresh weight of about 20–40 mg) or a piece of young leaf (0.3–0.4 cm^2 or fresh weight of 20–40 mg) from in vitro plantlet in the bottom of a 6 cm glass Petri dish. Add 100 μL extraction buffer (Partec CyStain UV Precise P Kit).
2. Break a double-edged razor blade (*see* **Note 10**) into four pieces. Mount one of them onto the tip of a hobby knife handle and secure tightly. The blade should be placed in the direction vertically to the handle to make a "T"-shaped chopping tool.
3. Chop the material for 30–60 s using the razor blade to release the nuclei from the tissue (*see* **Note 11**). Wash the blade with distilled water before chopping the next sample. Discard the razor blade after cutting 5–10 samples.
4. Cover the Petri dish containing the nuclei suspension and place on the surface of ice in the ice bucket at an angle so that all the chopped tissues are immersed in the buffer solution for a 15–20 min incubation.

5. Add 400 μL DAPI staining solution (Partec CyStain UV Precise P Kit) to 100 μL of extraction buffer containing the nuclei suspension, and shake carefully to mix the solution.
6. Filter the nuclei suspension through a 30 μm nylon mesh (CellTricsTM, Partec) and collect the filtrate into a 1.2 mL micro-dilution tube. Place the tube in the rack, cover with aluminum foil, and place on ice for 15–30 min before analysis by flow cytometry.

4.2.2 Preparation of Standard Samples

1. Select 3–5 pieces of PLBs or young leaves from different in vitro plantlets of the species of interest (*see* **Note 12**).
2. Use one piece of the above materials to prepare the nuclei suspension according to **steps 1–6** of Subheading 4.2.1 with the following exceptions: (1) for each sample, young leaf of 0.5 cm^2 in size should be used; (2) add 400 μL extraction buffer; (3) add 1600 μL staining buffer; (4) sample tube specific for flow cytometry (FC tube) instead of 1.2 mL micro-dilution tube should be used to collect the filtrate of nuclei suspension.
3. Repeat **step 2** by using tissues from other individual plants to prepare few more tubes of nuclei suspensions which are used as standard references for setting up the flow cytometer.

4.2.3 Preparation of the Flow Cytometer (Ploidy Analyser PA-I, Partec GmbH, Münster, Germany)

1. Check the fluid levels in the sheath and waste containers. Add double-distilled water to the sheath container if needed.
2. Turn on the power of the machine and then switch on the computer. Let the UV light system warm up for 30 min according to manufacturer's instruction. Clean the tubular system of the machine twice with double-distilled water at high speed (speed 10). Return to low speed (speed 1) for analysis of samples.
3. Set the lower limit (LL) of the machine at 20.
4. Place a FC tube of prepared standard sample on the cup head of the vortex mixer; resuspend the nuclei suspension briefly.
5. Install the FC tube onto the sample inject port (SIP) and adjust the flow speed (between 1 and 3) to have a flow rate of 50–100 particles/second until 2500–5000 nuclei are analyzed or a stable histogram appears on the screen of the machine.
6. Adjust the gain of the flow cytometer so that the mean of the first peak (i.e., the G0/G1peak) of the standard sample should be positioned at channel 50 or 100 (the total length is 1024 channels in a linear scale).
7. Once the setting is done, remove the tube from the cytometer. Press the button "Clean" on the control panel to remove the remains of the previous sample.

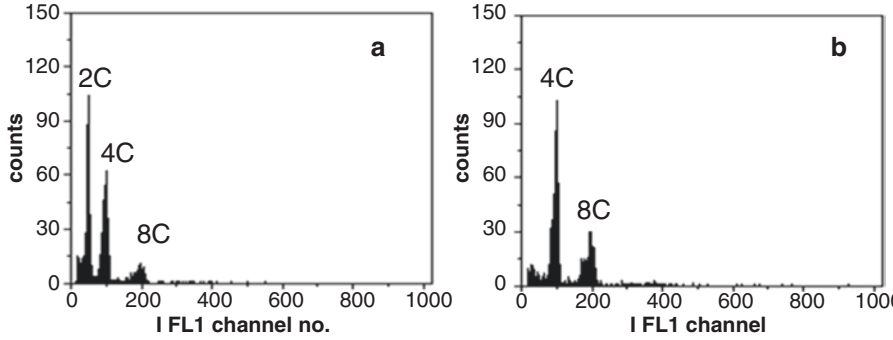


Fig. 3 DNA histograms of nuclei isolated from in vitro leaves of regenerated plantlets of *P. aphrodite*. (**a**) Diploid; (**b**) tetraploid

8. Repeat **steps 4** and **5** by using other four tubes of the standard sample to confirm the position of the G0/G1 peak is at approximately channel 50 or 100. This is a reference channel which represents the ploidy level before polyploidization of the species of interest.

4.2.4 Flow Cytometric Analysis of the Testing Samples

1. Keep track and record the number of the testing samples in a record sheet.
2. Select one sample of the nuclei suspension in a micro-dilution tube and vortex it. Then place it into a FC tube.
3. Set the speed of the flow rate of the machine to low (i.e., speed 1). Install the FC tube onto the sample injection port (SIP), and let it run until the appearance of a stable histogram on the screen (*see* **Note 13**). Record the mean of the G0/G1 peak in terms of channel number (*see* **Note 14**).
4. Compare the channel number of the testing sample with that of the standard sample to determine the ploidy level of the testing sample (Fig. 3).
5. Remove the tube, press the "Clean" button to remove the remains of the previous sample, and continue to analyze other testing samples of the same species.
6. At the end of the experiment, clean the tubular system of the machine with 5% Clorox and double-distilled water according to the instruction of the operating manual before turning off the computer and the power of the machine.

4.3 Recovery of Polyploidy Plants

1. Grow regenerated plantlets with polyploidization (Fig. 4) on T2 medium for 5 months or longer in the tissue culture room. Select some plants without polyploidization for regeneration as control for comparison.
2. Transplant the plantlets having 4–5 leaves (or larger) into plastic pots (3.5 cm wide × 4.5 cm high) filled with sphagnum moss.

Fig. 4 Diploid (left) and tetraploid (right) plantlets of *P. aphrodite* regenerated from the lower portion of PLBs after cutting

Fig. 5 Diploid (left) and tetraploid (right) flowers of *P. pulcherrima* var. *champornensis*

See Subheading 3.2 for the growing condition and management.

3. Evaluate and confirm ploidy level at the flowering stage of the plants (Fig. 5 and *see* **Note 7**).

5 Concluding Remarks

Tetraploids play an important role in variety improvement of *Phalaenopsis* orchids due to the advantages of large flower size and other characteristics. The protocol to produce polyploids presented in this chapter is a simple, effective, and reliable technique to produce a large amount of polyploid plants in *Phalaenopsis* orchids.

This method would broaden the genetic base of the breeding materials and be beneficial to orchid breeding programs especially for the interspecific hybridization between varieties having different chromosome sizes and ploidy levels. It might have a great impact for the new variety development of the orchid in the future.

6 Notes

1. For methods of production of protocorms or PLBs, *see* [17, 19, 27].
2. For general procedure of cultivation of orchid plants in the greenhouse, *see* [28] or cultivation manuals of orchid nurseries.
3. Chance of success for production of next-generation PLBs by **steps 1–3** in Subsection 4.1.1 depends on the species and the techniques of the operator. About 50% success and about 3–4 PLBs produced from each cut protocorm or PLB are assumed.
4. Selecting plant tissues for analysis by flow cytometry should be carried out under aseptic conditions, especially if they are to be used for further tissue culture development.
5. For practical purposes, plants that are triploids or preferably tetraploids should be selected for further development and use. Plants of higher ploidy levels are usually abnormal in plant growth.
6. The chance of obtaining the polyploids after the first cutting cycle according this protocol might vary from 10 to 45% depending on the species [22].
7. Preferably, confirmation of the ploidy level of a particular plant should be carried out using mature ovary of the flower before pollination by flow cytometric analysis [21, 22]. Another method is the measurement of stomata density of the mature leaf [22]. By this method, mature leaf of known diploid plant at similar developmental stage should be used for comparison.
8. According to the assumption made in **Note 3**, at least 100 protocorms from the same seed stock are used in the process in order to have sufficient number of polyploid lines for evaluation at the final stage of a breeding program.
9. Phenotypes of the polyploid plants developed from different lines according to this protocol could be evaluated directly for selection purpose because they are grown under normal tissue culture condition without any toxic effect, as in the methods of polyploid induction by anti-microtubule agents.
10. Double-edged razor blades of good quality should be used for the cutting.

11. Chopping of the tissues should be carried out vertically up and down with rapid and steady speed. Keep the cut tissue immersed and floating in the extraction buffer.
12. These samples are used for setting the baseline (i.e., the position of the G0/G1 peak on the scale of the screen of the flow cytometer) to represent the ploidy level before polyploidization. Three or more different PLBs or plantlets before or at the early stage of polyploid induction should be selected to prepare the samples because the chance of all of them which are polyploids is very low.
13. Adjust the flow rate to about 50–100 particles/second by turning up or down the speed of the machine. If the flow rate is too high when the speed is at 1 or less (i.e., the density of nuclei in the suspension is too high), use smaller pieces of tissue or reduce the time of chopping to decrease the nuclei density in the suspension in the next sample preparation. High flow rate may cause blocking of the tubular system of the machine or poor resolution of the histogram which should be avoided.
14. Keep records of the file number, means of the G0/G peak, percent, and CV value in the record sheet for each sample for future reference.

References

1. Christenson EA (2001) *Phalaenopsis*: a monograph. Timber Press, Portland, OR
2. Sattler MC, Carvalho CR, Clarinde WR (2016) The polyploidy and its role in plant breeding. Planta 243:281–296
3. Nakasone H, Kamemoto H (1961) Artificial induction of polyploidy in orchids by the use of colchicine. Hawaii Agric Ex Stn Tech Bull 42:27
4. Griesbach RJ (1981) Colchicine-induced polyploidy in *Phalaenopsis* orchids. Plant Cell Tiss Org Cult 1:103–107
5. Griesbach RJ (1985) Polyploidy in *Phalaenopsis* orchid improvement. J Hered 76:74–75
6. Silva PAKX, Callegari-Jacques S, Bodanese-Zanettini MH (2000) Induction and identification of polyploids in *Cattleya intermedia* Lindl. (Orchidaceae) by *in vitro* techniques. Ciência Rural 30:105–111
7. Barow M, Meister A (2003) Endopolyploidy in seed plants is differently correlated to systematics, organ, life strategy and genome size. Plant Cell Environ 26:571–584
8. Barow M (2006) Endopolyploidy in seed plants. Bioessays 28:271–281
9. Jones WE, Kuehnle AR (1998) Ploidy identification using flow cytometry in tissues of *Dendrobium* species and cultivars. Lindleyana 13:11–18
10. Lin S, Lee HC, Chen WH, Chen CC, Kao YY, YM F, Chen YH, Lin TY (2001) Nuclear DNA contents of *Phalaenopsis* sp. and *Doritis pulcherrima*. J Amer Soc Hort Sci 126:195–199
11. Lee HC, Chiou DW, Chen WH, Markhart AH, Chen YH, Lin TY (2004) Dynamics of cell growth and endoreduplication during orchid flower development. Plant Sci 166:659–667
12. Alvarez MR (1968) Quantitative changes in nuclear DNA accompanying postgermination embryonic development in *Vanda* (Orchidaceae). Amer J Bot 55:1036–1041
13. Lim WL, Loh CS (2003) Endopolyploidy in *Vanda* Miss Joaquim (Orchidaceae). New Phytol 159:279–287
14. Yang M, Loh CS (2004) Systemic endopolyploidy in *Spathoglottis plicata* (Orchidaceae) development. BMC Cell Biol 5:33–39
15. Chen WH, Tang CY, Lin TY, Weng YC, Kao YL (2011) Changes in the endopolyploid pattern of different tissues in diploid and tetraploid *Phalaenopsis aphrodite* subsp. *formosana* (Orchidaceae). Plant Sci 181:31–38
16. Jean GT, Kao YL, Tang CY, Chen WH (2011) Distribution of nuclei of different ploidy levels during ovule, seed and protocorm

development in *Phalaenopsis aphrodite* subsp. *formosana* (Orchidaceae). Amer. J Plant Sci 2:325–333

17. Tanaka M (1987) Studies on the clonal propagation of *Phalaenopsis* through *in vitro* culture. Memo Fac Agri Kagawa Univ 49:1–85
18. Murdad R, Hwa KS, Seng CK, Latip MA, Aziz ZA, Ripin R (2006) High frequency multiplication of *Phalaenopsis gigantea* using trimmed bases protocorms technique. Sci Hort 111:73–79
19. Paek KY, Hahn EJ, Park SY (2011) Micropropagation of *Phalaenopsis* orchids via protocorms and protocorm-like bodies. In: Thorpe TA, Yeung EC (eds) Plant embryo culture: methods and protocols. Springer, Dordrecht, pp 293–306
20. Doležel J, Bartoš J (2005) Plant DNA flow cytometry and estimation of nuclear genome size. Ann Bot 95:99–110
21. Chen WH, Kao YL, Tang CY, Tsai CC, Lin TY (2013) Estimating nuclear DNA content within 50 species of the genus *Phalaenopsis* Blume (Orchidaceae). Sci Hort 161:70–75
22. Chen WH, Kao YL, Tang CY (2014) Variation of the genome size among *Phalaenopsis* species using DAPI fluorochrome and flow cytometry. J Taiwan Soc Hort Sci 60:115–123
23. Chen WH, Tang CY, Kao Y (2009) Ploidy doubling by *in vitro* culture of excised protocorms or protocorm-like bodies in *Phalaenopsis* species. Plant Cell Tiss Org Cult 98:229–238
24. Chen WH, Kao Y, Tang CY (2013) Method for producing polyploid plants of orchids. US Patent no. 8383881B2
25. Lee YI, Hsu ST, Yeung EC (2013) Orchid protocorm-like bodies are somatic embryos. Amer J Bot 100:2121–2131
26. Lee HC, Lin TY (2005) Isolation of plant nuclei suitable for flow cytometry from recalcitrant tissue by use of a filtration column. Plant Mol Biol Rep 23:53–58
27. Chang WC (2007) *In vitro* morphogenesis and micro-propagation of orchids. In: Chen WH, Chen HH (eds) Orchid biotechnology. World Scientific, Hackensack, NJ, pp 45–64
28. Runkle E, Wang YT, Blanchard M, Lopez R (2007) Growing the best *Phalaenopsis*. Orchids 76(1):24–28

Chapter 18

Virus Detection

Chin-An Chang

Abstract

Orchids are appreciated for their delicate and enchantingly beautiful floral parts. Decades ago, orchids were more expensive than other ornamentals due to their limited supplies and difficulties in propagation and cultivation. Nowadays, using modern tissue culture technology, orchids are readily available commercially, and the price of plants is becoming more affordable. However, during mass propagation, virulent viruses may also be reproduced along with tissue-cultured clones and thus decrease the growth vigor and the ornamental value of the finished products. As a result, virus detection prior to orchid propagation is a vital process in modern orchid industry, in order to prevent viruses from spreading among the tissue-cultured plantlets. This chapter describes a technique, namely, an enzyme-linked immunosorbent assay (ELISA), which is currently widely adopted by most major orchid companies as a routine test for virus detection. The basic mechanism of the technique, materials and facilities needed, detailed protocols, result interpretation, and some tips to prevent background problems are presented in the text. We also detail and discuss some alternative methods that orchid growers can use in orchid virus detection.

Key words Orchid, Virus, Virus disease, Detection technique, ELISA

1 Introduction

The family Orchidaceae is probably the largest family of flowering plants, containing at least 25,000 species, and most of them can give enchantingly beautiful flowers during their life span [1]. Many orchid species are now popular ornamentals dominating the output value of global floral markets, while some of them are famous for their medicinal uses [1, 2]. Decades ago, the unit price for a pot of artificially cultivated orchids is more expensive than other common ornamentals due to their limited supplies [2]. However, nowadays most orchids have become more affordable and easy to purchase from commercial growers. This is primarily due to improved propagation methods; millions of plantlets can be reproduced from a single orchid plant by the use of tissue culture technology within 2–3 years. This efficient vegetative propagation technology has also significantly assisted the blooming development of the global orchid industry in recent decades. However, viruses and other diseases also

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_18,

accompanied this fast population increase and have become one of the major cultivation problems for orchids [2–4]. Once infected by viruses, the growth of orchid plants will usually be reduced. Some viruses can cause evident symptoms on the leaves or even on the floral parts that will significantly decrease their ornamental value. There have been at least 42 taxonomically explicit virus species found to infect orchids [2]. Fortunately, most of them are only endemic or sporadic cases with little economic significance [1, 2, 5, 6]. So far, nearly all relevant literature cited that *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CymMV) are the two most common and economically important viral agents for orchids [1, 2, 5, 6]. Therefore, the initial explants used for propagation have to be checked carefully, making sure that they are free from viral infections before mass propagation [2, 7, 8]. Since the early 1970s, some conventional techniques such as electron microscopy (EM) examination, differential host inoculation, and serological tests have been used for orchid virus detection [1, 2, 5, 9]. However, these traditional techniques are not sensitive in viral detection, and at the same time, these methods are labor intensive and are mainly suitable for smaller sample working environment [2]. Since year 2000, orchid production has been industrialized, and thousands of samples are processed daily in most orchid companies [2]. Thus, an efficient, cost-effective, sensitive, and dependable technique for detecting viruses needs to be employed. Currently, the enzyme-linked immunosorbent assay (ELISA), invented in 1977 by Clark and Adams, has become popular and widely applied in current orchid industry for virus detection [2]. In the following sections, a protocol for routine detection of orchid viruses by ELISA is detailed and its advantages are discussed.

2 Features of the Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is one of the serological techniques for virus detection based on antibody-to-antigen reaction mechanism [10–13]. ELISA uses enzyme-conjugated antibodies for detection [2, 10–13] unlike conventional serological tests, which depend entirely on precipitation phenomenon occurring between antibodies and their viral antigens. After reacting with their virus antigens, the reaction signal will be amplified by the continuous reaction of enzyme with its substrate molecules. This is how the sensitivity of ELISA is significantly improved [10]. Secondly, ELISA is performed in EIA plates with 96 sample wells, making it more efficient and cost effective than other techniques [10]. Thirdly, ELISA results are expressed by the color reaction of the enzyme with its substrate and read by an absorbance reading machine, not by technician's subjective judgment [2]. Lastly, the same ELISA system and protocols can easily be adopted for the detection of nearly all viruses including those of orchids, once the antibodies of targeted

viruses are available [2]. Nowadays most ELISA protocols can be completed in a robotic system, with the exception of some key steps such as sampling. With these improvements, ELISA has nearly dominated worldwide as the premier virus detection technique in the plant industry since the 1980s [2]. In Taiwan, our laboratory established ELISA detection of ORSV and CymMV in 1990, and since then the technique has become the most widely applied method in Taiwan's orchid companies [2, 3]. Routine detection of at least nine different orchid-infecting viruses has also been established in our lab based on this platform (*see* **Note 1**).

2.1 Basic Mechanism

The procedure described in this chapter is for an antigen coating type of ELISA also called indirect ELISA [2, 11]. The sap from test orchid samples is first coated on the EIA plate and then reacted with antibody against targeted virus. The trapped antigen and antibody complex on the plate will be detected using enzyme-conjugated secondary antibody prepared from a different animal. Commonly used universal conjugates are alkaline phosphatase-labeled goat anti-rabbit or anti-mouse immunoglobulins. This procedure is simpler than another technique known as direct ELISA or double-sandwich ELISA. In direct ELISA, the targeted virus coats the EIA plate, and after reacting and capturing the viral antigens, the same enzyme-conjugated antibody is used for detection without the need of a secondary antibody [8]. Since indirect ELISA routinely uses commercialized universal enzyme-conjugated antibodies, users do not have to conjugate the enzyme with the primary antibody themselves. Despite this difference, both ELISA formats are nearly identical in their protocols, mechanisms, and technical skills. However, after working on virus detection for almost 30 years, our experience and many others have shown that the reaction spectrum of indirect ELISA is somehow wider than the direct format [2, 7, 11]. In another words, using an antibody of a virus isolate, indirect ELISA can always detect nearly all related strains of the same virus, while direct format often detects only the homologous strain. The high specificity of direct ELISA makes it more suitable for the study of certain specific virus strain, while the broader specificity allows the indirect format to be more widely applied in plant industries that always concern with all possible virus strains [7].

3 Materials

1. Plastic sealed bags for preserving orchid test samples. Regular sandwich bags can be used for this purpose.
2. Knives or blades for sampling. We regularly use thin razor blades from Gillette or Lion. One razor blade can be divided into four separate parts for routine uses. All knives or blades need to be sterilized before reuse (*see* **Note 2**).

Fig. 1 A specially designed electric tissue roller for high-throughput sample sap preparation

3. Maceration bags for tissue grinding (*see* **Note 3**).
4. 96-well flat-bottomed polystyrene or polyvinyl EIA plates. We routinely use EIA microplate manufactured by Greiner Bio-One GmbH., Germany. Reusing EIA plates is not recommended.
5. Single- and multi-channel pipets.
6. Humid incubation boxes or chambers.
7. Handy tissue grinders or special designed automatic roller (Fig. 1) (*see* **Note 4**).
8. Temperature control incubators (37 °C and 25 °C).
9. Handy plastic washing bottles or high-throughput washing apparatus (*see* **Note 5**).
10. Rabbit immunoglobulin (IgG) specific to various orchid viruses with concentration mostly adjusted to 1 mg/mL (*see* **Note 6**).
11. Enzyme-conjugated antibody: Alkaline phosphatase-conjugated goat anti-rabbit IgG. We regularly use the affinity-purified enzyme conjugate (code no. 111-055-003) made by Jackson ImmunoResearch Laboratories, Inc., USA.
12. Coating buffer: 0.1 M carbonate buffer, adjusted to pH 9.6 (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ per L). An antimicrobial agent like 0.2 g/L of sodium azide (NaN_3) should be added if prolonged storage is anticipated. Please note that NaN_3 is highly toxic; therefore, manufacturer's guidelines should be strictly followed.
13. Conjugate buffer: PBS buffer [8 g NaCl, 0.2 g KH_2PO_4, 2.9 g Na_2HPO_4 $12H_2O$ per L (pH 7.4)] with the addition of 2 g/L ovalbumin (Sigma, Grade V) and 2% polyvinylpyrrolidone (mol. wt. 40,000). An antimicrobial agent like 0.2 g/L of sodium azide (NaN_3) should be added if prolonged storage is anticipated.

14. Substrate buffer: 1 M diethanolamine adjusted to pH 9.8 with 2 N HCl. Buffers should be kept refrigerated. An antimicrobial agent like 0.2 g/L of sodium azide (NaN_3) should be added if prolonged storage is anticipated.
15. Washing buffer: PBS-Tween [PBS: 8 g NaC1, 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4 \cdot 12H_2O$ per L (pH 7.4)] with the addition of 0.05% (v/v) of Tween-20. Washing buffer can be stored as a 10× concentrate stock without preservative and diluted before use.
16. Substrate: p-Nitrophenyl phosphate (pNPP) is normally used as a substrate for alkaline phosphatase enzyme. Some chemical companies provide powdered pNPP while some produce tablet forms. We prefer to use pNPP tablets (catalogue no. 0617-1KT) manufactured by AMRESCO Inc., USA. It is more convenient to use tablet form to prepare substrate solution of 1 mg/mL. Be sure to make substrate solution just before use.
17. Absorbance reading machine. There are different models of EIA reading machine available in the market, but we found EMax model from Molecular Devices LLC, CA, USA, is satisfactory.
18. Infected orchid virus tissue as positive control.
19. Healthy tissue as negative control.

4 Methods

4.1 Collection of Leaf Tissue and Extraction of Sap

To perform sampling in a nursery, usually collect an entire leaflet or leaf from an inspected plant, place into a plastic sealed bag, and bring to the lab for further tissue sampling. Always use sterilized small knives for tissue sampling. We prefer to use commercial razor thin blades. Divide each blade into four equal parts and use each piece for only one individual cut to prevent the spread of diseases. Save the used blades and reuse after sterilization (*see* **Note 2**). For some orchid species, such as *Phalaenopsis*, besides leaves, roots, flower spikes, or petals can also be used as target samples. If leaves are sampled, excise several leaves of different ages including young, fully grown, and old leaves and combine them as one sample. It is advisable to refrigerate collected samples at 4 °C if they are not processed for several days before ELISA determinations.

Perform more precise sampling in the laboratory. We routinely sample tissues from different leaves or from different parts of the same leaves to make a total of about 0.1 g of tissue and transfer them into another plastic bag (maceration bag) for maceration. In our laboratory, we routinely use a specially designed electric roller (Figs. 1 and 2) to grind the tissue (*see* **Notes 3** and **4**). After complete maceration, add an appropriate volume of coating buffer (2.4 mL in our case) into each maceration bag to make a 25× dilution

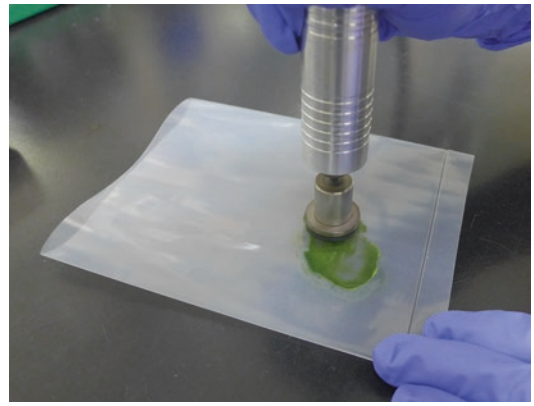


Fig. 2 The electric roller can compress orchid tissues inside the maceration bag easily and efficiently

of sap before transferring the aliquots to EIA plate wells (*see* **Note 5**). Be sure to minimize the interval from sap extraction to placement in wells. If prolonged interval is anticipated, store the extracted at 4 °C. Here we need to emphasize that key to get a correct ELISA detection result is to avoid virus cross contamination between test samples during sap preparation (*see* **Note 6**).

4.2 Designing an ELISA Experiment in an EIA Plate

When performing an ELISA experiment, an 8 × 12 well EIA plate made from polystyrene material is recommended**.** There are different brands of EIA plates available in the market. One should test the available brands before choosing a consistent one as routine test plate. Due to possible quality variation among plate wells, duplicate the test sample in at least two wells [11]. Therefore 48 samples at most can be analyzed in one 96-well EIA plate. However, each plate requires six control wells, i.e., two for healthy plant tissues, two for virus-infected tissues, and another two as blank controls [11] (*see* **Note 7**). These three controls are always set at the corners of each plate, for example, at lines no. 11 and 12 of rows F, G, and H. Therefore, a maximum of 45 test samples can be processed in one plate. If the total number of samples is less than 45, the wells at the edge of the plate should not be used, as these wells are more easily affected by the surrounding temperature, making the enzyme reaction possibly bias from the central wells.

4.3 Standard Procedures

1. Coating of antigens: Transfer 100 μL of expressed plant sap from each sample tissue bag to each well of an EIA plate. At least two replicate wells for each sample number are preferred. As indicated earlier, it is necessary to include healthy plant tissue as a negative control, a known infected plant tissue as a positive control, and at least two more wells as blank controls.

2. Incubation: Incubate at 37 °C for 4 h or at 4 °C overnight to allow for viral antigen trapping on the surface of an EIA plate.
3. Washing: Mechanically or manually empty coating solution from the wells and wash three times with washing buffer. One can use a plastic washing bottle or other commercial washing facilities to do plate washing. In our lab, we routinely use a commercial 12-channel tip apparatus fitting with a pumping system for washing the wells (*see* **Note 8**). Invert washed plates and tap onto tissue paper to dislodge droplets remaining in wells. After washing, use the plates immediately for the next step, or alternatively keep in moisture box to prevent drying, and store at 4 °C for a few days or up to 6 months at −20 °C.
4. Adding virus-specific IgG: Transfer 100 μL of virus-specific IgG (1:1000 diluted from the 1 mg/mL of stock rabbit IgG solution with conjugate buffer) to each well.
5. Incubate at 25 °C for 3–4 h or at 4 °C overnight to allow virus-specific IgG to react with trapped antigens on the surface of wells.
6. Washing as in **step 3**.
7. Adding alkaline phosphatase conjugated goat anti-rabbit IgG (second antibody): Transfer 100 μL of the second antibody solution diluted with conjugate buffer (follow manufacturer's instruction for appropriate dilution) to each well.
8. Incubate plates as in **step 5**.
9. Washing as in **step 3**: Sometimes, a 4× washing step is preferred at this stage in order to further reduce the background reaction.
10. Adding enzyme substrate: Transfer 100 μL of substrate solution into each well. Prepare fresh alkaline phosphatase (pNPP) substrate solution in the substrate buffer (1 mg/mL) at room temperature. Incubate at room temperature (25 °C) for the color development, or keep in a 25 °C incubator during cold winter season if necessary. During incubation, keep plates away from direct sunlight. Record absorbance readings at 405 nm in an automatic reader when color has developed, usually after 15–60 min. If necessary, stop the development by the addition of 50 μL of 3 N NaOH to each well.

5 Data Interpretation

1. It is generally accepted that two times the healthy control EIA readings is considered the threshold for a positive reaction, although some researchers prefer three times the healthy control readings as a threshold [2, 11]. Any sample with two duplicate wells having averaged reading higher than this threshold will be

considered as positively infected by the targeted virus. Some high-quality virus-specific antibodies react almost negatively with the healthy control sap so that their negative control EIA readings are always lower than 0.1 optical density (OD) value, no matter how long the reaction period is given. Under this rare situation, we recommend using a 0.2 OD value as threshold, above which is determined as positive reaction. In our lab, we always stop the reaction and take the final OD readings of the healthy control when OD readings approach 0.1, so that all samples with OD reading higher than 0.2 are considered as virus infected.

2. If the OD readings of two duplicate wells from the same test sample are highly varied, this is usually due to operation error. One possibility is spillover of enzyme conjugate or cross contamination of viral antigens from nearby wells. Incomplete washing during operation can also contribute to this error. Once this happened, these samples should be retested again.
3. If by chance that the two positive control wells give only low or no OD readings, this implies either the reactivity of antibody or the control antigen is poor. Under this situation, the whole experiment should be repeated and reactivity of antibody and the positive control viral antigen should be rechecked. If necessary, using a new lot of antibodies and/or antigen is preferred.
4. If both wells of the healthy control give significantly high readings, this indicates that the virus antibodies used may have cross-reacted with host antigens. If this happened, differentiation of positive from negative reaction becomes difficult. To deal with this problem, one can follow the tips described below to minimize the false-positive readings.

6 Tips to Deal with High Background Readings in ELISA

6.1 Minimize False Positive in Blank Control Wells

As mentioned earlier in each EIA plate, at least two wells should be set as blank control, in which only blank coating buffer is added at the first step. The final EIA readings in these two wells are used by the absorbance reading machine to calibrate the background reaction readings. However, sometimes contamination of enzyme conjugate spilled over from nearby test sample wells may cause abnormal high readings in these blank control wells. This will make the healthy control OD readings lower than 0.0 after calibration of the absorbance machine. Once this has happened, it will be difficult to determine the positive reaction of test samples. To prevent this from happening, we use conjugate buffer instead of coating buffer to fill these blank control wells. The ovalbumin protein in the conjugate buffer will block the plate surface, thus

preventing the spillover of enzyme conjugate from coating to the wells. With this modification, the false high EIA reading of control wells will be prevented.

6.2 Minimize False-Positive Problem Due to Cross Reactivity of Virus Antibodies with Host Plant Antigens

To perform a dependable virus detection using ELISA, it relies on high-quality virus-specific antibodies, which react specifically only with their homologous viral antigens (*see* **Note 9**). However, in reality most antibodies can react partly with host plant antigens, thus causing so-called background reading or false-positive or false-negative problems. These background readings will prohibit a clear differentiation of infected samples from the healthy ones. To solve this problem, we found that a minor modification of diluting the virus antibody with the conjugate solution can minimize the background reaction of the antibodies with host antigens. First, we grind the healthy plant tissue (the same host species as targeted sample) with the conjugate buffer (*see* Subheading 3) by the ratio of 1: 25 (W/V). After filtering through cheesecloth, the sap is then used for diluting virus IgG and virus detection in the following ELISA procedure. This is in fact a type of competition mechanism between viral and host plant antigens with the antibodies. This allows those antibodies that can react with host antigens to remain in the solution instead of trapping on the well surface and consequently washed away without further reacting with enzyme-conjugated secondary antibodies. We have successfully applied this modification and obtained good results minimizing background readings.

7 Other Alternatives for Orchid Virus Detection

The ELISA is the most popular method for orchid companies to handle hundreds or even thousands of samples daily. However, there are situations such as in small nurseries or amateur orchid hobbyists that only a few samples are required for testing. In this situation, ELISA is not necessarily the most appropriate choice. Instead, some techniques such as the polymerase chain reaction (PCR) and immune-strip tests are used as alternatives [4]. PCR aims to amplify certain region of the targeted orchid virus genome [2, 14–17]. By using two artificially synthesized oligonucleotide primers complementary to the flanking regions of the targeted sequence and through the action of a heat-stable DNA polymerase, DNA fragments with expected sizes will be efficiently amplified from the targeted virus genome. Subsequently they can be revealed by agar gel electrophoresis analyses [11, 16]. Multiplex techniques capable of detecting both ORSV and CymMV have also been developed and adopted by some orchid companies in Taiwan [2, 14, 18]. Under optimum conditions, PCR is generally accepted as a more sensitive method than ELISA and other commonly used

techniques [11]. However, there are limitations in conducting PCR. First, its high sensitivity of amplification will possibly result in false-positive reactions when there is cross contamination between samples and if precaution has not been taken [2, 11]. Secondly, procedure of PCR required tedious and sophisticated pipetting skills to handle trace volume of reactants. Thirdly, PCR procedures required the laboratory to meet higher sanitation and technical standard than those conventional methods like ELISA [2, 11]. Therefore, it is difficult for most orchid companies to adopt PCR as a routine detection method. Alternatively, orchid growers especially the hobbyists can select the immune-strip test for the detection of orchid viruses (*see* **Note 10**). Orchid virus immune-strips such as those of ORSV and CymMV are readily available from commercial biotech companies. Some companies even sell immune-strips that can detect both ORSV and CymMV in one strip. Although the cost for immune-strip test is much higher than that of ELISA, it has unique advantages such as performing virus detection on site where the suspected orchids are spotted in the nurseries. Simply by grinding the test tissue with buffer that come with the kit and then dipping the end of strips, sample sap moves upward by capillary action. Two puerperal lines will be visualized within 15–30 min in the infected sample sap. The pigmentation on the lines is due to gradual accumulation of gold-labeled virus-specific antibodies reacting with its viral antigens. This technique is very simple without the need of laboratory facilities. However, due to its theoretically lower sensitivity in virus detection, it is mostly recommended for identifying causal virus species on infected symptomatic orchids. This method is rarely suggested to apply in certifying the virus-free status of asymptomatic orchids. Another limitation is that, due to costs, strip test kits are not available for all orchid viruses.

8 Concluding Remarks

There have been numerous detection techniques developed since the early twentieth century when plant viruses had detrimental effects on crop production. In current orchid industry, ELISA is the most widely adopted method to detect viruses [2, 4, 7]. In addition to efficiency and cost-effectiveness, the flexibility of this method solidifies its important role in virus detection. Furthermore, after almost three decades of use, ELISA has been shown to be dependable enough to solve practical virus problems in orchid production. This chapter describes in detail the mechanisms of indirect ELISA, its operation protocols, materials and facilities needed, and some tips for troubleshooting for orchid propagators to perform virus indexing. We are confident that by following these protocols and suggestions, one should consistently obtain satisfactory

results of virus detection. However, there are still additional remarks that need to be emphasized.

1. By grouping and sampling together different parts of tissue and performing multiple trials, more correct, reproducible, and dependable detection results can be obtained. Our past experience indicated that the result of two repeated ELISA tests is often more dependable than that obtained from a single PCR experiment.
2. To ensure that selected orchids are indeed freed of viral infection, one dependable strategy is to retest your suspected orchid plants again after a few weeks or a month. However, you need to guarantee those plants are kept in an insect proof greenhouse and well separated from each other so that re-infection by viruses is prevented.
3. Finally, ELISA only indicates if a virus is present. In order to solve virus disease problems in orchid production, one must implement strict hygiene management to prevent orchids from becoming infected.

9 Notes

1. The aforementioned indirect ELISA protocol can be used to detect all orchid viruses once the specific antibody is available. We have successfully applied this protocol to detect at least nine orchid viruses, including ORSV, CymMV, *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Basella rugose mosaic virus* (BaRMV), *Carnation mottle virus* (CarMoV), and *Cymbidium ringspot virus* (CymRSV), *Turnip mosaic virus* (TuMV), and *Orchid fleck virus* (OFV). In our lab, the same ELISA system is routinely applied for the detection of more than 50 different plant viruses infecting various crops.
2. To prevent the knives or razor blades from cross contamination by viruses during tissue sampling, they should be sterilized before use and re-sterilized again for later applications. Sterilization is preferable using an autoclave apparatus setting at 121 °C for at least 20–30 min.
3. Plastic bag sized about 12 × 15 cm with a thicker plastic sheet is used for tissue maceration. Since we routinely use an electric roller to compress the tissue, thicker plastic sheet is needed to prevent bag breakage during maceration.
4. Depending on the number of samples needed to be processed for ELISA detection, there are different ways to perform tissue maceration. In the Netherlands, there are virus detection service companies that efficiently process thousands of samples in

a day. However, the facilities are highly expensive and not feasible or affordable for most research laboratories and orchid nurseries. Therefore, we designed a handy electric roller system (Fig. 1), which combines a commercial electric transmission shaft driving a roller head to compress orchid tissue in the maceration bag (Fig. 2). The running speed of roller is adjustable by the electric shaft that can save the labor for maceration. Hundreds or even thousands of samples can be easily processed daily by this system. This roller system has been released to a company [Accurate Co., Taiwan (accurat.ct27@msa.hinet.net)] for commercialization. Alternatively, for orchid nurseries dealing with very small sample number, it will be cost effective just to use regular mortar and pestle to do tissue grinding.

5. We recommend routine application of 25× dilution of the tissue sap for routine ELISA determination. This is the best way for sap preparation based on many years of experience. Actually, dilution factors from 25× to 100× work equally well for consistent virus detection by this ELISA system. But the 25× dilution consumes the least coating buffer volume and thus considered the most cost-effective way for routine tests. Furthermore, 100 μL of sample sap adding into the ELISA wells is also experimentally checked as an optimum volume which can be applied universally for almost all viruses.
6. When conducting sampling in orchid nursery, technicians should wear disposable gloves and avoid touching directly the test plants. This is to prevent cross contamination between samples and unexpected virus transmission among sampled plants during sampling. We always use plastic sample bags to hold the target leaf and cut to release it with the razor blade. This way, the sampled plant or the sample tissue will not be touched by the operators. To perform precise sampling in the lab, we routinely use individual weighing paper to hold and estimate the weight of each sample and then transfer them into the maceration bag. Be sure not to allow any possible cross contacts between sampled tissues by operator's fingers. Each razor blade can only be used specifically for one individual sample. Once the sampled tissues are placed inside the maceration bags, the chance for cross contamination will be minimized (Fig. 2). In addition, remember to keep the working benches and facilities free from contamination by orchid viruses, which may easily be brought into the lab through infected orchid tissues. This contaminant is the possible cause of ELISA experimental error, if they are not appropriately sanitized. We routinely sterilize the working benches and laboratory facilities with regular lab cleanser and then spray with 0.5% (v/v) of Clorox (sodium hypochlorite) solution to disinfect possible contamination of orchid viruses especially the ORSV and CymMV.

7. Virus-specific antibody is not the only crucial aspect for ELISA detection. The availability of good positive and healthy control antigens is also crucial. Although some positive control virus antigens can be purchased from biotech companies, they are fairly expensive and sometimes difficult to get. We suggest that routine ELISA users should prepare and maintain their own positive control virus antigen collections. To maintain ORSV and CymMV tissue antigens, for example, we always cut the infected leaves into 1 cm^2 leaf disc and immerse them into 50% (v/v) glycerol solution and store in glass bottles at −20 °C freezer. Under this condition, the stored tissue can stay green and fresh but not frozen for a long period of time and their virus antigenicity can be well maintained. After washing with tap water, the leaf disc can be ground with coating buffer and applied for reaction.
8. The washing apparatus contains a common 12-channel washing head connecting with the buffer tank and driven by a pumping system. One can easily purchase and combine these separate parts to make a handy washing system.
9. Antibodies against orchid viruses can be purchased from biotech companies specialized for plant disease diagnosis (such as Agdia Co., USA) or shared from various professional research laboratories. Actually, we produce our own antibodies for routine detection. These antibodies are also released to a biotech company, Accurate Co., Taiwan, for commercialization. Conventionally, most biotech companies provide orchid virus-specific antibodies in the form of purified immunoglobulin G (IgG) raised from rabbits with regular concentration at 1 mg/mL, so that the users can consistently prepare optimum dilution of antibody for reaction. The working concentration, i.e., the dilution factor, for different antibodies should follow producer's instruction. If the antibody is raised from mice, for example, the monoclonal antibodies, then the enzyme conjugate of goat anti-mouse antibodies should be used for detection.
10. Immuno-strip test kits for orchid viruses are available in some diagnostic companies such as Agdia Co., USA, and Rega Biotech Inc., Taiwan.

References

1. Lawson RH, Hsu HT (1995) Orchid. In: Loebenstein G et al (eds) Virus and virus-like diseases of bulb and flower crops. Wiley, West Sussex, pp 409–420
2. Chang CA (2017) *Phalaenopsis* viruses and their detection techniques. In: Chen WH, Chen HH (eds) Orchid biotechnology III. World Scientific Publishing Co., Singapore, pp 165–188
3. Chang CA (2008) Economically important orchid viruses; how to produce clean orchid plantlets. Orchids 77:668–671
4. Chang CA (2013) A review of how the orchid industry in Taiwan to tackle virus disease prob-

lem in the past two decades. Proceedings of promotion of competitiveness of ornamental plant industry in Taiwan. Taiwan Society for Horticultural Science, Taiwan, pp 49–76

5. Inouye N (2008) Viruses of orchids, symptoms, diagnosis, spread, and control. Blue Bird Publishers, Velsen-Zuid
6. Zettler FW, Ko NJ, Wisler GC, Elliott MS, Wong SM (1990) Viruses of orchids and their control. Plant Dis 74:621–625
7. Chang CA (2010) Some tips for choosing appropriate virus indexing techniques. ICOGO Bull 4:12–13
8. Hadidi A, Khetarpal RK, Koganezawa H (1998) Plant virus disease control. APS Press, St. Paul, MN
9. Van Regenmortel MHV (1982) Serology and immunochemistry of plant viruses. Academic Press, New York
10. Clark MG, Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J Gen Virol 34:475–483
11. Dijkstra J, de Jager CP (1998) Practical plant virology: protocols and exercises. Springer, New York
12. Hampton R et al (1990) Serological methods for detection and identification of viral and bacterial plant pathogens. APS Press, St Paul, MN
13. Matthews REF (1993) Diagnosis of plant virus diseases. CRC Press, Boca Raton, FL
14. Chang CA, Lin M, Hsu HT (2006) A sensitive simultaneous detection of two orchid viruses by DNA microchip array. Phytopathology 96:S21
15. Lim ST, Wong SM, Yeong CY, Lee SC, Goh CJ (1993) Rapid detection of cymbidium mosaic virus by the polymerase chain reaction. J Virol Methods 41:37–46
16. Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 155:335–350
17. Seoh ML, Wong SM, Zhang L (1998) Simultaneous TD/RT-PCR detection of cymbidium mosaic potexvirus and odontoglossum ringspot tobamovirus with a single pair of primers. J Virol Methods 72:197–204
18. Chang CA, Tseng YS, Chen CC, Deng TC (1999) Detection of *Odontoglossum* ringspot virus by DNA probe hybridization and reverse transcription-polymerase chain reaction. Plant Pathol Bull 8:29–36

Part IV

Cell and Molecular Biology and Transformation

Chapter 19

Genetic Transformation on Orchid Species: An Overview of Approaches and Methodologies

Masahiro Mii and Dong Poh Chin

Abstract

Genetic transformation technologies have been used to overcome the difficulty in achieving the breeding objectives by conventional methods. Although orchids have been rather difficult targets for genetic transformation, it is now feasible to transform some of the commercially important orchid species due to the progress made in technologies for overcoming some difficulties during the past two decades. In this chapter, we overview the past efforts in establishing genetic transformation methods on orchids and also summarize the important factors affecting successful transformation.

Key words Acetosyringone, *Agrobacterium tumefaciens*, Biolistic method, Beta-glucuronidase (GUS) gene, Carbenicillin, Cefotaxime, Co-cultivation, Embryogenic callus, Foreign gene, Gene isolation, Gene transfer, Genetic transformation, Green fluorescent protein (*GFP*), Hygromycin, Kanamycin, Meropenem, Particle bombardment, Plant regeneration, Plasmid, Protocorm, Protocorm-like body (PLB), Phosphinothricin (PPT), Ti plasmid, T-DNA

1 Introduction

Significant progress has been made in the past two decades on orchid transformation studies. Successes are reported in the literature regularly. This chapter provides an overview of approaches and methodologies used in orchid transformation. Some important factors in the genetic transformation of orchids are emphasized.

1.1 Principal Methods of Genetic Transformation

For the genetic transformation of orchids, two major strategies have been employed so far. One of these is the physical method called particle bombardment, microprojectile bombardment, or biolistic bombardment, which can deliver the genes coated on the surface of micro-gold particles into plant cells with the aid of the equipment named as particle gun or gene gun. The other strategy is to utilize *Agrobacterium tumefaciens*, a soilborne bacterium that causes crown gall disease, to transfer and integrate its own genes on the Ti plasmid into the plant genome.

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_19,

1.1.1 Particle Bombardment

In particle bombardment, the objective tissues or cells have to be hit by numerous micro-metal particles (ca. 1 μm in diameter) coated with plasmid DNA, at high frequencies and with even distributions. Since the air resistance is too strong for small particles to travel at such speeds, it is necessary to generate a vacuum inside the equipment. Gold is normally the particle used, since it has low toxicity to plant cells. After attaching the plasmid with objective DNA onto the surface of the gold particles, they are evenly attached on the surface of a thin membrane, which is then discharged by the pressure given by the helium gas. The membrane is immediately come into collision with the screen stopper. The particles are dislodged from the membrane at the collision and immediately hit the target plant materials such as PLBs, protocorms, and embryogenic calli laid at a few cm below. Since the area of particle dispersal is restricted, it is necessary to place the plant materials at an appropriate position. Successful transfer of the particles is usually affected by various factors such as the given pressure by helium gas, the distance of particle movement, and the surface structure and hardiness of the target plant material. If the force of the particles is too strong, tissues may be severely damaged, but, if the force is too weak, the particles may fail to penetrate. Only appropriate force can particles penetrate into the target tissue and remain in the cells or intercellular spaces. For successful genetic transformation, the particles with attached objective DNA must at least be delivered into the cells but outside the vacuoles to avoid the immediate degradation of the DNA. Therefore, successful delivery of the particles into the cytosol is required for having a chance for genetic transformation. Most of the foreign DNAs on the surface of particles are degraded in the cytosol, but some survived intact DNAs are successfully integrated into some parts of the chromosomes after passing through the nuclear membrane. It is also possible that the particles have a chance to deliver directly into the nucleus, where loss of the objective DNAs might be minimized. However, details on the sequential events of the foreign DNA integration are largely not yet clarified in the biolistic method.

For determining the optimum conditions of particle bombardment for a given target plant material, vector plasmid with a reporter gene such as beta-glucuronidase (GUS) gene is usually used. In this case, once the gene is introduced into the cell, the survived undigested gene can be instantly transcribed and translated to produce the enzyme beta-glucuronidase without incorporation into the chromosome. If the substrate X-Gluc is exogenously supplied, GUS can convert it into intense blue precipitate of chloro-bromoindigo. This blue dye expression can be used as an indication of successful delivery of the gene by the particles into the target cells, which are easily detected under a stereoscopic microscope as blue dots. Suitable conditions for the bombardment can be obtained to give the maximum number of

the blue spots by adjusting several parameters such as helium gas pressure (i.e., velocity of particle discharge) and the distance between stopper and plant material. After adjusting the parameters by observing such GUS expression, plasmids with the gene of interest and selectable marker gene are used for obtaining the objective transformants. However, such GUS expression usually occurs in the cytoplasm before the integration of the gene into genomic chromosomes, so that it is called as transient expression. Although transient GUS expression is a prerequisite for obtaining the transformants with the stable integration of the gene, it does not always mean that stable integration can be achieved successfully due to the various obstacles after gene delivery into the cytoplasm.

Particle bombardment method is applicable for any kind of plants, but the site of particle delivery is restricted near the surface of the target tissues. Therefore, it is rather difficult to obtain transgenic plants if plant regeneration from these areas is difficult. It also often results in the regeneration of chimeric transgenic plants in this method. Moreover, it is common to get transgenic plants with multi-copies of transgenes due to the delivery of numerous gene copies into the cell. In the case of multi-copy gene transformation, it is possible to obtain much higher gene expression than single copy one, but this can cause silencing of all copies of genes introduced, which ends up appearing as if no transformation occurred at all. If the gene copies are integrated at different chromosomal locations, the progenies obtained may show large variations in the number and location of the gene copies with different gene expression patterns, which makes programmed breeding difficult. In this method, therefore, it is important to select individuals with strong gene expression but with few gene copies, hopefully just a single one. Furthermore, problems with various types of incomplete gene integration can occur, which may result in the failure of gene expression. Despite these problems, the method is applicable to any kind of plant materials provided that plant regeneration systems from the cell and tissue cultures are available.

1.1.2 Agrobacterium-Mediated Method

In contrast to the physical method of particle bombardment, *Agrobacterium*-mediated method is a biological method utilizing the ability of the two species of *Agrobacterium*, *A. tumefaciens* and *A. rhizogenes*, which are soilborne bacteria with the ability to transfer the genes on their possessing large plasmid, Ti or Ri plasmid, to plants. Both species are now classified into the genus *Rhizobium* as *R. radiobacter* and *R. rhizogenes*, respectively, but the old genus name *Agrobacterium* is still used in the field of genetic transformation. Since *A. tumefaciens* has predominantly been used for the genetic transformation of plants, the methods using this species are the focus of this chapter.

A. tumefaciens is a soilborne bacterium which infects roots and stems nearby soil surface and causes tumor formation at the site of infection. The tumor is called crown gall and recognized as crown gall disease, which inhibits plant growth and sometimes results in the death of plants. Because of the difficulty to cure the disease, it has been recommended to remove the infected plants. The gall looks similar to the callus tissues obtained by tissue culture. Since callus formation is usually induced by the combination of two kinds of phytohormones, i.e., auxin and cytokinin, *Agrobacterium* was considered to give some stimulus to induce de novo phytohormone synthesis in the cells, which stimulates cell division, leading to callus formation. However, isolated crown gall tissue can continuously be subcultured on a phytohormone-free medium even after completely killing the contaminated microorganisms including *Agrobacterium*, which is usually not possible in callus cultures. These results suggest some permanent changes in the plant cells after the infection with *Agrobacterium*. Subsequent studies conducted in the 1970s finally revealed that some genes possessed by the bacteria are integrated into the plant genome and cause this mysterious phenomenon. These genes were subsequently determined to be located on the large plasmid named Ti (tumor-inducing) plasmid of this species. At the infection, only T-DNA (transferred DNA) region of the plasmid is excised and integrated into some region of the plant chromosomes. For the excision of the T-DNA, *vir* region (virulence region) of the Ti plasmid, which is located outside T-DNA region, is activated by some kind of phenolic substances such as acetosyringone secreted by plant cells and gives instructions to excise T-DNA region. Tumor formation is actually induced by the overexpression of the two genes for the synthesis of phytohormones, i.e., cytokinin and auxin, after the permanent integration into plant chromosomes. Continuous expression of the genes leads to the overproduction of two important phytohormones and results in the unorganized cell proliferation leading to the tumor formation. T-DNA region has one more important gene, which is the opine synthesis gene. Opine is a general name for the amino acids, which are not utilized as components of proteins but are served as nutrients for the bacteria. Therefore, *Agrobacterium* is considered to develop such gene integration mechanism to make plant cells as the source for their food production. Finding of such interesting mechanism led to the establishment of genetic transformation method of plants by replacing the gene of interest from the disease-causing genes originally existed in the T-DNA.

Compared to the particle bombardment method, *Agrobacterium*-mediated method can be used without any special equipment if the bacterial strain with a plasmid containing the objective gene is available. In this method, it has been confirmed to be rather easy to obtain transgenic plants with a single copy of the transgene.

Therefore, once the transgenic plant with a single gene insertion conferring sufficient gene expression is obtained, it can be efficiently used for further breeding program as a dominant single gene since no allele exists against the transgene. In this method, however, it is rather difficult to transform plants, which are not the natural hosts of *A. tumefaciens* such as monocots. The difficulty was partially improved in rice by using the selection of appropriate competent cells as the target, virulent bacterial strain with improved plasmid vector, and the use of acetosyringone [1], which triggered successful applications of *Agrobacterium* method for the other monocot species including ornamentals. Consequently, *Agrobacterium* method is now used as the most popular and important method for the genetic transformation of a wide range of plants.

2 History of Genetic Transformation Studies in Orchids

The first successful study of genetic transformation was reported at the 13th World Orchid Congress held in New Zealand by Chia et al. [2]. They showed fluorescing protocorms 6 weeks after particle bombardment of *Vanda* embryos with the luciferase gene of firefly. Thereafter, they applied this method to PLB of *Dendrobium* White Angel, in which they showed the usefulness of luciferase gene for the visual selection of transformed tissues by adding luciferin as the substrate into the medium [3]. On the other hand, Kuehnle and Sugii [4] succeeded to produce transgenic kanamycin-resistant plants with a coat protein gene of papaya ringspot virus (PRV) as a model gene by particle bombardment. Later, Anzai et al. [5] also showed the usefulness of particle bombardment for the transformation of *Phalaenopsis*. In this study, the *bar* gene, a herbicide resistance gene, was introduced as a selectable marker gene to PLBs derived from the leaf segment culture; the transgenic plants successfully showed resistance to the herbicide, bialaphos. Yang et al. [6] also succeeded in the transformation of *Cymbidium* by particle bombardment.

When transforming with particle bombardment, it is rather common to raise chimeric transgenic plants with non-transgenic tissues. This is probably due to the regeneration of adventitious shoots or somatic embryos from transgenic cells with adjacent non-transgenic cells. In adventitious embryo formation of *Panax ginseng*, Choi et al. [7] showed the increase in the frequency of single-cell-originated embryos by high-osmolarity treatment. By applying this method, Li et al. [8] obtained 3–4 times more single-cell-derived embryo formation as well as 15 times more PLBs with GUS expression in *Oncidium*. Men et al. [9] also succeeded in transforming *Dendrobium phalaenopsis* and *D. nobile* by applying high-osmolarity treatment just before bombarding PLBs although the mechanism of such high-osmolarity treatment is not clear.

The first successful result on the genetic transformation of orchids by *Agrobacterium*-mediated method was reported for *Phalaenopsis* by Belarmino and Mii [10], which was almost 10 years later than the success with the particle bombardment method. As described above, orchids are not the natural host of *Agrobacterium*, but prolonged infection period for 10 h of embryogenic cell clumps with *Agrobacterium* in the presence of acetosyringone led to the successful regeneration of transgenic plants expressing both *hpt* and *gus* genes. Chai et al. [11] also succeeded in transformation using four *Phalaenopsis* cultivars, in which transformation efficiency was increased by halving transversely the target PLB materials. Moreover, Mishiba et al. [12] showed an increased transformation efficiency by preculturing for 2 days on medium with acetosyringone before *Agrobacterium* infection treatment by using young protocorms obtained 21 days after sowing as the target material. In this study, they showed the effectiveness of a two-step selection system for obtaining non-chimeric transgenic plants, in which the second selection was conducted for the secondary PLBs induced after 1 month of culture on the hygromycin-free medium from dissected protocorms, which survived 2 months after selection with hygromycin. In the transformation of *Phalaenopsis* orchids, cefotaxime used as an antibiotic for elimination of *Agrobacterium* usually causes browning of the target tissues after infection, which results in a reduced transformation efficiency. However, the efficiency was increased by replacing cefotaxime by meropenem, which showed reduced occurrence of browning [13].

Besides *Phalaenopsis*, Yu et al. [14] reported successful transformation of *Dendrobium* by *Agrobacterium*-mediated method. They introduced an antisense gene of A class MADS box gene, *DOH1*, and showed abnormal growth of the transformants, i.e., multiple shoot formation, suggesting the involvement of this gene in morphogenesis. Although they did not use acetosyringone in this study, Men et al. [9] showed the effectiveness of this phenolic substance to increase the transformation efficiency by preculturing dissected PLBs on medium supplemented with BA and acetosyringone for 2 days before *Agrobacterium* infection of 30 min, which was the optimal time. In *Cymbidium*, another horticulturally important genus, Niimi et al. [15] showed the successful transformation of *C. niveo-marginatum*, a terrestrial orchid, by targeting rhizomes derived from in vitro seedlings by *Agrobacterium*-mediated method. Then Chen et al. [16] successfully obtained transgenic plants by using PLBs of a commercial cultivar. In *Oncidium*, Liau et al. [17] obtained transgenic plants by infecting Sherry Baby "OM8," in which only bacterial elimination was performed on medium containing timentin and cefotaxime, followed by selection medium with hygromycin. Shrestha et al. [18] reported the first successful *Agrobacterium*-mediated transformation of *Vanda*, and this is the first report on the use of

sonication-assisted *Agrobacterium*-mediated transformation in orchid. Another successful transformation of an important orchid, namely, *Cattleya*, was conducted by using PLBs as target materials [19]. More recently, Phlaetita et al. [20] showed a high-efficiency transformation system in *Dendrobium* orchid, with the highest transformation efficiency being 18.5%. The accumulation of the successful transgenic studies using *Agrobacterium*-mediated method in orchids suggests that this method will be continued to be used as the general method for the genetic transformation of various orchid species.

3 Practical Approaches and Important Factors in Genetic Transformation of Orchids

Agrobacterium-mediated method was first successfully applied for genetic transformation of plants by Horsch et al. [21] through the use of leaf disks. In this method, surface-sterilized leaf segments excised from the plants were immersed in the bacterial solution for ca. 10 min for allowing the adhesion of bacteria on the surface of leaf, blotted dry on paper to remove excess bacterial solution, and left on the medium for a few days for inducing the infection, i.e., integration of T-DNA into plant chromosomes. Then the leaf disks were transferred onto a culture medium with two kinds of antibiotics to remove the bacteria and to induce the preferential growth of the successfully transformed cells with antibiotic resistance gene and subsequent regeneration of transgenic plants. Owing to the kind of plant material or species, transgenic plants were directly obtained through the regeneration of adventitious shoots or somatic embryos from the infected explants. Various genetic transformation protocols with *Agrobacterium*-mediated method presently used are based on this first protocol.

For the *Agrobacterium*-mediated genetic transformation, at least two important requirements must be fulfilled. One is to establish the plant regeneration system from cell and tissue cultures, and the other is to develop the efficient method for infecting target plant cells with *Agrobacterium*. In some orchids such as *Phalaenopsis*, genetic transformation has become relatively easy by fulfilling these two important requirements. Here we describe the practical approaches and important factors for the genetic transformation of orchids with special references to *Phalaenopsis*.

3.1 Target Material for Infection

Irrespective of the methods used for transformation, particle bombardment method or *Agrobacterium*-mediated method, only small portions of cells in the target tissue could be transformed after the treatment. Moreover, the cells of target tissue must have the potential to regenerate plants since our purpose is breeding to obtain transgenic plants with the desired useful traits and not

unorganized-transformed tissues. Therefore, it is necessary to prepare the cells or tissues with highly regenerable potential as possible as you can. In orchids, it is usually difficult to regenerate plants from tissues excised from the cultivated plants or naturally grown plants; PLBs induced from the culture of meristems excised from the shoot apex have generally been used as the target material. In *Phalaenopsis*, however, the highly regenerable embryogenic callus cultures from shoot tip culture had been successfully utilized for transformation studies [10]. Subsequently, successful results of *Agrobacterium*-mediated genetic transformation were obtained by using PLB [11] and early stage of seedlings (protocorms) [12].

Since the family Orchidaceae is the largest family in angiosperm, it involves numerous species with wide variations in morphological and physiological characters. Consequently, response in tissue culture varies greatly depending on the species and genotypes, and still many of them are left recalcitrant for in vitro propagation. For some terrestrial orchids such as *Cypripedium* and *Paphiopedilum* species, induction of PLB or even survival of meristem explants is difficult. Moreover, even if the suitable method is once established for a species in these genera, the method may not always be applicable to the other species or cultivars in the same genus. Therefore, it is indispensable to establish the plant regeneration system in the particular genotype targeted for genetic transformation. In addition, particular attention must be paid for the possible occurrence of somaclonal variations, which is difficult to avoid in tissue culture. Even if transgenic plant with the objective gene is successfully produced, it is possible that it could not be used if important original characters are altered by somaclonal variation. Since the occurrence of somaclonal variations is affected by genotype [22], it is necessary to check the possible occurrence of variations in the target genotype by using the medium supposed to be used before conducting transformation study.

In *Phalaenopsis*, protocols for the PLB induction and its utilization for the commercial clonal multiplication from shoot tip [23] and leaf segments [24] have already been established. It is also possible to induce embryogenic callus directly from shoot tip culture or from PLB segments as described earlier. Consequently, these plant materials have been used as the target materials [10, 11, 13, 25, 26]. Information on micropropagation methods can be found in Section II of this book.

Genetic transformation of orchids is usually aimed at adding some novel useful traits to the special genotypes or cultivars with desirable or superior characters. In *Phalaenopsis*, therefore, PLBs derived from shoot tip culture have predominantly been used as the target material. Although protocorms have also been confirmed as the useful material for transformation studies [12], those obtained by a particular cross combination of commercial cultivars

or selected genotypes are considered to consist of heterogeneous individuals of different genotypes; such protocorms are of course not suitable for the particular genotype-targeted genetic transformation. The transformation of protocorms will give the chance to yield relatively high number of transgenic plants because one fruit of *Phalaenopsis* usually contains tens of thousands of seeds. Therefore, it is expected to obtain enough number of transgenic plants population for selecting the objective desirable genotypes even though the transformation rate of *Phalaenopsis* protocorm is not so high (1.3–1.9%). To ensure the efficient breeding by utilizing protocorm materials, it is of course indispensable to increase the transformation efficiency for realizing such breeding approach.

3.2 Selection of Bacterial Strains and Plasmids

Wild *Agrobacterium tumefaciens* involves various genetically different strains with different host range of plants and varied degree of virulence. For the infection, *vir* region in the Ti plasmid has an important role as described previously. Other plasmids and chromosomal DNA of *A. tumefaciens* are also known to affect the transformation efficiency [27]. Therefore, it is important to select the most suitable bacterial strain for the target plant or genotype. However, even if such strain is found from the natural population, it cannot be used directly for transformation since they possess the virulence gene causing crown gall disease. For the actual use of the wild *Agrobacterium* strain, the virulent genes must be removed from the Ti plasmid. Since the size of Ti plasmid is relatively large with ca. 200 thousand base pairs, it is not suitable for genetic manipulation. Therefore, the binary vector system, in which T-DNA and *vir* genes are separated into two plasmids as a binary plasmid and a helper plasmid, is now widely used. In this system, special strains of *A. tumefaciens* such as EHA101, EHA105, and LBA4404 are used as binary vectors, in which helper plasmid containing *vir* genes is always located. The binary plasmid containing oncogene-free but objective gene-integrated T-DNA is added and used for the genetic transformation. For the genetic transformation of *Phalaenopsis*, we initially used LBA4404 [10], but it was replaced by EHA101, which showed stronger infection ability than the former in the later study [12] and is now predominantly used for the genetic transformation of orchids including *Phalaenopsis*, *Cymbidium*, *Cattleya*, *Vanda*, *Dendrobium*, etc.

3.3 Selection of Promoter

For the efficient expression of the introduced foreign genes, it is important to select the appropriate promoter, a region of DNA that initiates transcription of a gene. Since any genes cannot be expressed without the promoter region, foreign genes of interest must be ligated to the appropriate promoter near the transcription start site of the genes. There are numerous kinds of promoters, and each one has organ, tissue, and developmental stage specificity for expressing the genes. Therefore, if the purpose is to alter the flower

color, for example, promoters isolated from the genes expressed in the flower organs must be used. However, few genes with strong expression at objective site or stage are available, so that at the initial trials, general-purpose constitutive promoters such as cauliflower mosaic virus (CaMV) 35S promoter have widely been used for the genetic transformation of various plants. Since CaMV is a kind of DNA virus with wide host range and constitutive expression of disease symptoms, its 35S promoter can be expressed in plant cells without tissue specificity and has been used in its native state as one of the most popular promoters for the transformation of various plant species for introducing various kind of genes. However, for some monocot plants such as those belonging to Poaceae, 35S promoter is not expressed adequately, so that promoters derived from gramineous plants such as ubiquitin promoter of maize were preferably used based on the results of comparative studies on several promoters [28]. In orchids, however, there have been only few reports, and Anzai et al. [5] showed that 35S promoter is more suitable than maize ubiquitin and rice actin promoters for the transformation of *Phalaenopsis*. Tee et al. [29] also showed the suitability of 35S than ubiquitin promoter for *Dendrobium*. Therefore, the use of 35S promoter for the transformation of orchids may have no problem to confirm the expression of genes of interest. After confirming the expression of the objective gene by using 35S, it might be replaced by other promoters with more specific and strong expressions at the next step if necessary since available promoters have been increasing year by year.

3.4 Bacterial Infection and Co-cultivation

Infection of plant cells with *A. tumefaciens* occurs when plant tissues are injured and several phenolic substances are secreted from the damaged tissue. These phenolic substances are captured by *Agrobacterium* and act as a trigger to activate *vir* genes. T-DNA is then excised by the enzyme produced by the activation of *vir* genes and transferred into plant cells and finally integrated into some part of chromosomes. Based on these infection mechanisms, acetosyringone, a phenolic substance, has been used for genetic transformation studies. Transformation efficiency increased in many plant species when acetosyringone was added into the co-cultivation medium. Especially, acetosyringone was confirmed to be effective for the monocot plants such as rice [1] and maize [30], which are usually not the natural hosts. The use of acetosyringone also contributed to the first successful genetic transformation of *Phalaenopsis* [10]. Acetosyringone is usually added at 100 μM to co-cultivation medium, which is also suitable for *Phalaenopsis* and other orchids [18–20, 31].

Agrobacterium is usually used for the infection for co-cultivation at OD_{600} = 0.5–0.6 (ca.10^8 bacteria/mL medium), which was usually attained 16 h of proliferation in a liquid suspension culture after inoculating a small amount of bacteria into LB medium at 28 °C. For this incubation, it is necessary to keep in

mind that overgrowth of bacteria by longer incubation may result in reduced infection activity. Proliferated bacteria are sometimes used after centrifugation and resuspension with plant culture medium for the infection but usually used after diluting with plant culture medium into ten times. In *Phalaenopsis*, the highest transformation efficiency was obtained when ten times diluted bacterial suspension was used in all the tested target materials, i.e., cell suspension culture, PLB, and protocorm. The period of incubation with bacterial solution is usually 5–15 min in most plant species. However, *Phalaenopsis* requires relatively longer infection period, i.e., 2 h for cell suspension culture [13] and ca. 7 h for PLB and protocorms [12].

After infection, bacterial solution attaching to the target plant material should be removed by blotting on filter paper and incubated on plant culture medium for allowing the transfer of T-DNA into plant cells, namely, for the infection. This short period of culture is called as co-cultivation. It is important to note that various environmental conditions such as light, temperature, and pH of culture medium can affect the infection process. In the plant species which are not the natural hosts of *Agrobacterium* such as *Phalaenopsis*, it is essential to add acetosyringone at the co-cultivation period. If overgrowth of bacteria occurs on and around the explants, it is possible to cause several serious problems such as browning of the explants, inhibition of organ formation, and difficulty for removing bacteria. Therefore, infection conditions might be determined by considering bacterial overgrowth as an important index.

To evaluate the success of infection in *Agrobacterium*-mediated method, GUS gene has predominantly been used as in the case of particle bombardment. If once optimum conditions for the transient GUS expression are obtained, stable expression of the gene is consequently expected in the same conditions. However, sometimes GUS expression fails in the callus or regenerated organs from the explants after several weeks of culture with selective agent. These problems might be caused by the failure of the gene to integrate into chromosome or the occurrence of gene silencing due to DNA methylation, etc. after gene integration. Since gene silencing is closely related to the combination between the promoter used and the plant species, one may need to test other promoters for transformation.

3.5 Bacterial Elimination and Antibiotics

The disadvantage of *Agrobacterium* method compared with biolistic method is the necessity to completely remove bacteria as soon as possible. In addition to the problems caused by the bacterial overgrowth, it is indispensable to prevent the unnecessary flow of foreign genes into natural environment, which is described in Cartagena Protocol and strictly regulated by the Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms.

Since *Agrobacterium* itself is sensitive to several antibiotics, it is rather easy to remove if it is in an isolated condition. However, in the case of genetic transformation, it is necessary to remove the bacteria without giving the damage to plant tissues. The antibiotics normally used are ß-lactams such as cefotaxime and carbenicillin which have been used to remove *Agrobacterium* as they are less toxic to plant cells. ß-Lactam antibiotics have the ability to inhibit cell wall formation of bacteria during cell division, thereby causing rupture of the cells. Since ß-lactams can degrade peptide glucan, which is the main component of bacterial cell wall, plant cell wall consisting of mainly cellulose is free from the damage. This is the reason why ß-lactam antibiotics have no harmful effect on plant cells. However, they sometimes have negative effects such as inhibition of cell proliferation and plant regeneration, which are the severe problems for genetic transformation in some plant species. As shown in the action mechanism of ß-lactam antibiotics, they have no effect on the nondividing or quiescent bacteria. Since some of the bacteria attaching to the plant tissue remain quiescent for a long time after inoculation treatment, it is necessary to add the antibiotic to the culture medium continuously to completely eliminate the bacteria even if they are not visible. Therefore, less harmful antibiotics to the plant cells are favored for successful genetic transformation. Although cefotaxime has extensively been used for eliminating *Agrobacterium*, it is known to have inhibitory effect on shoot regeneration from tissues infected in many plant species. In the first study on *Agrobacterium*-mediated genetic transformation of *Phalaenopsis*, we used 300 mg/L cefotaxime for eliminating bacteria, but it frequently caused tissue browning and gave low yield of transgenic plants (10/g cells). However, replacement of cefotaxime by meropenem, which was confirmed to be highly effective for elimination of *Agrobacterium* [32, 33], resulted in increased efficiencies of transgenic plant production (35/g cells) [13]. Since meropenem is effective at low concentration (5 mg/L) and has no appreciable inhibitory effect on plant regeneration, it has been used for genetic transformation of orchids [12, 18, 20, 31] as well as other plant species [34–37] in our laboratory.

Irrespective of the kind of antibiotics used, it is indispensable to confirm the complete elimination of bacteria from the transgenic plants before transfer from in vitro to ex vitro conditions. For this purpose, a small part of the plant is excised, ground, and pasted on the surface of a bacterial medium such as LB medium to observe the appearance of bacterial colonies. Since *Agrobacterium* can survive for several years without division, careful attention must be paid for evaluating the complete removal of the bacteria.

3.6 Methods for Selecting Transformants

In *Agrobacterium*-mediated method, successful genetic transformation is supposed to occur in a low population of cells with bacteria attached. Of course, transformation never occurs in most of

the remaining cells that never make contact with bacteria. Therefore, it is necessary to select and enhance the proliferation of the transformed cells with concomitant prevention of the growth of the remaining non-transgenic tissues. For achieving such purpose, there are two major strategies. One is the introduction of resistant genes to the harmful substances applied to the plant cells such as antibiotics and herbicides. As the antibiotics, kanamycin and hygromycin have predominantly been used and bialaphos or phosphinothricin as the herbicides; by adding these substances to the culture medium after *Agrobacterium* infection treatment, non-transformed cells cannot survive, but transformed cells can continue to grow by detoxifying these harmful substances. Continuous use of these substances through plant regeneration process usually leads to the production of transgenic plants. However, if the selection agent is added to the medium immediately after bacterial infection, the target cells will be killed before the occurrence of integration and expression of the gene. Therefore, selection agent is usually applied a few days after bacterial infection. The period of culture with selective agent-free medium may sometimes be prolonged depending on the species because the time required for sufficient expression of the resistant gene may differ depending on the species or genotypes. In such case, it is necessary to keep in mind that the chance to obtain chimeric transgenic plants with non-transformed tissues may increase because detoxifying enzymes produced by transgenic cells may be transferred to the neighboring non-transgenic cells.

3.7 Regeneration of Transgenic Plants

As described earlier, it is indispensable to establish plant regeneration system from the target material for genetic transformation since there is little use for transgenic tissues with no regeneration ability. For plant regeneration, usually two types have been recognized, direct plant regeneration and indirect plant regeneration, through callus proliferation by altering plant growth regulators used. For species or genotypes which have the ability to regenerate adventitious shoots or embryos, it is possible to obtain transgenic plants through the same manners on medium containing selective antibiotics after *Agrobacterium* infection. However, in most cases, it is also highly possible that they become chimeric with non-transgenic tissues because adventitious direct organogenesis or embryogenesis originates from several different cell clusters, some of which may not be transformed. Therefore, it is necessary to repeat the subcultures of axillary buds on medium containing selective antibiotics for selecting the non-chimeric transgenic shoots with normal growth. In the species with easy rooting, culture of the shoots showing normal rooting on medium with selection agent enables the selection of pure transformants. On the other hand, selection of pure transgenic plants seems to be relatively easy in the species with indirect plant regeneration process since callus proliferation can be selectively

induced only from the transgenic cells, from which plants will be regenerated. However, it is necessary to keep in mind that antibiotics used for the selection such as kanamycin and hygromycin sometimes show inhibitory effects on plant regeneration. Therefore, it is better to transfer the putative transgenic callus onto regeneration medium after several subcultures.

In orchids, PLBs and protocorms are mainly used as the target materials, and hence transgenic plants are obtained from secondary PLBs induced from the target materials. Therefore, problems with chimeras are mostly solved in this system. In the use of callus tissue in orchids, culture medium must be changed according to the different steps of culture. Callus proliferation from infected cell culture, PLB regeneration from callus tissues, shoot regeneration, and rooting for the efficient plant regeneration all require different culture media. Especially, in this system, replacement of carbon sources such as sugar and sugar alcohol is known to be useful for the rapid growth of transgenic tissues [38, 39]. Consequently, these culture conditions must be efficiently employed in the processes of selection and regeneration of transgenic tissues.

3.8 Confirmation of Gene Integration and Expression

It is natural to consider that the plants obtained through the selection with antibiotics are transgenic because of the expression of integrated antibiotic resistance gene. However, it is sometimes possible to get non-transgenic plants regenerated from the chimeric tissue with transformed tissue. Plant regeneration from such chimeric tissue may also result in the chimeric transgenic plants. Therefore, it is necessary to directly detect the objective foreign gene in the tissue of the putative transgenic plants. For this purpose, polymerase chain reaction (PCR) method has generally been used. By employing PCR method, some DNA sequence unique to the objective foreign gene can be preferentially amplified. The amplified DNA sequence can be detected as a unique band by staining with ethidium bromide after electrophoresis. However, it is necessary to keep in mind that the amplified DNA band might also be derived from the plasmid DNA of the bacteria used for transformation but still surviving in the plant tissue. Even if the plant is obtained through the antibiotic selection process, it is possible that the plant is not transformed but misjudged as transformant due to the contaminating bacteria. Consequently, it is necessary to further apply Southern blot analysis to confirm the integration of the objective gene. In this method, genomic DNA of the putative transformant is cleaved by restriction enzymes into small fragments, which are then unfolded into single strands by alkaline denaturation, and hybridized with a single-strand complementary DNA of the introduced gene labeled with digoxigenin (DIG) as a probe. Since the probe is labeled with DIG before hybridization, the objective gene can be detected by using anti-DIG-alkaline phosphatase F_{ab} fragments

followed by the fluorescence detection. Introduced genes are not always integrated into single site, but multiple insertions at different loci of the chromosomes occur frequently. Since each restriction enzyme can cut DNA only at the specific site by recognizing specific small base sequences, it gives varied sizes of the DNA fragments. After electrophoresis of these fragments, they migrate at different positions from the origin depending on the DNA sizes. The bands with inserted objective gene labeled with DIG can be detected with anti-DIG-alkaline phosphatase, which is visualized by its enzyme activity finally. Differences of insertion sites are recognized as bands with different positions, from which the number of insertion sites on the chromosomes is confirmed. Generally, each insertion site contains at least one gene copy. Therefore, the number of gene copies inserted is equal to or more than the insertion number. Multi-copy or multisite insertion of the gene often causes gene silencing. Moreover, these gene copies are inherited independently in the next generations, which makes programmed breeding difficult. Therefore, it is important to select individuals with strong expression of the introduced gene with one copy number. Compared to the biolistic method, *Agrobacterium*-mediated method usually yields low-copy-numbered transgenic plants, which is the advantage of this method. In orchids, however, commercial cultivars are mostly propagated vegetatively by tissue culture. Consequently, selection of individuals with strong expression of the transgene might be the priority irrespective of the copy number of the gene.

After confirmation of the integration of a foreign gene, it is necessary to confirm the transcription of the gene into mRNA and translation into protein by Northern analysis followed by Western blotting. Moreover, if the objective protein is an enzyme, activity of the protein must be confirmed. When using genetic transformation for practical breeding, evaluation of the resulted final characters is extremely important. Consequently, individuals with the most appropriate expression must be selected as in the traditional breeding methods. As pointed out previously, it is important to note the copy number of the gene or the number of integration sites for conducting the subsequent breeding program efficiently.

Toxicity of antibiotics to plant cells differs depending on the species. Especially, kanamycin is less toxic against monocots including orchids. Kuehnle and Sugii [4] succeeded to obtain transgenic plants of *Dendrobium* by using kanamycin; however, they needed a long period for the selection of transformants. Although Yang et al. [6] also used kanamycin for the transformation of *Cymbidium*, most of the other researches on genetic transformation of orchids used hygromycin for the selection [10, 40, etc.]. On the other hand, Anzai et al. [5] used *bar* gene, which confers a resistance against phosphinothricin (PPT), a nonselective herbicide, as a selection marker and succeeded in obtaining transformant with

GUS gene by using 1 mg/L bialaphos, a herbicide consisting of PPT with alanine, which was added 2 months after bacterial infection. Knapp et al. [41] also succeeded in selecting transformants using *bar* gene and 1–3 mg/L bialaphos.

Instead of using marker genes for chemical selection, visual selection markers have also been used in the transformation of orchids. The first example is the use of luciferase gene derived from firefly. Luciferase gives fluorescence by catalyzing the substrate luciferin into a fluorescent substance, oxyluciferin. Chia et al. [3] introduced this gene into the cells of *Dendrobium* PLB by particle bombardment and selected transgenic tissues based on the fluorescence under dark condition. This method is, however, difficult to apply for the practical selection of transformants because of the following reasons: (1) addition of luciferin into culture medium as the substrate is required, and (2) the fluorescence is too weak to make visual selection and needs the use of amplifier of the fluorescence. In contrast, green fluorescent protein (*GFP*) gene originated from a jellyfish, *Aequorea victoria*, which yields a protein that has green fluorescence under UV light conditions. No exogenous substrates are needed for this procedure. However, the fluorescence is sometimes not strong enough for the visual observation and generally becomes undetectable by the autofluorescence of chlorophyll if the tissues turn green. Consequently, *GFP* gene has not been used as a popular strategy for the selection of transformants. However, further improvement of the gene to give more stronger fluorescence is now in progress, which will have the possibility to make it more popular. Tee and Maziah [42] compared the transient expression between *GUS* and *GFP* and concluded that no difference was found between them as the marker gene for the confirmation of the transgenic event, but that GFP is better for the selection because it makes continuous observation of the transformed tissue without giving damage.

In our transformation system of *Phalaenopsis*, hygromycin resistance gene (*hpt*) is always used as a selection marker, and hygromycin is usually added from 2 weeks after co-cultivation period due to the reason described previously. When PLBs or protocorms are used as the target materials, non-transformed ones turn brown within a few days, whereas those with transformed cells remain green due to the detoxification role of the transgenic cells for the non-transformed cells. However, it is difficult to expect the regeneration of transgenic plants from the transformed cells, and some gradually turn brown subsequently, except when meristematic cells are transformed. Therefore, it is necessary to cut the original explants in order to enhance the chance to induce secondary PLB formation from the transformed quiescent cells. In such cases, it is better to remove hygromycin for inducing secondary PLB because direct culture of cut explants on medium with hygromycin usually results in the total death of the explants.

Usually after 1 month of culture on hygromycin-free medium, the cultures with secondary PLBs are transferred onto medium containing hygromycin. Interestingly, these secondary PLBs are mostly non-chimeric transgenic ones, suggesting that non-transformed surviving cells in the target original explants have already lost the dividing ability during the initial selection process.

4 Concluding Remarks

Genetic transformation systems have been established in some of the horticulturally important orchids as described in this review. By utilizing these systems, it is expected to produce novel cultivars with desirable or useful traits for commercial cultivation and improving ornamental values. Since Orchidaceae plants have unique morphological, physiological, and ecological characters, it is possible to clarify the functions of various genes involved in these unique characters with the aid of genetic transformation technologies. However, transformation frequencies in most of the systems are still low, and the protocols for genetic transformation available at present may not be applicable to many other orchid species with diversified and specified characters. Therefore, further improvements of the genetic transformation technologies are needed.

References

1. Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282
2. Chia TF, Chan YS, Chua NH (1990) Large-scale screening of *Cymbidium* mosaic and *Odontoglossum* mosaic ringspot viruses in cultivated orchids by nucleic acid spot hybridization. In: Bonham DG, Kemohan J (eds) Proceedings of the 13th World Orchid Conference. 13th WOC Proceedings Trust, Auckland, New Zealand, p 284 (Abstract)
3. Chia TF, Chan YS, Chua NH (1994) The firefly luciferase gene as a non-invasive reporter for *Dendrobium* transformation. Plant J 6:441–446
4. Kuehnle AR, Sugii N (1992) Transformation of *Dendrobium* orchid using particle bombardment of protocorms. Plant Cell Rep 11:484–488
5. Anzai H, Ishii Y, Shichinohe M, Katsumata K, Nojiri C, Morikawa H, Tanaka M (1996) Transformation of *Phalaenopsis* by particle bombardment. Plant Tiss Cult Lett 13:265–272
6. Yang J, Lee HJ, Shin DH, SK O, Seon JH, Paek KY, Han KH (1999) Genetic transformation of *Cymbidium* orchid by particle bombardment. Plant Cell Rep 18:978–984
7. Choi YE, Yang DC, Choi KT (1998) Induction of somatic embryos by macrosalt stress from mature zygotic embryos of *Panax ginseng*. Plant Cell Tiss Org Cult 52:177–181
8. Li SH, Kuoh CS, Chen YH, Chen HH, Chen WH (2005) Osmotic sucrose enhancement of single-cell embryogenesis and transformation efficiency in *Oncidium*. Plant Cell Tiss Org Cult 81:183–192
9. Men S, Ming X, Wang Y, Liu R, Wei C, Li Y (2003) Genetic transformation of two species of orchid by biolistic bombardment. Plant Cell Rep 21:592–598
10. Belarmino MM, Mii M (2000) *Agrobacterium*-mediated genetic transformation of a *Phalaenopsis* orchid. Plant Cell Rep 19:435–442
11. Chai ML, CJ X, Senthil KK, Kim JY, Kim GH (2002) Stable transformation of protocorm-like bodies in *Phalaenopsis* orchid mediated by *Agrobacterium tumefaciens*. Sci Hortic 96:213–224
12. Mishiba K, Chin DP, Mii M (2005) *Agrobacterium*-mediated transformation of *Phalaenopsis* by targeting protocorms at an

early stage after germination. Plant Cell Rep 24:297–303

13. Sjahril R, Mii M (2006) High-efficiency *Agrobacterium*-mediated transformation of *Phalaenopsis* using meropenem, a novel antibiotic to eliminate *Agrobacterium*. J Hortic Sci Biotech 81:458–464
14. Yu H, Yang SH, Goh CJ (2001) *Agrobacterium*-mediated transformation of a *Dendrobium* orchid with the class 1 *knox* gene *DOH1*. Plant Cell Rep 20:301–305
15. Niimi Y, Chen L, Hatano T (2001) Gene transformation by using *Agrobacterium* in some orchidaceous plants. In: Proceedings of the 7th Asia Pacific Orchid Conference. Nagoya, Japan, pp 95–98
16. Chen L, Hatano T, Niimi Y (2002) High efficiency of *Agrobacterium* mediated transformation by using rhizome of *Cymbidium* (Orchidaceae: Maxillarieae). Lindleyana 17:16–20
17. Liau CH, You SJ, Prasad V, Hsiao HH, Lu JC, Yang NS, Chan MT (2003) *Agrobacterium tumefaciens*-mediated transformation of an *Oncidium* orchid. Plant Cell Rep 21:993–998
18. Shrestha BP, Chin DP, Tokuhara K, Mii M (2007) Efficient production of transgenic plants of *Vanda* through sonication-assisted *Agrobacterium*-mediated transformation of protocorm-like bodies. Plant Biotechnol 24:429–434
19. Zhang L, Chin DP, Mii M (2010) *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cattleya*. Plant Cell Tiss Org Cult 103:41–47
20. Phlaetita W, Chin DP, Tokuhara K, Nakamura I, Mii M (2015) *Agrobacterium*-mediated transformation of protocorm-like bodies in *Dendrobium* Formidible 'Ugusu. Plant Biotechnol 32:225–231
21. Horsch RB, Fry JE, Hoffmann NL, Wallroth M, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231
22. Tokuhara K, Mii M (1998) Somaclonal variations in flower and inflorescence axis in micropropagated plants through flower stalk bud culture of *Phalaenopsis* and *Doritaenopsis*. Plant Biotechnol 15:23–28
23. Tokuhara K, Mii M (1993) Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. Plant Cell Rep 13:7–11
24. Park SY, Murthy HN, Paek KY (2002) Rapid propagation of *Phalaenopsis* from floral stalk-derived leaves. In Vitro Cell Dev Biol Plant 38:168–172
25. Liao LJ, Pan IC, Chan YL, Hsu YH, Chen WH, Chan MT (2004) Transgene silencing in *Phalaenopsis* expressing the coat protein of *Cymbidium* mosaic virus is a manifestation of RNA-mediated resistance. Molec Breeding 13:229–242
26. Chan YL, Lin KH, Sanjaya, Liao LJ, Chen WH, Chan MT (2005) Gene stacking in *Phalaenopsis* orchid enhances dual tolerance to pathogen attack. Transgenic Res 14:279–288
27. Ogawa Y, Mii M (2001) Ti- and cryptic-plasmid-borne virulence of wild type *Agrobacterium tumefaciens* strains CNI5 isolated from chrysanthemum (*Dendranthema grandiflora* Tvelev.) Arch Microbiol 173:311–315
28. Shrawat AK, Lörz H (2006) *Agrobacterium*-mediated transformation of cereals: a promising approach crossing barriers. Plant Biotechnol J 4:575–603
29. Tee CS, Marziah M, Tan CS, Abdullah MP (2003) Evaluation of different promoters driving the GFP reporter gene and selected target tissues for particle bombardment of *Dendrobium* Sonia 17. Plant Cell Rep 21:452–458
30. Ishida Y, Saito H, Ohta S, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat Biotechnol 4:745–750
31. Chin DP, Mishiba K, Mii M (2007) *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cymbidium*. Plant Cell Rep 26:735–743
32. Ogawa Y, Mii M (2004) Screening for highly active β-lactam antibiotics against *Agrobacterium tumefaciens*. Arch Microbiol 181:331–336
33. Ogawa Y, Mii M (2005) Evaluation of twelve β-lactam antibiotics for *Agrobacterium*-mediated transformation through in planta antibacterial activities and phytotoxicities. Plant Cell Rep 23:736–743
34. Azadi P, Chin DP, Kuroda K, Khan RS, Mii M (2010) Macro elements in inoculation and co-cultivation medium strongly affect the efficiency of *Agrobacterium*-mediated transformation in *Lilium*. Plant Cell Tiss Org Cult 101:201–209
35. Ntui VO, Khan RS, Chin DP, Nakamura I, Mii M (2010) An efficient *Agrobacterium tumefaciens*-mediated genetic transformation of "Egusi" melon (*Colocynthis citrullus* L.) Plant Cell Tiss Org Cult 103:15–22
36. Khan RS, Ntui VO, Chin DP, Nakamura I, Mii M (2011) Production of marker-free disease-resistant potato using *isopentenyl transferase* gene as a positive selection marker. Plant Cell Rep 30:587–597

37. Otani Y, Chin DP, Mii M (2013) Establishment of *Agrobacterium*-mediated genetic transformation system in *Dahlia*. Plant biotechnol 30:135–139
38. Islam MO, Ichihashi S (1999) Effects of sucrose, maltose and sorbitol on callus growth and plantlet regeneration in *Phalaenopsis*, *Doritaenopsis* and *Neofinetia*. J Japan Soc Hort Sci 68:1124–1131
39. Tokuhara K, Mii M (2003) Highly-efficient somatic embryogenesis from cell suspension cultures of phalaenopsis orchids by adjusting carbohydrate sources. In Vitro Cell Dev Biol Plant 39:635–639
40. Yu ZH, Chen MY, Nie L, HF L, Ming XT, Zheng HH, LJ Q, Chen ZL (1999) Recovery of transgenic orchid plants with hygromycin selection by particle bombardment to protocorms. Plant Cell Tiss Org Cult 58:87–92
41. Knapp JE, Kausch AP, Chandlee JM (2000) Transformation of three genera of orchid using the *bar* gene as a selectable marker. Plant Cell Rep 19:893–898
42. Tee CS, Maziah M (2005) Optimization of biolistic bombardment for *Dendrobium* Sonia 17 calluses using GFP and GUS as the reporter system. Plant Cell Tiss Org Cult 80:77–89

Chapter 20

Recent Protocols on Genetic Transformation of Orchid Species

Chia-Wen Li and Ming-Tsair Chan

Abstract

Gene transfer technology is an important tool for accelerating the process of breeding. *Agrobacterium*-mediated genetic transformation method has been applied to commercial orchid species, such as those in the genera *Cattleya*, *Cymbidium*, *Dendrobium*, *Oncidium*, *Phalaenopsis*, and *Vanda*. This chapter describes an efficient selection and plant regeneration protocol for *Agrobacterium*-mediated transformation of a model orchid, *Erycina pusilla*. Selection of suitable experimental materials, generation and propagation of explants, and components of the medium used in the serials of transformation procedures are detailed and compared with other orchid transformation protocols. In addition, some tips for improving the transformation efficiency are also included.

Key words *Agrobacterium tumefaciens*, *Erycina pusilla*, *Oncidium* orchid, *Phalaenopsis*, Protocorm-like body, Transformation

1 Introduction

Orchidaceae is the largest family in flowering plants. There are more than 25,000 species of orchid plant, and many of them are well-known economic ornamental plants. These important ornamental species belong to diverse genera, such as *Cymbidium*, *Dendrobium*, *Phalaenopsis*, *Cattleya*, and *Oncidium*. To speed up the breeding progress, genetic transformation strategies have been applied for orchid molecular breeding [1–7].

Conventional breeding techniques that are based on sexual crossing are widely used for generating new orchid varieties. The cross-pollination strategies enable intraspecific and interspecific hybridization of orchid species. Furthermore, polyploidization is the simplest way to induce variability in horticultural plants. Orchid protocorms or protocorm-like bodies (PLBs) can be treated with 0.1% (w/v) colchicine in seed germination medium. Combined with the micropropagation procedures and genetic transformation techniques, new orchid traits with potential economic value could be established efficiently.

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_20,

Genetic transformation is an important tool for improving cultivars and for studying gene function in plants. In orchids, the most common genetic transformation methods are biolistic-mediated method [8] and *Agrobacterium*-mediated procedures [1, 3, 9] (Table 1). Transgenic plants overexpressing antimicrobial peptide (pflp) and viral coat protein genes showed enhanced resistance to *Pectobacteria carotovora* (*Pcc*, formerly named *Erwinia carotovora* subsp. *carotovora*) and *Cymbidium* mosaic virus (CymMV) in *Phalaenopsis* and *Oncidium* [10, 11]. Overexpressing an *Arabidopsis methionine sulfoxide reductase 7* (*MSRB7*) in *Erycina pusilla* enhances tolerance to methyl viologen (MV, paraquat)-induced oxidative stress [12]. Thus, genetic transformation is a powerful tool for improvement of disease resistance and abiotic stress tolerance in orchid species [10, 12, 13].

This chapter provides a detailed *Agrobacterium*-mediated transformation protocol of a model orchid plant, *Erycina pusilla*. *E. pusilla* has several advantages as an orchid model plant. When

Table 1
***Agrobacterium tumefaciens* strains and binary vectors used for orchid genetic transformation**

Orchid species	Explants	*Agrobacterium* strain	Antibiotics to eliminate *Agrobacterium*	Selectable gene/agent	Reference
Erycina pusilla	Protocorms	EHA105[a]	40 mg/L meropenem	*hpt*[b]/20 mg/L hygromycin	[12]
Oncidium	PLBs	EHA105[a]	40 mg/L meropenem	*hpt*/20 mg/L hygromycin	[3]
Phalaenopsis	Protocorms or PLBs	EHA105[a]	40 mg/L meropenem	*hpt*/20 mg/L hygromycin	[3]
Cattleya	PLBs	EHA101	20 mg/L meropenem	*hpt*/10 mg/L hygromycin	[7]
Cymbidium	PLBs	EHA101	10–40 mg/L meropenem	*hpt*/20 mg/L hygromycin	[1]
Dendrobium	Protocorms	EHA101	20 mg/L meropenem	*hpt*/30 mg/L hygromycin	[5]
Vanda	PLBs	LBA4404	250 mg/L cefotaxime	*nptII*[c]/30 mg/L geneticin or 50 mg/L kanamycin	[15]
	PLBs	EHA101	10 mg/L meropenem	*hpt*/10 mg/L hygromycin	[6]

[a]Other strains of *Agrobacterium*, such as EHA101, LBA4404 and GV3101, can also be employed, while EHA105 demonstrated a better transformation efficiency as tested in *Erycina pusilla*, *Oncidium*, and *Phalaenopsis* orchids [3, 4]
[b]*hpt*, encode hygromycin phosphotransferase
[c]*nptII*, encode neomycin phosphotransferase II

compared with other orchids, this fan-shaped-blade miniature orchid has a smaller genome size (6 N), its chloroplast genome is well known [14], and its chromosomal genomic and transcriptomic databases are available. Mature *E. pusilla* plant has a small size, about 50 mm diameter, and it can be easily micropropagated and self-pollinated in vitro (see Chapter 13). Therefore, it is relatively easy to obtain transgenic homozygotes for further confirmation of the genotypes and observation of the phenotypes. In this protocol, we present a simple and efficient procedure for *Agrobacterium*-mediated transformation by using the protocorms obtained from self-pollinated *E. pusilla* as target material. The components used for genetic transformation of other orchid species have also been included for references.

2 Technical Comments

There are several checkpoints to enhance genetic transformation efficiency, such as the genotypes, wounding (sonication treatment for 5 min at 80% ultrasound power) [6], the presence of acetosyringone (AS) in the co-cultivation medium, different types and concentrations of phytohormones, and the carbon source in medium (Table 2). Transformation efficiency is also influenced by *Agrobacterium* density; the optimal density ranges from A_{600nm} 0.5 to 0.8 [15]. Modifying inoculation time of *Agrobacterium* solution apparently gives high transformation efficiency (Table 3). Two to three days of co-cultivation is standard for most transformation protocols. In addition, different kinds and concentrations of selectable agent and *Agrobacterium*-eliminated antibiotics also influence the transformation efficiency [1–3, 15]. Orchids are not the natural hosts of *Agrobacterium*. AS released from injured part plays a chemotactic role and induces the *vir* genes to initiate T-DNA transfer. The optional AS concentration is 100–200 μM. Overdosage of AS would increase the browning of PLB tissues. Addition of 100 μM AS at inoculation and co-cultivation increases the putative transformation efficiency in several orchid species belonging to *Cymbidium*, *Dendrobium*, *Phalaenopsis*, *Oncidium*, and *Vanda*. Conventional antibiotic or herbicide selection marker genes, such as *nptII*, *hpt*, and *bar* genes (encoding neomycin phosphotransferase, hygromycin phosphotransferase, and phosphinothricin acetyltransferase, respectively), are widely used (Table 1). Although kanamycin is one of the most widely used antibiotics as a selective agent for transformation of plants, it was often ineffective for monocot plants including some orchid plants such as *Cymbidium* [1]. Some plant-originated non-antibiotic selectable marker genes are reported useful for orchid transgenic selection, such as *pflp*/Pcc (selection gene/selectable agent) for *Oncidium* and *Phalaenopsis* [11] and MSRB7/MV for *E. pusilla* [12].

Table 2
Component of medium used for orchid genetic transformation

Step	Purpose	*Erycina pusilla*	*Oncidium*	*Phalaenopsis*	*Cattleya*	*Cymbidium*	*Dendrobium*	*Vanda*
	Initial material	**Silique/seeds**	**Stalk bud**	**Silique/seeds**	–[a]	**Seeds**	**Silique/seeds**	**Shoot tip**
1	Explant preparation (protocorm preparation or PLB initiation)	Hyponex medium/20 g/L sucrose	New Dogashima Medium (NDM)/ 0.4 mg/ L NAA and 0.1 mg/L BA	MS medium/20 g sucrose or liquid NDM/10 g/L sucrose	Liquid NDM	Liquid NDM/ 10 g/L sucrose	Liquid NDM	NDM/30 g/L maltose, 0.1 mg/ L NAA, and 1 mg/L BA
2	PLB propagation	–[b]	G10 solid medium	NDM solid medium	Liquid NDM/10 g/L sucrose, 1.0 mg/L NAA, and 0.1 mg/L BA	Liquid NDM	–[b]	Liquid NDM/ 30 g/L maltose, 0.1 mg/ L NAA, and 1 mg/L BA
3	Pretreatment medium	Liquid NDM/ 100 μM AS	Liquid NDM/100 μM AS	Liquid NDM/100 μM AS	Liquid NDM/100 μM AS	–[a]	–[a]	–[a]
4	Co-cultivation medium	NDM liquid medium	NDM liquid medium	NDM liquid medium	NDM liquid medium	NDM solid medium/ 100 μM AS	NDM solid medium/ 100 μM AS	NDM/0.1 mg/ LNAA, 1 mg/L BA, 30 g/L maltose, and 100 μM AS[c]

5	Washing solution	40 mg/L meropenem	40 mg/L meropenem	40 mg/L meropenem	Liquid NDM/10 mg/L meropenem	10 mg/L meropenem	20 mg/L meropenem	Solid NDM/ 5 mg/L meropenem for 2 weeks
6	Selective medium	Hyponex medium/ 20 mg/L hygromycin and 40 mg/L meropenem	G10 medium[d]/ 20 mg/L hygromycin and 40 mg/L meropenem	T2 medium[e]/ 20 mg/L hygromycin and 40 mg/L meropenem	NDM/10 g/L sucrose, 1.0 mg/L NAA, 0.1 mg/L BA, 10 mg/L hygromycin, and 20 mg/L meropenem	NDM/20 mg/L hygromycin and 10 mg/L or 40 mg/L meropenem	NDM/10 g/L maltose/ 30 mg/L hygromycin and 20 mg/L meropenem	NDM/30 g/L maltose, 10 mg/L hygromycin, and 10 mg/L meropenem[f]
Reference		[3]	[3]	[12]	[7]	[1]	[5]	[6, 15]

Note: *NDM* New Dogashima Medium containing 10 g/L maltose, *NAA* 1-naphthaleneacetic acid, *BA* N6-benzyladenine

[a]Not mentioned in the original report

[b]Skip this step, because explants are protocorms

[c]Additional protocol used modified Vacin and Went (VW) solid medium containing 15% coconut water, 30% tomato extract, and 200 μM AS

[d]G10 medium (1 L): 4.3 g Murashige and Skoog (MS) salts (Duchefa); 1 g tryptone; 20 g sucrose; 65 g potato tubers, pH adjusted to 5.4; 3 g Phytagel (Sigma); and 1 g charcoal

[e]T2 medium (1 L): 3.5 g Hyponex No. 1 (N-P-K 7–6-19, Hyponex Co., USA), 1 g tryptone, 0.1 g citric acid, 20 g sucrose, 1 g active charcoal, 20 g sweet potato, and 25 g unripened banana (outer coat peeled and fine paste prepared using a kitchen mixer) . The pH of the medium was adjusted to 5.4 with 0.1 N HCl or 0.1 N NaOH before autoclaving and gelling with 0.3% Phytagel (Sigma). For selection, the medium was supplemented with 40 mg/L meropenem and the optimal concentration of antibiotics

[f]Additional protocol used VW /15% coconut water, 30% tomato extract/30 mg/L geneticin, and 250 mg/L cefotaxime [15]

Table 3
Timetable for orchid genetic transformation

Procedure	*Erycina pusilla*	*Oncidium*	*Phalaenopsis*	*Cattleya*	*Cymbidium*	*Dendrobium*	*Vanda*
Explant preparation	3 M	4 M	5 M	–[a]	–[a]	21 d	–[a]
PLB propagation	–[b]	3 W	3 W	–[a]	4 W	–[b]	4 W
AS pretreatment	2 d	3 d	3 d	3 d	–[a]	–[a]	–[a]
Co-cultivation	4 h[c] + 1–2 d[d]	1–7 d[d]	1–7 d[d]	3 h[d] + 3 d[c]	7 h[d] + 3 d[c]	5 h[d] + 3 d	0.5 h[d] + 4d or 3d
Selection	5 M	5 M	5 M	6 M	4 M	5 M	4 M

[a]Not mentioned in the original reports
[b]Skip this step, because explants are protocorms
[c]Without shaking
[d]Mild agitation

3 Materials

3.1 Plant Materials for Initiation of Protocorm

1. Healthy protocorms that generated from *E. pusilla* seeds of 3–4 months post-pollinated siliques (Fig. 1) are used as explants for *Agrobacterium*-mediated genetic transformation.
2. Growth condition (for protocorms and plants): 24–26 °C, 70% relative humidity, with a 16-h photoperiod (approximately 80 μmol m^{-2} s^{-1}).

3.2 Agrobacterium tumefaciens Strains and DNA Constructs

1. The *A. tumefaciens* strain EHA105 or GV3101 harboring the pCAMBIA1301 or pH2GW7 series of binary vectors is tested (Table 1).
2. Binary vector pCAMBIA1301. This plasmid contains an antibiotic-selectable marker gene and a reporter gene. The antibiotic-selectable marker, *hygromycin phosphotransferase* (*hpt*), confers hygromycin resistance on the transformed plant cells. *β-Glucuronidase* (*GUS*) driven by the cauliflower mosaic virus *35S* promoter (in pCAMBIA1301) causes the transformed plant cells to appear blue in the presence of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc).

3.3 Culture Medium for Agrobacterium

1. *YEP solid medium* containing appropriate antibiotics (1 L): 10 g yeast extract, 10 g peptone, 5 g NaCl, and 15 g Bacto-agar; adjust pH to 7.0 prior to autoclaving. Add appropriate

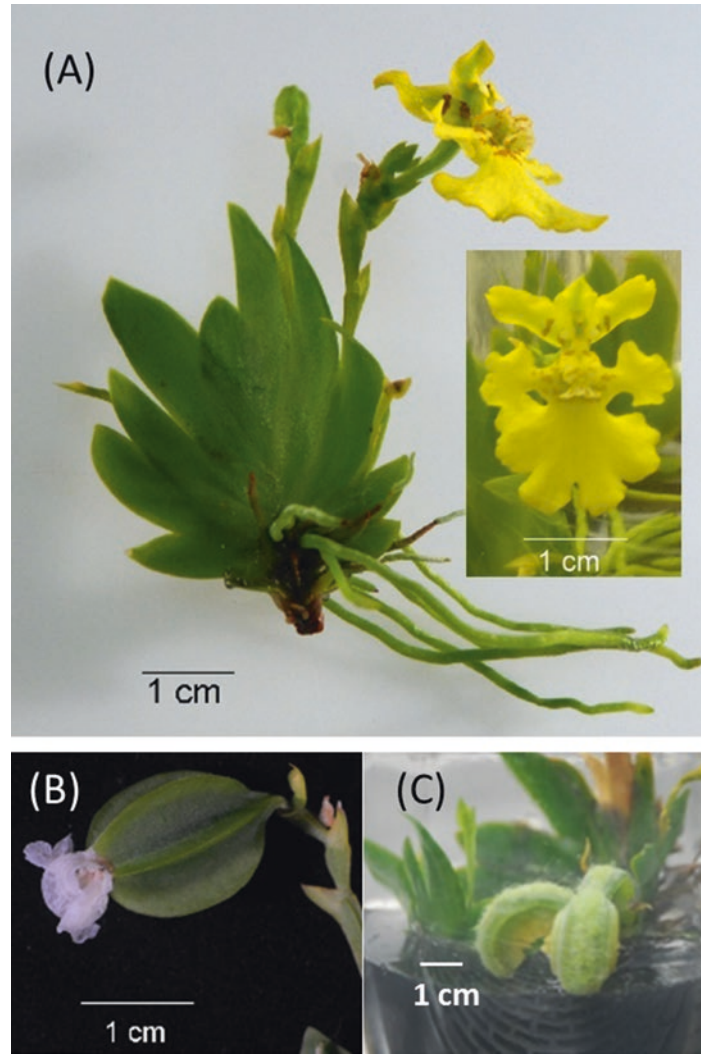


Fig. 1 *Erycina pusilla*. (**a**) Mature plant and flower. (**b**) Growing silique. (**c**) Mature burst opened silique

antibiotics such as 50 mg/L kanamycin for pCAMBIA1301 or 50 mg/L spectinomycin for pH2GW7.

2. *Mannitol-glutamate/Luria (MGL) broth medium* containing appropriate antibiotics (1 L): 2.5 g yeast extract, 5 g tryptone, 0.1 g NaCl, 5 g mannitol, 1 g L-glutamic acid, 0.25 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, and 1 μg biotin, pH 7.0, containing 25 mg rifampicin (for commonly used *Agrobacterium* strains), 25 mg/L gentamycin (for GV3101), and appropriate binary vector selective antibiotics such as 50 mg/L kanamycin for pCAMBIA1301 or 50 mg/L spectinomycin for pH2GW7 series vector.

3.4 Culture Medium for Orchids

1. *Hyponex medium* (*protocorm propagation medium*) (1 L): 3 g Hyponex No. 1 (N:P:K = 7:6:19, Hyponex), 2 g tryptone (Difco, Detroit, USA), 20 g sucrose, and 1 g activated charcoal (Sigma-Aldrich, St. Louis, MO, USA). Adjust the pH of the medium to 5.4 with 0.1 N HCl before autoclaving and gelling with 3 g/L Phytagel (Sigma-Aldrich, St. Louis, MO, USA) or 0.8 g/L agar (Amresco, Cleveland, USA) (*see* **Note 1**).
2. *New Dogashima Medium* (*NDM*) (1 L): 0.48 g NH_4NO_3, 0.2 g KNO_3, 0.47 g $Ca(NO_3)_2{\cdot}4H_2O$, 0.15 g KCl, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.55 g KH_2PO_4, 3 mg $MnSO_4{\cdot}4H_2O$, 0.5 mg $ZnSO_4{\cdot}7H_2O$, 0.5 mg H_3BO_4, 0.025 mg $CuSO_4{\cdot}5H_2O$, 0.025 mg $Na_2MoO_4{\cdot}2H_2O$, 0.025 mg $CoCl_2{\cdot}6H_2O$, 0.5 μL concentrated H_2SO_4, 0.1 g myoinositol, 1 mg nicotinic acid, 1 mg pyridoxine-HCl, 1 mg thiamine-HCl, 1 mg calcium pantothenate, 1 mg adenine, 1 mg cysteine, 0.1 mg biotin, 21 mg Fe-EDTA, and 10 g maltose; adjust pH to 5.4 and then autoclave the medium [16].
3. *Pretreatment medium*: NDM liquid medium with 0.1 mM acetosyringone (AS).
4. *Co-cultivation medium*: NDM liquid medium.
5. *Washing solution*: 40 mg/L meropenem (Myron, China Chemical and Pharmaceutical Co., Taiwan) in sterile ddH_2O (*see* **Note 2**).
6. *Selective medium*: Supplement the Hyponex medium with 40 mg/L meropenem and the appropriate selectable agent, such as 20 mg/L hygromycin for *hpt* selectable marker gene (*see* **Note 3**).

3.5 Antibiotics and Other Stock Solutions

1. Kanamycin stock solution: dissolve 50 mg/mL in ddH_2O, filter-sterilize, and dilute to 50 mg/L for working concentration.
2. Spectinomycin stock solution: dissolve 50 mg/mL in ddH_2O, filter-sterilize, and dilute to 50 mg/L for working concentration.
3. Hygromycin stock solution: dissolve 100 mg/mL in ddH_2O, filter-sterilize, and dilute to 25 mg/L for working concentration.
4. Meropenem (Myron, China Chemical & Pharmaceutical Co., Taiwan) stock solution: dissolve 40 mg/mL in ddH_2O, filter-sterilize, and dilute to 40 mg/L for working concentration.
5. Rifampicin stock solution: dissolve 25 mg/mL in dimethyl sulfoxide (DMSO), and dilute to 25 mg/L for working concentration.

6. Gentamycin stock solution: dissolve 25 mg/mL in ddH_2O, filter-sterilize, and dilute to 25 mg/L for working concentration.
7. α-Naphthaleneacetic acid (NAA) stock solution of 1 mg/mL: dissolve 50 mg NAA in few drops of 1 N NaOH, and bring to 50 mL with ddH_2O.
8. 6-Benzylaminopurine (BA) stock solution of 1 mg/mL: dissolve 50 mg BA in few drops of 1 N NaOH, and bring to 50 mL with ddH_2O.
9. Sodium hypochlorite working solution (1 L): 1% NaOCl (167 mL Clorox bleach per liter) and 0.05% Tween 20.

3.6 Reagents for Transgenic Plant Verification

1. CTAB DNA extraction buffer: 100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) hexadecyltrimethylammonium bromide (CTAB), and 0.3% 2-mercaptoethanol. For 100 mL: Add 1.21 g Tris base; 8.18 g NaCl; 4 mL 0.5 M EDTA, pH 8.0; and 2 g CTAB to a final volume of 100 mL ddH_2O, autoclave, and store at room temperature (RT). Add 0.3 mL 2-mercaptoethanol and 1 g polyvinylpyrrolidone (PVP-40) and warm the buffer to 60 °C before use.
2. Sterilized sea sand.
3. Liquid nitrogen.
4. Phenol/chloroform/isoamyl alcohol (25:24:1), pH 8.0.
5. Chloroform/isoamyl alcohol (24:1).
6. Na-acetate buffer: 3 M sodium acetate adjusted to pH 5.2 by 3 M acetic acid.
7. Isopropanol (IPA): 100%.
8. Ethanol (EtOH): 70%, 95%, and 100%.
9. Trizol (Invitrogen).
10. Diethylpyrocarbonate (DEPC)-treated ddH_2O: autoclave DEPC-treated ddH_2O before use.
11. TE buffer: 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA; autoclave before use.
12. RNase A: 10 mg/mL in TE buffer.
13. X-Gluc staining solution: 1 mM 5-bromo-4-chloro-3-indolyl beta-D-glucuronic acid cyclohexylammonium salt (X-Gluc, x-glucuronide); 100 mM sodium phosphate buffer, pH 7.0; 10 mM ethylenediaminetetraacetic acid (EDTA); 0.5 mM potassium ferricyanide; 0.5 mM potassium ferrocyanide; and 0.1% Triton X-100.
14. HealthView nucleic acid stain (Genomics, Taiwan)

4 Methods

4.1 Preparation of Protocorms

4.1.1 Self-Pollination of E. pusilla from In Vitro Grown Plants

After anthesis, self-pollinate flowers in vitro artificially. Silique maturation takes approximately 3–4 months. Sow mature seeds onto fresh Hyponex medium or MS medium for protocorm development.

4.1.2 Initiation of Protocorms from Seeds

1. If mature siliques are collected from greenhouse-cultivated plants, sterilize the siliques for 5 min in 70% EtOH and for 25 min in sodium hypochlorite solution (under vacuum for 10 min and with shaking at 50 rpm for 20 min), followed by five sterile water washes.
2. Sow the seeds on the Hyponex medium or MS medium (*see* **Note 4**).

4.2 Determination of the Minimal Inhibitory Concentration of Selection Agents

1. Transfer 3-month-old protocorms to selective medium (Hyponex medium supplemented with 0, 15, 20, or 25 mg/L hygromycin, respectively), and incubate the cultures at 24–26 °C under a 16-h photoperiod with a light intensity of 50–100 μmol m^{-2} s^{-1}. Use three replicate plates per biological sample for each treatment.
2. Subculture the protocorms to new selective medium once every 2 weeks. Determine the minimal inhibitory concentration of selection agents one and a half months later (Fig. 2).

4.3 Bacterial Strain Culture and Preparation

1. Streak *Agrobacterium* on YEP solid medium containing appropriate antibiotics (i.e., 25 mg/L rifampicin for *Agrobacterium* EHA105 and 50 mg/L kanamycin for binary vector pCAMBIA1301), and incubate the plates at 28 °C for 2 days in the dark.
2. Pick individual colonies from plates and inoculate into 3 mL MGL liquid or YEP broth supplemented with appropriate antibiotics. Maintain the cultures at 28 °C for 24 h with agitation (150 rpm).
3. Subculture 0.1 mL of the bacterial suspension the following day into 6 mL MGL medium supplemented with AS, and incubate at 28 °C overnight or until A_{600nm} reached 0.5–0.8.

4.4 Inoculation and Co-cultivation

1. Transfer healthy 3-month-old *E. pusilla* protocorms (about 2500 protocorms isolated from a single mature silique) into a 125-mL flask containing 30 mL pretreatment medium (NDM/AS liquid medium). Incubate the flask with shaking (70 rpm) in the dark at 26 °C for 2 days.
2. Add 1 mL of *A. tumefaciens* suspension (A_{600nm} = 0.5–0.8) to 30 mL of the pre-cultured protocorms. After addition, maintain the culture for 4 h without shaking and then followed by

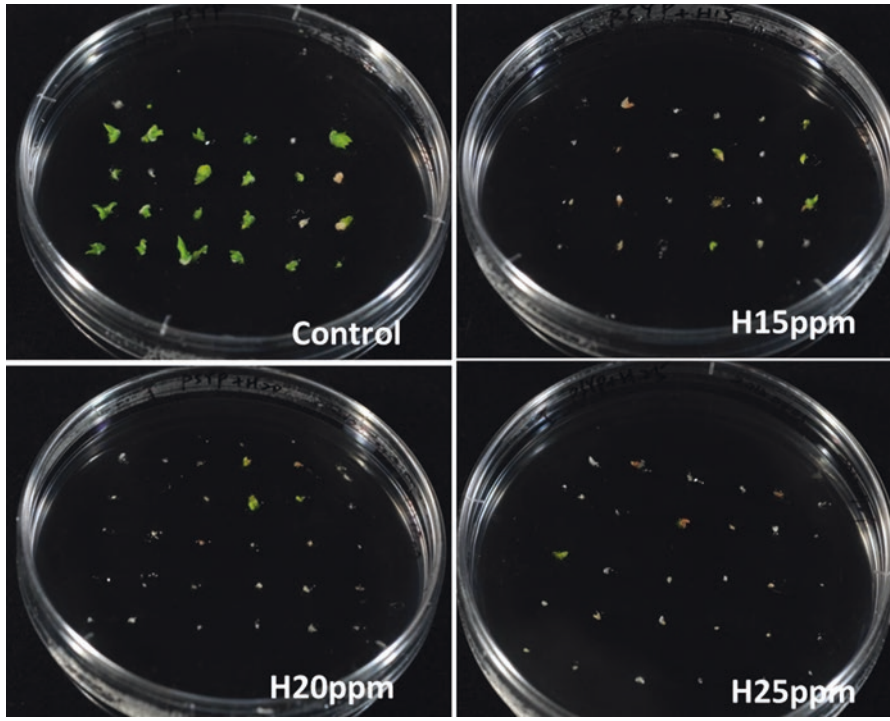


Fig. 2 Three-month-old protocorms that were exposed to various concentrations of hygromycin for 1.5 months, respectively. From upper left to lower right 0, 15, 20, and 25 mg/L hygromycin

co-cultivation with shaking (70 rpm) at 24–26 °C in the dark for 1–2 days until the concentration of the *A. tumefaciens* reached A_{600nm} = 0.5 (*see* **Note 5**).

3. After co-cultivation, wash the protocorms three times with sterile water and once with *washing solution* (sterile ddH_2O supplemented with 40 mg/L meropenem). All washings involve shaking (80 rpm) for 30 min (*see* **Note 6**).

4.5 Selection and Regeneration of Transformants

1. After co-cultivation and washing, blot dry the protocorms on sterile filter paper, and transfer to s*elective medium* (solid Hyponex medium supplemented with 20 mg/L hygromycin and 40 mg/L meropenem), and incubate at 24–26 °C under a 16-h photoperiod with a light intensity of 50–100 μmol m^{-2} s^{-1}.
2. Subculture protocorms ten times on fresh *selective medium* once every 2 weeks.
3. Select healthy growing seedlings, after 5 months of culturing on the *selective medium* (Fig. 3a), for molecular analysis (*see* **Note 7**).
4. Detach blades of the putative transgenic plants and subject to GUS histochemical staining (Fig. 3b) to check the transformation efficiency.

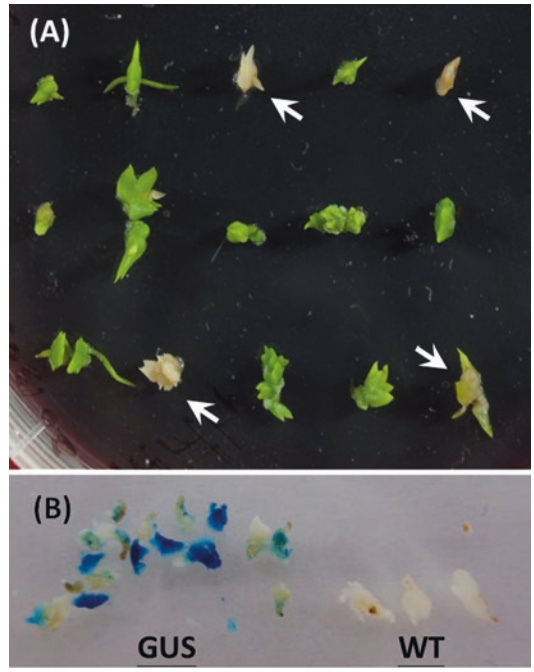


Fig. 3 Hygromycin selection. (**a**) Hygromycin selection for 5 months. Arrows indicate hygromycin-sensitive wild-type plants. (**b**) GUS staining of hygromycin-resistant *E. pusilla* plants. GUS, 1301 transformants; WT, wild type

4.6 Molecular Analyses

4.6.1 Isolation of Genomic DNA from Putative Transformants

The CTAB DNA extraction method is modified from Porebski et al. [17].

1. Ground orchid tissues (about 0.1 g) to fine powder with 50 mg sea sand in liquid nitrogen with a pestle and mortar or by using the TissueLyser II with Grinding Jar Set, s. steel (Qiagen), and further homogenize the sample with 1 mL of 60 °C-preheated CTAB DNA extraction buffer in 2-mL Eppendorf tube.
2. Heat the samples at 60 °C for 1 h with shaking (50 rpm) and then hold at RT for 2 min.
3. Centrifuge samples at 12,000 × *g* for 10 min at RT and transfer supernatants to new tubes.
4. Phenol/chloroform/isoamyl alcohol extraction: Add 1 mL phenol/chloroform/isoamyl alcohol shake for 5 min at 50 rpm, and centrifuge at 5000 × *g* for 10 min at RT, and transfer the aqueous phase to new tubes.
5. Chloroform/isoamyl alcohol extraction: Add 1 mL chloroform/isoamyl alcohol, shake for 5 min at 50 rpm, and centrifuge at 5000 × *g* for 10 min at RT, and transfer the aqueous phase to new tubes.

6. Add one-tenth of the volume (0.1 mL) of 3 M Na-acetate and one volume (1 mL) of 100% isopropanol to the aqueous phase, and follow by centrifugation at 16,000 × *g* for 10 min at RT.
7. Wash the pellet with cold 70% EtOH, rinse with 100% EtOH, and then air-dry.
8. Dissolve the DNA pellet in 200 μL TE at 65 °C for 10 min (optional: 4 °C overnight).
9. Centrifuge the samples at 5000 × *g* for 10 min at RT, and then transfer the supernatant into new 1.5-mL Eppendorf tube.
10. Add 1 μL 10 mg/mL RNase A and 1 μL 1 mg/mL proteinase K (optional) and incubate at 37 °C for 1 h.
11. Repeat **steps 4** and **5** to remove RNase A.
12. Add TE (50 μL) to the organic phase, mix well, and centrifuge at 16,000 × *g* for 15 min, and then combine the aqueous phase with the DNA fractions.
13. Add one-tenth of the volume of 3 M Na-acetate and 2 volumes 100% EtOH, mix well, and centrifuge at 16,000 × *g* for 20 min, and then discard the supernatant.
14. Wash the pellet with cold 70% EtOH, rinse with 100% EtOH, and then dry.
15. Dissolve the DNA in 30–50 μL TE and quantified by spectrophotometry (Nanodrop 2000, Thermo Scientific).

4.6.2 PCR Amplification

1. For 20 μL PCR, mix 0.1–0.4 μg orchid genomic DNA with 0.5 μL 10 μM forward primer, 0.5 μL 10 μM reverse primer, 2 μL 10× Taq buffer, 1 μL 2.5 mM dNTPs, and 0.2 μL Taq DNA polymerase (5 units/μL), followed by sterile ddH_2O to a final volume 20 μL.
2. PCR conditions: 95 °C for 3 min, 25–35 cycles of 95 °C for 25 s, 50–65 °C (Ta = primer Tm—5 °C) for 30 s, and 72 °C for 1 min/kb; 72 °C for 7 min.
3. Check the PCR products by 1% agarose gel electrophoresis and then stain with HealthView fluorescent dye.

4.6.3 RNA Extraction Using Rapid Trizol Method

1. Add fresh orchid tissues (0.1 g) to each tube with 1 mL Trizol reagent, 50 mg sea sand, and one 5-mm stainless steel bead, vortex (Qiagen TissueLyser II) vigorously for 5 min at 30 rpm (chill tubes on ice for seconds at 1 min intervals), and then incubate at RT for 5 min.
2. After centrifugation at 12,000 × *g* for 10 min at RT, transfer the supernatant into new tubes.
3. Add chloroform (0.2 mL) to tubes, shake the tubes vigorously by hand for 15 s, and then incubate at RT for 2 min.

4. After centrifugation at 12,000 × *g* for 10 min at RT, transfer the supernatant into new tubes.
5. Add isopropanol (0.5 mL) and incubate at RT for 10 min.
6. After centrifugation at 12,000 × *g* for 10 min at RT, discard the supernatant.
7. Wash the pellet with 0.5 mL 70% EtOH and centrifuge at RT for 5 min, and then discard the supernatant carefully.
8. Rinse the pellet carefully with 100% EtOH.
9. Air-dry the RNA pellets and then dissolve in 30 μL DEPC-treated ddH_2O.

4.6.4 Reverse Transcription (RT)-PCR

1. For 30 μL RT reaction, mix thoroughly 1–3 μg total RNA with 2 μL 10 μM $dT_{(15–18)}$ and H_2O to a final volume of 11 μL, and then incubate at 72 °C for 10 min.
2. Add RT mixture (19 μL comprising 7.4 μL H_2O, 6 μL 5× RT buffer, 5 μL 2.5 mM dNTP, 0.5 μL M-MLV reverse transcriptase (Promega), 0.1 μL RNase inhibitor (Takara)) and then incubate at 42 °C for 70 min.
3. Inactivate the RT at 72 °C for 15 min and then keep the cDNA samples on ice.
4. Add sterilized H_2O (70 μL) to each tube.
5. For PCR, use 1–5 μL diluted cDNA (the amount depends on target gene expression level) for each reaction.

4.6.5 GUS Histochemical Examination of Transformants

1. For GUS staining, soak antibiotic-selected PLBs or leaves of regenerated plants in X-Gluc staining solution (pH 7.0) overnight at 37 °C.
2. Remove the chlorophyll by soaking in 95% EtOH.

5 Conclusion

There are thousands of seeds in one orchid capsule. However, depending on the nutritional status, the number of seeds in the fruit will be slightly different. In vitro flowering, fruiting, and seed production of *E. pusilla* have been well studied. Typically, the number of protocorms produced by seeds from one to two ripe fruits is sufficient to perform one transformation experiment. In order to ensure that the plants obtained are transgenic plants, the healthy green protocorms were maintained and subcultured for at least 5 months at 2-week intervals on the appropriate selective medium. Based on three independent experiments, the transformation efficiency was 0.6 ± 0.1% [12].

In orchid, PLBs typically provide sufficient material for gene transformation, but the PLB generation stage is not necessary with *E. pusilla*, since it flowers in vitro and artificial pollination is relatively simple to perform. Thus, the gene transfer operation time (from material preparation to transgenic T_1 plants) can be significantly reduced as compared to the other economic orchids. The transition from explant preparation to flowering of the transgenic orchids takes approximately 17, 28, and 39 months for *E. pusilla*, *Oncidium*, and *Phalaenopsis*, respectively [12]. These characteristics, such as small genome size and plant size, short life cycle, year-round blooming, and well-established in vitro pollination and transformation system, make *E. pusilla* an effective orchid model plant for functional genomic and flowering studies [14].

6 Notes

1. The concentration of plant growth regulators for protocorms or PLBs initiation can differ among cultivars, i.e., 0.4 mg/L 6-benzylaminopurine (BA) and 0.1 mg/L naphthaleneacetic acid (NAA) for *Oncidium* PLB initiation.
2. Timentin (200 mg/L), cefotaxime (250 mg/L), or other antibiotics can be employed to replace meropenem (40 mg/L) to eliminate *Agrobacterium*, but a higher antibiotic concentration entails more cost.
3. The antibiotic used for transformation depends on the selection marker of the binary vector; the antibiotic concentration can differ among cultivars and should be determined before genetic transformation.
4. The 2- to 3-month-old protocorms are more appropriate for *Agrobacterium*-mediated transformation. The small size of 1-month-old protocorms causes them difficult to be handled in the transformation procedure, while 4-month-old protocorms have differentiated into seedlings (Fig. 4) and are less suitable as transformation materials.
5. It is better to keep the bacterial concentration below 0.6 A_{600nm}. Overgrowth of *Agrobacterium* needs to be prevented to avoid the death/desiccation of infected PLBs during the co-cultivation period.
6. Meropenem has excellent bactericidal activity and has been tested to eliminate *Agrobacterium* efficiently. However, other *Agrobacterium*-eliminating antibiotics, such as 100–200 mg/L Timentin or 250 mg/L cefotaxime, also can be used.
7. On the *selective medium* without containing any plant growth regulator, *E. pusilla* protocorms can directly regenerate into seedlings.

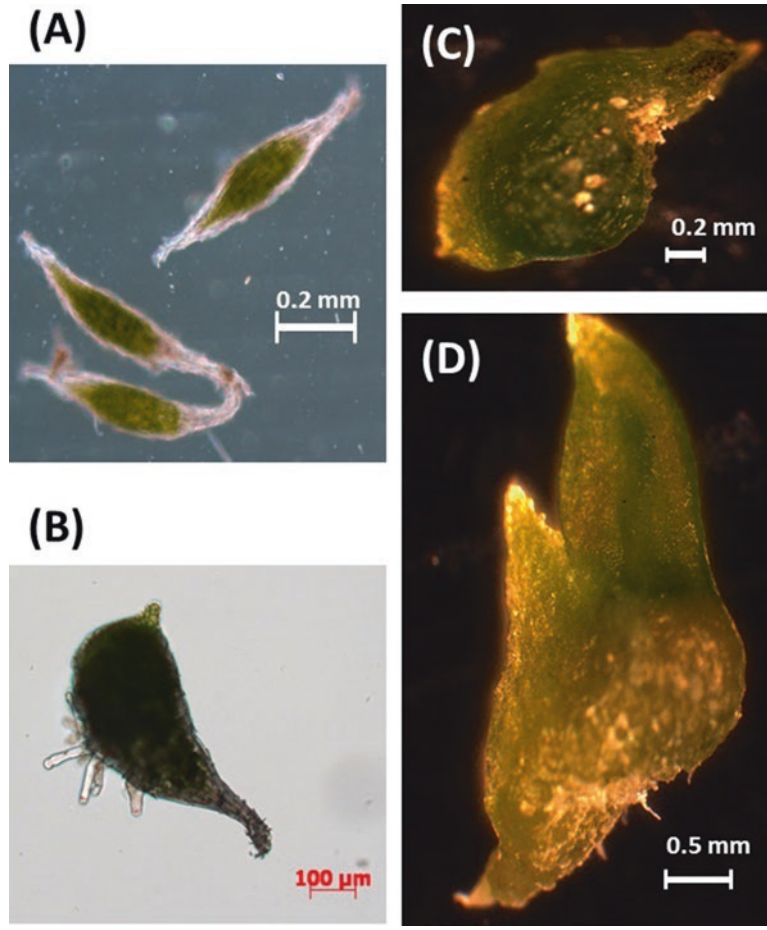


Fig. 4 Different developmental stages of *E. pusilla* plants. (**a**) One-month-old protocorms. (**b**) Two-month-old protocorms. (**c**) Three-month-old protocorms. (**d**) Four-month-old protocorm

Acknowledgments

This work was supported by grants from Academia Sinica and the National Science Council of the Republic of China.

References

1. Chin DP, Mishiba K, Mii M (2007) *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cymbidium*. Plant Cell Rep 26:735–743
2. Teixeira da Silva JA, Dobránszki J, Cardoso JC, Chandler SF, Zeng S (2016) Methods for genetic transformation in *Dendrobium*. Plant Cell Rep 35:483–504
3. Li C-W, Liao C-H, Huang X, Chan M-T (2015) Orchids (*Oncidium* and *Phalaenopsis*). In: Wang K (ed) *Agrobacterium* protocols: volume 2. Humana Press, Totowa, NJ, pp 331–346
4. Mishiba K, Chin DP, Mii M (2005) *Agrobacterium*-mediated transformation of *Phalaenopsis* by targeting protocorms at an early stage after germination. Plant Cell Rep 24:297–303
5. Phlaetita W, Chin DP, Tokuhara K, Nakamura I, Masahiro M (2015) Agrobacterium-

mediated transformation of protocorm-like bodies in *Dendrobium formidible* 'Ugusu'. Plant Biotechnol 32:225–231

6. Shrestha BR, Chin DP, Tokuhara K, Mii M (2010) *Agrobacterium*-mediated transformation of *Vanda* using protocorm-like bodies. Asia-Pacific J Molec Biol Biotechnol 18:225–228
7. Zhang L, Chin DP, Mii M (2010) *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cattleya*. Plant Cell Tiss Org Cult 103:41–47
8. Chai D, Lee SM, Ng JH, Yu H (2007) L-methionine sulfoximine as a novel selection agent for genetic transformation of orchids. J Biotechnol 131:466–472
9. Semiarti E, Indrianto A, Purwantoro A, Machida Y, Machida C (2011) *Agrobacterium*-mediated transformation of Indonesian orchids for micropropagation. In: INTECH Open Access Publisher, pp 215–240
10. Chan YL, Lin K-H, Liao L-J, Chen W-H, Chan M-T (2005) Gene stacking in *Phalaenopsis* orchid enhances dual tolerance to pathogen attack. Transgenic Res 14:279–288
11. You SJ, Liau C-H, Huang H-E, Feng T-Y, Prasad V, Hsiao H-H, J-C L, Chan M-T (2003) Sweet pepper ferredoxin-like protein (*pflp*) gene as a novel selection marker for orchid transformation. Planta 217:60–65
12. Lee S-H, Li C-W, Liau C-H, Chang P-Y, Lin C-S, Chan M-T (2015) Establishment of an *Agrobacterium*-mediated genetic transformation procedure for the experimental model orchid *Erycina pusilla*. Plant Cell Tiss Org Cult 120:211–220
13. Qin X, Liu Y, Mao S, Li T, Wu H, Chu C, Wang Y (2011) Genetic transformation of lipid transfer protein encoding gene in *Phalaenopsis amabilis* to enhance cold resistance. Euphytica 177:33–43
14. Pan IC, Liao D-C, Wu F-H, Daniell H, Singh ND, Chang C, Shih M-C, Chan M-T, Lin C-S (2012) Complete chloroplast genome sequence of an orchid model plant candidate: *Erycina pusilla* apply in tropical *Oncidium* breeding. PLoS One 7:e34738
15. Gnasekaran P, Antony JJJ, Uddain J, Subramanian S (2014) *Agrobacterium*-mediated transformation of the recalcitrant *Vanda* Kasem's Delight orchid with higher efficiency. Sci Worl J 2014:583934
16. Tokuhara K, Mii M (1993) Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. Plant Cell Rep 13:7–11
17. Porebski S, Bailey LG, Baum BR (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Molec Biol Reprod 15:8–15

Chapter 21

The Use of Laser Microdissection to Investigate Cell-Specific Gene Expression in Orchid Tissues

Raffaella Balestrini, Valeria Fochi, Alessandro Lopa, and Silvia Perotto

Abstract

In the past decade, laser microdissection (LMD) technology has been widely applied to plant tissues, highlighting the role of different cell-type populations during plant–microbe interactions. In this chapter, a method to apply the LMD approach to study gene expression in specific cell-type populations of orchid mycorrhizal protocorms and roots is described in detail, starting from the preparation of biological material to gene expression analysis by RT-PCR.

Key words Laser microdissection, Gene expression, Orchid, *Serapias vomeracea*, *Tulasnella calospora*

1 Introduction

Orchid seeds were named "dust seeds" because they contain little stored food reserves due to the lack of endosperm. Therefore, colonization by a compatible mycorrhizal fungus is essential in nature for the provision of major nutrients such as carbon (C) and nitrogen (N) during their germination ([1]; also *see* Chapter 1). Symbiotic germination supported by mycorrhizal fungi allows successful formation of protocorms leading to seedling development. The protocorm is a fully heterotrophic structure that generally lacks chlorophyll and is therefore completely fungus-dependent for organic C supply. This peculiar ability of the plant to receive C from its fungal partner is known as mycoheterotrophy [2, 3]. Continual development leads to the formation of shoot and roots and photosynthetic leaves. Moreover, some species completely lack or are only partially capable of photosynthesis; these orchids remain, therefore, as fully or partially mycoheterotrophic plants as adults. In all cases, roots of mature orchids are commonly found to associate with mycorrhizal fungi [1]. Inside either mycorrhizal protocorms or roots, fungal hyphae spread from cell to cell by

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_21,

penetrating the plant cell wall, forming elaborate coiled structures known as pelotons. However, symbiotic protocorms and colonized roots have a heterogeneous cellular environment with various cell types present [4]. Global transcriptome analyses show that several genes are activated in symbiotic protocorms [4–7], but interpretation of the results derived from whole protocorm expression profiling is complicated by the presence of multiple cell types. These include non-colonized as well as colonized cells; and among the latter, cells containing pelotons at different developmental stages of formation are also present. Each cell type is likely to have a different role(s) in the interaction, as already demonstrated for other symbiotic associations [8–10]. In order to obtain cell type-specific RNA and/or protein expression, alternative methods for isolating populations of specific cell types are needed. Laser microdissection (LMD) technology allows rapid isolation of specific cell-type populations from a tissue section that is composed of heterogeneous cell types [11]. DNA, RNA, proteins, and even metabolites can be then extracted from these homogeneous samples.

Over the past decade, LMD has been widely applied to plant tissues [12–17]. The advantage of LMD technology is that it can usually be applied to all cells that can be identified by conventional light microscopy, without the obligate use of specific cell markers or genetic lines [18, 19]. Possible disadvantages of LMD relate to histological fixation and processing, which can compromise the target molecules, i.e., RNA, DNA, proteins, and metabolites, and the high cost of the LMD instrumentation. Moreover, the preparation of plant samples, the different LMD systems, and the protocols for the downstream analyses have been extensively illustrated in several original publications [20–25] and reviews [12, 13, 15, 16, 26, 27]. It is important to note that there is no universal LMD protocol that can be applied to all plant tissues. Researchers should consider optimizing the fixation and processing protocols on a case-by-case basis [13]. Different protocols are now available, providing a good starting point for most applications (*see* **Note 1**).

Although some applications of LMD in combination with protein [28] and metabolite [29] analyses have been performed, the LMD approach in plant research has been mostly developed for the identification of differentially expressed transcripts. Thus, one of the foci in using LMD technology is to focus on RNA extraction and processing from LMD-collected cells [12–16, 26, 30, 31]. In order to have a satisfactory preservation of plant tissues for target identification, samples are generally embedded in paraffin after fixation [26]. Although the paraffin embedding method provides excellent morphological preservation, the RNA yield is reduced compared with that from frozen samples. Nevertheless, satisfactory amounts of RNA can be obtained from paraffin-embedded materials, and the improved morphology is essential to the identification of appropriate cell types. An important aspect of most LMD-based gene expression studies is that the RNA usually needs to be ampli-

fied in order to obtain a sufficient amount of material for further analyses. RNA yield from microdissected cells is generally sufficient for targeted molecular analyses like reverse transcription and amplification (RT-PCR), while one or two rounds of RNA amplification are required to obtain microgram amounts of mRNA needed for global profiling analyses [31–33].

The study of plant–microbe interactions is surely an area where LMD could have an impact because this technology provides information about the spatial expression of genes involved in the interaction. The responses of the two symbiotic partners can in fact be localized in specific plant cell types or microbial structures [8, 26, 34–36]. When LMD is used, cells associated with particular infection stages can be visualized under the microscope and harvested to verify the plant response in different cell types and during the progression of infection. The use of LMD, in combination with target gene expression and/or transcriptome analyses, has been successfully applied to the study of cell specificity in mycorrhizal symbiosis, mainly in arbuscular mycorrhiza [8–10, 37–39]. A LMD protocol has also been developed to investigate whether expression of some orchid genes was restricted to specific cell types in protocorms of *Serapias vomeracea* colonized by the mycorrhizal fungus *Tulasnella calospora* [7, 40]. One of the results shows that *SvNod1*, a *S. vomeracea* nodulin-like protein containing a plastocyanin-like domain, is expressed only in protocorm cells containing intracellular fungal hyphae [40].

In this chapter, we detail a method applying the LMD technology to study gene expression in specific cell-type populations in orchid mycorrhizal protocorms and roots, starting from the preparation of the biological material to gene expression analyses by RT-PCR. Some novel results on fungal and plant genes expressed in specific cell-type populations from mycorrhizal root samples will be also discussed.

2 Laser Microdissection to Study Cell Type-Specific Responses in Orchid Mycorrhiza: Technical Comments and Observations

2.1 Morphological Preservation and LMD of Orchid Protocorm and Root Tissues

In order to analyze cell-type-specific gene expression in orchid mycorrhiza with the use of LMD technology, it is necessary to have access to the target cells located deep within the tissues. Tissues need to be fixed and processed for paraffin embedding and sectioning in order to obtain tissues where preservation of cell structures is sufficiently good to recognize the different cell types and fungal structures (Fig. 1). The sections obtained from mycorrhizal protocorms and roots of *S. vomeracea* after ethanol/acetic acid (3:1 v/v) fixation and paraffin embedding ([7, 40]; *see* below) enable us to distinguish plant cells colonized by fungal pelotons and non-colonized cells (NM) (*see* **Note 2**). In addition, it is possible to

distinguish, without staining, plant cells containing either well-developed fungal coils (C) or more condensed or collapsing fungal coils (CC). Moreover, with *S. vomeracea* root samples where the fungal coils filled most root cells, this makes the collection of non-colonized cells difficult. In addition, with a low amount of adult *S. vomeracea* root material available, it is almost impossible to separate immature and mature pelotons from root cells (Fig. 1).

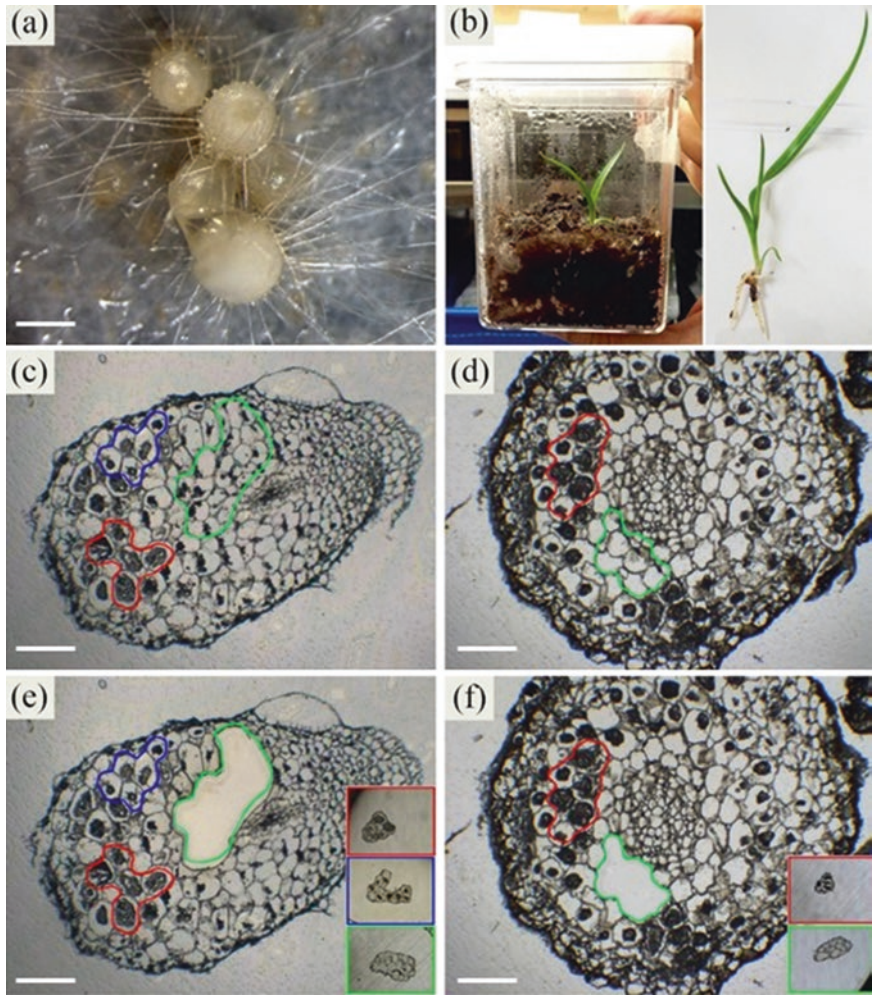


Fig. 1 (**a**) Mycorrhizal protocorms of *Serapias vomeracea* 30 days after sowing (stage 3) with *Tulasnella calospora*. Stereomicroscopic image of protocorms, showing well-developed rhizoids. Bar = 1 mm. (**b**) Magenta jar containing a 4-month-old plantlet. (**c**, **e**) Paraffin section of a *S. vomeracea* mycorrhizal orchid protocorm before (**c**) and after (**e**) cutting. Microdissection of non-colonized cells (NM, circled by a green line), cells containing young pelotons (C, circled by a red line), and cells containing collapsed pelotons (CC, circled by a blue line). (**d**, **f**) Paraffin section of a *S. vomeracea* mycorrhizal root before (**d**) and after (**f**) cutting. Pelotons formed by the fungus are well visible (C, circled by a red line). Cells without coils were also collected using LMD (NM, circled by a green line). Insets showing collected cells. Bars = 200 μm

2.2 RNA Extraction from Microdissected Cells

RNA is readily extracted from different microdissected cell-type populations isolated from both root and protocorm sections. NanoDrop spectrophotometric quantification indicates that a total RNA yield ranges from 46 to 220 ng per sample, depending on the cell type and the number of collected cell sections (approximately 1200–1500 sections). For example, RNA yield from cells containing well-developed coils (C in Fig. 1) is higher than yield from non-colonized cells, probably due to the presence of both plant and fungal RNA (Table 1).

DNase treatment needs to be performed on the eluates because previous studies [8] reported it to be more effective than DNase treatment in the column, as suggested by the manufacturer. To validate the presence of plant and fungal RNA in the extracts, as well as the presence of potential contaminating DNA, one-step RT-PCR experiments are performed with primers specific for plant and fungal housekeeping genes. Primers targeting two elongation factor genes have been used, *SvEF-1α* for the orchid and *TcEF-1α* for the fungus [4, 7]. As reported by Perotto et al. [4] and Fochi et al. [7], using primers specific for *SvEF-1α*, a PCR product of the expected size was detected in all the different RNA samples (C, CC, NM from protocorms; C, NM from roots). Using primers

Table 1
RNA extraction from LMD cells: RNA yield and concentration were measured in the different replicates with a NanoDrop spectrophotometer

	Populations	RNA yield (ng)	RNA (ng/μL)
Protocorm	*1st biological replicate*		
	C (1200 cells)	184	9.2
	CC (1200 cells)	78	3.9
	NM (1200 cells)	48	2.4
Protocorm	*2nd biological replicate*		
	C (1500 cells)	196	9.8
	CC (1500 cells)	100	5
	NM (1500 cells)	74	3.7
Root	*1st biological replicate*		
	C (1200 cells)	140	7
	NM (1200 cells)	46	2.3
Root	*2nd biological replicate*		
	C (1500 cells)	220	11
	NM (1500 cells)	54	2.7

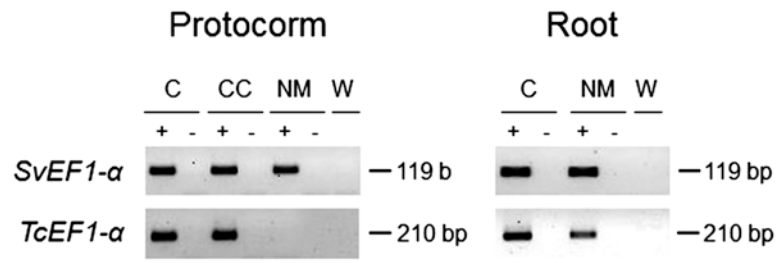


Fig. 2 One-step RT-PCR analysis of microdissected cells from *S. vomeracea* protocorms and roots colonized by *T. calospora*, using primers specific for the plant and fungal elongation factors, *SvEF1-α* and *TcEF1-α*, respectively, as housekeeping genes. Each sample was tested for potential DNA contamination by omitting the RT step. The presence of an amplified product in the RT sample (+) and its absence in the RT-negative reaction (–) excluded any DNA contamination. Water was used as a control for the reaction (W)

specific for the fungal housekeeping gene *TcEF-1α*, an amplified fragment of the expected size was detected in samples containing fungal structures (C and CC) in all replicates from protocorms, whereas an amplified fragment was only rarely observed in non-colonized protocorm cells. By contrast, an amplified band was always detected with the *TcEF-1α*-specific primers in the RNA extracted from root cells without apparent fungal colonization. This is probably caused by the presence of hyphae, though not readily visible on microscopic inspection (*see* Subheading 2.3). Potential DNA contamination is checked by avoiding the retro-transcription step (RT) in the same RT-PCR experiment. The absence of an amplified product in the RT reaction excluded any residual contamination by genomic DNA (Fig. 2).

2.3 Gene Expression in Specific Cell Types: A Targeted Approach

One-step RT-PCR assays are used to detect plant and fungal mRNAs in the specific cell-type populations collected by LMD [7, 40] (*see* **Note 3**). These two reports [7, 40] showed for the first time the successful application of LMD to study cell-specific gene expression in orchid mycorrhiza. Balestrini et al. [40] focused on some *S. vomeracea* genes that could have a role during the symbiotic interaction, whereas Fochi et al. [7] investigated genes involved in nitrogen uptake and metabolism, providing novel insights on the mechanisms of nutrients exchange between orchid and fungus. Based on the results of a large-scale transcriptomic experiment, Fochi et al. [7] selected some *T. calospora* genes for further investigations by one-step RT-PCR, i.e., ammonium transporters (*TcAMT1* and *TcAMT2*), glutamine synthetase (*TcGS*), urease (*TcURE*), arginase (*TcCAR*), ornithine aminotransferase (*TcOAT*), ornithine decarboxylase (*TcODC*), and some amino acid transporters, i.e., *TcAAT1*, *TcAAt2*, *TcAAT3*, *TcAAT4*, and *TcAAT5*. Using specific primers, results on laser-microdissected

cells from mycorrhizal protocorm sections allowed us to detect the presence of corresponding transcripts in cell-type populations containing fungal structures (C and CC) [7].

We also investigated the expression of these fungal genes in peloton-containing cells (C) from *S. vomeracea* plantlet roots, with the aim to verify whether changes occur in the expression of selected genes when the plant becomes photosynthetic. One-step RT-PCR of the fungal genes previously identified as being expressed in the protocorms [7] is also expressed in the root cells containing fungal pelotons (Fig. 3). Amino acid transport genes, i.e., *TcAMT2*, *TcAAT1*, *TcAAT3*, *TcAAT4*, *TcURE*, *TcCAR*, and *TcOAT*, are only detected in root cells containing fungal coils (C), whereas *TcGS*, *TcAAT2*, *TcAAT5*, *TcODC*, and *TcOAT* are expressed in cells containing the fungal coils (C) as well as in non-colonized (NM) root cells (Fig. 3). This is likely due to fungal hyphae/RNA contamination in these non-colonized cells that are collected together during LMD, as suggested by the results with the primer specific for *TcEF-1α*. This may also be due to the high colonization rate of mycorrhizal fungi (Fig. 1), and degenerating pelotons may go undetected in some cortical cells. In the arbuscular mycorrhizal (AM) symbiosis, the non-colonized cell-type population also shows varied results [41]. The slight variability observed in the molecular analyses could be due to the presence of fungal hyphae not visible under a microscope as well as the fact that the non-colonized cell-type population is formed by cortical cells that have already been activated [26]. The expression of fungal genes in plant cells in the absence of the fungus (NM) indicates difficulties in the collection of specific cell types from roots.

3 Materials

3.1 Biological Materials

1. Fungal mycelium*: Tulasnella calospora* (Boud.) Juel AL13/4D (accession number MUT4182) (*see* [7]). Experimental details for the growth of the free-living mycelium are detailed in [7].
2. Plant material: Mature *Serapias vomeracea* (Burm.f.) Briq. seeds. Obtain seeds from mature yellowing capsules from naturally pollinated orchids. Allow capsules to dry at room temperature for 2–3 weeks. After capsule dehiscence, clean seeds away from capsule debris and store in glass vials on silica gel at 4 °C [4].

3.2 Major Equipment

1. Laser microdissection system (6500 LMD system—Leica Microsystems or different suppliers).
2. PCR thermal cycler (different suppliers).
3. Rotary microtome to prepare paraffin sections (Leica Microsystems or different suppliers) using A35 durable low-profile microtome blades (Tissue-Tek—Electron Microscopy Sciences, #63069).

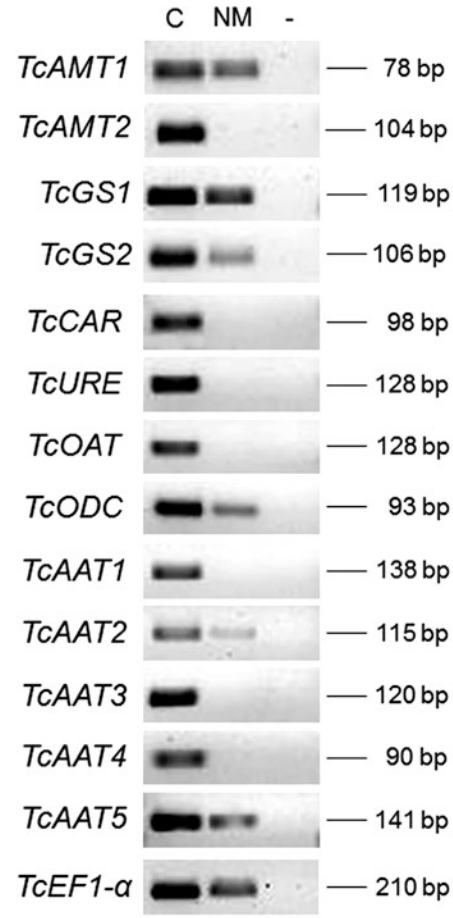


Fig. 3 One-step RT-PCR of *T. calospora* transcripts in different microdissected cell-type populations from mycorrhizal *S. vomeracea* roots: C, root cortical cells containing fungal coils; NM, non-colonized cortical cells from mycorrhizal roots. *TcEF-1α* was used as housekeeping gene. Water (–) was used as a control for the reaction

4. Clean bench- and tissue culture-associated equipment.
5. Slide warmer (different suppliers).
6. Binder oven (different suppliers).

3.3 Supplies for LMD and RNA Extraction

1. Membrane slides (poly-ethylene-naphthalate (PEN)-membrane 2.0 microns thick, Leica Microsystems, #11505189) (*see* **Note 4**).
2. Thin wall PCR tubes, 0.5 mL (Greiner Bio-One, #682201) (*see* **Note 4**).
3. RNA extraction buffer (PicoPure Kit, Arcturus Engineering).

4. PicoPure® RNA Isolation Kit (Thermo Fisher Scientific, # KIT0204) or equivalent RNA extraction kits from low amount of starting material (different suppliers).
5. One-step RT-PCR kit (Qiagen).
6. Turbo DNA-free kit (Ambion, Austin, TX, USA).
7. Chemical and supplies associated with the paraffin embedding method (*see* [42]).

4 Methods

4.1 Symbiotic Seed Germination

The interaction between the Mediterranean terrestrial orchid *Serapias vomeracea* and the basidiomycetous fungus *Tulasnella calospora* has been used as a model system to study mycorrhizal interactions in orchids. Mycorrhizal protocorms as well as colonized roots from photosynthetic plantlets, obtained from symbiotic protocorms as described below, have been used for laser microdissection studies.

4.1.1 Seed Germination and Protocorm Formation

Obtain *S. vomeracea* symbiotic protocorms by co-inoculation of mycorrhizal fungi and orchid seeds in 90 mm petri dishes, as described in Perotto et al. [4]. Surface sterilize seeds of *S. vomeracea* in 1% sodium hypochlorite and 0.1% Tween-20 for 20 min on a vortex, followed by three 5-min rinses in sterile distilled water. Re-suspend seeds in sterile water and drop on strips of autoclaved filter paper (1.5 × 3 cm) previously positioned on solid oat agar medium (OA, 0.3% milled oats and 1% agar). Place a plug of actively growing mycelium of *T. calospora* isolate AL13 in the center of each petri dish, and incubate the plates at 20 °C in total darkness for 30 days.

4.1.2 From Protocorms to Plantlets

The following protocol has been developed to obtain *S. vomeracea* roots colonized by fungal mycelium. After 30–45 days of incubation in the dark, symbiotic protocorms reach an adequate size (3–4 mm), with the first developing leaf primordium. At this stage, transfer plates containing the protocorms to light for about 20 days in a growth chamber (23 °C for 16 h light/21 °C for 8 h dark). Once leaf primordia turned green, transfer protocorms to magenta jars containing fresh OA medium with a reduce agar content (7.5 g/L agar and 3 g/L oat flour) and maintain in the growth chamber with the same parameters described above until a root appears at the base of the protocorms.

Once reaching the seedling stage (with developed leaf blades and rootlets), transfer seedlings to new magenta jars containing fresh OA medium (5 g/L agar and 3 g/L oat flour) supplemented with previously sterilized soil before use. Sterilize soil three times

before use (for the first two times, sterilize soil for 1 h at 100 °C with flowing steam and the third time at 121 °C for 21 min). Leave the soil for 1 day to dry at 70 °C in an oven prior to use. To prepare the magenta jars with the mixed substrate, add the OA medium on the sterilized soil previously placed inside the jar.

Finally, transfer vigorously grown seedlings (higher than 2 cm) to new magenta jars containing twice-sterilized natural soil, and water with a sterile solution of water containing oat flour (3 g/L). Maintain plantlets at 23 °C for 16 h in the light and 21 °C for 8 h in the dark. Approximately 4–5 months are needed to obtain small mature plants from symbiotic protocorms.

4.2 Fixation and Paraffin Embedding

Collect *S. vomeracea* symbiotic protocorms and root segments in RNase-free tubes containing cold freshly prepared Farmer's fixative (absolute ethanol/glacial acetic acid, 3:1 v/v) as described in Balestrini et al. [40]. Maintain the samples for 20 min at room temperature under vacuum, to facilitate the fixative penetration by removing air from the intercellular spaces of plant tissues. Replace with fresh fixative and incubate samples overnight at 4 °C. Dehydrate samples in a graded ethanol series (70%, 90%, and 100% twice) (*see* **Note 5**), followed by two changes in Neo-Clear® (Merck; *see* **Note 6**), each step of 30 min on ice. Replace Neo-Clear gradually with paraffin (Paraplast Plus). In detail, add a few chips of paraffin (approximately 10 pieces) to 20 mL of Neo-Clear, and maintain the samples at room temperature for about 2 h and then at 58 °C until the chips dissolve (about 3 h). Substitute the mixture with pure paraffin, previously melted and maintained at 58 °C. Incubate the tubes overnight at 58 °C. In the first step with pure paraffin, keep the tubes open to permit evaporation of remaining Neo-Clear from tissues. The following day, replace paraffin one to two times at approximately 6-h intervals. For embedding, pour the content into petri dishes (60 mm) and allow to solidify by cooling.

Section protocorms and root samples at 12 μm of thickness using a rotatory microtome. Place ribbons of sections on sterile double-distilled water (ddH_2O, filtered with a 0.2 μm filter) on the Leica RNase-free PEN foil slides (Leica Microsystems) and dry on a 40 °C slide warming plate. Store slides at 4 °C and use within 2 days (*see* **Note 7**).

4.3 Laser Microdissection (LMD)

A Leica LMD 6500 laser microdissection system (Leica Microsystems) is used to isolate cells from tissue sections (*see* **Note 8**). Just before use, deparaffinize paraffin sections with Neo-Clear for 8–10 min, followed by a fast rinse in 100% ethanol for 1 min, and then air-dry. For LMD, place the treated slides facedown on the microscope slide holder and place a 0.5 mL RNase-free PCR tube in a special collector so that the tube cap is just beneath the tissue section. Visualize the tissues on a computer monitor through a video camera and with the use of a software (Leica LMD

Software); encircle target cells on the computer screen. Cut target cells using a UV laser (337 nm wavelength).

Collect three different cell types separately from symbiotic protocorms: (1) cells with fungal coils occupying the whole plant cell, (2) cells with more condensed fungal coils, and (3) non-colonized cells; collect two different cell types separately from root sections: 1) colonized cells and 2) non-colonized cells. Optimize the LMD conditions to obtain a clean, narrow excision of selected cells: 6.3-XT objective at power 20–25 and at a speed of 3–4. Avoid damage to the target cells, carefully directing the laser to cut the cells immediately surrounding selected cells. Gravity will cause the dissected cells to fall into the cap of the 0.5 mL RNase-free PCR tube. After collection, add 10 μL of RNA extraction buffer (PicoPure Kit, Arcturus Engineering) to the tube cap containing cell sections (approximately 300 sections are collected in a day), and close the tube. Incubate samples at 42 °C for 30 min, centrifuge at 800 × *g* for 2 min, and then store at −80 °C.

4.4 RNA Extraction, DNase Treatment, and One-Step RT-PCR

For the following RNA extraction steps, samples containing the same dissected cell-type population in the extraction buffer are pooled in a single tube (about 1200–1500 sections in total for each cell-type population), with a final volume of 50 μL. Extract RNA using the PicoPure™ RNA Isolation Kit (Arcturus Engineering) with slight modifications. In particular, the DNase treatment is not performed on the isolation kit column, as described in the protocol provided by the manufacturer; instead, treat RNA with the Turbo DNA-free (Ambion) once eluted from the column (*see* Subheading 4.4.2).

4.4.1 RNA Extraction

Perform RNA extraction according to the protocol described in the PicoPure manual (protocol IV). Before adding the RNA sample to the column, precondition the RNA purification column. To this aim, add 250 μL of the conditioning buffer (CB) onto the purification column filter membrane (provided in the collection tube), and incubate the tubes for 5 min at room temperature and then centrifuge at 16,000 × *g* for 1 min. In the meantime, add 50 μL of 70% ethanol to each pooled sample and mix well by pipetting up and down. Wash and elute the total RNA (in 100 μL) bound to the preconditioned column according to the manufacture's protocol. Elute the RNA in 20 μL of elution buffer (EB) and collect into a new 0.5 mL micro-centrifuge tube. The isolated RNA is ready for DNase treatment.

4.4.2 DNase Treatment

To avoid the risk of DNA contamination, subject the RNA to a DNase treatment using Turbo DNA-free™ Kit (Ambion). In detail, add 1.5 μL of enzyme and 2.4 μL of 10× buffer to 20 μL of eluted RNA; mix samples gently and incubate at 37 °C for 30 min. Stop Turbo DNase incubation with 3 μL of DNase inactivation reagent,

and keep for 5 min at room temperature with occasional mixing. Centrifuge at 10,000 × *g* for 1.5 min to remove the inactivation buffer. Transfer the solution containing RNA to a fresh tube. Either use the treated RNA immediately for reverse transcription and amplification (RT-PCR) to verify the DNA contamination or store at −80 °C until use.

4.4.3 One-Step RT-PCR

The one-step RT-PCR kit (Qiagen) is used to investigate accumulation of the target gene transcripts in RNAs extracted for the different cell-type populations. This kit allows the reverse transcription (RT) and the subsequent cDNA amplification by PCR in a unique reaction. Carry out the reactions in a final volume of 10 μL containing 2 μL of 5× buffer, 0.5 μL of 10 mM dNTPs, 0.25 μL of each primer (diluted 1:10 from the 100 mM stock), 0.25 μL of one-step RT-PCR enzyme mix, and 0.5 μL of total RNA (generally diluted 1:5). For the RT-PCR analyses, incubate samples for 30 min at 50 °C, followed by 15 min of incubation at 95 °C. Run the amplification reactions for 40 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s using *T. calospora*- and *S. vomeracea*-specific primers [7, 40, 43].

Design primers to the plant and fungal elongation factor genes (*SvEF-1α*, *TcEF-1α,* respectively). Use these housekeeping genes to assess RNA amplification and possible contamination of RNA samples with genomic DNA. The absence of an amplified product in the RT-minus reactions excludes genomic DNA contamination (Fig. 2). Perform RT-PCR experiments on two biological replicates, and separate PCR products on 1.5% agarose gel.

5 Concluding Remarks

The results obtained so far demonstrate that LMD is a suitable technology to investigate the cellular complexity of orchid mycorrhizal tissues. However, some difficulties in the collection of homogenous cell-type populations from root tissues have been highlighted. A further challenging step will be to obtain, e.g., through RNAseq experiments on RNA extracted from microdissected cells, "global" transcriptomic profiles associated with the different cell types that occur in symbiotic orchid structures, like symbiotic protocorms.

6 Notes

1. The Science Lab portal by Leica Microsystems (http://www.leica-microsystems.com/science-lab/topics/laser-mic) provides useful information (e.g., short articles), showing several applications of laser microdissection that may be useful for

researchers working on this technology (independently from the LMD system used).

2. Alternative fixatives may be used instead of ethanol/acetic acid 3:1 (Farmer's fixative). For example, 100% ice-cold acetone or a mixture of 60% absolute methanol, 30% chloroform, and 10% glacial acetic acid (v/v/v) (methacarn fixative) can be used. On the contrary, fixatives containing formaldehyde/formalin should be avoided since formaldehyde breaks nucleic acids and is therefore not suitable for subsequent RNA and DNA analyses.
3. Quantitative RT-PCR (RT-qPCR) can be also performed using RNA extracted from LMD-collected cell-type populations. If there are problems, due to low quality or quantity of the extracted RNA, to obtain good results using a two-step protocol (i.e., a first RT reaction with random primers followed by a qPCR with specific primers), a one-step RT-qPCR protocol could be used (*see*, e.g., http://www.leica-microsystems.com/science-lab/laser-microdissection/quantitative-one-step-protocol-to-detect-transcripts-in-laser-microdissected-samples/).
4. The products cited in LMD supplies are specifically requested for the Leica LMD apparatus. Other LMD systems most likely require different products.
5. Use sterile water to prepare diluted ethanol solution.
6. Neo-Clear® is a mixture of aliphatic hydrocarbons and can be used for the same applications as xylene (e.g., paraffin embedding and deparaffinization). No changes in methods are necessary when Neo-Clear® is used instead of xylene. The evaporation rate is lower than xylene so that Neo-Clear® has less odor and the smell in the lab is reduced significantly (https://www.merckmillipore.com/GB/en/).
7. All the steps of fixation and paraffin embedding require the use of sterile materials (tweezers, scalpel, tubes, petri dishes, etc.) to avoid RNA degradation. Fixation as well as infiltration must be performed under a chemical hood. To better maintain RNA integrity, paraffin with a lower melting point is preferred, as it will minimize RNA degradation.
8. LMD parameters (e.g., which objective to select and cut the targeted cells, laser power, etc.) must be optimized depending on the biological material, the section thickness, as well as the LMD system used. Published protocols represent only a starting point and may need modifications to obtain the best results with different specimens. Any changes in tissue handling, such as different fixation times, may lead to different laser cutting parameters (e.g., longer fixation can change the hardness of the tissues). However, the procedure to prepare the biological materials can be the same for different LMD systems.

Acknowledgments

The LMD system upgrade was possible thanks to the financial support of the "Compagnia di San Paolo" (Torino, Italy).

References

1. Smith SE, Read D (2008) Mycorrhizal Symbiosis, 3rd edn. Academic Press, New York
2. Leake J (2004) Myco-heterotroph/epiparasitic plant interactions with ectomycorrhizal and arbuscular mycorrhizal fungi. Plant Biol 7:422–428
3. Hynson NA, Weiß M, Preiss K, Gebauer G, Treseder KK (2013) Fungal host specificity is not a bottleneck for the germination of Pyroleae species (Ericaceae) in a Bavarian forest. Mol Ecol 22:1473–1481
4. Perotto S, Rodda M, Benetti A, Sillo F, Ercole E, Rodda M, Girlanda M, Murat C, Balestrini R (2014) Gene expression in mycorrhizal orchid protocorms suggests a friendly plant–fungus relationship. Planta 239:1337–1349
5. Zhao M-M, Zhang G, Zhang D-W, Hsiao Y-Y, Guo S-X (2013) ESTs Analysis reveals putative genes involved in symbiotic seed germination in *Dendrobium officinale*. PLoS One 8:e72705
6. Liu S-S, Chen J, Li S-C, Zeng X, Meng Z-X, Guo S-X (2015) Comparative transcriptome analysis of genes involved in GA-GID1-DELLA regulatory module in symbiotic and asymbiotic seed germination of *Anoectochilus roxburghii* (Wall.) Lindl. (Orchidaceae). Int J Mol Sci 16:30,190–30,203
7. Fochi V, Chitarra W, Kohler A, Voyron S, Singan VR, Lindquist EA, Barry KW, Girlanda M, Grigoriev IV, Martin F, Balestrini R, Perotto S (2017) Fungal and plant gene expression in the *Tulasnella calospora–Serapias vomeracea* symbiosis provides clues about nitrogen pathways in orchid mycorrhizas. New Phytol 213:365–379
8. Balestrini R, Gómez-Ariza J, Lanfranco L, Bonfante P (2007) Laser Microdissection reveals that transcripts for five plant and one fungal phosphate transporter genes are contemporaneously present in arbusculated cells. Mol Plant Microbe Interact 20:1055–1062
9. Hogekamp C, Arndt D, Pareira PA, Becker JD, Hohnjec N, Küster H (2011) Laser microdissection unravels cell-type-specific transcription in arbuscular mycorrhizal roots, including CAAT-box transcription factor gene expression correlating with fungal contact and spread. Plant Physiol 157:2023–2043
10. Hogekamp C, Küster H (2013) A roadmap of cell-type specific gene expression during sequential stages of the arbuscular mycorrhiza symbiosis. BMC Genomics 14:306
11. Emmert-Buck MR, Bonner RF, Smith PD, Chauqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA (1996) Laser capture microdissection. Science 274:998–1001
12. Day RC, Grossniklaus U, Macknight RC (2005) Be more specific! Laser-assisted microdissection of plant cells. Trends Plant Sci 10:397–406
13. Nelson T, Tausta SL, Gandotra N, Liu T (2006) Laser microdissection of plant tissue: what you see is what you get. Annu Rev Plant Biol 57:181–201
14. Ramsay K, Jones MGK, Wang Z (2006) Laser capture microdissection: a novel approach to microanalysis of plant–microbe interactions. Mol Plant Pathol 7:429–435
15. Day RC, McNoe LA, Macknight RC (2007) Transcript analysis of laser microdissected plant cells. Technical focus. Physiol Plant 129:267–282
16. Balestrini R, Bonfante P (2008) Laser microdissection (LM): applications to plant materials. Plant Biosyst 142:331–336
17. Millar JL, Becker MG, Belmonte MF (2015) Laser microdissection of plant tissues. In: Yeung ECT, Stasolla C, Sumner MJ, Huang BQ (eds) Plant microtechniques and protocols. Springer International Publishing, Switzerland, pp 337–350
18. Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN (2003) A gene expression map of the *Arabidopsis* root. Science 302:1956–1960
19. Galbraith DW, Birnbaum K (2006) Global studies of cell type-specific gene expression in plants. Annu Rev Plant Biol 57:451–475
20. Asano T, Masumura T, Kusano H, Kikuchi S, Kurita A, Shimada H, Kadowaki K (2002) Construction of specialized cDNA library from plant cells isolated by laser capture microdissection: toward comprehensive analysis of the

genes expressed in the rice phloem. Plant J 32:401–408

21. Kerk NM, Ceserani T, Tausta SL, Sussex IM, Nelson TM (2003) Laser capture microdissection of cells from plant tissues. Plant Physiol 132:27–35
22. Klink VP, Alkharouf N, MacDonald M, Matthews B (2005) Laser capture microdissection (LCM) and expression analyses of *Glycine max* (soybean) syncytium containing root regions formed by the plant pathogen *Heterodera glycines* (soybean cyst nematode). Plant Mol Biol 59:965–979
23. Inada N, Wildermuth MC (2005) Novel tissue preparation method and cell-specific marker for laser microdissection of *Arabidopsis* mature leaf. Planta 221:9–16
24. Cai S, Lashbrook CC (2006) Laser capture microdissection of plant cells from tape transferred paraffin sections promotes recovery of structurally intact RNA for global gene profiling. Plant J 48:628–637
25. Tang W, Coughlan S, Crane E, Beatty M, Duvick J (2006) The application of laser microdissection to in planta gene expression profiling of the maize anthracnose stalk rot fungus *Colletotrichum graminicola*. Mol Plant Microbe Interact 19:1240–1250
26. Balestrini R, Gómez-Ariza J, Klink VP, Bonfante P (2009) Application of laser microdissection to plant pathogenic and symbiotic interaction. J Plant Interact 4:81–92
27. Gomez SK, Harrison MJ (2009) Laser microdissection and its application to analyze gene expression in arbuscular mycorrhizal symbiosis. Pest Manag Sci 65:504–511
28. Fiorilli V, Klink VP, Balestrini R (2012) Proteomic analyses of cells isolated by laser microdissection. In: Leung H-C (ed) Integrative proteomics. InTech. ISBN: 978-953-51-0070-6
29. Fang J, Schneider B (2014) Laser microdissection: a sample preparation technique for plant micrometabolic profiling. Phytochem Anal 25:307–313
30. Gautam V, Sarkar AK (2015) Laser assisted microdissection, an efficient technique to understand tissue specific gene expression patterns and functional genomics in plants. Mol Biotechnol 57:299–308
31. Nakazono M, Qiu F, Borsuk LA, Schable PS (2003) Laser capture microdissection, a tool for the global analysis of gene expression in specific plant cell types: Identification of genes expressed differentially in epidermal cells or vascular tissue of maize. Plant Cell 15:583–596
32. Klink VP, Overall CC, Alkharouf NW, MacDonald MH, Matthews BF (2007) Laser capture microdissection (LCM) and comparative microarray expression analysis of syncytial cells isolated from incompatible and compatible soybean (*Glycine max*) roots infected by the soybean cyst nematode (*Heterodera glycines*). Planta 226:1389–1409
33. Ithal N, Recknor J, Nettleton D, Hearne L, Maier T et al (2007) Parallel genome-wide expression profiling of host and pathogen during soybean cyst nematode infection of soybean. Mol Plant Microbe Interact 20:293–305
34. Hacquard S, Delaruelle C, Legué V, Tisserant E, Kohler A, Frey P, Martin F, Duplessis S (2010) Laser capture microdissection of Uredinia formed by *Melampsora larici-populina* revealed a transcriptional switch between biotrophy and sporulation. Mol Plant Microbe Interact 23:1275–1286
35. Chandran D, Inada N, Hather G, Kleindt CK, Wildermuth MC (2010) Laser microdissection of Arabidopsis cells at the powdery mildew infection site reveals site-specific processes and regulators. PNAS 107:460–465
36. Hacquard S, Tisserant E, Brun A, Legue V, Martin F, Kohler A (2013) Laser microdissection and microarray analysis of *Tuber melanosporum* ectomycorrhizas reveal functional heterogeneity between mantle and Hartig net compartments. Environ Microbiol 15:1853–1869
37. Gomez KS, Javot H, Deewatthanawong P, Torres-Jerez Y, Tang I, Blancaflor EB, Udvardi MK, Harrison MJ (2009) *Medicago truncatula* and *Glomus intraradices* gene expression in cortical cells harboring arbuscules in the arbuscular mycorrhizal symbiosis. BMC Plant Biol 9:10
38. Guether M, Neuhäuser B, Balestrini R, Dynowski M, Ludewig U, Bonfante P (2009) A mycorrhizal specific ammonium transporter from *Lotus japonicus* acquires nitrogen. Plant Physiol 150:73–83
39. Gaude N, Bortfeld S, Duensing N, Lohse M, Krajinski F (2012) Arbuscule-containing and non-colonized cortical cells of mycorrhizal roots undergo extensive and specific reprogramming during arbuscular mycorrhizal development. Plant J 69:510–528
40. Balestrini R, Nerva L, Sillo F, Girlanda M, Perotto S (2014) Plant and fungal gene expression in mycorrhizal protocorms of the orchid *Serapias vomeracea* colonized by *Tulasnella calospora*. Plant Signal Behav 9:11
41. Gómez-Ariza J, Balestrini R, Novero M, Bonfante P (2009) Cell-specific gene expression of phosphate transporters in mycorrhizal tomato roots. Biol Fertil Soils 45:845–853

42. Stasolla C, Yeung EC (2015) Paraffin and polyester waxes. In: Yeung ECT, Stasolla C, Sumner MJ, Huang BQ (eds) Plant microtechniques and protocols. Springer, New York, pp 45–66

43. Fochi V, Falla N, Girlanda M, Perotto S, Balestrini R (2017) Cell-Specific Expression of Plant Nutrient Transporter Genes in Orchid Mycorrhizae. Plant Sci 263, 39–45

Part V

Orchid Propagation and Maintenance

Chapter 22

Vegetative Propagation of Orchids

Yung-I Lee

Abstract

The conventional methods of vegetative propagation, such as division and cutting, provide simple ways for multiplication and maintenance of selected individuals with desirable traits for commercial applications, experimental studies, and germplasm conservation. Orchids are a diverse group of flowering plants which have different growth habits, i.e., sympodial and monopodial growths, and they are able to survive in a wide range of natural environments as terrestrial, epiphytic, and lithophytic forms. These features play important roles in the propagation and cultivation of different orchids. Due to the varied forms of orchids, it is not possible to demonstrate all the details of vegetative propagation methods in a chapter. Here we introduce the vegetative propagation methods of division, cutting, and keikis growing of sympodial and monopodial orchids. In addition, guidelines for the care of stocks before propagation and the propagules after operation are provided.

Key words Monopodial orchids, Sympodial orchids, Pseudobulb, Division, Cutting, Keiki, Potting mixtures, Vegetative propagation, Propagule

1 Introduction

The mass clonal propagation by tissue culture can generate numerous plantlets. Once individuals with desirable traits are identified, they need to be propagated and maintained. Although continual use of micropropagation methods guarantees further propagation of selected genotypes, regenerants may not necessary be true to type because of possible occurrence of mutations in the tissue during the micropropagation process. For the maintenance of valuable mother plants, multiplication by conventional methods of vegetative propagation, e.g., division and cutting, is perhaps the simplest way to ensure the maintenance of desirable stocks for future experimental studies and commercial applications.

Orchid family (Orchidaceae) comprises approximately 800 genera and at least 24,000 species that is one of the largest plant families [1]. In orchids, two major growth habits, i.e., sympodial growth and monopodial growth, can be observed. Besides,

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_22,

different life forms, including terrestrial, epiphytic, and lithophytic, exist in orchids according to their ecological niche in nature. These features play important roles in propagation and cultivation of different orchids. In the horticultural industry, the orchids, e.g., cattleyas, cymbidiums, dendrobiums, oncidiums, paphiopedilums, and phalaenopsis, have become very popular and important floricultural crops during the past few decades. Methods and procedures for propagation of plantlets and mature plants are readily available in the literatures [2, 3] and in websites of societies, such as the American Orchid Society (http://www.aos.org) and the Royal Horticultural Society (https://www.rhs.org.uk).

Different orchid genera or species may have their unique requirements for propagation and cultivation. It is not easy to manage the cultivation of them without any mistakes. In this chapter, we provide a brief overview and protocols on the conventional methods of vegetative propagation, including division and cutting for orchids of horticultural importance. Furthermore, the preparation of plants prior to vegetative propagation and the care of propagules after propagation are crucial to the success of the operation. We hope the guidelines and notes will provide practical information to researchers to minimize mistakes in vegetative propagation and cultivation of orchids.

1.1 Growth Habits and Life Forms

There are two primary growth habits of orchids, i.e., sympodial and monopodial forms [4]. The method of propagating mature plants of orchids depends on their growth habits. This relates to the location and the renewal ability of the axillary buds. In plants, every growing branch has an apical shoot meristem. An apical meristem is responsible for the formation of all the primary tissues of a plant. As leaves are initiated at the shoot apical meristem, axillary bud primordia are formed in the axil of leaves. Each axillary bud has its own apical meristem, responsible for the growth of the bud. Although axillary buds are present, they usually remain dormant. However, if excised from the mother plant, or under appropriate conditions, the axillary buds can be activated and give rise to new shoots (the vegetative growth) or inflorescences (the reproductive growth). The presence of axillary buds along the stem will determine how the plant should be divided.

Ecologically, different orchids can occupy different habitats and can be classified as terrestrial, epiphytic, and lithophytic forms [5]. The majority epiphytic and some lithophytic orchids have succulent leaves and possess crassulacean acid metabolism (CAM) that can help in adapting to water stress conditions, while most terrestrial orchids have thin leaves and possess C_3 metabolism that is less tolerant to water stress [6, 7]. The unique growth habits, adaptations, and physiological characters influence cultural practices after repotting the orchids.

1.2 Sympodial Orchids

For sympodial orchids, e.g., *Bulbophyllum*, *Cattleya*, *Cymbidium*, *Dendrobium*, and *Oncidium*, there are multiple shoots growing along a rhizome (Fig. 1). Each shoot usually has limited growth potential, having few internodes. A new shoot may arise from a rhizome where there is an axillary bud (usually from the base of a leading shoot or from any part of an older shoot). Hence individual shoots can be separated from one another, generating more individuals.

A number of sympodial orchids have thickened stem structures, called "pseudobulbs"(neither a bulb nor a tuber) (Fig. 2). A pseudobulb is composed of a single or several thickened internodes, while a bulb is structurally a short stem with fleshy leaf bases, referring only to the modified stem structure like that of the tulip or the onion. Among orchid species, pseudobulbs may be quite different in appearance and are known as "bulbs" or "canes." A pseudobulb, comprising of a lot of parenchyma cells, is an adaptive structure that can store nutrients and water for surviving the drought period and for the support of newly developing shoots. In addition to the storage function, pseudobulbs are important materials for vegetative propagation in horticultural operations, such as division and cutting [8].

In cattleyas, a pseudobulb with a section of rhizome is the basic unit for division. In greenhouse cultivation, it is very unusual to see the occurrence of adventitious shoots in orchids. Hence, the division unit should possess at least one "live eye" (i.e., a live dormant bud) along the rhizome to make sure that there will be a new shoot for continual growth [2, 3]. In dendrobiums, the pseudobulb is called a

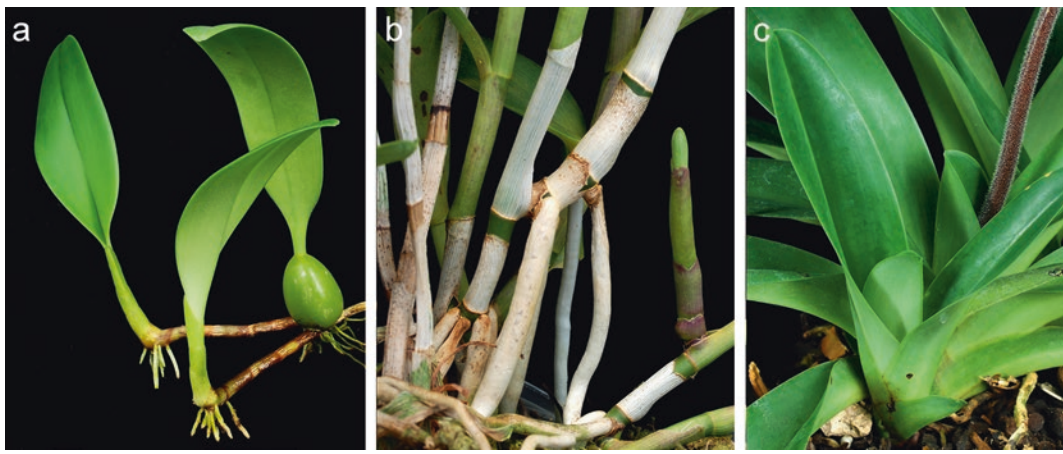


Fig. 1 Sympodial orchids. (**a**) *Bulbophyllum lasiochilum*. (**b**) *Epidendrum radicans* (the reed-stem *Epidendrum*). (**c**) *Paphiopedilum* hybrid

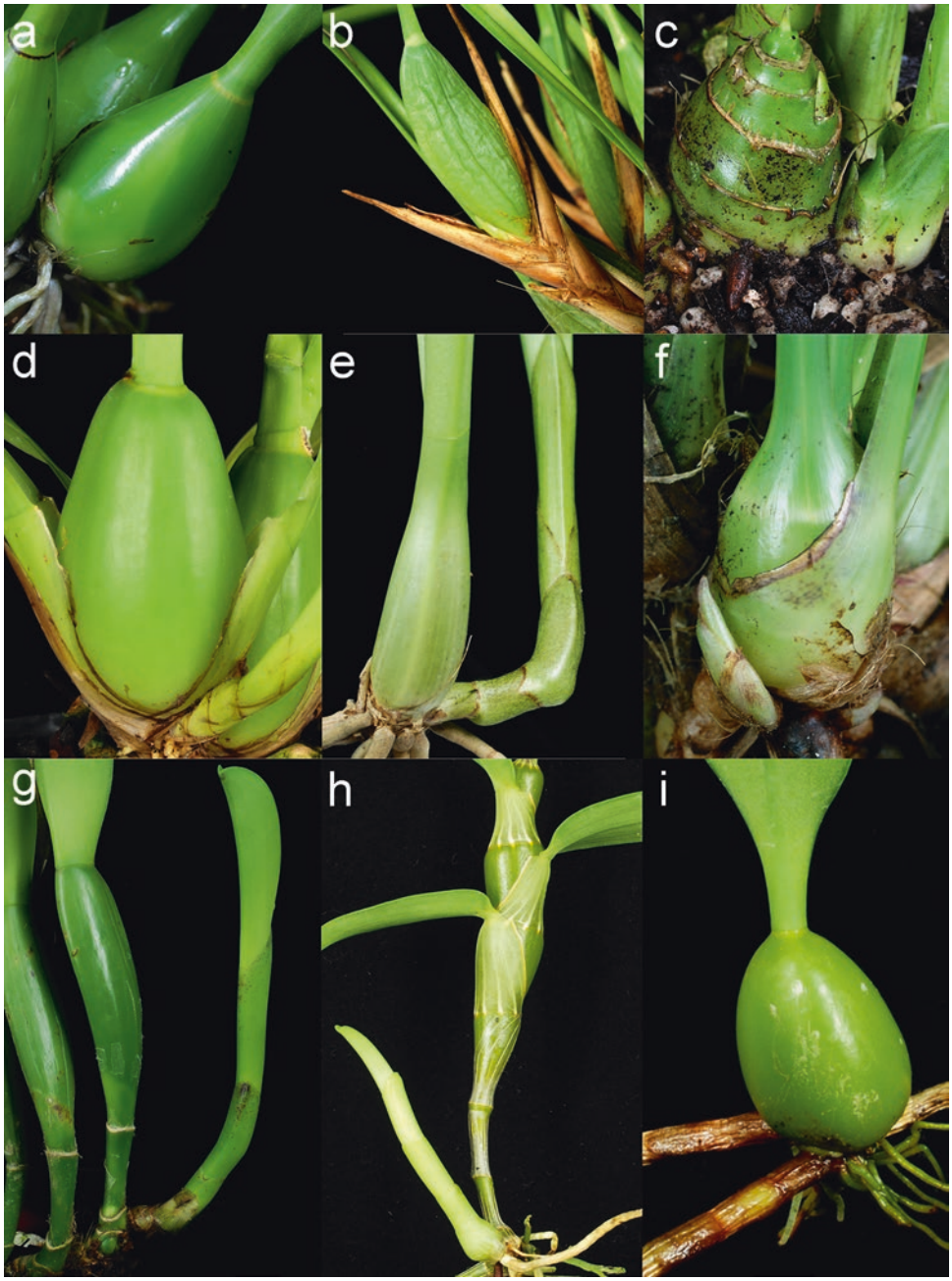


Fig. 2 Different types of pseudobulbs. (**a**) *Encyclia alata*. (**b**) *Maxillariella tenuifolia*. (**c**) *Phaius tankervilleae*. (**d**) *Oncidium sphacelatum*. (**e**) *Eria hyacinthoides*. (**f**) *Cymbidium ensifolium*. (**g**) *Cattleya purpurata*. (**h**) *Dendrobium nobile*. (**i**) *Bulbophyllum lasiochilum*

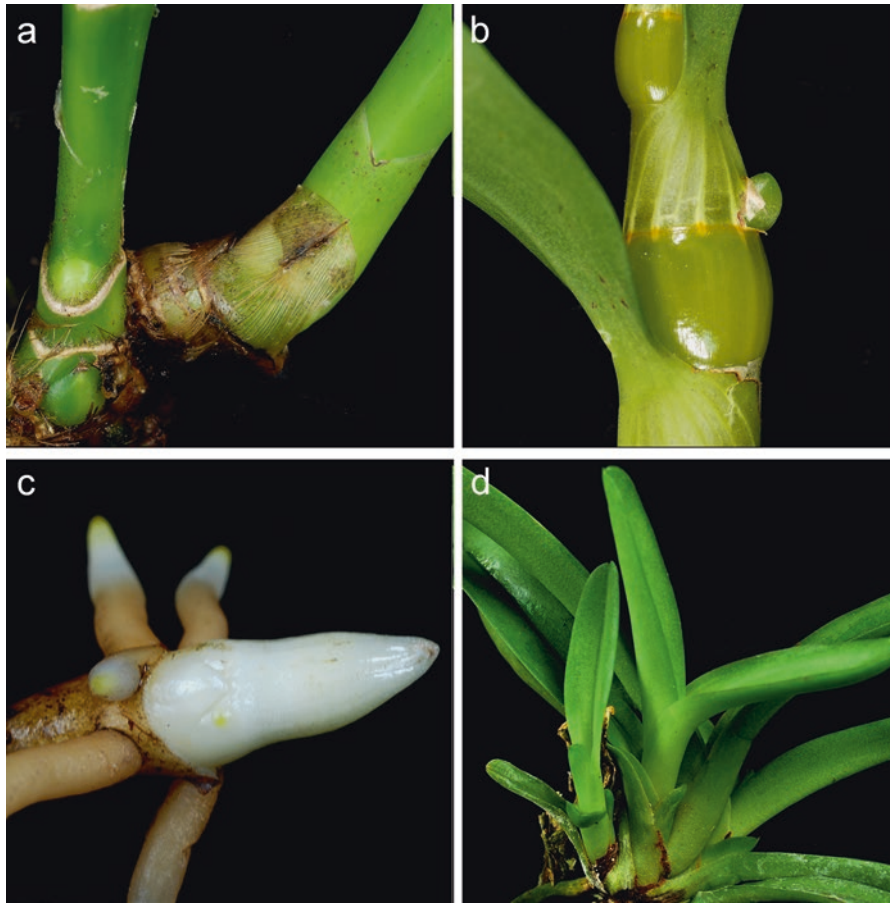


Fig. 3 The presence of axillary buds in different orchids. (**a**) *Cattleya purpurata*. (**b**) *Dendrobium nobile*. (**c**) *Cypripedium formosanum*. (**d**) *Vanda coerulescens*

"cane" and is composed of several nodes. There are dormant buds at the nodes along the mature pseudobulb, and a section of pseudobulb with at least one live dormant bud can be used for cutting. The production of plantlets from cutting is an easy and practical way in the industrial-scale multiplication of the nobile-type *Dendrobium*. In other *Dendrobium* species, such as *D. aphyllum*, *D. anosmum*, and the antelope-type dendrobiums, a few new shoots will come up if the cane is cut and fastened horizontally to a bed filled with moistened sphagnum moss or other potting media.

Slipper orchids, e.g., *Cypripedium*, *Paphiopedilum*, and *Phragmipedium*, are sympodial orchids without pseudobulbs (Fig. 1c). For nutrient storage, roots as well as thick leaves, e.g., *Paphiopedilum*, or rhizomes, e.g., *Cypripedium* (Fig. 3c), are important storage organs. *Paphiopedilum* and *Phragmipedium* are evergreen species native to subtropical and tropical areas, and, like

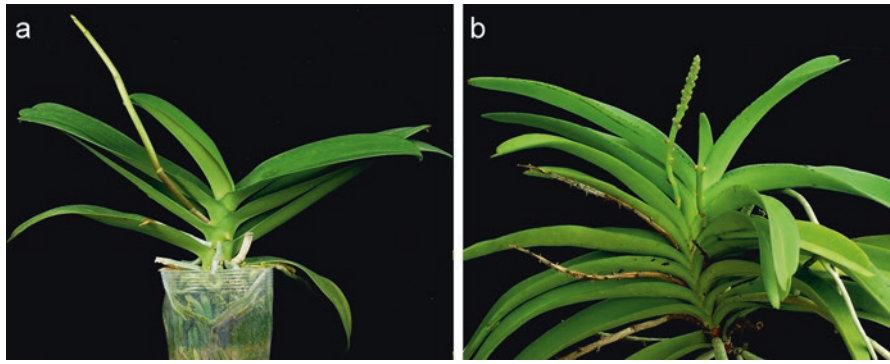


Fig. 4 Monopodial orchids. (**a**) *Phalaenopsis* hybrid. (**b**) *Rhynchostylis coelestis*

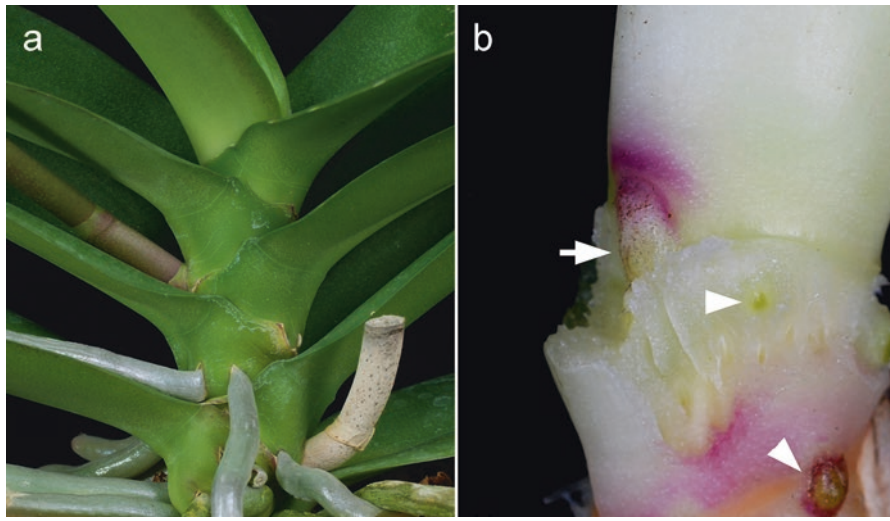


Fig. 5 The presence of axillary buds in a phalaenopsis hybrid. (**a**) Monopodial orchids, such as phalaenopsis, have a short and compressed stem surrounding by succulent leaves. Each leaf is accomplished with two axillary buds. (**b**) After removing leaves, an axillary dormant bud (arrow) and primordial roots (arrowheads) are visible

Phalaenopsis, their compressed stems are surrounded by leaves. For division, one to three shoots with short rhizome sections and healthy roots could be separated from the mother plants in spring. For *Cypripedium* species, since these are deciduous species that grow in temperate areas, for division, rhizome sections with a few buds could be cut from the mother plants in the dormant period.

1.3 Monopodial Orchids

Monopodial orchids, e.g., *Phalaenopsis*, *Rhynchostylis*, and *Vanda*, have a single main stem and the shoot apex has the potential for unlimited growth (Fig. 4a, b). In *Phalaenopsis*, the shoot apical meristem is deeply embedded in the terminal shoot, and the axillary bud develops in the axil of a leaf (Fig. 5). As a leaf matured,

axillary bud becomes dormant (Fig. 5b). Under the cool condition (about 20 °C), the dormant axillary bud will be activated and develop into a floral stem (Fig. 5a).

Pseudobulb is absent from orchids having a monopodial growth habit; instead, succulent leaves or roots are formed serving as storage organs. Since it has a single growing axis, usually it cannot be divided as for the sympodial orchids. Moreover, similar to the sympodial orchids, additional branches may arise from axillary buds on the main stem as the main terminal shoot apex becomes weak or after being damaged (Fig. 3d), and therefore, propagation by cutting is possible due to the presence of shoots from axillary buds.

1.4 Keikis

Some orchids, such as *Arundina*, *Dendrobium*, *Phalaenopsis*, and the reed-stem *Epidendrum*, are able to spontaneously produce keikis, i.e., offshoots from the nodes along the pseudobulbs or floral stems (Fig. 6). In the natural environment, the multiplication by offshoots is an important strategy for the survival and expanding

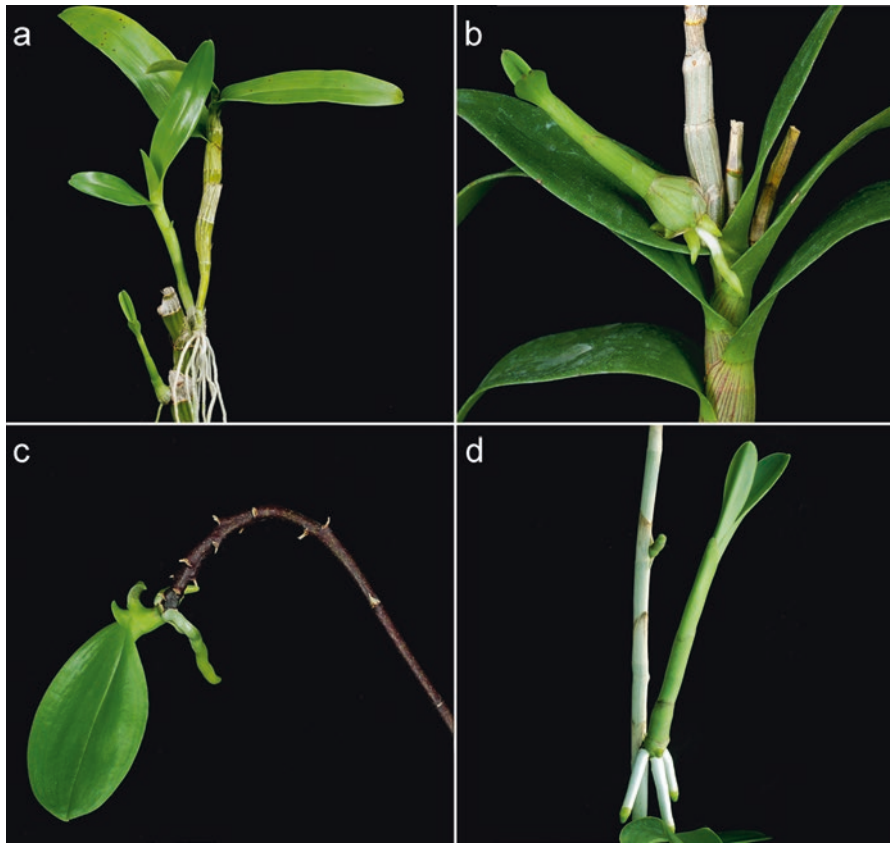


Fig. 6 Keikis growing from different orchids. (**a**) The nobile-type *Dendrobium*. (**b**) The phalaenopsis-type *Dendrobium*. (**c**) *Epidendrum radicans* (the reed-stem *Epidendrum*). (**d**) *Phalaenopsis equestris*

populations of some orchids asexually without pollinators and seed production. As compared to the sexual propagation by seeds, the vegetative propagules are larger in size, and they could have a greater potential than seeds for successful colonization and establishment because of higher photosynthetic ability and nutrients consumption [9]. Both *Arundina graminifolia* (common in tropical Asia) and *Epidendrum radicans* (common in tropical America, Fig. 6d) have reedy stems which produce a number of keikis and form large growing clumps in the field.

Some *Phalaenopsis* species, e.g., *P. equestris*, prefer to produce keikis at the tip of inflorescence near the end of blooming (Fig. 6c). Since the inflorescence of *Phalaenopsis* is indefinite, under the cooler environment, the flowers differentiate successively along the extending inflorescence. But in the warmer environment, the inflorescence stops growing, and its tip transforms from reproductive state to vegetative state. Besides, in *Phalaenopsis*, nodes with dormant buds along the floral stems also have potential to produce keikis. Production of keikis can be encouraged by application of plant growth regulator mixtures, such as keiki paste to the nodes of floral stem.

2 General Plant Maintenance Prior to Propagation

The use of a health plant (vigorous and disease-free) as the propagation stock is a good start of vegetative propagation. The fundamental consideration in growing any orchids is to know their natural habitats, including the elevation, seasonal changes in temperature, rainfall amounts, and light intensity. In addition, it is important to know whether they grow in soils, on trees, or on rocks. A better understanding of the local climates would provide an important appreciation of different and critical demanding cultural requirements of various species.

2.1 Temperature

The natural habitats of different orchids can differ widely, and different orchids have a temperature range in which they can grow. Below or above the temperature range, at the extreme temperatures, the plants will soon become weak. Orchids can be generally grouped into three different temperature categories, i.e., cool-, intermediate-, or warm-growing, depending on their temperature requirements. The common definitions of these groups are the following: warm growers need approximately 20–30 °C during the day and 20–25 °C at night; intermediate growers need approximately 15–20 °C during the day and 10–15 °C at night; and cool growers need daytime temperatures approximately 10–15 °C days and 5–10 °C at night. The representative cool-growing orchid genera are *Masdevallia*, *Miltoniopsis*, and *Odontoglossum*. The intermediate-growing orchids include *Oncidium*, *Paphiopedilum*,

and *Phragmipedium*. The warm-growing orchids include *Cattleya*, *Phalaenopsis*, *Vanda*, and some *Dendrobium* sections, such as phalaenopsis and antelope types. In horticultural markets, the popular orchid genera, such as *Cattleya*, *Cymbidium*, *Dendrobium*, *Oncidium*, and *Phalaenopsis*, are native to tropical forests, and they are considered intermediate to warm growers. Generally, they grow well between 20 and 30 °C during daytime. A 10–15 °C day/night differential is favorable to the growth of most of them (*see* **Note 1**).

2.2 Humidity

Most orchids would grow very well in the range between 60 and 80% humidity with good air circulation. Some species native to cloudy forests prefer a little higher humidity around 90%. Nonetheless, a high humidity in combination with poor air circulation and high temperatures will increase the risk of bacterial diseases. If the relative humidity is too high, increasing ventilation or installing a dehumidifier is necessary. On the contrary, if the relative humidity is too low, especially in combination with the higher temperature, it is required to install a high-pressure humidification system, a humidifier, sprinkler lines, or pad/fan systems, etc. to increase the humidity without wetting the leaves (*see* **Note 2**).

2.3 Light Intensity

Orchids rely on light to generate food from photosynthesis and to regulate their growth and development. Different orchids can thrive and flower under a wide range of light intensity. Meadow orchids, such as *Anacamptis*, *Dactylorhiza*, *Orchis*, and *Spiranthes*, like to grow under full sunlight, while those in the forest prefer different levels of shading. In addition, the requirement of light intensity also changes depending on the stages of development. Hence, knowing the natural growth habits of orchids is essential to the successful cultivation of orchids.

Orchids can be generally grouped by the amount of light requirements in order to grow well, i.e., the low-, medium-, and high-light orchids. The "low-light" orchids, such as Chinese cymbidiums (section *Jenosa*), the mottled-leaf paphiopedilums (section *Barbata*), and the complex-type paphiopedilums, require low light intensity which is generally under 1500 (foot-candle, abbreviated fc). The "medium-light" orchids, such as cattleyas, the standard-type cymbidiums, dendrobiums, oncidiums, and phalaenopsis, typically do well in light levels of 2000–4000 fc. Although oncidiums, phragmipediums, the strap-leaf paphiopedilums, and phalaenopsis are "medium-light" orchids, they prefer the lower end of the medium light intensity (2000 fc), while the cattleyas, the standard-type cymbidiums, and dendrobiums prefer the higher end of medium light intensity (4000 fc). The "high-light" orchids, such as *Papilionanthe teres* (previously known as *Vanda teres*) and the meadow orchids, love bright light above 4000–6000 fc or even full sunlight.

In summer, the outdoor light intensity of full sun on a clear day may reach 10,000 fc. Since most orchids cannot thrive in full sunlight, shading a greenhouse by screens or whitewashing is essential for orchid growing. In general, cattleyas, standard-type cymbidiums, and dendrobiums can take 30–50% shade to almost full sun. Oncidiums, the strap-leaf paphiopedilums, and phalaenopsis need 70% shading all day or full morning sun. Chinese cymbidiums, the mottled-leaf paphiopedilums, and most young seedlings require up to 80% shading (*see* **Note 3**).

During the winter, especially in the temperate region, light intensity and day length are below optimum for orchid growing in the temperate region. Using supplemental lighting in the greenhouse is essential to increase the photosynthetic rate of orchids. Fluorescent lamps and high-intensity-discharge (HID) lamps are commonly used in growing rooms and greenhouses. Recently, light-emitting diodes (LED), which are small in size and long life with very little heat generated, are popular in greenhouse lighting.

2.4 Watering

Watering management is important in growing orchids. As the proverb says, "if you can master watering, you can grow anything." The frequency of watering cannot be solely determined by growth habits or climates. Several factors, such as temperatures, light conditions, humidity, potting media, pot sizes, and plant growth conditions, can affect watering management (*see* **Note 4**).

For epiphytic orchids, especially those with pseudobulbs, they are air lovers and may withstand a slight dryness of their potting media; for terrestrial orchids, they usually prefer keeping the potting media slightly moistened at all times.

Conditions of high temperature, high light intensity, and low relative humidity increase water evaporation and plant transpiration; watering frequency needs to be increased. Conversely, in conditions of low temperature, low light intensity, and high relative humidity, watering frequency needs to be reduced. Besides, the ability of water retention by the potting media also influences the watering frequency. Coarse particles, e.g., bark, tree fern, charcoal, and diatomite, cannot retain water, requiring more frequent watering. Conversely, sphagnum moss, peat moss, and fine particles can retain more water; hence, watering frequency can be reduced. Moreover, the pot size and pot drainage can also play a role in watering frequency. Larger pots with less drainage holes require watering less frequently, whereas smaller pots and more drainage holes require more often watering.

The quality of water is crucial for growing orchids. It must be free of chemicals and visible contaminants. A good-quality water for irrigation should not contain an amount of elements such as sodium and chlorine in excess of 100 mg/L and not to contain an excessive amount of bicarbonate. Rainwater is a good source

for irrigation. In the absence of good-quality water supplies, it is necessary to make use of reverse-osmosis water [10, 11].

2.5 Fertilizing

Fertilizers contain elements that are essential to plant growth, such as nitrogen, phosphorus, potassium, calcium, sulfur, etc. For orchid growing, blended fertilizers with a fixed ratio of nitrogen–potassium–phosphate ($N–P_2O_5–K_2O$) can be used, and they are easily obtained from suppliers, such as Peters Professional fertilizers (The Scotts Company, Ohio), HYPONex® (Hyponex Japan Corp., Osaka, Japan), Orchid-Pro® (Dyna-Gro, California), etc. For phalaenopsis growing, a 20–20–20 fertilizer is commonly used from young to mature stages (*see* **Note 5**).

Recommended concentrations of fertilizer differ depending on the orchids, developmental stages, and the potting medium used. In general, it is acceptable to feed your orchids by adding 1 g/L of a 20–20–20 fertilizer (*see* **Note 6**), giving an electrical conductivity (EC) value near 1 mS/cm of the fertilizing solution (*see* **Note 7**). Feed the orchids frequently (every watering) when they are actively growing in spring and summer and leech out with plain water once every 4 weeks. You may feed the orchids less frequently in winter. Besides, controlled-release fertilizers, such as Osmocote® (The Scotts Company, Ohio) or Multicote® (Haifa Chemicals Ltd., Haifa, Israel), can be applied if fertilizer application with watering is not possible. This type of fertilizer will be released slowly and continuously over a few months.

3 Materials Needed for Plant Propagation

3.1 Potting Media

Orchids are versatile and can be grown in a variety of potting media [12]. Richter [12] wrote, "the great adaptability and vitality of orchids is evident in their ability to show excellent growth when grown in any number of different composts." Although different potting media can be used for growing orchids successfully, various media have different physical/chemical properties, such as retention of water and salts, pH value, and rate of decomposition. Sheehan [13] wrote a historical review on the evolution of potting mixes in growing orchid. The general requirements of a good potting mix include (1) durability (maintain its physical structure for at least 2 years), (2) moisture-holding capacity, (3) drainage and aeration, and (4) ability to supply some nutrients, (5) promote ionic exchange between the water and roots, and (6) deter the development of fungi and microbes. Finally, the availability and cost of the material are also important. The fresh, quick-draining but moisture-retentive media/mixes are essential to the healthy roots. The healthy root system is fundamental for cultivation of good-quality orchids. A variety of potting media are briefly described below.

Various potting media can be used for orchid growing, including barks from Douglas fir (Rexius, Eugene, Oregon) or *Pinus radiata* (Besgrow, Christchurch, New Zealand) (*see* **Note 8**); the sphagnum moss can be obtained from different areas, such as New Zealand (Besgrow, Christchurch, New Zealand), Chile (Commercial Mar Andino Ltda., Santiago, Chile), China (Hsien Chinis Trading Co., Ltd., Pingtung, Taiwan; Shine Ten Ti International Co., Ltd., Taichung, Taiwan), or any other brands/manufacturers with the same material; the peat can come from different areas or companies (such as BVB Substrate, Netherlands), and coconut husk chips or coirs; diatomite, pumice and aliflor can obtained from companies (such as rePotme.com, Georgetown, DE) (diatomite, pumice, and aliflor). It is also recommended to prepare a mixture that combines different ratios of materials, such as bark, peat, sphagnum moss, coconut husk, and perlite. The potting mixes are available from different companies, such as Infini-Mix (A&P Orchids, MA, USA), Debco Premium Orchid Mix (Debco Pty Ltd., Victoria, Australia), and Miracle-Gro Orchid Potting Mix (The Scotts Miracle-Gro Company, OH, USA).

For orchids growing in a greenhouse, using the same potting media is the simple way to handle the watering and fertilizing frequency. Nevertheless, it is necessary to consider different types of roots, e.g., fine-/thick-rooted species and terrestrial/epiphytic species, when choosing the potting media. Usually, the fine-rooted orchids, e.g., *Oncidium* and *Pleurothallis*, prefer small particle-sized mixes, while the thick-rooted orchids, e.g., *Phalaenopsis* and *Vanda*, prefer coarse to medium particle-sized mixes. For terrestrial orchids, they require much more moisture around their roots, while epiphytic orchids require good drainage and plenty of aeration around their roots.

3.1.1 Bark

Conifer barks from Douglas fir and pine trees are common potting media for orchid growing, and it provides some nutrients and root aeration for about 2 years. The finer the particles, the faster the nutrients are released [14] (*see* **Note 9**).

3.1.2 Peat

Different types of peats are available, such as peat moss (derived from sphagnum moss and hypnum moss), reed-sedge peat (derived from reeds, sedges, and marsh grasses), and peat humus (derived from highly decomposed peat moss and reed-sedge peat). Peat moss has a high water-holding capacity, and it has long been a recommended ingredient of potting mixes, especially for growing terrestrial orchids (*see* **Note 10**). But peat moss is naturally acid, and thus it requires the addition of lime for adjusting its low pH level before use (*see* **Note 11**).

3.1.3 Sphagnum Moss

It is versatile for growing most orchids and has been used extensively for planting deflasked orchid seedlings and for propagation of back bulbs. It has a low pH level of 3–4 and lasts for about 1 year. It can be used on its own as a potting medium (very common in Japan and Taiwan) or used as an ingredient to potting mixes. The common resources of sphagnum moss are from Chile, mainland China, and New Zealand [14] (*see* **Note 12**).

3.1.4 Coconut Husk Chip or Coir

They are ideal potting media for growing epiphytic orchids. They can keep moisture and nutrients, be slow to decompose, and last for at least 2 years (*see* **Note 13**).

3.1.5 Charcoal

It is produced from burnt wood or bamboo stems. It can absorb some chemicals from the breakdown of organic materials and does not decay. It is a common additive to potting mixes.

3.1.6 Diatomite and Pumice

They are porous, lightweight, and stable and thus provide good water retention and aeration and great support for orchid growing. They can be used as a sole orchid medium or used as an ingredient to potting mixes, especially for growing terrestrial orchids (*see* **Note 14**).

3.1.7 Aliflor

It is a porous expanded clay product that can be used on its own as a potting medium or used as an ingredient to potting mixes. It has a neutral pH value and is very long lasting.

3.1.8 Perlite

It is light and sterile and chemical inert, providing a good substitution for sands in maintaining good aeration in potting mixes (*see* **Note 15**). Since it is synthetic and inorganic, perlite does not easily break down.

3.1.9 Other Synthetic Materials

Besides the more common potting materials indicated above, other synthetic materials are commercially available such as Oasis blocks, rockwool, polystyrene foam, polyurethane, and polyether clumps. These materials are lightweight with good aeration properties that are used as ingredients in potting mixes. Recently, the shaped potting mixes, using coconut coirs and peat, etc., for orchid commercial productions are popular in the Netherlands, e.g., Fibre-Neth® plugs, Quick Plug®, and Jiffy®.

3.2 Pots

Anything that holds plants and potting media can be used as a pot for growing orchids, such as plastic pots, terracotta pots and baskets, etc. (*see* **Note 16**). Different sizes of pots are available from garden centers (*see* **Note 17**). Transparent plastic pots are popular in growing phalaenopsis and other epiphytic orchids because one can easily see what is going on with the roots and moisture content. It is important to note that growing orchids in transparent

plastic pots, roots of epiphytic orchids are capable in engaging in photosynthesis. Terracotta-tinted plastic pots are popular for the cultivation of terrestrial orchids (*see* **Note 18**).

3.3 Tools

The following is a list of tools that are useful for plant propagation use, e.g., knives, garden scissors (e.g., GARDENA®, Ulm, Germany, and Chikamasa Co., Ltd. Osaka, Japan), forceps, soap (brands such as Dove, DHC Pure Soap, or any other brands), iron wire, plant labels (e.g., Norwood Industries P/L, Victoria, Australia), permanent marker (Pentel Co., Ltd., Tokyo, Japan), and burner.

3.4 Chemicals

Pesticides, such as Mancozeb (DuPont, Wilmington, DE, USA), Physan 20® (Maril Products, Inc., Tustin, CA), or any other brand with the same effect, can be useful as disinfectants for a variety of microorganisms and algae (*see* **Note 19**). The commercial bleach solutions, e.g., Domestos® (Unilever, UK) and Clorox® (The Clorox Company, Canada) or any other brand containing approximately 5–10% of available chlorine, are good for general disinfection (*see* **Note 20**).

4 Methods

4.1 Dividing Mature Plants

Once a plant has outgrown its pot, it will need to be divided. The ideal timing to divide a plant is just before the leading pseudobulb begins forming new roots of the season (*see* **Note 21**). The process of division involves splitting sections of the rhizome of a plant with multiple pseudobulbs into two or more parts. Consequently, each new section of rhizome contains at least one pseudobulb with its own set of roots could be a new individual plant. The new growth arisen from the joints of the rhizome will develop into the leading pseudobulb. Sympodial orchids with pseudobulbs, e.g., cattleyas (Fig. 7), dendrobiums, oncidiums, and cymbidiums (Fig. 8), can be easily propagated by division (*see* **Note 22**).

1. Immerse the plant with its pot into water for 30 min for removing pot easily.
2. Gently remove the potting media using a forceps (*see* **Note 23**).
3. Carefully wash the leaves and pseudobulbs with a soap solution for expelling insects, e.g., scales, aphids, thrips, and mites (*see* **Note 24**).
4. Rinse the plant with tap water.
5. Trim off the dead roots, rotting old pseudobulbs, and old leaves.
6. Divide the plant by cutting the rhizome into different divisions (*see* **Note 25**), allowing for a lead division with at least three pseudobulbs (Fig. 9). Removing the front part will stimulate sprouting of dormant buds on the back bulb (*see* **Note 26**).
7. Apply fungicides on the wounds and place the divisions in the shade to dry for 3 h.

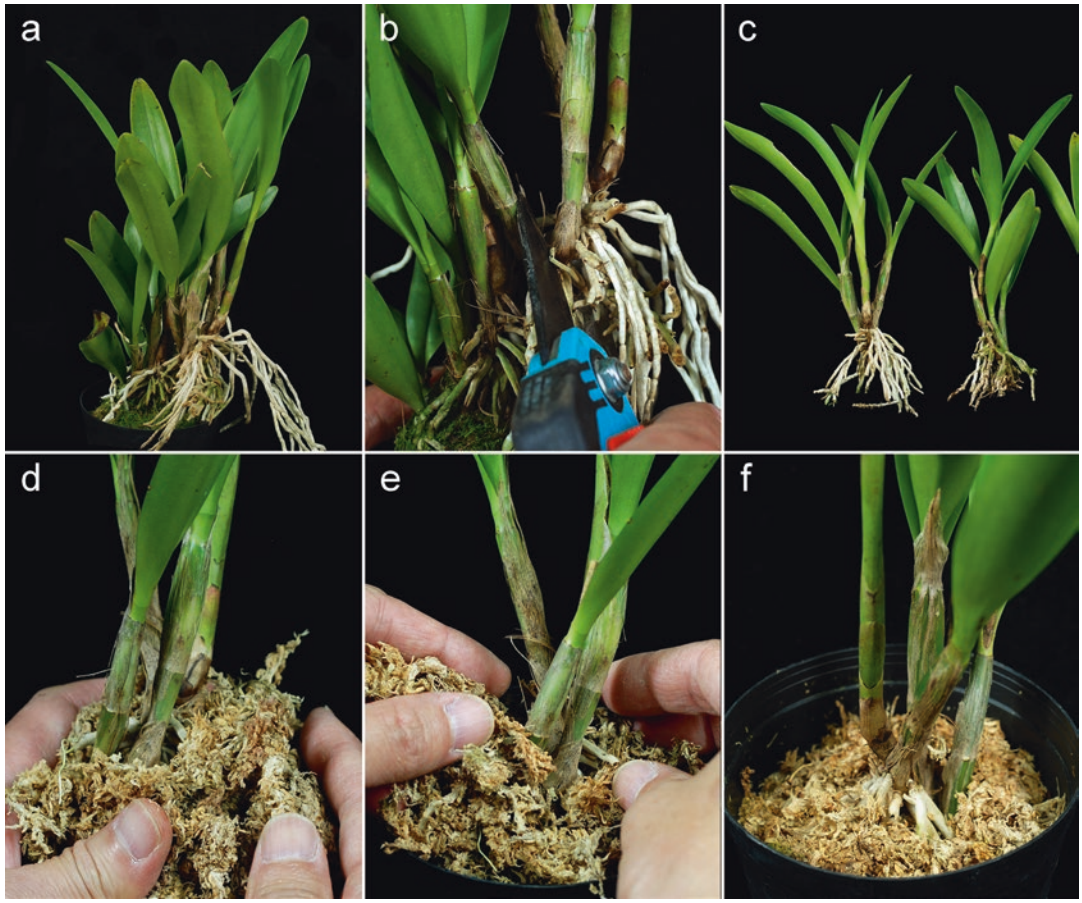


Fig. 7 The process of division for cattleyas. (**a**) A compact plant with a few leading growths. (**b**) After removing the old potting materials, this plant could be divided into two to three parts. (**c**) After trimming the roots, the divisions are ready for potting. (**d**) Hold the division and wrap the roots with fresh sphagnum moss. (**e**) Fill in the spaces around the roots with more sphagnum moss with your hands and ensure the sphagnum moss is pressed down tightly around the perimeter of the pot. (**f**) Using sphagnum moss as the potting medium, it is simple to make the division lifted by its stem. If the division has not many roots, it will make the division to be unstable and hurt the new root tips

8. Place the plant in its new pot with the older growth toward the edge of the pot. This allows more space for the growing of the new leading sprout.
9. Scoop the potting media into the pot, and shake the pot to help the potting material to settle tightly around the roots (*see* **Note 27**).
10. Insert a stick into the pot to support the newly planted pseudobulbs, or tie the pseudobulbs to the hanger of the pot.
11. Clearly mark the name of the clone and the date of division on the label.

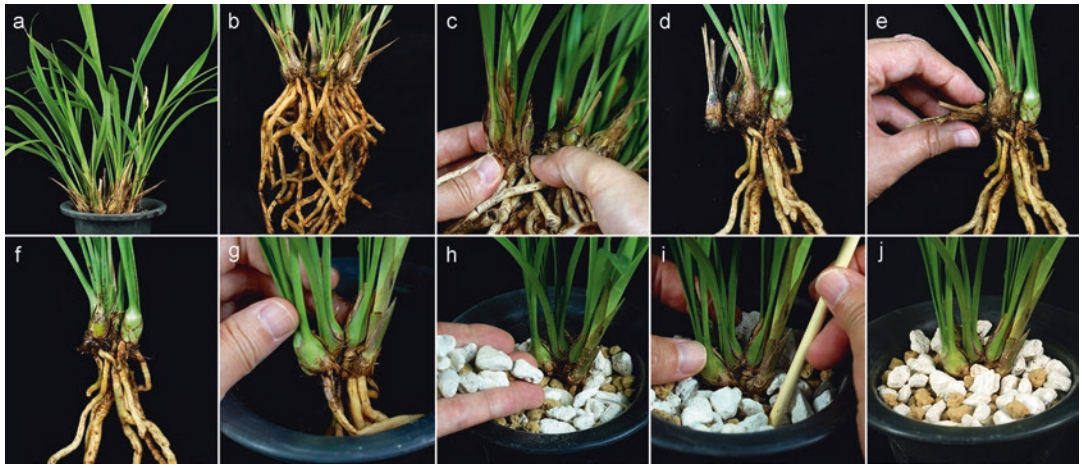


Fig. 8 The process of division for Chinese cymbidiums. (**a**) A plant with several leading growths. (**b**) Remove the old potting materials and trim the dead/weak roots. (**c**) Twist in the basal part of pseudobulb by hands to make a division of at least three pseudobulbs. (**d**) A division with old and dead back bulbs. (**e**) Remove the old and dead back bulbs. (**f**) After trimming the decayed roots, a division with three pseudobulbs and one new growing shoot is ready for potting. (**g**) Hold the division at the proper level in the pot. (**h**) Fill in the spaces around the roots with fresh potting media to hold the division at the desired level and position in the pot. (**i**) Gently firm the potting media with a stalk to ensure the potting media are pressed down tightly. (**j**) Loose potting media will cause the division to be unstable

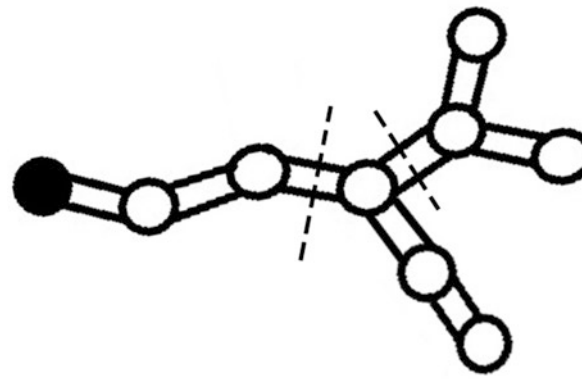


Fig. 9 The diagram demonstrating how to divide sympodial orchids. The open circle represents young pseudobulbs and the black circle represents the oldest pseudobulb. The dotted lines represent the cutting sites

4.2 Cuttings

Some pseudobulbs, e.g., dendrobium canes (Fig. 10) or vanilla vines (Fig. 11), with several nodes can be cut into sections and placed in the potting media for sprouting from the dormant nodes. The flower stems of *Phaius* with dormant nodes can be also cut similarly to produce new plantlets.

1. Cut a stem and divide it into sections that retain at least one dormant bud "live eye" (*see* **Note 28**).
2. Coat the cut ends of the sections with powdered fungicides and place the cuttings in the shade to dry for 3 h (*see* **Note 29**).

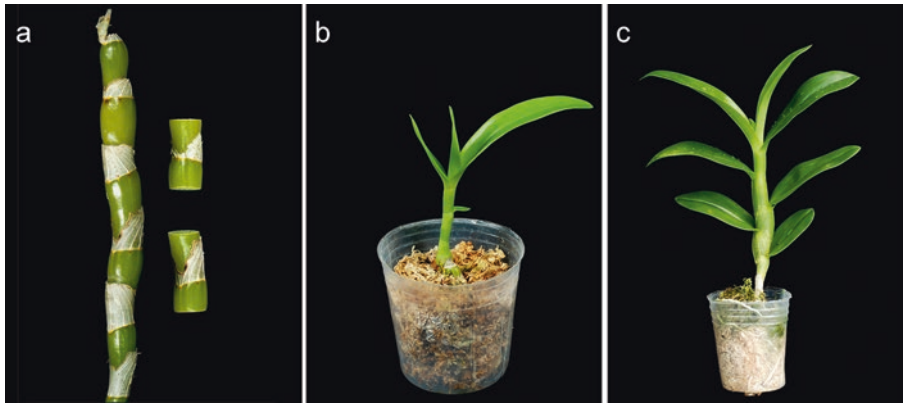


Fig. 10 The process of cutting for the nobile-type dendrobiums. (**a**) Cut a stem on into several 3–4-cm sections using a clean and sharp knife, and make sure each segment has at least one dormant bud. (**b**) The section wrapped by sphagnum moss has sprouted. (**c**) After about 6 months of culture, the new growth has matured with plump internodes

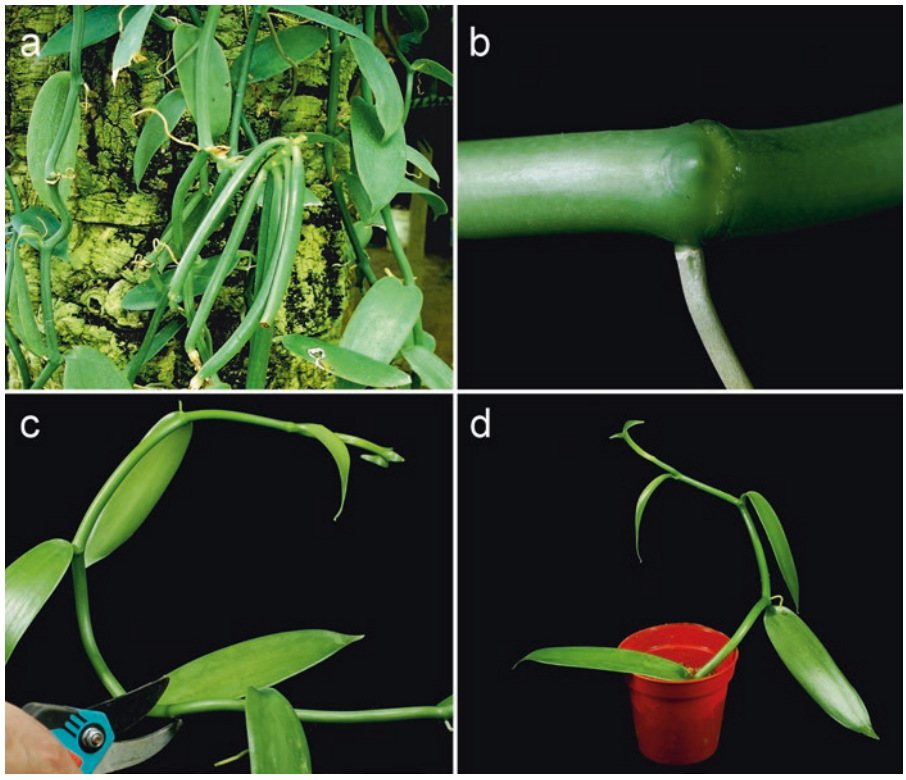


Fig. 11 The process of cutting for *Vanilla planifolia* vines. (**a**) The bean of *Vanilla planifolia* is one of the most popular spices in the world. (**b**) An axillary bud in a node appears after removing the leave. (**c**) A vine with four nodes in length can be cut by the garden shears as a propagule. (**d**) After curing or wound healing of the cut end, the lower tip of cutting is wrapped by sphagnum moss and planted in a plastic pot

3. Place the long cuttings horizontally in a tray, or a single node cutting vertically (Fig. 10a, b) in a 6-cm pot.
4. Fill the tray or the pot with potting medium, and tap the tray or the pot to help the potting material settling tightly around the cuttings.
5. Cover the tray with polyurethane plastic wrap and place in a warm greenhouse and out of direct sunlight.
6. Mark the name of the clone and the date of cutting on the label.

4.3 Keikis

Some orchids, such as phalaenopsis, dendrobiums, and *Pleione*, can spontaneously produce keikis, i.e., offshoots from the nodes along the pseudobulbs or flower stems. In some *Phalaenopsis* species, e.g., *P. equestris* and *P. lueddemanniana* and their progenies, it is easy to see spontaneous keikis formed from the tip of floral stems.

1. Allow the keikis to grow on the mother plants until they have roots about 1 cm long and with two or three leaves (*see* **Note 30**).
2. Cut the keikis from the mother plants by a sharp blade previously sterilized.
3. Coat the cut ends of the keikis with powdered fungicides (*see* **Note 31**).
4. Pot the keikis as described above.
5. Mark the name of the clone and the date of separating on the label.
6. In *Phalaenopsis*, the flower stem usually has a few dormant buds, and keikis can be induced from the dormant nodes by applying ointments with plant growth regulators (*see* **Note 32**).

4.4 Care After Potting

During the dividing or cutting process, there are wounds in the separated stem sections, and some of the roots have been bruised or broken. Some of the wounds are small and are not readily visible. In this situation, the divisions or cuttings are vulnerable to rotting if the potting medium is too wet. For the proper curing of wounds, we usually keep the potting medium dry until we observe the development of new roots (*see* **Note 33**).

Before new roots are visible, it is essential to protect the plants from water loss through their leaves (*see* **Note 34**). Water the plant sparingly. We usually place the plants in a humid environment with good aeration and with extra shade (80% shade of the full sunlight). In sunny days, it is required to give the plants a light mist spray in the daytime, just dampening the leaves, pseudobulbs, and the surface of potting media.

As new roots become visible, give a light watering, but not enough to soak the potting media thoroughly. Once the orchids start growing normally, irrigate orchids until water leaks from the

bottom drainage holes, and allow the potting media surface to dry out between waterings. Fertilize with a liquid fertilizer once every 2 weeks during the growing seasons.

5 Notes

1. In fact, there is no clear-cut line between these categories, and the temperature ranges of these categories may overlap somewhat. Some orchids are adaptable to a wide range of conditions, e.g., the nobile-type *Dendrobium*, while a few orchids, especially the cool growers, e.g., *Masdevallia* species, are very sensitive to heat and they will perish soon in hot climate. This is the reason why knowing the origin and habitat of the species is the most important consideration in growing them.

   Furthermore, different species within a genus may have different cultural requirements. In *Paphiopedilum*, the standard-type hybrids are intermediate growers, while the strap-leaf species, e.g., *P. rothschildianum* and *P. lowii*, are warm growers. In *Dendrobium*, the phalaenopsis and antelope types, e.g., *D. bigibbum* and *D. antennatum*, are native to the lowlands of Northern Australia and New Guinea that prefer the warm condition. On the contrary, *D. cuthbertsonii* and *D. lawesii* are native to mountains of New Guinea that prefer cool to intermediate conditions. In *Cattleya*, *C. intermedia* is a warm grower, while *C. coccinea* is a cool grower.
2. In actual fact, humidity in greenhouses is changeable and not easy to control. If installing an automated humidifier system is not possible, there are some simple ways to raise the humidity, such as placing large buckets of water in the greenhouse and placing plants on pebble trays filled with water. When water evaporates, the humidity will rise to the orchids above.
3. Leaf color provides a good indicator of the light intensity a plant received. Healthy leaves of orchids should have a bright green color. Dark green leaves indicate that a plant is too shady. Too much light will result in yellowish-green or red leaves. The bleached white leaves indicate that a plant is severely burned. Leaf temperature is also a clue of adequate light intensity. Excessive light will generate more heat, and thus it requires more shading, more air circulation, and cooling systems to maintain proper leaf temperature to avoid sunburn.
4. There some rules of watering management: (1) use a well-drained potting medium; (2) water thoroughly each time; (3) water just before moisture stress occurs; and (4) if it is hard to decide to water or not, it is always better to slightly underwater than overwater.

5. For different orchids or developmental stages, other ratios of $N–P_2O_5–K_2O$ of blended fertilizers can be obtained from manufacturers or by mixing with simple fertilizers.
6. Some orchid species or young plantlets prefer a diluted fertilizer solution at half- or quarter-strength (approximately 0.25 or 0.5 g fertilizer per liter).
7. The EC value represents the concentration of the total soluble salts in a solution that can be measured by an electrical conductivity meter.
8. Osmunda and tree fern used to be popular in growing orchids such as cattleyas. These materials are porous with excellent moisture-retention properties. However, the natural resources of osmunda and tree fern are rapidly diminishing. Availability is becoming an issue.
9. Barks are required to be partially composted prior to use as a potting medium. Because of its high carbon/nitrogen ratio, increasing the nitrogen fertilizer application is recommended.
10. A peat moss-based mix retains moisture well, but it is easy to coalesce and form an impervious mat on the surface of the potting mix after a few months of cultivation. With such a mat on the surface, good aeration cannot be achieved and the roots of orchids under such an anaerobic condition will soon perish. By adjusting the proportion of peat moss and the other ingredients with good aeration, this condition can be improved.
11. If the pH value is too low, trace elements such as manganese and zinc could easily show signs of being excessive.
12. In sphagnum moss, because of the survival of some microorganisms and weed seeds, it is recommended to pretreat sphagnum moss by immersing into 70 °C hot water overnight or immersing into a 2% $Ca(ClO)_2$ solution for about 6 h, followed by washing away the residual $Ca(ClO)_2$ with water before use.
13. Because coconut trees usually grow near seashore, the chips or coirs must be thoroughly washed to get rid of any salts before use.
14. Although most *Paphiopedilum* species like a bark-based mix (fine to medium grades), species in subgenus *Brachypetalum*, e.g., *P. godefroyae* and *P. niveum*, prefer the diatomite- or pumice-based mix (medium to coarse grades). Good aeration is very important for these *Brachypetalum* species [15].
15. Perlite is notorious for the overpowering cloud of dust in the process of mixing with other ingredients. This can be avoided with the spray of water.

16. Pots with good drainage with multiple holes are critical for orchids growing because waterlogging at the bottom of pots will damage the root system. Plastic pots are lightweight and low-priced. Color-tinted or transparent pots can be used. Terracotta pots are durable and their heavyweights can prevent the toppling of plants. Baskets are commonly used for growing *Rhynchostylis* and *Vanda*.
17. We usually use the pot that is just big enough to contain the roots without stuffing them. In phalaenopsis productions, 6-cm, 9-cm, 12-cm, and 15-cm transparent plastic pots are commonly used in different development stages. A small plant growing in a large pot will result in poor drainage and aeration, leading to root rotting.
18. Sometimes, we use transparent plastic pots for growing terrestrial orchids, and they also grow very well. It is convenient to observe the root system and the conditions of the potting mix.
19. As for any chemicals, it is important to read the manufacturer's instruction before use. If spraying chemicals in a greenhouse, covering one's face with a respirator is necessary. After spraying, the greenhouse should be ventilated by fans for several hours before reentering.
20. The concentrations of available chlorine will decrease after opening the bottle of bleach. It is important to close the cap securely after each use and replace with new bottles regularly.
21. The root tips are very tender and are easily broken. When a root tip is broken, the root ceases to grow from that point.
22. Before dividing, prepare a clean working area. Clean the workbench with diluted bleach solution. All necessary tools should be disinfected before use.
23. Use this opportunity to examine the health conditions of a plant, especially the root system.
24. It is common to observe scales and mites under leaves, near the base of leaves and at the joints of pseudobulbs or stems.
25. Tool sterilization is important before cutting because *Odontoglossum* ring spot virus (ORSV) and *Cymbidium* mosaic virus (CyMV) can be transmitted by handling during dividing, repotting, cutting, or any other management practice. Treating the tools with a 2% NaOH solution is sufficient to destroy virus. If it is not convenient to prepare NaOH solution, using a burner to destroy viruses on cutting tools by heat is a good alternative method.
26. The leading pseudobulb in each division should have a "live eye" (dormant bud), so that growth can continue. If there is no live eye on the leading pseudobulb, the division will probably not develop new shoots, and thus it should be discarded.

27. The lower part of the rhizome should be at the same level of the potting medium surface, and the new leading sprout should be just above the surface of the potting medium. If the bud is too deep (buried in the potting medium), the risk of becoming rotted will increase. On the other hand, if the bud is placed well above the potting mix, the new roots will grow on the surface, increasing the chance of falling over.
28. For dendrobiums, the long stem cuttings containing four to five nodes are commonly used in *D. anosmum*, *D. aphyllum*, and *D. loddigesii*, while the short stem cuttings containing only one node are often used in the commercial productions of the nobile-type dendrobiums.

    For cutting propagation of vanilla, a cutting with 8–12 nodes is preferred. Long cuttings with more nodes enable the vines to grow more vigorously resulting in earlier flower formation and capsule development. However, if the material is scarce, short cuttings with two to four nodes can be used.
29. The exposed wounds need to be protected to prevent rotting. It is also important to watch carefully if severe dryness occurs in the cuttings. Shriveled leaves or pseudobulbs indicate a lack of water to plants.
30. By the time they have reached a length near 3 cm, it is difficult to pot keikis without injuring them.
31. Use powdered fungicides, such as Mancozeb (DuPont, Wilmington, DE, USA).
32. Mix a small amount (2 mg/L) of 6-benzylaminopurine powder (BA) with lanolin (wool wax or wool grease), and smear it on the dormant bud of a phalaenopsis floral stem. The plant growth regulator will be absorbed and stimulate bud development. Some formulas of keiki paste include 0.5 mg/L of α-naphthaleneacetic acid (NAA) besides BA.
33. The control of watering is crucial after potting. Keeping the potting medium dry can prevent rotting and stimulate rooting of many epiphytic orchids. However, for some terrestrial orchids, e.g., paphiopedilums, the roots may not be able to endure dryness too long. After coating the cut ends with powdered fungicides and potting of plants, water them immediately.
34. The control of stable climate is important to the recovery of newly potted orchids. The shriveled leaves indicate a lack of water to plants. The immediate solution is to mist the plants and raise humidity (at least maintaining a 70% humidity) with good aeration. For some difficult-to-root species/cultivars, the application of auxin-containing products, e.g., indole-3-butyric acid or NAA in addition to nitrogen, may be helpful to stimulate root formation.

References

1. World Checklist of Monocotyledons (2006) The Board of Trustees of the Royal Botanic Gardens, Kew. Published online at: http://www.kew.org/wcsp/monocots
2. Northen RT (1990) Home orchid growing, 4th edn. Simon & Schuster, New York, NY
3. DeYoung G, Rowe B and Runkle E (2011) Propagating orchids. Orchids August. www.AOS.org
4. Dressler RL (1993) Phylogeny and classification of the orchid family. Dioscorides Press, Portland, OR
5. Withner CL (1974) The orchids: scientific studies. Wiley, New York, NY
6. Motomura H, Yukawa T, Ueno O, Kagawa A (2008) The occurrence of crassulacean acid metabolism in *Cymbidium* (Orchidaceae) and its ecological and evolutionary implications. J Plant Res 121:163–177
7. Hew CS, Yong JWH (1997) Physiology of tropical orchids in relation to the industry. World Scientific Publishing Co Pte Ltd., Singapore
8. Withner CL (1959) The orchids: a scientific survey. The Ronald Press Company, New York, NY
9. Yang YY, Kim JG (2016) The optimal balance between sexual and asexual reproduction in variable environments: a systematic review. J Ecol Environ 40:12
10. Nelson PV (1991) Greenhouse operation and management, 4th edn. Prentice Hall, Upper Saddle River, NJ
11. Van der Knaap N (2005) Cultivation guide *Phalaenopsis*: knowledge for professionals. Anthura B.V., Bleiswijk, Netherlands
12. Richter W (1977) Orchid care: a guide to cultivation and breeding. Van Nostrand Reinhold Co., New York, NY
13. Sheehan TJ (1997) Appendix: orchid potting mixtures-an abridged historical review. In: Arditti J, Pridgeon AM (eds) Orchid biology: reviews and perspectives VII. Kluwer Academic Publishers, Dordrecht, pp 317–362
14. Wang YT, Blanchard M, Lopez R, Runkle E (2007) Growing the best Phalaenopsis. Orchids February. www.AOS.org
15. Birk L (1984) The *Paphiopedilum* growers manual. Day Printing, Kelowna, Canada

Chapter 23

Orchid Cultivation in Taiwan: Conventional and Innovative Methods

Rong-Show Shen, Yu-Hsin Liao, and Kuang-Liang Huang

Abstract

Phalaenopsis orchids are one of the most popular potted plants of the world and are widely cultivated in greenhouses. Breeding, cloning cultivars, and improving cultivation techniques have contributed significantly to the *Phalaenopsis* industry both in Taiwan and abroad. This chapter uses *Phalaenopsis* as an example to thoroughly describe the seedling production process, including hardening-off, sorting, deflasking, potting, growth media, irrigation, and fertigation. The conventional cultivation model and techniques used in Taiwan are presented, and an accelerated two-step continuous cultivation protocol is also introduced.

Key words *Phalaenopsis*, Deflasking, Sorting, Hardening-off seedlings, Fertilization, Growth medium, Fertigation, Accelerated growing technique

1 Introduction

Phalaenopsis orchids are one of the most important potted plants in the floricultural markets of the world. The cultivation of *Phalaenopsis* involves a typical container nursery system, and it usually takes more than 1 year from young deflasked plantlets to the adult flowering plant stage. The length of the growth period varies according to different cultivation systems. The successful cultivation of *Phalaenopsis* relies on the exact control of environmental conditions to keep the light, temperature, humidity, ventilation, etc. as stable as possible. Although greenhouse management can be monitored and controlled by computer programs, the practical operation still requires experienced, well-trained growers.

The quality of plantlets is the critical factor affecting the success of *Phalaenopsis* cultivation. For example, at the beginning of cultivation, the young deflasked plantlets are very sensitive to handling practices and environmental changes. It is difficult for the plantlets to recover from physical and physiological damages.

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_23,

Therefore, careful observation of plantlet growth and quick adjustments of environmental conditions to fit the growth requirements of plantlets are important. Here, we demonstrate the techniques of *Phalaenopsis* cultivation including the sorting of plantlets, treatments of potting media, and fertigation management. The approaches and general principles can also be applied to the production of other commercial orchids.

2 Sorting and Potting of Deflasked Plantlets

Uniform plantlet establishment with vigorous growth is the first critical step for successful cultivation of *Phalaenopsis*. In commercial production, the plant materials are generally obtained from clonal propagation of selected elite individuals through tissue culture. For improving the production efficiency, young plantlets from tissue culture laboratories need to be "hardened-off" (*see* **Note 1**), carefully sorted, and graded before potting. The criteria of sorting young plantlets are (a) no yellow leaves or observable disease symptoms, (b) adequate plantlet height (6–8 cm) with good leaf/root ratio to indicate satisfactory growth potential, and (c) plantlets with three healthy leaves and three roots, with the longest root shorter than 4 cm, allowing for uniform potting (Fig. 1). For maintaining and improving the management and production efficiencies, plantlets that do not meet the above criteria should not be used.

The procedure of deflasking and potting young plantlets is illustrated in Fig. 2. For deflasking plantlets, it is important to remove the young plantlets from flasks gently with fingers; avoid breaking of leaves during the deflasking process. Then, use the reverse-osmosis water to wash off the tissue culture medium that adhered to the roots of young plantlets. After being cured for 90–120 min, wrap the deflasked plantlets with sterilized sphagnum moss and pot immediately (*see* **Note 2**). When potting the young plantlets, it is necessary to minimize root breakage to lower the possibility of infection by pathogens. This will result in a higher survival percentage (*see* **Note 3**).

Before the first new roots grow to the edge of the pot, mist the plantlets regularly to prevent dehydration of leaves after potting. During this period, keep the potting medium, i.e., sphagnum moss dry for the curing of wounded roots (the original roots in flasks) (*see* **Note 4**). After 70% of the deflasked plantlets with visible new roots that have grown to the edge of the pot, fertilize the plantlets normally by following a cultivation management schedule.

For the newly deflasked plantlets, set the light intensity in the range of 180–200 mol m^{-2} s^{-1} during the first 3 weeks after deflasking. The greenhouse environment should be well ventilated, and the day and night temperatures should be rigidly controlled at

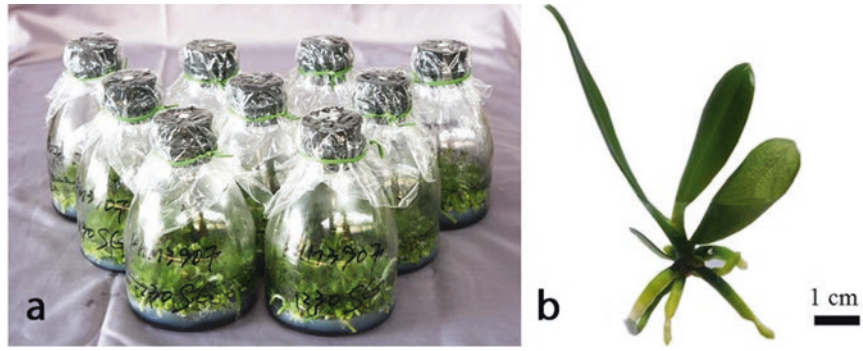


Fig. 1 Flask plantlets from mericlonal micropropagation (**a**) of commercial saleable and standard deflasked *Phalaenopsis* plantlet (**b**)

30 °C and 25 °C, respectively (*see* **Note 5**). If the aforementioned criteria are followed, a high survival percentage of deflasked plantlets is expected. Usually, the experienced *Phalaenopsis* growers fully understand that this is the critical stage with the highest potential of plantlet loss. Only those who thoroughly implement the guidelines of cultivation management will be able to succeed in obtaining healthy plants.

3 Potting Media

A variety of potting media can be used for growing *Phalaenopsis* successfully such as sphagnum moss and bark. However, selection of potting media needs to take into consideration the frequency of watering, the irrigation methods (e.g., ebb and flow, bottom irrigation, or top watering), the types of pot used, etc. The ideal potting media for growing *Phalaenopsis* must meet the following criteria:

1. Capable of retaining water and fertilizers
2. Easy to drain
3. Does not easily rot or decompose prematurely
4. Does not easily fall apart or disintegrate
5. Does not contain toxic substances
6. Provides sufficient support to the plant
7. Provides sufficient space for the growth of roots
8. Provide sufficient air space to allow for gaseous exchange

Since *Phalaenopsis* is an epiphytic orchid, the potting media with good drainage capacity and ventilation are critical to root growth. In greenhouse cultivation, because the roots are buried in the medium, the medium must contain enough oxygen to enable normal root initiation and growth.

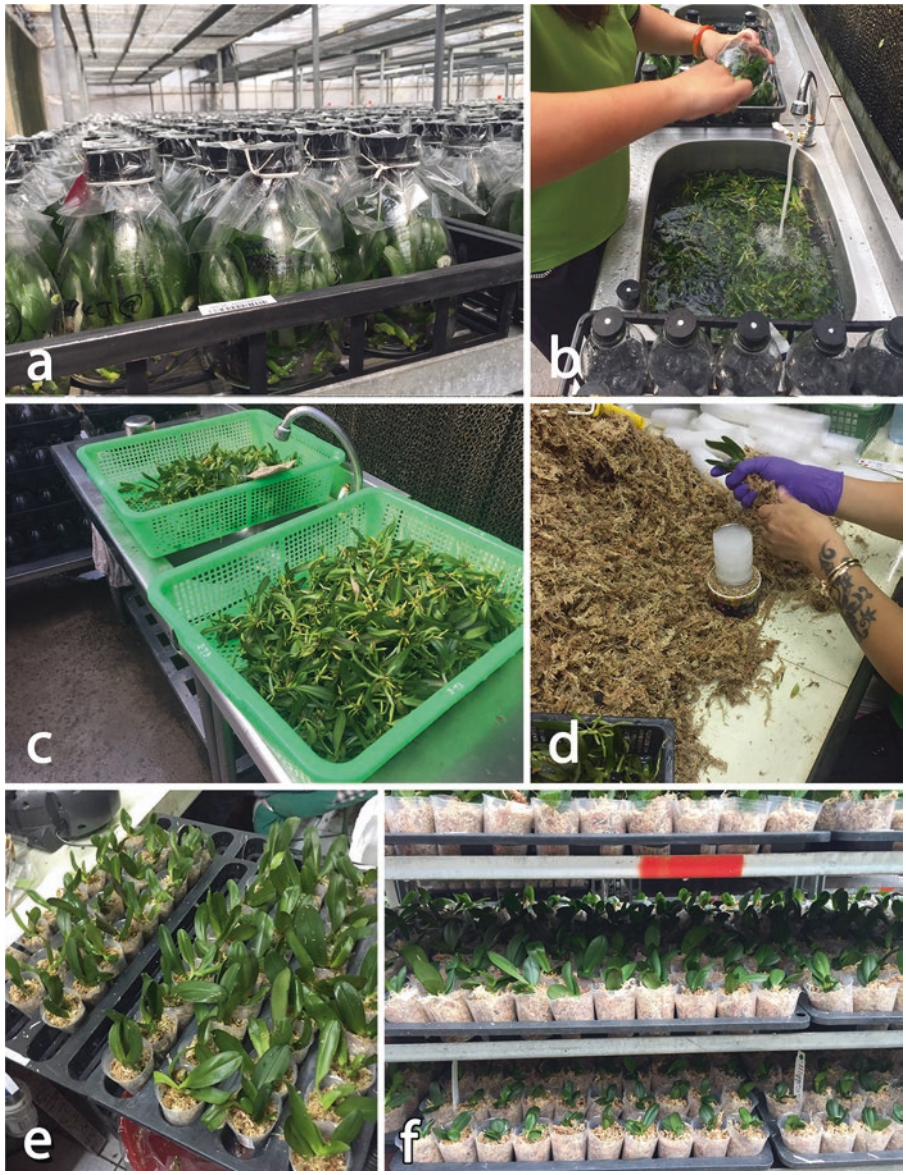


Fig. 2 Operating procedure in which the flask plantlets of *Phalaenopsis* are hardened, deflasked, washed, sorted, potted, and planted in transparent 5-cm-diameter plastic pots. (**a**) Flask plantlets are moved to the plantlet cultivation greenhouse with a light intensity of 180–200 μmol m^{-2} s^{-1} just prior to repotting, to be hardened for 14–21 days. (**b**) The plantlets are removed using fingers or tweezers, avoiding damage to the leaves and roots. After washing off the agar gel on the roots using reverse-osmosis water, the plantlets are sorted, avoiding crowding of seedlings. (**c**) Plantlets with adequate size and favorable roots and leaves are sorted and placed in a ventilated plastic basket. After 90–120 min of shade drying, the plantlets are potted on the same day. (**d**) The plantlets are carefully wrapped with sterilized sphagnum moss and then planted in the pot, in which 1–1.5 cm of space is reserved for draining stagnant water. (**e**) After potting, plantlets are placed in a communal pot on a movable trolley. (**f**) The pots are transported and placed on the bench

A wide variety of media can be used for greenhouse cultivation (also *see* Chap. 22). Choosing an appropriate medium should involve consideration of not only its capacity for plant growth but also the cost of production. Factors such as a lack of pathogens (insects), toxicity, stability, impact resistance, convenient operation, and easily obtained commercially also need to be considered.

3.1 Sphagnum Moss

Sphagnum moss is the major potting medium used for *Phalaenopsis* cultivation in Japan and Taiwan. According to a survey conducted by the Taiwan Orchid Growers Association, more than 90% of *Phalaenopsis* growers in Taiwan prefer to use sphagnum moss as the potting medium [1]. The annual demand of sphagnum moss for potting medium is approximately 1100 metric tons in Taiwan. The main sources of sphagnum moss in Taiwan are Chile, mainland China, and New Zealand. After harvesting from natural conditions, the sphagnum moss is required to be dried and compressed for shipping and storage. Hence, before the use in a potting operation, it is necessary to treat the sphagnum moss with the procedures of drenching, sterilization, and dehydration (*see* **Note 6**). For *Phalaenopsis* growers, the advantages of using sphagnum moss as a potting medium include (a) good moisture-holding and nutrient retention capacities and (b) the high plasticity for handling and supporting the plantlets in the potting operation. However, the availability of sphagnum moss is becoming limited due to the huge consumption and climate change. Furthermore, the quality of sphagnum moss is getting worse, i.e., becoming less durable in recent years.

Sphagnum moss is easy to use and handle during the potting of plantlets. Because of its high plasticity and ease of handling, this can result in different filling amounts and methods of plantlet packaging during potting. According to the study on *Phalaenopsis* cultivation in our laboratory, we investigated the physical properties of the sphagnum moss of various weights (i.e., various bulk densities) used in filling 5-cm-diameter pots. As shown in Fig. 3, the different weights of sphagnum moss used generate a wide range of gaseous, solid, and liquid phases surrounding the roots of the newly potted young plantlets. This can affect the uniformity of plant growth and development. Hence, it is important to standardize the filling amounts of potting medium used and the method in potting plantlets.

The optimum timing to fertigate is when the moisture content of the sphagnum moss is near 18–20% [2]. If there is no standard of filling amounts in a potting operation, the moisture content of sphagnum moss will vary among pots, and the frequency of fertigation will differ substantially among young plantlets (*see* **Note 7**). This would be a difficult situation for managing fertigation in the greenhouse cultivation.

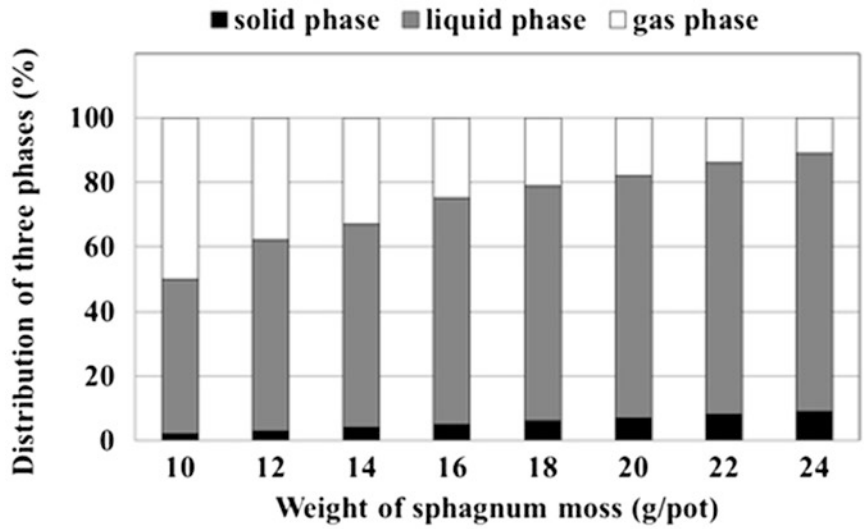


Fig. 3 Effects of the weight of sphagnum moss on three-phase distribution in a 5-cm-diameter potted *Phalaenopsis*

When sphagnum moss is used as the potting medium, in about 3 months, the pH value in the *Phalaenopsis* root zone is usually around 3. However, it seems that the low pH value does not affect the growth and blooming of *Phalaenopsis*. Usually after 6 months of growing, the sphagnum moss gradually decays, nutrient retention capacity reduces greatly, and this is the time to repot.

3.2 Bark

Bark of trees is also an ideal potting medium for *Phalaenopsis* growing, because it is durable for about 2 years, and it provides good root aeration. Bark of various sizes can be used alone or mixed with other potting materials. Conifer barks obtained from Douglas fir and pine trees are good potting media for orchid cultivation. Since bark is a by-product of lumbering, there is less problematic of resource shortage. As compared to sphagnum moss, the treatments of drenching, sterilization, and dehydration to bark are much easier. Bark or the bark-based potting mixes are loose pieces, and therefore, it is easy to handle during the repotting operation with less damage to roots. This decreases the risk of pathogenic infection. Furthermore, the procedures of potting and repotting can be handled automatically, which ensure uniformity in the filling amount and the compactness of barks among pots. Hence, the automatic fertigation system can be applied to the large-scale production of *Phalaenopsis* using bark as the potting medium.

Before using bark as a potting medium, some characteristics of bark are worthy of note (*see* **Note 8**). First, fresh bark may contain some pests and toxic compounds to plants, and it requires adequate composting for a few months (aged bark) or adjusting pH values before use [3]. Second, bark has a high carbon-nitrogen

ratio (C/N ratio) of approximately 300:1; it is necessary to add fertilizers during composting. Even the aged bark used in the potting mix and the slow decomposition by microorganisms may tie up the availability of nitrogen to *Phalaenopsis* plantlets, resulting in nitrogen deficiency. Although bark provides good root aeration, the water and nutrient retention capacities are poor when coarser bark pieces are used (*see* **Note 9**). Therefore, a higher frequency of fertigation, with added nitrogen, needs to be applied to plants potted in pure bark medium, preventing nutrient deficiency (*see* **Note 10**). Using the finer bark pieces or mixing with coconut fiber and/or sphagnum moss may assist to increase water and nutrient retention capacities [4].

3.3 Potting Mix

Potting mixes combining the advantages of different materials are commonly used in orchid cultivation. In Netherlands, the bark-based potting mix for *Phalaenopsis* contains bark (50%), coconut fiber (15%), perlite (20%), vermiculite (10%), and sphagnum moss (5%). In a previous investigation, we compare the characteristics between the bark-based potting mix and the sphagnum moss (Table 1) [5]. We also demonstrated that the performance of *Phalaenopsis* plantlets potted in the bark-based potting mix is as good as compared to those potted in the sphagnum moss (Fig. 4, unpublished data).

4 Watering, Fertigation, and Accelerated Growth Technique for *Phalaenopsis*

Proper watering/fertigation management is the fundamental factor of successful *Phalaenopsis* cultivation. In Japan, as the proverb says, the novice needs at least 3 years to learn the management of watering orchids. Optimizing the management of watering/fertigation (e.g., types and concentrations of fertilizers) is critical for accelerating the growth of *Phalaenopsis* plantlets and for improving the quality. For proper fertigation management, one needs to consider the following factors: (1) temperature, (2) light, (3) humidity, (4) ventilation in the greenhouse, (5) types of potting media, (5) concentrations of fertilizer, and (6) the growth conditions of plants. Before watering or fertigation, a grower needs to observe and check these conditions carefully (*see* **Note 11**). Since most *Phalaenopsis* growers in Taiwan prefer to use sphagnum moss as the potting medium, here, we use the example of sphagnum moss to illustrate the management of watering/fertigation in *Phalaenopsis* cultivation.

4.1 Water Quality

The quality of irrigation water is a critical factor affecting the growth of *Phalaenopsis* (*see* **Note 12**). Table 2 shows the grades of water quality for irrigation in *Phalaenopsis* cultivation. Irrigation water should be free of chemicals and any visible contaminants.

Table 1
Comparison of characteristics of the bark-based potting mix and the sphagnum moss medium

Items	Bark (mix)	Sphagnum moss
Cost	Low	High
Physical and chemical properties	Good ventilation and low possibility of salt accumulation and acidification	Poor ventilation and high possibility of salt accumulation and acidification
Stability	Decomposes with difficulty	Easily decomposes and acidifies
Determination of fertigation time	Easy	Difficult
Possibility of root growth failure	Low	High
Proportion of favorable plants	No significant difference in small and medium seedlings, but a high proportion of favorable large seedlings	No significant difference in small and medium seedlings, but a low proportion of favorable large seedlings
Flower life span	Short	Long
Occurrence of pests and diseases	Less frequent	Frequent
Control of production procedure	Easy; less likely to result in root growth failure because of incorrect judgment	Difficult
Automatic mass cultivation	Easy	Difficult
Production cost of mature plants	Low	High
Difficulty of sterilizing for quarantine	Pests hide in the pores and sterilization is difficult	Easy to sterilize
Market acceptance	Accepted by European countries and the United States but not well accepted in Asian countries where combination pots are popular	Highly accepted

Usually, rain water is a good source for irrigation. For well water, it is necessary to analyze the water quality before irrigation. The sources of water should be analyzed and monitored regularly, including the values of pH, electrical conductivity (EC), and the amount of elements present, e.g., Na^+, Cl^-, HCO_3^-, and Ca^{++}. The EC values of various sources of irrigation water used in Taiwan, e.g., well water, tap water, and reverse-osmosis water, are 0.75, 0.40, and 0.03 mS cm^{-1}, respectively [6]. EC represents the total

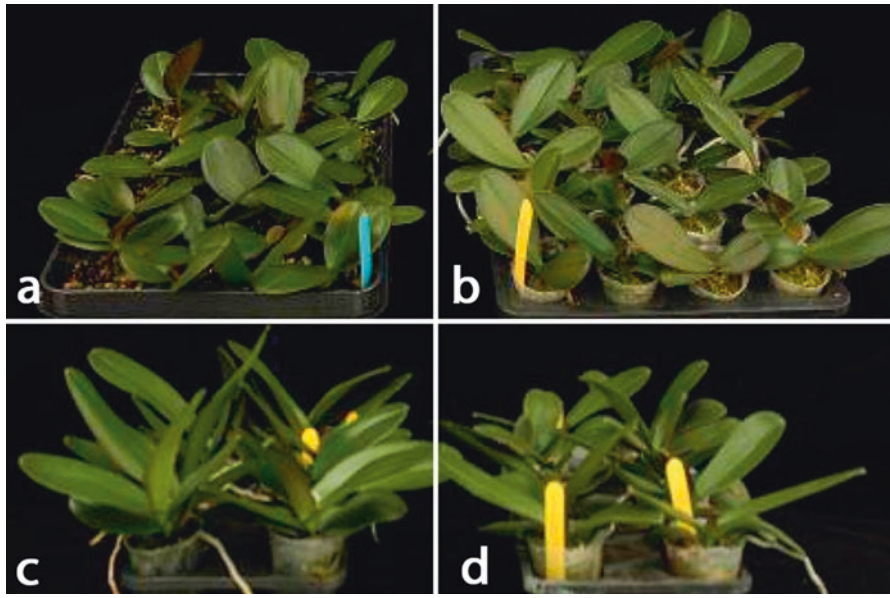


Fig. 4 Appearance of deflasked plantlets planted for 4 months in the 5-cm-diameter pot at the first stage (**a**, **b**) and 8-cm-diameter pot at the second stage (**c**, **d**) using the bark-based potting mix (**a**, **c**) and sphagnum moss medium (**b**, **d**)

concentration of soluble salts in the solution. If the solution around the potting media and roots contains excessive soluble salts, i.e., too high an EC value, it hampers the absorption of water and nutrients by roots because of the high osmotic potential. The fertilizer strength levels by each fertigation will also be affected (Fig. 5). The potting media with a high EC level can cause damage to *Phalaenopsis* roots. Under a high EC level for a period of time, the root tips start to turn brown, and the entire root system will become necrotic. In this situation, the leaves of *Phalaenopsis* still remain green but have lost vigor and will wither. The EC values of irrigation water can be classified into four levels according to the response of plant growth [6]:

1. When EC is < 0.75 mS cm^{-1}, the salt content does not have a negative influence on plant growth.
2. When EC is 0.75–1.50 mS cm^{-1}, the salt content poses a threat to plants sensitive to salinity; root tips start to turn brown.
3. When EC is 1.51–3.00 mS cm^{-1}, the salt content poses a threat to plants that have high salinity tolerance. These plants and those with lower salinity tolerances should be carefully managed.
4. When EC is 3.01–4.50 mS cm^{-1}, the salt content is extremely high. Most plants cannot survive, except for those with extremely high salinity tolerance.

Table 2
Grading standards for the EC and salt content of irrigation water

	EC	Na^+		Cl^-		HCO_3^-		Ca^{++}	
Water quality	**mS cm^{-1}**	**mmol L^{-1}**	**mg L^{-1}**	**mmol L^{-1}**	**mg L^{-1}**	**mmol L^{-1}**	**mg L^{-1}**	**mmol L^{-1}**	**mg L^{-1}**
Good	<0.5	<1.0	23	<1.0	35	<0.5	<31	<1.0	<40
Acceptable	0.5–1.0	1.0–3.0	23–69	1.0–3.0	35–105	0.5–1.0	31–62	1.0–2.0	40–80
Poor	>1.0	>3.0	>69	>3.0	>105	>1.0	>62	>2.0	>80

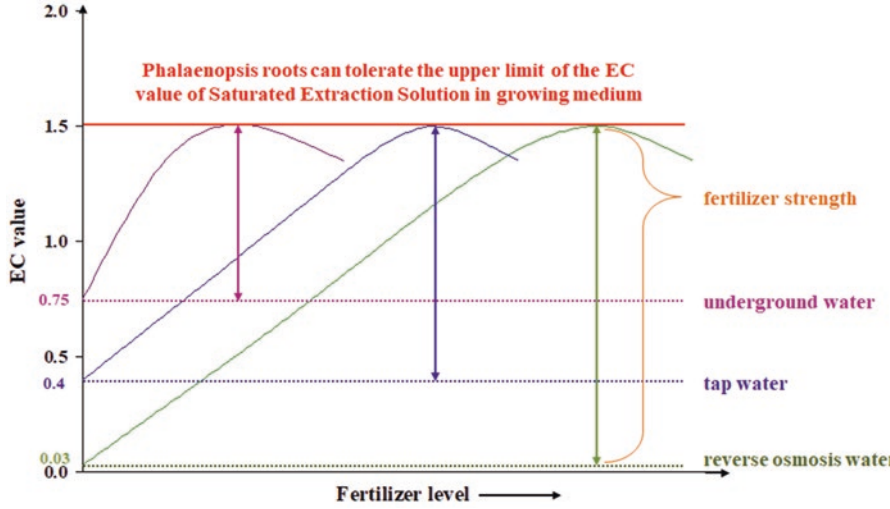


Fig. 5 Effect of the electrical conductivity of irrigation water on fertilizer strength (modified from Shen and Chen [6])

A number of studies reported the effect of EC levels on the growth of *Phalaenopsis* plants. Wang [7] used a potting mixture of bark (80%) and sphagnum moss (20%) for growing *P.* Tam Butterfly. When the EC value of the irrigation water was 1.4 mS cm^{-1}, the plant showed the highest defoliation percentage. According to investigation by Cui et al. [8], when the EC value of the fertigated solution within the potting medium reached 2.4 mS cm^{-1}, the stomata of *Doritaenopsis* Tinny Tender closed and resulted in decreases of the transpiration rate and photosynthetic efficiency [8]. According to the "Cultivation Guide for *Phalaenopsis*" [9], the appropriate EC values for fertigation solution should be within the range of 0.8–1.4 mS cm^{-1}. A desirable EC value of the water before adding any fertilizer is below 0.5 mS cm^{-1}. In our investigations, when the EC value of the fertigation solution within the potting media is lower than 1.5 mS cm^{-1} (using the saturated media extract method), this is a safe threshold [2]. If the EC value of the fertigation solution is higher than 1.5 mS cm^{-1}, less fertilizers can be added as the irrigation water already has a higher EC value (*see* **Note 13**). Currently, most professional *Phalaenopsis* growers in Taiwan use reverse-osmosis water for irrigation. Since the EC value of reverse-osmosis water is only about 0.03 mS cm^{-1}, the grower can add more fertilizers in the fertigation solution to improve the growth of *Phalaenopsis* plants [6] (Fig. 5).

4.2 Water Temperature and Watering Tools

Phalaenopsis is an epiphytic orchid native to tropical areas; hence the plants cannot be subjected to low temperatures, and the roots should be maintained above 20 °C. During winter, the fertigation solution temperature should be warmed to 20–22 °C before use.

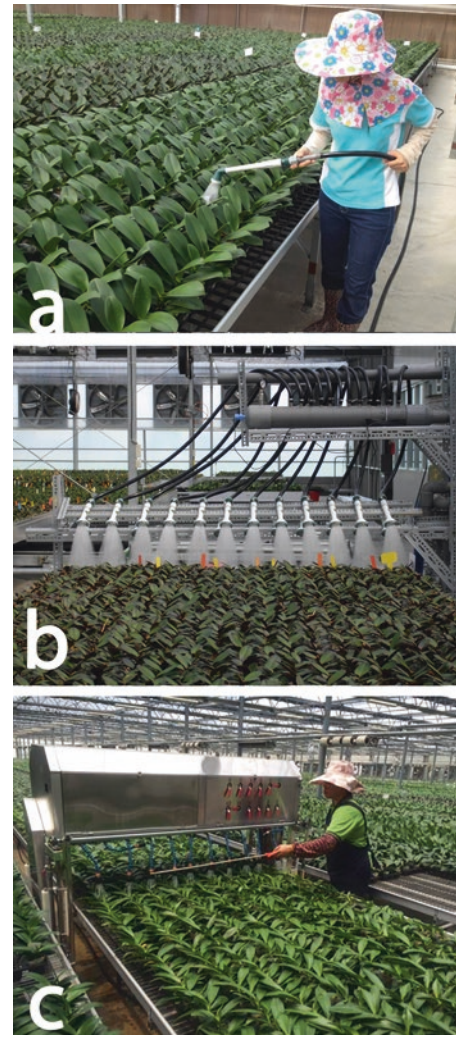


Fig. 6 Irrigation approaches commonly adopted in Taiwan. (**a**) Manual watering using a shower head. (**b**) Fixed watering using shower heads on a movable bench. (**c**) Semiautomatic movable irrigation waterwheel

The roots of *Phalaenopsis* prefer good aeration. Since the amount of oxygen dissolved in water is approximately one-thirtieth of that in the air, oxygen deficiency may occur in the roots when the potting medium has poor drainage issues. The long-term oxygen deficiency will cause the root to rot. For *Phalaenopsis* plants potted with sphagnum moss, the aeration around roots is not as good as those potted with bark; growers in Taiwan commonly use mist shower heads for watering to increase the amount of dissolved oxygen in water (Fig. 6).

4.3 Timing and Amount of Watering

The appropriate fertigation management is critical to provide an environment of mild, humid, and good aeration (*see* **Note 14**) for the growth of *Phalaenopsis* [6]. As indicated above, since there are

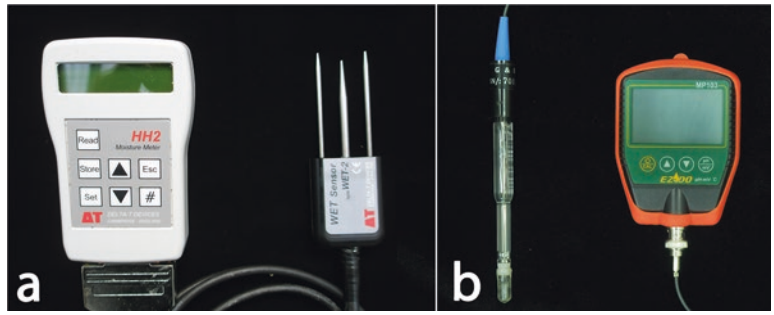


Fig. 7 Directly insertable moisture meter that can measure EC, moisture content, and temperature (**a**) and the embedded pH meter (**b**), all of which are commonly used in Taiwan

many factors affecting the timing of watering and fertilization in *Phalaenopsis* cultivation, a logical fertigation management program needs to be established according to the quality of water, the types of potting media, and the types of fertilizers used. The standard operating procedure should be designed based on data of scientific studies to ensure stable production of high-quality *Phalaenopsis*. In Taiwan and Japan, most *Phalaenopsis* plants are potted with sphagnum moss, and they are usually watered and fertilized manually, and this requires an experienced grower to provide all pots with the same amount of water (*see* **Note 15**). The potting medium with optimal moisture content is beneficial for the growth of *Phalaenopsis*. But the ability to retain moisture content varies among the types and compactness of potting materials [10–12]. Directly insertable meters can be used to measure EC, moisture content, temperature, and pH levels (Fig. 7). Based on the investigations in the moisture content of potting media in *Phalaenopsis* cultivation, as the moisture content of sphagnum moss decreased to 18–20%, it is time to water or to fertilize [2]. It is important to make sure that the potting medium has dried up slightly before the next irrigation.

5 Nutrient Needs

Nitrogen, phosphorus, and potassium are essential elements for plant growth and development. In Taiwan, *Phalaenopsis* growers usually use blended fertilizers with a fixed ratio of nitrogen–phosphate–potassium (N–P_2O_5–K_2O), e.g., a ratio of 20-20-20. Various brands, such as Peters Professional fertilizers (The Scotts Company, OH), HYPONex® (Hyponex Japan Corp., Osaka, Japan), and Orchid-Pro® (Dyna-Gro, CA), can be obtained from local suppliers. The effects of types, ratios, and concentrations of fertilizers on the growth of orchids have been studied extensively. Here, we

briefly summarize the requirement of nitrogen, phosphorus, and potassium from some reports.

The application of 200 mg/L of nitrogen (N) in the practice of fertigation is optimum for the growth of *Phalaenopsis* plants [13, 14]. The plants *P.* Tam Butterfly with higher leaf span, leaf number, leaf area, and fresh weight were obtained with the application of 200 mg/L of nitrogen, as compared to those fertilized with 100 mg/L of nitrogen [15]. Applying 1.0 g/L Peters fertilizer ($20N–20P_2O_5–20K_2O$) containing 200 mg/L of nitrogen (100 mg/L of urea nitrogen, 61.1 mg/L of nitrate nitrogen, and 38.9 mg/L of ammoniacal nitrogen) improved the vegetative growth of *Phalaenopsis* of different potting sizes [15–18]. In *P.* Taisuco Kochdian, the higher NO_3^-/NH_4^+ ratios are beneficial to vegetative growth, e.g., with larger leaf span, leaf length, and leaf width, as well as reproductive growth, e.g., the timing of flowering and the quality of flowers [18].

Previous reports have shown that the application of phosphorus (P) at 25–50 mg/L is sufficient for optimum vegetative and reproductive growths in *Phalaenopsis* [13, 14]. A lack of phosphorus in *Phalaenopsis* plants will result in a decrease in the number of new leaves and an increase in leaf abscission. It has been recommended that better flowering quality could be enhanced by applying high levels of phosphorus prior to flower initiation. However, in *Phalaenopsis* cultivation, the application of high levels of phosphorus before spiking does not improve flowering [18–21].

Little information is available concerning the effects of potassium (K) on the growth of orchids. The scientists in Taiwan and the United States began to pay attention to the role of potassium on the growth of *Phalaenopsis* because of the physiological disorders of *Phalaenopsis*. Near the year 2000, some white-flowered *Phalaenopsis* cultivars showed physiological disorders showing yellowing of lower leaves. The symptoms were severe in mature plants, especially after those plants that have been delivered to the cool room for inducing flowering [22–24]. The yellow leaf tissue contains an extremely low level of potassium (only 0.40–0.64% on a dry-weight basis), whereas the potassium content in healthy leaves is 3–8% [23]. A lack of potassium results in the death of *Phalaenopsis* plants, and a reduction of potassium causes the leaves to form yellow, brown, or gangrenous spots, indicating potassium is essential for the growth of *Phalaenopsis* [18]. Chang et al. [20] indicated that frequent applications of potassium resulted in an increased potassium content in *Phalaenopsis* leaves. The performance of both vegetative growth (e.g., leaf span, leaf length, and leaf width) and reproductive growth (e.g., flower number, flower size, the length, and the diameter of flower stalk) improves with the increase of potassium concentration (*see* **Note 16**).

6 Cultivation System

In Taiwan, it is common to use the three-step cultivation system for the growth of *Phalaenopsis* plants from plantlets to mature stages. Even though the use of sphagnum moss prevents the use of automated cultivation protocols, its use is still preferred. Here, we introduce the conventional three-step cultivation system and an improved two-step procedure.

6.1 The Conventional Three-Step Cultivation System

The main operation procedures of the conventional three-step cultivation system (Fig. 8) are briefly described as follows:

1. In the first step, each deflasked young plantlet is potted with sphagnum moss in a 5-cm-diameter pot (usually known as the small-sized plant).
2. In the second step, after 3–5 months of cultivation, the plant is then repotted in an 8-cm-diameter pot (usually known as the medium-sized plant) and is cultivated for a further 4–6 months.

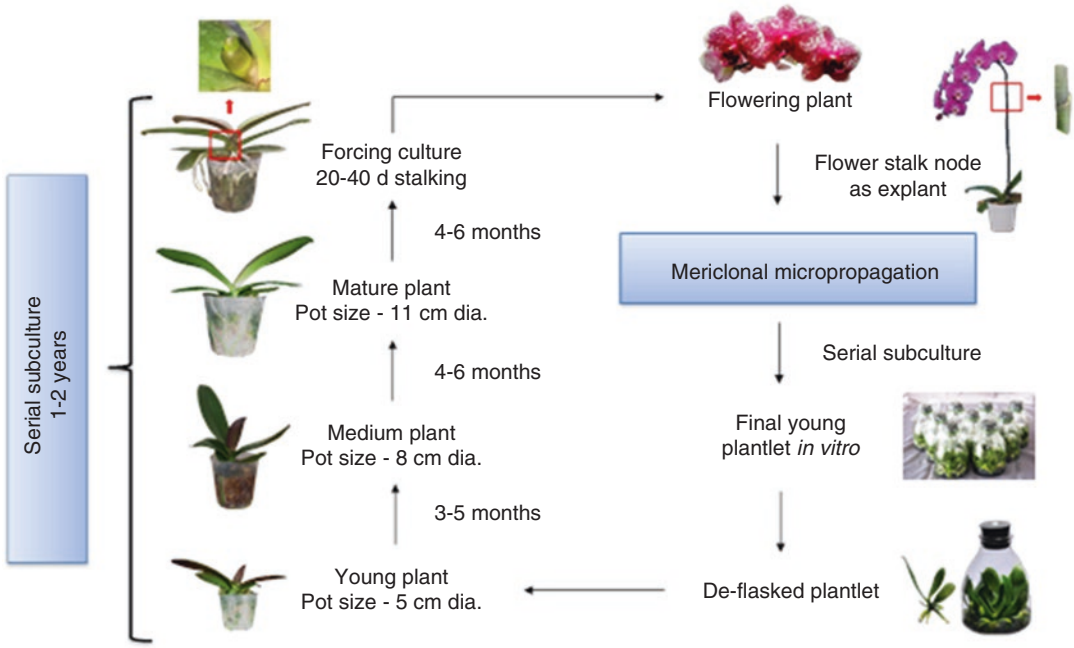


Fig. 8 A "three-step continuous cultivation" for *Phalaenopsis*: Flowering plants that show satisfactory horticultural traits can be used as parent plants, and the stalk buds are used as explant sources for the mericlonal micropropagation system. After repeated serial subcultures, the final young plantlets are then deflasked and potted in 5-cm-diameter pots for the three-step continuous cultivation regime. Sphagnum moss is used as the growth medium in all steps, and the plantlets are potted in transparent soft plastic pots of various diameters. The required length of cultivation varies according to the cultivars. Each step necessitates 4–6 months of continuous cultivation after repotting. Mature plants that meet export standards and are available for forcing culture can be obtained at the third step to form the main product exported from Taiwan

3. In the third step, the plant is subsequently repotted in an 11-cm-diameter pot for 4–6 months of cultivation until maturity (usually known as the large-sized plant). The large-sized plant is then moved to the cool room (at day/night temperatures of 25/20 °C) for spiking. Visible flower stalks are formed approximately 4 weeks after "flower forcing" [25].

6.2 Accelerated Growth Technique by a Two-Step Cultivation System

The three-step cultivation system indicated above requires three times of potting/repotting, which is labor intensive, and the plants often require about 2 weeks of recovery in each repotting practice due to repotting shock. If, despite reducing one step, the operation still produces quality *Phalaenopsis* mature plants, the total production cost will be significantly lowered. Here, we introduce a two-step cultivation system. In this system, it only requires one repotting after deflasking during the vegetative growth period, and the mature plants are then moved to the cool room for spiking [2] (Fig. 9). The main operation procedures are as follows:

1. In the first step, the deflasked young plantlets are potted with sphagnum moss in 6-cm-diameter pots. The plantlets can be fertilized with 0.5 g/L of Peters 20N–20P_2O_5–20K_2O and watered using reverse-osmosis water when the moisture content of sphagnum moss is near 18–20% (*see* **Note 17**).

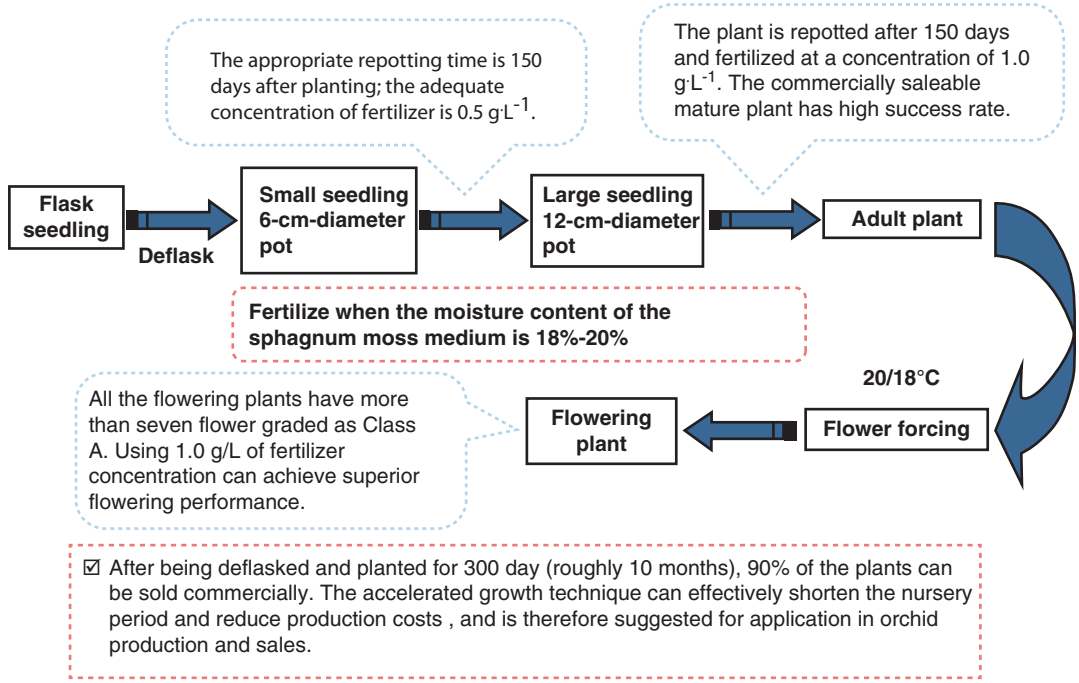


Fig. 9 Accelerated growth technique for *Phalaenopsis* through a two-step continuous cultivation protocol. All the flowering plants with more than seven flowers are graded as Class A

2. In the second step, as the leaf of the plant is larger than 15 cm (after 3 months of cultivation), it is then repotted with new sphagnum moss to a 12-cm-diameter pot. The plants are supplied with 1.0 g/L Peters 20N–20P_2O_5–20K_2O and watered using reverse-osmosis water when the moisture content of sphagnum moss is near 18–20% (*see* **Note 18**). With a strict fertigation regime based on monitoring of moisture content, the growth rate of the roots and leaves of the plantlets is optimized. After 5 months of cultivation, the plants mature and can be moved to a cool room for spiking.

7 Notes

1. Before deflasking, the plantlets need to be hardened. Move the flasks from the laboratory to the greenhouse and place under an 80% shade cloth for adapting to sunlight for 2 weeks.
2. Prepare a clean stainless steel bench for sorting the deflasked plantlets. Wash and sort qualified plantlets (with three healthy leaves and three roots). Place plantlets in ventilated polyethylene (PE) baskets (appropriate size: 30 × 60 × 10 cm) for the curing process. In the PE basket, do not overlap plantlets to prevent overcrowding. Avoid exposure to rain and direct sunlight. After curing (keep moist but not wet) for 90–120 min, pot the plantlets as soon as possible to prevent leaf-tip dieback due to over dehydration. Pot all the deflasked plantlets on the same day.
3. Gently wrap the roots of a deflasked plantlet with sterilized sphagnum moss, then put it into a pot, and minimize the breakage of roots. If the roots of a deflasked plantlet are too long, it is easy to break the roots when placing into a pot. Fewer wounds on the roots indicate a lower possibility of infection by pathogens and therefore a higher survival rate.
4. Although the plantlets are removed from flasks carefully, some wounding is unavoidably (many invisible wounds). Before the first new root appears, we do not water or fertilize the plantlets directly but give a light mist spray in the daytime, just dampening the leaves and the surface of sphagnum moss.
5. Because the young plantlets are just taken out of flasks, they are sensitive to the changes of the new environment. It is necessary to keep the greenhouse environment as stable as possible. The temperature should not be lower than 20 °C and higher than 30 °C; the light intensity has photon flux density of 180–200 μmol m^{-2} s^{-1}, PAR; the relative humidity should be maintained higher than 85% with good air circulation using fans. The exposures to direct sunlight and rain are strictly forbidden.

6. After harvesting sphagnum moss from the natural environment, if it is not properly processed and cleaned, the sphagnum moss can be mixed with soil and contaminated with pathogenic microorganisms and weed seeds. This will result in a low survival percentage of deflasked plantlets. At present, professional growers in Taiwan usually sterilize the sphagnum moss before use. The sphagnum moss can be treated with steam for 30 min, or 80 °C hot water for 30 min, or 0.05% hypochlorite solutions (calcium hypochlorite or chlorine dioxide) for 4 h. It is important to note that the sphagnum moss treated with steam for too long will decompose quickly after potting.
7. It will take a lot of time to check the moisture content of each pot and to rectify the moisture content with watering by hand. If the sphagnum moss is too wet, it is better to skip a week of watering until the sphagnum moss has dried up slightly.
8. In our investigation, the barks from different sources may have various pH values. For example, pine tree bark is acidic (pH = 5.5–6.5); the pH value should be adjusted by adding $Ca(OH)_2$ before use [3]. Moreover, because Douglas fir is the host for the pathogenic fungus *Phytophthora ramorum* and bark is porous that is difficult to sterilize, the strict quarantine law in some countries does not allow the import of *Phalaenopsis* plants potted in bark mix. In addition, bark has a high carbon-nitrogen ratio, and it may tie up some nutrients during decomposition. Hence, increasing the nitrogen fertilizer application is recommended. The symptoms of nutrient deficiency in *Phalaenopsis* plantlets potted in bark include yellow leaves, leaves losing luster, and a large number of roots growing beyond the edge of the pot [4].
9. Various sizes of bark pieces are available in the commercial market, and it should be carefully selected for different sizes of plants. The coarse piece of bark does not have much capillary action for transferring moisture. The fine piece of bark may hold too much water that resulted in severe root rot. Sheen [4] suggested using the small bark no. 8 (3–6 mm) for a deflasked plantlet potted in a 5–6 cm pot and using bark no. 9 (6–12 mm) for a plant potted in an 11–12 cm pot [3].
10. It is worthy to note that frequent watering of *Phalaenopsis* plants potted in bark usually results in a greenhouse environment being too humid (over 90% RH), and this increases the risk of pathogenic infection. In this situation, it is necessary to increase the ventilation of greenhouses by fans or install dehumidifiers.
11. For a trainee learning how to water, it is necessary to check frequently the root condition and moisture distribution in

potting medium within the pot. The appearance of the roots is a good index of watering. Under a proper watering management, the proximal section of the root will look grayish silver (but not shriveled), and the distal section (the root tip) will be green with a yellowish-white or a pinkish-white color. Through careful and regular observation, it is possible to obtain a more complete picture of watering management.

12. The roots of *Phalaenopsis* are sensitive to salinity, especially the young roots. As the roots have elongated and become mature, they are more tolerant to salinity changes. It is hard to recover, or it can take a lot of time to recover if the roots are damaged.
13. Over the course of *Phalaenopsis* growth, the change in the EC of the potting medium must be regularly measured. From analysis of the drainage water from the pot, it gives us a quick and simple data about the accumulation of elements in the potting medium, e.g., the excess or deficiency of elements.
14. In the practice of watering, each pot must be watered until water flows from the drainage holes of the pots. This not only forces unfavorable gases out the potting medium but also leaches out the extra salts. Typically, the adequate amounts of water required for each small (5–6 cm in diameter), medium (8–9 cm in diameter), and large (11–12 cm in diameter) pots are 100, 200, and 300 mL, respectively.
15. The frequencies of watering or fertigation of *Phalaenopsis* potted in sphagnum moss are as follows: (1) small plants are watered every 7–10 days, (2) medium plants every 10–14 days, and (3) mature plants every 20 days. The frequency of watering or fertigation is variable according to the seasons, the outside climates, and the environmental control parameters inside the greenhouse.
16. It is recommended that a potassium concentration of 200 mg/L in the solution at every irrigation is adequate for the growth and flowering of *Phalaenopsis*.
17. The moisture content of potting medium is measured by the HH2 moisture meter 2.1 (Delta-T Devices Ltd. Cambridge, UK). In our investigation, the potting medium must be allowed to dry at 18–20% of its moisture content before being irrigated again. The potting media, especially sphagnum moss, should not be allowed to dry out completely, because sphagnum moss is not easy to be moistened in a completely dry condition.
18. The EC of the medium should be lower than 1.5 mS cm^{-1}. If the EC is excessive, leaching should be performed using reverse-osmosis water. Without a proper leaching process, the roots will be damaged by salt accumulation in the potting medium.

References

1. Lin FP (2006) Taiwan orchid growers information manual. Taiwan Orchid Growers Association, Tainan, Taiwan
2. Su TB (2009) Effects of substrate water content, fertilizer concentration and culture duration on growth and flowering of two-step relay cultivation in Phalaenopsis hybrids. MS thesis. National Chiayi University, Chiayi, Taiwan
3. Lee N (1988) The growth and flowering physiology of Phalaenopsis plants. The symposium of improving to orchid cultivation in Taiwan. Taitung District Agr Res Ext Sta, Taiwan, pp 13–27
4. Shen TM (2008) The growing medium and its characteristic of physical and chemical in *Phalaenopsis*. In: Shen TM, Hsu ST (eds) *Phalaenopsis* cultivation. National Chiayi University, Chiayi, Taiwan, pp 19–28
5. Kuan SH (2007) Effect of growing media and fertilizer concentrations on the growth of Phalaenopsis. MS thesis. National Chiayi University, Chiayi, Taiwan
6. Shen RS, Chen GY (2008) Water management of *Phalaenopsis*, Phalaenopsis cultivation. In: Shen TM, Hsu ST (eds) *Phalaenopsis* cultivation. Natl. Chiayi University, Chiayi, Taiwan, pp 29–34
7. Wang YT (1998) Impact of salinity and media on growth and flowering of a hybrid *Phalaenopsis* Orchid. HortSci 33:247–250
8. Cui YY, Pandey DM, Hahn EJ, Paek KY (2004) Effect of drought on physiological aspects of crassulacean acid metabolism in *Doritaenopsis*. Plant Sci 167:1219–1226
9. Van der Knaap N (2005) Cultivation guide *Phalaenopsis*: knowledge for professionals. Anthura, B.V., Bleiswijk, Netherlands
10. Yu FL (2004) Effects of sphagnum moss, coir mix medium and enriched slow released fertilizers on the growth of Phalaenopsis. MS thesis, National Taiwan University, Taipei, Taiwan
11. Wang YT, Konow EA (2002) Fertilizer source and medium composition affect vegetative growth and mineral nutrition of a hybrid moth orchid. J Amer Soc Hort Sci 127:442–447
12. Key BD, Hajabbasi MA, Ying J, Tollenaar M (2006) Optimum versus non-limiting water contents for root growth, biomass accumulation, gas exchange and the rate of development of maize (*Zea mays* L.) Soil Tillage Res 88:42–54
13. Lo SS, Lin AP (1994) A study of drip trickle fertilization for *Phalaenopsis*. Res Bull Taitung District Agric Improvement Station 5:37–46
14. Wang YT (2005) Effect of temperature and nutrient on growth of Phalaenopsis cultivars. International symposium of Phalaenopsis, Paphiopedilum alliance, and Taiwan international orchid symposium. Taiwan Orchid Growers Assoc. pp 259–267
15. Wang YT (1996) Effects of six fertilizers on vegetative growth and flowering of Phalaenopsis orchids. Scientia Hort 65:191–197
16. Yoneda K, Suzuki N, Hasegawa I (1999) Effects of macroelement concentrations on growth, flowering, and nutrient absorption in an *Odontoglossum* hybrid. Sci Hortic 80:259–265
17. Wang YT (2007) Potassium nutrition affects *Phalaenopsis* growth and flowering. HortSci 42:1563–1567
18. Wang YT (2008) High NO_3-N to NH_4-N ratios promote growth and flowering of a hybrid *Phalaenopsis* grown in two root substrates. HortSci 43:350–353
19. Wang FN, Chang KH, Hsieh TF, Chung RS (2008) Effects of three fertilizer formulae on growth and nutrient uptake of *Phalaenopsis amabilis* var. *formosana*. J Taiwan Soc Hort Sci 54:231–246
20. Chang KH, Wang FN, Hsieh TF, Chung RS (2008) The effect of three fertilizer formulae on the growth and nutrient uptake of *Phalaenopsis amabilis* var. *formosana* in seedling stage. Taiwanese J Agric Chem Food Sci 46:57–69
21. Wang PH (2006) Effect of fertilizer concentration on vegetative growth of 4.5 cm and 7.5 cm pot seedlings of relay cultivation in Phalaenopsis hybrids. MS thesis. National Chiayi University, Chiayi, Taiwan
22. Chen YJ (2001) Effect of mineral nutrition on growth, mineral composition and leaf quality during stalk emergence of Phalaenopsis sp. MS thesis, National Taiwan University, Taipei, Taiwan
23. Lee N (2001) Research on photosynthesis, flowering physiology and leaf disorders of Phalaenopsis spp., Industrial Science Technology Development Academic Proceedings. Taiwan Sugar Co., Taiwan, pp 22–35
24. Li PC, Lee N (2000) A study of flower sensitivity to ethylene and flower longevity in *Phalaenopsis*. J Chinese Soc Hort Sci 46:491–492
25. Lee N, Lin GM (1984) Effect of temperature on growth and flowering of *Phalaenopsis* white hybrid. J Chinese Soc Hort Sci 40:223–231

Chapter 24

Strategies in Orchid Health Maintenance

Jiunn-Feng Su, Shu-Pei Chen, and Ting-Fang Hsieh

Abstract

In modern agricultural sector, orchid industry is one of the high value-added production systems. To produce healthy orchid plants is the best policy in commercial cultivation system; growers must learn how to create environmental conditions that are suitable for growth of orchid plants with minimum risks of outbreaks of orchid diseases and pests. Effective methods for quick diagnosis, identification, and forecasting of pests and diseases are critical and fundamental. This article is to present an overview of management strategies for problems of diseases and pests that are common in commercial production of orchids. Three major steps of management orchid diseases and pests will be discussed, including periodic inspection for pest problems, the use of clean and healthy seedlings, and effective methods for management of orchid greenhouses.

Key words Orchid, Plant disease, Plant pest, Greenhouse management, Periodic inspection, Clean and healthy seedlings, Integrated pest management

1 Introduction

In the cultivation of orchids, growers often encountered problems of diseases, pests, and environmental stresses, which must be properly managed during the growing season. The effect of environmental stresses could be corrected by growing orchids in environmentally controlled greenhouses. However, disease and pest problems are more complicate and difficult to control. Numerous diseases are known to attack *Phalaenopsis* orchids during the growing season. Orchid growers should inspect their plants frequently for fungal diseases such as anthracnose, leaf yellowing or Fusarium sheath rot, petal blight or gray mold, root rot and bud blight, southern blight, and black rot, bacterial diseases such as bacterial soft rot and bacterial brown spot, and virus diseases such as *Odontoglossum ringspot virus* (ORSV), *Cymbidium mosaic virus* (CymMV), cucumber mosaic virus (CMV), and *Capsicum chlorosis virus*-phalaenopsis isolate (CaCV), as well as pests such as Lepidoptera larvae, scale insects, mealybugs, thrips, fungus gnats, mites, and mollusks. To produce healthy orchid plants, growers

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_24,

must learn how to create environmental conditions that are suitable for growth of orchid plants with minimum risks of outbreaks of orchid diseases and pests. Effective methods for quick diagnosis, identification, and forecasting of pests and diseases are critical and fundamental [1, 2]. This chapter is to present an overview of management strategies for problems of diseases and pests that are common in commercial production of orchids.

2 Diseases and Pests of Orchids

2.1 Diseases

Orchid diseases frequently reported include fungal diseases such as Fusarium yellow, gray mold, anthracnose, *Phytophthora* bud blight, southern blight, and black rot; bacterial diseases such as brown spot, leaf spot, and soft rot; and viral diseases such as *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV). There are three species of *Fusarium*, including *F. solani*, *F. oxysporum*, and *F. proliferatum* that cause different symptoms on *Phalaenopsis* [3]. *Fusarium solani* (teleomorph *Haematonectria haematococca*) causes basal sheath rot (Fig. 1a) and root rot of *Phalaenopsis*, resulting in leaf yellowing and defoliation. In some cases of basal sheath rot, numerous orange-red perithecia were formed on the lesion. *Fusarium oxysporum* caused gray spot on leaves and sheaths of *Phalaenopsis*, resulting in appearance of irregular-to-round-shaped, dark brown color lesions, surrounded by yellow halo, and the lesion center turned into gray color. If the leaf base or sheath is infected, the plants will show symptoms of leaf yellowing. *Fusarium proliferatum* causes black spot of *Phalaenopsis*, resulting in formation of circular to irregular shape, black lesions on diseased sheaths and leaves. In some *Phalaenopsis* cultivars, the leaves of infected sheath often turned yellow. Gray mold of *Phalaenopsis* is caused by *Botrytis cinerea*. Symptoms include formation of water-soaked lesions with dense, gray mold on infected tissues [4]. Anthracnose of *Phalaenopsis* is caused by *Colletotrichum gloeosporioides* (Fig. 1b). Symptoms are formation of sunken, water-soaked, round to oval, regular to irregular, and brownish red to black spots on infected leaves [5]. Two major species of *Phytophthora*, *P. parasitica* and *P. palmivora*, have been reported on orchids [6]. All infected orchid plants, *Phalaenopsis*, *Dendrobium*, and *Oncidium*, develop water-soaked lesions initially. Disease lesions on leaves expand rapidly and turn to dark green or light brown color. Infected plants show symptoms of leaf yellowing and leaf dropping and eventually, plant death. However, *P. palmivora* induces typical black rot symptoms on leaves and pseudostems on affected *Cattleya*. *Phytophthora* spp. mainly attack pseudostems and young buds of *Cymbidium* spp., causing yellowing, wilting, and death of infected plants [6]. Southern blight caused by *Sclerotium rolfsii* is known to occur on several economically important orchid hosts. Symptoms

Fig. 1 The common diseases of *Phalaenopsis*. (**a**) Leaf yellowing of *Phalaenopsis* caused by *Fusarium solani* (teleomorph *Haematonectria haematococca*). (**b**) Anthracnose of *Phalaenopsis* caused by *Colletotrichum gloeosporioides*. (**c**) Leaf spot of *Phalaenopsis* caused by *Burkholderia gladioli*. (**d**) Soft rot of *Phalaenopsis* caused by *Pectobacterium chrysanthemi*

of southern blight are wilt at the apex and with formation of tan, soft, water-soaked lesions at the base of infected plants. As lesions progress, leaves begin to fall off, leaving bare stems. White, mycelial mats are present at the stem base with formation of numerous, tan- to brown-colored sclerotia on the surface [7].

The important bacterial diseases in *Phalaenopsis* are brown spot caused by *Acidovorax avenae* subsp. *cattleyae*, leaf spot caused by *Burkholderia gladioli* (Fig. 1c), and soft rot caused by *Pectobacterium chrysanthemi* [8] (Fig. 1d). *Phalaenopsis* plants infected by *P. chrysanthemi* show symptoms of water-soaked lesions and soft rot of leaf tissues, which turned into dark brown and dry out quickly under conditions of low relative humidity. Old leaves are more resistant to soft rot than young ones. The occurrence and development of symptoms of soft rot are affected by temperature, relative humidity, and inoculum concentration. Incidence of soft rot on *Phalaenopsis* was very high, usually more than 80%, under conditions of high temperatures (above 28 °C), high relative humidity (100%), and high inoculum concentration (1.0×10^{10} cfu/mL) [9]. Symptoms of bacterial leaf spot of *Phalaenopsis* appear

most frequently under humid and warm conditions. Plants are stunted and show soft rot symptoms. Leaf spots are brown in color, surrounded by yellow halos, circular shape or, less frequently, irregular shape, and, sometimes, with cracks at the lesion center under low humidity. Bacterial ooze leaks from the cracks of leaf spots under high humidity [10]. Symptoms of bacterial brown spot of *Phalaenopsis* are formation of dark brown, firm, circular lesions 1–2 mm in diameter on leaves. Lesions are usually surrounded by a chlorotic halo or a water-soaked margin, leading to dieback of infected leaves or peduncles [11]. Symptoms of bacterial brown spot and bacterial leaf spot on *Phalaenopsis* leaves are often similar in early stage of infection and, thus, uneasy to diagnose [8].

Cymbidium mosaic virus (CymMV) and *Odontoglossum ringspot virus* (ORSV) are two of the most prevalent viruses, causing diseases on orchids [12, 13]. CymMV causes chlorotic or necrotic sunken patches on orchid leaves and flowers. Infected flowers show symptoms of deformation and color variegation. ORSV induces streaks or stripe mosaic, mottling, or ringspots on orchid leaves. Infected flowers show symptoms of ringspots and color variegation on petals. Combined infection of CymMV and ORSV resulted in development of severe symptoms on orchid plants [14].

2.2 Pests

Lepidoptera larvae, scale insects, mealybugs, thrips, fungus gnats, and mites are common pests of orchids. In the greenhouses, small pests such as scale insects and mealybugs are usually found on the surface of leaves or flowers of orchids. They are sucking insects causing direct injury to orchid leaves and flowers. Meanwhile, scale insects also secret sticky honeydew that attract colonization and growth of sooty mold fungus on insect-infested orchid plants.

Thrips are sucking insects that are closely associated with orchid flowers. They will continue to grow and multiply on orchid flowers under mild temperature (20–25 °C) [15]. Infestation of flowers of *Cymbidium* spp. by thrips resulted in loss of cell contents of pollen grains and flower tissues, but thrip infestation is rare on flowers of *Phalaenopsis* orchids.

Caterpillars, Lepidoptera larvae, are chewing insects which often caused severe damage to young leaves and, therefore, reduced market value of orchid seedlings. Adult females usually lay eggs on dorsal surface of leaves, and larvae from hatched eggs caused damage to orchid plants by feeding on leaves from evening to dawn (Fig. 2a, b). Fungus gnats (*Bradysia* spp.) (Fig. 2c, d) are common pests of *Phalaenopsis* in gardens. It takes 20–25 days for fungus gnats to complete a life cycle. Larvae of fungus gnats feed on young seedlings, causing damage at seedling base and, eventually, death of the infested seedlings.

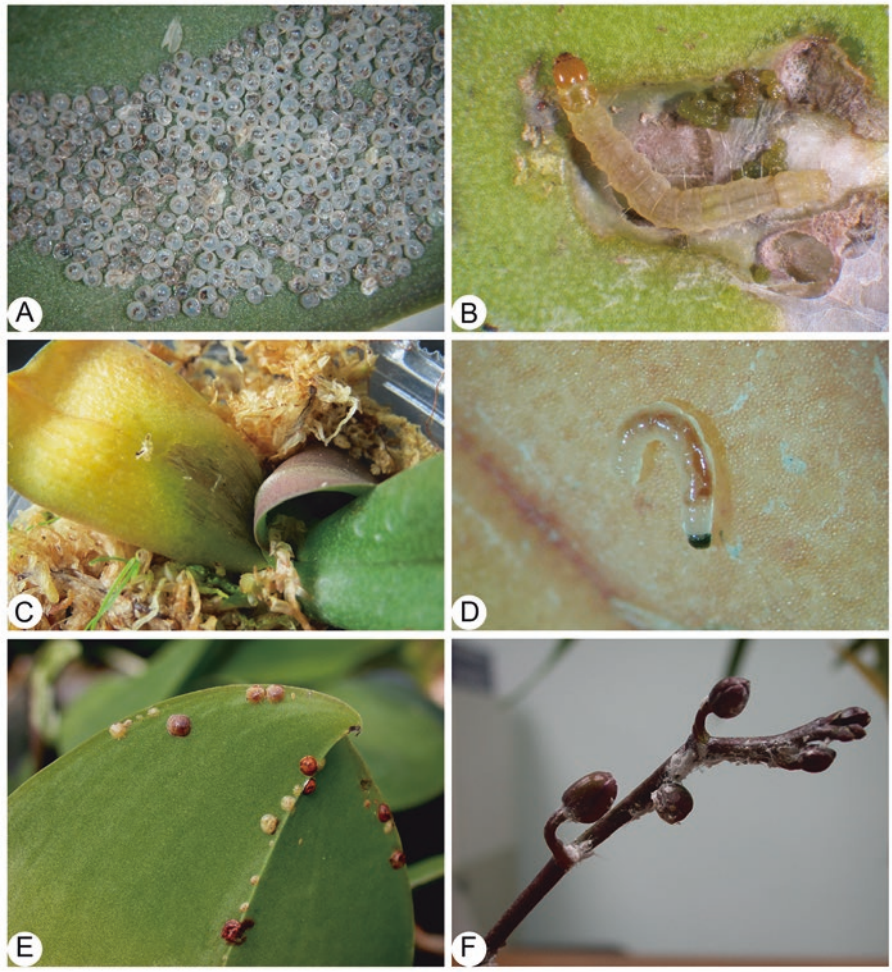


Fig. 2 The common pests of *Phalaenopsis*. Eggs (**a**) and larvae (**b**) of moth (Lepidoptera: Pyralidae) on leaves of *Phalaenopsis*. Yellow leaves of *Phalaenopsis* (**c**) caused by fungus gnats (*Bradysia* spp.) (**d**). Scale insects or mealybugs on leaves (**e**) and buds (**f**) of *Phalaenopsis*

Scale insects and mealybugs (Fig. 2e, f) are usually found on the surface of leaves or flowers of *Phalaenopsis* orchids. They cause damage by sucking juice from plant tissues. The sticky honeydew produced by these insect pests also attracts colonization of sooth mold fungi on leaves of *Phalaenopsis* plants [16].

Mites (Fig. 3a, b) are frequently found on the dorsal surface of leaves of *Phalaenopsis* under high temperature and low humidity environments. Mites feed and breed on both sides of a leaf but prefer the dorsal surface of leaves. They cause damage to *Phalaenopsis* leaves with orange-colored mites and eggs on leaf surface and numerous small, silver-colored spots on the dorsal surface of leaves. Severe infested leaves turn into yellow color and withered.

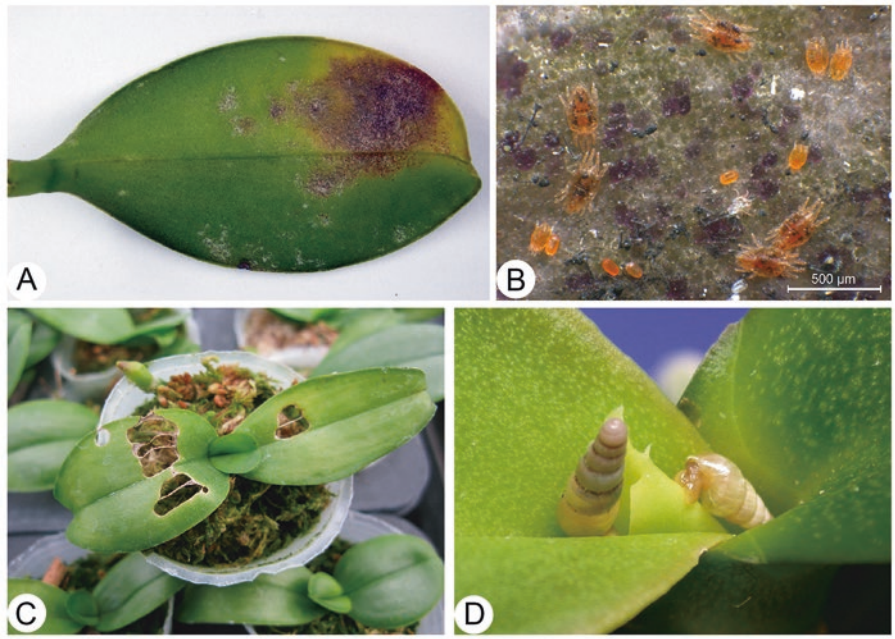


Fig. 3 The common pests of *Phalaenopsis*. Yellow or brown spots on a dorsal surface of *Phalaenopsis* leaf (**a**) caused by mites (**b**). Irregular-shaped holes on *Phalaenopsis* leaves (**c**) caused by snails (**d**)

Mollusks (snails and slugs) (Fig. 3c, d) can be found in humid areas of greenhouses. They are often present on plants in open fields during the day or at night or under objects such as wood logs and rocks. However, snails and slugs are rare in modern greenhouses [17].

3 Prevention and Control of Orchid Diseases and Pests

Over the agricultural history, progress of plant disease management has experienced four major phases: (i) limited intervention in ancient farming systems, (ii) mechanical and temporal disease suppression approaches (rogueing, plowing, rotations), (iii) widespread use of major gene resistances and chemical pesticides before and following the first green revolution, and (iv) integrated pest management and ecological management emphasizing synergic effects on economy, human society, agricultural environments, and natural environments [18]. Ecological management of plant diseases is to develop and apply disease control methods that are environmentally friendly and ecologically sound for maximizing crop production [19]. No single disease control strategy works for all plant pathogens or for one pathogen in all environments.

Disease control programs that combine strategies to reduce pathogen population sizes, limit pathogen dispersal, and force pathogens to endure fluctuating, diversifying selection forces have

the best prospects for reducing the emergence of new strains of pathogens that increased virulence to host plants and increased resistance to chemical pesticides. Such an integrated approach would have good potential for long-term management of plant diseases [19]. This article focuses the discussion on three major steps of management orchid diseases and pests, including periodic inspection for pest problems, use of clean and healthy seedlings, and effective methods for management of orchid greenhouses.

4 Periodic Inspection

4.1 Periodic Inspection of Greenhouse Facilities

Inspection of greenhouse facilities is of paramount importance to prevent orchid pests from entering into greenhouses that are used for orchid production. First step is to ensure structural integrity of the greenhouse. The ventilation ducts are completely covered with insect net without breakage to prevent pests such as moths or butterflies from flying into greenhouse. The hole of insect nets must be smaller than 0.6 mm. All the greenhouse doors are functioning normally. Ideally, concrete floors are used for the greenhouse floor for orchid production, and the floor must be cleaned and checked for cracks at all time.

4.2 Regular Inspection of Plant Bed

The plant beds for orchid production must be kept clean without contaminations of sand, weeds, and plant debris such as leaves or other planting substrates.

4.3 Periodic Inspection of Cultivation Medium

Sphagnum and bark are common growth substrates for orchids, and they must be treated with hot water or steamed at 80 °C for 30 min or treated with insecticide or fungicide before used for planting of orchids (Fig. 4a). After disinfection, the medium must

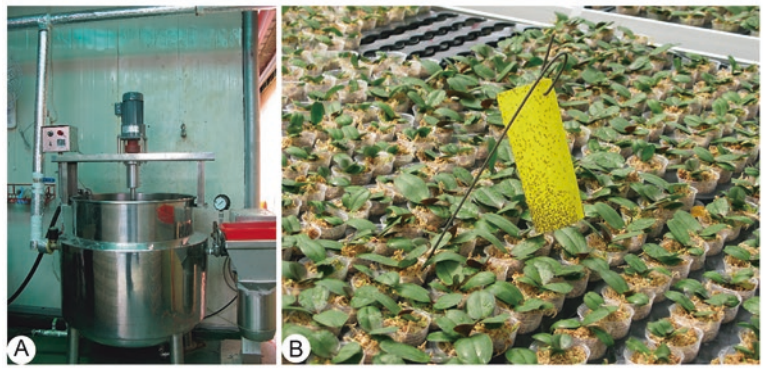


Fig. 4 (**a**) Sterilized tissue culture medium stored in a clean tank to prevent from contamination by pests or pathogens. (**b**) A paper covered with yellow sticky substance was hanging above *Phalaenopsis* seedlings to trap orchid pests

be stored in a clean area to prevent risks of contamination by pests or pathogens. Meanwhile, the growth medium in planting pots must be checked periodically for contamination of pathogenic fungi and parasitic nematodes.

4.4 Periodic Inspection of Irrigation Water

Water used for irrigation of orchid plants includes tap water, boiled or disinfested rain, or well water. Untreated river water is not recommended because of potential risks of microbial contaminations. It is important to test the irrigation water in orchid farms, periodically, for possible contamination of pathogenic fungi, parasitic nematode, or other microorganisms that are harmful to orchids.

4.5 Regular Inspection of Orchid Pests and Diseases in Greenhouse

Cardboards covered with yellow sticky substances are hanged inside and outside the greenhouse to trap orchid pests. These insect-trapping cardboards should be replaced monthly. Species of insects collected on the cardboards from inside and outside the greenhouse must be identified in order to identify the presence of harmful pests and develop proper pest control strategy. Also, UV lamps are used as insect traps for catching flies and moths that are present in the greenhouse. It is more effective to control orchids in the greenhouse by hanging UV lamps or sticky cardboards at 20–30 cm above the orchid plants (Fig. 4b).

4.6 Periodic Inspection of Log Sheet

Log sheet is used in the cultivation of *Phalaenopsis*. All the events involved in the operation of *Phalaenopsis* cultivation should be recorded in detail in the log sheet, including cleaning of greenhouse facilities, pest control, and so on. The log sheet should also contain detail information on time and number of orchid plants transferred between greenhouses.

5 Use of Clean and Healthy Seedling

Tissue culture techniques are used for asexual reproduction of orchid seedlings. In the culture process, virus-free testing is one of the most important events in indexing tissue culture materials for virus pathogens. Virus diseases caused by *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV) are economically important to orchid industry. Indexing techniques for producing virus-free seedlings of orchids are ELISA, immunoblotting test, DNA probe hybridization, and RT-PCR. Virus-free seedlings with 2–3 roots were selected, washed to remove residues of the culture medium on roots, and then transplanted in pots. Orchid plants showing symptoms of diseases should be eliminated throughout the entire cultivation process. Information of pest status is required when orchid seedlings are transferred from one greenhouse to another.

6 Greenhouse Management

6.1 Prevented Pests Entrance

Orchid workers may play a role in prevention of orchid pests from entering greenhouses. Proper inspection of orchid seedlings is required to ensure that only clean and pest-free seedlings are transferred from outside to the greenhouse.

6.2 Remove Disease Plants

Orchid plants in the greenhouse should be inspected periodically for symptoms of diseases, and the diseased plants should be removed immediately. For example, bacterial diseases are common on orchid plants in the greenhouse, and they should be eliminated to prevent further spread of these diseases to other healthy plants.

6.3 Disinfestation of Tools and Pots

Tools and pots for cultivation of orchids should be cleaned and treated with surface disinfectants. Disinfectants recommended for orchids are 5.23% bleach solution diluted with water at 1:100 ratio and chlorine dioxide at 50 ppm.

6.4 Precisely Regulated Temperature and Humidity

Temperature and humidity are important factors affecting occurrence and prevalence of orchid pests and diseases in the greenhouse. In general, higher temperature (above 30 °C) and lower humidity (below 70% RH) may cause a significant increase of mite population in the greenhouse [20]. Methods recommended for control of temperature and humidity in the greenhouse include increased greenhouse ventilation and convection, reduced frequency of watering of growing medium in pots, avoidance of watering on floor, and the use of dehumidifiers.

6.5 Adjust Plant Density

High plant density in greenhouse production of orchids is commercially profitable. However, high plant density is a conducive factor affecting outbreaks of orchid diseases and pests. Thus, avoiding high plant density by reducing leaf overlapping between plants is required to produce healthy orchid plants in commercial greenhouses.

6.6 Application of Fungicide and Insecticide

Chemical pesticides are used for effective control of plant pests and diseases. However, due to existence of a wide range of varieties of *Phalaenopsis*, growers must pay specific attention to whether the pesticide they applied can cause phytotoxicity on their orchids. Several fungicides are recommended for control of Fusarium diseases of orchids, including 50% prochlorate manganese WP (wettable powder) diluted 6000 times, 25% prochloraz EC (emulsifiable concentrate) diluted 2000 times, and 23.6% pyraclostrobin EC diluted 3000 times [21]. For control of anthracnose of orchids, the recommended fungicides are 25% prochloraz EC diluted 2500 times, 50% prochlorate manganese WP diluted 6000 times, 70% propineb WP diluted 500 times,

and 62.25% mancozeb + myclobutanil WP diluted 600 times. For control of gray mold of orchids, the recommended fungicides are 78% thiophanate-methyl WP diluted 2500 times, 50% iprodione WP diluted 1500 times, and 50% procymidone WP diluted 2000 times. For control of *Phytophthora* blight of orchids, the recommended fungicides are 66.5% propamocarb hydrochloride SL diluted 1000 times, 33.5% oxine-copper SC diluted 1500 times, 25% etridiazole EC diluted 1000 times, and 35% etridiazole WP diluted 1500 times. For control of southern blight of orchids, the recommended fungicides are 50% flutolanil WP diluted 3000 times, 75% mepronil WP diluted 1000 times, and 50% tolclofos-methyl WP diluted 1000 times. For control of bacterial diseases of orchids, the recommended bactericides are 30.3% tetracycline hydrochloride SP diluted 1000 times, 77% copper hydroxide WP diluted 400 times, 40% copper hydroxide + oxine-copper WP diluted 400 times, 68.8% thiophanate-methyl + streptomycin WP diluted 1000 times, 10% streptomycin + tetracycline hydrochloride SP diluted 1000 times, and 20% oxolinic acid WP diluted 1000 times.

A wide array of chemicals has been used to control pests of *Phalaenopsis*. For example, contact insecticides such as fluvalinate, malathion, and others are used for control of thrips and scale insects on orchid flowers, insecticidal soap for control of mealybugs and scale insects, and bifenthrin, cyhalothrin, cyromazine, deltamethrin, fenvalerate, malathion, mevinphos, and phosalone for control of thrips [22]. Snails are controlled by 6% paraldehyde bait, and slugs are controlled by calcium nitride, salt, and lime. Many chemicals with acaricidal properties are available, but only a few chemicals are effective in killing eggs and young and adult mites at the same time. For effective chemical control, careful considerations should be made, including pesticide application methods, potential problem of phytotoxicity on orchid plants, and potential danger of development of pesticide resistance to orchid pathogens and pests. Chemicals for *Phalaenopsis* pest and disease control are listed in Table 1.

6.7 Consideration of Chemical Control

Chemical pesticides are often used to control orchid diseases and pests when other pest management approaches are inadequate or failed. However, using a fixed and static strategy of pesticide application for control of orchid pests not only reduces management efficiency and increases costs but also brings numerous negative effects to the environment and the society, including potential problems of pesticide toxicity to human and livestock and ecological degradation [18]. During pesticide application, factors such as mode of pesticide action and problem of pathogen resistance should also be considered [23]. To maximize efficiency of a chemical pesticide and minimize its

Table 1
A list of chemicals for the control of pests and diseases of *Phalaenopsis* plants

Common names	Action	Formulation types	Trade name, for example	Supplier
Copper hydroxide	Bactericide	SC, WP	Coproxide	VAPCO
Cyhalothrin	Insecticide	EC, WP	Cyhalon	Syngenta
Cyromazine	Insecticide	SL, WP	Trigard	Syngenta
Deltamethrin	Insecticide	DP, EC, EG, EW, GR, HN, PO, SC, SL, TB, UL, WG, WP	Decis	Bayer
Etridiazole	Fungicide	DP, EC, ME, WP	Terrazole	Crompton
Fenvalerate	Insecticide	EC, SC, UL, WP	Arfen	Ramcides
Flutolanil	Fungicide	DP, GR, SC, UL, WP	Moncut	Gowan
Fluvalinate	Insecticide	EC, EW, UL, VP	Klartan	Makhteshim-Agan
Iprodione	Fungicide	DP, EC, FS, SC, SU, WG, WP	Amazzones	Rocca
Malathion	Insecticide	DP, EC, EW, UL, WP	Fyfanon	Cheminova
Mepronil	Fungicide	DP, SC, WP	Basitac	Kumiai
Mevinphos	Insecticide	EC, SL	Phosdrin	Amvac
Oxine-copper	Fungicide	DS, PA, SC, WP	Quinondo	Agro-Kanesho
Oxolinic acid	Bactericide	DP, SD, WP	Starner	Sumitomo
Prochloraz	Fungicide	EC, EW, FS, LS, WP	Sportak	BASF
Procymidone	Fungicide	DP, HN, SC, SP, WG, WP	Sumilex	Sumitomo
Propamocarb hydrochloride	Fungicide	SC, SL	Banol	Bayer
Propineb	Fungicide	DP, WG, WP	Antracol	Bayer
Pyraclostrobin	Fungicide	EC, SE, WG	F 500	BASF
Streptomycin	Bactericide	WP	Cuprimicin 17	Ingenieria industrial
Thiophanate-methyl	Fungicide	DP, PA, SC, WP	Topsin M	Cerexagri
Tolclofos-methyl	Fungicide	DP, EC, SC, WP	Rizolex	Sumitomo

negative impacts on the environment, information on pest forecasts and knowledge of population dynamics of the pest are required to determine the best time and frequency of pesticide application [19] and to choose the type and dosage of the pesticide for control of a specific pest [24].

7 Additional Comments and Notes

1. Once the pests and diseases occur, correct diagnosis will be done to make sure what kind of pest or disease causes this damage at first. Then try to identify the real causal agent by means of pest and disease checking list or with the help of microscopic observation. More often than most people imagine, the cause of damage often be misidentified. Damage can also be the result of other factors such as incorrect irrigation, herbicide toxicity, or physical damage.
2. Based on the diagnostic results, carefully select an appropriate chemical compound that is effective against your pest or disease and also poses the least risks to human health and the environment.
3. Before purchasing a pesticide, please check the label to make sure it is appropriate to use on orchid plants or treatment site. When purchasing chemicals, check the name, trade name, active ingredient, use targets, how much to use, how and when to apply, and manufacturer's information, and pay attention to the shelf life. In addition, it will be noticed how to take the emergency and first aid measures if someone has been exposed and how to properly store and dispose of the pesticide and empty containers. Before chemical application, recognize the label once it is needed to avoid misuse of pesticides.
4. Pesticides are toxic, so they need to be stored in the cabinet, and the cabinet is best to lock.
5. Orchids include a wide range of varieties; each orchid species has different responses to the pesticides according to the cultivation environment. It is necessary to check pesticide efficacy in a small area and to confirm it no injury to orchid seedlings before scale-up application a pesticide. Then it can be applied in a large-scale area.
6. Each application is preferable with a single dose of pesticides. When applied to leaves or petals, a spreading agent may be added.
7. Please check according to the recommended concentration of pesticides, and do not increase the concentration at will to make sure the operator can accurately deploy the concentration of drugs and the amount of application. At the same time, let the operator own the ability to adjust the machine, and the machine can be quantitative and uniform spraying liquid.
8. Wear protective clothing to protect operator from exposure even when applying the safest pesticides. Minimally, protective gear should include rubber gloves, eye protection, a long-sleeved shirt, long pants, and closed shoes.
9. Do not apply pesticide when rainy, windy, or high-temperature weather. The best time to apply pesticide is the period time of

after 3:00 pm and before sunset, and there is a need to close the shading net. Applying pesticide should always be in the direction of the wind, and do not stand at anti-wind direction.

10. Do not stay in the orchid garden after applying the pesticides, especially in the hermetic or semi-hermetic greenhouse.
11. All equipment should be thoroughly cleaned after each application to avoid the different pesticides mixed with each other and caused phytotoxicity to crop. Wash all the gloves, boots, waterproof clothing, and eye protection to avoid soaking as this can spread contamination over previously clean surfaces. Clean any reusable face masks, and replace any cartridge filters as required, following the manufacturers' instructions.
12. After application, operator should remove cloth immediately, clean contaminated cloth, and then take a bath. If in a case of suspected poisoning, seek medical help immediately.
13. All empty containers and waste must be collected safely and properly handled, rather than casually disposed of.
14. All of pesticide application processes must be recorded in detail as the reference for pest management.

8 Conclusion

The objective of this article is to discuss occurrence and management of diseases and pests of orchids. Major diseases important to commercial production of orchids are fungal diseases such as Fusarium yellow, gray mold, anthracnose, *Phytophthora* bud blight, southern blight, and black rot; bacterial diseases such as brown spot, leaf spot, and soft rot; and virus diseases such as *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus*. Major insect pests are Lepidoptera larvae, scale insects, mealybugs, thrips, fungus gnats, and mites. Methods of cultural practice, sanitation, and chemical control are used to minimize economic loss to commercial orchid productions caused by problems of diseases and pests. It is of paramount importance to develop and adopt integrated pest management (IPM) techniques that are not only economically feasible but also environmentally friendly and ecologically sound for commercial production of orchids.

References

1. Ann PJ, Hsieh TF (2004) The occurrence and management of orchid diseases caused by fungi and bacteria in Taiwan. In: Proceeding of the 8th Asia Pacific Orchids Conference, Tainan, Taiwan, pp 329–342
2. Miller SA, Beed FD, Harmon CL (2009) Plant disease diagnostic capabilities and networks. Annu Rev Phytopathol 47:15–38
3. Su JF, Lee YC, Chen CW et al (2012) Field surveys for Fusarium diseases of *Phalaenopsis* in

Taiwan. Plant Pathol Bull 21:115–130. (in Chinese with English abstract)

4. Baiswar P, Chandra S, Kumar R, Ngachan SV, Roy AR (2008) First report of leaf blight of Chinese ground orchid (*Phaius tankervilliae*) caused by *Botrytis cinerea* in India. Plant Dis 92:1586
5. Gautam AK (2014) *Colletotrichum gloeosporioides*: biology, pathogenicity and management in India. J Plant Physiol Pathol 2:2
6. Ann PJ (1995) *Phytophthora* disease of orchids in Taiwan. Plant Pathol Bull 4:152–162
7. Cating RA, Palmateer AJ, McMillan RT (2009) First report of *Sclerotium rolfsii* on *Ascocentrum* and *Ascocenda* orchids in Florida. Plant Dis 93:963
8. Hseu SH, Shentu H, Sung CJ (2012) Multiplex PCR-based rapid detection of bacterial diseases on *Phalaenopsis* orchid. Plant Pathol Bull 21:91–100
9. Wey GC, Chen WH (1998) Influence of temperature and relative humidity on the incidence of soft rot of orchid *Phalaenopsis*. Rep Taiwan Sugar Res Inst 159:43–57
10. Keith LM, Sewake KT, Zee FT (2005) Isolation and characterization of *Burkholderia gladioli* from orchids in Hawaii. Plant Dis 89:1273–1278
11. Hseu SH, Sung CJ, Shentu H (2011) A specific primer pair for diagnosis and detection of bacterial brown spot pathogen: *Acidovorax avenae* subsp. *cattleyae* on *Phalaenopsis* orchid. Plant Pathol Bull 20:52–64
12. Koha KW, Lua HC, Chana MT (2014) Virus resistance in orchids. Plant Sci 228:26–38
13. Zettler FW, Ko NJ, Wisler GC et al (1990) Viruses of orchids and their control. Plant Dis 74:621–626
14. Kim SM, Choi SH (2015) Simultaneous detection of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* in orchids using multiplex RT-PCR. Virus Genes 51:417–422
15. Tang CC (1974) Studies on the population dynamics and control of asparagus thrips. In: Research report of asparagus cultivation. Publ Dept of Agr and For, Taiwan Provincial Government, ROC, pp 60–62 (in Chinese with English summary)
16. Wong CY, Chen SP, Chou LY (1999) Guidebook to scale insects of Taiwan. (In Chinese) Taiwan Agricultural Research Institute, Taichung, Taiwan
17. Wen HC (1990) Seasonal occurrence and chemical control of *Bradybaena similaris* on sponge gourd. J Agri Res China 39:228–233 (in Chinese with English summary)
18. He DC, Zhan JS, Xie LH (2016) Problems, challenges and future of plant disease management: from an ecological point of view. J Integr Agric 15:705–715
19. Zhan J, Thrall PH, Papaïx J, Xie L, Burdon JJ (2015) Playing on a pathogen's weakness: using evolution to guide sustainable plant disease control strategies. Annu Rev Phytopathol 53:19–43
20. Gerson U, Weintraub PG (2012) Mites (*Acari*) as a factor in greenhouse management. Annu Rev Entomol 57:229–247
21. Su JF, Lee YC, Chien LY, Hsieh TF (2015) Evaluation of fungicides for growth inhibition of "Phalaenopsis" Fusarium pathogens. J Taiwan Agric Res 64:32–44. (in Chinese with English abstract)
22. Wang CL (1982) Chemical control of insects on chrysanthemum cutflower. J Agri Res China 31:339–346
23. Siegwart M, Graillot B, Lopez CB, Besse S, Bardin M, Nicot PC, Lopez-Ferber M (2015) Resistance to bio-insecticides or how to enhance their sustainability: a review. Front Plant Sci 6:381
24. van den Berg F, van den Bosch F, Paveley ND (2013) Optimal fungicide application timings for disease control are also an effective anti-resistance strategy: a case study for *Zymoseptoria tritici* (*Mycosphaerella graminicola*) on wheat. Phytopathology 103: 1209–1219

Part VI

Showcasing Orchids

Chapter 25

Macro Flower Photography: An Introduction to Photographic Principles and Techniques

Mendel Perkins

Abstract

Photography is a good way to capture and communicate scientific information. Knowledge of some key principles of photography may greatly improve the results achieved. Examples of the setup of photographic equipment and photographs are included as a guide to the reader.

Key words Photography, Digital cameras and accessories, Stereomicroscope, Orchid

1 Introduction

Like many other fields, science uses visual aids to help clarify a complex subject that large amounts of descriptive text may fail to adequately capture. Diagrams can be useful; however, they are limited by the author's drawing ability. Also, rendering of a diagram can introduce some subjectivity into the representation of the subject. Photography can be a more objective media, and information can be captured readily with proper photographic equipment.

1.1 The Importance of Scientific Photography

Photography can contribute to many aspects of scientific studies (*see* **Note 1**). In the study of orchids and plants in general, it is often relevant to consider the morphology and development of plants in the context of environmental and ecological interactions. For this reason, some information about the habitat of the plant is useful in studying the plant. Photographs can efficiently capture the range of information available at the site where the plant is found naturally. It would be difficult to verbally describe all the details that a picture can capture. A photograph also allows for re-examination and reinterpretation of the scene in a different way than notes and diagrams could afford. Photographs are important alongside diagrams in describing the characteristics of new species such as this recently described species from Vietnam [1]. In the

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_25,

study of development, such as changes during orchid seed germination and protocorm and plantlet formation, changes occur at a timescale of hours, days, months and years. It is often difficult to comprehend changes at this pace. Photography can be a valuable aid as it makes possible to show images taken throughout a long-time range side by side or in sequence. Time-lapse cameras have been used to study shrub and herb development over an entire growing season, and observations made in that manner were shown to be consistent with more conventional field observation techniques [2]. Modern photographic equipment can, to a certain extent, be automated allowing for capturing of relatively infrequent events such as pollination. For example, a waterproof camera with a timer was used in the habitat of the *Calanthe izuinsularis* orchid to capture the identity and behaviour of that species' pollinator [3]. It would have been difficult or impossible for the authors to take those photographs without potentially disturbing the pollinating insects.

Careful observation in conjunction to macrophotography is a simple and yet powerful approach in studying how plants or in vitro explants respond to the experimental treatment [4]. The specimens can be studied using a hand lens, a stereomicroscope or simple methods, such as freehand sections. The observation can be recorded using different types of cameras. Recommendations of photography through a stereomicroscope can be found in the literature [4, 5]. Through careful observations, additional experiments can be designed for detailed analysis, using other protocols.

1.2 Art and Science

With some modest effort, technically better and more aesthetically pleasing pictures can be taken. Very often in science, the subject of study is interesting in an academic and scientific sense, yet beautiful at the same time. Unfortunately, it is often the case that a good scientific story about a very beautiful species is presented in a respected journal with photographs of relatively low quality. It may be possible to raise the reader's interest in the paper and understanding of the claims by aspiring to higher-quality photographs wherever possible. In particular, the orchid family is so biologically interesting while, at the same time, offering a range of diverse beauty.

It is likely that at some point, a grower of orchids would like to take a descriptive and flattering photograph of his/her plants, particularly the flowers. Many people have some level of experience with photography and may even have a camera of fairly good quality. Flowers are often smaller than the subjects that most people have experience photographing. Most commonly, people have experience taking photos of landscapes and other people. When a camera is used to photograph something much smaller than typical subjects, such as a flower, some effects that are not very apparent

in more common circumstances can become very important to the quality of the image. Because people typically do not have experience with these effects when they attempt to take macro photos, the results may be disappointing.

The aim of this chapter is to give the lay photographer some information and techniques that may significantly improve their results when photographing flowers and also to introduce some slightly more advanced equipment and techniques for those seeking even higher levels of image quality. The techniques of photography through a stereomicroscope based on Yeung [5] are also included for completeness.

2 Photographic Principles Relevant to Macrophotography

Someone with an interest in flowers may want to capture the overall appearance of a flower or even move in closer to show details of the individual floral organs. In these cases, where the aim is to render the subject larger than it is in real life, magnification is required.

2.1 Magnification

2.1.1 Focal Length of the Lens

The magnification that can be achieved by a lens is determined by two primary factors: the focal length of the lens used and the minimum focussing distance of the lens. The larger the focal length of a lens, the closer the subject when viewed through the lens. For example, a sports photographer would use a long focal length lens to capture an image of a football player making a catch across the field, while a landscape photographer would use a short focal length lens to capture a broad view of a mountain range surrounding a valley.

2.1.2 Focussing Distance

The second factor is the focussing distance of the lens. Almost all lenses are capable of focussing at what is called infinite distance, which means that by default they can capture distant objects in sharp focus. What is most important to the macrophotographer is the minimum focussing distance that a lens can achieve. This can vary widely from one lens to another and is a major criterion in selecting a lens or camera for macrophotography.

It may seem that a very long focal length lens with a very close focussing distance would make a good macrophotography lens. In a simplistic sense in order for an optical system to focus on a more close-by object, the lens must move away from the optical plane of the optical sensor. The longer the focal length of the lens, the more the optical components need to move in order to focus. This combined with other optical properties of lenses means that there is a trade-off where the longer the focal length of a lens, the farther away its minimum focussing distance becomes. The result is that most macrophotography is done with more intermediate focal

lengths where the trade-off results in more optimal magnification. Another implication is that typically a lens capable of zooming, which is generally the type found on common compact cameras, will be able to focus closest at its widest zoom setting that encompasses the broadest field of view.

In fact, what is commonly referred to a camera lens is usually a collection of individual glass elements making up an optical system. Within a photographic lens there is a mechanism that will move either the whole lens assembly consisting of several individual glass pieces or a subset of the individual glass pieces in order to focus the lens. This mechanism will have a limited range of motion, and it is this range of motion that limits the available focussing distances of that particular lens. When selecting equipment, it is important to note the minimum focussing distance and magnification that can be achieved.

2.2 Lighting

It is important to remember that photography is a light centric pursuit. A literal translation of photograph is light-drawing. Often a photographer can be consumed with the choice of and arrangement of various equipment, accessories and subject matter. It is important to remember that the product of a photographic effort is determined entirely by the pattern of light captured by the camera and that all of the other details merely change aspects of the light that is captured by your camera.

The quantity, quality and direction of light falling on a subject will greatly impact the way that subject appears. The quantity of light simply refers to the intensity of a given light source. The quality of light is a much broader term that encompasses many factors. One of the most important factors is the hardness or softness of light. When you hold up your hand in the light cast by a bare light bulb, the shadow that is cast has a clearly defined edge and that is said to be hard light. If you hold up your hand below a common fluorescent fixture and look at the shadow, you would see a shadow, but the edges would be very diffuse, and you could say that is a soft light. In general, harder light sources tend to bring out the texture and finer details of subjects, while softer light sources tend to smooth out finer details. The hardness of a shadow is proportional to the size of the subject, so a light that may illuminate a human face in a very harsh way may appear fairly soft on a small flower.

The direction of light is also critical to the appearance of the subject. It is quite common for photographers to take photographs using the flash built into their cameras. These photos tend to have a particular look to them; they lack a sense of depth. When the main light source comes from the same direction that the picture is being taken, the shadows formed fall straight back from the subject, and little information on the depth and 3-D structure of the subject is recorded. During photography of a flower, there are usually some organs protruding outwards, some petals at different

angles and a great deal of fine detail to see. If the main source of light is off to one side of the subject, some of the shadows that form will be cast across the subject, emphasizing the dimensionality and surface character of the subject. For any given subject, it is a productive approach to move the light source from one side to the other and from different angles and heights until a desirable relative position between light and subject is found.

2.3 Depth of Field

The term depth of field refers to the range of distance in front of the lens that is rendered in focus. An image is said to have a wide depth of field if both close-by objects and far-away objects are rendered sharply. An image is said to have a narrow depth of field when only a certain portion of the image is in focus, while closer and farther away objects become blurry. When taking a photograph, three factors affect the depth of field: the focal length of the lens, the aperture of the lens and the focus setting of the lens. In general, the longer the focal length of the lens, usually expressed in mm, the narrower the depth of field. The wider the aperture of the lens, the narrower is the depth of field. Aperture refers to a variable opening within the lens assembly that can open and close to admit differing amounts of light. Aperture has a counter-intuitive scale where *f*2.8 represents a larger aperture than *f*4.0. The *f* stop value represents the denominator of a fraction of the relative radius of the hole admitting light. The focus setting of a lens also impacts the depth of field. The closer the lens is focussed, the narrower the depth of field gets.

2.4 Exposure Times

While it may seem instantaneous, a camera snap is actually a carefully measured time period where the sensor is exposed to the light coming in through the lens. Movement of either the camera or the subject during the time of the exposure could result in a blurry image. If the subject moves enough during the exposure, the motion will be rendered as blurry portions of an image. For example, many photographs of cars show some levels of motion blur in the wheels since they move enough even during a short exposure to be picked up as a blur. If the camera moved relative to the subject during the exposure, the entire frame might be blurred as the image projected on the sensor by the lens moves during the exposure. Many photographs are taken while the camera is held in the photographer's hands, which for relatively short exposures are still sufficient to yield sharp images. A general rule of thumb is that an exposure of 1/100th of a second or shorter with a handheld camera will yield a sharp picture. This rule of thumb is less useful at smaller scales. When the camera and subject are placed close together and the lens is focussed close-up to magnify the subject, any movement of either the camera or subject will be magnified as well, so more careful attention should be placed on limiting motion.

For example, if you were to take a photograph of a person where the person's height was the same as the frame and during the exposure that person's hand moved 1 mm during the exposure, the resulting blur would be nearly imperceptible. If you were instead taking an exposure of a floral organ where a small flower a few cm in size fills the frame and that floral organ moved 1 mm, there would be noticeable blurring of the subject.

The same 1/100th of a second exposure is therefore much more likely to capture motion blur from either movement of the subject or of the camera. For this reason attention needs to be paid to the shutter speed and stability of the subject and the camera. The easiest way to stabilize a camera is to use a tripod. Almost all cameras have an industry-standard threaded hole in the base that fits a standard threaded bolt on most tripods. A small tabletop tripod can be used with smaller compact cameras. SLR cameras, which use the single-lens reflex viewfinder system, require more robust tripods with metal or carbon fibre legs. Stronger and heavier tripods generally minimize vibration of any type of camera.

Self-timer functions can also be helpful, if it is available on the camera. When pressing the shutter button, the camera usually moves and vibrates a little, even if it is attached to a tripod. By using the timer, the button may be pressed, several seconds will pass, and then the exposure will be taken. By allowing some time to pass between touching the camera and the exposure, any vibrations and movements in the camera may dissipate, and a sharper image may result. An alternative is to use an accessory, called a cable release; it allows the photographer to trigger the shutter using a button connected to the camera with a section of cable that isolates hand movements from the camera.

2.5 Camera and Accessories

The most cost-effective approach to taking relatively high-quality macro-images is to use a mid- to higher-calibre compact consumer digital camera. One advantage that small cameras have is small sensor sizes and small lenses. Therefore, the focussing mechanism can have a wide range of motion, and quite a close focus can usually be obtained. The all-in-one nature of these cameras does mean that whatever capability the camera has is fixed since the lens cannot be removed or altered. The small flash unit that is usually included in a compact camera is usually of little use to a macrophotographer. It only illuminates from the front, and the lens protruding from the front of the camera will even shade very close subjects. The characteristics to look for when choosing a camera are presence of a tripod mount, an ability to override automatic focus systems, a self-timer function and the minimum focussing distance.

2.5.1 Compact Cameras

2.5.2 SLR Cameras

SLR cameras are cameras that use the single-lens reflex viewfinder system, which means that when you look through the viewfinder, the image that you see is coming from the same lens that will take the final image. There is an angled mirror in place between the lens

and the sensor that redirects the light to the viewfinder eyepiece. When an exposure is taken, this mirror will move up and out of the way allowing the sensor to be exposed to the light. Almost always SLR cameras have interchangeable lenses, which is their most important characteristic.

Using a camera with interchangeable lenses means that the photographer has the opportunity to use different lenses for different purposes. By using lenses suited to each purpose, a superior result can be achieved. In addition, the removable nature of the lenses means that other accessories can be used.

SLR cameras typically have larger and higher-quality sensors. While there is a broad range of nuance within optical sensors, there are a few general trends. Generally, the larger the sensor is, the better the image can be. Higher-quality sensors also have noticeable differences in the rendition of colours. Better sensors can capture a wider and more realistic range of colours.

2.5.3 Add-on Macro Lenses

Dedicated macrophotography lenses: there are lenses that are produced exclusively for high-magnification macrophotography. These lenses typically have limited utility for any other purpose and are generally quite expensive. The use of these lenses is likely beyond the intended reader of this publication.

There are certain products available online for relatively low prices that claim to be high-quality macrophotography lenses. In the world of photographic equipment, as with most things, you usually get what you pay for, and these lenses are typically of very low quality and not worth the trouble.

3 Equipment

3.1 General Equipment

1. Cloth or card for setting up an even background: these can be black, white or any other colour.
2. Blinds or curtains on windows in the room to block out sunlight.
3. Binder clips and tape to hold cloth and paper in place.
4. A variety of stands and supports which may be purpose-built photographic tripods and light stands or improvised supports such as lab equipment stands, stacks of books, a colleague with a free hand or anything else that works.

3.2 Example Equipment (See **Note 2***)*

3.2.1 Modest Equipment (as Used in Fig. 2b)

1. Compact digital camera (Canon G16)
2. Piece of white card stock
3. Section of black cloth
4. Household LED lamp (IKEA Jansjö)
5. Tabletop tripod

3.2.2 High-End Equipment (as Used in Fig. 3b)

1. High-end SLR camera (Canon 5d mk II)
2. Canon 24–105 mm f4 L lens
3. External flash unit (Canon 430EX)
4. Full-size tripod with ball head for camera
5. Flash attachment cable (Canon OC-E3)
6. Small tripod for supporting flash
7. Remote switch (Canon RS-80N3)

4 Methodology

4.1 Photographing Natural Habitat

It may be necessary to take photographs of a specimen in its natural habitat or descriptive photographs of the habitat. When out in the natural environment, a photographer loses direct control of the lighting of their scene. By timing the photographs at various points in the day, the photographer can choose from nature's range of lighting conditions. Direct midday sunlight is a very challenging environment to produce either flattering or descriptive photographs. The light is very hard and bright resulting in very bright highlights and very dark shadows. Good outdoor light is better found during the beginning and end of the day or during overcast weather. Overcast clouds give a soft and even light that reduces the overly high contrast of direct sun. Lighting around morning and evening comes from a lower angle, which can be more flattering to the landscape and is more gentle and lower in contrast than midday light.

The point of view from which a photo was taken will have a large impact on the outcome of the image.

4.2 Photographing Flowers

Figure 1 was taken with little thought by handholding a camera with all automatic settings. Figure 2a demonstrates what is possible with some thought and preparation and a minimum of equipment. The camera was first placed relative to the subject to make the desired composition (Fig. 2b). The light was adjusted such that the illumination gave a sense of depth and dimension without shadowing in an unpleasant way. A piece of white paper was first used to set the white balance of the camera for accurate colour reproduction, and then it was placed on the table to reflect some of the light back to fill in the shadows, so they were not too dark. The final piece to position was the black cloth in the background. It was hung over a clothes hanger some distance away from the table (Fig. 2b). This ensured the black cloth was not illuminated at all by the light and was fully out of focus, so even if it was slightly wrinkled or uneven, it would render as black. The aperture was closed as far as possible, in this case f8, to extend the depth of field as far as possible rendering as much of the flower sharply as possible. The exposure was taken using the self-timer, and the exposure time

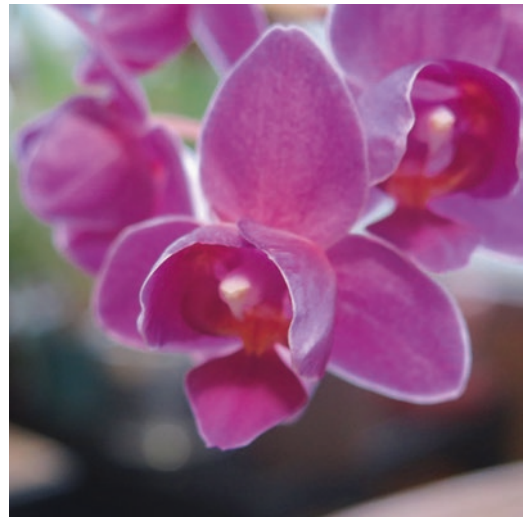


Fig. 1 An example of an image of a *Phalaenopsis* flower captured with a handheld compact camera (Canon G16) using automatic exposure settings and ambient overhead lighting

Fig. 2 (**a**) An example image of a *Phalaenopsis* flower captured using the same compact digital camera as in Fig. 1 (Canon G16). The differences in image quality that were achieved demonstrate what is possible with some thought and preparation and a minimum of equipment. (**b**) A schematic of the placement of the camera, subject, lighting and background used to capture the image in (**a**). A section of black cloth was placed behind the subject, a white card underneath to reflect light into the shadows and the camera attached to a tripod for stability, and a household floor lamp with an adjustable neck was used to illuminate the subject. Not shown is the placement of the subject on a small table leaving room on all sides for the placement of the other equipment

was set manually using a series of test exposures as a guide to the correct exposure.

Figure 3a was generated with higher-end photographic equipment. More attention was paid in arranging the equipment and background setting to achieve a superior result. Perhaps most striking

Fig. 3 (**a**) An example image of a *Phalaenopsis* flower captured using a professional quality SLR camera (Canon 5D Mk II) and lighting. This image demonstrates the level of quality that can be achieved with a higher complexity and accordingly higher-cost assembly of equipment. (**b**) As in Fig. 2b, a section of black cloth was placed behind the subject, a white card underneath to reflect light into the shadows and the camera attached to a tripod for stability. A photographic flash unit was used and the light modified by placing a translucent scrim in front of the flash. Not shown is the placement of the subject on a small table leaving room on all sides for the placement of the other equipment

is the difference in colour rendition between the two examples. Although both images were taken with correct white balance settings, the higher-end camera does a much better job of rendering the intense colour in the petals. The arrangement of equipment is described in Fig. 3b. The use of flash instead of continuous illumination allows for the use of short shutter speeds. This has the benefit of preventing unwanted light from impacting the image and limiting the impact of vibrations on blurring the image. The flash unit was manually set to 1/16th of maximum output. A sheet of white paper was placed ~15 cm away from the front of the flash unit. The effect was to subtly soften and diffuse the light. The use of a cable attached to shutter button vibrations was further minimized adding to the sharpness of the image. High-quality equipment does have a significant impact; however, even with more limited equipment, good-quality results can be achieved. It is most important to be creative, experimental and thoughtful about the process.

4.3 Photographing Pressed and Dried Herbarium Specimens

For photographing pressed specimens on a flat surface, it may be best to employ what is known in the photography as copy lighting. It is a standard lighting setup used for photographing documents, paintings and drawings. In the case of a biological specimen, it is more likely that the subject is quite small, so the above general

principles of macrophotography apply as well. It is quite simple to set up a standard copy arrangement. Ideally the camera would be on a tripod that can point the camera straight down. The item to be photographed would then be placed on the floor or a table depending on the size. Best results in a true reproduction can be obtained when the camera is pointed directly at the subject. The camera could be placed on a tripod pointing straight down at a table, and the specimen would be centred in front of the lens. This arrangement limits distortion and results in the truest reproduction possible. If multiple specimens are to be photographed, they can be moved in front of the camera in succession. Two light sources are required in order to make the illumination even across the specimen. The most convenient way would be the use of bendable arm desk lamps as they are readily available and allow for repositioning of the light source.

4.4 Photographing Through a Stereomicro-scope (Revised from [5])

For detailed examination and photographing small objects, a stereomicroscope with an attached digital camera is preferred. The size of the object can be increased using the "zoom" function of a stereomicroscope, and the image can be captured using a digital camera attached. There are cameras that are purposely built for use with a microscope manufactured by the microscope companies as well as third-party companies. Additionally by the use of the correct adapter, general purpose SLR cameras can be attached to a microscope (*see* **Note 3**).

1. For photographing in vitro explants, one can remove them from culture vessels and take photographs accordingly. Or, one can examine the explants without removing them from the culture vessels, especially if Petri dishes are used. Using this approach, one can follow the developmental changes through the course of the experiment without disturbing the in vitro culture environment. Condensation often occurs on the lid of a Petri plate impeding observation. It is possible to remove the condensation by using a hair dryer to evaporate the condensate from the surface of the Petri dish or culture vessel just prior to photography. Culture vessels do impede the passage of light, and since the surface may not be particularly smooth, this will limit the resolution possible at higher-magnification settings.
2. For better close-up observations, especially for smaller specimens, such as seeds and floral parts, it is advisable to take a closer look without the obstruction of the Petri plate or the container. Selected explants can be removed and placed directly on a black agar plate (*see* **Note 4**). The black agar plate provides a better contrast for the specimen. The moist agar medium also prevents the specimen from drying during

examination. Since agar is soft, the specimen can be partially submerged to allow for a sharper focus of surface features.

3. For determining the actual size of a specimen and for future reference, a small scale bar should be placed close to the specimen and photographed together. The scale bar can be created by photocopying a ruler, cutting off a centimetre portion of the image and placing it next to the specimen.
4. When illuminating smooth and reflective materials such as the smooth surface of agar media or the surface of a culture vessel or plate or even a highly glossy leaf, the specular reflection of the light source becomes more apparent. Dull surfaces, such as paper, reflect light but do so in a diffuse way. Shiny surfaces will directly reflect some of the light in what it called a specular reflection. An example of this would be the bright glare that is reflected by passing vehicle windows in bright sunlight; the windows are projecting an image of the sun directly to your eye. This specular reflection of a light source will appear in the image when the angle between the camera and the light source is the same. If a bright unwanted glare is observed instead of adjusting the light intensity, try adjusting slightly the angle of the light. Proper lighting can be provided using a ring light as it can provide an even illumination over the entire specimen when examining the specimen using a stereomicroscope. The positioning of the gooseneck arms and/or any other lights can create shadows and provides a better contrast of the specimen. Try different positions by moving the gooseneck arms or other light sources to obtain the best desirable image that showcases the feature of interest (*see* **Note 5**).

4.5 Additional Resources

1. Cornell has a website with a range of plant photography-related information. Although their focus is plant pathology, the website had useful lighting setups and example images. http://www.plantpath.cornell.edu/PhotoLab/index.html.
2. *National Geographic* has a guide to photographing orchids in their natural habitat with numerous examples. Elsewhere on the site are other photography advice materials that may be useful. http://www.nationalgeographic.com/photography/photo-tips/2017/photographing-orchid-flowers/.
3. A wide range of books on the general principles of photography are available at any library or bookstore ranging from small concise guidebooks to lengthy and highly technical texts.
4. A relatively concise guide with much more technical detail regarding macrophotography: *Closeup Shooting: A Guide to Closeup, Tabletop and Macro Photography* by Cyrill Harnischmacher (Rocky Nook, Inc. Santa Barbara, CA,USA, 2007).

5 Notes

1. In this digital age, rigorous academic and personal integrity must be applied to the creation of scientific imagery. It is possible and relatively easy to make changes to the appearance and even content of a photograph with image manipulation software. Most academic journals have quite strict guidelines of which manipulations may be acceptable and which are prohibited. In general, it is advisable to avoid manipulations completely or severely limit manipulations to modest overall contrast, brightness and colour adjustments that move the image towards a truer representation of reality.
2. Although Canon photographic equipment is named, similar equipment can be obtained from other manufacturers including but not limited to Nikon, Sony and Olympus. Given the rapidly advancing field of digital photography equipment, specific equipment models should be used as a guide. Advice on current models should be sought from trusted photography-specific retailers, online reviews and knowledgeable individuals.
3. Martin Microscope Company (www.martinmicroscope.com) provides useful information showing the different types of cameras and stereomicroscopes at a lower budget. When purchasing a new camera setup, if possible, test the camera with your microscope system before purchasing it to ensure compatibility. Software is usually purchased or included with the camera. Again, it is important that the software be compatible with one's computer system.
4. To prepare black agar plates, add approximately 4% (w:v) charcoal powder (e.g. Sigma cat. no. 161551) to a 2% agar solution before solidification. Agar plates can be prepared ahead of time and kept in a refrigerator. They store well. Allow the plates to warm up to room temperature before use. Excess water condensation can be removed using absorbent paper or a paper towel.
5. Different illumination systems are available from different sources. The new "mini-light" from Electron Microscopy Sciences offers high light intensity with a small footprint that fits into most locations. LED illuminators are becoming more common. Additional information can be obtained from website such as the Electron Microscopy Sciences website (www.emsdiasum.com) and Martin Microscope Company (www.martinmicroscope.com).

References

1. Averyanov LV, Ormerod PA, Duy NV, Tien TV, Chen T, Zhang DX (2016) *Bidoupia phongii*, new orchid genus and species (Orchidaceae, Orchidoideae, Goodyerinae) from southern Vietnam. Phytotaxa 266:289–294
2. Laskin DN, McDermid GJ (2016) Evaluating the level of agreement between human and time-lapse camera observations of understory plant phenology at multiple scales. Eco Inform 33:1–9
3. Suetsugu K, Nakahama N, Ito A, Isagi Y (2017) Time-lapse photography reveals the occurrence of unexpected bee-pollination in *Calanthe izuinsularis*, an endangered orchid endemic to the Izu archipelago. J Nat Hist 51:783–792
4. Gray DJ (2005) Photographic methods for plant cell and tissue culture. In: Trigiano RN, Gray DJ (eds) Plant development and biotechnology. CRC Press, Boca Raton, FL
5. Yeung EC (2015) A guide to the study of plant structure with emphasis on living specimens. In: Yeung ECT, Stasolla C, Sumner MJ, Huang BQ (eds) Plant microtechniques and protocols. Springer, New York, NY

Chapter 26

An Introduction to Chinese Orchid Paintings

Patrick I. Siu

Abstract

Orchids have been painted by artists throughout centuries. They are often associated with elegance, virtues and integrity. Orchids are one of the most popular topics for Chinese painting. Two main methods of painting orchids are discussed and illustrated in this chapter, i.e. *gongbi* painting (detailed painting) which requires high artistic skill and *literati* painting (freestyle painting) which requires more calligraphic skill instead. *Literati* painting gives artists more freedom to express themselves through the vivid brushstroke despite the fact that the final products may deviate a lot from the real specimens. Painting the orchids gives fun, satisfaction and intellectual aspirations to artists, amateurs and even beginners.

Key words Cymbidium, Chinese painting, Gongbi painting, Literati painting

1 Introduction

Botanical illustrations and accompanying records provide useful information about plants of interest. This is one of the key characteristics and usefulness of herbals that were published in the past hundreds of years. With the different media such as water colour and printing methods used in botanical illustrations, besides providing useful scientific information about plants, botanical drawings can also serve as an art form, as the illustrators can emphasize the aesthetic values of plants.

In botanical illustrations, drawing usually implies working in pencil, pen, charcoal, pastel and even ink on paper. The outlines of the objects are clearly shown. Usually a lot of the paper is left blank. On the other hand, painting entails the use of a brush or palette knife and liquid paint on paper, canvas or other materials. The details of the objects are shown and the colourful pigment covers most if not all of the painting. Moreover, the differences between drawing and painting can be subtle and have changed over time. Some drawings may contain washes that cover more area of the paper. Botanical drawings are always meticulous illustrations containing fine details in colour, with the background in white and not

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_26,

coloured [1]. A drawing may be considered the 'skeleton' of a painting as a preliminary step.

Chinese paintings are done by brushes usually with thick strokes of ink. Traditionally they are called paintings rather than drawing although they contain a lot of blank spaces or voids. To many, Chinese paintings look like unfinished work. Chinese culture based on Confucianism is also greatly influenced by Taoism and Buddhism which emphasized on 'emptiness'. It may be said that in general, Chinese art depicts spirit, while Western art depicts form. A well-executed brushstroke, expressing beauty and joy through movement, involves not only the actions of the artist's fingers, wrist and arm, but also his mental, emotional and psychic state [2].

In order to appreciate orchids from a different perspective, this chapter provides a brief introduction to the history of orchids in Chinese paintings and gives an overview of the materials used in Chinese painting. A simple step-by-step guide is detailed in Subheading 4 in how to paint a cymbidium orchid.

1.1 Chinese Orchid Paintings

Orchids are not only subjects of scientific research, but they also feature in Chinese painting at least over the last 1000 years. Zhao Shigeng (趙時庚), a noted Chinese scholar and botanist, wrote *Manuals on Jinzhang Orchid* (金漳蘭譜) in 1233. The manuals in three volumes describe the morphological features of more than 30 varieties of orchids. This is known as the first book extant in China on orchids. In 1805 Xu Jilou (許霽樓) wrote the *Handbook on Orchids* (蘭蕙同心錄) giving a detailed account of his orchid planting experience, and how to identify and classify orchids [3]. As photography has not been invented in those days, botanical drawing is the only way to show the appearance of plants. Without botanical drawing, identification and classification are not possible.

The cymbidium orchid is a perennial and terrestrial evergreen herb. The leaves are usually long and slender and leathery in texture and bend like an arc. The orchid usually flowers in spring with a faint fragrance. The flowers are delicate, exotic and graceful. Many orchids live in deep valleys or on steep cliffs. They grow on poor soil and do not need too much minerals or sunlight but they thrive in high humidity. Too much water, minerals and sunlight can kill the orchids.

One day, Confucius (孔子) (551–479 BCE), the great Chinese scholar, philosopher and educator, passed by a valley with his students and they suddenly smelled a lovely flower fragrance. They pursued its source and found a large number of orchids blooming among the grass. Confucian exclaimed on the spot, 'Orchid should be used to adorn the noble, but now it thrives alone in the small valley, mixed with the weeds. It is like a virtuous person born at the wrong time, thus living together with the vulgar' (*see* **Note 1** for the original text in Chinese).

Later the Confucian culture relates the characters of orchids with the noble character of a learned gentleman who will not lose his moral principle even in destitution. Ever since, orchids have been associated with elegance, virtues and integrity. Over the centuries, numerous literary and philosophical works attributed other virtues to orchids, such as friendship, loyalty and patriotism. Because of these associations, members of the scholar-officials (士大夫) came to identify strongly with the plant (*see* **Note 2**). Many cymbidium orchids have been moved from their natural habitats and cultivated in gardens and homes for more than 2000 years in China.

Flowers and birds form an independent subject of Chinese painting during the Song dynasty (960–1279). The earlier part of Song dynasty enjoyed political stability and the emperors appointed painters and calligraphers to become members of the Hanlin Calligraphy and Painting Academy (翰林院), providing a stable environment and fostering great devotion to painting. Emperor Huizong (徽宗) of Song, whose name was Zhao Ji (趙佶) (1082–1135), was a great patron of art and literature. He himself was a noted artist in calligraphy and painting. Huizong encouraged his court painters to use fine brush techniques and detailed painting [4].

Orchid drawing is an important element in flowers and bird paintings. Artists created meticulous depictions of the orchid employing outline and colour. This kind of painting is known as *gongbi* painting (工筆畫), literally meaning painting with detailed strokes. The strokes delimit details very precisely and without independent or expressive variation. This kind of detailed painting is similar to the botanical drawing of the Western art. Figure 1a is an example of a *gongbi* painting which is redrawn from a masterpiece of a *gongbi* orchid painting of the Song dynasty (960–1279) (*see* **Note 3**).

Parallel to this *gongbi* (detailed) painting, some scholars, probably led by the great scholar Su Shi (蘇軾) (1037–1101), began to develop another style of painting based on their calligraphy technique. This style of painting may look like sketches or unfinished drawings. People who appreciate Chinese calligraphy will also appreciate the beauty and elegance of the brushstrokes. This kind of painting is known as *literati* painting (文人畫) or painting in freestyle (意筆畫). It is much less concerned with the accuracy of the form and shape, but places more attention to the brushstrokes like Chinese calligraphy. The practice of using only ink or diluted ink became a major trend. Nothing, they felt, should distract from the beauty of monochrome brushstrokes. The various shades of grey were said to reveal colours to the sensitive eye [5]. This kind of painting is very suitable for showing the interlace of the long and slender leaves of Chinese orchids. People are attracted by the graceful way the leaves sway in the wind.

Fig. 1 (**a**) An example of a *gongbi* painting of orchid. This is redrawn from an orchid painting of the Song dynasty (960 CE–1279 CE) and the original artist is unknown. (**b**) An example of a *literati* painting (freestyle painting) of orchid. This is redrawn of a freestyle orchid painting by Zheng Sixiao (鄭思肖) (1239–1316)

Figure 1b is a redrawing of a freestyle orchid painting by Zheng Sixiao (鄭思肖) (1239–1316). Zheng was a great painter as well as a poet. He used the orchid to express his bitter feelings about the devastation of his country by the Mongolians. The leaves and flowers of his orchids are rendered in watery, abbreviated strokes. There is no root to hold the plant securely to the ground. The soil has been taken by the foreign invaders [6] (*see* **Note 4**).

Following the Mongol conquest of the Song in 1279 and the founding of the Yuan dynasty, China went into a period of turbulence and the Hanlin Calligraphy and Painting Academy closed down. Many scholars devoted themselves to writing and teaching. They refused to serve in the new regime. *Literati* painting became more popular. The 'ink orchid' took on strong overtones of loyalty to the fallen regime. During the late Ming and early Qing dynasties (16th–17th centuries) many masterpieces of orchid paintings were drawn [7].

1.2 Two Main Methods of Painting Orchids

Gongbi painting (工筆畫) (detailed painting) requires high skill and patience. Because it attempts to capture the fine details of the botanical specimen, it usually takes hours or even days to finish a painting. Before photography was invented, this style of painting allowed accurate and even scientific records to be kept.

Literati painting (文人畫) or painting in freestyle (意筆畫) allows a painting to be completed in a short time. The painting may not look exactly like the object depicted. An experienced artist may be seen to be able to complete a painting within a few minutes. However, this betrays the many years of study and practice required to be able to produce a good painting. Beginners may find this more satisfying as a painting with a few strokes would be more achievable than one with intricate details.

2 Some Technical Issues, Especially for Beginners

To practise Chinese calligraphy or painting, one has to learn the proper way to hold the brush. The brush needs to be held vertically in order to draw fine solid and steady lines (Fig. 2).

Hold the brush about midway, with the first two fingers (index and middle fingers) to the front and the thumb nail facing your face. The fourth finger (ring finger) rests behind the brush, the fingernail almost touching the base of the thumb. Curl the little finger away. Now bring the hand upright to make the brush

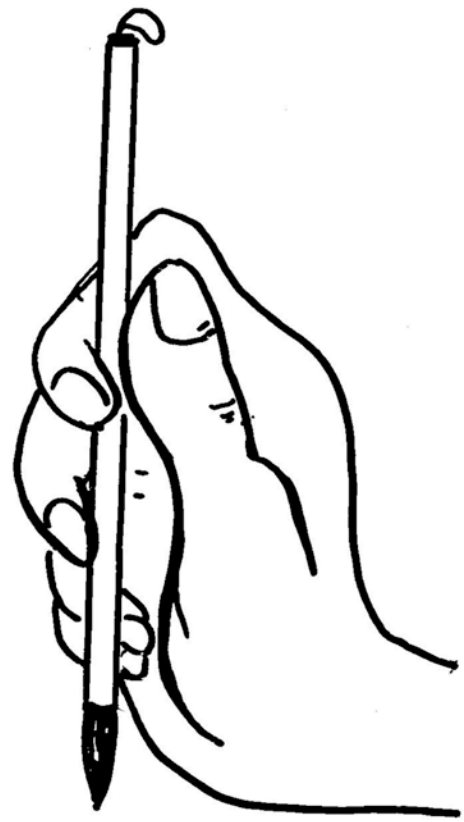


Fig. 2 Holding a brush with a right hand

perpendicular to the arm. If the brush is held properly in the right place, the tip of the brush is just about to touch the paper. If it does not, fiddle around on the handle either up or down until it feels right; otherwise proper strokes cannot be made.

Beginners need to practise drawing some simple horizontal lines and vertical lines. If the brush is well controlled, the thickness of the strokes will be uniform. It is normal that the strokes drawn by beginners may be shaky and crooked (*see* **Note 5**). Practice makes perfect. There will be a lot of fun once the brush is under control.

3 Materials for Chinese Painting

There are four main groups of materials for Chinese painting:

- Brushes
- Ink
- Colours
- Paper

3.1 Brushes

Brushes come in different sizes, big, medium or small. Most calligraphy and painting are written with a medium-sized brush. The smallest brushes are used for fine and detailed work. Bigger brushes are for writing larger characters or drawing large paintings.

According to the material used, brushes can be divided into two main types, soft (軟毫 ruǎnháo) brushes and hard (硬毫 yìngháo) brushes (Fig. 3). The common types are goat hair brushes (羊毫筆) for soft brushes. Yellow weasel hair brushes (黃鼠狼毫筆) are hard and stiffer brushes. They are easier to write especially with miniature work (*see* **Note 6**).

3.2 Ink

Ink comes in two forms: ink stick (墨條) and liquid ink (墨汁). Ink sticks are made mainly of soot and animal glue, with other ingredients such as incense as preservatives. Fine ink sticks give high-quality ink which can also be diluted to a whole spectrum of grey tones. Once the fresh ink gets dried on paper or silk, it will stay on the medium permanently (Fig. 4a). An ink stone (硯, *yàn*) is a stone mortar for the grinding and keeping of ink (Fig. 4a). Ink stones come in different shapes, round, oval or rectangular.

Liquid ink is ready to use (Fig. 4b). It saves the troubles of grinding an ink stick on an ink stone (*see* **Note 7**).

3.3 Colours

Ochre (brown), anthocyanin (blue), cinnabar orange (orange), gamboge (yellow), anthocyanin (blue), malachite (opaque green), azurite (opaque blue) and white are commonly used in traditional Chinese painting. In the past, colours were purchased in the form of pigments or pans. They are natural plant materials or minerals.

Fig. 3 The five brushes on the top are soft brushes made of goat hair. The other brushes are made of yellow weasel hair. They were all made in China

Nowadays colours are mass produced by modern technology and they come in tubes (Fig. 5a). They are much cheaper but the quality may not be as desirable as the pigments. The differences in the colour quality are very subtle. A full spectrum of colour in tubes can be purchased. They are similar to watercolour or gouache (Fig. 5b) (*see* **Note 8**).

3.4 Paper

Rice paper or shuen (xuan) paper (宣紙) is used for Chinese calligraphy and painting. The paper is not made from rice, but traditionally from tree bark. Usually the paper is made to the standard dimensions of 136 × 68 cm. It can be cut into smaller sizes. There are two main types of rice paper—unsized and sized. Each may be used for a different purpose.

Unsized rice paper, sometimes called raw rice paper (生宣紙), is absorbent. The moisture goes through the paper. It is best for spontaneous painting with bold and fast brushstrokes in the abstract style showing the dynamics of painting in freestyle. As it is highly absorbent, it requires a firm and experienced brush

Fig. 4 (**a**) Ink sticks and ink stones. (**b**) Liquid ink. The one on the left was made in Japan and the other two were made in China

handling, fast movements of the brush and a good moisture control; otherwise ink and colours will spread out unwieldly.

Sized rice paper is less absorbent because it is coated with an alum (potassium aluminium sulphate) solution (明礬). Artists call the sized paper alum-treated paper (礬紙) or commonly 'mature' rice paper (熟宣紙). The ink does not penetrate the paper; it stays on the outside and shines therefore outwardly. This rice paper is best for more detailed, delicate painting, where the brush work is done with slow and multiple strokes.

On the whole sized rice paper is easier to handle for beginners. Less experience in brush handling and moisture control is

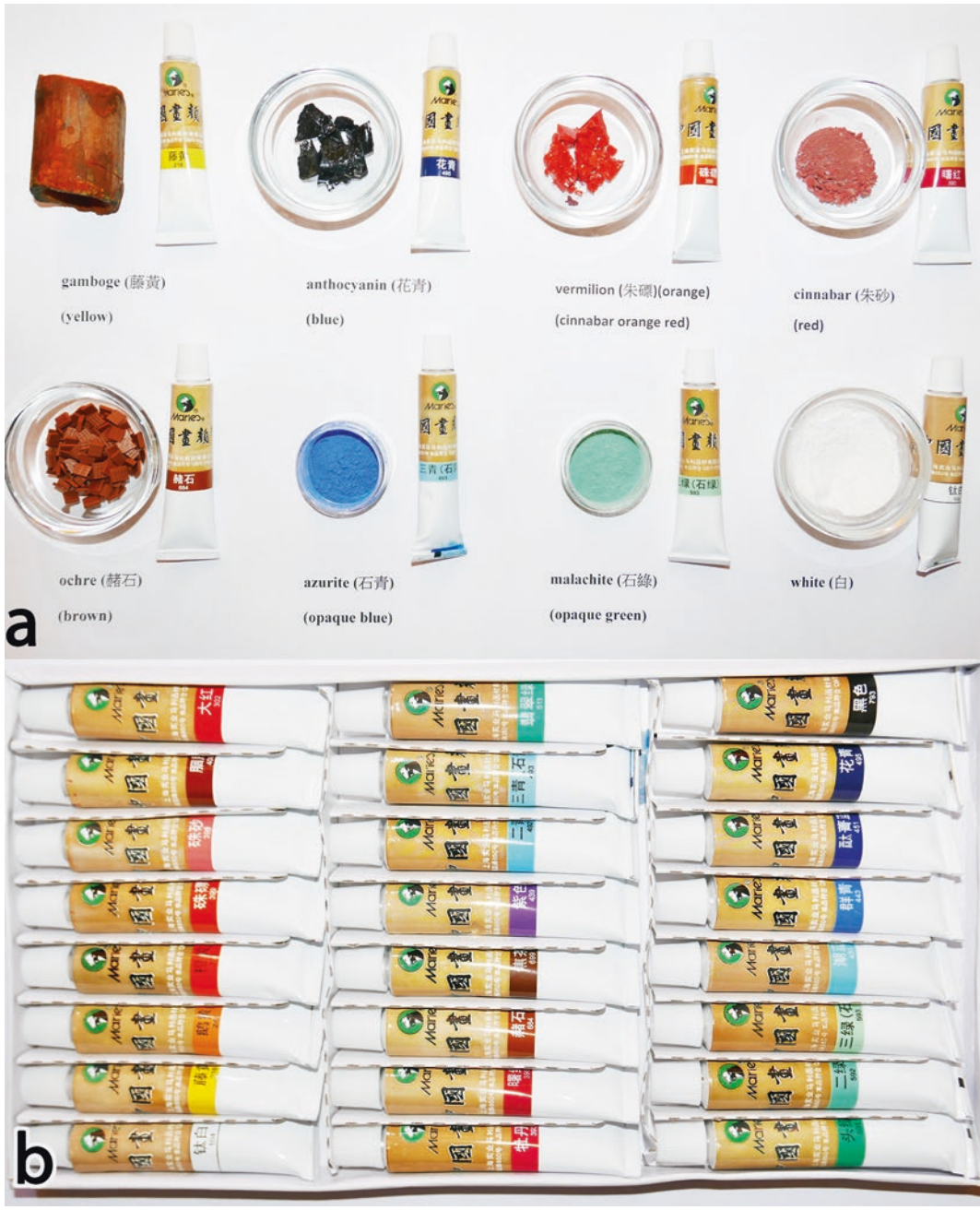


Fig. 5 (**a**) Commonly used paints in Chinese painting. (**b**) Modern paints in tubes

needed as ink and colours do not spread freely. It lends itself to multiple washes, which can be applied one after the other. However, different shades of the ink and colours on sized paper are not as vibrant as on unsized paper because of the alum coating (*see* **Note 9**).

4 Methods

4.1 Ink Preparation

Apply some water onto the ink stone. Gradually grind the ink in a circular motion with the end flat on the surface to produce ink (Fig. 6a). Keep on grinding until the ink is thick and dark enough, showing some ripples (Fig. 6b). The thickness of the ink can be tested using a brush and rice paper.

4.1.1 Preparation of a Spectrum of Grey Colour

Diluting the ink with water gives various shades of grey colour (Fig. 7a).

4.1.2 Preparation of Different Shades of Green Colour

A green colour is made by mixing blue (anthocyanin) and yellow (gamboge) together. Various shades of green can be made from the combinations of these two colours (Fig. 7b).

4.2 Step-by-Step Guide to Paint Orchids

The following is a step-by-step guide to paint orchids in *gongbi* painting and *literati* painting methods.

4.2.1 Gongbi Painting (Detailed Painting)

Gongbi painting (工筆畫) shows the fine details of the botanical specimen. This kind of painting is similar to botanical drawing. Sized paper is used for this type of painting.

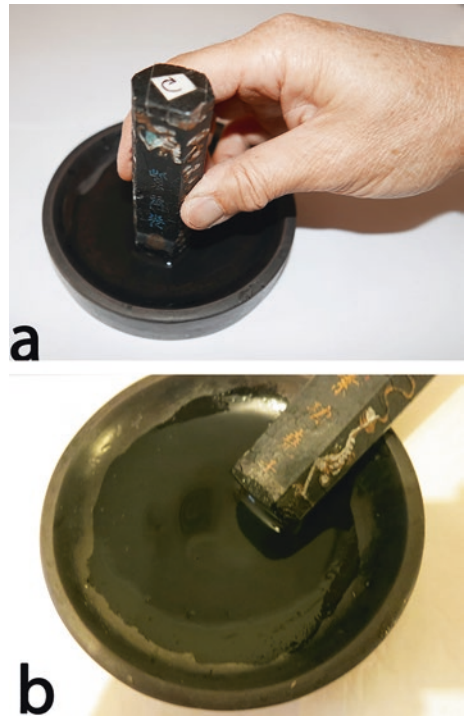


Fig. 6 (**a**) Grinding ink from an ink stick with some water on an ink stone. (**b**) The ink needs to be thick and dark enough for painting

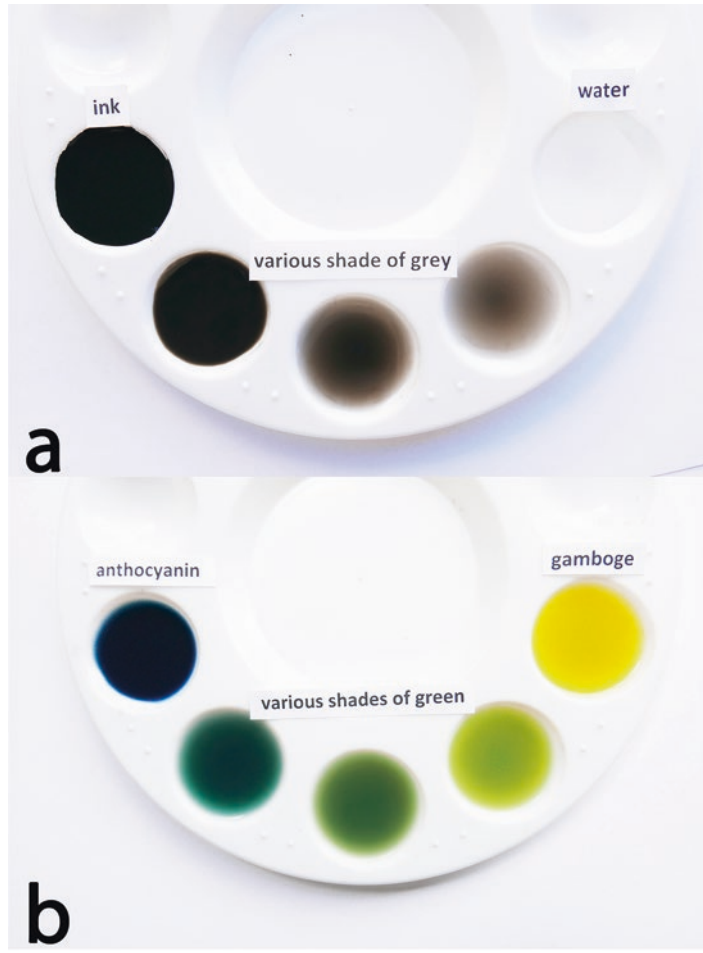


Fig. 7 (**a**) Diluting the ink with water gives various shades of grey colours. (**b**) Mixing different amounts of anthocyanin with gamboge gives various shades of green

Step 1. Draw the outline of the first two flowers with a fine brush using ink diluted with a drop of water (grey ink) (Fig. 8a).

Step 2. Draw the pedicels (flower stalks) and the peduncle (main flower stalks) of the inflorescence. Add two flower buds at the top (Fig. 8b).

Step 3. Draw the third flower behind the peduncle. Some parts of the flowers should overlap (Fig. 8c).

Step 4. Draw the outline of the leaves. Some of the leaves mainly show the upper epidermis, and some show more of the lower epidermis (Fig. 8d).

Step 5. Colouring: The finished painting shows the fine detail of the flowers and leaves (Fig. 8e).

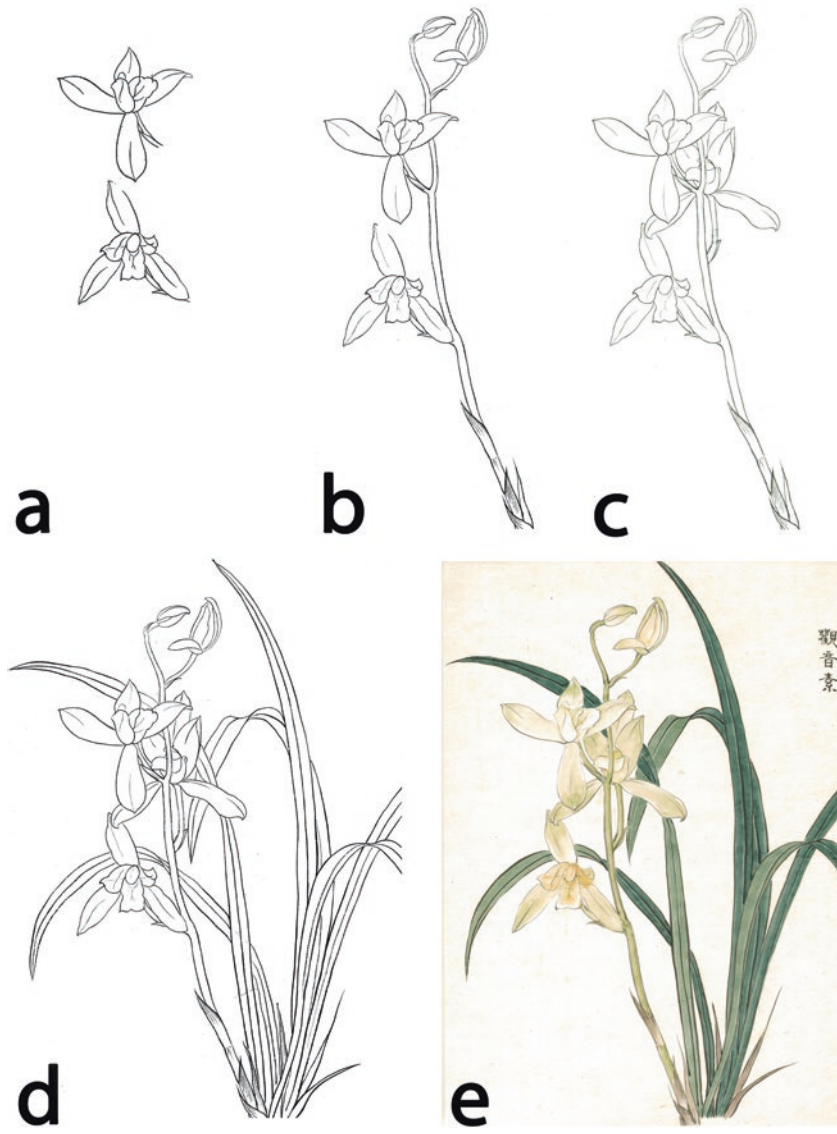


Fig. 8 (**a**–**d**) **Steps 1–4** of *gongbi* painting. (**e**) *Gongbi* painting of orchid in Chinese watercolour

4.2.2 Literati Painting (文人畫)

Literati painting refers to painting in freestyle (意筆畫). Even without formal training in drawing, everyone can draw an orchid by following some simple steps. It is quite easy to learn, but difficult to excel. Unsized paper is used for this type of painting.

Step 1. Start with the leaves. Use a big brush and dip the bristles in ink. Start very lightly from the bottom on the left-hand side and sweep the brush upward to the right. The leaf shows variations in thickness. Apply pressure to the bristles to make the stroke thicker, and reduce pressure to make the stroke thinner. The tip of the leaf should be thin (Fig. 9a).

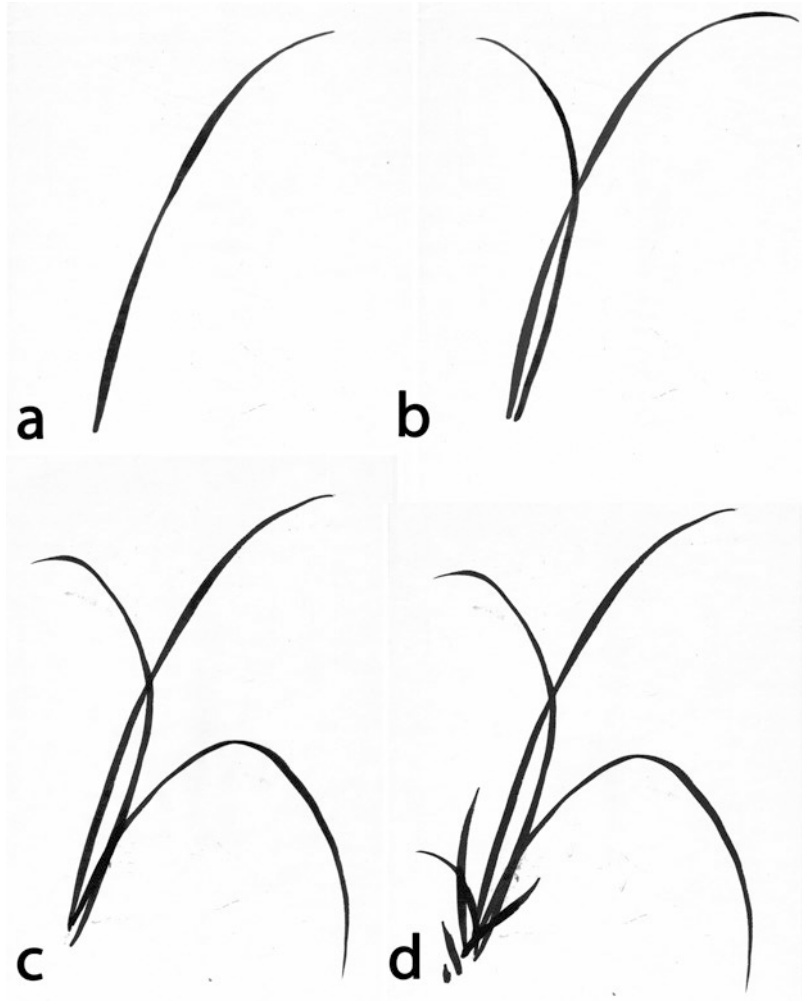


Fig. 9 (**a**–**d**) **Steps 1**–**4** in *literati* painting

Instead of starting from the left, you can also start from the right and sweep the brush upward to the left. The whole painting can be laterally inverted. *See* **Note 10** on strokes to avoid mistakes in painting orchids.

Step 2. Recharge the brush with ink and draw the second leaf. This leaf will curve to the left. The two leaves cross each other. Usually it looks better if the crossing is at the lower parts (Fig. 9b).

Step 3. The third leaf shows a turned-over orchid leaf. This creates more variations in the leaf arrangement (Fig. 9c).

Step 4. A few short leaves are added. In grouping many leaves, each leaf should be distinct and separate from the others so that they do not look jumbled or too crowded (Fig. 9d). There are a few things in painting the orchid leaves that should be avoided (*see* **Note 11**).

Step 5. There are two ways to paint the flower.

Method 1: Dilute the ink as in Fig 7. Draw the five strokes according to the sequence Fig. 10 a1–a6 from outside to inside as indicated by the arrows. The five strokes represent all the different parts of the flower including the labellum, the other two petals, three sepals, etc.

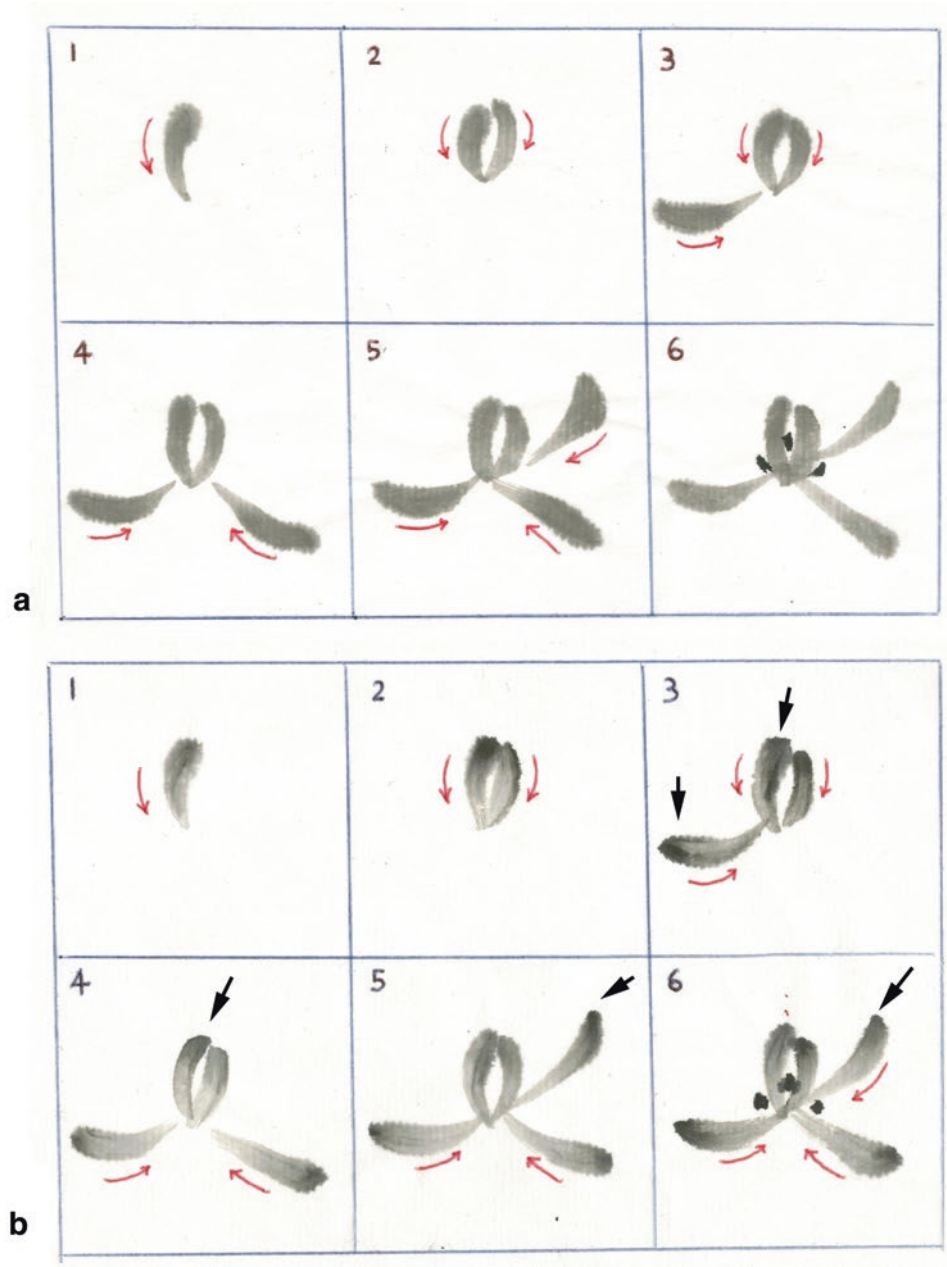


Fig. 10 (**a**) Steps in drawing the flowers using uniform grey ink. (**b**) Steps in drawing the flowers with various shades of grey ink

Method 2: Use a clean brush, roll the bristles in pale grey and then tip the bristles in a darker grey. Make sure that the bristles are not too wet. A good stroke should show some variations of grey tones (Fig. 10 b1–b6).

Step 6. Three flowers are first drawn (Fig. 11a). The shape and orientation of the flowers need to be different from one another. A few flower buds are then added to the end of the peduncle (main flower stalk). Each flower should then be connected to the peduncle by pedicels (individual flower stalks) (Fig. 11b).

Fig. 11 (**a–c**) **Steps 6–7** in *literati* painting. (**d**) Another version of the same painting in a better ink tone

Step 7. A series of three dots in dark black ink are added to the centre of each flower. The dots represent the column, pollinia or other small but prominent parts of the orchid flower. Only one dark black dot is needed for the flower bud (Fig. 11c). Another version of the same painting in a better ink tone is represented by Fig. 11d.

4.2.3 Comparing the Two Methods

The two methods of painting have their own advantages and disadvantages. *Gongbi* (detailed) painting gives an accurate record of the objects but the painting may be monotonous. *Literati* (free-style) painting gives the artists more freedom to express themselves through the vivid brushstrokes. The paintings may deviate too much from the real objects.

Developing a method in between may have the advantages of both methods. Artists are encouraged to draw from life objects but in less fine details rather than purely from their imaginations. The wonders of painting lie somewhere between likeness and unlikeness with the subject.

5 Concluding Remarks

Flowers are like messengers of beauty, greeting viewers with their myriad forms and colours. Indeed, their splendour is very moving. Artists all over the world continue to be attracted to painting flowers, creating many beautiful masterpieces.

Artists not only portray the outer beauty of flowers, but they also express their personal spirit and feelings. Paintings may show flowers in bloom or withered, allowing the artist to convey joyful or sorrowful feelings. More examples of orchid painting can be found in *The Mustard Seed Garden Manual of Painting* (芥子園画譜) [8].

Photography is popular and can easily capture images of different shapes or forms. However, a painting still offers a strong medium for an artist to depict the characters of the object to the full and convey inner feelings.

By learning how to draw orchids especially using the literati painting method, one can appreciate more about the graceful interlace of the long and slender leaves and the beauty of the flowers to the full. As we do not need to care about the likeness of the paintings with the actual botanical specimens, we can relax when we paint. The process itself can give artists, amateurs and even beginners many enjoyable hours with orchids. The process of literati painting can serve as an art therapy.

6 Notes

1. 孔子曰: 夫蘭當為王者香, 今乃獨茂, 與眾草為伍, 譬猶賢者不逢時, 與鄙夫為倫也。
2. Scholar-officials were civil servants appointed by the emperor of China to perform day-to-day governance from the Han dynasty to the end of the Qing dynasty in 1912, China's last imperial dynasty.
3. A good image of the original painting was published in 宋人画册 (1959) 中國古典藝術出版社. This book is out of print. A smaller image can be found in 陳履生, 張蔚星 (2000) 中國花鳥画 - 唐宋卷, painting 147, 廣西美術出版社 ISBN 7-80625-744-6. Similar painting by Ma Lin (circa 1180–after 1256) can be found on Maxwell K. Hearn's (2008) *How to read Chinese Paintings,* page 69, Metropolitan Museum of Art, ISBN 978-1-58839-281.
4. A good image of this painting was published in Zhang Hongxing's (2013) *Masterpieces of Chinese Painting, 700–1900*, page 221, V & A Publishing, ISBN 978-1-851-77756-3.
5. Various kinds of brushstrokes (*see* Fig. 12a).
6. Brushes made by Shanghai Gongyi (上海工藝) are highly reliable and the prices are also reasonable. There are many other good brands from various provinces. Most Japanese brands are of high quality but they are much more expensive. Most Taiwanese brands provide fine brushes too. Chinese art speciality shops can be found in most major cities in China, for instance, Duo Yun Xuan (朵雲軒) in Shanghai, and Man Luen Choon (文聯莊) and Yau Sang Cheong (友生昌) in Hong Kong. Their addresses can be found through Google. A huge range of fine brushes can also be bought online. Again just Google for more information. Examples of some online stores that sell Chinese painting stationery are as follows:

   ebay stores—http://stores.ebay.com.au/wenfangsibaowu?_trksid=p2047675.l2563

   Blue Heron Arts—https://www.blueheronarts.com/
7. Grinding ink from an ink stick is a bit tedious unless artists would like to get tonnes of grey in the highest quality. Liquid ink from a bottle is much more convenient. Japanese-made ink is highly recommended, e.g. 玄宗墨液 made by 墨運堂 Boku-Undo Co. Ltd, Japan. Yi De Ge (一得閣) is a famous Chinese brand. It produces high-quality Chinese ink. Ink can also be purchased online.

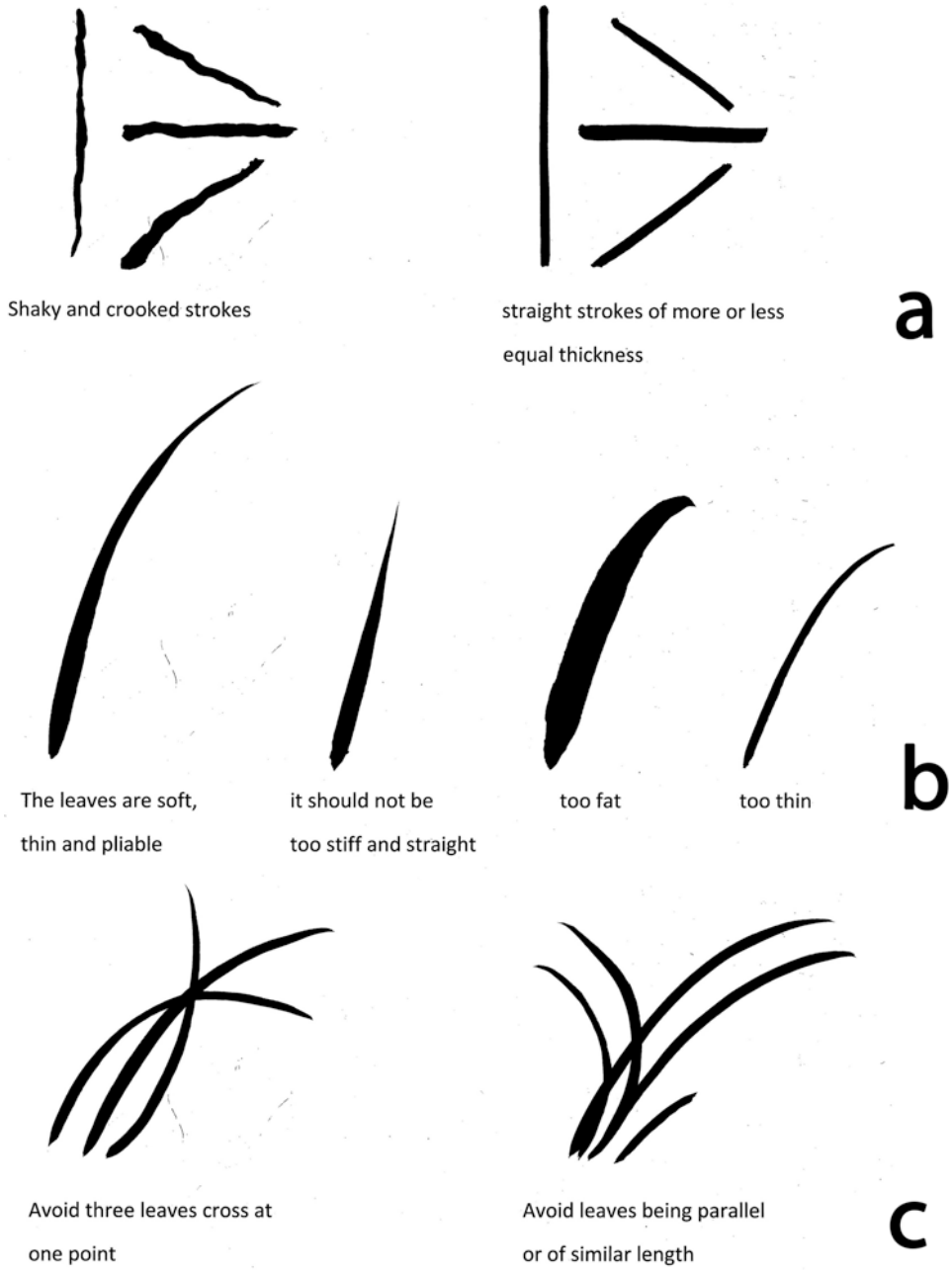


Fig. 12 (**a**) Various kinds of brushstrokes. (**b**) Differences in the thickness of leaves. (**c**) Crossing of leaves

8. Solid paints in pans or powders made by Jiang Si Xu Tang, Su Zhou (蘇州姜思序堂), provide colours of the highest quality. They are rather expensive. Small quality of water is added onto the paints before applying onto the paper. Powders like malachite (opaque green) or azurite (opaque blue) cannot be applied directly onto paper. Special thin liquid glue has to

be added to the powders first before applying to the paper so that the powders can adhere firmly onto the paper. The procedures are quite complicated. Nowadays most artists use colours that come in tubes. Marie's Chinese Painting Colour (馬利牌中國**画顏**料) is the most popular brand. The price is also quite reasonable and affordable. Again these colours can be purchased online. Winsor & Newton Water Colour is also highly recommended for botanical drawings as it produces a full spectrum of colours.

9. Red Star Brand (紅星牌) rice (xuan) paper of Jing County, Anhui Province (安徽涇縣), is the best known brand of brush painting paper. The paper is usually sold in bundles of 100 sheets but smaller quality can also be purchased. Besides the Red Star Brand there are many other brands of rice paper manufactured in Jing County. Some other provinces in China also made fine rice paper. Japan produces high-quality rice paper too. Rice paper can easily be purchased online.
10. Differences in the thickness of the leaves (Fig. 12b): The leaves should be soft, thin and pliable. It should not be too stiff, too fat or too thin [9].
11. Crossing of the leaves (Fig. 12c): Avoid crossing three or more leaves at one single point. The leaves should not be parallel and of similar length.

References

1. Morris C, Murray LJ (2016) The Florilegium—The Royal Botanic Gardens Sydney Celebrating 200 years. The Florilegium Society at the Royal Botanic Gardens Sydney Inc
2. Fong WC (1984) Images of the mind: selections from the Edward L. Elliot family and John B. Elliot Collections of Chinese Calligraphy. The Art Museum, Princeton University Press, Princeton, USA
3. Wang J (2014) Plum blossom, orchid, bamboo and chrysanthemum. Huangshan Publishing House, Purple Culture.net
4. Cahill J (1977) Chinese painting. Rizzoli International Publications, New York
5. Weng W-G H (1979) Chinese painting and calligraphy—a pictorial survey. Dover Publications Inc., New York
6. Zhang H (2013) Masterpieces of Chinese painting, 700–1900. V & A Publishing, London, UK
7. Zhou S (2006) Book of orchid. Yi Shu Tu Shu Publisher, Art Book Co. Ltd, Taipei
8. Sze M (1978) The mustard seed garden manual of painting. Princeton University Press, Princeton, USA
9. Cameron AS (1968) Chinese painting techniques. Dover Publications, New York

Chapter 27

Recent Advances in Orchid Seed Germination and Micropropagation

Yuan-Yuan Li, Colin Chan, Corrine Stahl, and Edward C. Yeung

Abstract

This chapter provides recent information focusing on different seed germination and micropropagation methods for various species of orchids. The three primary methods, i.e., symbiotic seed germination, asymbiotic seed germination, and micropropagation, are emphasized. Additionally, some recent studies on artificial seeds for germination are included. The information is presented in a table form in order to provide a quick overview on recent publications at a glance.

Key words Micropropagation, Asymbiotic seed germination, Symbiotic seed germination, Artificial seeds

1 Introduction

Orchids are extremely important economically. Their stunning blooms have mesmerized human for hundreds of years, and naturally, people have been seeking ways to reliably germinate and grow these plants for flowers for centuries. It was not until the work of Lewis Knudson in 1922 that a reliable protocol for asymbiotic germination of orchids was demonstrated [1, 2]. With subsequent discoveries and improvements in plant tissue culture techniques, propagation through seed germination and micropropagation is now readily achieved ([3, 4], also *see* Chapters 5, 6, 7, and [8]). However, seeds of terrestrial orchids are difficult to germinate asymbiotically [4, 5]. It was recognized early by Noel Bernard in the late 1800s that mycorrhizal fungi play an essential role in seed germination (*see* [4]). In the past few decades, especially with a better understanding of the mycorrhizal fungi, an increased number of publications on symbiotic seed germination appear in the literature. With a concerted effort, seed germination studies, albeit asymbiotic and symbiotic, will provide valuable information and methods toward orchid conservation efforts.

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0_27,

This chapter aims to serve as a gateway to the vast orchid literature by providing samples of the most recent publications in seed germination and micropropagation of orchids. The information is presented in a table form, summarizing key information, i.e., media used, growth regulators, and additives. However, due to space constraint, the information in the tables is by no means comprehensive, and it is recommended that readers consult the original publication (listed in the table) to obtain detailed methods and procedures for interested studies.

For earlier literature, readers can consult the reviews by Chugh et al. [6] and Teixeira da Silva [7]. The latter review provides extensive citations on regeneration and seed germination studies up to the year 2004. For more updated information, readers are referred to the Kew Orchid Research Newsletter [8], which is published twice a year. This newsletter aggregates and summarizes recent orchid literature. Additionally, for tissue culture, the recent publication by Herman [9] provides a good overview of the recent protocols and technologies used for orchid tissue cultures. The *Orchid Biology: Reviews and Perspectives* series with Professor Joe Arditti as the founding editor is a rich source of information on different aspects of orchid biology.

2 Symbiotic Germination (Table 1)

Symbiotic germination refers to using mycorrhizal fungi to aid in seed germination and early growth. For most protocols in this chapter, this refers to sowing the seeds on medium already inoculated with a sample of mycorrhizal fungi (one-step process). However, some protocols germinate the seeds asymbiotically, and soon after germination, they transfer the plant to medium containing the fungi (two-step process). Some protocols focused on conservation of orchids also utilize a method of symbiotic germination, referred to in this chapter as seeds on tree or seeds in soil, where they place the seeds in a packet with holes large enough for fungi from the environment to enter but not large enough for the seeds to escape. The pouches are then planted in the soil or attached to a tree in the orchid's natural environment, so the seed can be exposed to the mixture of fungi and bacteria it would have in the wild. Please refer to Chapters 2 and 3 for more information.

3 Asymbiotic Germination (Tables 2 and 3)

Asymbiotic seed germination is an efficient way of germinating many orchids. This method of germination allows for more controlled germination conditions, which make this method favorable

Table 1
Samples of symbiotic germination protocols from 2012 to 2017

Orchid species	Symbiont(s)	Method of infection	Germination (%)	Reference
Aa achalensis	*Thanatephorus cucumeris*	One step	30	[10]
Anoectochilus formosanus	*Thanatephorus*, *Ceratobasidium*	One step	44–91	[11]
Anoectochilus roxburghii	Not specified	One step	–	[12]
Bipinnula fimbriata	*Tulasnella*	One step	65	[13]
Bletilla striata	Fungal isolates (*Tulasnella calospora*) from roots of wild *Pecteilis radiata* (Thunb.) Raf.	One step	44.9	[14]
Caladenia huegelii	Fungal isolates from wild plants	One step	98	[15]
Caladenia latifolia	Stored cultures of mycorrhizal fungus from wild *Caladenia latifolia*	One step	95	[16]
Caladenia latifolia	Fungal isolates from wild plants	One step	100	[15]
Chloraea bletioides	*Bipinnula fimbriata* (*Tulasnella*)	One step	55	[13]
Chloraea chrysantha	*Tulasnella*	One step	65	[13]
Chloraea crispa	*Tulasnella*	One step	55	[13]
Chloraea gavilu	*Tulasnella*	One step	60	[13]
Chloraea gavilu	*Tulasnella*	One step	58	[13]
Chloraea grandiflora	*Tulasnella*	One step	58	[13]
Chloraea longipetala	*Ceratobasidium*	One step	62	[13]
Chloraea riojana	*Rhizoctonia*-like strains	One step	–	[17]
Coelogyne nervosa	*Epulorhiza*	One step	62	[18]
Cremastra appendiculata	*Coprinellus domesticus* and other members of *Coprinellus*	One step	37.8; 33.3	[19]
Cynorkis purpurea	*Sebacina*,*Tulasnella*, *Ceratobasidium*	Two step	34–76	[20]
Cyrtopodium glutiniferum	*Epulorhiza*	One step	51.18–86.67	[21]
Cyrtopodium glutiniferum	*Epulorhiza*	One step	–	[22]
Cyrtosia septentrionalis	*Armillaria gallica*, *Armillaria mellea* subspecies nipponica, *Armillaria tabescens*, Polyporales TK-10, *Xylobolus annosus*	Seeds in soil	0.02–63.5	[23]

(continued)

Table 1
(continued)

Orchid species	Symbiont(s)	Method of infection	Germination (%)	Reference
Dendrobium pulchellum	*Epulorhiza*	One step	–	[24]
Dendrobium aphyllum	*Rhizoctonia*	One step	21.69	[25]
Dendrobium aphyllum	*Tulasnella*	Seeds on tree	45	[26]
Dendrobium chrysanthum	*Rhizoctonia*	One step	13.58–90.5	[27]
Dendrobium crepidatum	*Epulorhiza*	One step	–	[24]
Dendrobium crystallinum	*Epulorhiza*	One step	–	[24]
Dendrobium devonianum	*Tulasnella* sp.	One step	0–83	[28]
Dendrobium findlayanum	*Epulorhiza*	One step	–	[24]
Dendrobium friedericksianum	*Tulasnella violea*, *Epulorhiza repen*, *Trichosporiella multisporum*	Seeds in soil	57.7	[29]
Dendrobium nobile	*Agrococcus*, *Sphingomonas*, *Mycobacterium*, *Bacillus pumilus*	One step	88–94	[30]
Dendrobium officinale	*Tulasnella*	One step	81.05; 98.47	[31]
Dendrobium officinale	*Sebacina*	One step	–	[32]
Dendrophylax lindenii	*Ceratobasidium*	One step	84	[33]
Encyclia tampensis	*Tulasnella irregularis*	One step	50.4–84.3	[34]
Epidendrum amphistomum	*Tulasnella irregularis*	One step	3.4; 3.0	[34]
Epidendrum nocturnum	*Tulasnella irregularis*	One step	68.8; 72.3	[34]
Gastrodia elata	*Mycena*	One step	60.1; 47.0	[35]
Gastrodia elata	*Mycena osmundicola*	Two step	40.4	[36]

(continued)

Table 1
(continued)

Orchid species	Symbiont(s)	Method of infection	Germination (%)	Reference
Gavilea australis	*Thanatephorus cucumeris*, *Ceratobasidiaceae*, *Tulasnella calospora*, *Ceratobasidium albasitensis*, *Ceratobasidium*	One step	42–70	[37]
Microtis media	Fungal isolates from wild plants	One step	93	[15]
Microtis media	*Sebacinaceae*, *Tulasnellaceae*	Seeds in soil	4–53	[38]
Paphiopedilum villosum	*Tulasnella*, *Ceratobasidium*, *Flavodon*	One Step	0.21–14.98	[39]
Papilionanthe teres	*Epulorhiza*	One step	93	[40]
Prosthechea cochleata	*Tulasnella irregularis*	One step	11.5; 15.5	[34]
Pterostylis sanguinea	Fungal isolates from wild plants	One step	98	[15]
Rhynchostele cervantesii	*Tulasnellaceae*, *Ceratobasidiaceae*, and moss from *Nostoc*	Seeds on tree	–	[41]
Serapias vomeracea	*Tulasnella calospora*	One step	60–95	[42]
Spathoglottis plicata	*Epulorhiza*	One step	37.8–67.6	[43]

for scientific studies. Two tables are provided below, one for asymbiotic germination using immature seeds and the other for germinating mature seeds. The main difference between using mature and immature seeds is that the seed coats of mature seeds often contain phenolic compounds that may hinder germination if left untreated. Therefore, many protocols will treat mature seeds with a pretreatment step in order to make the seeds more suitable for germination. Please see Chapter 5 for more information.

4 Micropropagation (Table 4)

Micropropagation is a method to multiply plants using explants to grow a new plant. This method takes advantage of the unique ability of plants to regenerate a whole plant from just an organ or a group of cells. Micropropagation is especially useful for generating new individuals from endangered or nearly extinct species as well

Table 2
Sample literature of asymbiotic germination from immature seeds (2012–2017)

Plant species	Medium composition	Germination efficiency (%)	Reference
Acampe praemorsa	MS, 2.0 mg/L BA	85	[44]
Acampe papillosa	M, 15% CW	70.75	[45]
Acampe praemorsa	MS, 15% CW	70.7	[46]
Acampe praemorsa	PM, 2% sucrose	96.8	[47]
Aerides maculosa	VW, 15% CW, 1 mg/L BA, 1 mg/L NAA	81.8 (callus)	[48]
Aerides multiflora	PDA, 0.1% CH, 0.2% AC	99.50	[49]
Agrostophyllum khasianum	PM, 2% sucrose	97.6	[47]
Coelogyne breviscapa	LOM, 0.088 M sucrose	88	[50]
Coelogyne flaccida	M, 2% sucrose	84.50	[51]
Coelogyne flaccida	MS, 3% sucrose, 0.5 mg/L BA, 0.5 mg/L NAA	n.s.	[52]
Coelogyne fuscescens	MS, 3% sucrose, 1 mg/L BA, 0.5 mg/L NAA	90	[53]
Coelogyne stricta	MS, 3% sucrose, 1 mg/L BA, 1 mg/L NAA	n.s.	[54]
Cymbidium aloifolium	MS, 30 g/L sucrose, 0.5 mg/L BA, 0.5 mg/L NAA	98	[55]
Cymbidium aloifolium	MS, 0.5 mg/l BA, 0.5 mg/L NAA	95	[56]
Cymbidium bicolor	LOM, 3% sucrose	96.6	[57]
Cymbidium faberi 'Jiepeimei' × *C.sinense* 'Qijianheimo'	KC, 30 g/L sucrose	17	[58]
Cymbidium iridioides	MS, 2% sucrose, 3.0 mg/L BA, 3.0 mg/L NAA	95	[59]
Cymbidium lowianum	1/2 MS, 3% sucrose, 1.0 mg/L BA, 0.5 mg/L NAA, 0.3% AC	95	[60]
Cypripedium macranthos	Modified Harvais medium	25.8	[61]
Cypripedium macranthos	¼ MS, 10 g/L sucrose, 100 mL/L CW or 100 mL/L sap (birch or maple sap)	70.8	[62]
Cypripedium macranthos	¼ MS, 10 g/L sucrose	68	[63]
Dendrobium aggregatum	MS, 3% sucrose, 1.5 mg/L BA, 15% CW	n.s.	[64]
Dendrobium aqueum	LS, 0.088 M sucrose	98	[50]
Dendrobium fimbriatum	MS, 3% sucrose, BA or NAA or both	90–100	[65]

(continued)

Table 2
(continued)

Plant species	Medium composition	Germination efficiency (%)	Reference
Dendrobium nobile hybrids	Modified (Hyponex, MS)	80.03; 83.41; 90.06	[66]
Dendrobium palpebrae	PM	–	[56]
Dendrobium wangliangii	N1 medium (3 g/L Hyponex-1, microelements and organics as in MS, 0.5 mg/L NAA, 0.5 g/L AC)	92	[67]
Dimorphics lowii	VW, 10% CW or 10% PH or 10% TJ	–	[68]
Esmeralda clarkei	MS, 0.5 mg/L BA, 0.5 mg/L NAA	n.s.	[69]
Flickingeria nodosa	LOM, 0.088 M sucrose	68	[50]
Hoffmannseggella cinnabarina	20 g/L sucrose, BA, MS or KC or VW	n.s.	[70]
Malaxis acuminata	MS, 3% sucrose, 4 μM NAA	85	[71]
Paphiopedilum armeniacum	1/8 MS, 20 g/L sucrose, 0.5 mg/L NAA, 10% CW, 1.0 g/L AC	96.2	[72]
Paphiopedilum hangianum	H026, 20 g/L sucrose, 0.5 mg/L NAA, 10% CW, 1.0 g/L AC	72.67	[73]
Paphiopedilum insigne	Modified Burgeff medium	88.5	[74]
Paphiopedilum SCBG Red Jewel	Hyponex N026, 0.5 mg/L NAA, 10% CW, 1.0 g/L AC	39.9	[75]
Paphiopedilum wardii	1/2MS, 0.5 mg/L NAA, 10% CW, 1.0 g/L AC	65.33	[76]
Phalaenopsis cornorerris	PM, 2% sucrose	98.2	[47]
Rhynchostylis retusa	VW, 15% CW, 1 mg/L BA, 1 mg/L NAA	80 (callus)	[48]
Spathoglottis plicata	P723 (PhytoTechnology Laboratories), 2% (w/v) sucrose, 1.0 mg/L BA	70±2.3	[77]
Vanda coerulea	MS	n.s.	[78]
Vanda dearei	KC, 1% sucrose, 0.5% yeast extract	98.3	[79]
Vanda tessellata	MS, 3% (w/v) sucrose	90	[80]
Xenikophyton smeeanum	VW, 2 mg BA, 1.5 mg NAA, 50 mL coconut milk	95	[81]

Table 3
Sample literature of asymbiotic germination protocols using mature seeds (2012–2017)

Plant species	Pretreatment and medium composition	Germination efficiency (%)	Reference
Aerides maculosum	Sterilized capsule MS, 3% sucrose, 10% CW, 2 mg/L 6-BA, 500 mg/L CH	95.6	[82]
Aerides ringens	Sterilized capsule KC, 4.44 μM BA, 500 mg/L peptone	89.28	[83]
Ansellia africana	Sterilized capsule ½ MS, 3% sucrose, SW 1:250 (v/v)	–	[84]
Aspasia variegata	Not specified KC, 20 g/L sucrose	–	[85]
Bletilla formosana	1% sodium hypochlorite (NaOCl) for 10 min SD medium, 0.5 mg/L niacin, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HC, 100 mg/L myoinositol, 15% CW, 0.6% potato powder, 0.1% peptone, 2% sucrose	91	[86]
Brasiliorchis picta	70% ethanol 1 min then 0.75% NaOCl 5 min WPM, 3% sucrose, 0.1 g/L inositol	76	[87]
Bulbophyllum lilacinum	Sterilized capsule PM, 2% sucrose	80	[88]
Caladenia latifolia	2% available chlorine solution 10 min ½ MS, 20 g/L sucrose, 5% (v/v) CW	87–92	[89]
Cattleya intermedia	2% NaOCl solution for 15 min ½ MS, 30 g/L sucrose, 3 g/L AC	–	[90]
Cattleya intermedia	Sterilized capsule MS medium with 50% of the original formulation of macronutrient salts, 30 g/L sucrose, 10 g/L AC	–	[91]
Cattleya mendelii	Sterilized capsule MS, 30 g/L sucrose, 1.5 μM of GA_3, 0.5 μM of NAA	95.2	[92]
Cattleya quadricolor	Sterilized capsule MS, 30 g/L sucrose, 1.5 μM of GA_3, 0.5 μM of NAA	96.4	[92]

(continued)

Table 3
(continued)

Plant species	Pretreatment and medium composition	Germination efficiency (%)	Reference
Cattleya warneri	2% NaOCl solution for 15 min ½ MS, 30 g/L sucrose, 3 g/L AC	–	[90]
Coelogyne nervosa	Sterilized capsule MS, 30% CW	96	[93]
Cymbidium aloifolium	Sterilized capsule PM, 2% sucrose	90	[88]
Cypripedium debile	Sterilized capsule Liquid: 1/4 MS, 1 g/L tryptone, 1 g/L yeast extract, 20 g/L sucrose, 100 ml/L CW	98.3	[94]
Cymbidium mastersii	Not specified MS, 3% sucrose (w/v)	93.58	[95]
Cyrtopodium saintlegerianum	Not specified KC, 20 g/L sucrose, 3 g/L AC	100	[96]
Dendrobium aphyllum	Sterilized capsule PM, 2.0 g/L peptone	100	[97]
Dendrobium aqueum Lindley	Sterilized capsule MS	97.75	[98]
Dendrobium hookerianum	Sterilized capsule MS	95.27	[99]
Dendrobium lasianthera	Sterilized capsule VW, 2 g/L peptone	100	[100]
Dendrophylax lindenii	A solution of (5 mL 100% ethanol, 5 mL 8.25% NaOCl, and 90 mL sterile water) for 1 min P723 medium (PhytoTechnology Laboratories)	45	[33]
Dendrobium ovatum	Sterilized capsule WPM, 0.5 mg/L NAA, 1 mg/L BA, 30% CW	–	[101]
Epidendrum fulgens	Sterilized capsule Liquid MS	83.5	[102]

(continued)

Table 3
(continued)

Plant species	Pretreatment and medium composition	Germination efficiency (%)	Reference
Epidendrum nocturnum	Disinfection agent (16.6 g/L disodium salt of dichloroisocyanuric acid dissolved in sterile distilled water) 8 min B5 macro-salts, MS micro-salts, 25 mg/L $Na_2FeEDTA$, 25 g/L sucrose	70	[103]
Eulophia cullenii	Sterilized capsule M, 0.05% CH	70	[104]
Grammatophyllum speciosum	15% NaOCl 10 min ½ B5, 0.1% AC	97	[105]
Habenaria edgeworthii	2% NaOCl solution for 5 min MS, 1.0 μM NAA	94.7	[106]
Hadrolaelia grandis	70% ethanol for 1 min, 0.75% NaOCl 5 min WPM, 3% sucrose, 0.1 g/L inositol	94.71	[107]
Hoffmannseggella cinnabarina	0.4% NaOCl 10 min MS, 20 g/L sucrose	98.3	[70]
Limodorum abortivum	5% NaOCl with a drop of Tween 80 BM-1	2.63	[108]
Limodorum brulloi	1% NaOCl with a drop of Tween 80 BM-1	8.7	[108]
Limodorum trabutianum	5% NaOCl with a drop of Tween 80 BM-1	63	[108]
Maxillaria rufescens	Disinfection agent (16.6 g/L disodium salt of dichloroisocyanuric acid dissolved in sterile distilled water) with a drop of Tween 20 B5 macro-salts, MS micro-salts, 25 mg/L $Na_2FeEDTA$, 25 g/L sucrose	60	[103]
Oberonia ensiformis	Sterilized capsule M, 3% sucrose, 0.5 g/L myoinositol, 1.0 g/L CH, 0.5 g/L L-glutamine, 250 mg/L peptone, 0.2 g/L p-aminobenzoic acid, 0.1 g/L biotin, 10% SW	85	[109]

(continued)

Table 3
(continued)

Plant species	Pretreatment and medium composition	Germination efficiency (%)	Reference
Oberonia recurva	0.1% mercuric chloride 3 min then 70% ethanol 30 s Modified Burgeff medium	86.66	[110]
Orchis coriophora	H_2O_2 30 min Orchimax medium (Duchefa (the Netherlands)), 2.0 g/L AC, 1 mg/L IAA	46.2	[111]
Paphiopedilum spicerianum	1% NaOCl 40 min 1/4 MS, 20 g/L sucrose, 10% CW	79	[112]
Paphiopedilum wardii	0.5% NaOCl 60 min 1/2MS, 0.5 mg/L NAA, 10% CW, 1.0 g/L AC	70.33	[76]
Phalaenopsis Blume	1.6% dichloroisocyanuric acid 10 min P1056 media (Sigma-Aldrich)	43.1	[113]
Platanthera chapmanii	Not specified P723 medium (PhytoTechnology Laboratories), 12 weeks of cold moist stratification	35.1	[114]
Prosthechea garciana	Disinfection agent (16.6 g/L disodium salt of dichloroisocyanuric acid dissolved in sterile distilled water) with a drop of Tween 20 B5 macro-salts, MS micro-salts, 25 mg/L $Na_2FeEDTA$, 25 g/L sucrose	70	[103]
Paphiopedilum armeniacum	1% NaOCl 9 min or 40 kHz of ultrasound 8 min 1/4 MS, 0.5 mg/L NAA, 10% CW, 1.0 g/L AC	25.4	[72]
Paphiopedilum liemianum	Sterilized capsule VW, 10% CW, 2.5 μM NAA, incubation in the dark for 4 weeks	76	[115]
Rhynchostylis retusa	Sterilized capsule KC, 3.0 mg/L NAA, 5.0 mg/L BA, 1.0 mg/L GA_3	75–95	[116]
Serapias vomeracea	10% NaOCl 12 min KC	83.44	[117]
Spathoglottis plicata	Sterilized capsule KC	90	[118]

(continued)

Table 3
(continued)

Plant species	Pretreatment and medium composition	Germination efficiency (%)	Reference
Thunia marshalliana	Sterilized capsule MS, 3% sucrose, 10% CW	78.32	[119]
Trichoglottis tenera	0.1% NaOCl 2 min then 0.1% $HgCl_2$ 30 s 1/2 MS, 0.4% banana powder	90	[120]
Vanda testacea	Sterilized capsule 1/2 MS	90	[121]
Vanda roxburghii	Sterilized capsule MS, 0.1 mg/L NAA, 15% CW	–	[122]

AC activated charcoal, *B5* Gamborg B5 medium (*see* Chapter 6), *BA* 6-benzylaminopurine, *BM-1* basic medium modified according to Van Waes and Debergh (*see* Chapter 6), *CH* casein hydrolysate, *CW* coconut water, *EDTA* ethylenediaminetetraacetic acid, GA_3 gibberellic acid A3, *H026* H026 sowing media [123], *IAA* indole-3-acetic acid, *KC* Knudson's C medium (*see* Chapter 6), *KN* kinetin, *LM* Linsmaier and Skoog medium [124], *LOM* Lindemann orchid medium (*see* Chapter 6), *M* Mitra medium (*see* Chapter 6), *MS* Murashige and Skoog medium (*see* Chapter 6), *OMR* orchid maintenance/replate medium (PhytoTechnology Laboratories, USA), *PDA* potato dextrose agar medium, *PH* potato homogenate, *PM* Phytamax® (Sigma, USA), *SD* 1/2 MS salts modified medium, *SW* smoke water [125, 126], *TJ* tomato juice, *VW* Vacin and Went medium (*see* Chapter 6), *WPM* woody plant medium [127]

as multiplying existing plants from species. Table 4 provides selected recent publications. For additional information, readers can consult Yam and Arditti [128] and Chapter 7.

5 Artificial Seeds (Table 5)

Artificial or synthetic seed technology developed for somatic embryos sees applications in orchid propagation [159]. A recent review [160] summarizes recent development in synthetic seed technology. The procedures generally consist of immersing explant tissue or protocorms in a gel matrix, normally sodium alginate, and solidifying the matrix with tissue present using a solution of calcium chloride. Conditions for storage and germination of synthetic seeds need to be optimized for individual tissues. This technique is often combined with cryopreservation (*see* Chapter 15) to create a system that allows artificial seeds to be preserved for a long time. Table 5 provides a sample of recent publications related to this method of micropropagation.

Table 4
Sample literature of micropropagation protocols from 2012 to 2017

Plant species	Explant source	End result	Basal medium	PGR and additives	Reference
Aerides odorata	In vitro leaf and shoot tips from 38-week-old cultures	Callus	1/2 MS	0.1–4.0 mg/L TDZ and 0.5–8.0 mg/L BA	[129]
Brassidium Shooting Star orchid	PLBs	PLBs	1/2 MS	1 mg/L (w/v) BA	[130]
Calanthe davidii	Nodal sections of shoots	PLBs	VW medium (semisolid)	2.27 μM TDZ, 0.06 M sucrose	[131]
Cattleya hybrid (Blc. Cherry Comton × Mem. Robert straight)	Lateral buds	Shoots	MS	20 g/L sucrose, 150 mg/L citric acid, 0.3 mg/L NAA, 1.0 mg/L kinetin	[132]
Coelogyne flaccida	Pseudobulbs (cut in half)	Plantlets	MS	4.44 μM BA, 5.37 μM NAA, 10% CW	[133]
Cymbidium aloifolium	Leaves	Shoot buds	MS	3% (w/v) sucrose, 6 μM NAA, 9 μM BA	[134]
Cymbidium dayanum	PLBs	Shoots	MS pH 5.5–5.8	0.1 mg/L chondroitin sulfate, green LED light, 412.5 mg/L ammonium nitrate, 950 mg/L potassium nitrate, 20 g/L sucrose (or 20 g/L trehalose or 10 g/L sucrose with 10 g/L trehalose)	[135]
Cymbidium finlaysonianum	PLBs	Shoots	MS pH 5.5–5.8	0.1 mg/L chondroitin sulfate, green LED light, 412.5 mg/L ammonium nitrate, 950 mg/L potassium nitrate, 20 g/L sucrose (or 20 g/L trehalose or 10 g/L sucrose with 10 g/L trehalose)	[136]
Cymbidium giganteum	Pseudostem segments with nodes	PLBs	Half-strength MS salts, full-strength MS vitamins	2 g/L tryptone, 30 g/L sucrose, 1.5 g/L activated charcoal, 0.909 μM TDZ	[136]

(continued)

Table 4
(continued)

Plant species	Explant source	End result	Basal medium	PGR and additives	Reference
Cymbidium iridioides	Leaves	Shoot buds	MS	3% (w/v) sucrose, 100 mg/L casein hydrolysate, 15% CW, 6 μM NAA, 9 μM BA	[134]
Cymbidium Sleeping Nymph	Transverse thin cell layers of PLBs	Plantlets	MS pH 5.8	10% CW	[137]
Cymbidium Waltz 'Idol'	Meristem culture	Shoots	MS pH 5.5–5.8	412.5 g/L ammonium nitrate, 950 mg/L potassium nitrate, 20 g/L sucrose, 1 mg/L hyaluronic acid (93.3%), 1 mg/L chitosan, 0.1 mg/L BA	[138]
Cyrtopodium brandonianum	Root tip	Adventitious shoots or roots	1/2 MS	0.5 mg/L TDZ (for shoots), 6% sucrose, and 1 mg/L NAA (for roots)	[139]
Dendrobium Alya Pink	Shoot tips	PLBs	1/2 MS pH 5.75	10% (v/v) CW, 2% (w/v) glucose	[140]
Dendrobium Caesar × *Dendrobium Tomie Drake* (Dendrobium sonia-28)	Nodal segment	PLBs	½ strength liquid or semisolid MS	4.44 or 8.88 μM BA and 8.88 μM NAA. 10 g/L sucrose	[141]
Dendrobium chrysotoxum	PLBs	Plantlets	VW medium	1 mg/L NAA, 1 mg/L BA	[142]
Dendrobium crepidatum	Nodal segment	Plantlets	MS	100 mg/L (w/v) myoinositol, 3% (w/v) sucrose, 2 mg/L TDZ, and 0.5 mg/L NAA	[143]
Dendrobium crumenatum	Bisected protocorms	Callus	VW	2% sucrose, 2% peptone, 0.5 mg/L NAA, and 1 mg/L BA	[144]
Dendrobium densiflorum	Shoot tips	Plantlets	MS pH 5.8	2 mg/L BA, 0.5 mg/L NAA	[145]
Dendrobium huoshanense	Root tip and stem segments all 1 cm long	Callus	1/2 MS medium pH 5.2	1 mg/L 2,4-D and 0.5 mg/TDZ (root tip), 1 mg/L 2,4-D, and 0.1 mg/L TDZ (stem node)	[146]

(continued)

Table 4
(continued)

Plant species	Explant source	End result	Basal medium	PGR and additives	Reference
Dendrobium nobile	Nodal segment	Plantlet	MS pH 5.8	3% sucrose, 3 mg/L TDZ, and 0.5 mg/L NAA	[147]
Dendrobium officinate	Shoot tips	Callus	MS medium pH 5.8	0.5 mg/L BA, 0.1 mg/L NAA, 3% w/v sucrose, 0.03% (w/v) activated charcoal	[148]
Dendrobium primulinum	Shoot tip	Shoots or roots	MS medium	1.5 mg/L BA (for roots), 1.5 mg/L BA, and 0.5 mg/L NAA (for shoots)	[149]
Dendrobium sonia-28	PLBs	Plantlets	1/2 MS	2% sucrose, 1 mg mL/L BA	[150]
Eulophia andamanensis	Juvenile shoots, pseudo bulbs, and leaf dormant buds	Plantlets	MS pH 5.8	2% sucrose, 2 mg/L BA, 0.5 mg/L NAA	[151]
Eulophia nuda	Tuber	Shoot buds	MS pH 5.8	44.4 μM BA, 283.8 μM ascorbic acid, 67.8 μM adenine sulfate, 143.5 μM arginine	[152]
Mokara Broga	Shoot tips	PLBs	1/2 MS	1 mg/L BA, 2% (w/v) sucrose, pH 5.8	[153]
Oncidium Gower Ramsey and Sweet Sugar	Tips of young leaf (stromal cells)	Plantlets	MS	1 mg/L 1-aminocyclopropane-1-carboxylic acid	[154]
Orchis catasetum	Protocorms	Plantlets	MS Medium pH 5.7	0.5 mg/L BA, 0.5 mg/L NAA, 3% (w/v) sucrose, and 0.8% agar-agar	[155]
Paphiopedilum hangianum	Seed-derived protocorms	Callus and PLBs	1/2 MS pH 5.8	5.0 mg/L 2,4-D, 1.0 mg/L TDZ	[73]
Renanthera Tom Thumb 'Qilin'	Leaves	Callus	VW	1.0 g/L peptone, 10% (v/v) CW, and 1.0 mg/L TDZ	[156]
Vanda coerulea	Shoot tips	Plantlets	MS or VW	1 mg/l BA, 0.5 mg/l NAA	[157]

(continued)

Table 4
(continued)

Plant species	Explant source	End result	Basal medium	PGR and additives	Reference
Vanilla planifolia	Nodal segments (1.5–2.0 cm)	Shoots	MS pH 5.8	1.0 mg/L BA and 15% CW. Add 10.0 μM sodium nitroprusside to gain a larger number of shoots which are smaller	[158]

BA 6-benzylaminopurine, *CW* coconut water, *2,4-D*, 2,4-dichlorophenoxyacetic acid, *MS* Murashige and Skoog medium, *NAA* 1-naphthaleneacetic acid, *PLBs* protocorm-like bodies, *TDZ* thidiazuron, *VW* Vacin and Went medium

Table 5
Sample protocols for growing orchids through artificial seeds

Species	Encapsulation method	Explant used	Germination medium	Reference
Aranda Wan Chark Kuan 'Blue' x *Vanda coerulea* Grifft. ex. Lindl.	3% sodium alginate and 75 mM $CaCl_2 \cdot 2H_2O$	In vitro shoot tips (3–4 mm)	¼ strength MS	[161]
Aranda Wan Chark Kuan 'Blue' × *Vanda coerulea* Grifft. ex. Lindl.	3% sodium alginate and 75 mM calcium chloride	Encapsulated PLBs (capsules)	Hormone-free ½ MS	[162]
Catharanthus roseus	2.5% sodium alginate and 100 mM calcium chloride. 3% sucrose	Somatic embryos from embryogenic callus derived from hypocotyls	MS medium, 1.34 μM BA, 1.10 μM NAA	[163]
Cymbidium aloifolium	3% sodium alginate and 0.2 M $CaCl_2$ left for at least 30 min	Protocorms	Hormone-free MS medium	[164]
Cymbidium aloifolium	4% sodium alginate and 0.2 mol/L calcium chloride solution	Protocorms	MS liquid medium, 0.5 mg/L BA, 0.5 mg/L NAA	[165]
Dendrobium nobile	3% sodium alginate solution dropped into 100 mM $CaCl_2 \cdot 2H_2O$ for 15–20 min. Adding 7.5–12.5% sucrose or mannitol minimizes seed bursting	60-day-old PLBs of *D. nobile*	MS medium, 2% sucrose (w/v), 1 mg/L BA, 0.1 mg/L IAA	[166]

(continued)

Table 5
(continued)

Species	Encapsulation method	Explant used	Germination medium	Reference
Dendrobium White Fairy orchid	3% sodium alginate and 100 mM $CaCl_2$ incubated for 40 min	12-week-old *Dendrobium* White Fairy orchid PLBs	½ MS medium pH 5.2, 15% (v/v) CW, 2% (w/v) sucrose	[167]
Dendrobium Shavin White	3% sodium alginate and dropped in 74 mM calcium chloride for 15–20 min	PLBs with 3–5 mm shoots grown from shoot tip cultures	Semisolid ½ MS medium	[168]
Rhynchostylis retusa	3% sodium alginate and 100 mM $CaCl_2$ incubated for 40 min	Organogenetic PLBs	M medium, 2% sucrose, 1–10 mg/L NAA, BA and kinetin	[169]
Vanda coerulea	3% sodium alginate and 100 mM of $CaCl_2 \cdot 2H_2O$ for 30 min	PLBs of approximately 3.0 mm in diameter from leaf explants	Hormone-free medium	[170]

BA 6-benzylaminopurine, *CW* coconut water, *IAA* indole-3-acetic acid, *MS* Murashige and Skoog medium, *NAA* 1-naphthaleneacetic acid, *PLBs* protocorm-like bodies

References

1. Knudson L (1922) Nonsymbiotic germination of orchid seeds. Bot Gaz 73:1–25
2. Arditti J (1990) Lewis Knudson (1994–1958): his science, his times, and his legacy. Lindleyana 5:1–79
3. Yam TW, Arditti J (2009) History of orchid propagation: a mirror of the history of biotechnology. Plant Biotechnol Rep 3:1–56
4. Yam TW, Nair H, Hew CS, Arditti J (2002) Orchid seeds and their germination: an historical account. In: Kull T, Arditti J (eds) Orchid biology: review and perspectives, VIII. Kluwer, The Netherlands, pp 387–504
5. Stoutamire WP (1974) Terrestrial orchid seedlings. In: Winther CL (ed) The orchids: scientific studies. Wiley, New York, NY, pp 101–128
6. Chugh S, Guha S, Rao IU (2009) Micropropagation of orchids: a review on the potential of different explants. Sci Hortic 122:507–520
7. Teixeira da Silva JA (2013) Orchids: advances in tissue culture, genetics, phytochemistry and transgenic biotechnology. Floricult Ornament Biotechnol 7:1–52
8. Royal Botanical Gardens Kew (2016) Orchid research newsletter. http://www.kew.org/science/who-we-are-and-what-we-do/publications/orchid-research-newsletter. Accessed 14 Jun 2017
9. Herman EB (2014) Recent advances in plant tissue culture XIX. Orchid tissue culture: 2007–2014. Agritech Consultants, Inc., Mohegan Lake, New York
10. Sebastián F, Vanesa S, Eduardo F, Graciela T, Silvana S (2013) Symbiotic seed germination and protocorm development of *Aa achalensis* Schltr., a terrestrial orchid endemic from Argentina. Mycorrhiza 24:35–43
11. Jiang JH, Lee YI, Cubeta MA, Chen LC (2015) Characterization and colonization of endomycorrhizal Rhizoctonia fungi in the medicinal herb *Anoectochilus formosanus* (Orchidaceae). Mycorrhiza 25:431–445
12. Liu SS, Chen J, Li SC, Zeng X, Meng ZX, Guo SX (2015) Comparative transcriptome analysis of genes involved in GA-GID1-DELLA regulatory module in symbiotic and asymbiotic seed germination of *Anoectochilus roxburghii* (Wall.) Lindl. (Orchidaceae). Int J Mol Sci 16:30,190–30,203
13. Herrera H, Valadares R, Contreras D, Bashan Y, Arriagada C (2017) Mycorrhizal compatibility

and symbiotic seed germination of orchids from the Coastal Range and Andes in south central Chile. Mycorrhiza 27:175–188
14. Yamamoto T, Miura C, Fuji M, Nagata S, Otani Y, Yagame T, Yamato M, Kaminaka H (2017) Quantitative evaluation of protocorm growth and fungal colonization in *Bletilla striata* (Orchidaceae) reveals less-productive symbiosis with a non-native symbiotic fungus. BMC Plant Biol 17:50
15. Nikabadi S, Bunn E, Stevens J, Newman B, Turner SR, Dixon KW (2014) Germination responses of four native terrestrial orchids from south-west Western Australia to temperature and light treatments. Plant Cell Tiss Org Cult 118:559–569
16. Bustam BM, Dixon KW, Bunn E (2014) *In vitro* propagation of temperate Australian terrestrial orchids: revisiting asymbiotic compared with symbiotic germination. Bot J Linnean Soc 176:556–566
17. Fracchia S, Aranda-Rickert A, Rothen C, Sede S (2016) Associated fungi, symbiotic germination and *in vitro* seedling development of the rare Andean terrestrial orchid *Chloraea riojana*. Flora 224:106–111
18. Sathiyadash K, Muthukumar T, Murugan SB, Sathishkumar R, Pandey RR (2014) *In vitro* symbiotic seed germination of South Indian endemic orchid *Coelogyne nervosa*. Mycoscience 55:183–189
19. Yagame T, Funabiki E, Nagasawa E, Fukiharu T, Iwase K (2013) Identification and symbiotic ability of Psathyrellaceae fungi isolated from a photosynthetic orchid, *Cremastra appendiculata* (Orchidaceae). Amer J Bot 100:1823–1830
20. Rafter M, Yokoya K, Schofield EJ, Zettler LW, Sarasan V (2016) Non-specific symbiotic germination of *Cynorkis purpurea* (Thouars) Kraezl., a habitat-specific terrestrial orchid from the Central Highlands of Madagascar. Mycorrhiza 26:541–552
21. Pereira MCCAA, Rocha DI, Veloso TGR, Pereira OL, Francino DMT, Meira RMSA, Kasuya MCM (2015) Characterization of seed germination and protocorm development of *Cyrtopodium glutiniferum* (Orchidaceae) promoted by mycorrhizal fungi *Epulorhiza* spp. Acta Bot Brasilica 29:567–574
22. Guimarães FAR, Pereira MC, Felício CDS, Torres DP, Oliveira SF, Veloso TGR, Kasuya MCM (2013) Symbiotic propagation of seedlings of *Cyrtopodium glutiniferum* Raddi (Orchidaceae). Acta Bot Brasilica 27:590–596
23. Umata H, Ota Y, Yamada M, Watanabe Y, Gale SW (2013) Germination of the fully myco-heterotrophic orchid *Cyrtosia septentrionalis* is characterized by low fungal specificity and does not require direct seed-mycobiont contact. Mycoscience 54:343–352
24. Swangmaneecharern P, Serivichyaswat P, Nontachaiyapoom S (2012) Promoting effect of orchid mycorrhizal fungi *Epulorhiza* isolates on seed germination of *Dendrobium* orchids. Sci Hortic 148:55–58
25. Harshani HC, Senanayake S, Sandamali H (2014) Host tree specificity and seed germination of *Dendrobium aphyllum* (Roxb.) C.E.C. Fisch. in Sri Lanka. In: J Nat Sci Found, vol 41, pp 71–86
26. Zi XM, Sheng CL, Goodale UM, Shao SC, Gao JY (2014) *In situ* seed baiting to isolate germination-enhancing fungi for an epiphytic orchid, *Dendrobium aphyllum* (Orchidaceae). Mycorrhiza 24:487–499
27. Hajong S, Kumaria S, Tandon P (2013) Compatible fungi, suitable medium, and appropriate developmental stage essential for stable association of *Dendrobium chrysanthum*. J Basic Microbiol 53:1025–1033
28. Shao CS, Burgess KS, Cruse-Sanders JM, Liu Q, Fan XL, Huang H, Gao JY (2017) Using *in situ* symbiotic seed germination to restore over-collected medicinal orchids in Southwest China. Front Plant Sci 8:888
29. Khamchatra N-M, Dixon K, Chayamarit K, Apisitwanich S, Tantiwiwat S (2016) Using *in situ* seed baiting technique to isolate and identify endophytic and mycorrhizal fungi from seeds of a threatened epiphytic orchid, *Dendrobium friedericksianum* Rchb.f. (Orchidaceae). Agri Nat Res 50:8–13
30. Tsavkelova EA, Egorova MA, Leontieva MR, Malakho SG, Kolomeitseva GL, Netrusov AI (2016) Dendrobium nobile Lindl. Seed germination in co-cultures with diverse associated bacteria. Plant Growth Regul 80:79–91
31. Tan XM, Wang CL, Chen XM, Zhou YQ, Wang YQ, Luo AX, Liu ZH, Guo SX (2014) *In vitro* seed germination and seedling growth of an endangered epiphytic orchid, *Dendrobium* officinale, endemic to China using mycorrhizal fungi (*Tulasnella* sp.) Sci Hortic 165:62–68
32. Chen J, Wang H, Liu SS, Li YY, Guo SX (2014) Ultrastructure of symbiotic germination of the orchid *Dendrobium officinale* with its mycobiont, *Sebacina* sp. Aust J Bot 62:229–234
33. Hoang NH, Kane ME, Radcliffe EN, Zettler LW, Richardson LW (2017) Comparative seed germination and seedling development of the ghost orchid, *Dendrophylax lindenii* (Orchidaceae), and molecular identification of its mycorrhizal fungus from South Florida. Ann Bot 119:379–393
34. Zettler LW, Corey LL, Jacks AL, Gruender LT, Lopez AM (2013) *Tulasnella irregularis* (*basidiomycota*: *tulasnellaceae*) from roots of *Encyclia tampensis* in south Florida, and confirmation of its mycorrhizal signifi-

cance through symbiotic seed germination. Lankesteriana 13:119–128
35. Park EJ, Lee WY (2013) *In vitro* symbiotic germination of myco-heterotrophic *Gastrodia elata* by *Mycena* species. Plant Biotech Rep 7:185–191
36. Park EJ, Lee WY, Ahn JK (2012) *In vitro* propagation of myco-heterotrophic *Gastrodia elata*. Hort Environ Biotech 53:415–420
37. Fracchia SCA, Aranda-Rickert A, Flachsland E, Terada G, Sede S (2014) Mycorrhizal compatibility and symbiotic reproduction of *Gavilea australis*, an endangered terrestrial orchid from south Patagonia. Mycorrhiza 24:627–634
38. Long JRD, Swarts ND, Dixon KW, Egerton-Warburton LM (2012) Mycorrhizal preference promotes habitat invasion by a native Australian orchid*: Microtis media*. Ann Bot 111:409–418
39. Khamchatra N, Dixon K, Tantiwiwat S, Piapukiew J (2016) Symbiotic seed germination of an endangered epiphytic slipper orchid, *Paphiopedilum villosum* (Lindl.) Stein. from Thailand. S Afri J Bot 104:76–81
40. Zhou X, Gao J (2016) Highly compatible Epa-01 strain promotes seed germination and protocorm development of *Papilionanthe teres* (*Orchidaceae*). Plant Cell Tiss Org Cult 125:479–493
41. Cruz-Higareda JB, Luna-Rosales BS, Barba-Álvarez A (2015) A novel seed baiting technique for the epiphytic orchid *Rhynchostele cervantesii*, a means to acquire mycorrhizal fungi from protocorms. Lankesteriana 15:67–76
42. Ercole E, Rodda M, Girlanda M, Perrotto S (2015) Establishment of a symbiotic *in vitro* system between a green meadow orchid and a *Rhizoctonia*-like fungus. Bio-protocol 5:1–7
43. Aewsakul N, Maneesorn D, Serivichyaswat P, Taluengjit A, Nontachaiyapoom S (2013) *Ex vitro* symbiotic seed germination of *Spathoglottis plicata* Blume on common orchid cultivation substrates. Sci Hortic 160:238–242
44. Sathiyadash K, Muthukumar T, Murugan SB, Sathishkumar R, Uma E, Jaison S, Priyadharsini P (2013) *In vitro* asymbiotic seed germination, mycorrhization and seedling development of *Acampae praemorsa* (Roxb.) Blatt. & Mc Cann, a common south Indian orchid. AsPac J Reprod 2:114–118
45. Hossein P, Promila P, Bhanwra RK (2013) Asymbiotic germination of immature embryos of a medicinally important epiphytic orchid *Acampe papillosa* (Lindl.) Lindl. Afr J Biotechnol 12:162–167
46. Suja RM, Williams BC (2016) Micropropagation, phytochemical screening and antioxidant potential of a wild epiphytic orchid *Acampe praemorsa* (roxb) of Kanyakumari district, India. Euro J Pharm Med Res 3:572–576
47. Bhattacharjee B, Islam SMS (2014) Development of an efficient protocol for *in vitro* germination and enhancing protocorm-like body development in three indigenous orchid species in Bangladesh. AsPac J Mol Biol Biotechnol 22:209–218
48. Parab GV, Krishnam S (2012) Rapid *in vitro* mass multiplication of orchids *Aerides maculosa* Lindl. and *Rhynchostylis retusa* (L.) Bl. from immature seeds. Indian J Biotechnol 11:288–294
49. Verma J, Bhatti SK, Sembi JK (2015) Asymbiotic germination of immature seeds in an ornamentally important 'Fox-tail' Orchid, *Aerides multiflora* Roxb. Vegetos Int J Plant Res 28:162–168
50. Wesley PS, Devi C, Shibu BS, Moin S (2013) In vitro propagation of *Coelogyne breviscapa* Lindl., *Dendrobium aqueum* Lindl., and *Flickingeria nodosa* (Dalz.) Seidenf. via asymbiotic seed germination. AsPac J Mol Biol Biotechnol 21:26–32
51. Kaur S, Bhutani KK (2014) *In vitro* conservation and asymbiotic propagation of *Coelogyne flaccida* (Lindl.): a threatened orchid. Plant Biosystems 148:935–944
52. Parmar G, Pant B (2015) *In vitro* seed germination and seedling development of *Coelogyne flaccida* lindl. (*Orchidaceae*). Adv Forestry Sci 2:85–88
53. Koirala D, Pradhan S, Pant B (2013) Asymbiotic Seed germination and plantlet development of *Coelogyne fuscescens* Lindl., a medicinal orchid of Nepal. Sci World 11:97–100
54. Gaurav P, Bijaya P (2016) *In vitro* seed germination and seedling development of the orchid *Coelogyne stricta* (D. Don) Schltr. Afr J Biotechnol 15:105–109
55. Pradhan S, Regmi T, Parmar G, Pant B (2013) Effect of different media on *in vitro* seed germination and seedling development of *Cymbidium aloifolium* (L.) Sw. Nepal J Sci Technol 14:51–56
56. Bhowmik TK, Rahman MM (2017) *In vitro* seed germination and flowering of *Dendrobium palpebrae* Lindl. Orchid. J Global Biosci 6:4748–4757
57. Mahendran G, Muniappan V, Ashwini M, Muthukumar T, Bai VN (2012) Asymbiotic seed germination of *Cymbidium bicolor* Lindl. (*Orchidaceae*) and the influence of mycorrhizal fungus on seedling development. Acta Physiol Plant 35:829–840
58. Li F, Bao J, Sun Y, Liu C, Ma H, Zhang T, Chen X (2016) Embryo development and

corresponding factors affecting in vitro germination of *Cymbidium faberi* × *C. sinense* hybrid seeds. Arch Biol Sci 68:541–550
59. Deb CR, Pongener A (2013) A study on the use of low cost substrata against agar for non-symbiotic seed culture of *Cymbidium iridioides* D. Don. Aust J. Crop Sci 7:642–649
60. Wang Y, Li Z, Huang L, Su J (2013) *In vitro* mass scale propagation of wild *Cymbidium lowianum* with a rare and endangered plant. Amer. J Plant Sci 4:1500–1507
61. Zhang Y, Lee YI, Deng L, Zhao S (2013) Asymbiotic germination of immature seeds and the seedling development of *Cypripedium macranthos* Sw., an endangered lady's slipper orchid. Sci Hortic 164:130–136
62. Huh YS, Lee JK, Nam SY, Paek KY, Suh GU (2016) Improvement of asymbiotic seed germination and seedling development of *Cypripedium macranthos* Sw. with organic additives. J Plant Biotechnol 43:138–145
63. Huh YS, Lee JK, Nam SY, Hong EY, Paek KY, Son SW (2016) Effects of altering medium strength and sucrose concentration on in vitro germination and seedling growth of *Cypripedium macranthos* Sw. J Plant Biotechnol 43:132–137
64. Vijayakumar S, Rajalkshmi G, Kalimuthu K (2013) Propagation of *Dendrobium aggregatum* by green capsule culture. Lankesteriana 12:131–135
65. Acharya R, Parmar G (2016) *In vitro* seed germination of endangered Nepalese orchid species: *Dendrobium fimbriatum* Hook. Adv J. Seed Sci Technol 3:72–74
66. Udomdee W, Wen PJ, Lee CY, Chin SW, Chen FC (2014) Effect of sucrose concentration and seed maturity on *in vitro* germination of *Dendrobium nobile* hybrids. Plant Growth Regul 72:249–255
67. Zhao D, Hu G, Chen Z, Shi Y, Zheng L, Tang A, Long C (2013) Micropropagation and *in vitro* flowering of *Dendrobium wangliangii*: a critically endangered medicinal orchid. J Med Plant Res 7:2098–2110
68. Bakar B, Latip MA, Gansau JA (2014) Asymbiotic germination and seedling development of *Dimorphics lowii* (*Orchidaceae*). Asian J Plant Biol 2:28–33
69. Paudel M, Pradhan S, Pant B (2013) *In vitro* seed germination and seedling development of *Esmeralda clarkei* Rchb.f. (Orchidaceae). Plant Tiss Cult Biotechnol 22:107–111
70. Suzuki RCAM, Moreira VC, Pescador R, Ferreira WDM (2012) Asymbiotic seed germination and *in vitro* seedling development of the threatened *orchid Hoffmannseggella cinnabarina*. In Vitro Cell Dev Biol Plant 48:500–511
71. Arenmongla T, Deb CR (2012) Germination of immature embryos and multiplication of *Malaxis acuminata* D. Don, an endangered therapeutically important orchid, by asymbiotic culture in vitro. Indian J Biotechnol 11:464–469
72. Zhang YY, KL W, Zhang JX, Deng RF, Duan J, Teixeira da Silva JA, Huang WC, Zeng SJ (2015) Embryo development in association with asymbiotic seed germination *in vitro* of *Paphiopedilum armeniacum* S. C. Chen et F. Y. Liu. Sci Rep 5:1–15
73. Zeng S, Wang J, Wu K, Teixeira da Silva JA, Zhang J, Duan J (2013) *In vitro* propagation of *Paphiopedilum hangianum* Perner & Gruss. Sci Hortic 151:147–156
74. Diengdoh RV, Kumaria S, Tandon P, Das MC (2017) Asymbiotic germination and seed storage of *Paphiopedilum insigne*, an endangered lady's slipper orchid. S Afri J Bot 112:215–224
75. Jiang N, Zhang JX, Teixeira da Silva JA, Duan J, Liu HT, Zeng SJ (2016) Stimulatory effects of sodium hypochlorite and ultrasonic treatments on tetrazolium staining and seed germination *in vitro* of *Paphiopedilum* SCBG Red Jewel. Seed Sci Technol 44:77–90
76. Zeng S, Wu K, Teixeira da Silva JA, Zhang J, Chen Z, Xia N, Duan J (2012) Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. Sci Hortic 138:198–209
77. Hossain MM, Dey R (2013) Multiple regeneration pathways in *Spathoglottis plicata* Blume—a study *in vitro*. S Afric J Bot 85:56–62
78. Hrahsel L, Thangjam R (2015) Asymbiotic *in vitro* seed germination and regeneration of *Vanda coerulea* Giff. Ex. Lindl., an endangered orchid from Northeast India. J Plant Sci Res 2:1–5
79. Jualang AG, Devina D, Hartinie M, Sharon JS, Roslina J (2014) Asymbiotic seed germination and seedling development of *Vanda dearei*. Malays Appl Biol 43:25–33
80. Prakash B, Bais RT, Singh P, Khan S (2013) Effect of different pH on *in vitro* seed germination of *Vanda tessellata* (Roxb.) Hook. Ex.G an endangered medicinal orchid. Adv Life Sci Technol 8:4–7
81. Sagaya M, Divakar KM (2015) *In vitro* seed germination of *Xenikophyton smeeanum* (rchb.f.) Garay: a rare orchid of Western Ghats, Karnataka. Int Res J Eng Technol 2:280–286
82. Deepti S, Gayatri MC, Sitikantha S (2013) *In vitro* seed germination as an aid to conserve *Aerides maculosum* lindl., an endemic and

endangered orchid of western Ghats. Indian Int J Pharm Bio Sci 4:478–486
83. Srivastava D, Gayatri MC, Sarangi SK (2015) *In vitro* seed germination and plant regeneration of an epiphytic orchid *Aerides ringens* (Lindl.) Fischer. Indian J Biotechnol 14:574–580
84. Papenfus HB, Naidoo D, Pošta M, Finnie JF, Staden JV (2016) The effects of smoke derivatives on *in vitro* seed germination and development of the leopard orchid *Ansellia africana*. Plant Biol 18:289–294
85. Neto VBDP, Campos GDO, Boaretto AG, Zuffo MCR, Torrezan MDA, Benetão J (2013) *In vitro* behaviour of *Aspasia variegata*, an epiphytic orchid from the Brazilian Cerrado. Ciência Rural 43:2178–2184
86. Hu W, Yang Y, Liaw S, Chang C (2013) Cryopreservation the seeds of a Taiwanese terrestrial orchid, *Bletilla formosana* (Hayata) Schltr. by vitrification. Bot Stu 54:33
87. Santos SAD, Smidt EC, Padial AA, Ribas LLF (2016) Asymbiotic seed germination and *in vitro* propagation of *Brasiliorchis picta*. Afric J Biotech 15:134–144
88. Hoque MM, Al-Forkan M (2016) Role of basal media, carbon sources and growth regulators in micropropagation of two valuable medicinal orchids of Bangladesh. Int J Sci Res 5:1022–1026
89. Bustam BM, Dixon K, Bunn E (2014) Proliferation and harvesting of secondary protocorms as a novel means for improving propagation of terrestrial orchids. Aust J Bot 62:614–621
90. Schneider L, de Paula Araujo JS, Zaffari GR (2014) Seed germination of *Cattleya intermedia* and *Cattleya warneri* in alternative culture media. Amer Int J Contemp Res 4:60–66
91. Sasamori MH, Endres Júnior D, Droste A (2015) Asymbiotic culture of *Cattleya intermedia* Graham (Orchidaceae): the influence of macronutrient salts and sucrose concentrations on survival and development of plantlets. Acta Bot Brasilica 29:292–298
92. Díaz-Álvarez EA, Torres-Galeano C, Rojas-Cortés ÁP, De La Barrera E (2015) *In vitro* germination and development of two endangered endemic Colombian orchids *Cattleya mendelii* and *Cattleya quadricolor*. Gayana Bot Concepcion 72:213–220
93. Abraham S, Augustine J, Thomas TD (2012) Asymbiotic seed germination and *in vitro* conservation of *Coelogyne nervosa* A. Rich. an endemic orchid to Western Ghats. Physiol Mol Biol Plants 18:245–251
94. Gogoi K, Kumaria S, Tandon P (2012) *Ex situ* conservation of *Cymbidium eburneum* Lindl.: a threatened and vulnerable orchid, by asymbiotic seed germination. 3 Biotech 2:337–343
95. Mohanty P, Paul S, Das MC, Kumaria S, Tandon P (2012) A simple and efficient protocol for the mass propagation of *Cymbidium mastersii*: an ornamental orchid of Northeast India. AoB Plants 2012:pls023
96. Rodrigues LA, Neto VBDP, Boaretto AG, Oliveira JFD, Torrezan MDA, Lima SFD, Otoni WC (2015) In vitro propagation of *Cyrtopodium saintlegerianum* rchb. f. (Orchidaceae), a native orchid of the Brazilian savannah. Crop Breed App Biotechnol 15:10–17
97. Hossain MM, Sharma M, Pathak P (2012) In vitro propagation of *Dendrobium aphyllum* (Orchidaceae)—seed germination to flowering. J Plant Biochem Biotechnol 22:157–167
98. Parthibhan S, Benjamin JHF, Muthukumar M, Sherif NA, Kumar ST, Rao MV (2012) Influence of nutritional media and photoperiods on in vitro asymbiotic seed germination and seedling development of *Dendrobium aqueum* Lindley. Afric J Plant Sci 6:383–393
99. Paul S, Kumaria S, Tandon P (2012) An effective nutrient medium for asymbiotic seed germination and large-scale *in vitro* regeneration of *Dendrobium hookerianum*, a threatened orchid of northeast India. AoB Plants 2012:plr023
100. Utami ESW, Hariyanto S, Manuhara YSW (2017) *In vitro* propagation of the endangered medicinal orchid, *Dendrobium lasianthera* J.J. Sm through mature seed culture. Asian Pac J Trop Biomed 7:406–410
101. Thejaswini R, Narasimhan S (2017) Undefined organic additives stimulates *in vitro* seed germination of *Dendrobium ovatum* (Willd.) Kraenzl, a medicinal orchid. Int J Pharma Med Biol Sci 6:29–31
102. Voges JG, Benevenuto RF, Fritsche Y, Guerra MP (2014) Protocorm development of *Epidendrum fulgens* (Orchidaceae) in response to different saline formulations and culture conditions. Acta Sci Biol Sci 36:287–292
103. Jevšnik TCBE, Luthar Z (2015) Successful disinfection protocol for orchid seeds and influence of gelling agent on germination and growth. Acta Agri Slovenica 105:95–102
104. Gangaprasad A (2013) Rapid *in vitro* multiplication of *Eulophia cullenii* (Wight) Blume-a rare, endemic and exquisite orchid of southern western Ghats. Int Agri Environ Biotechnol 6:721–727
105. Samala S, Te-chatob S, Yenchonb S, Thammasiri K (2014) Protocorm-like body proliferation of *Grammatophyllum speciosum* through asymbiotic seed germination. SciAsia 40:379–383

106. Giri L, Jugran A, Rawat S, Dhyani P, Andola H, Bhatt ID, Rawal RS, Dhar U (2012) *In vitro* propagation, genetic and phytochemical assessment of *Habenaria edgeworthii*: an important Astavarga plant. Acta Physiol Plant 34:869–875
107. Vudala S, Ribas L (2017) Seed storage and asymbiotic germination of *Hadrolaelia grandis* (Orchidaceae). S Afr J Bot 108:1–7
108. Magrini S, Vitis MD (2017) *In vitro* reproduction of three *Limodorum* species (Orchidaceae): impacts of scarification methods and nitrogen sources on mature seed germination and seedling development. Plant Biosyst 151:419–428
109. Malabadi RB, Meti NT, Mulgund GS, Nataraja K, Kumar SV (2012) Smoke saturated water promoted *in vitro* seed germination of an epiphytic orchid *Oberonia ensiformis* (Rees) Lindl. Res Plant Biol 2:32–40
110. Thakur U, Dongarwar N (2013) A new report of *in vitro* flowering and multiple shooting in a wild epiphytic orchid *Oberonia recurva* Lindl. from asymbiotically germinated seedlings. Plant Knowledge J 2:113–118
111. Bektaş E, Cüce M, Sökmen A (2013) *In vitro* germination, protocorm formation, and plantlet development of *Orchis coriophora* (Orchidaceae), a naturally growing orchid species in Turkey. Turkish J Bot 37:336–342
112. Chen Y, Goodale UM, Fan XL, Gao JY (2015) Asymbiotic seed germination and in vitro seedling development of *Paphiopedilum spicerianum*: an orchid with an extremely small population in China. Global Ecol Conserv 3:367–378
113. Lesar H, Čeranič N, Kastelec D, Luthar Z (2012) Asymbiotic seed germination of *Phalaenopsis* Blume orchids after hand pollination. Acta Agric Slovenica 99:5–11
114. Poff KE, Sharma J, Richards M (2016) Cold-moist stratification improves germination in a temperate terrestrial orchid. S Appalachian Bot Soc 81:292–301
115. Utami ESW, Purnobasuki H, Soedarti T, Hariyanto S (2015) Asymbiotic seed germination and *in vitro* seedling development of *Paphiopedilum liemianum* Fowlie, an endangered terrestrial orchid in northern Sumatra, Indonesia. J Plant Sci 10:25–34
116. Kalita R, Das AK, Gogoi H, Veer V (2013) Effect of growth hormones on *in vitro* mass-propagation of orchid species *Rhynchostylis retusa*. J Cell Tiss Res 13:3711–3714
117. Cevdet G, Sebnem E (2012) Seed germination and development of *Serapias vomeracea* (Burm. fil.) Briq. ssp. orientalis Greuter in tissue culture. Res J Biotechnol 7:4–8
118. Sebastinraj J, Muhirkuzhali S (2014) Asymbiotic seed germination and micropropagation of *Spathoglottis plicata* Blume. Int J Adv Phar Biol Chem 3:495–501
119. Singh M, Kumaria S, Tandon P (2014) *In vitro* multiplication of *Thunia marshalliana* Rcbh. f. through mature seeds and pseudonodes. Indian J Biotechnol 13:268–273
120. Bastin M, Jeyachandran R (2015) *Ex situ* conservation of *Trichoglottis tenera* (Lindl.) a threatened, and endangered orchid of western Ghats using asymbiotic seed germination technique. Int J Recent Sci Res 6:3488–2496
121. Sebastinraj J, Britto SJ, Kumar DV, Robinson JP, Thangavel P (2014) Rapid propagation of *Vanda testacea* (Lindl.) Rchb. F—a highly medicinal value epiphytic orchid of India. World J Agric Sci 10:223–230
122. Islam R, Kabir KR, Hossain S, Khalil I (2014) Efficient *in vitro* cultural techniques for seeds germination of *Vanda roxburghii*. World J Agric Sci 10:163–168
123. Zeng SJ, Chen ZL, KL W, Zhang JX, Bai CK, Teixeira da Silva JA, Duan J (2011) Asymbiotic seed germination, induction of calli and protocorm-like bodies, and *in vitro* seedling development of the rare and endangered *Nothodoritis zhejiangensis* Chinese orchid. HortScience 46:460–465
124. Linsmaier EM, Skoog L (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127
125. Thomas TH, van Staden J (1996) Dormancy break of celery (*Apium graveolens* L.) seeds by plant derived smoke extract. Plant Growth Regul 17:195–198
126. Malabadi RB, Teixeira da Silva JA, Mulgund GS (2008) Smoke-saturated water influence *in vitro* seed germination of *Vanda parviflora* Lindl. Seed Sci Biotechnol 2:65–69
127. McCown BH, Lloyd G (1981) Woody plant medium (WPM)—a mineral nutrient formation for microculture for woody plant species. Hort Sci 16:453
128. Yam TW, Arditti J (2017) Micropropagation of orchids, 3rd edn. Blackwell, Cambridge
129. Devi HS, Devi SI, Singh TD (2013) High frequency plant regeneration system of *Aerides odorata* Lour. through foliar and shoot tip culture. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 41:169–176
130. Mubbarakh SA, Rahmah S, Rahman ZA, Sah NNM, Subramaniam S (2014) Cryopreservation of *Brassidium* Shooting Star orchid using the PVS3 method supported with preliminary histological analysis. Appl Biochem Biotechnol 172:1131–1145
131. Lin L, Xu Q, Ma J, Yuan B, Li W (2014) Cryopreservation of protocorm-like body-derived shoot tips of *Calanthe davidii* by droplet-vitrification. Cryo-Letters 35:129–137

132. Dewir YH, El-Mahrouk ME, Murthy HN, Paek KY (2015) Micropropagation of *Cattleya*: improved *in vitro* rooting and acclimatization. Hortic Environ Biotechnol 56:89–93
133. Kaur S, Bhutani K (2013) *In vitro* mass propagation of ornamentally and medicinally important *Coelogyne flaccida* Lindl. through pseudobulb segments. Plant Tiss Cult Biotechnol 23:39–47
134. Deb CR, Pogener A (2013) *In vitro* regenerative competence of foliar explants of *Cymbidium aloifolium* (L.) and *C. iridioides* D. Don: two horticultural important orchids. Indian J Biotechnol 12:402–408
135. Nahar SJ, Haque SM, Kazuhiko S (2016) Application of chondroitin sulfate on organogenesis of two *Cymbidium* spp. under different sources of lights. Notulae Sci Biol 8:156–160
136. Roy AR, Sajeev S, Pattanayak A, Deka BC (2012) TDZ induced micropropagation in *Cymbidium giganteum* Wall. Ex Lindl. and assessment of genetic variation in the regenerated plants. Plant Growth Regul 68:435–445
137. Vyas S, Guha S, Kapoor P, Rao IU (2010) Micropropagation of *Cymbidium* Sleeping Nymph through protocorm-like bodies production by thin cell layer culture. Sci Hortic 123:551–557
138. Kaewjampa N, Shimasaki K, Nahar SJ (2012) Hyaluronic acid can be a new plant growth regulator for hybrid *Cymbidium* micropropagation. Plant Tiss Cult Biotechnol 22:59–64
139. Flachsland E, Terada G, Fernandez JM, Medina R, Shinini A, Rey H, Mroginski L (2011) Plant regeneration from root-tip culture of *Cyrtopodium brandonianum* Barb. Rodr. (Orchidaceae). Propag Ornam Plants 11:184–188
140. Nambiar N, Tee CS, Maziah M (2012) Effects of organic additives and different carbohydrate sources on proliferation of protocorm-like bodies in *Dendrobium* Alya Pink. Plant Omics 5:10–18
141. Julkiflee AL, Uddain J, Subramaniam S (2014) Efficient micropropagation of *Dendrobium* sonia-28 for rapid PLBs proliferation. Emir J Food Agric 26:545–551
142. Atichart P (2013) Polyploid induction by colchicine treatments and plant regeneration of *Dendrobium chrysotoxum*. Thai J Agric Sci 46:59–63
143. Bhattacharyya P, Kumaria S, Job N, Tandon P (2016) *En-masse* production of elite clones of *Dendrobium crepidatum*: a threatened, medicinal orchid used in traditional Chinese medicine (TCM). J App Res Med Aromatic Plant 3:168–176
144. Kaewubon P, Hutadilok-Towatana N, Teixeira da Silva JA, Meesawat U (2014) Ultrastructural and biochemical alterations during browning of pigeon orchid (*Dendrobium crumenatum* Swartz) callus. Plant Cell Tiss Org Cult 121:53–69
145. Pradhan S, Paudel YP, Pant B (2013) Efficient regeneration of plants from shoot tip explants of *Dendrobium densiflorum* Lindl., a medicinal orchid. Afr J Biotechnol 12:1378–1383
146. Lee PL, Chen JT (2014) Plant regeneration via callus culture and subsequent *in vitro* flowering of *Dendrobium huoshanense*. Acta Physiol Plant 36:2619–2625
147. Bhattacharyya P, Kumaria S, Tandon P (2016) High frequency regeneration protocol for *Dendrobium nobile*: a model tissue culture approach for propagation of medicinally important orchid species. S Afr J Bot 104:232–243
148. Qian X, Wang C, Ouyang T (2014) *In vitro* flowering and fruiting in culture of *Dendrobium officinate* kimura et migo. (*Orchidaceae*). Pakistan J Bot 46:1877–1882
149. Pant B, Thapa D (2012) *In vitro* mass propagation of an epiphytic orchid *Dendrobium primulinum* Lindl. through shoot tip culture. Afr J Biotechnol 11:9970–9974
150. Poobathy R, Sinniah UR, Rathinam X, Subramaniam S (2013) Histology and scanning electron microscopy observations of cryopreserved protocorm-like bodies of *Dendrobium* sonia-28. Turkish J Biol 37:191–198
151. Singh DR, Baskaran V, Abirami K, Simhachalam P (2016) *In vitro* micropropagation of *Eulophia andamanensis*—a tropical orchid. Int J Trop Agric 34:2173–2177
152. Panwar D, Ram KH, Shekhawat N (2012) *In vitro* propagation of *Eulophia nuda* Lindl., an endangered orchid. Sci Hortic 139:46–52
153. Antony JJJ, Sundarasekar J, Rathinam X, Marimuthu K, Subramaniam S (2014) Microscopical analysis of *in vitro Mokara* Broga Giant orchid's PLBs. Emir J Food Agric 26:73–81
154. Chen J, Hong P (2012) Cellular origin and development of secondary somatic embryos in *Oncidium* leaf cultures. Biol Plant 56:215–220
155. Baker A, Kaviani B, Nematzadeh G, Negahdar N (2014) Micropropagation of *Orchis catasetum*—a rare and endangered orchid. Acta Sci Pol Hortorum Cultus 13:197–205
156. Wu K, Zeng S, Teixeria da Silva JA, Chen Z, Zhang J, Yang Y, Duan J (2012) Efficient regeneration of *Renanthera* Tom Thumb 'Qilin' from leaf explants. Sci Hortic 135:194–201

157. Jitsopakul N, Thammasiri K, Ishikawa K (2013) Efficient adventitious shoot regeneration from shoot tip culture of *Vanda coerulea*, a Thai orchid. Sci Asia 39:449–455
158. Tan BC, Chin CF, Alderson P (2013) Effects of sodium nitroprusside on shoot multiplication and regeneration of *Vanilla planifolia* Andrews. In Vitro Cell Dev Biol Plant 49:626–630
159. Saiprasad GVS (2001) Artificial seeds and their applications. Reson 6:39–47
160. Sharma S, Shahzad A, Teixeira da Silva JA (2013) Synseed technology—a complete synthesis. Biotechnol Adv 31:186–207
161. Gantait S, Sinniah R (2013) Storability, post-storage conversion and genetic stability assessment of alginate-encapsulated shoot tips of monopodial orchid hybrid *Aranda* Wan Chark Kuan 'Blue' X *Vanda coerulea* Grifft. ex. Lindl. Plant Biotechnol Rep 7:257–266
162. Gantait S, Bustam S, Sinniah UR (2012) Alginate-encapsulation, short-term storage and plant regeneration from protocorm-like bodies of *Aranda* Wan Chark Kuan 'Blue' × *Vanda coerulea* Grifft. ex. Lindl. (Orchidaceae). Plant Growth Regul 68:303–311
163. Maqsood M, Mujib A, Siddiqui ZH (2012) Synthetic seed development and conversion to plantlet in *Catharanthus roseus* (L.) G. Don. Biotechnology 11:37–43
164. Pradhan S, Tiruwal BL, Subedee BR, Pant B (2014) Micropropagation of *Cymbidium aloifolium* (L.) SW., a medicinal orchid by artificial seed technology. J Nat Hist Museum 28:42–48
165. Pradhan S, Tiruwal BL, Subedee BR, Pant B (2016) Efficient plant regeneration of *Cymbidium aloifolium* (L.) Sw., a threatened orchid of Nepal through artificial seed technology. Am J Plant Sci 7:1964–1974
166. Mohanty P, Nongkling P, Das MC, Kumaria S, Tandon P (2012) Short-term storage of alginate-encapsulated protocorm-like bodies of *Dendrobium nobile* Lindl.: an endangered medicinal orchid from North-east India. 3 Biotech 3:235–239
167. Siew WL, Kwok MY, Ong YM, Liew HP, Yew BK (2014) Effective use of synthetic seed technology in the regeneration of *Dendrobium* White Fairy Orchid. J Ornam Plants 4:1–7
168. Bustam S, Sinniah UR, Kadir MA, Zaman FQ, Subramaniam S (2012) Selection of optimal stage for protocorm-like bodies and production of artificial seeds for direct regeneration on different media and short term storage of *Dendrobium* Shavin White. Plant Growth Regul 69:215–224
169. Sharma V (2014) *In vitro* propagation of endangered orchid taxa using alginate-encapsulated protocorm-like bodies (PLBs). Nature Environ Pollut Technol 13:401–404
170. Sarmah DK, Borthakur M, Borua PK (2010) Artificial seed production from encapsulated PLBs regenerated from leaf base of *Vanda coerulea* Grifft. ex. Lindl.—an endangered orchid. Curr Sci 98:686–690

INDEX

Yung-I Lee and Edward Chee-Tak Yeung (eds.), *Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols*, Springer Protocols Handbooks, https://doi.org/10.1007/978-1-4939-7771-0,

D

E

F

G

H

I

R

S

T

U

V

W