Tulasi Satyanarayana
Sunil K. Deshmukh
B. N. Johri *Editors*

Developments in Fungal Biology and Applied Mycology

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To late Prof. S. B. Saxena, whose birth centenary has been celebrated during 2016–2017

Preface

Fungal biology deals with the study of fungi, including their growth and development, their genetic and biochemical characteristics, their taxonomy and genomics, and their use to humans. The current research focuses on mushrooms which may have hypoglycemic activity, anticancer activity, anti-pathogenic activity, and immune system-enhancing activity. A recent research has found that the oyster mushroom naturally contains the cholesterol-lowering drug, lovastatin, that mushrooms produce large amounts of vitamin D when exposed to UV light, and that certain fungi may be a future source of taxol. To date, penicillin, lovastatin, cyclosporine, griseofulvin, cephalosporin, ergometrine, and statins are the most famous pharmaceuticals which have been isolated from fungi.

Fungi are fundamental for life on earth in their roles as symbionts (e.g., in the form of mycorrhizae, insect symbionts, and lichens). Many fungi are able to break down complex organic biomolecules such as lignin, and pollutants such as xenobiotics, petroleum, and polycyclic aromatic hydrocarbons. By decomposing these molecules, fungi play a critical role in the global carbon cycle.

The kingdom fungi encompasses an enormous diversity of taxa with varied ecologies, life cycle strategies, and morphologies ranging from unicellular aquatic chytrids to large mushrooms. Little is, however, known about their true biodiversity, which has been estimated at 1.5 to 5 million species, with about 5% of these having been formally classified. Advances in molecular genetics have opened the way for DNA analysis to be incorporated into taxonomy, which has sometimes challenged the historical groupings based on morphology and other traits. Phylogenetic studies published in the last decade have helped reshape the classification within kingdom fungi, which is divided into one subkingdom, seven phyla, and ten subphyla.

The human use of fungi for food preparation or preservation and other purposes is extensive and has a long history. Mushroom farming and mushroom gathering are large industries in many countries. The study of the historical uses and sociological impact of fungi is known as ethnomycology. Because of the capacity of this group to produce an enormous range of natural products with antimicrobial or other biological activities, many species have long been used or are being developed for industrial production of antibiotics, vitamins, and anticancer and cholesterol-lowering drugs. More recently, methods have been developed for

genetic engineering of fungi, enabling metabolic engineering of fungal species. For example, genetic modification of yeast species, which are easy to grow at fast rates in large fermentation vessels, has opened the way for pharmaceutical production that are potentially more efficient than production by the original source organisms.

Several pivotal discoveries in biology have been made by researchers using fungi as model organisms, which grow and sexually reproduce rapidly in the laboratory. For example, the one gene–one enzyme hypothesis was formulated by scientists using the bread mold *Neurospora crassa* to test their biochemical theories. Other important model fungi are *Aspergillus nidulans* and the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, each with a long history of use to investigate issues in eukaryotic cell biology and genetics, such as cell cycle regulation, chromatin structure, and gene regulation. Other fungal models have more recently emerged that address specific biological questions relevant to medicine, plant pathology, and industrial uses; examples include *Candida albicans*, a dimorphic, opportunistic human pathogen; *Magnaporthe grisea*, a plant pathogen; and *Pichia pastoris*, a yeast widely used for eukaryotic protein production. Fungi are used extensively to produce industrial chemicals such as citric, gluconic, lactic, and malic acids, and industrial enzymes such as lipases used in biological detergents, cellulases used for making cellulosic ethanol and stonewashed jeans, and amylases, invertases, proteases, and xylanases. Several fungi such as *Psilocybe* mushrooms (colloquially known as magic mushrooms) are ingested for their psychedelic properties, both recreationally and religiously.

We are grateful to Prof. G. P. Mishra, Prof. R. S. Mehrotra, and Dr. Shashi Rai for their constant encouragement in bringing out this book on the occasion of the birth centenary of late Prof. S. B. Saksena.

The book is an attempt in collating recent developments in fungi from various environments: their diversity and potential applications. We greatly appreciate the efforts of experts in contributing on various aspects of fungi. The opinions expressed by the authors are their own. We wish to thank all the contributors for readily accepting our invitation and Springer for publishing the book.

New Delhi, India — Tulasi Satyanarayana
New Delhi, India — Sunil K. Deshmukh
Bhopal, India — B. N. Johri

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About the Editors

Prof. Tulasi Satyanarayana became a faculty fellow at the Division of Biological Sciences and Engineering, Netaji Subhas Institute of Technology (affiliated to the University of Delhi), New Delhi, after retiring from Department of Microbiology, University of Delhi South Campus, New Delhi, in 2016. He has over 270 scientific papers and reviews, six edited books, and three patent to his credit. He is a fellow of the National Academy of Agricultural Sciences (NAAS), the Association of Microbiologists of India (AMI), the Biotech Research Society (I), and the Mycological Society of India (MSI). He has 40 years of research and teaching experience and was the president of the AMI and MSI. His research has focused on understanding the diversity and applications of yeasts, thermophilic fungi, and bacteria and their enzymes as well as carbon sequestration using extremophilic bacterial carbonic anhydrases.

Dr. Sunil K. Deshmukh is a fellow and area convenor at the Nano-Biotechnology Research Centre, The Energy and Resources Institute (TERI), New Delhi. He was an assistant director (natural products) at Piramal Enterprises Ltd., Mumbai. He has broad industrial experience in the field of applied microbiology. He is now the president of the Mycological Society of India. He has 100 publications and eight patent to his credit. He has also edited seven books.

Dr. B. N. Johri is a professor and NASI senior scientist at the Department of Biotechnology, Barkatullah University, India. He has been the recipient of many academic awards, including the Indian National Science Academy's Young Scientist Medal, Rafi Ahmad Kidwai Memorial Award, and Acharya PC Ray Fellowship (MPCST). He is a fellow of the National Academy of Sciences (I), National Academy of Agricultural Sciences, and National Institute of Ecology. He has extensive teaching and research experience and has 148 research publications and three edited books to his credit.

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Significant Contributions of Prof. S.B. Saksena to Indian Mycology

1

R. S. Mehrotra, M. R. Siddiqui and Ashok Aggarwal

Abstract

Late Prof. S.B. Saksena was born on 10th Aug. 1917, therefore, his birth centenary has been celebrated during Aug. 10, 2016–Aug. 10, 2017. On this occasion, his students and associates fondly remembered his endearing qualities, commitment to teaching and research, and significant contributions to Indian mycology. A brief account of his life and achievements are discussed.

Keywords

Prof. Saksena · *Saksenaea vasiformis* · *Gliocladiopsis sagariensis*
Zygomycosis Soil fungi · *Trichoderma viride*

Let us first thank the organizers of the Centenary Celebration Committee of the birth of the celebrated botanist, late Professor Shyam Bahadur Saksena, as 2016–2017 mark the centennial year of the birth of late Professor Saksena. It is a measure

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of scholarly stature, breadth of learning and his human nature that the former students, colleagues, friends and well-wishers have decided to celebrate the centenary of the birth of Professor Saksena. We at the outset state that we feel privileged to write about the life and work of late Professor Saksena. Not only Professor Saksena was an eminent mycologist of international stature but was equally good as an ecologist especially with regard to the soil microorganisms, especially fungi. Late Professor S.D. Garrett, FRS of the Botany School Cambridge, wrote in the Festschrift Volume in the year 1980 (Garrett 1980), and we thought nothing can give better appreciation of the scholarly achievements of late Professor Saksena than the quotation which was given in the INSA Memoir Volume 17. Professor Garrett wrote, "It is a privilege to have been invited to contribute to the Festschrift Volume of essays assembled in honour of Prof. Saksena in recognition of his international standing as a mycologist. The writing of this article has been a pleasure for me, because for many years Prof. Saksena and I have shared an interest in soil fungi, including pathogenic root-infecting fungi. This is why he was an honoured guest, for the academic year 1958–1959, in our sub-department of Mycology and Plant Pathology at the Cambridge Botany School. Unlike me, however, Professor Saksena has the further distinction of being an internationally known authority on the taxonomy of soil fungi, to which he has added several new genera and species. This taxonomic competence was an essential ingredient of his distinguished early studies on the synecology of the Indian soil mycoflora".

In 1950s University of Saugar, Sagar had one of the finest faculty of teachers headed by Prof. R. Misra an outstanding ecologist of the country, Dr. Shyam Bahadur Saksena an eminent mycologist, Dr. T.V. Deshikachary an eminent phycologist of the country, Dr. Y. Sundar Rao, an eminent cytogeneticist, Dr. L.P. Mall an eminent autecologist and Dr. Y.D. Tiagi an eminent morphologists of the country.

But before we write anything further about the scientific achievements of Professor Saksena, we would prefer to write about his early years. Shyam Bahadur Saksena was born on 10, August, 1917 at Jabalpur (M.P.) in a middle class family. He lost his mother at the age of eight years. His father, late Munshi Ram Gulam Saksena, was at that time in the Excise department of the old province of C.P., and Berar. Shyam Bahadur was sent to Gwalior for his education under the care of his sister who was nearly 20 years elder to him. He completed his primary and secondary education in the municipal school at Lashkar (Gwalior). It is said that as a young boy he was very much attracted towards zoos and botanical gardens. The headmaster of his school had a very long-lasting influence on his personality. It is also said that young Saksena took interest in the ongoing national movement for independence. This made him quite bold, assertive and confident in his statements, and this trend continued in his future life. Shyam Bahadur joined Victoria College, Gwalior, for his intermediate and B.Sc. examinations. In intermediate he got second division, but in his B.Sc. Examination he secured first division and first position in the Biology Group of the College. It was under Agra University that he took his B.Sc. degree from Victoria College in the year 1937. Because of his brilliance, his family members and well-wishers of the family including his teachers advised him to join Agra College, Agra, for his M.Sc. degree in Botany. Shyam Bahadur did his

M.Sc. under the care of renowned Botanist Late Professor K.C. Mehta, FNA of Agra College, Agra. He passed M.Sc. examination with first division and with first position in the University (Agra University). After passing his M.Sc. examination, he joined the department of Agriculture of Gwalior State in 1939 as a research botanist. He was married to Smt. Sarla Saksena of Gwalior State. Shyam Bahadur Saksena joined the Dept. of Botany of the University of Saugar, Sagar in the year 1948. It is here that he came in close contact with Professor R. Misra an eminent ecologist of the country who laid the firm foundation for research and teaching at Sagar. As a young lecturer in Botany, Shyam Bahadur was encouraged to get registered for the Ph.D. degree of the University of Sagar under the external guidance of Late Professor Ram Kumar Saksena of Allahabad University, Allahabad. He did his work on the ecology, morphology and taxonomy of the fungi of local forest soils and which became a classical work. Dr. Saksena selected the study area for his Ph.D. work, a part of the forests neighbouring the Sagar town on the eastern side which is known as Patharia Forest. Initially when the Saugar University was founded by Late Sir Hari Singh Gour, the old university site was situated near a village Makronia, a few kilometres away from the main town, but now the university campus is situated on a hillock very near to the Patharia Forest. Patharia forest site for Ph.D. study was selected because of a number of interesting features discernible in a small area from the point of view of geology, topography, soil types and the variety of fungal flora. This forest was previously studied by Misra and Joshi in 1952 with respect to higher vegetation (Misra and Joshi 1952). They (Misra and Joshi) collected and studied general data on climate, soil characteristics, physiographic and biotic communities recognized by them.

After Dr. R. Misra left Sagar in the year 1955 as Professor of Botany, Bananas Hindu University, Varanasi, Dr. Saksena took over as Reader and Head of the Department. Dr. Saksena was elevated to the position of Professor in the year 1963. He remained Professor and Head of the department for nearly 15 years and finally retired from the University of Saugar, Sagar in the year 1977. He, however, continued as visiting Professor at Sagar for one more year and then joined Jiwaji University, Gwalior, for four years as Principal Investigator of the UGC Project entitled "Fungi of Madhya Pradesh". Thus, Saksena stayed in Sagar for about 30 years. Professor Saksena produced around 35 Ph.D. students including one from Ravi Shankar University, Raipur. The process continued to produce three more students from Jiwaji University, Gwalior. A list of his Ph.D. students is given in Table 1.1. His first Ph.D. student was Dr. M.R. Siddiqui, who did excellent monographic work on the genus *Alternaria* in India and it is due to the efforts of Dr. Siddiqui, the Department of Botany got built a fully air-conditioned Glass House with temperature and humidity control. Dr. Siddique is the oldest student of Professor Saksena and is residing in New Delhi after retirement as Professor and Project Coordinator in the Division of Seed Science and Technology, Indian Agriculture Research Institute, New Delhi. Reverting back to Professor Shyam Bahadur Saksena's Ph.D. work, it may be said that Dr. Saksena used the phytosociological methods used for vegetational analysis of higher plants towards the study of determination of frequency, abundance and total number of fungi in the

Table 1.1 List of Ph.D. thesis which have been completed under the guidance of Prof. S.B. Saksena

1.	M.R. Siddiqui	1960	Taxonomy and pathogenicity of genus *Alternaria* with special reference to Indian species
2.	R.S. Mehrotra	1961	Studies on soil fungi from *Piper betle* orchards with special reference to the diseases caused by *Phytophthora parasitica* var. *piperina* Dastur and their control
3.	K. Lily (Ku.)	1961	Ecological studies on soil fungi with special reference to the ecology of *Trichoderma viride* Pres. ex. Fries
4.	V.R. Ghurde	1962	Studies on foot-rot disease of wheat in Saugor
5.	P.K. Shetye	1962	Studies in soil fungi: seasonal variation in fungal flora of Vindhyan sand stone and basaltic soils of Sagar
6	A. Thammayya	1964	Ecological studies on soil microorganisms with special reference to their antibiotic activities
7.	G.V. Thampi	1964	Studies on the Fusaria of Sagar with special reference to their taxonomy, pathogenicity and interaction with other microorganisms
8	O.P. Mall	1965	Wilt disease of coriander with special reference to the rhizosphere studies
9	A.S. Saxena	1967	Studies on soil microorganisms with special reference to Actinomycetes and their antibiotic products against soil borne pathogens
10.	Sudha Srivastava (Ku.)	1967	Studies on rhizosphere flora of Potato with view of investigating its relationship with some soil borne diseases (*Rhizoctonia solani* and *Fusarium* spp.)
11.	S.S. Singh	1967	Taxonomical and ecological studies on soil fungi of Chhatarpur
12.	B.S. Somal	1968	Studies on *Helminthosporium* and *Curvularia*
13.	S.C. Aggarwal	1969	Studies on litter fungi of Saugar with special reference to cellulolytic forms
14.	H.N. Satya	1970	Studies on taxonomy and pathogenicity of genus *Pestalotia*
15.	S.S. Ali	1970	A study on post-harvest fruit rot of Musambi (*Citrus sinensis* Linn.) caused by *Rhizoctonia* sp.
16	Manjit Kaur Randhawa (Ku.)	1970	Studies on root nodule bacteria of Sagar soil
17.	Shashi Prabha Singh (Ku.)	1970	Studies on Indian Penicillia with special reference to their antibiotic products.
18	P.K. Rai	1971	Studies on soft rots of Papaya (*Caica papaya* L.) with special reference to the physiological and biochemical aspects
19	Shashi Rai Mrs.	1971	Studies on pathogenesis by *Colletotrichum capsici* (Syd) Butler and Bisby causing fruit rot of chillies (*Capsicum annum* L.)
20.	K.M. Vayas	1971	Studies on ecology and physiology of microorganisms with respect to a soil borne plant disease
21.	Miss. K. Singhai	1973	The phenomenon of soil fungistasis and study of certain aspects.

(continued)

Table 1.1 (continued)

22.	Mrs. M. Choudhari	1973	Study on fungal metabolites with special reference to plant growth regulators produced by some fungi
23	M.C. Kanchan	1973	Studies on soil mciroorganisms with special reference to Bhindi
24.	A.D. Adoni	1975	Study on microbiology of Sagar lake
25.	D.C. Garg	1977	Biochemical and pathological study of fruit rot fungi
26.	D.V.S. Balyan	1977	Studies on isolation of Actinomycetes from soil and their antagonistic activity in relation to pathogens
27.	S.P. Dubey	1977	Studies on production of metabolites with special reference to citric acid production from soil fungi
28.	T.S. Thind	1977	Physiological and pathological investigations on *Clathridium* rot of apple caused by *Clathridium corticola*
29.	D.P. Sharma	1979	Studies on cellulose decomposition by microorganisms
30.	Mrs. N. Pathak	1979	Investigation of leaf surface fungi of *Mangifera indica* L.
31.	J.P.N. Pandeya	1980	Studies on soil microbiology with special reference to litter decomposition
32.	A.K. Kher	1982	Effect of chemical fertilizers and fungicides on soil microorganisms
33.	B. Sundara Singh	1982	Ecological and biological studies of Penicillia
34.	Kartik Ghosh	1987	Limnology of some aquatic ecosystems with special reference to microbes and benthos

world for the first time. This work of Dr. Saksena paved the way for the Banaras School, the initiators being Dr. R.Y. Roy and his student Dr. R.S. Dwivedi and others to do similar type of synecological studies in grasslands of Varanasi and other places in India and other parts of the world. Dr. Saksena discovered several new genera and species of fungi, named a new genus *Saksenaea*, with a new species *S. vasiformis*, a new genus *Gliocladiopsis* with a new species *G. sagariensis*, another new genus *Monocillium* with a new species *Monocillium indicum*, a new species of *Paecilomyces* namely *Paecilomyces fusisporus* and another new species of *Cephalosporium* namely *Cephalosporium roseogriseum*. The genus *Saksenaea* was first placed in the family Mucoraceae by Hesseltine and Ellis (1973) and Ellis and Hesseltine (1974). Ellis and Hesseltine created a new family Sakseneaceae with *Saksenaea* and *Echinosporangium.* The research paper of Ellis and Hesseltine got published in the journal Mycologia a year later, but in the Volume IVB of the edited book entitled "THE FUNGI" by Ainsworth et al. This information was published in the year 1973. It may be pointed out that Canon and Krick (2007) and in the 10th edition of the Dictionary of fungi by Kirk et al. (2008); the genus *Saksenaea* has been placed in the family Radiomycetaceae of the order Mucorales, and they have not recognized the family Sakseneaceae (nomen nudum, without a Latin diagnosis; Art. 36.1 of the ICBN, McNeill et al. 2006)

The discovery of the fungus *Saksenaea* has made Dr. S.B. Saksena immortal due to the two reasons, one that this is a very interesting mucoraceous fungus, and secondly of late it has been found to be pathogenic to humans, and the first report came from Ajello et al. in 1976 from USA. This was published in the journal Mycologia. A complete case history of this case was published in American Journal of Medicine by Dean et al. (1975). Professor Saksena discovered this fungus in 1953a, b. Saksena gave a challenging presidential address in the Botanical Section of 66th Session of the Indian Science Congress held at Osmania University, Hyderabad, in the year 1979 where he stated "It was in 1953 when I was working on the ecology of soil fungi of the local forests of Sagar, I found that some of the fungal isolates did not sporulate despite every treatment of temperature, pH, nutrition, etc. and I was greatly puzzled about one of them. When several months passed and I was on the brink of throwing them away, an idea struck me. Since I had some experience on dealing with aquatic species of fungi, I thought that it may be a fungus of aquatic bearings. So I floated small pieces of agar bearing the fungus on the surface of water in Petri dishes. To my great bewilderment, very beautiful flask shaped structures appeared on the surface, which I later named *Saksenaea vasiformis* in honour of Professor R.K. Saksena of Allahabad University under whose guidance I was carrying out this work". This fungus resembles very much with the fungus *Nowakowskiella* of Chytridiomycota. Saksena hypothesized that mucorales or members of Zygomycota have directly evolved from Chytridiomycetous ancestors like *Nowakowskiella* and not from Saprolegniales of Oomycota. There is another sequel to this discovery. Dr. S.B. Saksena sent his paper to the journal Mycologia for publication in 1953, but this fungus was also discovered almost at the same time by Farrow in 1954 from Panama Canal Zone from Barro Colorado Island, but his paper reached the journal Mycologia Editor one month later than that of Professor Shyam Bahadur Saksena, and thus, he got the priority of discovering this new genus. Since then this fungus has been reported from different parts of India and abroad. But the human infection aspect has also become important. Vega et al. (2006) reviewed the previously reported cases of *S. vasiformis* infection, and both immune competent and immunosuppressed patients have been reported from the world including some from India. The first case of subcutaneous zygomycosis in India was reported by Padhye et al. (1988) in a rice mill worker. The infection involved the foot and multiple sinuses. Amputation of the forepart of the foot followed by a split thickness graft and treatment with potassium iodide cured the infection. However, Padhye et al. (1988) in their paper stated that in fact Chakrabarti et al. (1997) reported this fungus from Chandigarh earlier and comparatively recently in 2006 by Padmaja et al. from Vishakhapatnam. Since 2006, *S. vasiformis* has been reported from different places causing serious human infections specially those who were involved in automobile accidents or of several burn cases. A very interesting paper was published by Alvarez et al. (2010) in the Journal of Clinical Microbiology, wherein on the basis of molecular phylogeny, they proposed two more new species of the emerging pathogenic fungus *Saksenaea* (Fig. 1.1). They proposed *Saksenaea oblongispora* characterized by oblong sporangiospores and unable to grow at 42 °C and *Saksenaea erythrospora*

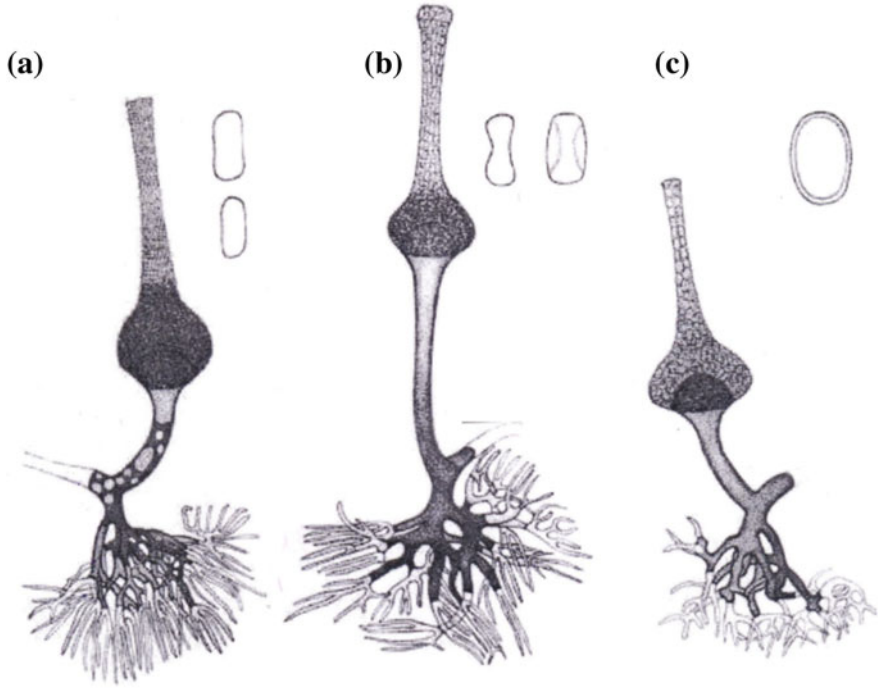

Fig. 1.1 Sporangiophores and sporangiospores of **a** 1 *Saksenaea vasiformis* **b** *S. oblongispora* **c** *S. erythrospora*. *Source* Alvarez et al. (2010)

characterized by large sporangiospores and sporangia and by ellipsoid sporangiospores, biconcave in lateral view. The optimum growth temperature was 25 °C and the minimum was 15 °C. The growth temperature requirements were also different for the two species. Sporangiospores of *Saksenaea vasiformis* were mainly cylindrical with rounded ends. A total of 11 strains from different culture collections of the world were included. None was included from any culture collection from India, but it is presumed that the culture taken from NRRL 2443 which was available in the ARS (NRRL) Culture Collection, Peoroa, Illinois, was the type strain discovered by Professor S.B. Saksena as it was from soil of India. No zygospore formation was reported by them or elsewhere in mating combination so far. So that sexual stage of the fungus is still not discovered or known.

Professor Saksena discovered two more genera, viz. *Monocillium* and *Gliocladiopsis*. The first one *Monocillium* with its species *indicum* was discovered in the year 1955 from the grassland soil of the Patharia Forest, in Sagar. This was however later believed to be the anamorphic stage of the Ascomycetous fungi such as *Nisselia* and *Hyaloseta*. The second new genus was *Gliocladiopsis* which was discovered in the year 1954 with a new species *sagariensis* (Saksena 1954). Agnihothrudu (1959) synonymized the genus with *Cylindriocarpon*. However, the good information is that the Lombard and Crous in 2012 resurrected the genus

using a global set of isolates and phylogenetic approach employing DNA sequence data from five genes (β-tubulin, histone H3, internal transcribed spacer region, 28S large subunit region and translation elongation factor $1-\alpha$); the taxonomic status of the genus *Gliocladiopsis* was re-evaluated, and *Gliocladiopsis sagariensis* was reinstated as a type species for the genus, which proved to be distinct from its former synonym *G. tenuis.*

Saksena discovered a new species of *Paecilomyces* namely *P. fusisporus* in 1953a, b from Patharia Forest soil. The new species has very characteristic fusiform or top-shaped conidia. Samson (1970), however, named the fungus as *Acrophialophora fusispora* (Saksena) Samson in 1970 [synonyms *Acrophialophora nainiana* Edwards (1961) *Massoniella indica* M.A. Salam and P. Rama Rao (1960), *Paecilomyces fusisporus* Saksena (1953a, b)]. It may be mentioned that *Acrophialophora fusispora* is an ascomycetous fungus that is a plant and human pathogen. Saksena described a new species of *Cephalosporium* as *C. roseogriseum* in 1955a, b, c from soil. The mycoparasitic behaviour of this fungus was shown by Chaturvedi and Dwivedi in 1982. Another new species reported by Professor Saksena (Saksena 1965) was *Sporothrix albicans* which was later thought to be synonymous with *Sporothrix schenckii.*

In 1958–1959, Dr. Saksena visited Cambridge University and worked in association with Late Professor S.D. Garrett on the biology of the fungus *Trichoderma viride*. Saksena (1960) showed conclusively that *T. viride* became the dominant colonizer of fumigated and steam sterilized soils in Evan's soil recolonization tubes not only due to its resistance to fumigant tolerant fungal species, but also due to its high growth rate in comparison with other fumigant tolerant species. Here again Dr. Saksena's insight into the ecological characteristics of a fungal species is clearly visible. Thus, Professor Saksena's proposal that the success of *T. viride* in fumigated soils was due to its combination of moderate but sufficient degree of fumigant tolerance with high growth rate through the soil, when the fumigant was dispersing in the soil and his interpretation of data made a significant advancement in our understanding of the problem of dominance of *T. viride* in fumigated soils. One of the Ph.D. students of Saksena and Lily (1967) later showed that in alkaline soils the role of *Penicillium nigricans* parallels with that of *T. viride*.

On his return from Cambridge, Prof. Saksena became interested in biological control of soil-borne plant pathogens and quite a number of his students in his laboratory worked on the biological control of soil-borne plant pathogens. Interest of Prof. Saksena diversified from soil fungi to soil-borne plant pathogens, aquatic fungi, and leaf litter-decomposing fungi, the dermatophytes and the post-harvest pathogens. The leaf rot and foot rot of Pan (*Piper betle*) was considerably investigated in his laboratory. One of us (RSM) worked in detail the role of cuttings and survival of the pathogens in soil. Dr. D.P. Tiwari showed that the pathogens survive in the soil in the form of the chlamydospores, and isolated the pathogen by using the selective medium of pimaricin, vancomycin and PCNB. *T. viride* was utilized for controlling the disease. Another student of Dr. Saksena, late Dr. Vyas and his research associate later found that streptomycin sulphate can be used for controlling *Phytophthora* on pan. Prof. Saksena was elected as President of the Indian

Phytopathological Society in the year 1975 and gave a very thought provoking address in the Bangalore session of the society. The title of his address was "*Phytophthora parasitica* the scourge of Pan (*Piper betle*)" in which the work done by his research associates and students was summarized. He made a fervent appeal for the establishment of Indian Pan Research Institute in the country.

Many honours came to Professor Saksena. He was elected Fellow of the Indian National Science Academy in 1971, President of the Indian Botanical Society, in 1971, President of the Indian Phytopathological Society in 1975, President Mycological Society of India in 1978 and President, Botany Section of the Indian Science Congress in the year 1979. He was a Fellow of the National Academy of Sciences, Allahabad. He was a Fellow/member of the Mycological Society of America and Transactions British Mycological Society of Great Britain. Professor Saksena was looked upon by eminent Botanists of the country with great respect.

As already been stated, Shyam Bahadur Saksena was married to Smt. Sarla Saksena, daughter of Shri Gopal Sahay Saksena who retired as IG Police of the then Gwalior state. They remained issueless. It may be mentioned that Mrs Saksena left for heavenly abode on 8th Feb, 1999. Both, the husband and the widowed wife, took interest in teaching poor children of the locality at their house. Some of the students of Prof. Saksena were entirely supported by them. Prof. Saksena's nephew Dr. D.N. Saksena who retired as Professor in Zoology at Jiwaji University, Gwalior, was one of them. He looked after the widow till her death in Feb, 1999 in the house built by Prof. Saksena. Late Prof. Saksena named his house as Vigyan Kutir. Another name worth mentioning is of Dr. S.C. Garg who studied at Sagar and did his Ph.D. in Chemistry and later became Professor. He is the son of one of his old friends. He was closely associated with Dr. Saksena at Sagar and is now settled in Bhopal.

Professor S.B. Saksena had gradually built up a good library of his own. It was donated to Jiwaji University, Gwalior, which happened to be his last working place.

Professor Saksena had been continuously in touch with his old students and corresponded with most of them enquiring about their family welfare, progress in career and research, and he took pride in talking about them with other scientists.

Prof. Saksena was very regular in habits and strict disciplinarian. He never missed his classes. He took tea usually at 2 pm with his students and colleagues, when he used to be at his best with humour and wit. He would discuss with them about their problems.

Professor Saksena laid great emphasis on field studies and field trips made with colleagues and students. Every year the first field trip was usually to Garhpera Forest about 23–30 km on Jhansi Road. A long excursion was also used to be taken and at least 3–4 teachers, research scholars and M.Sc. students would accompany the trip. Dr. Saksena very often was the leader of the trip. Dr. Saksena was not only interested in fungal collection but was very good in the taxonomy of higher plants. Collecting plants was a fun due to his sense of humour, his enthusiasm and ability to make his students feel that finding even the most common fungus or plant was a discovery. He would very often announce prizes for best collections made. Thus, a competitive spirit was aroused among the students for good collections. Wives of

the teachers often accompanied the trips, and cooking was done while on excursions by the excursion party itself which also included two to three peons or laboratory staff. A family atmosphere was seen during the excursions. Every one cooperated in the successful completion of the trip.

Prof. Saksena became diabetic rather early in life, and his health was deteriorating fast during the last 3 years of his life. He used to tell his friends and admirers that he received two warnings and the bell is ringing, the cruel death can snatch him at any time. On the 21, March, 1988, apparently healthy looking Prof. Saksena left his house 51, Shri Ram Colony, Jhansi Road to show himself to a specialist doctor unaccompanied by his wife. A severe heart attack struck him while going to the first floor of the Medical College which brought an end of this noble soul. Prof. Saksena was a rare combination of scholarship with humility, intelligence with honesty, strong will with compassion, sweetness in behaviour with straight forwardness (Mehrotra 1992/1993).

In an obituary published in The Journal Indian Phytopathological Society, Late Prof. B.S. Mehrotra (1989) of Allahabad University stated that his academic attainments, his amiable temperament and helpful attitude towards his students and colleagues endeared him to all who came in contact with him. We need more mycologists and human like him.

Before close, we want to emphasize that not only Professor Shyam Bahadur Saksena built the Department Botany, University of Sagar (Now known as Dr. Hari Singh Gour Central University, Sagar), but also he and his colleagues especially late Professor Y.D. Tiagi built a botanical garden in the new campus which will remain a living testimony of the love and care that Dr. Saksena had for plants.

Professor Saksena was a scholar and a thorough gentleman. Among the contemporary mycologists and plant pathologists, he can be ranked as one of the topmost. We pay our sincere tribute to him on this auspicious occasion of his Birth Centenary Year.

For the late Professor C.V. Subramanian FNA, it had been said that he started as a plant pathologist and turned into a mycologist, while late Prof. Saksena started as a mycologist and turned into a plant pathologist.

Acknowledgements R.S. Mehrotra, Retd. Professor of Botany, Kurukshetra University, has written about the scientific achievements and later part of personal life of Prof. S.B. Saksena. He was his second Ph.D. student and subsequently a junior colleague of Prof. Saksena. The association lasted for 15 years at Saugar. M.R. Siddiqui, former Professor and Project coordinator in the Division of Seed Science and Technology of Indian Agricultural Research Institute, happened to have obtained Ph.D degree as Prof. Saksena's first student. He had compiled the bibliography. The third author is Ashok Aggarwal, Professor of Botany, Kurukshetra University, Kurukshetra, who has collected the latest information about the discovery of the two species of the genus *Saksenaea* and the latest information on the other new genera or species and the preparation and typing of the manuscript. Most of the personal information of Dr. Saksena has been taken from the memoir of Dr. Saksena published in 1993 (Mehrotra et al. 1993) by Indian National Science Academy, New Delhi, which is duly acknowledged. The personal information was collected by late Prof. R. Misra of Bananas Hindu University, Varanasi, which is also gratefully acknowledged.

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Biology and Significance of *Saksenaea vasiformis*

2

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Abstract

The status of *Saksenea vasiformis* discovered by S.B. Saksena from soil of Sagar forests in 1953, now found distributed all over the world as serious pathogen, is reviewed. Its morphology, clinical aspects, pathogenecity, case reports, antibiotic sensitivity, treatment and molecular phylogeny is also reviewed. Almost all the citations dealing with fungus are included.

Keywords

Saksenaea vasiformis · Sporulation · Sporangia · Rhizoids · Mucormycoses

Introduction

Saksenaea vasiformis S.B. Saksena was first isolated from forest soil of Sagar MP, India, by S.B. Saksena in 1953. This fungus belongs to subphylum Mucomycotina. This is the only species of the genus Saksenea. Later Ajello et al. (1976), Chien et al. (1992), Pillai and Ahmed (1993) reported it from wood and grains. The characteristic features of this fungus are typically flask-shaped sporangia, sporangiophores, oval sporangiospores, and dark rhizoides (Fig. 2.1).

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The flask-shaped sporangia and inability to sporulate on normal media are special features of *Saksenaea vasiformis*. However, Czapeks Dox agar and agar blocks or nutrient deficient media may be favorable for sporulation (Ellis and Ajello 1982; Padhye and Ajello 1988). These workers reported fast growing white colonies without any pigmentation. Aseptate broad hyphae bearing typical flask-shaped with long neck, dividing singly or dichotomously branched hyphae rhizoides are darkly pigmented.

Fig. 2.1 *Saksenea vasiformis* Saksena. X1000

Saksenaea vasiformis can be isolated on several media but grows without sporulation. Only agar blocks and nutrient deficient media favor its sporulation. However, Czapeks dox agar may also favor sporulation but not always. Bearer et al. (1994) and Vega et al. (2006) studied 11 strains from different reference culture collections for DNA extraction, amplification, and sequencing, which were provided by the American Type Culture Collection.

Routine mycological media support mycelia growth of *Saksenia vasiformis* without sporulation. Nutritionally deficient media such as agar blocks stimulate its sporulation and favor development of flask-shaped sporangia. Sterilized yeast extract and/or sterile distilled water is mostly used for development of sporangia (Bearer et al. 1994). A total of 11 strains from different reference culture collections were studied by Vega et al. (2006) for DNA extraction, amplification, and sequencing, which they obtained from the American Type Culture Collection, (ATCC) Manassas, VA; the Centraalbureau voor Schimmelcultures (CBS) Utrecht, Netherlands; the National Reference Center for Mycoses and Antifungal Agents (NRCMA), Institut Pasteur, Paris, France; the Facultad de Medicina de Reus (FMR), Reus, Spain; the Fungus Testing Laboratory at the University of Texas Health Science Center (UTHSC), San Antonio, TX; and the ARS (NRRL) Culture Collection, Peoria.

Reports revealed that these strains were cultured and maintained on PDA, MEA, and CZA at 37 °C. The growth rate on above media temperature ranging 4–50 °C and carbon source assimilation profile were measured by using method described by Schwarz et al. (2007).

Al-Hedaithy (1998) have reported two new species namely *S. oblongispora* and *S. erythrospora* based on molecular phylogeny. Induction of sporulation in the fungus is successful in tap water agar and exposure to sunlight was used.

Clinical Aspects, Pathogenecity and Case Reports

Kaufman et al. (1988) and Tanphaichitr et al. (1990) further clarified its thermo-tolerant nature growing at 25 and 44 °C and reported to cause infection in immunocompromised and immunocompetent human hosts. Mucormycosis caused by *S. vasiformis* most often occurs after traumatic implantation of the fungus but can also be due to inhalation of spores (García-Martínez et al. 2008), spider bites, insect stings, and the use of indwelling catheters (Chakrabarti et al. 1997; Lechevalier et al. 2008). Clinical cases seem to be more common in tropical and subtropical climates than elsewhere and have been reported from Australia (Ellis and Kaminski 1985; Gonis and Starr 1997; Holland 1997; Stewardson et al. 2009; Wilson 2008), India (Baradkar and Kumar 2009; Baradkar et al. 2008; Chakrabarti et al. 1997; Padhye et al. 1988), the USA (Ajello et al. 1976; Bearer et al. 1994; Oberle and Penn 1983; Pierce et al. 1987; Toreil et al. 1981), Thailand (Tanphaichitr et al. 1990), Tunisia (Lechevalier et al. 2008), the Middle East (Al-Hedaithy 1998), and Central and South America (Blanchet et al. 2008;

Vega et al. 2006). Other workers also reported *S. vasiformis* from several clinical samples (Upton et al. 2002; Wilson 2008; Mogbil Al Wedaithy 1988; Parker et al. 1986; Robeck and Dalton 2002).

The first human infections due to *S. vasiformis* were described by Ajello et al. in 1976. *Saksenaea vasiformis* is most often associated with cutaneous or subcutaneous lesions after trauma. *Apophysomyces elegans* is often being reported (Adam et al. 1949). Up to now, approximately 40 cases of zygomycete infections, mostly cutaneous infections, have been attributed to *Saksenaea* (Vega et al. 2006; Trotter et al. 2008; Baradkar and Kumar 2009), although for the reasons indicated above, it is likely that the actual number of clinical cases has been underestimated. To avoid difficulties in the detection and identification of *Saksenaea* in clinical samples, several authors have emphasized the need for special culture techniques, such as the use of floating agar blocks on water, or the use of Borelli's lactrimel agar (Ellis and Ajello 1982; Lye et al. 1996) to induce sporulation. In contrast, the use of Czapek agar, a culture medium traditionally used for the phenotypic characterization of *Aspergillus* and *Penicillium* species, produced good in vitro sporulation of the *Saksenaea* strains isolated from clinical sources. *Saksenaea vasiformis* was found implicated in human infections of cutaneous and subcutaneous lesions as studied by Ajello et al. (1976). Emerging prominence of cutaneous infections has also been reported by Adam et al. (1949). About 40 cases of cutaneous infections by *Saksenaea vasiformis* reported so far (Vega et al. 2006; Trotter et al. 2008; Baradkar and Kumar 2009). But it appears that clinical cases reported so far are as much lesser than expected. Attempt to induce sporulation of this fungus several techniques such as floating agar blocks on water was suggested (Ellis and Ajello 1982; Lye et al. 1996) in order to correct identification of *Saksenaea vasiformis.* The CZA also proves a good medium for sporulation for *Saksenaea vasiformis.*

Saksenaea vasiformis is an emerging pathogenic fungus of order Mucorales and appeared to be involved in immunocompromised patients such as with leukemia, diabetes, and under treatment and under treatments with corticosteroids. Infection of this fungus is diagnosed by angio-invasion leading to tissue necrosis; also cutaneous and subcutaneous involvement is noted. This is being associated with traumatic inoculations. Alvarez et al. (2010) have reported rhino-orbito-cerebral and disseminated infections. The clinical relevance and incidence of infections are not yet very clear, but *S. erythrospora* was found associated in a wounded person in Iraq.

The association of *Saksenaea vasiformis* always in infection through soil is not very well recognized. The first report of subcutaneous zygomycosis by *Saksenaea vasiformis* was from Vishakhapatnam, India by Padmaja et al. (2006). Ellis and Kaminski (1985), Oberle and Penn (1983) reported 28 cases of *Saksenaea vasiformis* infection of which 11 died. Out of 11 deaths six were observed immunocompetent hosts (Padhye et al. 1988; Patino et al. 1984; Kaufman et al. 1988; Hay et al. 1983; Solano et al. 2000; Oberle and Penn 1983), while three were found with malignancies as described by Toreil et al. (1981), Ellis and Kaminski (1985), Gonis and Starr (1997). And two were in diabetics (Chakrabarti et al. 1997; Campelo et al. 2005). Most of these infections were cutaneous and localized. *Saksenaea vasiformis*

also causes rhinocerebral diseases (Kaufman et al. 1988; Gonis and Starr 1997). Solano et al. (2000) reported some cases of pulmonary sinusitis which were from Spain. These infected patients died in spite of debridement and anti-fungal therapy. Amphotericin B treated patients were recovered. Some cases responded to debridement alone (Padhye et al. 1988; Holland 1997; Chakrabarti et al. 1997; Pritchard et al. 1986).

Subcutaneous zygomycosis is often found due to *Saksenaea vasiformis* starting with mild to fatal infections. Padmaja et al. (2006) reported that early diagnoses and treatment may give better results. Since this species normally do not sporulate on common media and it takes time on nutritionally deficient media to sporulate and diagnose.

The infections caused by *Saksenia vasiformis* are subcutaneous, disseminated, rhinocerebral, and fatal disseminated (Toreil et al. 1981; Hay et al. 1983). Subcutaneous infections reported in 3-month- and 11-year-old children. This fungus was also able to invade tissue in traumatic injury. Padhye et al. (1988) reported subcutaneous zygomycosis by this fungus in India for the first time in a foot infection rice mill worker revealing multiple sinuses. However, this infection was cured by split thickness graft and potassium iodide treatment. Cranial wound, subcutaneous tissue necrotizing cellutites, and disseminated infections were also reported but are not common in clinical laboratories. (Ajello et al. 1976; Ellis and Kaminski 1985; Oberle and Penn 1983; Toreil et al. 1981).

Al-Hedaithy (1998) reported rhinocerebral infection by *Saksenea vasiformis,* while cutaneous infection suggested to be primary or secondary infection was suspected from some other sources. Hay et al. (1983) reported fatal disseminated infection in a woman. A three-month-old infant and 11-year-old child were reported to have subcutaneous infection (Al-Hedaithy 1998). *S. vasiformis* is filamentous fungus belonging to Mucorales causing human infection. Ajello et al. (1976) reported to cause human infection for the first time.

Alvarez et al. (2010) reported a cutaneous lesion of the abdominal wall in a woman in French Guinea which was due to zygomycosis and was treated with liposomal amphotericin B and itraconazol. Vega et al. (2006) found a child in Paris who developed lesion due to scorpion sting with many areas of necrosis and non-specific inflammation from where later on zygomycetous branched, non-septate fungal hyphae were observed. These were failed to sporulate. Fluorescent microscopy after proper staining with calcofluor white also revealed zygomycete hyphae.

Some animal cases of *S. vasiformis* in Taxas, Australia, were reported including marine mammals which were found to develop various symptoms as a result of fungus infection.

In 2010 *Saksenaea oblongispora* Alvarez, Stchigel, Cano, Garcia-Hermoso, et Guarro sp. nov. MycoBank MB 518626; *Saksenaea erythrospora* Alvarez, Cano, Stchigel, Garcia-Hermoso, et Guarro sp. nov. MycoBank MB 518627 were discovered. *Saksenaea oblongispora*, characterized by oblong sporangiospores and unable to grow at 42 °C, and *Saksenaea erythrospora*, characterized by large

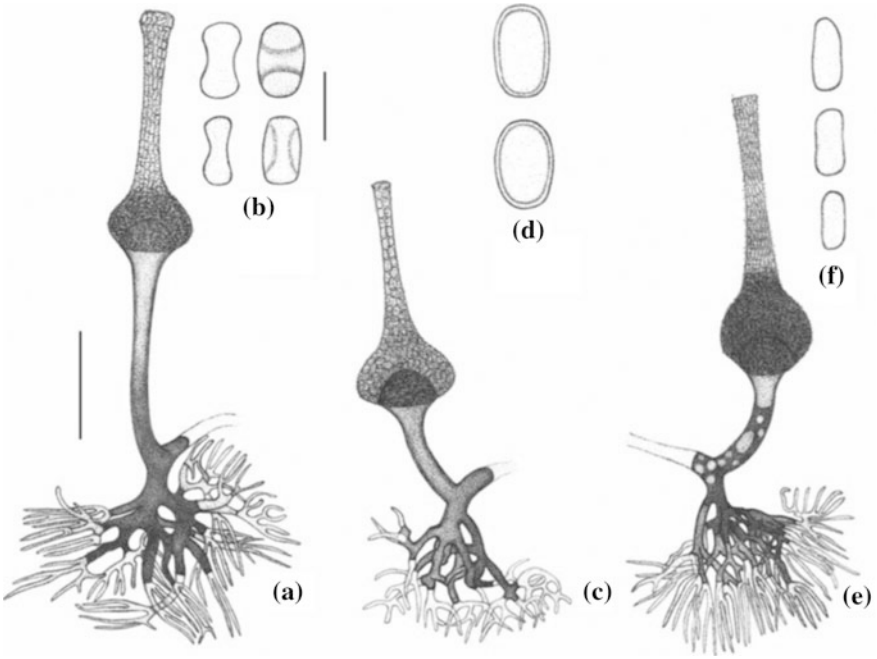

Fig. 2.2 *Saksenaea erythrospora* (**a**, **b**). *Saksenaea oblongispora* (**c**, **d**). (*Saksenaea vasiformis* (**e**, **f**). Bars, 50 μm for (**a**, **c**, and **e**); 5 μm for (**b**, **d**, and **f**) (Alvarez et al. 2010)

sporangiophores and sporangia and by ellipsoid sporangiospores, biconcave in the lateral view (Alvarez et al. 2010) (Fig. 2.2).

deHoog et al. (2000) and Ribes et al. (2000) reported often fatal infections caused by species of *Rhizopus, Mucor, Absidia, Rhizomucor, Apophysomyces* including *Saksenaea.* These infections may be localized or disseminated and most commonly rhinocerebral form of diseases. Ribes et al. (2000) discussed predisposing factors. The published first report of *Saksenaea vasiformis* infection is of Ajello et al. (1976) along with 26 more cases. This is rare fungus worldwide in distribution causing mild to chronic infection leading to fatal acute infection (Dean et al. 1977).

Phylogenetic Studies

Alvarez et al. (2010) mentioned small number of isolates availability hinders correctization on the basis of phenotypic characters. In the case of *S. vasiformis,* molecular studies were based on internal transcribed spacer (ITS) sequences which revealed relatively high specific intraspecific diversity (Blanchet et al. 2008; Lechevalier et al. 2008). This study disclosed more than one phylogenetic

species of *S. vasiformis*. DNA sequences generated should be useful for further characterization of clinical isolates and/or identification of causative species from tissue biopsy specimens. They may also allow the identification of other species within the genus or the *S. vasiformis* complex. *S. vasiformis* was redefined as a complex, and two new cryptic species were identified, *S. erythrospora* and *S. oblongispora* (Alvarez et al. 2010). These workers showed that possibility exists as a complex species (Fig. 2.3).

Al-Hedaithy (1968), Blanchet et al. (2008), Padmaja et al. (2006) reported 35 cases of *S. vasiformis* infection. Vega et al. (2006) reported 22 cutaneous infections, three sinusitis and three disseminated infections. Infection also occurred after insect and spider bites (Padhye et al. 1988). Study of Padmaja et al. (2006) showed that most of the infections are from USA, Australia, New Zealand, Colombia, Ecuador, French Guiana, Israel, Thailand, Spain, India, and Iraq which are from tropical subtropical regions. In addition to above 35 cases of infection, one more case of *S. vasiformis* infection was reported from Tunisia. In case of non-sporulation of the fungus identification relies on molecular analysis directly on the infected tissue. Frozen- or paraffin-embedded tissues are generally used for these analysis (Vega et al. 2006) of *S. vasiformis* infected tissue.

Alignment of the sequences of other descried species with *S. vasiformis* sequences allowed us to identify the fungus with confidence as the species *S. vasiformis*. It is interesting that there are relatively large genetic distances between isolates of *S. vasiformis*, as previously noted (Padmaja et al. 2006). A more comprehensive phylogenetic study of this rare genus would be of interest (Alvarez et al. 2010; Castresana 2000). *S. vasiformis* ITS sequence available with the gene bank database is suggestive of more and more phylogenetic study of the genus.

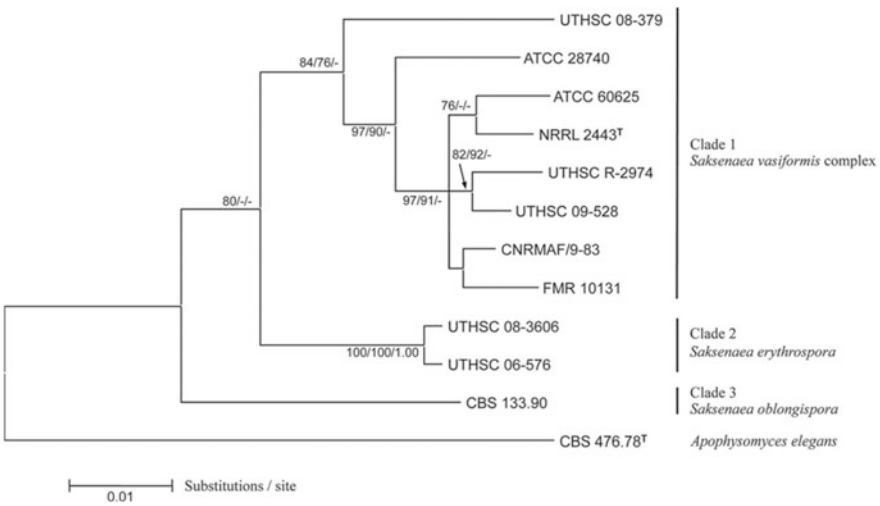

Fig. 2.3 Maximum-likelihood (ML) tree obtained from the combined DNA sequence data from three loci (ITS, D1/D2, EF-1-1α) (Alvarez et al. 2010)

Vega et al. (2006) studied genetic and phenotypic diversity among 11 strains of and showed useful markers for species label identification of genus Saksenea. The genus proved to be genetically heterogenous having more than one species. The most important characteristic features were shape, size, thermotolerance, carbon assimilation for the species differentiation.

Antibiotic Sensitivity and Treatment

Sporangiospore suspension was counted microscopically and adjusted to the required density. Rodriguez-Tudela et al. (2008) screened *S. vasiformis* against amphotericin B, itraconazole, voriconazole, posaconazole, and caspofungin and found the MIC of 0.5 μg/mL for posaconazole effective. Alvarez et al. (2010) found AMB and PSC effective against *S. vasiformis.*

Antifungal susceptibility testing of above strains was carried out on CZA for 7–20 days at 30 °C or 37 °C. Sporangiospores were then collected in water, and the suspension was adjusted to 2×10^4 CFU^5/ml per well. Pure active powders, of known potency, of amphotericin B (Sigma-Aldrich, Saint Quentin Fallavier, France), voriconazole (Pfizer Central Research, Sandwich, United Kingdom), itraconazole (Janssen-Cilag, Issy-les-Moulineaux, France), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ), terbinafine (Novartis Pharma AG, Basel, Switzerland), caspofungin (Merck & Co., Inc., Rahway, NJ), micafungin (Astellas Pharma, Osaka, Japan), and anidulafungin (Pfizer) were used by Alvarez et al. (2010). And antifungal susceptibility testing was performed by a broth microdilution technique according to the guidelines of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing for the testing of conidium-forming molds, with some modifications (Alvarez et al. 2010).

More aggressive approach is required to induce sporulation of zygomycetes that are initially sterile. *S. vasiformis* infections are now found globally, but still the genus is very poorly studied and a very few numbers are preserved in culture collections. Unfortunately no any culture is available in India.

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History of Mycology from India—Some Glimpses

3

C. Manoharachary

Abstract

Fungi are known to be nonchlorophyllous and true nucleated organisms possessing cell wall of chitin and osmotrophic. Around 1.5–5.1 million fungi are estimated. So far one lakh fungal species have been identified in the world, and 29,000 fungi are identified and described from India. 32% of Indian fungi have been discovered by Subramanian (1971, 1992), and remaining 68% are described by others. All these fungi belong to Zoosporic group, Zygomycota, Ascomycota/Basidiomycota and anamorphic fungi. Presently anamorphic fungi are merged with Ascomycota/Basidiomycota as per one fungus-one name concept of Melbourne code and new classification. This review presents the historical perspective emphasizing on the contributions made by Indian mycologists.

Keywords

Diversity · Characterization · Fungi · Groups of fungi · History

Subject knowledge on living biota proceeds in different states: (i) simple observation (ii) identification and characterization (iii) study of novel molecules (iv) metabolic processes. Fungi and their utilization are known to humans since times immemorial. The utility of fungi began when men lived as hunter gatherers, and some information was known in Greek and Roman civilizations. The basic work of Micheli (1729) has established methodological approach on study of fungi. The developments in microscopy lead to the understanding of biology of fungi. Federicocesi illustrated around 1620 species of fungi observed in Italy. Saccardo (1882–1931) compiled the descriptions of all such fungi described till 1931 in 26 volumes entitled "Sylloge fungorum omnium hucusque cognitorum". The naming

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of fungi was introduced by Persoon (1801). Vuillemin (1910) considered conidial development as stable character for anamorphic fungi.

Sculptures, etchings and paintings of mushrooms which date back to 1300 B.C. were found in Mexico, Guatemala in ancient Egypt, Indian and Sumerian civilizations. The ancient Stone Age and Neolithic Era through Mesolithic Era have witnessed that primitive people were familiar with edible and poisonous mushrooms with reference to their domestication, edibility, nutrition and poisonous nature. The cavemen discovered that *Amanita muscaria var. muscaria* (L) Lam as medicinal/poisonous mushroom. This hallucinogenic mushroom was shown as "Round Heads" as pictoral stages by archaeologists in ancient times. In Denmark, the fungus *Fomes fomentarius* (L.) Fr. of Polyporaceae was found associated with fragments of pyrites and silica in 6000 BC and also in Yorkshire (U.K). A megalithic monument found in Kerala namely Kudakkallu (Umbrella Stage) has been common in Trichur.

The use of fungi is known since ancient times. Further, the documentation by Wasson (1957) indicated that Roman Emperor Claudius Caesar (AD 54) was murdered by offering poisonous mushroom, *Amanita phalloides* (Veill ex. Fri.) Link as mix with edible mushroom food in order to occupy the throne. Greek Physician by name Dioocordieshas listed out the differences between edible and poisonous mushrooms. The warning that comes out of these studies is that edibility of a mushroom has to be certified by scientists (mycologist), and a certificate needs to be obtained by mushroom growers to this effect. Most of the mushrooms are death traps but for few like button mushroom, oyster mushroom, paddy straw mushroom, Morel or Guchi (*Morchella esculenta* (L.) Pers.) and few others which are edible and nutritious and are commercially produced but for *Morchella*.

There are few records available about mushrooms as Soma and about some plant diseases caused by fungi in the Vedas and Hindu scriptures, respectively (122 BC). The discovery of microscope in seventeenth century by Leeuwenhoek laid the foundation for the discovery of microbes and fungi. The Greek Theophrastus, disciple of Aristotle, defined fungi as "Imperfect plant, without roots, leaves, flowers and fruits". Samorin (2002) studied mushroom sculptures and paintings of ancient times and noted the drawings of *Amanita muscaria*, *Psilocybe* and others. One wall painting in central France showed that entry of Christ into Jerusalem was greeted with a painting having mushroom like formations on tree. Andrea Caesalpino described fungi as plants without fruits and seeds. Hooke (1665) was the first botanist to draw the illustrations of sporangia of *Mucor* and teleutospores of rust fungus namely *Phragmidium.* The contributions of eminent mycologists are provided in Table 3.1.

Besides the above some strong schools have been working on fungi of India and these include Prof. M.S. Patil on Rust fungi; Prof. L.V. Gangawane on soil and rhizosphere fungi; Dr. D.K. Agarwal, Rusts, Smuts, General fungi; Dr. S.K. Singh, Fungal Taxonomy, Dr. R.V. Gandhe, Smut fungi; Dr. M.C. Srinivasan on *Entomophthora*; Prof. D.S. Mukadam on seed-borne fungi; Prof. G.J. Vaidya, Higher Fungi; Dr. P.G. Patwardan on Lichens; Prof. D.D. Awasthi on Lichens;

Table 3.1 Enlists the glimpses of some important mycological contributions from India

Year	Name	Contribution
1843–1914	D.D. Cunningham	Aeromycology, mucorales, rusts, smuts,
1874–1943	E.J. Butler	*Pythium, Allomyces* fungi of India, HCIO, plant diseases
1931–1960	R.S. Vasudeva	Fungi of India
1888–1970	S.R. Bose	*Polyporaceae*
1982–1950	K.C. Mehta	Wheat rust
1895–1942	M. Mitra	Cereal rusts, karnal burnt of wheat
1896–1952	B.B. Mundkar	Smuts fungal diseases of crops, smuts, quarantine regulations
1917–1988	S.B. Saksena	Soil fungi, *Saksenaea vasiformis*
1908–1973	K.D. Bagchee	Soil borne diseases
1959–1961	M.N. Kamat	Cytology of fungi
1920–1978	K. Ramakrishnan	Coelomycetes
1917–1991	K.S. Thind	Aphyllophorales
1913–2001	T.S. Sadasivan	Root-borne and Soil-borne diseases Fungal enzymes
1914–1999	M. J. Thirumalachar	Rusts, smults, antibiotics
1902–1990	S.N. Das Gupta	Aquatic fungi, medical mycology
1903–1999	R.N. Tandon	Physiology of fungi, mucorales
1933–1996	K.S. Bilgrami	Physiology of Fungi, mycotoxigenic fungi
1924–2016	C.V. Subramanian	Hyphomycetes taxonomy, biology
1918–2010	K.S. Bhargava	Saprolegniaceae, cytology of fungi
1926–1992	B.S. Mehrotra	Mucorales
1932–till date	R.S. Dwivedi	Rhizosphere fungi
1929–2016	G.P. Agarwal	Entomogenous fungi
1923–2010	K.G. Mukerji	Mycorrhizae, fungal taxonomy *Chaetomium*, myxomycetes, laboulbeniomycetes
1919–2005	M.S. Pavgi	Smuts, fungi imperfecti, *Synchytrium,* chytrids, *Physoderma*
1932–2015	Ram Dayal	Aquatic fungi, nematode trapping fungi
1945	C. Manoharachary	Soil fungi, hyphomycetes, mycorrhizal fungi, agaricales, lichens, aquatic fungi.
1997–2009	D.J. Bhat	Hyphomycetes
1942–2008	K. Natarajan	Agaricales
1930–1999	V. Agnihotrudu	Fungi imperfecti
2001	R.S. Mehrotra	*Phytophthora*
1997	T.N. Lakhanpal	Myxomycetes, agaricales

Dr. D.K. Upreti, Lichens and metabolites; Dr. K.P. Singh on Lichen taxonomy; Prof. Bharat Rai, Ecology of Fungi; Prof. D.K. Arora Molecular taxonomy of fungi; Prof. H.C. Dubey, Plant—microbe interaction; Prof. T.S. Suryanarayana on Endophytes; Prof. R.N. Kharwar, Endophytic fungi, Hyphomycetes; Prof. R.S. Upadhyay, Soil-borne diseases, Biocontrol; Prof. N. Samajpati, Taxonomy of fungi; Prof. R.P. Purkayastha, Phytoalexins; Prof. S.M. Baruha, Phylloplane fungi; Prof. Dhruv Kumar Jha, Diversity of fungi, Mycorrhizae; Dr. U.N. Saikia, Hyphomycetes; Prof. P.C. Trivedi, Nematode-trapping fungi; Prof. S.K. Hasija, Prof. R.C. Rajak, Prof. A.K. Pandey, Dr. Jamaluddin on Entomogenous fungi Diversity and Taxonomy of fungi, Chytrids, Coelmycetes, weed fungi, Forest fungi; Prof. Kamal, Taxonomy of Cercospraceous and Allied fungi; Dr. V. Mohanan and Dr. K.V. Sankaran, Forest fungi; Dr. S.T. Tilak, Aeromycology; Dr. P.N. Chowdhry, Pythiaceous fungi, General fungi; Prof. I.B. Prashar on Higher fungi and Discomycetes; Prof. D.S. Dargan, *Xylaria;* Prof. G.S. Dhingra, Corticioid fungi; Dr. Alok K. Srivastava, on biocontrol fungi, culturing of fungi, endophytes; Dr. Susheel Kumar, Taxonomy and culturing of fungi; Prof. Raghaveer Rao, Hyphomycetes; Prof. P. Rama Rao, Soil fungi; Prof. S.M. Reddy, Mycotoxigenic fungi: Prof. K. R. Aneja, fungi on weeds, Diversity of fungi: Prof. B.L. Jalali, Mycorrhizal fungi; Late Dr. S.Y. Padmanabhan, Rice diseases; Prof. H.C. Govindu, Diseases of cereals; Prof. T.N. Lakhanpal, Mushrooms Myxomycota; Prof. Indira Kalyanasundaram, Myxomycetes; Prof. T. Satyanarayana, Thermophilic fungi; Prof. B.N. Johri, Thermophilic fungi, Prof. R.R. Mishra, Soil fungi, Prof. S.C. Agarwal, Medical mycology, Prof. R.K.S. Chauhan and Dr. Sashi Chauhan, seed fungi, Mycotoxigenic fungi, Prof. Sudha Mall, Prof O.P. Mall seed-borne fungi, root diseases, Dr. Sunil Kumar Deshmukh on Keratinophilc fungi, Dr. A.K. Roy on mycotoxigenic fungi, Prof. S.R. Niranjana on biocontrol fungi, Dr. A.N. Mukhopadhya on *Trichoderma*, Dr. Rashmi Aggarwal on Rust fungi, Prof. N.K. Dubey on plant extracts and fungi, Dr. S. Raghukumar Thraustochytrids, marine fungi, Dr. C. Raghu Kumar Marine fungi, Dr. Ramdayal nematode fungi, Prof. N. Raman ectomycorrhizae, Dr. K.S. Vrinda on edible mushrooms, Dr. J.R. Sharma on higher fungi, Dr. V.B. Hosagoudar on Meliolales. Prof. N.S. Atri on edible fungi, Dr. Kanad Das, Gasteromycetes, Dr. Prameela Devi on *Chaetomium* and *Trichoderma;* Dr. M.V. Deshpande, Entomogenous fungi; Prof. B.F. Rodrigues on AM fungi. Dr. Alok Adholeya on AM fungi and technology, Dr. G.S. Prasad, yeasts and others.

There are many eminent mycologists and young mycologists who have contributed for the growth of Mycology in India. India is a vast country and there might have been some left out names of mycologists. Further, it is impossible to cover all such contributions. In the last ten years, mycological research has been slowed down and number of senior mycologists either might have left this world or retired.

The available number of such eminent mycologists in the taxonomy of fungi is reduced to 10–15, thus not only taxonomy of fungi has become forgotten science but the subject of mycology has been getting raw real and step motherly treatment at different levels. However, the biodiversity of fungi is the resource material for

Table 3.2 Cell wall taxonomy of fungi

Chemical category	Taxonomic group
Cellulose-glycogen	Acrasiales
Cellulose-glucan	Oomycetes
Cellulose-chitin	Hyphochytnidomycetes
Chitosan-chitin	Zygomycetes
Chitin-glucan	Chytridomycetes, ascomycetes, basidiomycetes, deuteromycetes
Mannan-glucan	Saccharomycetaceae, cryptococcaceae
Mannan-chitin	Sporobolomycetaceae
Polygalactosamine-galactan	Rhodotorulaceae, trichomycetes

biotechnology and biotechnological products are worth of million(s) of dollars, (Wildman 2001) hence requires attention.

Characterization of Fungi

Morphotaxonomic criteria which include holocorpic and eucarpic nature, hyphal septation, vegetative, reproductive structures (sclerotia, rhizomorphs, etc.) asexual reproductive bodies/units (zoospores. sporangiospores, conidia, conidiomata, synnemata, acervuli) sexual fruit bodies (cleistothecia, perithecia, apothecia, pseudothecia, ascostroma, basidiocarp) and sexual units of reproduction (oospore, zygospore, ascospore, basidiospore) and nature of other perennating structures like chlamydospore, sclerotia and rhizomorphs form important diagnostic features in identifying and classifying fungi besides the chemical composition of cell wall as given in Table 3.2.

Molecular Taxonomy

Methodologies in the field of systematics have undergone rapid and phenomenal changes during the past two decades. DNA-based molecular methods are rapidly replacing and supplementing the conventional methods such as microscopy, cultural and biochemical characterization. Nucleic acid probes are used for rapid identification of fastidious and fast-growing microorganisms. Polymerase chain reaction (PCR) offers several advantages over other methods and has become a powerful tool in recent times. Several versions of PCR are now available. Similarly, the molecular techniques on mutation detection, RAPD, RFLP, DGGE, DNA sequencing, genomic finger-printing, biotyping and DNA barcoding have become necessary for taxonomic studies and can generate valuable information.

Zoosporic Fungi

Lower fungi form a dominant group of fungi in aquatic habitat with zoospores which were earlier included in the Phyla Chytridiomycota, Hyphochytriomycota, Oomycota and Plasmodiophoromycota (Kirk et al. 2008). They are found not only in water, but also in humid soils, insects, on keratin, chitin, angiospermic debris, pollen grains and on others living either as saprophytes or parasites. Such fungi have been arbitrarily grouped under lower fungi on the basis of zoospore and oospore. Presently zoosporic fungi are classified under Chytridiomycota and Straminopila. Approximately 900 species are reported. Butler and Bisby (1960) have enlisted Indian fungi. Aquatic fungi were studied by Hamid from India and Pakistan followed by Ramdayal on chytrids, Karling on Indian chytrids, Khulbe and his associates on Saprolegniaceae, M.S. Pavgi on *Physoderma* and Synchytrium, S.D. Patil on aquatic fungi from Maharashtra, K.S. Bhargava on Saprolegniceae, R.S. Mehrotra on *Phytophthora* and others have made significant contributions. The above account clearly indicates that still many in-depth studies covering all biogeographic regions are essential. Accordingly present classification which is based on molecular data includes the Chytridiomycota, Hyphochytridiomycota and Oomycota under Zoosporic fungi. Manoharachary et al. (2005) studied aquatic fungi from Telangana and Andhra Pradesh (India) exhaustively.

Marine Fungi

These fungi are commonly found in oceans and estuaries colonizing, decomposing organic matter such as drift and intertidal wood and other materials. Initial studies of marine fungi in India were on surface seawater, marine sediments and mangrove mud. An extensive survey of marine fungi was made from south India by Raghukumar (1996) and from Maharashtra by Borse (2002). Marine fungi and marine yeasts are to be explored extensively.

Zygomycota

Fungi under this group are commonly found in soil, dung, organic debris, air as saprophytes and few as biotrophs on plants and animals. Another interesting group of fungi such *Entomophthora* spp. are associated with insects and *Trichomycetes* live in the insect guts. Approximately 900–1000 species belonging to *Zygomycota* are reported from India. These fungi are important in food industry and form biotechnologically important in agriculture, industry, medicine, etc., and genetics. *Saksenaea vasiformis*, which was discovered by late Prof. S.B. Saksena, has become important in medical mycology. Valuable contribution was made from

India on Zygomycota by R.N. Tandon, B.S. Mehrotra, A.K. Sarbhoy and few others and much needs to be done on this group by Indian mycologists.

Ascomycota

Ascomyceteous fungi are a larger group among fungi. It includes fungi which differ in morphology, physiology, biochemistry and genetic makeup besides occurring in diversified habitats. Ascomycota encompasses 6355 genera and 64,143 species (Kirk et al. 2008). Members of *Saccharomyces* are commonly found on surgery substances, on environments like plant surfaces and fruits, but are also found in terrestrial and marine habitats. Importance of yeasts in industrial fermentation, like brewing and bakery, is well known. *Emerciella*, *Talaromyces*, *Chaetomium*, *Xylaria*, *Neurospora*, *Sordaria, Cochiobolus* and *Ascobolus* are commonly found in soil, plant and animal remains and on others. *Lulworthia* and others are common on decomposed organic material, because of their ability to adjust in estuarine environment. All these fungi are biotechnologically important. Truffels like Tubers form ectomycorrhiza on forest trees *Tuber* spp. ate known for their delicacy hence form prized commodity. Species of *Arthroderma* and *Nanninzzia* are pathogenic and cause diseases. Species of *Ceratocystis*, *Claviceps*, *Erysiphe*, *Phyllactinia*, *Sphaerotheca*, *Taphrina,* etc., parasitize plants and cause losses. Ascomycetes form approximately 60% of the total fungi.

Eminent Indian mycologists who have contributed significantly on Ascomycetous fungi include Kamat on *Phyllachora*, Lodha on Coprophilous Ascomycetes, Alka Pande on Ascomycetes of Western India, K.G. Mukerji on *Chaetomium* and Laboulbeniomycetes, Manoharachary on Indian *Thielavia*, M.P. Sharma on Discomycetes and Hosagoudar on Meliolales.

Basidiomycota

This group comprises largely Agarics, polypores, toadstools, fairy clubs, puff balls, stinkhorns, earth stars, *Cyathus* and Jelly fungi that are either parasites, biotrophs or saprophytes. Fungi like *Amanita, Laccaria, Pisolithus,* etc., form ectomycorrhizae with gymnosperms and woody trees and help in the transport of phosphorus and other elements as symbionts. *Armillaria mellea* occurs on a wide range of woody plants and cause damage (Singer 1989).

Edible fungi are widely distributed in India. They colonize diversified substrates such as soils, decaying organic matter, wooden stumps, etc. They are found generally after monsoon when organic matter or nutrients are easily available. More than 2000 species of edible mushrooms are from different parts of the world. Mushrooms alone are represented approximately by 850 species in India including extended surveys of the Himalayan region (Lakhanpal 1997) and others.

Rusts and smuts are the plant pathogenic fungi of Basidiomycota of causing huge losses in millets, legumes, orchids ferns and grasses. These are mostly obligate parasites producing more than one spore form in their life cycle. More than 160 rust fungal genera comprising 7000 species exist world over, out of which 4600 are monotypic. Geographically, rusts are distributed all over the world except in Antarctica. The most widely occurring rust genera are *Puccinia, Uromyces, Phragmidium, Melampsora* and *Tranzschelia* and other rust pathogens.

Several smuts are known to occur in nature and cause considerable economic loss to cultivated plants like wheat, jowar, pearl millet, members of Caryophyllaceae and others.

Mushrooms are known to contain more fibre, protein, minerals, water and lesser amount of carbohydrates and fat. Edible mushrooms are medicinally important, and mushroom cultivation is a multibillion industry in Europe. No smut fungus is known to occur in the plant family Orchidacae. Teliospore forming smuts (Ustilaginomycetes) comprising 62 genera and 1113 species are parasitic on herbaceous, non-woody plants, while those lacking teliospores (Microstromatales) comprises of 4 genera and 10 species. Exobasidiales comprises of 17 genera and 83 species mostly parasitize woody plants. Rusts, smuts and Karnal bunt fungi from India have been worked out by B.B. Mundkar, M.J. Thirumalachar, K.C. Mehta, D.K. Agarwal, S.S. Chahal, ManoranjanMisra, K.D. Bagchee, M.S. Pavgi, Ramachar, A.V. Sathe and others. The higher fungi such as Agaricales, Ectomycorrhizal fungi, Edible Mushrooms, Aphyllophorales and Morels have been surveyed, identified and characterized in an authentic manner by B.K. Bakshi, Sujan Singh, K.S. Thind, S.S. Rattan, T.N. Lakhanpal, K. Natarajan, S.R. Bose, M.S. Patil, J.R.Sharma, H.S. Sohi, N.S. Atri, I.B. Prashar, R.S. Upadhyay, K.B. Vrinda, N. Raman, Kamal and few others.

Anamorphic Fungi

Conidial fungi represent the anamorphic stages of either Ascomycota or Basidiomycota, besides being second largest group of fungi in numericals. These fungi occur in/on litter, air, soil, water, coprophilous and other habitates. These fungi are observed in situ or isolated on nutrient media. Their classification is mainly based on conidial ontogeny, asexual fruit bodies, and in recent times molecular approaches are also made. This group includes around 1700 anamorphic genera of conidial fungi and 700 genera of coelomycetes, which are now merged mostly with Ascomycota and to some extent with Basidiomycota. Anamorphic fungi play an important role in biodegradation of cellulose, lignin, pectin, protein and polysaccharides and others. They are also important in wastewater treatment, recycling of raw materials, breaking down of xenobiotic compounds and other processes which are important in agriculture, medicine, pharmaceuticals, etc.

Conidial fungi and Coelomycetous fungi of India have been worked out with in-depth analyses by C.V. Subramanian, V. Agnihothrudu., C. Manoharachary, D.J. Bhat, J.N. Kapoor, D.K. Agarwal, S.K. Singh, V.G. Rao, B.P.R. Vittal, U.N. Sakia, J. Muthumary, T.R. Nagaraj and others.

Prof. S.B. Saksena (1917–1988) was an eminent mycologist and established a strong school at the University of Sagar (M.P.), India. His contributions on soil fungi, fungal ecology, discovery of some new taxa and discovery of *S. vasiformis* which has created history in medical mycology are worth mentioning. He had his post-doctoral training with Late Dr. S.D. Garrett at University of Cambridge and worked on an important fungus, *Trichoderma aviride*, besides his work is related to biocontrol, aquatic fungi and litter fungi which have also earned much recognition.

Conclusions

India is the rich source centre for fungi as 1/3 of global estimate of fungi exists in India. Ascomycota followed by Basiodiomycota and other groups form the sequence of occurrence. It is estimated that world fungal biotechnological products which are important in agriculture, healthcare, industry, pharmaceuticals, waste management, food security and environment stabilization and others account for US $42,840 million (Wildman 2001).

The present review has given an in-depth analysis of historical account of Indian mycology as contributed by eminent Indian mycologisis.

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Various Aspects of Ammonia Fungi

4

Akira Suzuki

Abstract

Ammonia fungi are a chemoecological group of fungi, which sequentially occur after a sudden addition of ammonium-nitrogen that reacts as a base, or of alkalis. Sequential occurrence (= succession) of ammonia fungi proceeds anamorphic fungi, fungi in Ascomycota (mostly Discomycetes), fungi having smaller fruit bodies in Basidiomycota (all fungi belonging to these successional stages are saprobic species), and fungi having larger fruit bodies in Basidiomycota (mostly ectomycorrhizal species in Agaricales). They colonize into ammonium-nitrogen-disturbed sites as pioneer fungal species instead of those pre-inhabitants (non-ammonia fungi). The saprobic ammonia fungi well adapt or tolerate to high concentration of ammonium-nitrogen under neutral and weak alkaline conditions. Most of saprobic ammonia fungi effectively decompose cellulose and hemicellulose, but do not remarkably decompose lignin. This replacement described may be viewed as a kind of "compensation process" in nutrient cycle in terrestrial ecosystems.

Keywords

Biogeographical distribution · Disturbance · Ecophysiology · Enzyme activity Fungal community · Nitrogen enrichment · Pioneer fungi · Succession

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Introduction

Terrestrial ecosystem comprises various biotic communities such as the communities of organisms in eukaryotes (Opisthokonta, Chromalveolata, Archaeplastida, etc.) and those in prokaryotes (Adl et al. 2005). Among them, terrestrial true fungal community composes of different kinds of true fungi which have been categorized by different ecological standpoints such as nutritional modes, i.e., biotrophy (including mycorrhizal symbiosis and parasitism) and necrotrophy (including saprotroph), and/or various responses to different environmental stresses, such as highly alkaline, highly acidic, high temperature, and low-temperature conditions, and/or disturbances of inhabiting soils by fire and by scratching of animals (cf., Sagara 1975, 1992). "Ammonia fungi" are one of ecological groups of true fungi that are characterized by the disturbance of high nitrogen such as urea and ammonia (Sagara 1975). Research history of ammonia fungi is as follows. Sagara and Hamada applied urea on the forest floor and found the occurrence of three species of fungi (Sagara and Hamada 1965). Thereafter, Sagara continued to apply different kinds of chemicals on various habitats and found an assemblage of true fungal species specific to each chemical in terrestrial ecosystems (Sagara 1975). He focused on the assemblage of fungal species which were specific to disturbance by ammonium-nitrogenous materials and termed them "ammonia fungi" (Sagara 1975). Later, he proposed a natural ecological group of fungi, "postputrefaction fungi,"[1] which were recognized by the observation of their occurrences from animal waste decomposing sites such as urine, feces, dead bodies, or deserted middens (latrines) of mammalian animals in the terrestrial ecosystems (Sagara et al. 2008). A set of the fungal species composing "postputrefaction fungi" absolutely includes a set of the fungal species composing ammonia fungi. However, some postputrefaction fungi such as *Rhopalomyces strangulatus* and *Hebeloma radicosum* cannot be called ammonia fungi since they have not been found by a large input of urea in the field.[2] Physiological characteristics of ammonia fungi have been examined by several researchers. However, we have not yet have enough physiological and ecophysiological data to describe the decomposition processes of feces and dead bodies by comprehensive activities of microbes including postputrefaction fungi. Therefore in the following, I describe various aspects of "ammonia fungi" which we have more ecophysiological data for discussion.

[1] "Postputrefaction fungi" was first proposed in Japanese as "Fuhaiatokin" by Sagara (1997). "Fuhaiatokin" in Japanese means "postputrefaction." Later, Tibbett and Carter separately defined such ecological group of fungi as "postputrefaction fungi" in English (Tibbett and Carter 2003).

[2] "Ammonia fungi" was redefined as a chemoecological group of fungi which occur after urea treatment. According to the new definition, the species in "postputrefaction fungi" which do not occur after urea treatment cannot be called "ammonia fungi," even when they occur by the treatment of nitrogenous materials such as peptone and different kinds of corpses.

Definition of Ammonia Fungi

"Ammonia fungi" are a chemoecological group of fungi which sequentially develop reproductive structures exclusively or relatively luxuriantly in the soil after a sudden addition of ammonia, or some other nitrogenous materials that react as bases by themselves (Fig. 4.1) or on decomposition, or of alkalis (Sagara 1975).

Mycobiota of Ammonia Fungi

Mycobiota (species assemblage) of ammonia fungi composes of saprobic fungi in anamorphic fungi such as *Amblyosporium botrytis*, *Cladorrhinum foecundissimum*, and *Doratomyces microsporus*, saprobic fungi in Ascomycota (mostly Discomycetes) such as *Ascobolus denudatus*, *Humaria velonovskyi*[3], *Peziza moravecii*, and *Pseudombrophila petrakii*, saprobic fungi having smaller fruit bodies in Agaricomycetes (Basidiomycota) such as *Lyophyllum tylicolor*[4] and *Coprinopsis phlyctidospora*, and fungi having larger fruit bodies in Agaricomycetes (Basidiomycota) such as *Hebeloma spoliatum* sensu Hongo, *Hebeloma vinosophyllum* (Fig. 4.1), and *Laccaria bicolor* (cf., Sagara 1975, 1992; Sagara et al. 2008).

Sequential Occurrence of Ammonia Fungi

The sequential appearance of reproductive structures (= succession) of ammonia fungi generally proceeds as follows: saprobic anamorphic fungi → saprobic fungi in Ascomycota → saprobic fungi in Basidiomycota having smaller fruit bodies (the fungi belonging to these three phases are described as early phase fungi—EP fungi) → mostly biotrophic fungi having larger fruit bodies and a few possibly saprobic fungi (the fungi belonging to this phase are described as late phase fungi—LP fungi) (Sagara 1975; Yamanaka 1999; Imamura and Yumoto 2004; cf., Tibbett and Carter 2003).

Invasion and/or colonization time of each ammonia fungus was examined by the cultivation of urea-treated soils collected at different days after urea treatment. It suggested that invasion and/or colonization of ammonia fungi into urea-treated soil initiated firstly by *A. botrytis* and followed quickly by *A. denudatus*, *Peziza moravecii*, *Coprinopsis phlyctidospora,* and then by *L. tylicolor* (see Footnote 4). The invasion/colonization sequence of the saprobic species speculated by the incubation experiments described above was similar as that of the appearance of reproductive

[3]A saprobic ammonia fungus *Humaria velonovskyi* occurs continuously from EP to LP (Sagara et al. 2008).

[4]According to current classification, *Lyophyllum tylicolor* and *Lyophyllum gibberosum* are named *Sagaranella tylicolor* and *Sagaranella gibberosa*, respectively (Hofstetter et al. 2014).

Fig. 4.1 Occurrence of an ectomycorrhizal LP fungus *Hebeloma vinosophyllum* in the field. A plot (1 m × 2 m) was placed on the forest floor of an evergreen forest (*Castanopsis cuspidata* var. *sieboldii* and *Distylium racemosum* dominated mixed forest) in Tosashimizu, Kochi, Japan, and 800 g urea was applied on June 4, 1995. A large number of fruit bodies of *H. vinosophyllum* were observed on the whole surface of the urea plot on October 12, 1995

structures of those fungi in the field although *L. tylicolor* occurred earlier than *C. phlyctidospora* (Suzuki et al. 2002b).

Vegetative growth and reproductive structure formation of each ammonia fungus are affected by the other species of ammonia fungi and non-ammonia fungi. The interactions among them varied with the changes in nitrogen concentration and pH value (Suzuki 2006; Licyayo and Suzuki 2006; Barua et al. 2012). For example, ascomata of saprobic EP/LP fungus *P. petrakii* are formed on the co-culture with the late-stage of saprobic EP fungus *Coprinopsis phlyctidospora* at pH 9.0 (Fig. 4.2), but not on monoculture.

These suggest that the successional occurrence of ammonia fungi would be caused by the combination of time required for fruiting of each fungus, which is associated with sequential invasion and/or colonization, and interaction among ammonia fungi and other microbes (Suzuki 1989, 2006).

Biogeographical Distribution of Ammonia Fungi

Until now, about 70 species of ammonia fungi have been collected by urea or ammonium-nitrogen application in the field and/or in the laboratory experiments [incubation of urea-treated soils (the mixture of A_0 horizon and upper part of A horizon) collected from different geographical areas; cf., Sagara 1975, 1976, 1992; Suzuki 2006]. However, we have not yet got enough collection records of ammonia

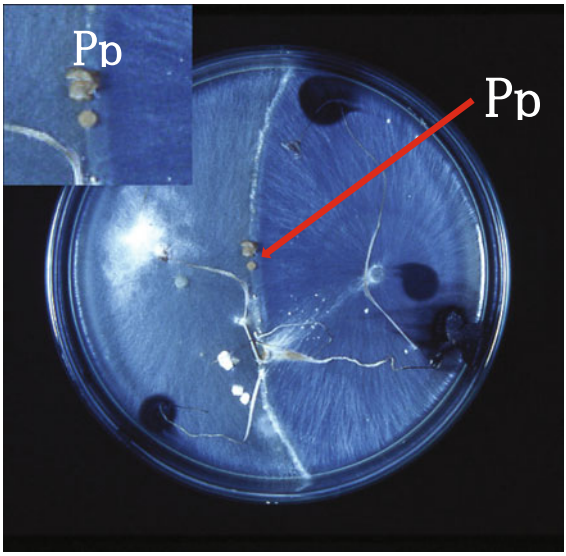

Fig. 4.2 Ascomata of *Pseudombrophila petrakii* (left) formed by co-culture with *Coprinopsis phlyctidospora* (right). Ascomata of the early-stage EP fungus *P. petrakii* (Pp) were formed at the contact zone with another late-stage EP fungus *C. phlyctidospora* after 28 days of cultivation at 20.0 ± 0.3 °C on MY agar medium (initial pH was adjusted to pH 9.0 by NH_4OH-NH_4Cl buffer) under light and dark regime (Light 2 h/Dark 22 h)

fungi to discuss biogeographical distribution pattern of ammonia fungi since those of ammonia fungi can be obtained from mostly by a large amount of ammonium-nitrogen application. The surveys of mycobiota of ammonia fungi after ammonium-nitrogen enrichment, mostly done by urea treatment, have been done in various geographical areas in Japan (Sagara 1975; Fukiharu and Hongo 1995; Yamanaka 1995a, b, c; Fukiharu et al. 1997, 2014b; Suzuki et al. 2002b; Imamura and Yumoto 2004, etc.) and New Zealand (Suzuki et al. 2002a, 2003; Fukiharu et al. 2011). Surveys of mycobiota of ammonia fungi by urea application have been also done in very limited area of Taiwan (Wang and Sagara 1997), UK (Sagara 1992), The USA (Sagara 1992), Australia (Suzuki et al. 1998, 2002a, 2003; Nagao et al. 2003; Fukiharu et al. 2011), Canada (Raut et al. 2011a, b, 2015), Thailand (Manusweeraporn et al. 2013), Vietnam (Ho et al. 2014), and China (Fukiharu et al. 2014a), etc. Therefore, biogeographical distribution of ammonia fungi tends to be described by the distribution of urea-applied sites since the number of those sites where a large input of ammonium-nitrogen in the field is not enough at a global scale. All collection records of fungal species which had been already known as ammonia fungi are also added for estimating their biogeographical distribution even when they were collected from the sites without the recognition of nitrogen enrichment sites. The collection records of ammonia fungi without urea application have been mostly obtained from the survey of other ecological groups of fungi such as coprophilous fungi and pyrophilous fungi (cf., Sagara 1975).

Tentatively, distribution type of ubiquitous species of ammonia fungi can be categorized into five types, namely absolutely worldwide distribution type (Fig. 4.3, cf., Table 4.1), the northern hemisphere distribution type (probably it will be Holarctic ecozone type), Eurasia distribution type (probably it will be Palearctic ecozone type), East Asia distribution type (probably it will be subregion of Asia bioregion type in Palearctic ecozone type), and Australia and New Zealand distribution type (probably it will be Australasia ecozone type) (cf., Olson et al. 2001). For example, two ammonia fungi *A. denudatus* and *Coprinopsis cinerea* which had been also categorized as coprophilous fungi show the most worldwide distribution (Suzuki et al. 2003). Probably, coprophilous fungi having long-lived pigmented spores expanded the distribution area in recent time with human migration and had the most worldwide distribution (cf., Suzuki et al. 2002a).

Coprinopsis phlyctidospora sensu lato collected from different geographical areas at a global scale have been examined by ITS rDNA sequence analysis, morphological features, and compatibility among those isolates. It comprised of at least four cryptic species *Coprinopsis phlyctidospora* sensu stricto (Suzuki et al. 2002a), *Coprinopsis austrophlyctidospora* (Fukiharu et al. 2011), *Coprinopsis neophlyctidospora* (Raut et al. 2011a), and *Coprinopsis asiaticiphlyctidopora* (Fukiharu et al. 2014b) inhabiting separately in Eurasia (based on collection records from temperate broad-leafed tree forests and temperate coniferous tree forests), Australasia (based on collection records only from temperate broad-leafed mixed forests in New Zealand and Australia), western Canada (based on collection records from boreal forests), and southern part of Japan (based on collection records form subtropical broad-leafed mixed forests), respectively. Ectomycorrhizal LP fungi in *Hebeloma* section *Porphyrospora*, *Hebeloma aminophilum* (collected from temperate region to tropical region) and *H. vinosophyllum* [collected from temperate region (Fig. 4.1) in East Asia to highland of tropical region in Vietnam] have also been examined to elucidate the existence of cryptic species by ITS rDNA sequence analysis and morphological features of those isolates. Both *Hebeloma* species did not comprise any cryptic species although the isolates of each *Hebeloma* species examined were collected from different vegetations in geographical areas far apart from each other (Suzuki et al. 2003; Ho et al. 2014).

The collection records of ammonia fungi up to now suggest that most ammonia fungi are ubiquitous species at different levels and a few would be endemic species. In conclusion, biogeographical distribution of ectomycorrhizal ammonia fungi appears to be more restricted than that of saprobic ammonia fungi (Suzuki et al. 2003).

Community Structure of Ammonia Fungi

Species-area curve of EP fungi reaches to stationary state by 0.5 m × 1.0 m or less (Sagara 1976), whereas that of LP fungi does not often reach to stationary state by the quadrat size. The apparent population sizes of saprobic EP fungi are estimated

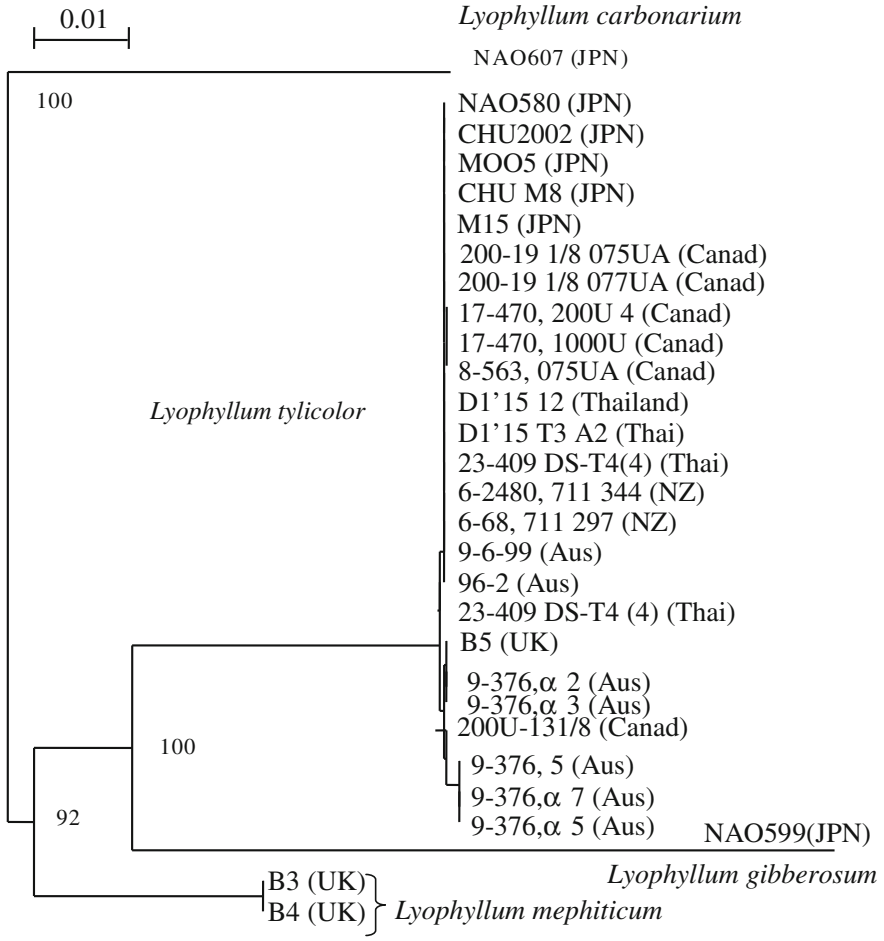

Fig. 4.3 Phylgenetic tree constructed by neighbor-joining method for 29 collections of *Lyophyllum* species based on nucleotide sequences of the ITS region. The values at the nodes are the confidence levels from 1000 replicate bootstrap sampling. The distance corresponding to 10 base changes per 1000 nucleotide positions is indicated by the bar. *Aus* Australia, *Canad* Canada, *Jpn* Japan, *NZ* New Zealand, *Thai* Thailand, *UK* United Kingdom. *Lyophyllum carbonarium* (syn: *Tephrocybe anthracophila*), *Lyophyllum tylicolor* (syn.: *Sagaranella tylicolor*), *Lyophyllum gibberosum* (syn.: *Sagaranella gibberosa*), *Lyophyllum mephiticum* (syn.: *Tephrocybe mephitica*) (Suzuki et al., unpublished data)

9–143 individual/plot (50 cm × 100 cm) by the cultivation of 200 soil blocks from a forest floor subsequent to urea application in vitro, but the apparent population density of each EP fungus varied remarkably in each collection site (Suzuki et al. 2002b; Suzuki 2006).

Table 4.1 Mycobiota of ammonia fungi in each habitat

Fungal Species	Successional stage	Vegetation							
		P. bambusoides	*A. australis* and *L. ericoides*	*E. marginata* and *E. calophylla*	*Pi. densiflora*	*Pi. luchuensis*	*C. cuspidata*	*C. cuspidata*	*Q. serrata*
Amblyosporium botrytis	EP	○			○			○	○
Cladorrhinum foecundissimum	EP					○	○		
Ascobolus denudatus	EP		○	○	○	○		○	○
Ascobolus hansenii	EP						○	○	○
Humaria velenovskyi	EP/LP								
Peziza moravecii	EP		○	○	○			○	○
Peziza urinophila	EP							○	○
Pseudombrophila petrakii[a]	EP				○			○	○
Thecotheus urinamans	EP			○					
Lyophyllum tylicolor	EP		○	○	○			○	○
Coprinopsis cinerea	EP	○					○		
Coprinopsis echinospora	EP				○				
Coprinopsis austrophlyctidospora[b]	EP		○	○					
Coprinopsis neolagopus	EP							○	○
Coprinopsis phlyctidospora	EP	○				○	○	○	○
Crucispora rhombisperma	EP	○				○			
Panaeolina sagarae	EP							○	
Collybia cookei	LP				○				
Hebeloma aminophilum	LP			○					

(continued)

Table 4.1 (continued)

Fungal Species	Successional stage	Vegetation							
		P. bambusoides	*A. australis* and *L. ericoides*	*E. marginata* and *E. calophylla*	*Pi. densiflora*	*Pi. luchuensis*	*C. cuspidata*	*C. cuspidata*	*Q. serrata*
Hebeloma lactariolens	LP					○	○	○	○
Hebeloma luchuense	LP						○		
Hebeloma radicosoides	LP						○	○	○
Hebeloma spoliatum	LP							○	○
Hebeloma vinosophyllum	LP				○			○	○
Laccaria amethystina	LP								○
Laccaria bicolor	LP				○			○	○
References		Fukiharu et al. (1997)	Suzuki (1992)	Suzuki et al. (1998) and Nagao et al. (2003)	Yamanaka (1995c)	Fukiharu and Hongo (1995)	Fukiharu and Hongo (1995)	Imamura and Yumoto (2004)	Imamura and Yumoto (2004)

A: *Phyllostachys bambusoides* grove in Japan, B: *Agathis australis* and *Leptospermum ericoides* dominated forest in New Zealand, C: *Eucalyptus marginata* and *Eucalyptus calophylla* forest in Australia D: *Pinus densiflora forest* in Japan, E: *Pinus luchuensis* plantation in Japan, F: *Castanopsis cuspidata* dominated forest in Japan, G: *Quercus serrata* dominated forest in Japan

[a]Yamanaka (1995c) as *Pseudombrophila deerata*; [b]Suzuki et al. (1998) as *Coprinopsis phlyctidospora* (sensu lato). *Hebeloma lactariolens* (syn: *Alnicola lactariolens*)

Physiological characteristics of ammonia fungi and apparent population densities of ammonia fungi described above suggest that ammonia fungi inhabit as remarkable small mycelia and/or spores although the latent form(s) of each ammonia fungus in the field has not been directly confirmed.

The collection records obtained from various habitats in different geographical areas, *A. botrytis, A. denudatus*, and *L. tylicolor* (see Footnote 4), especially the latter two species would be basic component species of fungal community of ammonia fungi since they have been recorded at high frequencies from urea plots in different geographical areas, irrespective of vegetation (Table 4.1). It means that fungal community of ammonia fungi in each habitat composes several or more ubiquitous species in anamorphic fungi, Ascomycota, and Basidiomycota, and sometimes, one or more endemic species in Ascomycota and/or Basidiomycota although those ubiquitous species have different level of biogeographical distribution.

Physiological Characteristic of Ammonia Fungi

Germination of sexual spores (ascospores and basidiospores) of most EP fungi and LP fungi are stimulated by 10–300 mM NH_4-N at pH 7–10. Germination of asexual spores (conidia) of the early-stage EP fungus *A. botrytis* is stimulated by 300 mM NH_4-N at pH 8 (Suzuki 1989, 2006, 2009a, b). Most of them germinate rapidly and show high germination percentage (cf., Figure 4.4; Suzuki et al. 1982; Deng and Suzuki 2008a; Suzuki 2009a; Raut et al. 2011b).

The early-stage EP fungi (saprobic ammonia fungi) grow well at pH 6–8 and the late-stage EP fungi (saprobic ammonia fungi) at pH 6–7, whereas ectomycorrhizal LP fungi show optimum growth at pH 5–6 (Yamanaka 2003).

These suggest that, at vegetative stage, the EP fungi prefer the condition from weak alkaline to neutral, whereas the LP fungi are tolerant to weak alkaline and neutral conditions. The early-stage EP fungus *A. botrytis* and the late-stage EP fungi *L. tylicolor* and *Coprinopsis echinospora* grow vigorously in ammonium salts, L-asparagine and urea, but not in nitrate (Yamanaka 1999). The ectomycorrhizal LP fungus *H. vinosophyllum* grows well both in inorganic nitrogen such as NH_4Cl, KNO_3, KNO_2, and organic nitrogenous materials such as urea and L-asparagine. Another ectomycorrhizal LP fungus *L. bicolor* also grows well in ammonium salts, nitrate salts, and urea, but not in L-asparagine (Yamanaka 1999). Ammonia fungi would be divided into three groups in the standpoint of response to ammonium-nitrogen concentration for vegetative growth, namely (1) The early-stage EP fungi such as *A. denudatus* and *P. petrakii,* and the late-stage EP fungi such as *Coprinopsis phlyctidospora* and *Coprinopsis austrophlyctidospora* (Fig. 1 and Tables 1–3 in Licyayo and Suzuki 2006 as *Coprinopsis* sp.), and the ectomycorrhizal LP fungi such as *H. vinosophyllum* and *H. aminophilum* do not grow at high concentration of ammonium-nitrogen (grow up to 0.6 M NH_4Cl; (2) The late-stage EP fungus *L. tylicolor* and the late-stage EP/LP fungus

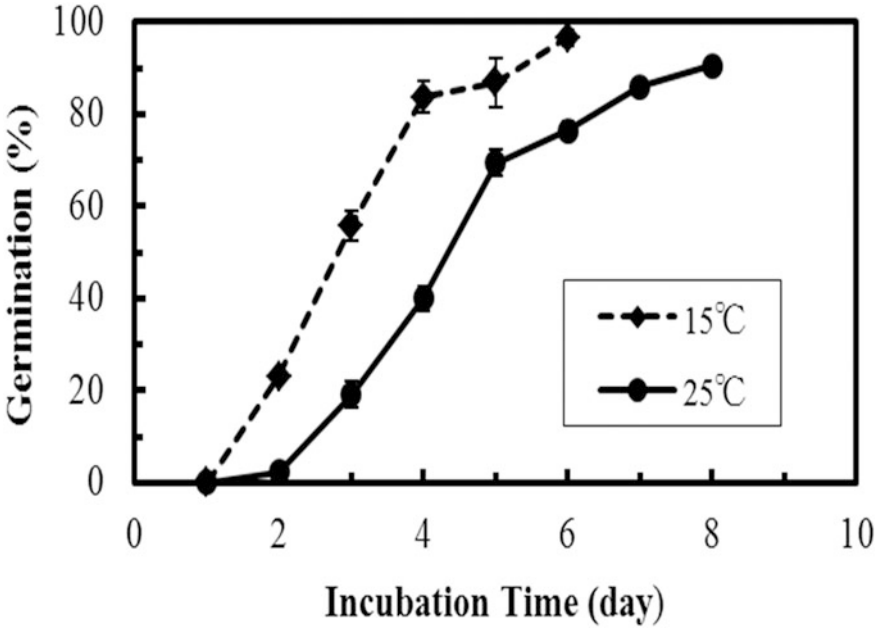

Fig. 4.4 Spore germination of *Hebeloma vinosophyllum*. The basidiospores of *H. vinosophyllum* NBRC107913 (the isolate deposited as NBRC31231 in Suzuki 2006) were collected from mature fruiting bodies after 14 days of cultivation at 25.0 ± 0.3 °C on MY agar medium. The basidiospores were suspended in 100 mM $(NH_4)_2HPO_4$ water solution. The density was adjusted at ca. 10^6 spores/mL. $n = 3$. The spore suspensions were separately incubated at 15.0 ± 0.3 °C and 25.0 ± 0.3 °C in darkness. Spore germination at each incubation time was shown as an average of three replicates with the standard error (vertical bar)

H. velonovskyi (see Footnote 3) grow well in wide range concentrations of ammonium-nitrogen (growth maximum at 0.01–0.1 NH_4Cl and 0.003–0.3 NH_4Cl, respectively, and grow up to 1.6 M NH_4Cl); and (3) The early-stage EP fungus *A. botrytis* grows well at high concentration of ammonium-nitrogen (growth maximum at 0.3–1.1 M NH_4Cl, grows up to 1.1–1.3 M NH_4Cl) (Table 4.2; Licyayo and Suzuki 2006).

Ammonium-nitrogen induces fruiting of the early-stage EP fungus *Coprinopsis cinerea* in darkness, but is not necessary for its fruiting in light (Morimoto et al. 1981). Urea stimulates both vegetative growth and fruiting of another early-stage EP fungus *Coprinopsis tuberosus* in light (Morimoto et al. 1982; as *Coprinus stercorarius*). These suggest that, probably, ammonium-nitrogen would be not the key affecting factor for reproductive structure formation of ammonia fungi in the field.

The late-stage EP fungus *L. tylicolor* in Basidiomycota forms reproductive structures like anamorphic fungi during linear growth phase, whereas other ammonia fungi in Basidiomycota such as *C. phlyctidospora* and *H. vinosophyllum* form reproductive structure only after reaching to stationary phase of growth curve

Table 4.2 The effect of NH_4-N concentration on vegetative growth of ammonia fungi

Fungal species	Nutritional	Successional	Concentration of NH_4Cl (M)		
	Mode	Stage	Optimum	Relative growth rate[a]	Upper limit
Amblyosporium botrytis	Saprotrophy	EP	0.3–1.1	D	1.1–1.3
Ascobolus denudatus	Saprotrophy	EP	0.003–0.03	A–B	0.6
Peziza moravecii	Saprotrophy	EP	0.01–0.1	B–C	1.3
Psudombrophila petrakii	Saprotrophy	EP	0.03–0.1	B	0.6
Humaria velenovskyi	Saprotrophy	EP/LP	0.003–0.3	A	1.6
Lyophyllum tyliclor	Saprotrophy	EP	0.01–0.1	B–C	1.3–1.6
Coprinopsis phlyctidospora	Saprotrophy	EP	0.003–0.1	B	0.6
Coprinopsis austrophlyctidospora[b]	Saprotrophy	EP	0.01–0.03	B	0.6
Hebeloma aminophilum	Biotrophy	LP	0.003–0.01	C	0.6
Hebeloma vinosophyllum	Biotrophy	LP	0.003	B	0.6

EP: Early phase, LP: Late phase, EP/LP: Early phase to Late phase
[a]A: above 50%, B: 20–49%, C: 10–19%, D: Less than 9% (Relative growth rate = Biomass obtained from cultivation at the lowest concentration of NH_4Cl/Biomass obtained from cultivation at optimum concentration of NH_4Cl) × 100 (%)
[b]Licyayo and Suzuki 2006 as *Coprinopsis* sp.
(Modified from Licyayo and Suzuki 2006)

(Suzuki 1989). *L. tylicolor* forms basidia directly on mycelia as well as on gills in fruiting bodies (Yamanaka and Sagara 1990). The ectomycorrhizal LP fungus *H. vinosophyllum* forms fruiting bodies not only from dikaryotic mycelium but also from homokaryotic mycelium (Deng and Suzuki 2008b). The shortcut basidium formation would be advantage for sustaining suitable population size of latent propagules even under non-ammonium-nitrogen-amended conditions (cf., Suzuki 2009b).

Enzyme Activity

Most saprobic ammonia fungi have faint chitinolytic and ligninolytic enzyme activities as well as a certain cellulolytic, proteolytic, and lipolytic enzyme activities (Yamanaka 1995a; Soponsathien 1998a, b). Many late-stage EP fungi (saprobic ammonia fungi) have relatively strong activities of hydrolases such as cellulolytic, ligninolytic and chitinolytic enzymes (Yamanaka 1995a; Soponsathien 1998a, b). An ectomycorrhizal LP fungus *H. vinosophyllum* has weak ligninolytic enzyme activity and faint activities in other hydrolases (Yamanaka 1995a; Soponsathien 1998a, b). Another ectomycorrhizal LP fungus *L. bicolor* does not show cellulolytic, ligninolytic, and chitinolytic enzyme activities (Yamanaka 1995a;

Soponsathien 1998a, b). Saprobic LP fungi *Calocybe constricta* (as *Calocybe leucocephala* in the following reference), *Lepista nuda*, and *Lepista sordida* (misidentified as *L. nuda* in the following reference) have strong cellulolytic and ligninolytic enzyme activities (Soponsathien 1998a). In contrast, a nectotrophic LP fungus *Collybia cookei* does not show cellulolytic and ligninolytic enzyme activities (Soponsathien 1998a). The pH optima for cellulolytic enzymes of EP fungi are between 7 and 9 (Enokibara et al. 1993). In general, cellulolytic, hemicellulolytic, and ligninolytic enzyme activities of the saprobic ammonia fungi are stronger than those of ectomycorrhizal ammonia fungi (Yamanaka 1995a; Soponsathien 1998a, b).

Ecophysiology of Ammonia Fungi

pH, water content, and ammonium-nitrogen concentration of the soil always increase rapidly after urea application in the habitats of acidic condition. Thereafter, pH and water content decline associated with continuous decrease in ammonium-nitrogen concentration through its oxidation probably by bacteria. Nitrate-nitrogen reaches maximum concentration at around the period of the first occurrence of LP fungi and gradually declines. The pH, water content, ammonium-nitrogen, and nitrate-nitrogen return to each control level, in that order. The changes in the abiotic environments disappear within 2 years after urea application (Yamanaka 1995a, b, c; Suzuki 2000; Suzuki et al. 2002b; He and Suzuki 2004). EP fungi would colonize continuously during the decrease in ammonium-nitrogen concentration accompanying declining of pH lead to the control level (Yamanaka 1995a, b, c; Suzuki 2000; Suzuki et al. 2002b). In other words, the principal nitrogen source for vegetative growth of EP fungi would be ammonium-nitrogen under a neutral to weakly alkaline conditions, whereas that for vegetative growth of LP fungi would be nitrate-nitrogen under acidic condition (cf., Yamanaka 1995a, b, c, 1999, 2003; Suzuki 2000, 2006; Suzuki et al. 2002b). Screening in activities and pH spectra of hydrolases in ammonia fungi (Enokibara et al. 1993; Yamanaka 1995a; Soponsathien 1998a, b; Suzuki 2009a; Manusweeraporn et al. 2013) and the results of litter bag experiments (He and Suzuki 2004; Suzuki 2006) indicate that some late-stage EP fungi effectively decompose cellulose and hemicellulose and weakly decompose lignin. Remarkable decomposition of lignin would be restored when fungal community of ammonia fungi is replaced again with that of pre-inhabiting fungi (non-ammonia fungi). Ectomycorrhizal LP fungi probably slowly grow in the territories of EP fungi and keep their territories by using monosaccharide and/or disaccharide derived from the decomposition of polysaccharide by the hydrolases of other organisms. The LP fungi would soon form ectomycorrhizal symbiosis to obtain carbon source at higher efficiency by evasion of nutritional competition with other saprobic microbes including EP fungi since pre-inhabiting ectomycorrhizal fungi (non-ammonia fungi) had been damaged seriously by high concentration of ammonium-nitrogen

associated with alkaline condition which is caused by the nitrogen enrichment. Namely, it would be the strategy of LP fungi to gain carbon source efficiently through gap of mycorrhizal symbiosis caused by the disturbance of nitrogen enrichment (cf., Suzuki 2006, 2009b).

Future Perspectives

1. **Research in Basic Field**

   Further collection of fungi after and the disturbance by a large input of ammonium-nitrogen in unexplored terrestrial ecoregions are required to elucidate the mycobiota of ammonia fungi at worldwide scale. It would contribute to elucidate the structure of fungal community of true fungi in terrestrial ecosystems as well as relationship between biogeographical distributions of ammonia fungi in ecoregions categorized within 14 biomes and 8 biogeographical based on flora or fauna in each habit (Olson et al. 2001).

   Longevity of basidiospores in ammonia fungi have been examined only two ectomycorrhizal LP fungi, *H. vinosophyllum* (Deng and Suzuki 2008a) and *Hebeloma spoliatum* sensu Hongo (Suzuki 2009b). Researches in spore longevity of other species in ammonia fungi, especially EP fungi, are important problem to speculate the community structure of ammonia fungi in non-disturbed period. The survey of microfungi in soil and litter after a disturbance by a large input of nitrogenous materials is indispensable to elucidate the community structure of ammonia fungi in each habitat since researches in ammonia fungi have been done mostly focused on macrofungi (cf., Sagara 1975; Sagara et al. 2008). Survey of microbes in soil and litter by next-generation PCR would contribute to reveal the population density of fungi including ammonia fungi in terrestrial environments when data base about morphological features of ammonia fungi increase. Advances in examination of enzyme activities of ammonia fungi would also contribute to reveal the dynamics in community structure of ammonia fungi in different habitats, based on elucidation of equivalent species of ammonia fungi in each vegetation.
2. Any loss of diversity of mycorrhizal species is harmful, considering the crucial roles of these fungi play in forest ecosystems (Egli 2011). For example, the observation of occurrence of ectomycorrhizal fungi (EMF) in the white spruce (*Picea glauca*)-dominated forests supports the hypothesis that long-term nitrogen inputs (soil acidification) may lead to loss of EMF sporocarp diversity (Lilleskov et al. 2001). The evaluation of the degree of forest healthiness would be possible based on the survey of species richness of fungi, especially that of

mycorrhizal fungi in each habitat. The survey of fungal fruiting in each habitat is troublesome work since the occurrence periods of fungi are short and difficult to estimate those fruiting time. The survey of community structure of ammonia fungi, especially ectomycorrhizal ammonia fungi, gives an advantage comparing with that of non-ammonia fungi for evaluation of the degree of forest healthiness since the community structure of ammonia fungi in each habitat is simpler than that of non-ammonia fungi. Moreover, the occurrence time of each ammonia fungus can be estimated by the succession after urea application. Global warming gradually affects the distribution area of vegetation. It is difficult to find it at latent time, but would be able to diagnose it by the changes in fungus community, especially by those in ectomycorrhizal fungi (EMF) community. The gradual migrating of EMF to high latitudes would be indicated by examining the changes in the distribution limitation of ectomycorrhizal ammonia fungi. In conclusion, survey of species diversity of ammonia fungi, especially ectomycorrhizal ammonia fungi (ectomycorrhizal LP fungi) would expect to contribute as a suitable indicator for detection of changes in forest environments even at latent stage (no symptoms in shoots). In other words, ammonia fungi would be suitable organisms as indicators for forests' healthiness and global warming.

3. The isolates of *Coprinopsis phlyctidospora* and *H. vinosophyllum* grow vigorously on various media (cf., Suzuki 2006, 2012; Deng and Suzuki 2008a; Raut et al. 2011b). *H. vinosophyllum* forms easily mycorrhizal association with the seedlings of its host plants in vitro (Fig. 4.5) and keeps high fruiting ability. Based on our experiences, both species sustain enough fruiting abilities more than 40 years even under continuous subcultures on nutrient agar media such as MYA and PDA. For example, an isolate of ammonia fungus *C. phlyctidospora* keeps high fruiting ability for longer periods than that of a famous model organism *Coprinopsis cinerea* ecologically belonging to both ammonia fungi and coprophilous fungi. The homokaryotic isolates and dikaryotic isolates of ectomycorrhizal ammonia fungus *H. vinosophyllum* grow rapidly and fruits easily on nutrient media including synthetic media (cf., Suzuki 2006, Deng and Suzuki 2008b).

   Both fungi would be suitable model organisms for experiments in vitro separately as a saprobic species and an ectomycorrhizal species. For example, a dikaryotic isolate of *H. vinosophyllum* was conducted to examine the effect of coexisting elements as competitors on the cesium uptake in order to establish the controlling procedure for the accumulation of radioactive cesium by mushroom fruit bodies in radioactive cesium-polluted area (Ho et al. 2013) since *H. vinosophyllum* has high accumulation abilities of cesium (Ban-nai et al. 2005).

Fig. 4.5 Fruit body formation of *Hebeloma vinosophyllum* by mycorrhizal association with *Pinus densiflora* in vitro. An isolate *H. vinosophyllum* collected from Vietnam (Ho et al. 2014) and a seedling of *P. densiflora* were inoculated at the same time on sterilized 35 mL substrate placed in a test tube (30 mm in diameter), corked with a Silicosen plug (Shin-Etsu Polymer, Tokyo, Japan). The substrate consisted of 200 g of dried vermiculite-sphagnum moss (Iris Ohyama, Sendai, Japan/Lixil Viva Corporation, Ageo, Japan), in a 49:1 w/w mixture, with 500 mL of glucose-free Modified Melin–Norkans liquid medium (MMN: Marx 1969). They were cultivated at 20 ± 3 °C in a light (85 μmol m^{-2} s^{-1} on the substrate surface)/dark cycle of 12 h for 8 weeks (After Ho 2013)

Conclusions

Community structure of ammonia fungi in each habitat composes more than several saprobic and sometimes with a few ectomycorrhizal species although the mycobiota of ammonia fungi is different in each habitat (cf., Sagara 1975; Suzuki et al. 2003; Suzuki 2009a). The reproductive structures of ammonia fungi appear sequentially. The successive occurrence of ammonia fungi would be mainly caused by the time required for reproductive growth of each fungal species accompanied by interactions among ammonia fungi and non-ammonia fungi (Suzuki 1989, 2006; Suzuki et al. 2002b; Licyayo et al. 2007; Barua et al. 2012). Both early-stage and late-stage of early phase of ammonia fungi (EP fungi) well adapt or somewhat tolerate to high concentration of ammonium-nitrogen under neutral and weakly alkaline conditions. The former colonize as *S-R* strategists and the latter colonize as *C-R* strategists (cf., Cooke and Rayner 1984; Suzuki 2009b). Among them, the late-stage EP fungi

weakly decompose lignin as well as effectively decompose cellulose and hemicellulose. Namely, the late-stage EP fungi could be principal litter and wood decomposer instead of pre-inhabiting saprobic non-ammonia fungi in the nitrogen-disturbed site (cf., Suzuki 2006). The ectomycorrhizal ammonia fungi (LP fungi) would be *C-R* strategists (cf., Cooke and Rayner 1984; Suzuki 2009b) and quickly colonize into the territories of ectomycorrhizal non-ammonia fungi by the disturbance derived from a large input of ammonium-nitrogen. Ammonia fungi colonize as pioneer fungal species in the site of nitrogen disturbance and would sustain nutrient cycle, especially, carbon and nitrogen cycles by replacement of the activities of non-ammonia fungi. The inhabiting forms of EP fungi and LP fungi in non-disturbed site have not been elucidated by direct observation in the field. The EP fungi and LP fungi would survive by spores and/or small pieces of mycelia speculating from the physiological characteristics (cf., Sagara et al. 2008; Suzuki 2009a). In other words, EP fungi and LP fungi have no abilities to colonize vigorously into the sites of low concentration of ammonium-nitrogen under acidic condition, especially under the competition with non-ammonia fungi. A large amount of ammonium-nitrogen and nitrate-nitrogen caused by nitrogen enrichment would be immobilized by EP fungi and LP fungi, respectively. The immobilization would contribute to stimulate re-colonization of non-ammonia fungi through the weakening of inhibitive effects caused by high concentration of ammonium-nitrogen subsequent to that of nitrate-nitrogen. The decomposition of cellulose and hemicellulose in litter and wood after ammonium-nitrogen enrichment would be mostly done by the early-stage of EP fungi and that of lignin would be done by the late-stage of EP fungi. Namely, both EP fungi and LP fungi would have roles in carbon and nitrogen cycles in terrestrial ecosystems after the nitrogen disturbance caused by animal wastes on the litter such as animal excreta and animal dead bodies (cf., Sagara 1975, 1992, 1995; Sagara et al. 2008). The ecophysiological replacement could be considered as "compensation process" in nutrient cycle (Suzuki 2002).

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Marine Filamentous Fungi: Diversity, Distribution and Bioprospecting

5

K. R. Sridhar

Abstract

Marine fungi constitute a model mycobiota to evaluate several fundamental assumptions and adaptations to extreme habitats. They are ecologically diverse and cosmopolitan in distribution in all oceans as endemics and indigenous communities. Molecular techniques are revealing high diversity and distribution than assessment based on morphological characterization. They have the capability to grow and process a wide variety of detritus and substrata in saline habitats. Besides, they also colonize live parts especially mangrove plant species, seaweeds and sea grasses as endophytes. Majority of studies on marine fungi are confined to the Europe, North America and Southeast Asia, while meagre information is available from the Indian Subcontinent in spite of its rich marine habitats. In the recent past, marine fungi became a focal point of investigation especially for bioprospecting. Marine fungi are capable of yielding a variety of new compounds worth exploring for their bioactive potential especially enzymes, antibiotics, anticancer properties and bioremediation. The current knowledge is mainly from culture-dependent marine fungi, while understanding culture-independent marine fungi needs sophisticated methods like metagenomics. Besides, metagenomics enhances our knowledge on marine fungal diversity and distribution and helps in cloning precise DNA of culture-independent fungi to harness bioactive principles.

Keywords

Marine fungi · Detritus processing · Food chain · Endophytes · Metabolites Bioremediation

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Introduction

Although marine habitats embody a plethora of microorganisms, a fraction of them have been identified and characterized. Marine filamentous fungi are ecologically distinct assemblage adapted to grow and sporulate on a wide range of substrates in saline habitats (marine, intertidal, estuarine and mangrove) (Kohlmeyer and Kohlmeyer 1979). Owing to their saprotrophic ability, they constitute a major segment in nutrient, biogeochemical and food web dynamics in marine ecosystem. Kohlmeyer and Kohlmeyer (1979) grouped marine fungi into three geographical groups: (i) cosmopolitan species; (ii) temperate-water species and (iii) species from tropical and subtropical waters. The latest statistics on marine fungi reveals occurrence up to 530 species (in 321 genera), which includes Ascomycota (424 sp. in 251 genera), Basidiomycota (12 spp. in 9 genera) and mitosporic fungi (94 sp. in 61 genera) (Jones et al. 2009). Reports on obligate marine fungi are steadily increasing from mid-eighteenth century (1840) to the recent past (2009) (Jones et al. 2009). But possibility of occurrence of all marine fungi raised up to 10,000 (Jones 2011a), and Jones and Pang (2012) suggested exploration of cryptic fungi indifferent ecosystems: marine fungi (560), facultative marine fungi (100), marine yeasts (1500), misidentified fungi (100), marine-derived fungi (1500), deep-sea fungi (300), planktonic fungi (500), endophytic, algicolous and cryptic species (7500). Fossil evidences suggest that fungi existed in late Proterozoic era (900–570 mya) (Taylor et al. 1992, 1995) and molecular evidences based on 18S rDNA (800 mya) almost match with the fossil records (Berbee and Taylor 1993). The environmental changes resulted in transformation of marine fungi in different pathways, which occurred in 11 lineages distributed in six classes (Jones and Pang 2012). Such evolutionary changes in marine fungi might be responsible for their wide distribution in all the oceans as they evolved prior to the continental drift. Interest in studying marine fungi is increasing steadily as they occur in extreme conditions and they possess novel and new metabolites functioning in harsh conditions (Hyde and Pointing 2000). However, in spite of studies on marine fungi for more than a century (Jones 2011a), their taxonomy, distribution, ecology and bioprospecting still remained as a black box. This mini-review discusses briefly on the diversity, distribution, ecology and bioprospecting of marine filamentous fungi based on recent literature.

Diversity and Distribution

Substrata

Organic matter in the ocean environment serves as major recyclable resource of energy, which includes woody litter, leaf litter, sedge detritus, fruits, seeds, sea grasses, microalgae, seaweeds, sea grasses, sediments and animal remains (corals,

exoskeletons and keratin-like substrata) (Kohlmeyer and Kohlmeyer 1979). Lignocellulosic materials in the ocean environments serve as major substrate for mineralization and energy flow. Export of plant detritus and increase in faunal biomass have been well addressed by Lee (1995). In spite of slow degradation, woody debris serve as long-term source for the food webs as well as nutrient cycles especially N, P and K. Along with woody litter, leaf litter constitutes a major source of carbon in mangrove habitats. Grasses and sedges also serve as major part of detritus in coastal and mangrove habitats. Opposing to mangroves, in subtropical and temperate habitats, salt marshes serve as major source of organic matter. Woody litter, leaf litter, grasses, sedges and salt marshes provide vertical zonation and niches for a number of fauna in marine habitats (Sridhar et al. 2012a). Besides, mangrove plant species, lignocellulosic substrates, seaweeds, sea grasses, salt marshes and animal remains, sediments in different marine habitats provide several niches for filamentous fungi.

Decomposition of detritus in marine habitats depends on a variety of biotic and abiotic factors (Jones 2000), and details of pattern of decomposition of detritus in mangroves are dealt by Sridhar (2012). Lignocellulosic materials showed the greatest diversity of filamentous fungi, while a few species colonize calcareous materials or sandy habitats. Besides abiotic factors, competition among fungi markedly influences fungal diversity and species composition (Sridhar and Maria 2006). Temperature and salinity serve as important factors in determining the diversity of marine fungi. No single factor could be accounted for the diversity of marine fungi, which differs from ocean to ocean, mangrove to mangrove and shore to shore. Further probing into the biotic and abiotic factors governing the diversity and distribution of marine fungi will allow us to develop management strategies for conservation of marine habitats.

Mangroves and Palms

Mangroves are characterized by different habitats like estuaries, backwaters, deltas and lagoons constituting second important ecosystem after coral reefs (Qasim and Wafar 1990; Alongi 2002). Mangrove flora consists of true mangroves (~ 80 sp. of trees and shrubs), minor vegetation and mangrove associates (salt-tolerant) responsible for the structure of mangrove forests (Tomlinson 1986; Field 1995). From mangrove vegetation, the annual litter production varies between 0.011 t/h (*Ceriops tagal*; Kenya) and 23.7 t/h (*Rhizophora stylosa*; Australia) (see Sridhar et al. 2012a). Such detritus provide ecological niches for a variety of filamentous fungi. From 72 mangrove plant species, 278 ascomycetes, 277 anamorphic fungi and 30 basidiomycetes have been documented by Schmit and Shearer (2003). Among ten mangrove habitats in a wide geographic region, meiosporic ascomycetes/anamorphic ascomycetes/basidiomycetes ranged between 0/1/1 (in Madagascar) and 225/190/33 (in tropical Asia) (Shearer et al. 2007). This is mainly due to the diversity of mangrove plant species and extent of surveys performed.

Rhizophora sp. constitute excellent host for marine fungi and so far as many as 201 filamentous marine fungi have been reported (Sarma 2012).

Common foliar endophytic fungi include *Sporormiella minima*, *Acremonium* sp., *Phomopsis* sp. and *Phyllosticta* sp., and the latter two genera are cosmopolitan in distribution (Suryanarayanan and Kumaresan 2000). In senescent standing stems of *Acanthus ilicifolius*, *Acremonium* and *Colletotrichum* were dominant and serve as saprophytes on senescence (Kumaresan and Suryanarayanan 2002). Some endophytes in mangroves serve as saprotrophs or opportunistic pathogens (e.g. *Chaetomium globosum* and *Paecilomyces variotii*) (Ananda and Sridhar 2002; Arnold et al. 2007; Naik et al. 2007; Hyde and Soytong 2008; Vega et al. 2008). Many typical plant pathogenic (e.g. *Alternaria alternata, Curvularia clavata* and *Drechslera halodes*) and toxigenic (e.g. *Aspergillus flavus, A. ochraceus* and *Trichoderma harzianum*) fungi are endophytic in mangrove plant species (Anita and Sridhar 2009; Anita et al. 2009). Besides, some fungi are also entomopathogenic (e.g. *Paecilomyces* sp.; Ananda and Sridhar 2002; Maria and Sridhar 2003). Many foliar endophytes are dominated by single species in mangrove plants like *Avicennia marina* (*Phoma* sp.), *Bruguiera cylindrica* (*Colletotrichum gloeosporioides*), *Rhizophora apiculata* (*Sporormiella minima*), *Rhizophora mucronata* (*Sporormiella minima*) and *Suaeda maritima* (*Camarosporium palliatum*) (Suryanarayanan et al. 1998; Suryanarayanan and Kumaresan 2000; Kumaresan and Suryanarayanan 2001). But multiple species dominance was also seen in *Avicennia officinalis, Lumnitzera racemosa, Rhizophora mucronata* and *Sonneratia caseolaris* (Kumaresan and Suryanarayanan 2001; Ananda and Sridhar 2002). Up to 619 endophytic fungi have been isolated from the mangrove plant species from Thailand with a highest colonization in *Bruguiera cylindrica* (Sakayaroj et al. 2012).

Palms constitute prominent tree species in the mangrove habitats in tropical and subtropical regions. Among the palms, *Nypa fruticans* is most prominent in brackish water habitats and ranges from Bangladesh to Pacific Islands (Tomlinson 1986). Up to 139 species of fungi are known to be associated with *N. fruticans* of South East Asia (see Loilng et al. 2012).

Seaweeds and Sea Grasses

Fungi from marine algae and endomycobiota in seaweeds have been excellently reviewed by Jones et al. (2012) and Suryanarayanan (2012a). Fungi on algal hosts consist of saprophytic, parasitic, endophytic, lichens and mycophycobionts (Kohlmeyer and Kohlmeyer 1979). Seaweeds cover large areas of sea floor and amount to 74,000 t of wet biomass (Nedzarek and Rakusa-Suszczewski 2004). They are consistent substrates for colonization of marine fungi on live as well as dead parts. The algicolous fungi account to 79 species without prevalent species. Evaluation of seaweed litter from the Portuguese coast on damp incubation yielded 29 species comprising 16 ascomycetes, two basidiomycetes and 11 mitosporic fungi (Sridhar et al. 2012b). On comparison of seaweed and woody litter of Portuguese coast, 15 species of ascomycetes, two species of basidiomycetes, and four

species mitosporic fungi were common. On these substrates, two species of arenicolous fungal dominance corroborating with earlier observations (Sakayaroj et al. 2011; Hong et al. 2015).

Seaweed endophytes were studied in length by Zuccaro et al. (2003) and Suryanarayanan et al. (2010). The number of marine fungi was higher in washed thalli than in dead thalli of *Fucus serratus* (67 vs. 36 sp.) (Zuccaro et al. 2003). Evaluation of green (11 sp.), red (8 sp.) and brown (6 sp.) algae in the East Coast of India resulted in isolation of 72 endophytic fungi. Comparatively, the brown algae supported the highest endophytes (25 sp.) (Suryanarayanan et al. 2010). Surprisingly, among 50 fungal strains derived from brown alga *Sargassum* sp. based on ITS, LSU and β-tubilin region, an arenicolous fungus *Corollospora angusta* was the most dominant species (Hong et al. 2015). Another arenicolous fungus *Corollospora maritima* was most frequent among 79 ascomycetes obtained from 1900 samples in a Marine National Park of Thailand (Sakayaroj et al. 2011). The common endophyte *Mycophycias ascophylli* grows mutually with *A. nodosum* and *Pelvetia canaliculata* and remains associated with algal host throughout its life cycle (Kohlmeyer and Kohlmeyer 1979; Stanley 1991; Kohlmeyer and Volkmann-Kohlmeyer 1998; Ainsworth et al. 2001).

Studies on endophytic fungi in sea grasses are sporadic, and a few sea grasses have been studied in Bermuda, Hong Kong, India, Puerto Rico and Thailand (Sakayaroj et al. 2012; Venkatachalam et al. 2016). From seven investigations, only 10 species have been fully identified and many were identified only up to generic level. Nearly 26 species of endophytic fungi have been reported in three sea grasses (*Thalassia testudinum*, *Zostera japonica* and *Z. marina*) by Alva et al. (2002). *Aspergillus* and *Penicillium* were dominant endophytes in sea grasses of southwest India (Venkatachalam et al. 2016).

Salt Marshes

Salt marshes being most productive dominant ecosystems in the temperate and high-latitude marine habitats provide vital ecological niches for innumerable number of microbes and fauna (Allen and Pye 1992). The emergent macrophytes survive and sustain in salt marsh ecosystem (e.g. *Juncus* sp., *Phragmites* sp. and *Spartina* sp.). A variety of biotic and abiotic factors govern colonization and functioning of filamentous fungi on the salt marshes: host specificity, competition, susceptibility/resistance for colonization and availability of specific conditions (e.g. water, oxygen, dissolved organic matter, salinity and temperature) (see Calado and Barata 2012). There is substrate specificity among the fungi associated with salt marshes; for instance, 332 species occurred on three genera of salt marsh plants (*Spartina* sp., *Juncus* sp. and *Phragmitis* sp.), 89% confined to one, 9% confined to two and 2% confined to three hosts (Calado and Barata 2012). There occurs a successive phase in colonization of decaying salt marshes: first phase with a pioneer community (a few species) followed by second phase with mature community (high diverse competitive species) and third phase with impoverished community.

Interestingly, 48 new species (in 14 new genera) and a new family of filamentous fungi have been reported from the salt marsh macrophyte *Juncus roemerianus* along the US Southeast coast which denotes the potentiality of salt marshes in supporting filamentous fungi (Kohlmeyer and Volkmann-Kohlmeyer 2001).

Animals and Their Remains

Suryanarayanan (2012b) reviewed the diversity and importance of fungi associated with marine sponges. Hawaiian sponges (*Gelliodes fibrosa, Haliclona caerulea* and *Mycale armata*) were colonized by culture-dependent ascomycetes (25 genera) and a basidiomycete (Li and Wang 2009). From marine sponge (*Hyatella cribriformis*) in Gulf of Mannar of southern India, nearly 15 genera of fungi were identified for the first time and *Aspergillus nomius* showed bioactive potential against many human pathogens (Meenupriya and Thangaraj 2012). Ten marine sponges of Rameshwaram coast of southern India harboured filamentous fungal symbionts (Thirunavukkarasu et al. 2012). *Acremonium, Alternaria, Aspergillus, Cladosporium, Fusarium* and *Penicillium* were frequent, while *Aspergillus* sp. dominated and co-dominated in all the sponges surveyed. A few fungal isolates of sponges produced acetylcholinesterase inhibitors. More evidences are accumulating on the endosymbiosis of fungi with sponges and their usefulness in bioprospecting. Gorgonian sea fans (*Annella* sp.) were also hosts for several fungi and based on morphological characteristics. Fifty-one fungi have been reported in sea fan tissues after tsunami in 2004 in Mu Ko Similan National Park, Andaman Sea, in Thailand (Phongpaichit et al. 2006).

Filamentous fungi are also known to invade a wide range of animal substrata like corals, shells (e.g. balanids, bivalves, foraminifers and snails), exoskeletons (e.g. bryozoans, crabs, hydrozoans, shipworms and tunicates), cuttlefish endoskeletons, feathers, snake skin, beetle wings, horse hair and teredinid tunnels (Rees and Jones 1985; Kohlmeyer and Volkmann-Kohlmeyer 1990, 1992; Rosello et al. 1993; Ananda et al. 1998; Ananda and Sridhar 2001; Nambiar and Raveendran 2015). Crab exoskeletons in mangroves serve as potential source of nutrients for chitin-degrading fungi (Grant et al. 1996). In three mangroves of southwest coast of India, Ananda and Sridhar (2001) recorded five ascomycetes and 16 mitosporic fungi. Feather samples were colonized by 14 filamentous fungi with dominance of *Corollospora intermedia* followed by *Aspergillus* sp., *Epicoccum nigrum* and *Scolecobasidium* sp. The arenicolous fungus *Corollospora intermedia* was also dominant on bivalve shells and cuttlefish endoskeletons. Such animal remains collected from beaches require long-term incubation (Ananda et al. 1998). Animal cellulose (tunicin) present in tunicates is known to be degraded by marine fungi (Kohlmeyer and Kohlmeyer 1979). However, our knowledge is poor on the role of fungi in the decomposition of animal remains in marine habitat.

Deep-Sea Habitats

With extreme conditions of temperature and hydrostatic pressure, deep-sea habitats cover up to 65% area on the total earth surface (Svendrup et al. 1942). First report on the occurrence of fungi in deep sea was on shells collected from 4610 m depth (Höhnk 1961). Subsequently, fungal occurrences in deep-sea substrates of subtropical Atlantic Ocean, Pacific Ocean and Indian Ocean have been reported (see Singh et al. 2012). Nagahama and Nagano (2012) reviewed diversity in deep sea by conventional culture and culture-independent methods. Using different techniques (e.g. dilution plating, particle plating and pressure enrichment), about 200 fungi have been isolated from the deep-sea sediments of the Indian Ocean (Damare et al. 2006). Occurrence of fungi in sediments has been demonstrated directly by immunofluorescence technique, and culture-dependent and culture-independent approaches were also employed to recover fungi from deep sea (Singh et al. 2011). Hydrothermal vents also constitute potential sites for fungal occurrence (see Singh et al. 2012). In spite of anoxic nature, several fungi have been detected by molecular techniques (Jebaraj et al. 2010).

Arctic and Antarctic Habitats

Marine fungal investigations in the polar region are scanty. Polar habitats harbour far greater fungal resource than expected, and woody litter and macroalgae provide potential shelter for many cosmopolitan and endemic fungi of environmental and biotechnological interest. From eight marine sediments in high Arctic (Kongsfjorden, Svalbard), fungal communities have been reported using different molecular techniques (Zhang et al. 2015). Of 113 operational taxonomic units (OTU), 62 belonged to Ascomycota, 26 to Basidiomycota, two to Chytridiomycota, one each to Zygomycota and Glomeromycota and 21 belonged to unknown fungi. Among 30 fungal genera found in the Arctic, 14 have been isolated from the marine sediments and *Fusarium* was widespread and found in all eight Arctic sediments. On the driftwood collected from Arctic waters in Norway (Tromsø and Longyearbyen), six marine fungi were recovered and four were new to science (Pang et al. 2011). Further experiments on the fungal isolates suggested that these fungi involve in degradation of wood in polar conditions. In Arctic region of north Norwegian coast, investigation of 50 intertidal and sea-floor logs resulted in 577 pure cultures assigned to 147 OTU with dominance of Ascomycota (Rämä et al. 2014). Obligate as well as facultative marine fungi were derived from woody materials, and about half of OTU belonged to non-marine origin.

Godinho et al. (2013) surveyed diversity of macrofungi associated with eight macroalgae along the rocky coastline in Antarctica using molecular methods. This study yielded 148 fungal isolates belonging 50 species (in 21 genera). Cold-adapted macroalgae in Antarctica are composed of cold-adapted endemic fungi, which are rich source of bioactive compounds. Molecular methods of assessment of endemic macroalgae from Antarctic Peninsula revealed 239 fungal isolates representing

48 species (in 18 genera) (Furbino et al. 2014). The community composition consists of endemic, indigenous and cold-adapted cosmopolitan species. It was hypothesized that the cold-adapted cosmopolitan fungi might be useful chronometers of climate change in maritime Antarctica.

Bioprospecting

There is a wide scope to use marine fungi as natural source of drugs in human health, industrial and environmental rehabilitation point of view. In developing novel drugs from marine source, natural products rather than synthetic products play a vital role. Until 2001, investigations on marine-derived fungi revealed about 272 new natural products and additional 240 new structures were found during 2002–2004 (Ebel 2006). Saleem et al. (2007) reviewed more than 23,000 bioactive microbial products from marine fungal origin (e.g. antifungal, antibacterial, antiviral, cytotoxic and immunosuppressive agents). New compounds obtained from marine-derived fungi are constantly increasing, and a number of compounds reached up to 200 during 2009 (see Ebel 2012). Statistics of new compounds from marine-derived fungi till recently reveal a highest of 41% polyketides followed by alkaloids (20%), peptides/terpenoids (14% each), prenylated polyketides (8%), shikimates (2%) and lipids (1%). Up to 80% of new compounds were derived from the living matter from the marine habitats. Of the marine sources, algae constitute the highest new compounds (21%) followed by sponges (19%), mangrove habitats/sediments (16% each), mollusks/wood and other plants of non-mangrove (6% each), corals/other marine invertebrates (4% each), fishes/ascidians (3% each) and water/undisclosed (1% each). Some compounds from marine-derived fungi (especially *Aspergillus* and *Penicillium* spp.) have entered clinical trials (see Ebel 2012). Damare et al. (2012) reviewed the current applications of marine-derived fungi (enzymes, hydrocarbon degradation, heavy metal sequestering, biosurfactants, omega-3 fatty acids and other secondary metabolites). Suryanarayanan et al. (2009) reviewed the scope of bioprospecting of fungal endophytes.

Enzymes

Marine fungal-derived enzymes have potential uses in catabolism, bioremediation and biomedical applications (Velmurugan and Lee 2012). Many marine fungi have the ability to rapidly utilize cellulose under a wide range of salinities (0–34‰); among 45 marine fungi, 89 and 84% showed cellulolytic and xylanolytic activities under in vitro conditions, respectively (Bucher et al. 2004). Lignin-degrading enzymes are highly useful in bioremediation of industrial effluents especially those possessing high chloride and sulphide contents or dyes or heavy metals (Raghukumar 2005). *Flavodon flavus* and *Podospora anserina* are known to produce lignin-modifying enzymes. High xylanase activity in marine and mangrove

fungi (*Aigialus mangrovei*, *Astrosphaeriella mangrovei* and *Halorosellinia oceanica*) has been demonstrated by Raghukumar et al. (1994). D'Souza et al. (2006) showed decolorization of black liquor, textile dye wastes and molasses spent wash by laccase producing marine fungi. Fungal isolates of marine origin (e.g. *Aspergillus niger* and *F. flavus*) are also known to produce thermostable and wide pH-tolerant cellulase-free xylanases, which are highly desired for bleaching of paper pulp and textiles (Raghukumar et al. 1999). Among the 117 isolates from marine algae and sea grasses, *Cladosporium* sp. and *Penicillium* sp. showed high chitinase activity (Venkatachalam et al. 2015). Other potential enzymes of interest from marine fungi include amylases, 1,3-β-D-glucanases, 1,6-β-D-glucanases, β-D-glucosidase, *N*-acetyl β-D-glucanases, β-D-galactosidases and β-D-mannosidases (see Velmurugan and Lee 2012).

Other Metabolites

Endophytic fungi derived from marine algae and sea grasses produce a variety of metabolites including broad-spectrum antimicrobial compounds (see Raghukumar 2008; Zhang et al. 2009). *Ascochyta salicorniae* associated with *Ulva* sp. showed antimicrobial alkaloid and anti-plasmodial activity (against *Plasmodium falciparum*) (Osterhage et al. 2000). Among 50 isolates of fungi derived from the brown alga (*Sargassum* sp.), *Arthrinium* sp. exhibited strong antifungal activity against several plant pathogenic fungi and *Arthrinium saccharicola* possesses high radical scavenging activity and in addition endoglucanase and β-glucosidase activities. *Dendryphiella salina* associated with brown seaweeds produced a wide range of bioactive compounds (Guerriero et al. 1989). Endophytic *Pestalotiopsis* spp. are known for antifungal, antioxidant and anticancer metabolites (Strobel et al. 2002; Mayer and Gustafson 2003). *Penicillium* sp. of marine origin produced epolactaene, which required treating many neurodegenerative diseases (Kakeya et al. 1995). From Caribbean Sea, *Emericella variecolor* yielded varitriol which acts against renal, central nervous system and breast cancer cell lines (Malmstrom et al. 2002). Some sponge-associated fungi also serve as source of acetylcholinesterase inhibiting properties worth further exploration towards treatment of Alzheimer's disease (Shi et al. 2009; Thirunavukkarasu et al. 2012).

Concluding Remarks

Marine fungal investigation is a fascinating field of mycology and widely appreciated in understanding diversity, distribution, ecology and harnessing new metabolites. Biodiversity research continues to progress as a few marine habitats are inventoried. Availability of substrate for marine fungi is the basis for their diversity, distribution and interaction. Besides dead plant, algal and animal remains (e.g. wood, leaf, seaweed, sea grasses, salt marshes and calcareous/chitinous substrates)

and live flora and fauna (e.g. mangrove plant species, palms, seaweeds, sea grasses, salt marshes and sponges) provide stable and persistent niches for marine fungi to perpetuate as saprophytes or endophytes or symbionts. According to Jones (2011b), the number of endophytes in marine habitats might reaches up to 6000 thus warrants further probing of plant and animal communities in marine habitats. Mangroves, deep-sea and polar region have already proved existence of a variety of endemic and cosmopolitan fungi possessing high bioprospecting potential. They could serve as source of new drugs, enzymes and biosurfactants which also facilitate in pollution monitoring or abatement (e.g. hydrocarbon degradation and heavy metal sequestering).

Although several endophytic fungi have been isolated from the plant species from marine habitats, many of them do not sporulate in cultures. Such situations demand molecular approaches for precise identification and to update the diversity status. Most of our knowledge on marine fungi comes from culture-dependent approaches. Culture-independent approaches need sophisticated methods like metagenomics. Besides providing an insight on the culture-independent fungi in marine habitats, metagenomics serves as resource as well as a potential tool in cloning environmental DNAs of culture-independent organisms to facilitate discovery of novel biocatalysts and metabolites (Almaabidi et al. 2015). In spite of vast marine resources available in the Indian Subcontinent, investigations on marine fungi are moderate. There is a vast difference in marine habitats of West Coast and East Coast of India, and there are no precise comparisons on marine fungi denoting wide scope for further investigation.

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Keratinophilic Fungi Distribution, Pathogenicity and Biotechnological Potentials

6

Shilpa A. Verekar and Sunil K. Deshmukh

Abstract

This chapter discusses the occurrence of keratinophilic fungi in different habitats wherein the population of these fungi is expected to occur: air, soil from various environments like gardens, caves, cold and saline habitat. The ability to degrade the keratinic material and mechanism of action is discussed. Importance of these fungi in petroleum hydrocarbon degradation is discussed. Various metabolites with antibacterial, antifungal, antitumor and related compounds produced by this group of fungi are documented. Examples of synthesis of silver nanoparticles are also included. The need for an extensive survey of keratinophilic fungi from unexplored substrates and habitats and biochemical potentialities is highlighted. It is concluded that fungi other than dermatophytes can be opportunistic pathogens. The need for a culture collection of this group of fungi is also highlighted.

Keywords

Keratinophilic fungi · Extreme climate · Secondary metabolites
Culture collection · Keratin degradation · Silver nanoparticles · Database

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Introduction

Keratin is a natural fibrous protein which forms the outermost keratinized layer of the skin and its appendages in man and animals. Keratin contains high sulphur-containing aminoacid, e.g., cysteine and methionine which makes it resistant to microbial degradation. Keratin is colonized and degraded by soil microorganisms, principally keratinophilic fungi. The presence of disulphide bridges makes keratin poorly biodegradable (Gopinath et al. 2015). These fungi have a number of common morphological and physiological characters and are members of the primitive Ascomycetes family, Gymnoascaceae. Within this family, there are number of species which are able to decompose keratin while they are still a part of man and animals. This article covers the different habitats from where these fungi are frequently recorded followed by the pathogenicity of these fungi, their ability to degrade keratin, petroleum products and to produce bioactive metabolites and synthesis of nanoparticles.

Distribution of Keratinophilic Fungi

Soil in General

The distribution of dermatophytes in soil was reported by Vanbreuseghem (1952). Later on, various researchers isolated these fungi from soils of different countries, i.e. Australia (McAleer 1980), Egypt (Abdel-Hafez and El-Sharouny 1987; Zaki et al. 2005), Nigeria (Ogbonna and Pugh 1987), West Bank of Jordan (Ali-Shtayeh 1989), Kuwait (Al-Musallam 1989), Spain (Calvo et al. 1984; Ulfig et al. 1997), New Guinea (Filipello Marchisio et al. 1991), Malaysia (Soon 1991), Antarctica (Mercantini et al. 1993), France (Agut et al. 1995), Italy (Caretta et al. 1990; Papini et al. 1998), Palestine (Ali-Shtayeh et al. 2002), Bahrain (Deshmukh et al. 2008), Iran (Kachuei et al. 2012), West Indies (Gugnani et al. 2012), Jamaica (Gugnani et al. 2014), Argentina (Sarmiento et al. 2015) and Tunisia (Anane 2012; Anane et al. 2015).

Dey and Kakoti (1955) reported *Microsporum gypseum* for the first time from the soils of Dibrugarh district of Assam, India. Later on these fungi were isolated from various Indian states which included Delhi (Randhawa and Sandhu 1965), Madhya Pradesh (Kushwaha and Agrawal 1976; Deshmukh and Agrawal 1983a), Bihar (Verma et al. 1982), Jharkhand (Kumar et al. 2012), Uttar Pradesh (Nigam 1987; Deshmukh and Verekar 2011a), Jammu and Kashmir (Kaul and Sumbali 2000a; Deshmukh 2002a), Rajasthan (Jain and Sharma 2011), Karnataka (Deshmukh et al. 2000; Vidyasagar et al. 2005), Orissa (Roy et al. 1972; Ghosh and Bhatt 2000), Goa (Deshmukh and Agrawal 1983b), Maharashtra (Padhye et al. 1966, 1967; Deshmukh 1999; Deshmukh and Verekar 2014a), Madras (Ramesh and Hilda 1998–99; Deshmukh and Verekar 2011b), Kerala (Deshmukh 2002b), the Andamans (Dixit and Kushwaha 1990), Himachal Pradesh (Deshmukh and Verekar 2006a),

Ladakh (Deshmukh et al. 2010; Kotwal and Sumbali 2014), Gujarat (Deshmukh and Verekar 2014b), Assam (Deshmukh et al. 2017) and Chhattisgarh (Deshmukh and Shukala 2000). Some of the dermatophytes and other keratinophilic fungi are responsible for superficial infections in human and animals and are therefore of possible significance in human welfare (Emmons et al. 1977; Monga and Mohapatra 1980; De Hoog and Guarro 1995).

Various distribution patterns of occurrence of these fungi are reported. These fungi are mainly reported from the locality frequented by man and animals. In most of these studies of keratinophiles, soil samples were taken randomly from the surface and at a depth not exceeding 3–10 cm from cattle farms, gardens, roadsides, forests, watercourse banks, grasslands, animal house floors and poultry farms. These studies established universal distribution pattern of keratinophilic fungi in soils with high frequency in the habitats frequently inhabited by humans and animals. As keratinophilic fungi have been reported from soils of various habitats of different geographical areas of the world, hence of epidemiological significance. Some of the habitats from where these fungi are prevalent, are described below.

Alkaline Soil

Usar soil is an alkaline soil and cover barren lands in India. Usar soil is commonly found in the area with poor/no drainage and less percolation. The soil is white alkaline type with pH of 7.5–11.0. Areas with alkaline soils show prominent whitish salt excess, with low moisture content, high temperature, drought and intense solar radiations for the most of the months of the year. These soils have scanty vegetation in the form of patches of poor grass growth. Deshmukh and Verekar (2011a) reported *Chrysosporium indicum, C. lucknowense, C. pannicola, C. tropicum, Chr.* state of *Ctenomyces serratus, Gymnascella dankailensis, Gymnoascus reessii, Microsporum gypseum, Trichophyton mentagrophytes* and *T. terrestre* from *Usar* soils of Lucknow, Bareilly, Azamgarh, Balia and Pratapgarh districts of Uttar Pradesh. Similarly, *Trichophyton verrucosum*, *T. mentagrophytes, Microsporum audouinii*, *M. canis* and *Chrysosporium tropicum* were isolated from the soils collected from the vicinity of Jaipur where pH ranges from 6.5 to 8.5 (Jain and Sharma 2011).

Public Parks

The local residents frequently visit public parks especially for morning/evening walks. Many of them bring their pets along with them. Part of the park is completely dedicated to children having swings, slides, small playground, etc. Due to lack of proper fencing these parks are some time invaded by animals such as buffalo's, cows, bullocks, horses and pigs. The rat's borrows are also seen in the

parks. The common birds are also seen in this park. Due to human and animal activity, the keratinous material is added to the park soil. The organic matters like keratin, faeces of animals and other organic matters residues provide suitable environment for the growth of these fungi with resultant health risk. There are reports of these fungi from public parks of Agra, Kanpur and Lucknow, and the list includes *C. carmichaelii, C. georgii, C. indicum, C. keratinophilum, C. queenslandicum, C. tropicum, C. xerophilum, C. zonatum, Ctenomyces serratus, Geomyces pannorum, Gymnoascus hyalinospora, M. gypseum, M. fulvum, M. vanbreuseghmii, M. nanum, Malbranchea pulchella, Myceliophthora vellera, Nannizzia gypsea, T. verrucosum* and *T. mentagrophytes* (Singh and Kushwaha 2010). While surveying keratinophilic fungi from the five public parks of Mumbai 11 species of 8 genera were recorded, viz. *Arthrographis kalrae*, *Auxarthron conjugatum*, *C. indicum*, *C. queenslandicum*, *C. zonatum*, *Gymnascella dankaliensis*, *G. hyalinospora, M. gypseum*, *Myriodontium keratinophilum*, *Trichophyton mentagrophytes* and *Uncinocarpus reesii*. Occurrence of keratinophilic fungi in public parks of Mumbai is of public health concern (Deshmukh and Verekar 2012).

More recently Pakshir et al. (2013) isolated keratinophilic fungi from soils of number of parks in Shiraz, and the list includes *Chrysosporium* sp., *Microsporum gypseum*, *M. fulvum*, *Malbranchea* sp. *Phialophora reptans, Bipolaris spicifera, Bionectria ochroleuca, Ochroconis constricta, Scedosporium dehoogii, Cephalosporium curtipes, Nectria mauritiicola, Scedosporium apiospermum,* along with the species of *Fusarium, Acremonium, Penicillium, Chaetomium Scopulariopsis* and *Tritirachium* sp. Majority of these fungi were recovered from the soils where pH ranges from 7 to 8. These reports highlighted that the soils of public parks are heavily contaminated with dermatophytes and other keratinophilic fungi.

Grazed Pastures

Arthroderma multifidum, A. uncinatum, Clonostachys rosea, Engyodontium album, Chrysosporium sp., *Chrysosporium keratinophilum, Malbranchea* sp., *Microsporum* sp., *M. vallerea*, *Paecilomyces carneus*, *P. lilacinus*, *Pochonia chlamydosporia*, *Trichophyton ajelloi*, *T. terrestre* and *Verticillium* sp. were recovered from the soils of long-term fold-grazed pastures in national parks of Slovakia and non-fold-grazed pasture in sierra Stolicke vrchy. *Trichophyton ajelloi* and *P. lilacinus* were prevalent in all the soil samples. In the fold-grazed pasture, keratinophilic fungi were more abundant in compared to non-fold-grazed pasture. Substantially lower presence of the other keratinophilic fungi in non-fold-grazed pasture may be because of low pH of these soils (Javoreková et al. 2012).

Potted Plants

Hedayati et al. (2004) isolated *Microsporum gypseum*, *M. cookei* and *Chrysosporium* sp. from the soils samples of potted plants from hospitals in Sari, Iran. The presence of these fungi in potted plants in hospitals could be a potential source of infection. Similarly, the soil samples of potted plants inside houses, hotels and offices from 15 localities of Kanpur, India, yielded *Acremonium* sp., *Acremonium implicatum, A. hennbertii, Aphanoascus terreus, A. keratinophilic, Arthroderma cunniculi, Botryotrichum piluliferum, Chrysosporium indicum, C. keratinophilum, C. queenslandicum, C. pannicola, C. sulfureum, C. merdarium, C. zonatum, C. tropicum, Ctenomyces serratus, Malbranchea pulchella, Microsporum gypseum, Trichophyton vanbreseghmii* and *Verticillium tenuipes*. Occurrence of these fungi in soils of indoor plants may be the source of dermatomycosis and other diseases in human (Singh et al. 2009a, b).

Waterlogged Conditions

Paddy fields are in waterlogged condition during different stages of cultivation and personnel involved in planting rice sapling spend 8–10 h per day in warm and wet conditions in the field that favours growth of these fungi. Sundaram (1987) reported *Microsporum gypseum, M. canis, Trichophyton terrestre, Trichophyton* sp., *T. ajelloi, Chrysosporiurn keratinophilum, Aspergillus fumigatus, Penicillium* sp. and *Mycelia sterilia* from the rice field near Madras. *Chrysosporium* anamorph of *Arthroderma cuniculi*, *C.* anamorph of *Arthroderma curreyi*, *C. carmichaeli*, *C. georgii*, *C. gourii*, *C. indicum*, *C. keratinophilum*, *C. lobatum*, *C. merdarium*, *C. pannicola*, *C. queenslandicum*, *C. tropicum*, *C.* anamorph of *Pechtinotricum illiase*, *C.* anamorph of *Rollandina vriesii* were isolated from paddy fields during different stages of cultivation, eg., transplanting, tillering, milking and maturation (Shrivastava et al. 2008). *Chrysosporium keratinophilum* followed by *C. tropicum* was the most dominating geophilic species. These fungi were most prevalent during the milking stage (100%), followed by the maturation stage (89.47%) of paddy cultivation.

Sediments

Abdel-Hafez and el-Sharouny (1990) isolated *Chrysosporium* state of *Arthroderma tuberculatum*, *C. asperatum*, *C. georgii*, *C. indicum*, C. *keratinophilum*, *C. pseudomerdarium*, *C. queenslandicum*, *Chrysosporium* state of *Thielavia sepedonium, C. tropicum, Microsporum cookei, M. gypseum, Myceliophthora* anamorph of *Corynascus novoguineensis, M. vellera* and *Trichophyton terrestre* from sewage sludge samples from Upper Egypt. Similarly, from the sediment samples collected from the Shatt Al-Arab River, and its creeks yielded *Aphanoascus fulvescens, A. durus, Chrysosporium crassitunicatum, C. keratinophilum, C. tropicum, M. fulvum,*

M. gypseum and *T. verrucosum* (Abdullah and Hassan 1995). In other study, sewage sludge samples collected from seven wastewater treatment plants in Sari city, Mazandaran Province, Islamic Republic of Iran yielded *Microsporum gypseum*, *Chrysosporium* spp. and *Geotrichum* spp. using the hair-baiting technique (Hedayati and Mirzakhani 2009). Ulfig et al. (2006) observed that there was a significant increase in the population of actidione-resistant keratinophilic fungi in the sludge during open-air drying and change in the numbers. Occurrence of these fungi in sewage slug/sediments can be a source of infection to man and animals (Kushwaha 2014).

Cold Desert

Ladakh "land of high passes" in Karakoram mountain ranges in the Indian state of Jammu and Kashmir is the highest plateau with height of 3000 ft and is situated between latitudes 30°N–36°N and longitudes 76°E–79°E. The temperature in this area ranges from 3–35 °C in summer to −20 to −45 °C in winter. *Amauroascus kuehnii, Aphanoascus keratinophilus, A. terreus, Auxarthron alboluteum, A. conjugatum, Chrysosporium articulatum, C. minutisporosum, C. mephiticum, C. siglerae, Chrysosporium* sp. *C. tropicum, C. submersum, C.* state *of Ctenomyces serratus, Geomyces pannorum* were isolated from the soils of cold desert of Ladakh. The samples were collected from places like Pangong Tso, Chang La, Durbuk, Khardung La, Tangste, Lukung, Magnetic Hill, Phey village, Leh and Nimmu and were from pastures, glacier, roadside, bank of lake, uncultivated soil, cultivated soil, river bank and roadside (Deshmukh et al. 2010). *Chrysosporium inops, C. merdarium*, *C. queenslandicum*, *Chrysosporium* anamorph of *Gymnoascus demonbreunii* along with other fungi were isolated from the soils collected from Khardung (14,738 ft) (Kotwal and Sumbali 2011, 2014).

Antarctic Environment

Antarctica is the coldest, windiest and the driest continent of the Earth, located at the South Pole. In Antarctic environment, the temperature in winter is as low as −63 °C and winter mean temperatures are −57 °C and lower. The temperature in summer is unlikely to be warmer than −18 °C. Low temperatures, high aridity, low and sporadic availability of nutrients are the major stress factors. In such extreme environment, fungi and bacteria can grow because of their oligotrophic nature (Parkinson et al. 1989). *Geomyces pannorum, Malbranchea gypsea, Malbranchea* sp, *Microsporum gypseum, M. megellanicum, Trichophyton mentagrophytes* and *T. terrestre* along with other fungi have been reported from Antarctica (Caretta and Piontelli 1977; Mercantini et al. 1989b; Mario Comerio and Mac Cormack 2004).

Keratinophilic fungi were reported from the dust samples collected in various rooms on the Italian scientist-base and from soil in close proximity to the base of Antarctica. The keratinophilic fungi isolated were *Malbranchea gypsea,*

T. mentagrophytes, *Aphanoascus fulvescens*, *C. carmichaelii*, *Geomyces pannorum* var *pannorum and G. pannorum* var *vinaceus* (Mercantini et al. 1993). Keratinophilic fungi found in polar region might have been tuned to the environmental conditions and adapted to lifecycle, genetic and physiological modifications.

Wildlife Sanctuary

In the wildlife sanctuary, the keratinous material, viz. hair and feathers, are added to the environment and keratinophiles survive on these substrates. Deshmukh (2002a) recorded *Arthroderma simii, Chrysosporium indicum, C. keratinophilum C. pannicola, Gymnoascella hylinospora, Malbranchea aurantiaca, Microsporum gypseum* complex and *Trichophyton terrestre* in the soils collected from "The Periyar Wildlife Sanctuary" (Kerala, India). The sanctuary is known for rich fauna including elephants, tigers, bisons and spotted deer. The occurrence of these fungi in "The Periyar Wildlife Sanctuary" can be due to the presence of animals and birds with less human interference (Deshmukh 2002b). Deshmukh and Verekar (2006a) isolated *Chrysosporium queenslandicum, C. xerophilum* and *Malbranchea gypsea* from the soils of Pin Valley National Park (Himachal Pradesh, India) famous for herds of Asiatic ibex (*Capra ibex*), Snow Leopard (*Uncia uncia*) and Himalayan blue sheep (*Pseudois nayaur*). Their occurrence is an indication of their adaptation to cold climate. While screening soils of Sanjay Gandhi National Park Mumbai, Deshmukh and Verekar (2014a) reported the presence *Aphanoascus durus*, *Arthroderma corniculatum*, *Auxarthron umbrinum, Chrysosporium evolceanui, C. indicum, C. tropicum, C. zonatum, Chrysosporium* state of *Arthroderma tuberculatum, Chrysosporium* state of *Ctenomyces serratus, Gymnascella dankaliensis, Microsporum gypseum, Myriodontium keratinophilum* and *Trichophyton mentagrophytes.* Deshmukh and Verekar (2014b) also reported *Aphanoascus durus, A. fulvuscence, Arthrographis kale, Auxarthron conjugatum, Chrysosporium indicum*, *C. tropicum*, *C. zonatum, Chrysosporium* state of *Ctenomyces serratus*, *Microsporum gypseum* and *Trichophyton mentagrophytes* from the soils of Gir Forest National Park and Wildlife Sanctuary, Gujarat (India), known for Asiatic Lions (*Panthera leo persica*). Similarly, a new fungus *Auxarthronopsis bandhavgarhensis* was isolated from Bandhavgarh National Park situated in the central India state Madhya Pradesh (Sharma et al. 2013). The occurrence of these fungi in these national parks indicates that these fungi are associated with the birds and animals of the national parks.

Poultry Farm

Poultry farm serves as a reservoir of keratinophilic and toxigenic fungi. There are reports on the occurrence of these fungi in poultry environment which include birds, cages, transporting trucks and dumping grounds. Various researchers have reported elevated occurrence of keratinophilic fungi in poultry farm soils

(Jain et al. 1985; Deshmukh 1999; Kaul and Sumbali 2000a; Anbu et al. 2004), which are neutral to weakly alkaline in nature and rich in organic matter. A high content of organic carbon, nitrogen, phosphorus, potassium, magnesium, calcium and iron is present in this habitat (Kaul and Sumbali 1998–99). Deshmukh (1999) recovered *C. indicum, C. lobatum* and *Microsporum gypseum* from poultry farm soil while surveying keratinophilic fungi from Mumbai and its vicinity. *Chrysosporium indicum, C. lucknowense, C. pannicola, C. queenslandicum, Chrysosporium* I, *Chrysosporium* II, *C. tropicum, C. zonatum, C.* state of *Arthroderma tuberculatum, Geomyces pannorum, Malbranchea pulchella, Microsporum gallinae, Trichophyton mentagrophytes* and *T. simii* were isolated from poultry farm soils from Bilaspur district of Chhattisgarh (India) (Deshmukh and Shukala 2000). Kaul and Sumbali (2000a) recorded very high per cent of keratinophilic fungi which were attributed to higher influx of poultry droppings and other remains (feathers and claws) in this soil. Keratinophilic fungi were also recorded from poultry farm from Namakkal and feather dumping sites from Chennai. The isolated fungi were *M. gypseum*, *Trichophyton mentagrophytes*, *Chrysosporium keratinophilum*, *Chrysosporium* state of *Arthroderma tuberculatum*, *Geomyces pannorum* (Anbu et al. 2004). Kaul and Sumbali (2000a, b) isolated *C. keratinophilum, C. queenslandicum, C. tropicum, C. indicum* and *Malbranchea chrysosporoidea* from feathers of poultry birds (*Gallus domesticus*). They also found *C. keratinophilum, C. queenslandicum, C. tropicum, C. pannorum* and *Malbranchea flava* from soils of poultry farm. The study indicates that colonization of these fungi on bird feathers is because they spent more time on ground.

Piggery Soils

Fifteen different keratinophilic fungi were reported from the soil samples collected from the piggeries of Ranchi, Jharkhand (India) (Kumar et al. 2012). The reported fungi were *Aspergillus niger*, *A. terreus A. flavus*, *Penicillium chrysogenum*, *Penicillium* sp., *Alternaria alternata*, *Fusarium oxysporum*, *F. solani*, *Trichoderma harzianum*, *T. reesei, T. viride, Curvularia lunata, Chrysosporium* sp. *Mucor pusillus* and *Rhizopus stolonifer*. The majority of them were secondary colonizer on keratin baits. More attention is needed to isolate the true species of keratinophiles from this environment.

House Dust

Chrysosporium carmichaelii, C. evolceanui, C. indicum, C. keratinophilum, C. merdarium, C. pannicola, C. queenslandicum and *C. tropicum* and other fungi were isolated from house dust of Kanpur (Nigam and Kushwaha 1990, 1993). From the dust samples collected from hospitals and houses in Kanpur, *Acremonium*

implicatum, *A. strictum*, *Aphanoascus fulvescens*, *Arthroderma simii*, *C. queenslandicum*, *C. indicum*, *C. pannicola*, *C. tropicum*, *Ctenomyces serratus*, *Gymnoascus reessii*, *Malbranchea fulva*, *Malbranchea pulchella*, *Microsporum gypseum*, *Microsporum cookie*, *M. fulvum*, *Paecilomyces lilacinum*, *Penicillium expansum*, *Trichophyton mentagrophytes* and *T. terrestre* were recorded (Singh et al. 2009a, b). In hospitals, *C. serratus* was less prevalent while the *A. simii* was the most. In houses, *C. queenslandicum* was less prevalent and *C. tropicum* the most (Singh et al. 2009a, b). While surveying keratinophilic fungi from Jaipur, Jain and Sharma (2011) reported *Trichophyton terrestre* and *C. tropicum* from house dust. Similarly, Vidyasagar et al. (2005) recovered *Microsporum gypseum*, *Chrysosporium keratinophilum*, *Trichophyton mentagrophytes*, *M. nanum* and *C. tropicum* from corridor dust of hospitals and soils of public places from Gulbarga in Karnataka (Vidyasagar et al. 2005). These data show the capability of the long-term existence of pathogenic fungi in the soil of indoor environments and is the main link in the human circulation of these pathogens.

Chrysosporium asperatum, *C.* state of *Arthroderma tuberculatum*, *C. indicum*, *C. inops*, *C. keratinophilum*, *C. merdarium*, *C. pannorum*, *C. queenslandicum*, *C. tropicum* and *C. xerophilum* along with *Trichophyton verrucosum* and *Trichophyton* sp. were isolated from air dust particles from Egypt (Abdel-Hafez et al. 1990a). Moubasher et al. (1990) reported *Chrysosporium asperatum, C. indicum, C. keratinophilum, C. merdarium, C. pannorum* and *C. tropicum* from the atmosphere of hay or winnow sites and from combine harvester wheat and sorghum air dust.

Epidermophyton floccosum, *M. canis*, *M. gypseum* and *T. mentagrophytes* along with species of *Chrysosporium* were isolated from the dust of ferry boats of Italy. *C. pannorum* was the most prevalent species amongst *Chrysosporium*. In the same study, *E. floccosum*, *T. mentagrophytes*, *T. tonsurans*, *T. ajelloi*, *Trichophyton* spp., *M. canis* and *M. gypseum* and seven different species of *Chrysosporium* were isolated from the dust of railway cars; *C. pannorum* was the most numerous one amongst the *Chrysosporium* sp. (Mercantini et al. 1989a).

Meteoritic Crater

Deshmukh and Verekar (2006b) reported *Aphanoascus durus, A. punsolae, Auxarthron kuehnii, C. indicum*, *C. tropicum*, *Chrysosporium* sp., *Chrysosporium* state of *Ctenomyces serratus* from Lonar meteorite crater in Maharashtra state (India). Lonar lake water is ten times saltier than drinking water (pH 10.5) and contains salts and minerals of sodium, chloride, carbonates, fluorides and bicarbonates. The area in and around is lush green with dense forest with numerous species of birds and animals. The occurrence of these fungi in such environment shows the addition of keratin material to the crater by birds and animals.

Veterinary Clinic

Microsporum canis, M. gypseum, T. ajelloi, T. mentagrophytes, T. terrestre, C. keratinophilum, C. pannorum, C. tropicum, Chrysosporium sp., *C.* state of *Arthroderma tuberculatum* were isolated from the veterinary clinic (Mancianti and Papini 1996). Dermatophytes survives for longer period in dorminant form in the clinic and clinical materials is the matter of concern. The clinical materials can come in the direct contact and be the means of transmission of dermatophytes. The presence of mites on animals can be another source of transmission of these fungi in veterinary clinics. Therefore, such clinics may represents sites where pets and human are exposed to the risk of infection of such pathogenic fungi.

Coastal Habitats

The coastal areas are an environment for migratory birds and burrowing animals. Occurrence of these fungi in such coastal soils supported the view that this habitat is a rich source of keratinophilic fungi. Deshmukh (2002b) reported *Chrysosporium indicum*, *C. keratinophilum*, *C. tropicum*, *Chrysosporium* state of *Ctenomyces serratus*, *Microsporum gypseum* complex, *Trichophyton mentragrophytes* and *T. terrestre* from Kovalam beach, Kerala (India). Occurrence of keratinophilic fungi in saline areas may be attributed to the preference of keratin material in such habitats. Many investigators studied the effect of salinity on the growth and survival of fungi and demonstrated that keratinophilic fungi are common in saline environments (Kishimoto and Baker 1969; Deshmukh and Agrawal 1983b; Larrondo and Calvo 1989; Ulfig et al. 1997; Deshmukh 1999). Katiyar and Kushwaha (1997) isolated *C. keratinophilum, C. tropicum and Chrysosporium* sp. from sand of the Mediterranean Sea, which is poor in organic matter. Pugh and Mathison (1962) found maximum distribution of *Chrysosporium* in coastal soils due to enrichment by the droppings and fish debris, which favours more the growth of aleurosporic fungi. Relatively high density of these fungi in coastal soils might be due to the human activity and presence of marine birds and burrowing animals.

Salt Pan Soils

Salt pans are very common in the coastal habitats and are part of the salt industry responsible for production of tons of salt around Mumbai and supplied to all over India. Seawater flows into the salt pans and brings keratinic material such as human hair, animal hair and wool fragments from blankets from neighbouring villages during high tide. Moulted feathers from various migrating birds also constitute part of the keratinic material in salt pans. The formation of salt takes 3–4 months.

During that period decomposition of keratin by keratinophilic fungi and related dermatophytes takes place. Deshmukh (2004a) reported *Chrysosporium indicum, Microsporum gypseum* complex, *C. tropicum*, *Chrysosporium* state of *Ctenomyces serratus, C. fluviale, Malbranchea aurantiaca, Trichophyton mentagrophytes, T. terrestre, Malbranchea* sp. and *Uncinocarpus reesii* from the soils around salt pans in Mumbai. This investigation indicates that soil of salt pans and surrounding areas provides an unusual ecological environment for keratinophilic fungi and related dermatophytes.

Animals

Keratinophilic fungi are reported from hairs of various animals which include cows, donkeys, rabbits, cats, dogs, goats and wild boars. The most frequently isolated species are *Trichophyton mentagrophytes, T. verrucosum, T. ajelloi, T. terrestre, Microsporum gypseum, M. nanum, M. canis, Arthroderma cuniculi*, *A. curreyi* and a number of species of *Chrysosporium* (Ali-Shtayeh et al. 1988, 1989; Abdel-Hafez et al. 1990b; Al-Musallam 1990; Mancianti et al. 1997; Guzman-Chavez et al. 2000). Mite *Tyrophagus putrescentiae* was found associated with *M. canis* and may be responsible for transmitting fungi amongst animals (Caretta et al. 1989). The occurrence of these fungi on these animals can be a risk of dermatophytic infections to man and animals.

Birds and Its Environment

The birds act as reservoirs of zoophilic keratinophiles and transport them from one place to another. It is thought that some fungal association with birds are species specific. These fungi occur in nests and can be transmitted from parents to off springs (Pugh 1972). There are several reports of occurrence of these fungi from birds and its environment (Sur and Ghosh 1980; Dixit and Kushwaha 1991; Sarangi and Ghosh 1991; Deshmukh 2004b). The most frequently isolated species include *Chrysosporium evolceanui*, *C. indicum*, *C. tropicum, Microsporum gypseum*, *M. fulvum*, *Microsporum* sp, *Trichophyton mentagrophytes, Malbranchea pulchella, Aphanoascus reticulisporus*, *Gymnoascoideus petalosporus*, *Malbranchea fulva*, *Pseudoarachniotus flavoluteus*, *P. roseus*, *R. hyalinospora*, *Rollandina capitata*, *Myriodontium keratinophilum.* While surveying keratinophilic fungi from different migratory bird *Chrysosporium keratinophilum, C. tropicum, C. merdarium, C. luteum*, *C. parvum*, *C. pruinosum*, *C. asperatum, Scopulariopsis* sp., *Trichophyton terrestre, Arthroderma tuberculatum, Sepedonium* spp. were isolated from 10 birds (Budgerigar, Ring neck, Lovebird, Pigeon, Alexandrian parrot, Amazon parrot, African grey, Quail, Duck and Chicken) from different countries coming to Bahrain (Mandeel et al. 2011). Similarly, *Auxarthron conjugatum*, *Chrysosporium fluviale*, *C. indicum*, *C. tropicum*, *Chrysosporium* state of *Ctenomyces serratus*, *Gymnoascus petalosporus* and *Microsporum gypseum* complex

were recorded from various sites in the vicinity of Vedanthangal Water Bird Sanctuary. From this study, we can conclude that migratory birds can be a mode of transport of variety of keratinophiles from one place to another (Deshmukh and Verekar 2011b).

Pathogenic Fungi

Species of *Chrysosporium* are reports from immuno-compromised patients causing various types of mycoses (Anstead et al. 2012). From a female in Chiba and from a male in Kyushu, *Chrysosporium zonatum* was isolated from bronchial lavage (Sigler et al. 1998). Roilides et al. (1999) reported *Chrysosporium zonatum* as the causative agent of chronic granulomatous disease. *Myriodontium keratinophilum* teleomorph of *Neoarachnotheca keratinophila* was found to cause sinusitis (Maran et al. 1985). There was a report of isolation of *Gymnascella dankaliensis* from superficial infections in human (De Hoog and Guarro 1995). Other species of *Gymnascella*, i.e. G. *hyalinospora* was reported from invasive pulmonary infection in a patient with acute myelogenous leukaemia (Iwen et al. 2000). Lysková (2007) reported *Chrysosporium queenslandicum*, *C. sulfureum*, *C. tropicum*, *Malbranchea pulchella*, *Myriodontium keratinophilum* from infections of the skin and nails of patients in the Moravian-Silesian Region of Czech Republic. *Geomyces pannorum* var. *pannorum* was isolated from superficial infection of the skin of a healthy man (Gianni et al. 2003). Zelenková (2006) reported *Chrysosporium pannorum* (Link) Hughes from finger nails of male and female patients from Slovak Republic. Saidi et al. (1994) reported *Chrysosporium tropicum* from the comb lesion in two breeds of chicken in India.

There are reports of infection of *Chrysosporium* species in multiple reptile species. *Chrysosporium guarroi* was isolated from pet green iguanas (*Iguana iguana*) from different areas of Spain (Abarca et al. 2010) and Turkey (Kahraman et al. 2015). *Chrysosporium queenslandicum* was isolated from a garter snake (Vissiennon et al. 1999). In the review by Paré and Jacobson (2007), it is reported that *Chrysosporium keratinophilum*, C. *tropicum* and *Chrysosporium* spp. can be a causal agents of cutaneous and systemic mycoses in reptile species. *Chrysosporium* anamorph of *Nannizziopsis vriesii* (CANV) was found to be a causal agent of dermatitis in multiple reptile species such as chameleons (Paré et al. 1997), similarly, it was identified from snakes (Bertelsen et al. 2005; Nichols et al. 1999), salt-water crocodiles (Thomas et al. 2002), bearded dragons (Bowman et al. 2007; Hedley et al. 2010), green iguanas (*Iguana iguana*) (Han et al. 2010) and girdled lizards (Hellebuyck et al. 2010). A *Chrysosporium* spp. related to *Nannizziopsis vriesii* was responsible for causing skin lesions in a pet bearded dragon (Abarca et al. 2009). Mycotic granuloma of a black rat snake was caused by *Chrysosporium ophiodiicola* (Rajeev et al. 2009). These reports indicate that the species of *Chrysosporium* may be regarded as opportunistic pathogens.

Biodegradation of Keratin

The invasion of hair by dermatophytes has been investigated with light and electron microscopy under natural and experimental conditions. English (1963) described the stages by which detached hair is attacked by keratinophilic fungi in following sequence (a) Cuticle lifting, (b) Cortical erosion, (c) Production of penetrating organism and (d) Colonization of medulla. Kanbe and Tanaka (1982) also recognized these stages, but they overlap one another during the process of infection with *M. gypseum*. Deshmukh and Agrawal (1982, 1985) studied the in vitro degradation of human hair by various soils inhabiting keratinophilic fungi. They observed that the in vitro breakdown of hair is characterized by the release of protein, peptides, amino acid and resulted in the marked alkalinization of the medium. Microscopic examination showed extensive hair damage and extensive rupturing of the hair cuticle. It was hypothesized that the reducing agent responsible for this process is sulphate, which is produced by oxidation of cysteine during the process of keratinolysis in dermatophytes (Kunert 1972). The enzyme responsible for production of sulphite from cystine/cysteine is cysteine dioxygenase (CDO), which reduces and degrade keratin. CDO is responsible for keratinolysis which is an important part in dermatophyte pathogenesis. CDO along with the sulphite transporter SSU1 is probably one of the most important dermatophyte virulence factors (Grumbt et al. 2013; Kasperova et al. 2013). Huang et al. (2015) reported the production and characterization of keratinolytic protease produced by *Onygena corvine* and suggested that *O. corvine* can be used for bioconverting feather waste into high-value products.

Bioactive Metabolites from Keratinophilic Fungi

A number of antimicrobial substances have been reported from *M. gypseum* and *Trichophyton violaceum*. Fusidic acid, penicillins, quinone antibiotics, gypsetin, 6 APA are some of the major antimicrobial compounds produced by *M. gypseum* (Uri et al. 1963; Elander et al. 1969; Shinohara et al. 1994). *T. violaceum* and *T. rubrum* are known to produce quinone antibiotics (Subrahmanyam 1980). *T. violaceum* produces vioxanthin and xanthomagnin possessing antimicrobial properties while *T. mentagrophytes* was found to produce antibiotics of penicillin group. *Epidermophyton floccosum*, an anthropophilic dermatophyte is also known to synthesize floccosin, an antifungal and floccosic acid, an antibacterial compound (Blank et al. 1969). Some non-dermatophytic keratinophilic fungi are also known to produce certain antibiotics. Production of anthraquinones (questin and questinol) and asteric acid by *Chrysosporium merdarium* is reported by Haskins (1971). *C. indicum* produces L-Arginyl-D-allothreonil-L-phenylalanine, an antifungal compound (Koenig et al. 1973). *C. pannorum* produces cryscandin, antibacterial and an anticandidal compound and pannorin, 3-hydroxy-e-methylglutaryl coenzyme, a reductase inhibitor (Yamashita et al. 1984; Ogawa et al. 1991). *Amauroascus niger* produces zaragozic acids, D and D2, which inhibits farnesyl transferase with IC_{50} values of

100 nM, (Dufresne et al. 1993). *Malbranchea aurantiaca* produces 1-hydroxy-2-oxoeremophil-1(10), 7(11),8(9)-trien-12(8)-olide and penicillic acid which causes significant inhibition of radicle growth of *Amaranthus hypochondriacus* with IC_{50} values 6.57 and 3.86 μM, respectively. In addition, 1-hydroxy-2-oxoeremophil-1(10), 7(11),8(9)-trien-12(8)-olide inhibited activation of the calmodulin-dependent enzyme cAMP phosphodiesterase (IC_{50} =10.2 μM) (Martinez-Luis et al. 2005).

Compounds produced by other non-dermatophytic keratinophilic fungi such as *Arachinotus aureus*, *Arachinotus flavoluteous*, *Pseudoarachniotus roseus*, *Malbranchea* species and *Auxarthron umbrinum* with antimicrobial and antitumor properties include: aranorosin, aranorosinol A, aranorosinol B, aranochlor A and aranochlor B (Roy et al. 1988, 1992; Mukhopadhyay et al. 1998), malbranchin (Chiung et al. 1993) and rumbrin (Yamagishi et al. 1993). Rumbrin a cytoprotective substance was isolated from *Auxarthron umbrinum* (Yamagishi et al. 1993; Alvi and Rabenstein 2004). *Malbranchea cinnamomea* is known to produce malbranchin, a quinone antibiotic with both antimicrobial and cytotoxic activity (Chiung et al. 1993). Premabai and Narsimharao (1966) isolated malbranchin A and B from *Malbranchea* sp which possesses antimicrobial properties.

The production of antiviral substances, i.e. acetyl aranotin, aporaratrotin and aranotin A by *Arachniotus aureus* and antifungal compounds such as aranorosin, aranorosinol A and aranorosinol B by *Pseudoarachniotus* and L-Arginyl-D-allothreonil-L-phenylalanine by *Chrysosporium indicum* are of great significance in pharmaceutical industries as a lead molecule for development of drugs. The overall metabolic potential of keratinophilic fungi is immense because of their specific nature. Keratinophilic fungi if explored extensively for metabolite production can be a novel source of many promising molecules of medical, biotechnological and agrochemical utility.

Keratinophilic Fungi for Green Synthesis of Nanoparticles

The keratinophilic fungi and related dermatophytes are also known to synthesize silver nanoparticles. It is reported that silver nanoparticles (SNPs) display effective antimicrobial properties (Panacek et al. 2006). The biosynthesis of SNP using three dermatophytic fungi, i.e. *Trichophyton rubrum*, *T. mentagrophytes* and *Microsporum canis* was reported by Moazeni et al. (2012). It was also found that species within the genus of the fungi have the capability to synthesize SNP with different efficiency and physical property (size and shape). Similarly, uniform, spherical SNP in the range of 20–50 nm were synthesized by *C. tropicum* (Soni and Prakash 2011).

Role of Keratinophilic Fungi in Petroleum Hydrocarbon Degradation

Keratinolytic fungi are found in the biopile at the refinery, with *Trichophyton ajelloi* as the most dominant species which may be due to the presence of human population in the vicinity and their activity (Ulfig et al. 2003). The keratinolytic and keratinophilic fungi like *Microsporum* sp., *Trichophyton* sp. and *Chrysosporium* sp. are reported in petroleum hydrocarbon degradation (Davies and Westlake 1979; Ulfig 2000). Biopile soil does not favour the growth of dermatophyte and *Trichophyton ajelloi*, which is a predominating species rarely cause infection (de Hoog and Guarro 1995); therefore, the biopile bioremediation process for petroleum hydrocarbon degradation can be regarded as safe from the fungal epidemiological point of view.

Outlook

Occurrence of these fungi has been studied in many environments including various types of soil, birds, bird nests and animal farms. Besides, we do not yet know how many novel strains of keratinophilic fungi are hidden in other habitats with varied physical and chemical environmental conditions and how many of them are pathogenic to both man and animals. The ecological factors responsible for the distribution of fungi and various conditions for the formation of anamorph and teleomorph need more attention. Hence, a systemic distribution pattern of dermatophytes and related keratinophilic fungi from unexplored areas such as heated and self-heated materials, environments with low and extreme pH values, high salt and solute concentrations needs attention.

It more than 65 years, scientists are working in this field and isolated keratinophilic fungi from soil, birds and their environment, healthy animal skin and disease specimens but are not available as in India as reference. The availability of these fungi as specimens will definitely be of immense value to younger scientists with new ideas and the ability to get much better results using biotechnological methods. It is of high importance to have culture collections that carry the standard strains and biodiverse isolates. Taxonomic identification by both classical criteria and molecular biological tools is a must in such culture collection centres.

It is evident that keratinophilic fungi have been prolific producers of numbers of important pharmaceutical compounds and novel skeletons. This makes it very important to have a systematic classification of the different active molecules identified from various species related to their ecological prevalence. This group of fungi is known to degrade keratinic material into peptides and amino acids by the action of enzyme keratinase. Keratinase is used industrially to remove hair from a hide in the preparation of quality leather. Certain genetically modified strains are

required which can decompose tons of waste keratin that generates into peptides and amino acids. The hydrolytic products can be used as poultry and animal feed whereas the enzyme keratinase can be used in leather industry.

The ability of dermatophytes and related keratinophilic fungi can be explored to produce metallic nanoparticles which can have various pharmaceutical and agriculture applications. Keratinophilic fungi have the ability to remove petroleum hydrocarbons and can be used as cost-effective way to naturally remediate petroleum-contaminated soils.

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Fungal World of Cave Ecosystem

7

Seema Rawat, Rachna Rautela and B. N. Johri

Abstract

Caves represent a unique ecosystem whose physical and chemical conditions are quite stable. They are considered as extreme environment having severe abiotic conditions, viz., oligotrophic conditions, low temperature, low light intensity, and high humidity yet they harbor a very rich diversity of microbial population. The secondary metabolites secreted by microbial flora of caves may cause chemical reactions with the substrate, contamination of the substrate by pigments and lithogenic processes, litholytic processes, as well as cavern enlargement or speleotherm deposition. Fungi are the important component of cave microbiota playing role as decomposers and parasites. *Geomyces destructans* and *Histoplasma capsulatum* are the most widely studied fungi of cave ecosystem. The microbial count and microclimatic conditions of cave are subjected to change with the frequent visitation of tourists in the caves resulting into destabilization of biological equilibrium of the caves and also induction of biochemical changes on the rock surface.

Keywords

Cave · Speleotherm · *Geomyces destructans* · *Histoplasma capsulatum*
Karst terrains · Stalactites

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Introduction

The subsurface of the Earth is one of the major habitats harboring a significant proportion of microbial life (Roussel et al. 2008). However, the existing knowledge about the life-forms and biogeochemical processes contained within it is very little primarily due to difficulties in approaching this habitat. The investigation of karst terrains, which expand over ~20% of the Earth's subsurface, is a relatively easy way for approaching this habitat (Ford and Williams 2007). The most prominent features of karst terrain are caves which may serve as noteworthy entries and virtual "windows" into subsurface habitats (Engel et al. 2008). The classification of caves is based on several criteria including the type of rock/bedrock, the way the cave is formed, and the proximity to the groundwater table (Lee et al. 2012). They constitute oligotrophic ecosystems containing less than 2 mg of total organic carbon (TOC) per liter. The average number of microorganisms growing in these ecosystems is quite high, i.e., 10^6 cells/g of rock, despite the oligotrophic conditions (Barton and Jurado 2007). Caves offer examples of possible past or present geomicrobiological interactions as they harbor diverse microbial populations and are active mineral precipitation sites (Baskar et al. 2006). The characterization of microbial diversity and resilience of different life-forms in caves would aid in recognizing biosignatures for subsurface life on other planetary bodies (Engel et al. 2003). Moreover, it is also of interest for microbial ecologists to understand the cave microbial dynamics and reevaluate the ecological model of competitive exclusion which does not hold true for cave microbiota as they work in a cooperative network to utilize all the available energy sources (Barton 2006). Fungi constitute the most important members of cave microbiota as they play a significant role in the feeding strategies of cave fauna (Nováková 2009). The various methods used for culturing fungi from caves are: culturing methods, trapping spores using sampling machines, baiting method for dermatophytes and keratinophilic fungi, and genetic methods (Wang et al. 2010; Docampo et al. 2011; Porca et al. 2011; Lindner et al. 2011). The bias and limitations of cultivation approaches may give only limited insights into the microbiota present in caves, and therefore, it necessitates the use of cultivation-independent approaches which will not only give a true picture of diversity but also help in determining the time-dependent changes in the microbial community inside the caves. This chapter emphasizes on the diversity and ecology of fungi in cave ecosystem with some limited insights into bacterial world.

Caves as Ecosystem

Caves are usually considered as extreme environment because total darkness or low light intensity, low temperature, high humidity, and lack of photosynthesis in most areas of the cave make them nutrient-poor biotopes (Barton and Northup 2007). Photosynthetic activity usually occurs at the entrance to a cave where light has access and sometimes may also occur inside the cave if artificial lights are present,

mounted for the public. The chemolithoautotrophic microorganisms produce the primary organic matter. They derive energy from binding to hydrogen, nitrogen, or volatile organic compounds as well as from oxidation of reduced metal ions (e.g., manganese and iron) (Tomczyk-Żak and Zielenkiewicz 2016).

The biological activity can be observed usually in all subterranean voids yet productivity and biomass are low and organisms are usually small (Hobbs and Culver 2009). The human visitation inside the caves may result in changes in its microclimatic conditions like increase in air temperatures and CO_2 concentration (Hoyos et al. 1998) and may serve as a source of organic matter and new microflora.

The cave has four zones based on the availability of light: (i) Entrance zone, (ii) Twilight zone, (iii) Middle zone in which relative darkness prevails with fluctuating temperature, and (iv) Dark zone in which total darkness and constant temperature prevail (Koilraj and Marimuthu 1998). There are four types of caves based on rock type and method of formation, namely lava caves, sea caves, ice caves, and limestone caves (Palmer 1991). The caves formed in limestone and other calcareous rocks, and as lava tubes in basaltic rock, are the most common types of caves (Northup and Lavoie 2001).

There are many caves all over the world. Mammoth cave is largest known cave in the world situated in Kentucky, USA. Magura cave in Bulgaria, Lower Kane cave in USA, Altamira cave in Spain, Frasassi caves in Italy, Cesspool cave in Virginia, Movile cave in Romania, and Lechuguilla cave in New Mexico are the popular caves all over the world. In India, more than 1545 cave sites (Deshmukh 1994) have been discovered but most of them remain unexplored due to infancy stage of geomicrobiology in India. The popular caves in India are Borra caves in Visakhapatnam, Ajanta, Elephanta, and Ellora caves in Maharashtra, Mahabalipuram cave in Tamil Nadu, Khasi and Jaintia Hill cave systems in Meghalaya, and Amaranth cave in Jammu and Kashmir.

Microbial Diversity of Caves

A very high level of biodiversity within the bacterial and fungal domain as well as the representatives from the Archaeal domain has been reported from caves (Tables 1 and 2). Such high biodiversity in the caves is a paradox according to the ecological principles of competitive exclusion which states that the two species cannot coexist if they utilize the same nutrient source, which is deficient (Tomczyk-Żak and Zielenkiewicz 2016). It is unlikely that a single microorganism can perform all high-energy reactions necessary for growth due to the availability of low amounts of nutrients which are a chemically complex source of carbon and energy (Barton and Jurado 2007). The resident microbial populations of caves overcome these limitations by forming collective structures (different types of biofilms) in which they cooperate and enter into mutualistic relationships rather than competing for nutrients (Tomczyk-Żak and Zielenkiewicz 2016).

Table 7.1 Reported microflora from caves in India

S. no.	Name of cave	Sample	Identified species	References
1	Sahastradhara cave/Uttarakhand	Stalactite, Moonmilk	*Bacillus anthracis*, *B. cereus*, *B. circulans*, *B. pumilus*, *B. lentus*, *B. sphaericus*, *B. thuringiensis*	Baskar et al. (2006, 2014)
2	Krem Mawsmai cave/Meghalaya	Stalactite	Actinomycetes, *Bacillus cereus*, *B. licheniformis*	Baskar et al. (2009)
3	Krem Phyllut cave/Meghalaya	Stalactite, Cave wall deposit	*Bacillus cereus*, *B. licheniformis*, *B. mycoides*	Baskar et al. (2009)
4	Krem Mawmluh cave/ Meghalaya	Moonmilk, Moonmilk water	Actinomycetes, *Bacillus cereus*, *B. licheniformis*, *B. pumilus*, *Bacillus* spp., *Micrococcus luteus*	Baskar et al. (2011)
5	Borra cave/Andhra Pradesh	Biofilm	α-, β-, γ- and δ-Proteobacteria, *Leptothrix* spp.	Baskar et al. (2012)
6	Mandeepkhol cave/Chhattisgarh	Guano deposits	*Alternaria* spp., *Aspergillus* spp., *Cladosporium* spp., *Curvularia* spp., *Drechslera* spp., *Emercilla* spp., *Fusarium* spp., *Mucor* spp., *Penicillium* spp., *Rhizopus* spp., *Talaromyces* spp.	Karkun et al. (2012)
7	Mawsmai cave/East Khasi Hills/Meghalaya	Stalactite, Cave wall deposits	*Acinetobacter johnsonii*, *Kocuria rosea*, *Lysinibacillus macroides*	Banerjee and Joshi (2014)
8	Mawjymbuin cave/East Khasi Hills/Meghalaya	Cave wall deposits	*Bacillus cereus*, *Lysinibacillus parviboronicapiens*	Banerjee and Joshi (2014)
9	Mawmluh cave East Khasi Hills/Meghalaya	Cave wall deposits	*Bacillus halodurans*	Banerjee and Joshi (2014)
10	Dam cave/East Khasi Hills/Meghalaya	Cave wall deposits	*Brevibacillus agri*	Banerjee and Joshi (2014)
11	East Jaintia Hills/Meghalaya	Stalagmite	*Bacillus thuringiensis*	Banerjee and Joshi (2014)

The various factors, viz., pH, availability of nutrients, light, oxygen, sulfur, and compounds of other metals, humidity, and susceptibility of the substrate to colonization influence the structure of microbial communities. Any alteration of physicochemical conditions inside and outside caves can influence a change in species composition. Jones and Bennett (2014) reported that the water, rich in sulfur but containing small amounts of oxygen, flowing directly from the spring orifices in the Lower Kane cave was dominated by ε-Proteobacteria while the water flowing from the cavern to outside containing large amounts of oxygen and low concentrations of sulfur was dominated by γ-Proteobacteria (Fig. 7.1).

Table 7.2 Abundance of fungal family reported from caves worldwide

Phylum	Family	Abundance	Ecology
Ascomycota	Trichocomaceae	620	Saprophytic, rarely on living plants and animals
Zygomycota	Mucoraceae	148	Saprophytic in soil and organic materials, many species are proteolytic
Ascomycota	Nectriaceae	106	Saprophytic, mycoparasitic, and phytoparasitic
Ascomycota	Laboulbeniaceae	105	Entomoparasitic
Mycetozoa	Dictyosteliaceae	87	Phagotrophic on organic debris and other microorganisms
Ascomycota	Pleosporaceae	86	Saprotrophic or nectrotrophic on living and dead plants
Ascomycota	Microascaceae	77	Saprotrophic on organic materials
Ascomycota	Hypocreaceae	77	Saprotrophic on plant materials or mycoparasitic
Ascomycota	Cordycipitaceae	76	Entomoparasitic
Ascomycota	Chetomiaceae	72	Saprotrophic, most species are strongly cellulolytic
Ascomycota	Bionectriaceae	70	Saprotrophic, mycoparasiticor phytoparasitic
Ascomycota	Davidiellaceae	64	Saprotrophic and phytoparasitic
Basidiomycota	Polyporaceae	50	Saprotrophic on wood
Ascomycota	Myxotrichaeceae	50	Saprotrophic or mycorrhizal
Ascomycota	Arthrodermataceae	48	Saprotrophic often keratinolytic
Zygomycota	Mortierellaceae	44	Saprotrophic often chitinolytic
Ascomycota	Clavicipitaceae	33	Entomoparasitic, phytoparasitic or endomutualistic on plants
Basidiomycota	Fomitopsidaceae	30	Saprotrophic, causing brown rot of wood
Basidiomycota	Psathyrellaceae	29	Saprotrophic in soil, dung, and wood
Basidiomycota	Mycenaceae	28	Saprotrophic in wood and leaf litter

Abundance is the number of times genera in each family were reported among 225 cave mycological studies
Source Vanderwolf et al. (2013)

The effect of human intervention on the microflora of caves is yet not very clear. Rusznyak et al. (2012) observed no unique phylogenetic patterns in Herrenberg cave as compared to other caves when it was accidently discovered during the digging of a new railway tunnel in the Thuringian Forest in Germany, free from the presence and interference of animals and humans. However, Adetutu et al. (2012) observed consistently higher bacterial counts in those regions of Naracoorte caves in Australia which were accessible to tourists as compared to undisturbed areas of the caves. The differences in the microbial diversity of cave sediments could be attributed to the presence of exogenous organic matter of human origin.

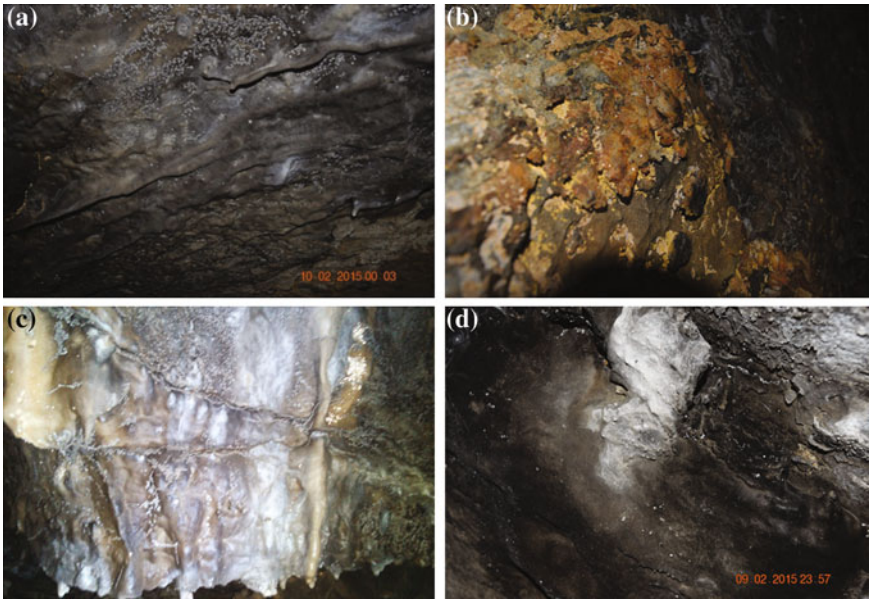

Fig. 7.1 Mineral structures of biogenic origin inside Bhadra cave of Garhwal Himalayas **a** Microbial mat, **b** Iron deposits, **c** Calcified structures, **d** Black deposits

Bacterial Diversity

Proteobacteria are the most dominant group in caves all across the world. It was found to be the dominant group in the aggregations colonizing rocks and water dripping from the walls of Altamira cave which has famous Paleolithic paintings (Laiz et al. 1999; Portillo et al. 2009). It has also been documented to be the most dominant population of the rocks in caves (Schabereiter-Gurtner et al. 2004), in microbial mats on basalt walls of lava caves (Hathaway et al. 2014), on the stalactites of the Herrenberg cave (Rusznyak et al. 2012), in the soil of the Niu cave (Zhou et al. 2007), sediments of the Wind Cave (Chelius and Moore 2004), and in pools of the Barenschacht cave (Shabarova and Pernthaler 2010). It also constitutes the largest taxonomic group of extreme environments like sulfurous caves (Jones et al. 2010). Macalady et al. (2006) observed that Proteobacteria represented >75% of the clone library prepared from the 16S rRNA genes of the organisms of water mat in Frasassi cave system. They have also been reported to be dominant in the biofilm, hanging from the walls of the cave in Grotta del Fiume having an extremely acidic pH (0–1) (Macalady et al. 2007).

The representation of particular classes of microbes varies in different caves. α-Proteobacteria usually constitute a small proportion of microbial populations inhabiting the caves except caves containing Paleolithic paintings, iron–manganese deposits of Lechuguilla and Spider caves (Northup et al. 2003) and in the microbial consortium growing on a chemically complex rock containing hematite, magnetite,

tourmaline, zircon, rutile, apatite, and epidote in the Carlsbad Cavern (Barton et al. 2007). α-Proteobacteria constitute the second most abundant group of yellow (13%) and white mats (15%) of caves (Hathaway et al. 2014). A small proportion of this class has been documented from sediments in the Wind Cave (Chelius and Moore 2004), water mats of sulfur-rich caves like Lower Kane (Engel et al. 2010), fluvial sediments and stalactites of the Herrenberg cave (Rusznyak et al. 2012), ferro-manganese deposits of the Carter Saltpeter cave (Carmichael et al. 2013), and slimes in Weebubbie cave (Tetu et al. 2013).

β-Proteobacteria constitute the abundant group in the water mat of the Movile cave (Chen et al. 2009) which is an isolated cavern having neutral pH, 20 °C temperature, water is rich in ammonium, methane, and reduced sulfur compounds while the atmosphere of cave contains methane, CO_2, and hydrogen sulfide. *Nitrosomonas* spp. constituted the major group of nitrifying microflora in the Movile cave (Chen et al. 2009). *Massilia* was documented to be most abundant on chemically complex rocks while *Comomonas* spp. was common on limestone rock in Carlsbad cavern (Barton et al. 2007). The presence of *Herbaspirillum frisingense* and *Janthinobacterium agaricidamnosusm* in Carlsbad cavern is indicative of nitrogen assimilation in this ecosystem (Barton et al. 2007). *Aquaspirillum* spp. and *Variovorax* spp. have been reported from Lechuguilla and Spider caves.

γ-Proteobacteria constitute an important component of biofilms developing in cave waters rich in sulfur. They also inhabit cave rocks. Engel et al. (2001) reported that the half of the 16S rRNA sequences analyzed from the biofilm of the Cesspool cave, containing high sulfur concentration, could be assigned to the group of γ-Proteobacteria (Engel et al. 2001). It constituted the second largest taxonomic class in the Movile cave. The species of genera *Beggiatoa*, *Halothiobacillus*, *Thioploca*, *Thiothrix*, and *Thiovigra*, members of Xanthomonodales family, and sulfur-oxidizing bacteria have been reported from Movile cave (Chen et al. 2009). γ-Proteobacteria constitute the dominant group of cotton-type biofilm in Grotta Sulfurea which is likely due to high concentrations of dissolved oxygen, a limiting factor for the growth of Ɛ-Proteobacteria (Nelson et al. 1986). Baker and Banfield (2003) reported the frequent occurrence of γ-Proteobacteria in Grotta del Fiume of the Frassi cave which is extremely acidic (pH 0–1), rich in sulfur compounds, and poor in iron ions.

Tomczyk-Żak and Zielenkiewicz (2016) reported δ-Proteobacteria to be the second abundant group in cotton-type water mats in the stream of Frasassi cave. The species of genera *Desulfocapsa* and *Desulfonema* were the most abundant constituting almost half of the 16S rRNA sequences analyzed. Additionally, the Geobacteriaceae family along with species of genera *Desulfoarculum*, *Desulfobacter*, *Desulfomonile*, *Syntrophobacter*, and *Syntrophus* was identified. ε-Proteobacteria was reported to be the second largest taxonomic group in the feathery biofilm of the Grotta Sulfurea (Tomczyk-Żak and Zielenkiewicz 2016). The majority of 16S rRNA sequences were assigned to the genus *Arcobacter* and other sulfur-oxidizing microflora. This group constituted a meager proportion of the population of sulfur-rich Movile cave (Chen et al. 2009). *Desulfobulbus* spp. and *Sulfuricurvum* spp. have been reported from the cave. *Nitrospirae* constituted the

third most abundant group in the Lechuguilla cave. The maximum homology of 16S rRNA sequences from cave was observed with *Leptospirillum ferrooxidans* and *Nitrospira marina* (Northup et al. 2003). *Nitrospira* type was documented to be highly represented group in the Pajsarjeva jama (Pasic et al. 2010) and moderately represented group in Lava caves (Hathaway et al. 2014) while this group is poorly represented in Lower Kane cave (Engel et al. 2010).

Actinobacteria have been isolated frequently from the microbial consortia of Pajsarjeva jama (Pasic et al. 2010), Wind Cave (Chelius and Moore 2004), and Altamira and Tito Bustillo caves (Schabereiter-Gurtner et al. 2002a). 80% of the population on calcium carbonate rocks in Carlsbad cavern comprised of actinobacteria (Barton et al. 2007). *Pseudonocardia* spp. constituted 50% of the actinobacteria inhabiting this cave. Actinobacteria have also been reported to be the second largest taxonomic group comprising of 16.3% of the 16S rRNA sequences in Pajsarjeva jama and Frasassi cave (Pasic et al. 2010). Actinobacteria have also been reported to be an important member of microbial population from ecological viewpoint in Altamira cave (Schabereiter-Gurtner et al. 2002a), Tito Bustillo cave (Schabereiter-Gurtner et al. 2002b), and La Garma cave (Schabereiter-Gurtner et al. 2004) as well as in the soil of the Niu cave (Zhou et al. 2007). Carmichael et al. (2013) reported Bacteroidetes phylum as the largest group of biofilms from the ferromanganese deposits of the Carter Saltpeter cave. Bacteroidetes, represented mostly by *Flavobacterium*, was documented to be the second largest group of microorganisms inhabiting the rocks in Altamira cave (Portillo et al. 2009). 20% of the 16S rRNA sequences of the bacterial population of sediments of Herrenberg cave were assigned to Bacteroidetes (Schabereiter-Gurtner et al. 2004).

The microbial populations inhabiting the surface of rock walls and sediments in caves are represented mostly by Firmicutes. Some of these microorganisms like *Desulfatomaculum* spp. and *Sulfobacillus acidophilus* are able to reduce or oxidize sulfur (Macalady et al. 2007). This group has been documented to be the dominant group in the medium and low tourist traffic areas, 52 and 66% of recovered isolates, respectively in the Kartchner caves (Ikner et al. 2007). Urzi et al. (2010) reported Firmicutes to be the dominant population inhabiting the biofilm in the cave of bats (49.5% of 16S rRNA sequences) with medium intensity of visitation of tourists (20,000 visitors per year). *Bacillus*, *Paenibacillus*, and *Staphylococcus* have been identified in this biofilm.

The phylum Verrucomicrobia, represented by a small number of microorganisms, has been reported from Altamira cave (Portillo et al. 2009), Barenschacht cave (Shabarova and Pernthaler 2010), Carter Saltpeter cave (Carmichael et al. 2013), Grotta Sulfurea Frasassi cave system (Macalady et al. 2006), Herrenberg cave (Rusznyak et al. 2012), Lava cave (Hathaway et al. 2014), Lower Kane cave (Engel et al. 2010), Movile cave (Chen et al. 2009), Pajsarjevajama (Pasic et al. 2010), and Wind Cave (Chelius and Moore 2004).

Planctomycetes have been reported to be the second most abundant group in the white colonies of the Altamira cave (18.8% of 16S rRNA sequences) (Portillo et al. 2009) and the third most abundant group in the soil of Niu cave (9% of 16S rRNA sequences) (Zhou et al. 2009). The noncultured members of *Chloroflexi* have been

isolated from the gray water mats of the Lower Kane cave (Engel et al. 2010). The rarely encountered group in cave ecosystems is Gemmatimonadetes whose members have been reported from soil of Niu cave (Zhou et al. 2007), Altamira cave (Portillo et al. 2008), walls of Pajsarjeva jama (Pasic et al. 2010), yellow and white mats of Lava caves (Northup et al. 2011; Hathaway et al. 2014), and fluvial sediments of Herrenberg cave (Rusznyak et al. 2012). Another rare group in cave ecosystems is Spirochetes, represented by a meager population in the water mats of various caves, viz., Grotta Nuova di Rio Garrafo (Jones et al. 2010), Movile (Chen et al. 2009), Lower Kane (Engel et al. 2010), and Wind (Chelius and Moore 2004). The ecological role of Planctomycetes, Verrucomicrobia, Chloroflexi, Gemmatimonadetes group, and Spirochetes in cave ecosystems is not yet known. Urzi et al. (2010) reported cyanobacteria belonging to the orders Chroococcales and Nostocales from the phototrophic biofilm growing on the rocks at the entrance and exit of the cave of bats. The ecological role of rarely found *Chlorobi* and *Fibrobacteres* in cave ecosystem is still unknown. Very few observations about the occurrence of Archaea in cave ecosystem have been reported. Members of Crenarchaeota and Euryarchaeota have been frequently reported from the water mats of Movile cave and sediments of Wind Cave (Chelius and Moore 2004; Chen et al. 2009).

Fungal Diversity

Fungi are the most important component of cave as they participate in the feeding strategies of cave fauna among other decaying organic material (Nováková 2009) and also play an important ecological role in the structuring of cave community. The dots on cave surface, precipitates, unusual coloration of speleotherms, corrosion residues, and presence of biofilm are evidences of fungal activity in caves (Barton 2006). The long-term viability of fungal spores helps them to survive in drastic conditions of caves. Cave-dwelling fungus generally functions as parasite or decomposer (Benoit et al. 2004; Santamaria and Faille 2007), although fungi may also help in formation of speleothems such as secondary mineral precipitation (Bindschedler et al. 2012).

Due to low temperature and lack of organic substrate, caves favor psychrotolerant and oligotrophic fungi. Cave-dwelling fungi are dependent on inputs of organic matter from the outside environment due to scarcity of organic matter inside the cave. Fungi contribute a major role in maintenance of biological diversity of cave.

Fungi are inhabitants of rocks, sediments, air, and biota of caves (Nieves-Rivera et al. 2009; Nováková 2009; Bastian et al. 2010). About 64,163 species of Ascomycota, 31,515 Basidiomycota, 1065 Zygomycota, Mycetozoa, and 956 Oomycota have been reported by Kirk et al. (2008). The comparative rarity of Basidiomycota versus Ascomycota may be either due to lack of nutrient-rich substrates in caves as former is often associated with nutrient-rich substrates or difficulty in culturing and identification of Basidiomycota. Zygomycota is easy to detect due to abundant spore production and rapid growth which may account for their overestimation in the caves. The majority of commonly reported fungal taxa

from caves are widespread and opportunistic saprotrophs associated with plant material, soils, and insects (Table 7.2).

The most frequently isolated genera from the air, flora, and fauna of the caves in Slovakia were *Aspergillus* spp. and *Penicillium* spp. (Nováková, 2009). Karkun et al. (2012) reported a total of 54 fungal species from Mandeepkhol cave, Chhattisgarh, India. Among them, Deuteromycotina fungi dominated over the member of Ascomycotina and Zygomycotina. Out of total isolates, four species (three genera) belonged to Zygomycotina, two species (two genera) belonged to Ascomycotina, and 48 species (12 genera) belonged to Deuteromycotina. Ogórek et al. (2013) isolated nine species of filamentous fungi and a yeast species from the air inside Niedźwiedzia cave in Poland. They also reported nine species of filamentous fungi and two species of yeasts from the cave rocks. *Rhizopus stolonifer* was the most frequently isolated species from the air and rocks while *Sclerotinia sclerotorium* was the least isolated species from the cave air. The least frequently collected species from rock was *Fusarium oxysporum* and the yeast *Rhodotorula rubra*.

Vanderwolf et al. (2013) documented 1029 species of 518 genera of fungi, slime molds and yeasts from caves (Man et al. 2015). The fungal taxa of caves were categorized as 69.1% Ascomycota, 20% Basidiomycota, 6.6% Zygomycota, 2.6% Mycetozoa, 1% Oomycota, and 0.8% other members (Amoebozoa, Chytridiomycota, Micrisporidiomycota, and Percolozoa). A total of 85 isolates have been reported from 21 sediment samples of Heshang cave of central China. The phylogenetic analysis grouped them in the phylum Ascomycota (87%), Basidiomycota (9%), and Zygomycota (4%). The genera were *Acremonium*, *Acrostalagmus*, *Alternaria*, *Arthrinium*, *Aspergillus*, *Auxarthron*, *Botrytis*, *Bjerkandera*, *Ceriporia*, *Chaetomium*, *Coprinellus*, *Emericellopsis*, *Fusarium*, *Geomyces*, *Monascus*, *Microdiplodia*, *Mortierella*, *Mucor*, *Myriodontium*, *Paecilomyces*, *Paraphaeosphaeria*, *Penicillium*, *Phoma*, *Stilbella*, *Tolypocladium*, *Trametes*, *Trichoderma*, and *Trichosporon*. The fungal spores can be transported to caves by organisms like invertebrates, bats, rodents, humans, and other animals, as well as by air circulation and water sources (Fig. 7.2; Northup et al. 1994).

Geomyces destructans is one of the commonly reported fungi from caves all across the countries in the eastern Canada, northeastern USA, and many European countries (Martinkova et al. 2010; Puechmaille et al. 2010; Wibbelt et al. 2010; Kubatova et al. 2011; Turner et al. 2011).

Its ubiquity in caves may be accounted to its unique ability to utilize live, hibernating bats as a nutrient source while other fungal species can grow only on dead bats in caves (Voyron et al. 2011). This fungus, being pathogenic, infects bats which do not have any innate defense against this fungal infection during the winter, although the impact of *G. destructans* varies among bat species. It caused mass mortality of bats in North America while no significant mortality or morbidity among European bats was documented (Puechmaille et al. 2010, 2011; Turner et al. 2011). The lack of mortality of European bats is may be due to differences in cave environment, host immune response, and other aspects of physiology and behavior, or variation in associated microflora or fauna (Cryan et al. 2010; Wibbelt et al. 2010). The widespread occurrence of *G. destructans* in Europe may be accounted to

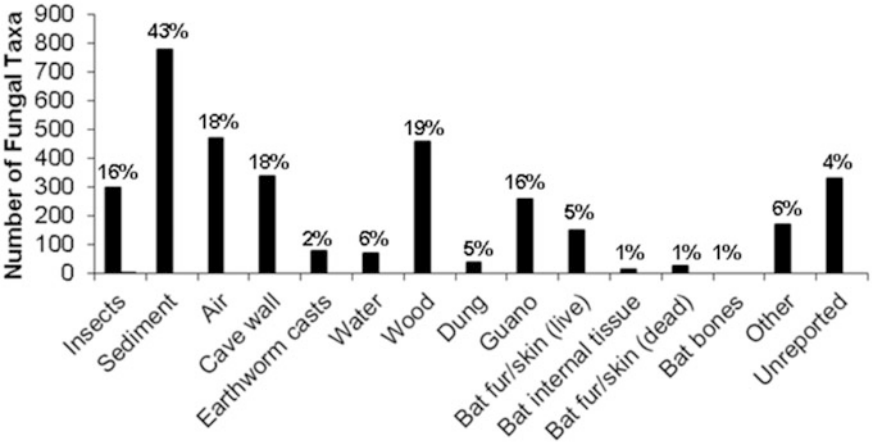

Fig. 7.2 Number of fungal taxa recorded from various cave substrates. Each taxon in a study is counted as 1 report; therefore, the same species may be reported from the same substrate multiple times by different studies. The percentage of papers sampling that substrate is given above the bars, most papers sampled >1 substrate type. n = 225 studies for the bars, n = 126 for the percentages. Many of the studies excluded from the percentage calculations did not report the substrate sampled. *Source* Vanderwolf et al. (2013)

availability of other cave substrates upon which this fungus grows (Puechmaille et al 2010; Wibbelt et al. 2010). This fungus can grow on other cave substrates but other expeditious growing fungi seem to compete *G. destructans* in these micro-habitats. Lindner et al. (2011) documented several species of *Geomyces* distinct from *G. destructants* in cave sediments in USA.

Another most widely studied fungus of caves is *Histoplasma capsulatum* which is most frequently found in bat or bird guano-enriched soil, both inside and outside of caves. It has also been isolated from bats, water, air, and sediment from caves of North America, South/Central America, Australia, Israel, Malaysia, New Guinea, Nigeria, Tanzania, and Romania (Ajello et al. 1960; Ponnampalam 1963; Alteras 1966; Tesh and Schneidau 1967; Disalvo et al. 1969; Lewis 1974; Quinones et al. 1978; Hunt et al. 1984; Quilici et al. 1984; Gugnani et al. 1994; Taylor et al. 1994; Lyon et al. 2004; Ulloa et al.2006; Neuhauser et al. 2008; Gonzalez-Gonzalez et al. 2012). Taylor et al. (1999) observed that the infection of bats with *H. capsulantum* is favored by the large guano deposits and small distance between ceilings with floor of the cave. The antibiosis between *H. capsulatum* var. *duboisii* and other fungi in the cave environments was investigated by Muotoe-Okafor and Gugnani (1997). Flooding was observed to control the growth of this fungus in caves. Shacklette and Hasenclever (1968) observed that *H. capsulatum* could not be recovered from cave soil for a duration of 2–3 months after a flooding event.

The other species frequently reported from caves are: *Altenaria alternata*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ustus*, *Aspergillus versicolor*, *Aureobasidium pullulans*, *Beauveria bassiana*, *Cephalotrichum stemonitis*, *Cladosporium cladosporioides*, *Cladosporium herbarum*,

Fusarium solani, *Geomyces pannorum*, *Paecilomyces lilacinus*, *Penicillium brevicompactum*, *Penicillium chrysogenum*, *Penicillium simplicissimum*, *and Rhizopus stolonifer* var. *stolonifer* (Vanderfolf et al. 2013). The occurrence of these most frequently documented taxa is not necessarily the reflection of biological patterns in caves. They can be the mere reflection of methodologies used for studying cave fungi (Vanderwolf et al. 2013). The isolation of many of the most commonly documented fungi is favored by the incubation temperature over 20 °C and high nutrient-rich media. The distribution of cave fungi is ubiquitous both within and among caves as suggested by the presence of some fungal species from the different sampling substrate in different caves. It appears that a core group of fungi is ubiquitous in cave environments accompanied by few rarely isolated species. Grishkan et al. (2004) reported a core population of nine fungal species present in all the three sampling periods. This type of pattern is common in mycological studies of both cave and non-cave environments (Christensen 1989; Kuzmina et al. 2012; Vanderwolf et al. 2013).

Fungal Ecology in Caves

Fungi in caves are generally either parasites or decomposers; however, their roles may not be mutually exclusive. They are opportunistic saprotrophs associated with soil, plant material, and insects. Mycorrhizal fungi have also been documented, whose source is the plant roots which penetrate into shallow caves (Lamout and Lange 1976; Jasinska et al. 1996). Many fungal species isolated from caves may be present only as spores carried by air current, water, and animals as these are not able to grow in the cave environment. The regular invasion of the caves may account for the presence of these fungal species. The fungi may act as major food sources for protozoans, as well as nonpredacious troglobitic invertebrates like isopods and collembolans inside the caves (Sustr et al. 2005; Walochnik and Mulec 2009; Bastian et al. 2010). Various fungal species in caves are reported to parasitize cave insects (Benoit et al. 2004; Santamaria and Faille 2007; Yoder et al. 2009). Solubilization of bedrock by cave fungi can serve as an inorganic nutrient pool for other cave-dwelling lives (Cubbon 1976). The cave mycota may also participate in speleothems formation, such as secondary calcium carbonate deposits (needle fiber calcite) (Bindschedler et al. 2012).

The distribution pattern of fungi is not even in the caves like that of bacteria. Bacteria are more uniformly distributed than fungi, and their biomass and diversity is much higher than cave fungi (Khizhnyak et al 2003; Urzi et al. 2010). However, Mulec et al. (2012) reported that fungi are more abundant in cave air as compared to bacteria except in the areas with frequent tourist visitation. Fungi are more diverse in wet than in dry cave passages, while reverse pattern was observed for bacteria (Dickson and Kirk 1976). The diversity and distributional pattern of fungi in caves are influenced by microenvironmental conditions, by the host material to be

colonized and by parameters such as porosity, rock permeability, and mineral composition (Gorbushina 2007).

Caves usually lack the rich fungal diversity generally found in nature except the diversity observed on dung in caves (Dickson and Kirk 1976). The diversity of fungal species in the soil outside the caves has been observed to be more than that inside the caves (Hsu and Agoramoorthy 2001). The diversity and biomass has been observed to decrease from the entrance to deep zone of the caves (Mulec et al. 2012). Kuzmina et al. (2012) observed a dominance of mesophilic species close to the cave entrance while the abundance of psychrotolerant micromycetes increased with the depth of cave. Koilraj et al. (1999) observed more dominance of some fungal genera like *Absidia*, *Chaetomium*, *Mucor*, *Rhizopus*, and *Sepedonium* in the interior of the cave while some genera such as *Aspergillus* and *Penicillium* were more abundant in the entrance compared to the interior in Indian cave. Fernández-Cortès et al. (2011) reported more abundance of spores of *Cladosporium* in the cave air at the entrance compared to the interior while reverse pattern was observed for *Penicillium.*

The scarcity of organic matter in caves made fungi dependent on outside environment for nutrient inputs (Jurado et al. 2010). The organic inputs inside the caves from external sources may affect the diversity of cave fungi (Chelius et al. 2009). Min (1988) observed that the conidia of *Aspergillus* sp., *Penicillium* sp., and *Mucor* sp. rapidly covered the organic debris in cave. The sudden availability of food source inside caves may result in opportunistic proliferation of several fungal species (Cubbon 1976).

The interdependency between bacteria and fungi was observed in Lechuguilla caves of New Mexico. The populations of chemolithoautotrophic bacteria support the populations of chemoheterotrophic bacteria and several species of fungi (Cunningham et al. 1995). A similar situation was documented in a Romanian mine by Gherman et al. (2007). Hose and Pisarowicz (1999) observed that the abundant chemoautotrophic microbes, viz., fungi, multiple insect species, and cave fish, act as primary producers in a Mexican cave.

Another nutrient source for fungi can be fauna present in the caves though all fauna cannot be easily accessed. The occurrence of fungi on insects and bats has been widely reported. Rimer and Briggler (2010) reported the presence of *Batrachochytrium dendrobatidis* from amphibians in the Missouri cave. Insects are the best host for fungi in caves. There are multiple fungal species like species of *Isaria*, *Beauveria,* and *Rhachomyces* that can utilize cave insects as nutrient sources. The transmission of these infectious fungal spores can be among and between insect species, or can be acquired from the cave environment. Bats can significantly affect the fungal diversity of cave through deposition of guano and carcasses and by transporting fungal spores. Bats may act as vectors for fungal spores inside and outside of caves (Vanderwolf et al. 2012). Nováková (2009) reported that the maximum diversity of fungal species occurred on bat guano as compared to other cave substrates in Slovakian cave. Min (1988) reported that guano can be a major factor influencing the structure of mycota of caves. Sustr et al. (2005) observed that fungi on bat guano had a higher biomass as compared to the rest of the cave. Bat

guano has been reported to be the most common source of organic matter in tropical caves, especially dry ones (Ferreira and Martins 1999; Fenolio et al. 2006). It may form the trophic base for many invertebrates in caves which may eat the guano directly or the fungi that grow on the guano (Moulds 2006; Ribeiro et al. 2012). Mites have been reported as the most diverse invertebrate taxa in bat guano, with *Sancassania* sp. representing the most abundant microarthropod (Estrada-Barcenas et al. 2010). These mites eat microfungi, including *Histoplasma capsulatum*, and may provide a natural biological control for this pathogen, as well as regulating the numbers of cave microfungi.

The dung of birds and rodents can also influence the mycoflora of caves (Vanderwolf et al. 2013). Insects on dung in caves are exposed to a high diversity as well as a large number of fungi. Some species are known to produce the antimicrobial substances to prevent infection (Ribeiro et al. 2012). Insects may introduce fungi into caves by transporting spores inside and outside the caves. Dickson (1975) observed a positive correlation between populations of invertebrates and fungi in sediments of Virginia cave. Cave crickets (*Ceuthophilus gracilipes gracilipes*) are believed to be vectors of dictyostelid cellular slime molds within caves (Stephenson et al. 2007).

The human interventions in caves may also affect the fungal diversity. Vaughan-Martini et al. (2000) reported more yeast strains from an Italian cave with high human visitation (~400,000 visitors/year) than the two other caves with low human visitation (a few speleologists/year). Similar pattern has been reported for cave fungi by Mosca and Campanino (1962). Wang et al. (2010) reported a positive correlation between the quantities of airborne fungal spores in Chinese cave with the number of human visitors. An alteration in the composition of fungal species with increasing human visitation has also been reported (Adetutu et al. 2011).

Kuzmina et al. (2012) recorded the highest number of heterotrophic fungi from the areas of caves with frequent human visitation. However, Shapiro and Pringle (2010) reported lower fungal diversity from caves with frequent human visitation as compared to caves with low visitation. Adetutu et al. (2011) observed similar patterns in Australian caves when samples were cultured; however, the genetic methods reveal the reverse pattern. The influence of human visitation on cave microbiota is still not very clear. There was no effect detected when caves with a few hundred human visitors per year were compared to caves with less than hundred visits (Vanderwolf et al. 2013). The human visitors may influence the quantity and diversity of microbiota in caves. The threshold(s) at which these influences occur is yet to be determined.

The changes in the abundance and diversity of cave mycota can be attributed to the organic inputs, viz., lint, hair, and dander coming along with human visitation as well as to the introduction of new spores (Chelius et al. 2009). The fast-growing species proliferate rapidly and mask slow-growing oligotrophic species due to the presence of organic matter. Northup et al. (2000) observed that the organic inputs from human visitation supported the growth of fungi alien to the oligotrophic cave

environment which eliminated the native species of caves resulting into reduced biodiversity. The changes in microclimate of caves due to human visitation and lighting systems could also have significant impact on fungal populations (Pulido-Bosch et al. 1997).

It appears that the majority of fungi reported from caves originate from the non-subterranean environment. The most commonly reported fungal taxa from caves are also common in environment aboveground. The air currents entering and circulating within caves can distribute spores from surface environment, suggesting that the study of cave structure and airflow is important for determining the origin of cave microbiota. The seasonal variation in the diversity of cave microfungi confirms the effect of outside influences. Wang et al. (2010) reported a greater abundance and diversity of microfungi in summer as compared to winter in the air of Chinese cave. Similar pattern has been documented in Spanish cave air by Docampo et al. (2010). Borda et al. (2004) observed more population of fungi in the air of Romanian caves in summer season as compared to winters. This seasonal pattern has also been observed among the microfungi outside caves. Docampo et al. (2011) observed a significant positive correlation between outside rainfall and concentrations of certain airborne spore types inside a Spanish cave.

The quantity of airborne fungal spores in Chinese caves was found to be positively correlated with temperature (inside and outside the cave) and negatively correlated with relative humidity and outside rainfall (Wang et al. 2010). The composition of fungal genera inside these caves was found to be identical to that outside the caves. The geographical location, season, and especially the outdoor climate affect the types of fungal spores indoors (Sterling and Lewis 1998). However, the indoor microclimate is also important for the growth of spores. The major factors driving the formation of unique fungal assemblages of caves are microclimate and substrates found in caves (Vanderwolf et al. 2013). The absence of sunlight inside caves may also be an influencing factor as it has often been reported to be the cause of deformities in the mushrooms growing in mines (Dobat 1970).

The existence of true troglobitic fungi is yet unknown. Certainly, there are species which have been exclusively isolated from caves like *Aspergillus baeticus* (Novakova et al. 2012), *Aspergillus spelunceus* (Marvanova et al. 1992), *Aspergillus thesauricus* (Novakova et al. 2012), *Chrysosporium chiropterorum* (Beguin et al. 2005), *Chrysosporium speluncarum* (Novakova and Kolarik 2010), *Microascus caviariformis* (Vanderwolf et al. 2013), *Mucor troglophilus* (Gunde-Cimerman et al. 1998), *Ochroconis anomala* (Martin-Sanchez et al. 2012), *Ochroconis lascauxensis* (Martín-Sánchez et al. 2012), *Ombrophila speluncarum* (Lagarde 1913), *Trichosporon akiyoshidainum* (Sugita et al. 2005), *Trichosporon cavernicola* (Sugita et al. 2005), and *Trichosporon chiropterorum* (Sugita et al. 2005). However, this may reflect inadequate sampling outside the caves. *Geomyces destructans* has frequently been isolated from bats outside caves (Dobony 2012).

Role of Cave Microbiota in Biomineralization

The occurrence of bacteria and fungi in cave sediments and water suggests the participation of microorganisms in deposition of cave speleothems such as carbonate speleothems (Went 1969) and saltpeter (Faust 1968). However, it was hypothesized in early studies that the translocated soil and surface microbial groups were brought into the cave via water droplets, animals, and air currents. This hypothesis made the researchers to think that the study of microbial community of caves was not very much scientifically relevant until the exploration of the deep-sea hydrothermal vent microbial system (Jannasch 1985) and the discovery of the chemoautotrophically sustained Movile cave in Romania (Sarbu et al. 1996) which marked the beginning of a new era of cave microbiology.

Speleotherms are the secondary mineral deposits. Stalactites are the most familiar speleothems which look like carrots hanging from ceilings of cave. Stalactites are generally composed of calcite, but may consist of iron or other minerals. The growth of all types of stalactite begins as hollow soda straws. At first, water coming through cracks in the rock or by condensation collects on the cave ceiling as droplets. A thin film of carbonate material precipitates and coats the outer side of water droplets. In case of arid cave environment, a thin coating of noncarbonated material like sulfate covers the surface of water drop. Due to accumulation of more water, the drop becomes heavier and starts to oscillate and causes the film of material to move toward ceiling of cave where it gets attached by surface tension. The material of film as round rim is left on the ceiling after the drop falls onto the floor resulting into the initial growth ring of soda straw (Hill and Forti 1997). Moonmilk is a white, pasty, and creamy substance found inside the cave which is usually composed of fine carbonate crystals like calcite, hydromagnesite, aragonite, and monohydrocalcite. In wet condition, it is soft and pasty while in dry condition, it is powdery and crumbly. Bacterial and fungal biomass is prevalent in moonmilk (Baskar et al. 2011; Sanchez-Moral et al. 2012). Canaveras et al. (1999) reported that the association of different species of genus *Streptomyces* with needle-fiberagonite and hydromagnesite may play a vital role in moonmilk formation in Altamira cave.

Calcium carbonate speleothems are predominant in most of the caves. The concentration of calcium and dissolved inorganic carbon (DIC), availability of nucleation site, and pH are key factors which govern calcium carbonate mineralization (Hammes and Verstraete 2002). Some researchers believe that the precipitation is a specific process with ecological benefits for the mineralizing organisms (Ehrlich 1996; Mc Connaughey and Whelan 1997) while others think that the precipitation is undesirable and accidental products of the microbial metabolism (Knorre and Krumbein 2000). The precipitation of carbonate in caves is governed by bacteria, algae, and fungi (Went 1969; Danielli and Edington 1983; Cox et al. 1995). Ercole et al. (2007) experimentally proved the direct role of capsular polysaccharides and exopolysaccharides secreted by *B. firmus* and *B. sphericus* in in vitro calcium carbonate precipitation. Laiz et al. (2003) reported that 61% of the

Actinobacteria recovered from cave produced crystal of calcium carbonate in culture media. Baskar et al. (2006) reported *Bacillus* species in Shastradhara cave and demonstrated that *B. thuringiensis* and *B. pumilis* precipitate calcite under well-defined conditions. Rusznyak et al. (2012) isolated *Arthrobacter sulfonivorans* and *Rhodococcus globerulus* from Herrenberg cave which were able to precipitate calcium crystal on B4 medium.

Deposits and biosignatures of biogenic iron in caves can provide information related to source of energy in dark and nutrient-poor environment for supporting microbial life and microbial metabolism (Angert et al. 1998). In cave ecosystems, iron-oxidizing microbes have received rare but increasing attention. Many studies have revealed the presence and association of bacteria with iron mineralization in cave by applying metagenomics and scanning electron microscopy (Caldwell and Caldwell 1980; Dyson and James 1981; Peck 1986; Jones and Motyka 1987; Davis et al. 1990; Provencio and Polyak 2001; Spilde et al. 2005).

Peck (1986) identified *Galionella ferruginea* and *Leptothrix* sp. from mud and wall crusts which were involved in iron precipitation in Level Crevice cave in Iowa. "Rusticles" of iron oxides and organic filaments in Lechuguilla cave, New Mexico, were identified by Davis et al. (1990) by using scanning electron microscopy. Later on, Provencio and Polyak (2001) studied these structures and reported them as fossilized *Clonothrix* sp. or similar iron-oxidizing bacteria. Baskar et al. (2012) suggested that the iron-oxidizing bacteria, namely *Leptothrix* sp., *Siderooxidans* sp., *Crenothrix* sp., *Comamonas* sp., and *Dechloromonas* sp., were involved in the formation of iron precipitates in Bora caves of India.

Mn(III, IV) is the oxidized form of manganese and serves as a source for various biological processes like scavenging of reactive oxygen species, photosynthesis, carbon fixation, and other cellular functions. The oxides of manganese act as second strongest naturally occurring oxidizing agents. They control the distribution of many trace metal by participating in numerous redox adsorption reactions. A wide range of bacteria can oxidize Mn(II) for energy and shows chemolithoautotrophism.

Many researchers have reported the occurrence of manganese-oxidizing bacteria in cave. Moore (1981) identified *Leptothrix* sp. present in birnessite a Mn(IV)-oxide, coating stream cobbles in Matts black cave in West Virginia. Northup et al. (2000) reported the occurrence of manganese and iron-oxidizing bacteria in Guadalupe Mountains caves by using X-ray diffraction techniques, bulk chemistry, molecular biology techniques, transmission electron microscopy, and scanning electron microscopy.

Fungus also plays an important role in oxidation of Mn(II). Well-known fungal enzyme laccase can oxidize Mn(II) to Mn(III). Hofer and Schlosser (1999) have reported *Trametes versicolor* as a laccase enzyme producer for manganese oxidation. Schlosser and Hofer (2002) produced extracellular laccase from *Stropharia rugosoannulata* for Mn(II) oxidation. Deposition of ferromanganese is observed in several caves around the world (Onac et al. 1997; Northup et al. 2003). Black/chocolate brown biofilms and mineral-rich crust on cave wall are identified as ferromanganese deposits (Carmichael et al. 2013).

Future Perspectives

Cave microbiology is still in its infancy stage. Caves, though, being a unique system are drawing attention but majority of caves are unexplored due to problems with approachability to the remote caves. Majority of the caves which have been studied till date are close to human reach and are the places of frequent human visitation; therefore, the picture of microbial diversity revealed from these caves does not reflect the truly indigenous microflora of caves. It is thus imperative to explore the caves which do not attract the tourists so that a real picture of cave microbiota can be obtained. The origin and ecological role of cave microbiota need to be understood. Study of bacterial flora of caves is more extensive as compared to fungi, and thus, it necessitates to emphasize upon speleomycological studies as fungi are the important component of cave microbiota. It is important to conserve the gene pool of cave fungi as otherwise the anthropogenic effect could alter the native fungal diversity. The biomineralization ability of cave microbiota can be used for various biotechnological applications like calcium-precipitating cave microflora can be used in preservation of ancient marble monuments and statue from continued erosion. There is great potential of finding out novel microorganism from caves which can be exploited in various biotechnological applications like biomining and bioremediation technologies.

Conclusions

Caves are open natural ecosystems constituting an extreme environment whose microbial community cause changes in the cave structure due to their involvement in various biogeochemical processes and also lead to its enlargement. A high microbial diversity is observed in caves along with preferential occupancy by particular taxonomic groups. The picture of microbial diversity of caves is still far from complete due to inaccessibility of caves, insufficient sampling, and bias of approaches used for studying the diversity. The high throughput-sequencing technologies need to be employed to have a deeper insight into the diversity of microbiota of unexplored caves. The changes in the microclimatic conditions of cave lead to fluctuations in the biotic population. The human impact on microbial diversity of caves can be limited by putting a check on the influx of organic matter with human visitation. The geological, climatic, and anthropogenic factors may result changes in the diversity and may lead to elimination of some species, and thus, it necessitates the conservation of the gene pool.

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Anaerobic Gut Fungi

8

D. N. Kamra and Birbal Singh

Abstract

The ruminants are fed on fibrous roughage feeds like straws and stovers, which can be rarely consumed by monogastric animals and human beings. The complex microbiome of the gastrointestinal tract of ruminants and non-ruminant herbivores serves as an exceptionally effective system for saccharification and fermentation of ingested plant biomass and converting it into microbial proteins, ammonia, short chain volatile organic acids and gases such as CO_2, H_2 and CH_4. The anaerobic rumen fungi play a vital role for making the lignocellulosic feed ready for hydrolysis by bacteria and protozoa. These three rumen microbes, which are directly involved in fibre degradation, do not compete with each other for the feed, rather they help each other and synergistically degrade the feed. The fourth microbial group (archaea) helps indirectly by using the end product of fermentation for reducing oxidized compounds like CO_2, SO_4, NO_3 and organic compounds with double/triple covalent bonds and does not allow end product inhibition. The inoculation of the rumen of domesticated animals with superior strains of anaerobic rumen fungi results in a significant increase in fibrous feed components and elimination of fungi from the rumen that results in a significant depression in fibre degradation.

Keywords

Ruminants · Anaerobic fungi · Zoosporangia · Zoospores · Fibrolytic bacteria Lignocellulosics · Cellulosomes

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Introduction

The way the chemical composition of feed of herbivorous animals differs from that of monogastric animals, the site of digestion and the mechanisms are also fascinatingly different. Crop residues, fibrous agro-industrial by-products, and forages including tree leaves, grasses, plants, and bushes are fed to animals in tropical countries. The digestion in herbivores is the net result of a mixture of processes occurring in different segments of the gastrointestinal tract. The primary fermentation of feed takes place in reticulo rumen, with enzymatic hydrolysis and degradation which is continued in abomasum and small intestine. Finally, fermentation is completed in caecum and large intestine. When cellular and structural carbohydrates are fermented, a significant part of energy is excreted in domestic and wild ruminants, rest of the substrate is recovered in the form of volatile fatty acids (VFA), which are readily metabolized by the host (Ulyatt et al. 1988) as an energy source.

Why are Gut Microbes Essential?

The diets of herbivorous animals including insects, feral and domesticated herbivores and ruminants comprise fibrous plant biomass that contains various phytometabolites. The microbiome within the gastrointestinal tract might be considered a metabolically active organ with its wide biodiversity in terms of species and the high number of cells that can reach up to 10^{14} per gram of the rumen content to digest this highly diverse rumen, omasum, abomasum feed. The microbiome of the rumen is exceedingly diverse and contains representatives of all three domains of life—*eucarya*, *archaea* and *bacteria*. The microorganisms have evolutionarily come up to form highly stable mutualistic relationships, which might vary and involve different functions in different hosts. The microorganisms provide the host essential amino acids and volatile fatty acids as source of energy and detoxify some of the anti-nutritional plant metabolites.

Anaerobic fungi have been isolated from different parts of the intestine and like faeces of different domesticated and wild herbivorous animals. The dust containing faeces of such excreta can also be used as an inoculum to isolate these fungi. The vegetative growth of mycelium might take place in different ways. The zoospore germinates, and a tubular structure emerges out of that. When the nucleus remains inside the zoospore and rhizoids are anucleate, such a mycelium is classified as endogenous and monocentric.

In the second type, the tip of zoosporangium gets enlarged and nucleus of zoospore cyst migrates into it and it develops into sporangium. The development of zoosporangium is known as exogenous and monocentric as the sporangium development is outside the zoosporangium and only one sporangium develops from each zoospore. The third type of thallus development is one sided and germ tube braches, in which nucleus migrates and divides repeatedly in the germ tube. It forms zoosporangium either at the tip or in between the mycelium. The details are presented in Table 8.1.

Table 8.1 Characteristics of rumen anaerobic fungi

Characteristics	*Neocallimastix frontalis*	*Piromyces* (*Piromonas*) *communis*	*Orpinomyces bovis*	*Caecomyces/Sphaeromyces communis*	*Cyllamyces*	*Anaeromyces* sp.
Observed in	Sheep, goat, llama, camel	Sheep	Cattle	Horse	–	–
No. of flagellates/ml	10^2–10^5	10^3–10^4	ND	ND	–	–
Zoospore size (μm)	20.6 × 8.7	14.6 × 7	5 × 10	4 × 5–6 × 10	–	–
No. of flagellates/zoospore	2–14	1	10–25	1	Uni-, bi- and quadriflagellate	Uniflagellate
Length of flagella (μm)	36.6	28.8	33–46	25		
Rhizoids	Filamentous, anucleate	Filamentous, anucleate	Filamentous nucleate	Bulbous in shape	Bulbous in shape	Filamentous
Zoosporangium	Endo, exo	Endo	Exo	Endo, exo		
	Mono	Mono	Poly	Mono	Poly	Poly
	Size	21 × 9–75 × 52	66.35–95 × 49	45 × 90–95 × 42	–	–
Zoospore/zoosporangium	2–38	2–78		114	–	–
Release of zoospores						
Temperature (°C)	39	39	39	39	39	39
pH	6.5–7.0	6.5	6.5	6.5	6.5	6.5
Gas phase	CO_2	CO_2	–	–		
Toxic effect of oxygen	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible
References	Orpin (1975), Gold et al. (1988)	Gold et al. (1988)	Barr et al. (1989)	Gold et al. (1988)	Ozkoseet al. (2001), Sridhar et al. (2014)	Breton et al. (1990)

Phylogeny and Classification of Anaerobic Gut Fungi

For a long time, rumen microbial population was considered to be composed of diverse populations of anaerobic bacteria and protozoa. Orpin (1975) for the first time unravelled that fungi are also the integral component of gut microbiome of herbivores. The series of publications thereafter elucidated that these are true fungi. Molecular phylogenetic studies have documented global phylogenetic diversity of gut fungi that has important function in initiating plant fibre degradation during fermentative digestion in ruminants and non-ruminant herbivores.

Based on ultrastructural characteristics of zoospores, the anaerobic fungi were assigned to the order Spizellomycetales and the family, Neocallimasticaceae (Orpin 1975; Barr et al. 1989). The family has three genera comprising monocentric species, *Neocallimastix*, *Piromyces* (previously, *Piromonas*) and *Caecomyces* (previously, *Sphaeromonas*) (Gold et al. 1988) and three genera of polycentric fungi are *Orpinomyces* (Barr et al. 1989), Anaeromyces (Breton et al. 1990) and *Cyllamyces* (Ozkose et al. 2001).

Genome-enabled mycology classified the anaerobic fungi as a single-order Neocallimastigales within the recently postulated phylum Neocallimastigomycota (phyla nov.) (Hibbet et al. 2007). Based on the analysis of DNA sequence, six genera are found within the Neocallimastigales—*Neocallimastix*, *Piromyces*, *Orpinomyces*, *Anaeromyces*, *Caecomyces* and *Cyllamyces*. The gut fungi in ruminants are associated with the ingested feed particles, where they synthesize highly active fibre-degrading enzymes, while their free-living zoospores are found in fluid phase. Before genomic studies focused on their unusual biology and metabolism, a large part of subsequent studies have emphasized the biotechnological potential of their cellulases, xylanases and phenolic esterases. In addition, the gut fungi serve as important source of hydrolytic enzymes for industrial applications. Although multiple uncharacterized isolates are reported from various laboratories, eight genera and around 20 species of anaerobic fungi are documented so far (Griffith et al. 2009).

Mutualism Between Fungi and Animals

The anaerobic gut fungi are unique in combining the resilience and invasiveness of fungi with the metabolic activities of anaerobic fermentative bacteria. The anaerobic gut fungi occupy a unique niche in the gut of large herbivorous animals and are considered to act as primary colonizers of ingested plant material during digestion. Interestingly, the anaerobic fungi do not possess mitochondria, but instead have hydrogenosomes, which form hydrogen and carbon dioxide from pyruvate and malate during fermentation of carbohydrates (Ljungdahl 2008).

The rumen fungi are the only known obligately anaerobic fungi; thus, these fungi are hampered by difficulties in culturing them in vitro. The gut fungi are very oxygen- and temperature-sensitive, and their DNA has an unusually high A + T

content ranging from 72 to 87-mol%. Anaerobic rumen fungi first described by Orpin (1975) are reported to live in close contact with bacteria and other microbes in the rumen and caecum of herbivores. Six different genera are reported so far from around fifty animal species (Table 8.2).

They possess remarkable cellulolytic and hemicellulolytic enzyme profiles for faster and sustained degradation of ingested plant biomass. By colonizing the plant biomass, they excrete extracellular enzymes that mobilize structural plant polymers to be available for other gut microbes. They penetrate the cuticle and attack recalcitrant plant cell wall, thereby weakening the forage fibre making it accessible to fibrolytic bacteria. Ruminal fungi account for up to 3–4% of the microbial biomass in the rumen. But the absence of this small quantity of fungal biomass might result in a significant decrease in fibre degradation as the fibrous substrate is not made ready for degradation by bacteria and protozoa. Experimental validation,

Table 8.2 Fungal diversity of gut ecosystem of herbivorous animals

Fungus	Host	References
Aspergillus niger	Cattle	Tannin acyl hydrolase-producing fungi (Bhat et al. 1996)
Neocallimastix frontalis	Cow	Existence of anaerobic fungi in rumen of cattle (Orpin and Mann 1986)
Neocallimastigomycota Phylum	Green iguana, American elk, pronghorn, goral, bontebok, southern gerenuk and Nile lechwe. Inferred by culture-independent analysis using rRNA ITS-1 as phylogenetic marker	The comprehensive study on the existence of gut fungi in wide diversity of herbivores (Liggenstoffer et al. 2010)
Neocallimastix patriciarum	Sheep	Orpin and Mann (1986)
Neocallimastix hurleyensis	Cattle	Webb and Theodorou (1988)
Sphaeromonas communis (*Caecomyces communis*)	Cattle	Orpin (1976), Wubah and Fuller (1991)
Caecomycesequi	Horse	Gold et al. (1988)
Orpinomyces bovis	Cattle	Barr et al. (1989)
Piromyces spp.	Blue bull	Paul et al. (2004)
Anaeromyces mucronatus (*Ruminomyces mucronatus*)	Cattle	Breton et al. (1990)
Ruminomyces elegans	Cattle (polycentric rumen fungi)	Ho and Bauchop (1991)
Piromyces communis, P. mae, P. dumbonica	Horse, elephant (uniflagellate fungi)	Li et al. (1990)

ITS-1—internal transcribed spacer region, rRNA—ribosomal RNA

biochemical evaluation of fungal enzymes and genomic analysis of their enzymes have clearly supported the adaptive metabolic capabilities of gut fungi (Youssef et al. 2013). It has been noted that fibre-based diet stimulates the fungal growth in bubaline rumen in comparison with diets rich in easily fermentable carbohydrates (Paul et al. 2003) and that fibrolytic activity is higher in fungal strains isolated from wild herbivores compared to those from domesticated ungulates (Paul et al. 2010).

Effect of Fungi on Digestion Performance of Animals

The anaerobic rumen fungus, *Piromyces* sp. FNG5 (an isolate from nilgai, *Boselaphus tragocamelus*), has been found to be tolerant to phenolic monomers released in the rumen by degradation of lignocellulosic poor-quality feeds. The phenolic monomers varied in their potential to inhibit the secretion of carboxymethylcellulose, xylanase, β-glucosidase and acetylesterase activities with catechol being the most inhibitory and vanillic acid being the least inhibitory. It is concluded that the anaerobic fungus *Piromyces* sp. FNG5 was tolerant to phenolic monomers and had the ability to degrade them. Therefore, such anaerobic fungi might play an important role in fibre and tannin degradation in the rumen (Paul et al. 2003).

The anaerobic fungi are superior to the rumen bacteria in their ability to break down and degrade the structural barriers in plant materials. The gut fungi can be selectively removed from the digestive tract by administering antibiotics such as cycloheximide. The removal of rumen fungi was found to increase the number of bacteria in rumen content, thereby increasing the population of rumen protozoa; however, the study could not infer the direct relationship of fungi with protozoa (Li and Hou 2007). When fungi were excluded from the rumen, feed intake and fibre digestibility were decreased, though total viable bacterial or ciliate protozoal populations remained unaffected (Gordon and Phillips 1993).

Anaerobic Fungi as Probiotic in the Diet of Animals

As indicated above, the anaerobic rumen fungi are superior fibre degraders, and the isolates of wild ruminants degrade lignocellulosics better than those from domesticated microbes. The wild isolates have better enzyme profile (Paul et al. 2010). There was a significantly higher digestibility of lignocellulose-containing feed components like dry matter, cellulose, hemicellulose, acid and neutral detergent fibres (Paul et al. 2004) as a result of which there is an improvement in fibre intake and body weight gain (Sehgal et al. 2004) (Table 8.3).

Table 8.3 Effect of fungal feed additives on nutrient utilization in ruminants

Animal	Microbial feed additive	Effect	Reference
Cattle calves	*Neocallimastix* sp. from sheep	35% increase in forage intake	Theodorouet al. (1990)
	Anaerobic fungal isolate	Increase in in vitro digestibility	Lee et al. (2000)
Buffalo	*Piromyces* strain FNG5 from *Boselaphus tragocamelus* (nilgai)	Increase in in vivo digestibility of DM, OM, NDF and ADF; VFA, cellulolytic and hemicellulolytic bacteria, fungi, cellulases, esterases, etc.	Paul et al. (2004, 2010)
Crossbred cattle calves	*Orpinomyces* sp.	Increase in body weight gain (15.37%), VFA and fungal count in rumen liquor increased	Dey et al. (2004)

Biotechnological Potential of Anaerobic Fungi

Fungi have contributed to shaping of humankind's welfare since the beginning of civilization. The anaerobic fungi, being the efficient plant biomass degraders, represent promising candidates for a variety of industrial applications. The cellulolytic arsenal of anaerobic fungi consists of both secreted enzymes and highly active extracellular multienzyme complexes called cellulosomes, which can degrade amorphous and crystalline cellulose. The anaerobic fungi have a potential for making the substrate ready for biogas production from municipal solid waste (MSW) and animal wastes, lignocellulose-rich materials of various industrial processes, agriculture, forestry, pulp and paper industries.

The rumen fungi are of high interest to enzymologists and microbial ecologists as they degrade lignocellulose and produce enzymes needed to hydrolyze cellulose and hemicellulose efficiently. The anaerobic fungus *Anaeromyces mucronatus* KF8 grown in batch culture is found to produce a broad range of enzymes including cellulase, endoglucanase, xylanase, α-xylosidase, β-xylosidase, α-glucosidase, β-glucosidase, β-galactosidase, mannosidase, cellobiohydrolase, amylase, laminarase, pectinase and pectate lyase that assist in degradation of plant structural and storage polysaccharides. Although some of these enzymes are found free in the rumen liquor, most of them are found as cellulosomal and polycellulosomal complexes wherein the enzymes are attached through fungal dockerins to scaffolding proteins; this is similar to what has been found for cellulosomes from anaerobic bacteria. Although cellulosomes from anaerobic fungi share many properties with cellulosomes of anaerobic cellulolytic bacteria, their structures differ in amino acid sequences and post-translational modification.

Opportunities and Challenges

The in vivo fibre degradation with fungi is more efficient as compared to degradation of plant biomass by bacteria or protozoa. This is because the bacteria, protozoa and fungi synergistically digest the plant fibrous feed. It is envisaged that microbial pre-treatment or rumen microbial inoculum might possibly increase degradation of recalcitrant substrate.

When an elite culture of anaerobic fungi (*Piromyces* sp. FNG5; isolated from faeces of wild blue bull), having higher lignocellulolytic activities than those normally isolated from domesticated ruminants, was fed to buffaloes and resulted in increased digestibility of NDF and ADF as well as N retention. This resulted in a substantial increase in VFA concentration, population of rumen fungi and cellulolytic, hemicellulolytic and total bacterial numbers. Results of this laboratory (Paul et al. 2004) demonstrated that administration of the fungal strain with superior lignocellulolytic activity caused an increase in digestibility of lignins and tannins and enhanced tolerance to their monomers.

On studying the functional diversity of rumen, twenty-two clones representing the hydrolytic enzyme activities (12 esterases, nine endo-β-1,4-glucanases and one cyclodextrinase) were characterized. All the three enzymes, especially the first one is responsible for breaking of the bond between lignin and carbohydrates, and therefore, is able to make carbohydrates free from feed and digestibility of lignocellulosic feeds improve and release of energy is enhanced. The sequence analysis of these enzymes indicated that 36% were new and not reported earlier and therefore might have a different mechanism of action and might also have new practical application in livestock production.

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Fungal Endophytes Representing Diverse Habitats and Their Role in Plant Protection

9

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Abstract

Fungal endophytes are commonly considered all those highly diverse fungi that colonize internal tissue of plants either part or complete their life without causing any negative symptoms of disease. Researches on structural and functional diversity of fungal entophytes have developed great appreciation among the scientific community because its promises plethora of advantages to host plants which most of them we are not aware and to be potential source of novel metabolites that could serve as new drugs of pharmaceutical and agricultural importance. Endophytic fungi are reported from each group of plants from thallophytes to spermatophytes (algae to angiosperms) and from aquatic to xerophytic plants. Taxonomically they are categorized into two different phylogenetic groups i.e. clavicipitaceous and non-clavicipitaceous group that may be transmitted horizontally or vertically from plant to plant. Endophytism is classical example of mutualistic symbiosis which has evolved from the balanced antagonism between microbes and host plant during the course of time. Past researches on fungal endophyte mainly focused on diversity and its secondary metabolites; many aspects of their role in plant protection against biotic and abiotic stresses are less explored. Since the majority of these groups of microbes

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are mysterious and their other hidden potential values are yet to be discovered which need more attention. Past fragmentary reports on role of fungal endophyte in plant protection give enough clue regarding scope and application of these microbes as tools for the improving fitness of plant in terms of quality and quantity of their productivity. The present article mostly focuses to review the status on diversity of fungal endophytes in different groups of plants, and their spatio-temporal distribution including the role in plant protection.

Keywords
Fungal endophyte · Endophytism · Abiotic stresses · Secondary metabolites Diversity

Introduction

In nature, every living organism lives by making interaction with other flora and fauna. Plants are not different, and they have been naturally evolved by making symbiosis with different types of microorganisms in various ways. Plants and microbes are not only fulfilling our basic needs i.e. food, clothes and shelter, but also provide us life-saving oxygen and useful pharmaceuticals of great value. Microbes play an important role in biosphere because of its capacity to grow anywhere and transform every process around them. In 1879, de Bary used the term "symbiosis" to describe the situation when two dissimilar organisms live together regardless of their nature of interaction. Plants have variety of interactions with microbial system. Some microbes grow inside the plant tissues either intercellular (apoplast) or intracellular (symplast) spaces without causing any harm to them. Microorganisms particularly from the soil, the plants and human pathogens have been recognized, acknowledged and applied in scientific research extensively, but enough attention has not been given to microbes that are growing inside plant tissue without showing any negative effect to host i.e. endophytic microorganisms. These endophytic microbes may help the plants by improving the fitness of host from various biotic and abiotic stresses, increase the productivity in crops and improve the quality of pharmaceuticals extracted from medicinal plants.

Endophytic Microorganisms: Definition

For the first time, de Bary (1866) introduced the term endophyte and since then it become deeply rooted in the literature. Basically, endophyte is primarily applied to any microorganisms that grow within a plant (Wilson 1995). As per Bacon and White (2000) "All the microbes that colonizing living, internal tissues of plant without causing any immediate, overt negative effect is called endophytes". These endophytes may be bacteria, actinomycetes and fungi or any other microorganisms. Endophytes are the endosymbionts and ubiquitous and have been found in all the

species of the plants studied till date from cryptogams to angiosperms (Stone et al. 2004; Guo et al. 2006; Ghimire and Hyde 2004).

With the advancement of researches, the concept of endophyte has been changed over the time (Siegel et al. 1984; Ghimire and Hyde 2004). According to Petrini (1991), endophytes are all microbes which live inside the plant and they may colonize internal plant tissues any point of time of their life without showing any symptoms of disease. This has become popular definition of endophytes which includes the latent pathogens and microbes that also show epiphytic phase in their life (Petrini 1991; Schulz et al. 1998). Microbes of different levels of interactions with plants are known ranging from fully mutualist to weak pathogen to aggressive parasites (Jersch et al. 1989; Kehr and Wulf 1993; Williamson 1994; Agrios 2005). In general, endophytes are beneficial in different way to host and non-aggressive and non-pathogenic to the host plant and because of this, several workers had shown their disagreement on the Petrini (1991) wider concept of endophytes (Freeman and Rodriguez 1993; Tyler 1993; Stone et al. 2004; Sinclair and Cerkauskas 1996). Wilson (1995) put forth working definition of endophytes with a wider consideration of their relationship with host plant as "endophytic microbes are fungi or bacteria which live inside host tissue all or part of their life and cause no apparent symptoms of the disease". He also proposed operational definition of endophytic fungi as "fungus which grows out from a piece of healthy-looking surface-sterilized plant material is usually considered as endophytes". Nature of plant–microbes interaction varies from one environmental condition to other; one microbe which is endophytic (mutualistic) to one host at a time may be act as saprobes or parasite to other host or invarying environmental settings (Boddy and Griffith 1989; Petrini 1991).

Epiphytic microorganisms are different from endophytes by being only inhabitants in the phyllosphere/pylloplane of the plants (Leben 1965; Kharwar et al. 2010). Plant leaves have endophytic microorganisms inside them and epiphytic microorganisms on them. Endophytes are usually occurred in above ground plant tissues, but also intermittently reported in roots while mycorrhizae only confined to roots of higher pants and are distinguished from endophyte by having special structure like external hyphae or mantels and VAM.

Discovery of Fungal Endophytes

The association of fungi and bacteria with plants perhaps dates back to the emergence of vascular plants (Rodriguez and Redman 1997). Evidences found in the fossilized tissues of different plant parts like stems and leaves of plant-associated microbes have revealed that endophytic microbial associations with plant may have evolved from the time when higher plants first appeared on the Earth (Zhang et al. 2006). Although it is well established that the term endophyte was introduced quite earlier by de Barry in 1866, while few years back to him, Leveille and other workers acknowledged the fungi in wheat leaves as endophytic fungi (Leveille 1846; Riesen and Close 1987), but the existence of fungi inside plant without any

harm has been confirmed by Guerin (1898). For the first time, Freeman (1904) described entire life of *Lolium temulentum* with fungal endophyte in Darnel, New Zealand. Except a few infrequent works reported earlier only by the end of twentieth century, the endophytic study received the attention of scientists with a various prospects like bioactive metabolites, plant protection, ecology and evolution of endophyte. The fungal endophytes have been given more attention rather than bacterial or other microbial endophytes because of their more applicability. The year 1977 became an important year in the history of endophytic research when Bacon and his colleagues discovered endophytic fungus *Neotyphodium coenophialum* which is causative agent of "fescue toxicosis" (Bacon et al. 1977). Later on, it was confirmed that toxicity in plant was due to the toxic alkaloid "peramin" produced by the fungus *N. coenophialum*. The discovery of taxol, an anticancer drug from *Taxomyces andreanae* and an endophytic fungus of *Taxus brevifolia*, by Stierle et al. (1993) led the search worldwide for potential and novel endophytic microbes for better understanding of their ecological role and ability to produce potential bioactive natural products from such untouched and fascinating group of microorganisms.

Biodiversity of Fungal Endophytes and Distribution

Fungal endophytes are reported from every plant examined till date; however, an assessment of real fungal diversity of any plant is a major challenge to mycologist. Large numbers of fungal species have already been identified, and many folds are predicted to exist in diverse niches on this globe (Gunatilaka 2006). Now, it has been accepted that more than 270,000 existing plants harbour at least one or more than one species of endophytes (Strobel and Daisy 2003; Huang et al. 2007). The two-thirds of the total known mycoflora exist as symbionts of other living plant or animals as parasitic or commensalist, or mutualist (Pirozynski and Hawksworth 1988). Most studied fungus–plant association is mycorrhizae and plant pathogens (parasites). Over one million species are estimated to reside as endophytes in plants out of the total estimated 1.5 million fungal species on Earth (Dreyfuus and Chapela 1994). It means the majority of the fungal species are endophytes. They have ubiquitous existence and live in all vascular and non-vascular terrestrial as well as in aquatic plants (Petrini et al. 1992; Carroll 1988; Kharwar et al. 2011).

Endophytic fungi are a group of polyphyletic and highly diverse organisms that occur within asymptomatic tissues of plant. Temperate plants have been the most surveyed for endophytic fungal communities (Bills 1996), but now several tropical plants have also been investigated for their endophytic fungal suit with various applications (Cannon and Simmons 2002; Verma et al. 2007; Gond et al. 2011; Kharwar et al. 2011; Suryanarayanan et al. 2012; Mishra et al. 2012, Verma et al. 2014). They are found associated with every group of plants studied such as algae, bryophytes (liverworts, hornworts and mosses), pteridophytes (lycophytes, equisetopsids, ferns), gymnosperms and angiosperms (monocots and dicots) from the

extreme cold arctic to the warm tropics, and from agricultural fields to the most biotically diverse tropical forests (Hawksworth 1987; Kharwar et al. 2011). Endophytic fungi may be grouped according to their distribution in different groups of host plants.

Fungal Endophytes from Algae

Fungal endophytes are recovered from higher group of marine algae and establish endosymbiotic association with them. They are isolated from green algae, red algae and brown algae with antioxidant, anticancer, antimicrobial, antifungal, cytotoxic and other bioactivities (Suryanarayanan and Johnson 2014). More than 125 morphologically distinct endophytic fungi were isolated from different marine red, green and brown algae such as *Codium* sp., *Gracilaria* sp., *Halimeda* sp., *Caulerpa* sp., *Gelidium* sp., *Chaetomorpha antennina*, *Ulva fasciata, Sargassum* sp., *Turbinaria* sp. *Halimeda macroloba* with antimicrobial and enzyme activities (Mathan et al. 2013, Thirunavukkarasu et al. 2011). *Chaetomium globosum*, an endophytic fungus, is isolated from tissue of marine red alga *Polysiphonia urceolata* with several secondary metabolites (Wang et al. 2006). *Geniculosporium* sp., is reported as fungal endophyte of red alga *Polysiphonia* sp. (Krohn et al. 2005). An unidentified endophytic fungus is isolated from seaweed *Sargassum* sp., with significant antimicrobial activity (Yang et al. 2006). Interestingly, a novel isorhodoptilometrin-1-methyl ether with seven known compounds was isolated from *Aspergillus versicolor,* an endophyte of marine alga *Halimeda opuntia*. The biological property of ethyl acetate extracts and certain purified metabolites were screened for antimicrobial activity, anticancer activity and against Hepatitis C virus protease (Usama et al. 2012).

Fungal Endophytes from Bryophytes

The comprehensive study of endophytic association with bryophytes is lacking. Glomeromycotean fungi are widespread in symbiosis with the Marchantiales, Metzgeriales and Anthocerotales are the most primitive in all terrestrial fungal symbiosis (Duckett et al. 2004). Diversity and phylogeny of endophytic fungi were described very well in the tissues of liverworts and mosses in boreal, temperate and tropical forests and also compared with lichen fungal community structure (Davis et al. 2003; Kauserud et al. 2008; U'Ren et al. 2010). Some fungal endophytes are restricted to rhizoids of mosses only while some have whole thallus colonization. It was found that fungal endophytes isolated from rhizoids are ascomycetous while those from thalli are basidiomycetous or glomalean fungi (Boullard 1988). *Hymenoscyphus ericae* is isolated endophytically from Antarctic liverwort (*Cephaloziella exilifora*) (Chambers et al. 1999). Ultrastructures of fungal endophytes have been observed in *Phaeoceros laevis* (L) Prosk, an anthocerotophyte (Ligrone 1988). A cytological study has been done on the basidiomycetous

endophytes of jungermannialean (leafy) liverworts (Duckett et al. 2006). Many fungal endophytes recovered from three bryophyte species including *Barbilophozia hatchery* (liverwort), *Chorisodontium aciphyllum* and *Sanionia uncinata* (mosses) in the King George Island, maritime Antarctica, by culture-dependent method (Zhang et al. 2013).

Fungal Endophytes from Pteridophytes

Fungi as mycorrhizal association are well known to colonize the roots of sporophytic pteridophytes and rhizoids of fern prothallii. Fern gametophytes develop fungal symbiosis as mycorhizae to absorb nutrients and water from soil. Fungal association with lower pteridophyte ranges from obligatory in Psilophyta to leptosporangiate ferns (Boullard 1979). Some comprehensive studies are reported to demonstrate the endophytic fungal assemblage to roots of pteridophyte sporophytes. Kumaresan et al. (2006) reported endophytic fungi from primitive pteridophytes *Psilotum nudum.* Many fungal endophytes have been recovered from five species of ferns namely *Adiantum* sp., *Gleichenia linearis*, *Lygodium flexuosum*, *Pteris* sp. and *Selaginella* sp. (Kumarsan et al. 2013). Several endophytic fungi comprising *Absidia cylindrospora* and *Mortierella* of zygomycetes, few species of anamorphic ascomycetes and a sterile fungus of basidiomycete were recovered from the root of *Pteridium aquilinum* (Petrini et al. 1992). Association between fungal endophyte and achlorophyllous gametophyte of *Lycopodium clavatum* is identified by Schmid and Oberwinkler (1993) and is termed as lycopodioid mycothallus interaction. Endophytic fungi have been isolated from surface-sterilized gametophytes of *Schizaea pusilla* (Swatzell et al. 1996). Number of species belonging to 9 genera of aquatic hyphomycetes, viz. *Alatospora, Anguillospora, Campylospora, Clavariopsis, Heliscus, Lunulospora, Pestalotiopsis, Tetrachaetum* and *Tetracladium* were recorded as root endophytes of pteridophytes *Equisetum* sp., *Botrychium* sp. and some other ferns (Sati et al. 2009; Sati and Belwal 2005).

Fungal Endophytes from Gymnosperm

Fungal endophytes of gymnosperms especially conifers are well documented in past. Presence of fungal endophytes is very common to gymnosperm and recovered from every plant studied till date (Sieber 2007; Rodriguez et al. 2009). Several reports with diverse aspect of fungal endophytes–host relationship and their ecology in conifers foliage have been studied from a large and diverse assemblage of plants with evergreen leaves (Carroll and Carroll 1978). Carroll and Petrini (1983) have described very well the utilization of substrate by endophytic fungi in some conifer needles. Some endophytic fungi recovered from gymnosperms have been shown to produce secondary metabolites with very strong antifungal, antibacterial and anticancer activity (Tan and Zou 2001; Strobel and Daisy 2003). Some works on

changes in species composition and diversity of fungal endophytes in gymnosperm with spatial (bio geographic location) and temporal (season) variation are also reported (Sahashi et al. 1999; Rodriguez et al. 2009; Thongsandee et al. 2012).

Fungal Endophytes from Monocots

Many researches are available on endophytic fungi of monocot plants, especially grass endophytes. Grass endophytes harbour in the different tissues of grass species, which can play essential ecological roles in plant communities. It has been reported that these are systemic and often mutualistic for cold season grasses, especially in sub-family Pooideae (Clay 1991). Toxic alkaloids produced by grass endophytes are supposed to enhance resistance against herbivores (Clay and Schardl 2002). These fungi play a vital role and enhance the fitness of plant through drought resistance (Elmi and West 1995), by helping nutrient uptake and resistance against heavy metal (Malinowski et al. 2004). In India, *Balansia andropogonis* grass endophyte was first reported from *Andropogon aciculatus* by P. Sydow in 1914. Janardhanan and Ahmad (1997) reported *Balansia* and its anamorphic state *Ephelis* from various grasses found in Indian states of Bihar, Uttar Pradesh, Karnataka and Tamil Nadu. Besides agronomical important grasses, endophytic fungi *Epichloë* and their asexual *Neotyphodium* forms are also thought to interact symbiotically with woody plant and other grasses and have also been reported to be protected against herbivores and pathogens by their fungal endophytes produced antiherbivory and antimicrobial alkaloids.

Fungal Endophytes from Dicots

Fungal endophytes are surprisingly common in dicotyledonous plants. Large numbers of fungal species are being reported frequently from different plant parts of dicotyledonous tree from all over the worlds (Lin et al. 2007). Endophytic fungi associated with dicotyledonous plants are diverse and may be more diverse in tropical forests. Tropical plant fungal endophytes coexist with number of species often highly localized infections within individual leaves (Arnold et al. 2000). Many fungi isolated as endophyte from woody angiosperms appear to be very much related to pathogens (Saikkonen et al. 1998). Evidence to prove defensive mutualism with regard to herbivores resistance or abiotic stress is fragmentary. Therefore, it is generally thought that endophytes associated with leaves of woody angiosperms are unlikely to play protective or mutualistic roles with regard to the host plants they inhabit (Faeth and Fagan 2002). Arnold et al. (2003) have shown that endophytic fungi of angiosperm improve the fitness of host plant by reducing leaf injury and negative effect due to pathogen attack in *Theobroma cacao* a economically important tree of malvaceae family. Some studies on isolation and diversity of fungal endophytes are available from Indian angiospermic plants such as *Azadirachta indica, Aegle marmelos, Catharanthus roseus*, *Eucalyptus*

citriodora, Terminalia arjuna, Adhatoda zeylanica, Bauhinia phoenicea, Callicarpa tomentosa, Clerodendron serratum, Lobelia cotinifolia, *Crataeva magna, Adenocalymma alliaceum, Madhuca indica* and *Tinospora cordifolia* (Rajgopal and Suryanarayana 2000; Nalini et al. 2005; Raviraja 2005; Tejesvi et al. 2005; Verma et al. 2007; Gond et al. 2007; Kharwar et al. 2008, 2010, 2011; Mishra et al. 2012; Verma et al. 2014).

Fungal Endophytes from Plants of Unusual Habitats

Now many plants growing in extreme, harsh, or unusual habitats such as marine, halophytes, cryophytes, xerophytes, epiphytes and complete parasite plants have been reported for their endophytic colonization (Suryanarayanan et al. 2005). Some marine plants and weeds have been reported to harbour fungal endophytes (Alva et al. 2002; Suryanarayanan et al. 1998). Suryanarayanan and Kumaresan (2000) recovered many endophytic fungi from four halophytes *Acanthus ilicifolius* (Acanthaceae), *Arthrocnemum indicum*, *Suaeda maritima* (Chenopodiaceae) and *Sesuvium portulacastrum* (Aizoaceae) growing in estuarine mangrove forest of India. Few species of halophytes from temperate regions have also been studied for fungal endophytes (Petrini and Fisher 1986; Pelaez et al. 1998).

Except few preliminary reports of the occurrence of endophytic fungi in cactus species of *Opuntia*, there are very less works available on fungal endophytes of desert and arid area plants (Bills 1996; Fisher et al. 1994). Few fungal endophytes from desert plants have been reported and analysed their association with host; however, endophyte colonizations are thought to decrease rapidly with reducing the relative humidity and rainfall (Bills 1996). Around 23 species of fungal endophyte were isolated from different tissues of complete parasite plant *Balanophora japonica* (Ikeda et al. 2016). Species of *Phomopsis, Cladosporium*, *Colletotrichum* with many other sterile morphotypes were isolated and reported from complete parasite of angiosperm *Cuscuta reflexa* (Suryanarayanan et al. 2000).

Taxonomy and Evolution of Endophytic Fungi

Taxonomically, endophytic fungi are mostly belong to Ascomycotina or Basidiomycotina, but may also include the members Oomycetes (Sinclair and Cerkauskas 1996). Diversity of fungal endophytes within the plant parts and indifferent plants may be very high, but it was also found that the some closely related endophytes may dominate in particular host plant of the same family. Similarity among the fungal endophytes was found less in distantly related host tree plants (Sieber 2007). Differences in species composition of endophytes of two different groups of plant are more prominent. Studies suggest that Diaporthales group of fungi are dominant endophytes of the broad-leaved angiosperms including from plant of Aceraceae, Betulaceae and Fagaceae, respectively, whereas Helotiales were found dominant in

gymnosperms including plant from Cupressaceae and Pinaceae. Interestingly, during evolution divergence between higher plants into gymnosperms and angiosperms, the ascomycetous Diaporthalean and Helotialean were reported to occurred at the same period, about 300 million years ago based on molecular data and carbon dating (Schneider et al. 2004; Berbee and Taylor 2007; James et al. 2006).

Bitunicate Dothideales, Pleosporales, Mycosphaerellales and the Xylariales ascomycetes are found prominently in both the gymnosperms and angiosperm. This probably suggests the divergence of "Bitunicate" before the 300 million years ago from the common ancestor and, accordingly, before the gymnosperms–angiosperms diverged from their ancestor (Sieber 2007). It was also found that some pathogenic fungus of some host may act as symptomless endophyte on other host. This idea is supported by one study in which one *Colletotrichum* species causing disease to its main host but unable to produce disease symptoms on alternate host species. Furthermore, *Colletotrichum* was found enhancing growth and protect plant from biotic and abiotic stresses (Redman et al. 1999, 2001). Switch in behaviour of pathogen to mutualist on the other host might result from change in gene(s) expression pattern, or gene related to pathogenicity may be switched off in different environmental settings (Sieber 2007). Pathogenic behaviour is highly specific and depends upon plant defence system and how plant responded to pathogens. Mutualistic relationship of endophytic fungi with the host has been evolved from parasitic or pathogenic fungi (Saikkonen et al. 1998). However, possibility of reverse direction of evolution is also reasonable. Symbioses of plant roots with fungi have existed since the evolution of land plants on Earth. Fungal endophyte may have evolved similarly in aerial parts of the plant. The nature and direction of relationship from pathogenic to non-pathogenic and vice versa may have switched several times during course of evolution in response to changing environmental pressures which is major selection force for evolution (Carroll 1988; Sieber 2007). It was found that endophytes and congeneric pathogen of same host often are more closely related than to congeneric fungal endophytes of other host. In a study, relatedness of *Pinus* sp., fungal endophyte *Lophodermium pinastri* is found more to its pathogenic congeneric species *L. seditiosum* than to *Picea* species endophytes *L. piceae*. From this study, one can easily conclude that congeneric endophytes and pathogen of same host might have evolved from the same ancestor (Sieber 2007).

Species diversity of endophytic fungi in higher woody plants is more than grass endophytes. Woody plant endophytes colonize in wider range of diverse host plants than grasses fungal endophytes. Generally, woody plant fungal endophytes are not specific and have their diverse presence in different hosts unlike grass endophytes which are mostly specific to the host (Petrini 1991; Petrini et al. 1992). However, Petrini et al. (1992) have also discussed about the presence of many host specific endophytes in woody plants similar to systemic endophytes of grasses.

Based on phylogeny and life history traits, initially two groups of fungal endophyte have been recognized, as clavicipitalean (C-endophytes) and non-clavicipitalean (NC-endophytes) endophytes (Carroll 1988; Petrini 1991; Schulz and Boyle 2005; Stone et al. 2004). Endophytic fungi of the Clavicipitaceae

group form close association with their grass hosts. Endophytic species of grass colonize the aerial part of their hosts systemically and most of them show vertical transmission from one generation to next generation of host through the host plant seeds. Non-clavicipitalean endophytic species are non-systemic and their colonization restricted to certain part of the plant (Stone 1988). NC-endophytes represent a diverse range of species from several groups of ascomycetina, to deuteromycetina and have been reported from all plant species covering the range of habitat from temperate to tropical. They have also been reported from grasses (Sieber 2007; Stone et al. 2004). Rodriguez et al. (2009) further classified NC-endophytes into three different functional groups based on their life history, features and their ecological importance.

Clavicipitalean endophytic fungi are few in numbers, but phylogenetically related species, and they are very particular in growing on medium and restricted their presence in warm and cool season grasses (White 1993). Usually C-endophytes live systemically in the aerial part of the plant not to the root. The NC-endophytes are represented by three different groups based on their colonization, mode of spread and transmission from one generation to next generation in host plant biodiversity and phylogeny, including the biological roles (Table 9.1). First group of NC-endophytes have been reported from both root and shoot system while other two groups are restricted to either shoot or root tissues only. These endophytes are differing in the colonization pattern as well from much localized to

Table 9.1 Fungal endophytic classes based on different symbiotic criteria (after Rodriguez et al. 2009)

Serial no.	Criteria	Clavicipitalean (C-endophytes)	Non-vlavicipitalean (NC-endophytes)
1.	Host range	Highly specific, narrow range	Not specific, broad range
2.	Colonization (tissue/plants)	Both shoot and root systems, extensive colonization	Mostly shoot, sometimes confined to roots or shoot only, limited colonization
3.	Biodiversity and phylogeny	Less diverse, phylogenetically related group	Highly diverse, phylogenetically unrelated group
4.	Transmission and spread	Mostly vertical by mycelia and spores, systemic in nature	Mostly horizontal by spores, non-systemic in nature
5.	Biological roles	Specific, ecophysiological roles, improving the fitness of host plants	Not specific, undefined diverse roles in plant fitness improvement
6.	Future prospects	Mechanism of molecular interaction between host and endophytes	Diversity, phylogeny and their bioprospecting for novel active metabolites
7.	Examples	Hypocreales (Ascomycotina): *Balansia* spp., *Epichloë* spp. and *Claviceps* spp. etc. (Bacon and White 2000)	Mostly Hyphomycetes and Coelomycetes (Deuteromycotina): *Curvularia* spp., *Taxomyces andreanae*, *Pestalotia* spp., *Phoma* sp.

widespread plant colonization (Arnold et al. 2003; Rodriguez et al. 2009). They usually transmitted horizontally their spores; however, some are transmitted vertically by host seeds and rhizomes. Functionally, they are very important for the ability to provide the fitness of plant against biotic and abiotic stresses (Rodriguez et al. 2009).

Penetration, Colonization and Establishment of Endophyte–Host Relationship

The endophyte–host relationship is supposed to be complex and may vary from plant to plant and microbe to microbe depending upon condition. Diverse symbiotic (living together of different organisms; de Bary 1879) lifestyles occur among endophytes with their hosts. Plant–endophyte interactions have been referred as a continuum because the outcome of interaction can range from antagonism to mutualism (Saikkonen et al. 1998; Schulz and Boyle 2005). Endophytic microbes enter in host tissue same as the pathogenic microorganisms do enter into the plants. But, because of weak pathogenic behaviour of endophytes, plant produces defensive molecules in lesser amount around vicinity of infection which does not create hurdles in entry of endophytes compared to pathogens. Fungal endophytes enter into the plants via natural openings such as stomata, lenticels or wounds or directly through breaking the cuticle, epidermal cell walls (Bernstein and Carroll 1977; Petrini 1991, 1996). In order to penetrate the cuticle and cell walls, endophytic fungi can produce hydrolytic enzymes such as cutinase, pectinase, cellulase, hemicellulase, protease and lignin-peroxidases. Endophytic fungi are well known to produce extracellular enzymes of great importance (Choi et al. 2005).

After penetration, endophytes live intercellularly or intracellularaly or some time they restrict their growth to few cells around the point of penetration of host. Non-clavicipitaceous endophytes have been reported to be inter- or intracellular and often localized to single cell (Stone 1988; Suske and Acker 1989; Cabral et al. 1993; Ghimire and Hyde 2004). Endophyte after infection initially may colonize asymptomatically within host tissue, but they may be latent pathogen and may produce symptoms of disease in adverse condition of host or may be latent saprophytes by this way showing antagonistic behaviour on the other end of the continuum. Beneficial role of some endophytes to their hosts are well studied. For example, *Neotyphodium* and *Epichloë* endophytes of grasses confer the host tolerance against herbivory, salt, drought, heat, metals and also help in nutrient mobilization to host plant (Schardl et al. 2004; Redman et al. 2011). Studies from grass-endophyte systems suggest that endophytes are herbivore antagonists and enhance plant growth by showing mutualism with host (Clay 1991) and this is because of being host specific and systemic in nature. Tree endophytes are not host specific, exhibit discrepancy in nature of association, and they may switch from mutualist to antagonist depending upon environmental pressure or become saprophytic when plant starts senescence or dies (Sieber 2007).

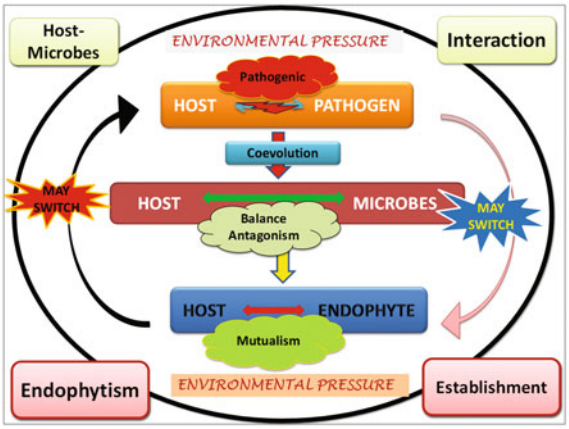

Fig. 9.1 Picture showing establishment of endophytism

Studies hypothesized that both the pathogen–host and endophyte–host interactions engaged constant mutual antagonisms, endophyte provides better fitness to the host, and in turn it utilizes nutrients from the concerned host. It is believed that pathogen–host interaction is imbalanced association and resulted into symptoms of disease. However, endophytes–host association is balanced, and it is beneficial relationship. Successful establishment of endophyte–host relationship depends upon internal and external conditions of host and microbes. Mutualistic symbiotic lifestyle may be a result of some biochemical and/or genetic communications occur between microbe and host plant. Finally, after the establishment of mutualistic relationship they benefit each other (Fig. 9.1). Host–endophyte relation may switch to host–pathogen or vice versa due to some environmental pressure that could lead them to a genetic modification at certain locus and established respective nature of relationship (Fig. 9.1). The genotype of host and fungus is an important factor to determine the establishment of symbiotic relation. Freeman and Rodriguez (1993) experimentally shown that mutation at a single genetic locus in *Colletotrichum magna* could change isolate from a pathogen to a non-pathogenic mutualistic endophytes.

Modes of Transmission of Endophytic Fungi and Host Specificity

Endophytic fungi have two modes of transmission i.e. vertical transmission and horizontal transmission. Vertical transmission occurs when endophytic fungi move from one host to their progeny via host tissues like host seeds, vegetative propagules, means from one generation to next generation. Vertical transmission is found in systemic infected endophytic fungi which are restricted to certain group of grasses. They show monophyletic evolution of life. In horizontal transmission,

fungus travels by its sexual or asexual spores. Horizontally transmitted endophytes form local latent infections and are found almost in all plants. They are non-specific, are highly diverse and show polyphyletic origin of life.

Grass endophytes are systemic and transmitted vertically through host seeds or vegetative propagules. *Neotyphodium* (formerly *Acremonium*) is a vertically transmitted fungal endophyte (Clay and Schardl 2002). *Epichloe*, the sexual form of *Neotyphodium,* transmits vertically, but can also be transmitted horizontally via fungal spores formed around the developing inflorescence of the host (Saikkonen et al. 2004). *Epichloe* forms stromata in developing inflorescence causing choke disease in host plant. The choke disease suppresses seed formation of the host plant. This gives an example of the latent pathogenicity of endophytes. Thus plant–endophyte associations may be mutualistic to antagonistic. According to Petrini (1991), endophytes are either avirulent or less virulent, or virulent in a latent phase, in which pathogenicity is checked by exogenous or endogenous physiological or ecological changes.

In grasses, infections of fungal endophytes are systemic throughout the host life. *Epichloe* and *Balansia*, two common genera of clavicipitaceae (Ascomycotina), are systemic endophytes of grasses (White 1993). *Ephelis* is anamorphic form of *Balansia*. *Balansia claviceps* and *B. obtecta* are mostly found on host inflorescences. Grasses have also non-systemic, non-seed-born endophytic fungi. Such non-systemic fungal endophytes are specially epiphytes, as *Alternaria alternata*, *Cladosporium* species and *Epicoccum purpurascens* or many pathogenic fungi of grasses (Stone et al. 2004). Barley leaves are occupied by pathogenic fungi *Didymella phleina*, *Alternaria* sp. and *Stemphylium botryosum* (Riesen and Close 1987). A very common leaf-and-culm-blotch-disease-causing fungus, *Phaeosphaeria nodorum,* is found as endophyte in winter wheat (*Triticum aestivum*) (Riesen and Sieber 1985).

Biogeographical and seasonal distribution of endophytic fungi in plants depends mainly on their modes of transmission. The vertically transmitted grass endophytic fungi have relatively long generation time covering several generations of grass. In contrast, horizontally transmitted endophytes colonize mostly in the aerial part of the plants throughout the growing season (Faeth and Fagan 2002). Many endophytic fungi which produce slimy spore masses may easily be dispersed by rain (Slippers and Wingfield 2007). Because of the localized infections of the horizontally transmitted fungal endophytes, the dispersal of spores takes place through mostly senescent and abscised leaves or other parts of plant.

Spatial and Temporal Variation of Endophytic Fungi

Ubiquitous occurrence of endophytes is true, and all plant species studied so far harbour one or more fungal endophytes in their different parts (Stone et al. 2000). Endophytic fungal diversity believed to be more in tropical forests (Frohlich and Hyde 1999), which follow the similar pattern of diversity of other organism in

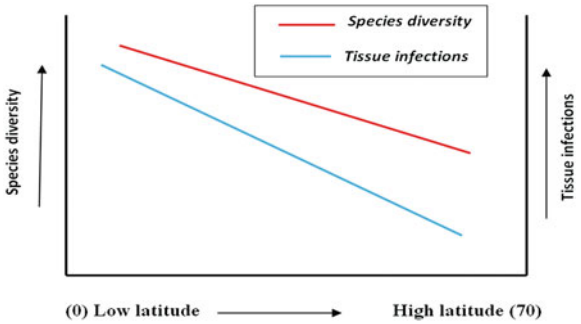

Fig. 9.2 Latitudinal gradient of endophyte infection and endophyte diversity (adopted from Arnold and Lutzoni 2007)

tropics. Presence of highly diverse mycoflora as whole from different sources in tropical rain forest supports these studies (Hawksworth 2001). Overall occurrence of endophytes–host plant ratio in temperate is 6:1 (Hawksworth 2001), however for tropical plants this ratio supposed to increase to 33:1 endophytes–host plant. Arnold and Lutzoni (2007) in their surveys showed that factors responsible for plants–microbes association differ in their different biogeographical location. They found significant increase in endophytic fungal colonization from 1% in arctic plant tissues to 99% in the plant from tropics. Following the trend of other organism diversity, foliar endophyte diversity in terms of both species richness and evenness in same host or host community found increasing from higher latitude to lower latitude (Fig. 9.2). Endophytic diversity also varies from tissue to tissue even in same host plant, and this may be because of difference in chemical environment of particular tissue.

Some studies on endophytic fungal assemblage, the diversity, and their pattern with temporal changes in deciduous and non-deciduous plants are available (Sahashi et al. 1999; Rodriguez et al. 2009; Thongsandee et al. 2012; Mishra et al. 2012). Influence of temporal variation on composition of total fungal endophytes has been recorded along with different sampling dates for all three different organs i.e. leaves, petioles and twigs of *Ginkgo biloba* (Thongsandee et al. 2012). Two dominant endophytes, *Phyllosticta* and *Phomopsis,* showed different pattern of distribution. *Phyllosticta* sp. was isolated from both leaves and petioles tissue but only in period between August and October; however, *Phomopsis* sp. was isolated only from twigs tissue but throughout the growing season of the host plant. This study explains the tissue specificity of endophytes and variation of their dominance temporally. Mishra et al. (2012) have also observed more pronounced variation in endophytic fungal diversity and distribution in season and tissue type compared to location of *Tinospora cordifolia*.

Role of Fungal Endophytes in Plant Protection

Endophytic microbes have been shown their ability to benefit host plant growth, provide defence, and can be source of various agriculturally and medicinally important secondary metabolites. Fungal endophytes benefit plant by modulating plant growth (Dai et al. 2008), improving resistance against multiple (biotic and abiotic) stresses (Lewis 2004; Malinowski et al. 2004; Redman et al. 2002, 2011) and protection from pathogens and insects (Wilkinson et al. 2000; Tanaka et al. 2005; Vega et al. 2008). By several means endophytic fungi may protect plant from pathogen, which can be categorized as direct means, indirect means and ecological means (Gao et al. 2010). It has been suggested that colonization of fungal endophytes in plant may induce the phytoalexin production by which they protect plant from pathogen infection and disease (Gao et al. 2010).

In direct way, endophytic microbes suppress pathogens and other microbes either by producing antimicrobial agents or by secreting varieties of hydrolytic enzymes. However, communication between endophytic fungi and host along with pathogens is very important in determining plant susceptibility to any disease (Arnold et al. 2003). In the indirect way, endophytes may protect host plant by conferring resistance, by producing secondary metabolites and by stimulating growth and physiology of plants (Fisher et al. 1984). Extract and pure compounds isolated from several endophytic fungi have been found very active against many phytopathogens tested. The endophytic fungi, *Colletotrichum gloeosporioides,* was isolated from healthy leaves of *Cryptocarya mandioccana* and found inhibiting fungal phytopathogens *Cladosporium cladosporioides* and *C. sphaerospermum* (Inacio et al. 2006). *Cryptosporiopsis quercina,* an endophytic fungus of *Tripterygium wilfordii,* produced an antimycotic compound, cryptocandin, which was found able to inhibit many plant pathogenic fungi as well as human pathogens including *S. sclerotiorum*, *Botrytis cinerea* and *Candida albicans* (Strobel et al. 1999). A number of antifungal compounds like indole derivatives, indole-3-acetic acid and indole-3-ethanol, a sesquiterpene, and a diacetamide have been reported by Yue et al. (2000) from the cultures of *Epichloe* and *Neotyphodium* species which showed antifungal activity against *Cryphonectria parasitica*. The metabolites, ethyl 2,4-dihydroxy-5,6-dimethylbenzoate and phomopsilactone, have been isolated from *Pestalotia cassiae,* an endophytic fungus of *Cassia spectabilis,* which showed strong antifungal activity against the phytopathogens, *C. cladosporioides* and *C. sphaerospermum* (Silva et al. 2005).

In addition to suppression of phytopathogens through different mechanisms, the endophytes also increase the concentration of host phytochemicals and improve plant defences against plant pathogens by modulating the host metabolism (Giménez et al. 2007). Endophytes are well known to improve plant growth and development. An increase in plant growth will makes plant more fit against variety of abiotic and biotic stresses, reflecting plant fitness or perseverance against stress (Kuldau and Bacon 2008). Narisawa et al. (2000) found that the root endophytic fungi, *Heteroconium chaetospira*, inhibited the growth of one of most destructive

pathogen *Verticillium* sp. in cabbage plant. Several endophytic fungi i.e. *H. chaetospira, Phialocephala fortinii, Fusarium, Penicillium* and *Trichoderma* after being inoculated on to axenically reared aubergine seedlings able to suppressed completely the pathogenic symptoms of a post-inoculated, virulent strain of *V. dahliae* (Narisawa et al. 2002). Endophytic fungi are now established for their role against plant pathogenic nematodes. An endophytic fungus *F. oxysporum* and an arbuscular mycorrhiza *Glomus coronatum* showed nematicidal activity against the important nematode *Meloidogyne incognita* in tomato plant (Diedhiou et al. 2003).

Endophytic niche becomes established after successful colonization of endophytic microbes in host tissue. Competition for colonization space and nutrition, hyperparasites and predation are between endophytes and pathogens are ways to check pathogenic infection and help in plant protection. "Endophytic microbes" recognition and colonization rapidly occupy ecological niche in host tissue and put down less pace for pathogenic microbes to colonize; by this way, endophytic fungi reduce the chances of infection by pathogens and protect host (Pal and Gardener 2006). Endophytic fungi protect plant against biotic and abiotic stresses by several means as discussed above. Successful identification and selection of endophytic fungi of specific crop plants are the need of today which can replace the use of chemical pesticides and fertilizers with these endophytes which would be more sustainable and economical.

Conclusion

Fungal endophytes are now well established and recovered microbial flora from every group of plants from thallophytes to angiosperm, from xerophytes to aquatic plants for their potential use for human mankind. Since there is large gap exists between estimated diversity (1.5 millions) of fungi and described fungi (0.1 million), working with endophytic fungal diversity gives great hope to discover new group of fungi and would fill the breach between known and unknown with novel value. Bioactivities shown by secondary metabolites produced by fungal endophytes already give us new alternative to isolate and characterize the novel and host mimetic potential drugs to combat day by day emerging pathogenic and threatening diseases. Although many preliminary reports are available regarding bioactive metabolites, it is a great challenge for the biologist, chemist, corporate and government to commercialize the final product to the society which needs really more intensive work with collaboration. The problem that put the researchers associated with fungal endophytes under stress is the production of desired or host mimetic compounds continuously for several generations. To overcome this hurdle, recently researchers from across the globe adopting the epigenetic modulation either through chemical/or food component modifiers to induce the gene/or gene clusters through DNA methylation, demethylation, histone modification, chromatin remodelling and miRNA. Epigenetics is the study of changes in the expression and regulation of the genes that are not dependent on DNA sequences. The epigenetic changes may

induce many 'cryptic metabolites' isolation which are not produced under normal conditions, and these changes may be either heritable or reversible. It may also enhance the production of known compounds many fold to reduce the unavailability of required secondary metabolites.

Another fascinating aspect of fungal endophyte is their role in plant protection against biotic and abiotic stresses. Fragmentary reports are available on thermal tolerance, metal tolerance, salt tolerance and fitness of plant against pests, but to know the exact molecular mechanism that endophytes conferring tolerance in the plant against diverse stresses need much focused and precise researches. Working on any aspect of the fungal endophytes has great future and most important its colossal outcome that could answer problems of basic science of plant–microbe interaction to fulfil necessity of the society.

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Fusarium oxysporum: Genomics, Diversity and Plant–Host Interaction

10

Anjul Rana, Manvika Sahgal and B. N. Johri

Abstract

Fusarium oxysporum is amongst the most important and diverse phytopathogenic fungi infecting almost 150 plant species, pathogen of each being specific and identified as formae speciales. It is a broad host range pathogen employing various infection strategies. Considering the economic importance and availability of sequenced genomes of several *Fusarium* species, its interaction with plant host is under intense investigation. Comparative genomics of four *Fusarium* species (*Fusarium graminearum*, *Fusarium oxysporum* f.sp. *lycopersici*, *Fusarium solani* and *Fusarium verticillioides*) have led to identification of basic and specialized/dynamic pathogenicity genes that confer host specialization. Fungal pathogenicity mechanisms, rapid emergence of pathogenic lineages and polyphyletic origins of host specialization have been identified but regulation of host and tissue specificity is still not known. Although comparative genomics, transcriptomics and proteomic analysis have greatly accelerated the identification of fungal functional genes, but assigning definitive roles is still a challenging task.

Keywords

Fusarium oxysporum · Diversity · Fungal–plant interactions · Genomics Pathogenicity genes · *Six* genes

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Fusarium: An Overview

Fusarium is a filamentous fungi (*Sordariomycetes: Hypocreales: Nectriaceae*) containing phytopathogenic and toxigenic species. The genus *Fusarium* wasfirst described by Link in 1809 as *Fusisporium* and is presently known as *Fusarium*, referred to as *Fusarium* sensu Wollenweber (Wollenweber 1931; Wollenweber and Reinking 1935). The genus is highly diverse with twenty monophyletic species complex and outgroups of nine species. Infestation of *Fusarium* coincides with that of the flowering plants nearly 91.3 million years ago (Fig. 10.1). *Fusarium* species are distributed on the plants, in soil and in water either as parasites, endophytes or saprophytes. Plant pathogenic *Fusarium* species cause wilts, blights, rots and cankers affecting field, horticultural, ornamental and forest crops in both agricultural and natural ecosystems. Fusaria also produce diversified toxic secondary metabolites (such as trichothecenes and fumonisins that can contaminate agricultural product, making them unsuitable for food and feed; trichothecenes can also act

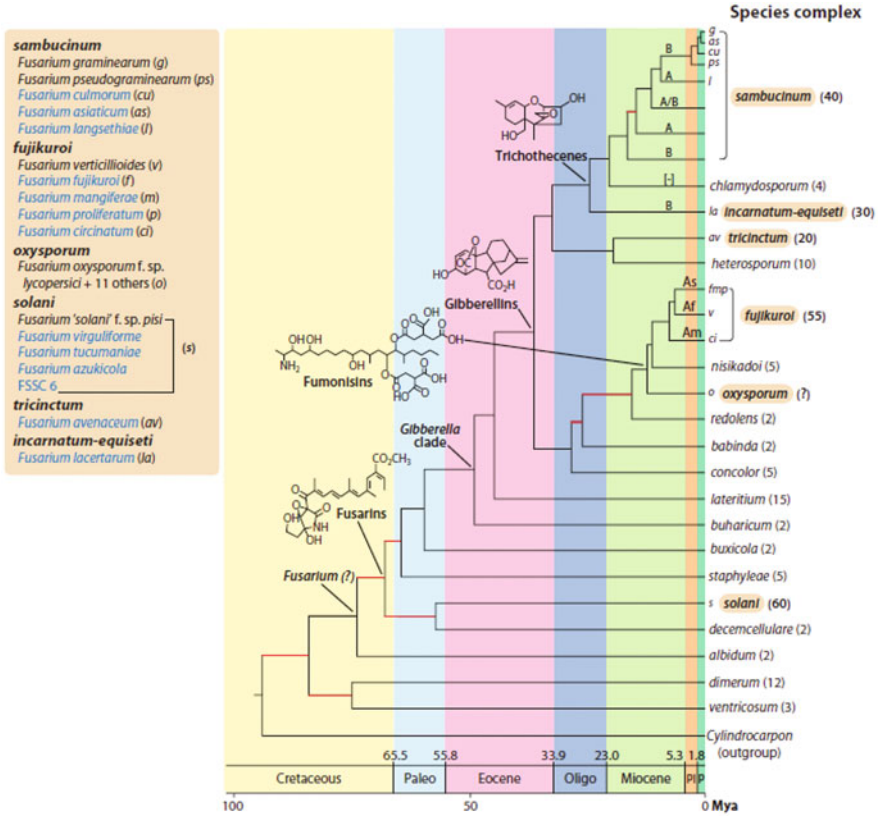

Fig. 10.1 Evolutionary diversification of the 20 *Fusarium* species. (reproduced from Ma et al. 2013, which was modified with permission from O'Donnell et al. 2013)

as virulence factor in plant diseases. A few *Fusarium* species are opportunistic human pathogens also causing corneal infections (O'Donnell et al. 2004).

Diversity Amongst *Fusarium* Species

Fusarium pathogens have diverse life cycles, niche specialization, host adaptation and specificity. *Fusarium graminearum* (Fg) and *Fusarium verticilloides* (Fv) are a narrow host range pathogens infecting predominantly the cereals, whereas *Fusarium oxysporum* (Fo) has a broad host range and infects both monocotyledonous and dicotyledonous plants (Armstrong and Armstrong 1981); besides, it is also an emerging pathogen on immuno compromised patients (O'Donnell et al. 2004) and other mammals (Ortoneda et al. 2004). *Fusarium* species vary in reproduction strategy; Fo is asexual, others are both asexual and sexual with either self-fertility (homothalism) or obligate out-crossing (heterothalism). *Fusarium* species produce meiotic (sexual) spores and at least three types of mitotic (asexual) spores. However, all *Fusarium* species do not produce all type of spores: Also, less than 20% of *Fusarium* species reproduce sexually (Fig. 10.2).

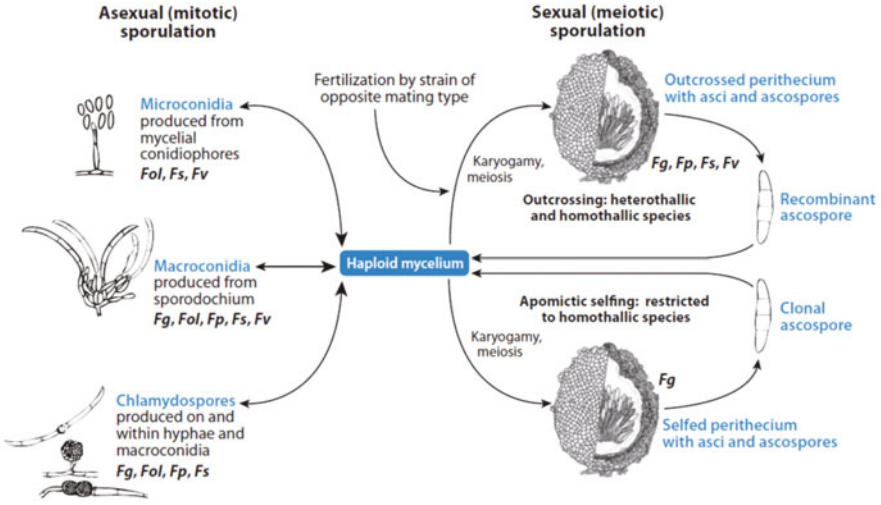

Fig. 10.2 Generalized life cycle of *Fusarium* depicting varying reproduction strategy (*Source* Ma et al. 2013) *Abbreviations* Fg, *F. graminearum*; Fol, *F. oxysporum* f. sp. *lycopersici*; Fp, *F. pseudograminearum*; Fs, *F. solani* f. sp. *pisi*; Fv, *F. verticillioides*

Fusarium oxysporum

Fusarium oxysporum Schlechtend, Fr. emended by Snyder and Hansen (1940) is an anamorphic species within the genus *Fusarium*. It is genetically heterogenous, polytypic morphospecies (O'Donnell and Cigelnik 1997; Waalwijk et al. 1996) which represents most abundant and ubiquitous soil-borne fungus; few strains have been reported from tundra soils as well (Stoner 1981) which exist as saprophytes and pervasive plant root endophytes. During saprophytism mode, *Fusarium* species degrade lignin (Rodriguez et al. 1996; Sutherland et al. 1983) and complex carbohydrates associated with soil debris (Christakopoulos et al. 1995, 1996). Root endophytic *Fusarium* species may be pathogenic or beneficial. A few strains are also pathogenic on gymnosperms. Pathogenic species within *F. oxysporum* have been differentiated into opportunistic, true pathogens and obligate pathogens based on the level/specialization of fungal–plant interactions (Scheffer 1991). Opportunistic parasites colonize weakened host plants or enter through wounds, have broad host range and exhibit low virulence. True pathogens require living plants for their growth; however, it can survive outside their hosts also, but are highly virulent on few host species. Obligate pathogens essentially require living host plant to complete their life cycle. They utilize host plant metabolism for their own growth, and in process alters plant growth pattern and morphology (Jackson and Taylor 1996). Members of *F. oxysporum* species complex are capable of causing vascular wilt diseases in over one hundred agronomically important plant species. However, individual *F. oxysporum* isolates are characterized by a high degree of host specificity; isolates that are pathogenic on single host are grouped into a forma specialis, e.g. *F. oxysporum* forma specialis *lycopersici* for tomato pathogens. Several *F.oxysporum* formae speciales consist of multiple independent lineages that have evolved polyphyletically. Interestingly, substantial genetic diversity has been revealed by molecular phylogenetic studies amongst isolates, supporting the present view that *F. oxysporum* represents a species complex (FOSC).

Reproduction

Fusarium oxysporum reproduces asexually, and its sexual state has never been observed (Booth 1971); it produces chlamydospores, microconidia and macroconidia (Nelson et al. 1983). Microconidia are uninucleate which germinate poorly with germination efficiency ranging from 1 to 20% (Ebbole and Sachs 1990). Macroconidia are multinucleate and germinate rapidly. Chlamydospores are resulting from the structural modification of vegetative hyphae or a thick-walled conidial cell and accessory spores (Schippers and van Eck 1981); *F. oxysporum* is diversified on shape of macroconidia, structure of micro-conidiophores, formation of chlamydospores (Beckman 1987).

Formae Speciales

Pathogenic and non-pathogenic *F. oxysporum* species cannot be distinguished morphologically unless pathogenic tests are performed. Pathogenic isolates of *F. oxysporum* (Fo) exhibit high level of host specificity which is directly linked to its pathogenicity to various plant species (Fravel et al. 2003). There are over 150 described formae speciales for Fo (Gordon 1965; Michielse and Rep 2009). Single forma specialis consist of isolates with the ability to cause wilt on a unique host or group of plant host species (Table 10.1). Thus, formae speciales is defined as an informal rank in classification scheme assigned on ability to cause disease in a unique host. Preliminarily, a forma specialis may be assigned to a strain based on the host from which a *F. oxysporum* isolate was recovered. It has been assumed that members of a forma specialis (f. sp.) are closely related and may have arisen from a common ancestor (Correll 1991; Kistler 1997). However, considerable genetic diversity has been reported within representative isolates of a forma specialis based on sequence comparisons in conserved regions of mitochondrial and nuclear DNA (Guadet et al. 1989; O'Donnell 1993). Kim et al. (1992, 1993) have analysed mt DNA of five formae speciales within cucurbitaceae, f. sp. *cucumerinum*, f.sp. *lagenaria*, f.sp. *luffae*, f.sp. *melonis* and f.sp. *niveum* that are pathogens of cucumber, calabash gourd, vegetable sponge, muskmelon and watermelon, respectively, and identified, fourteen mt DNA haplotypes within each forma specialis, out of which a few were common across formae speciales. Thus, all five formae speciales within cucurbitaceae are monophyletic (Kim et al. 1993). Similarly, Fo f. sp. *melonis* isolates were placed in two separate clusters, one closely related to Fo f. sp. *lagenaria* and other to Fo f. sp. *melonis* cluster. On the other hand, *F. oxysporum* isolates pathogenic to banana (f.sp. *cubense*) are more divergent than those of cucurbits. Koenig et al. (1997) identified 72 RFLP haplotypes in 165 isolates of f. sp. *cubense* and placed them into two major groups and seven lineages. Two lineages of Fo *cubense* were genetically similar to an isolate of f. sp. *niveum*, than to each other. Moreover, *F. oxysporum* f. sp. *cubense* strains from all over the world were placed in 10 clonal lineages. Thus, it is concluded that Fo *cubense* has polyphyletic origin, and pathogenicity to banana is acquired independently.

The isolates within forma specialis also have overlapping host ranges. Gerlagh and Blok (1988) reported that Fo causing wilt in cucumber was pathogenic to both muskmelon and watermelon and grouped it as f. sp. *cucurbitacearum*.

Pathogenic Races

Pathogenic races are sub-divisions of individual forma specialis based on differential virulence to various cultivars of the same host (Correll 1991). *F. oxysporum* forma specialis *cucumis* (Armstrong and Armstrong 1978) constitute, five races (0, 1, 2 and 1, 2), and their pathogenicity to different melon cultivars varies (Risser et al. 1976). All pathogenic races within a forma specialis might have a single

Table 10.1 List of *Fusarium oxysporum* formae speciales along with its host plant

S. no	Formae speciales (f.sp.)	Habitat/crop hosts	
		Botanical name	Common name
1.	*anoectochili*	*Anoectochilus formosanus*	Jewel orchid
2.	*aechemeae*	*Aechema fasciata*	
3.	*albedinis*	*Phoenix dactylifera*	*Date palm*
4.	*anethi*	*Anethum graveolens*	*Dill*
5.	*apii*	*Apium graveolens* *Tithonia rotundifolia*	*Delery* *Mexican sunflower*
6.	*asparagi*	*Asparagus officinalis*	*Asparagus*
7.	*batatas*	*Ipomoea batatas* *Nicontiana tabacum*	*Sweet potato* *Tobacco*
8.	*betae*	*Beta vulgaris*	*Beet root*
9.	*callistephi*	*Calistephuschinensis*	China aster
10.	*cannabis*	*Cannabis sativa* L.	Hemp
11.	*carthami*	*Carthamus tinctorium* L.	Safflower
12.	*cassiae*	*Cassia tora*L.	
13.	cattleyae	*Cattleya* spp.	Orchid
14.	*cepae*	*Allium* spp.	Onion
15.	*chrysanthemi*	*Chrysanthemum* spp.	
16.	*ciceris*	*Cicerarietinum*	Chickpea
17.	*coffeae*	*Coffea arabica* L.	Coffee
18.	*conglutinans*	*Brassicaoleracea* L. var.*capitat*	Cabbage
19.	*crassulae*	*Crassula ovata*	
20.	*cubense*	*Musa* spp.	Banana
21.	*Cucumerinum*	*Cucumis sativus* L.	Cucumber
22.	*Cyclaminis Gerlach* earlier known as *aurantiacum*	*Cyclamen persicum* Mill	Cyclamen
23.	*delphini* Laskaris	*Delphinium cardinale*	Forking larkspur
24.	*dianthi*	*Dianthus* spp. *Lychnis chalcedonica* L.	Carnation Maltese cross
25.	*echeveriae*	*Echeveria gavoides*	
26.	*elaeidis* Toovery	*Elaeis guineensis* Jacq.	Oil palm
27.	*eucalyptis* Arya & jain	*Eucalyptus gomphocephala D.C, E. rudis Endl.*	
28.	*fragariae*	Strawberry	
29.	*gerberae*	*Gerbera jamesonii* Hook	
30.	*gladioli*	*Gladiolus* spp., *Babina* spp. *Crocus* spp., *Freesia* spp., *Iris* spp., *Ixia* spp., *Sparaxis* spp., *Streptanthera* spp., *Tritonia* spp., *Watsonia* spp.	Gladioli and other flowers

(continued)

Table 10.1 (continued)

S. no	Formae speciales (f.sp.)	Habitat/crop hosts	
		Botanical name	Common name
31.	*hebae* Raabe	*Hebe buxifolia* (=*Veronica buxifolia* Benth.)	
32.	*herbemontis*	*Vitis aestivatis* Michx. *V. cinerea* *V. vinifera* L. hybrids	Herbemont grapes
33.	*lathyri* Bhide & Uppal	*Lathyrus sativus* L.	Lantana
34.	*lentis*	*Lens esculenta* Moench.	Lentil
35.	*lilii* Imle	*Lilium spp.*	Lily
36.	*lini*	*Linumusitatissimum* L.	Flax
37.	*lupini*	*Lupinus luteus*, *L. albus*, *L. angustifolia, L. mutabilis*	Lupine varieties
38.	*luffae*	*Luffa cylindrica*	
39.	*lycopersici*	*Lycopersiconesculentum*	Tomato
40.	*mathioli* Baker	*Mathiola incana var annua L.*	Stock
41.	*medicaginis* Weimer	*Medicago sativus L.*	Alfalfa
42.	*melongenae*	*Solanum melongena* L.	Egg plant
43.	*melonis*	*Cucumis melo* L.	Muskmelon
44.	*momordicae*	*Momordica charantia*	Balsam pear
45.	*narcissus*	*Narcissus pseudo-narcissus* L.	Daffodil, trumpet narcissus
46.	*nelumbicolum*	*Nelumbo nucifera* Gaertn	Lotus
47.	*nicotianae* Johns.	*Nicotiana tabacum* L.	Tobacco
48.	*niveum*	*Citrullusvulgaris* Schrad.	Water melon
49.	*opuntiarum* Pettinari	*Opuntia fucus-indica* Mill.	Spine less cactus alongwith other cactus
50.	*passiflorae* Gordon apud Purss	*Passiflora edulis*	Passion flower
51.	*palmae*	*Syagrus romanzoffiana* *Washingtonia robusta*	(Queen palm) (Mexican fan palm)
52.	*papaveris*	*Papaver nudicaule*	Iceland poppy
53.	*perniciosum*	*Albizzia* spp.	Mimosa
54.	*phaseoli* Kend. & Snyd.	*Phaseolus vulgaris L.*	Kidney bean
55.	*pini*	*Coniferae*	Conifers
56.	*phormii*	*Phormium tenax Forst.*	New Zealand flax
57.	*pisi*	*Pisum* spp.	Pea
58.	*psidii* Prasad, Mehta & Lal	*Psidium guajava* L.	Guava

(continued)

Table 10.1 (continued)

S. no	Formae speciales (f.sp.)	Habitat/crop hosts	
		Botanical name	Common name
59.	*querci*	*Quercus* spp.	Oak
60.	*radici-lupini* Weiner	*Lupinus angustifolius* L., *L. luteus* L., *L. albus* L.	
61.	*raphani* Kendr, Snyd.	*Raphanus sativus* var. *longi pinnatus* Bailey	Radish
62.	*racini*	*Ricinus communis L.*	Castor bean
63.	*rhois*	*Rhus typhina L.*	Slaghorn sumac
64.	*sedi* Raabe	*Sedum amecamecanum*	
65.	*sesami* Castell.	*Sesamum indicum L.*	Sesame
66.	*sesbaniae* Singh	*Sesbania aegyptiaca*	
67.	*spinaciae* (Sherb.) S & H	*Spinacia oleracea L.*	Spinach
68.	*stachydis*	*Stachys sieboldii Miq.*	Japanese artichoke
69.	*tracheiphilum*	*Vigna sinensis* *Glycine max*	Cowpea Soybean
70.	*trifolii* Bilai	*Trifolium* spp.	Clover
71.	*tuberosi*	*Solanum tuberosum* L.	Potato
72.	*tulipae*	*Tulipa* spp.	Tulip
73.	*vanillae*	*Vanilla planifolia*	Vanilla
74.	*vasinfectum*	*Gossypium* spp.	Cotton

ancient ancestor (monophyletic forma specialis) or affiliated to distinct clades (polyphyletic forma specialis).

Vegetative Compatibility Groups (VCGs)

Vegetative compatibility group (VCG) comprises of isolates that undergo somatic fusion and form stable heterokaryons. They are genetically similar and represent a clonal population (Puhalla 1985; Kistler 1997; Gordon and Martyn 1997). Moreover, a VCG could also be correlated with virulence (Katan et al. 1989; Manicom et al. 1990).

There is a correlation between forma specialis, VCG and pathogenic races. Sometimes all isolates of a forma specials correspond to a single VCG (Puhalla 1985). Within certain formae speciales, vegetative compatibility could be used as a method for identifying and differentiating pathogenic races. For example, a large collection of isolates each of Fo *apii* race 2, a pathogen of celery and Fo *vasinfectum* race 3, a pathogen of cotton from diverse geographical locations correspond to a single VCG. FOX isolates pathogenic to crucifers are placed in three distinct VCGs, each containing isolates pathogenic to a specific host. All Fo *niveum* isolates

are placed in three VCGs. VCG1 consist of all race 2 isolates, VCG 2 comprises of race 1 isolates from USA (all areas except Florida), Australia and Taiwan. VCG3 includes isolates from Florida.

Vegetative compatibility cannot always be used to identify races within a forma specialis because more than one race has been reported in a single VCG; also, isolates of single race may as well belong to different VCGs, e.g. four VCGs have been identified in f.sp.*pisi*, races 1 and 6 constitute single VCG, race 5 another and race 2 isolates were placed in two VCGs. Eight VCGs are identified in Fo*melonis,* out of which one contains isolates of different pathogenic races (Jacobson and Gordon 1988). Eleven VCGs have been identified in Fo *cubense* isolates from all over the world (Ploetz 1990; Ploetz and Correll 1988); a single VCG comprises of multiple races and a given race may belong to multiple VCGs. Similarly, in Fo *lycopersici,* three known races form single VCG as well isolates of one race are placed in multiple VCGs viz., race1 isolates belong to 41 different VCGs (Elias and Schneider 1991) and 46 distinct VCGs have been identified amongst a collection of isolates pathogenic to asparagus during greenhouse assay for testing pathogenicity (Elmer and Stephens 1989).

The high degree of VCG diversity has been observed in pathogenic and non-pathogenic strains of *F. oxysporum*. The mutations amongst isolates of single VCG lead to changes in virulence, which could be strong, weak, or non-existent (Leslie 1993, 1996). In general, RFLP patterns of mitochondrial (mt) DNA are identical within a VCG but vary between different VCGs of same formae speciales. All 44 isolates of Fo *albedinis* recovered from entire geographical range of disease occurrence were represented by a single VCG because of similarity in mt and nuclear DNA (Tantaoui et al. 1996). Four different models have been proposed to explain evolutionary relationships between VCG-race diversity in *F. oxysporum* (Kistler and Momol 1990). The VCG-race diversity is supported by genomic DNA and mitochondrial DNA (mt DNA), restriction fragment length polymorphism (RFLP) profiles. In *F. oxysporum, f. sp. melonis*a single VCG is shown to be associated with multiple races, e.g. VCG 0134 is associated with all four known races race 0, race1 and race1, 2 within f.sp. *melonis*; these races have identical mt DNA (Jacobson and Gordon 1990a, b) and nuclear DNA haplotypes (Schroeder and Gordon 1993). Minor genetic variation results in one pathogenic race giving rise to another; for example, race 3 was first identified in a field where race 2 of f.sp. *lycopersici* was already present. Isolates of race 2 and 3 constitute a single VCG (Elias and Schneider 1991) with identical isozyme (Elias and Schneider 1992) and nuclear DNA profiles. Co-occuring pathogenic and non-pathogenic Fo strains have similar mt DNA haplotype (Gordon and Okamoto 1992) or IGS haplotype (Appel and Gordon 1995) and placed in single VCG.

At times, non-pathogenic and pathogenic isolates are vegetatively compatible owing to a coincidental sharing of alleles at the loci-governing vegetative compatibility. The inter-isolate transfer of mt DNA through hyphal anastomosis has been reported. For example, in California, eight non-pathogenic isolates from a single field exhibited identical mtDNA haplotype and varying nuclear DNA fingerprints. Similarly, Fo f.sp. *vasinfectum* (Katan and Katan 1988) and f.sp. *spinaciae*

(Fiely et al. 1995) were different from root colonizing non-pathogenic *F. oxysporum* isolates based on vegetative compatibility, whereas non-pathogenic isolates of *F. oxysporum* associated with cyclamen were similar to pathogenic isolates based on polymorphisms in the intergeneric spacer region (IGS) of nuclear rDNA (Woudt et al. 1995). Hence, VCGs are not markers for pathogenicity.

Diversity of Plant–Fungal Interactions

Fungal–plant interactions are complex, diverse and give rise to morphological and physiological alterations in both partners. Fungi produce species-specific signals and employ species-specific mechanisms during interactions with plant host. The outcome of plant–fungal interactions can be saprophytic, symbiotic and pathogenic based on receptors and expression pattern of defence-related plant proteins which interact with specific fungus-derived molecules (Grigoriev 2013). Fungal endophytes become pathogenic if they are able to evade the plant's innate immunity that comprises physical barriers, mechanism of programmed cell death and production of antimicrobial compounds (Dangl and Jones 2001; Brundrett 2004).

The interactions between plants and their pathogens are constantly evolving, wherein pathogens employ innovative strategies to cause vascular infection. The process of vascular infection by *F. oxysporum* is complex and requires a series of highly regulated processes, adhesion, penetration and colonization. Pathogenic fungi form feeding structures similar to symbiotic fungi to establish obligate relationships with plants (Corradi and Bonfante 2012). Fungal pathogens after adhesion gain access into plant interior through stomata and wounds in leaf and stem tissue. However, in several cases, cell wall degrading enzymes (CWDEs) and secondary metabolites secreted by fungus facilitate penetration. Once pathogen penetrates host, it secretes protein effectors that suppress plant defence responses and promotes invasion (Lo Presti et al. 2015). Moreover, several morphological and biochemical alterations occur so that pathogenic fungi take over and utilize host metabolic pathways for their growth and development (Zeilinger et al. 2015).

Plant pathogenic fungal species have been classified as biotrophs, hemibiotrophs and necrotrophs, each interacting differently with their host plants. Pathogenic fusarium employ various infection strategies like biotropic (pathogen that colonizes living plant tissue and obtains nutrient from them), necrotrophic (pathogen that kills host cell and obtains nutrient from dead cells) and hemibiotrophic (pathogens that are initially biotrophic and subsequently necrotrophic) varying in mode of interaction. Biotrophic pathogens interact with the host through specialized hyphae which secrete host-specific effectors that suppress host immunity at interfacial zone (Perfect and Green 2001; Yi and Valent 2013). For example, powdery mildews develop primary and appressorial germ tubes on the plant cuticle and breach the cell wall using a combination of mechanical force and CWDEs (Takahashi 1985; Pryce-Jones et al. 1999). After plant cell wall penetration, a close metabolic interaction between plant host and biotrophic pathogen is established

(Horbach et al. 2011). Subsequently, the aim of fungus is to block host defence and utilizes host processes for feeding and growth (Giraldo et al. 2013; Yi and Valent 2013). In necrotrophic pathogens, virulence has been correlated with toxin synthesis (Wang et al. 2014). A combination of CWDEs, reactive oxygen species (ROS) and or toxins destroys host cells, their nutrients are released (Kistler and Momol 1990) which results in colonization of plant host (Wolpert et al. 2002). Hemibiotrophic pathogens are initially biotrophic later switching to a necrotrophic lifestyle (Struck 2006; Gardiner et al. 2013). The biotroph–necrotroph switch in hemibiotrophs depends on molecular and physiological factors. Several hemibiotrophs require extended periods to establish infection while for others, the switch to necrotrophy is rapid (Kabbage et al. 2015). From evolutionary perspective, biotrophy is primitive while necrotrophy is a recent phenomenon (Pieterse et al. 2009); hemibiotrophy is a transitional infection strategy for pathogenic fungi (Horbach et al. 2011). The infection strategy of necrotrophic fungi is less complex than that of obligate biotrophs. Necrotrophs exhibit restricted physiological interaction with plant host on account of poorly developed infection structures and smaller number of biochemical compounds required for host penetration.

A mutation in either, fungal pathogen or host receptor genes alters pathogen–plant interactions from resistant to susceptible or vice versa (Stracke et al. 2002; Giraldo and Valent 2013).

Fusarium Pathogenicity and Pathogenicity Factors

Pathogenesis is the complete process describing disease development in the host, from initial infection to production of symptoms (Lucas 1998), and pathogenicity is the ability for pathogenesis. *F. oxysporum* initially penetrates roots asymptomatically; subsequently, it colonizes vascular tissue and triggers massive wilting, necrosis and chlorosis of aerial produce. Certain species-producing toxin, fusaric acid initially infect floral tissue during anthesis, spreads to flower through central axis of inflorescence, eventually damaging and contaminating grains with toxins (Gardiner et al. 2013).

Fusarium pathogens use both general and specific pathogenicity factors/mechanisms to invade their hosts (Fig. 10.3). Hydrolytic enzymes involved in plant cell wall degradation and components of cellular signalling pathways, which are often required for systemic pathogen invasion, comprise general pathogenicity factors, whereas production and secretion of effectors and host-specific toxin are specific pathogenicity factors. The counter defence mechanism of plants plays significant role in pathogenesis and categorised as general and specific (Poppenberger et al. 2003). General defence mechanisms encompass production of antifungal proteins and activation of defence signalling pathways, whereas pathogen-specific include recognition of specific pathogen effectors by plant resistance gene products and detoxification of pathogen-specific toxins (Proctor et al. 2007).The specific properties that discriminate endophytic strains

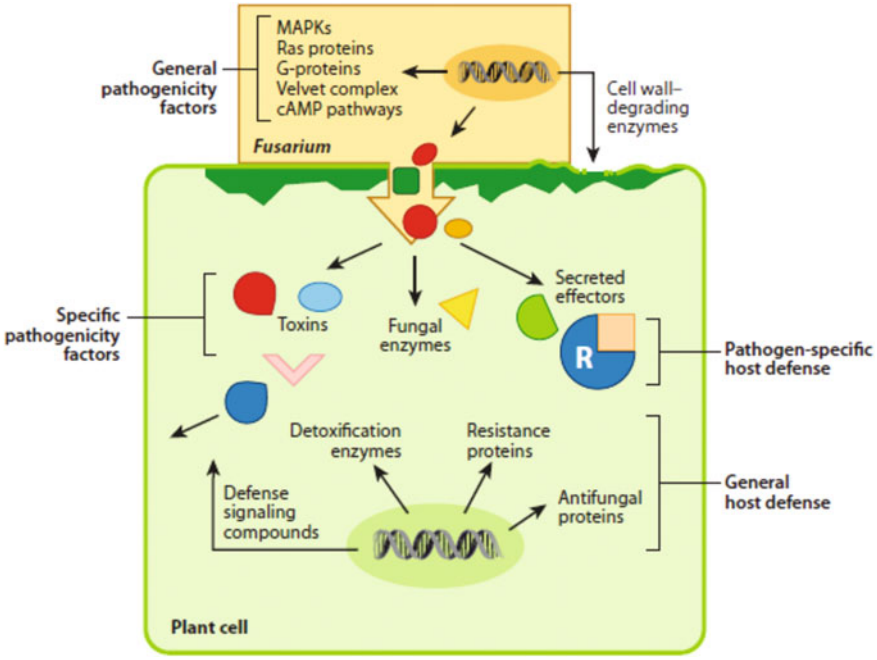

Fig. 10.3 Pathogenicity factors and host defence mechanisms during *Fusarium*–plant interactions (reproduced from Ma et al. 2013)

from closely related non-endophytic strains have been identified in several studies (Taghavi et al. 2010; Mitter et al. 2013; Amadou et al. 2008; Tisserant et al. 2013; Tian et al. 2012; Karpinets et al. 2014).

Comparative studies analysing genomic and metabolic network revealed major difference in cellular processes and metabolic capabilities of pathogenic ($n = 36$) and mutualistic ($n = 28$) plant microbes (Tian et al. 2012). Genes regulating biosynthetic processes and functions were enriched and more diversed amongst plant mutualists, while those controlling degradation and host invasion were detected in phytopathogens. Pathogens possess genes and regulons required for plant penetration and colonization (Wright et al. 2013). Moreover, mutualists utilize more stress-related compounds, whereas pathogens compound from plant cell wall. Genes encoding secretion systems, required to invade the host plant, were present in pathogen genome, while those encoding nitrogen fixation proteins and ribulose bisphosphate carboxylase/oxygenase (RuBisCO) proteins were specifically present in mutualistic bacteria (Karpinets et al. 2014). Bacteria with relatively large genomes often successfully colonize a wide range of unrelated plant hosts and soils, whereas strains with smaller genomes have a narrow host range (Mitter et al. 2013).

Fungi generally secrete a mixture of CWDEs to enter plant cells and secrete effectors, toxins or plant hormone-like compounds that manipulate the plants' physiology for its invasion and growth.

Cell Wall Degrading Enzymes

Fungal cell wall degrading enzyme (CWDE) system comprises of peroxidases and laccases for the degradation of lignin and glycoside hydrolases. Fungus secretes cellulases, hemicellulases and pectinases for degradation of cellulose, hemicellulose and pectin, respectively (Kubicek 2013). Genomics analysis of 103 fungi revealed that a large number of carbohydrate-active enzymes (CAZymes) such as carbohydrate esterase and pectate lyases (PL) are present in fungal pathogen compared to saprophytic fungi (Liao et al. 2013). Thus there is an upregulation of genes encoding CWDEs in *Fusarium graminearum,* the hemibiotrophic pathogen and *Magnaporthe oryzae* during infection of plant hosts (Kawahara et al. 2012; Zhao et al. 2013). In contrast, biotroph genomes possess few plant CWDEs encoding genes which completely lack glycoside hydrolase family 6(GH6) endoglucanase and cellobiohydrolase genes (Zhao et al. 2013).

Effector Proteins

Small effector proteins deregulate plant immune responses and facilitate pathogen in colonizing plant host (Rovenich et al. 2014). Fungal effectors are either apoplastic, those secreted into the plant extracellular component and cytoplasmic, those accumulated in plant membrane rich structure associated with invasive fungal hyphae (Giraldo et al. 2013). Apoplastic effectors include protease inhibitors that destroy host proteases. Plant proteases protect fungal cell walls against plant chitinase and small molecules minimising ROS levels. Host plant resistance (R) proteins recognize cytoplasmic effectors, thereby triggering the hypersensitive response (HR), a reaction characterized by rapid cell death in local infection region and thus blocks pathogen growth and spread (Giraldo and Valent 2013). Avirulence proteins are a type of cytoplasmic effectors. The interaction between an *avr* gene of pathogens and cognate resistance (R) gene of host leads to HR-mediated activation of host defence mechanism which prevents the pathogen invasion. This is an effector-triggered immunity (ETI) and is exemplified by C*f* 9 and *avr* 9 genes for the *Cladiosporum fulvum*-tomato pathosystem. The product of fungal race-specific *avr* 9 gene induces HR on tomato plants carrying the complementary resistance gene *Cf* 9. The fungal races virulent on *Cf* 9 tomato genotypes lack *avr* 9 gene. The genome analysis predicts that biotrophic maize pathogen *Ustilago maydis* encodes ~550 secreted proteins. Several of these are upregulated during host colonization (Djamei and Kahmann 2012). *U. maydis* secretes 'core' and organ-specific effectors. Core effectors suppress plant defence during the penetration stage and organ-specific effectors infect different plant tissue (Skibbe et al. 2010; Djamei and Kahmann 2012). *U. maydis* genome has effector-encoding gene clusters. There are 23 genes in the largest effector gene cluster, 19A. These are differentialy induced when different plant organs are colonized. It has been observed that deletion of complete 19A cluster abolished tumour formation in maize plants, whereas deletion of individual genes showed minor reduction in virulence (Kamper et al. 2006; Brefort et al. 2014).

Although, effectors are mostly proteins but a few are metabolites also. Fungal pathogens of genera *Cochliobolus*, *Alternaria* and *Pyrenspora* species secrete host-specific toxins (Tsuge et al. 2013), e.g. *Fusarium verticillioides* produces fumonisin (Arias et al. 2012) and *M. oryzae* pyrichalasin H and Ace 1 (avirulence conferring enzyme 1). Secondary metabolite-synthesized by *ace* 1 has not been identified yet (Collemare et al. 2008; Yi and Valent 2013). Pathogenic fungi deliver small non-coding RNAs into plant host cell to suppress plant immunity. *Botrytis cinerea* small RNAs silence genes confer immunity in *Arabidopsis* and tomato through hijacking host RNA machinery (Weiberg et al. 2013). Pathogens, e.g. *Cladosporium fulvum* and *M. oryzae* escape plant defence by secreting LysM effectors that bind to soluble chitin fragment and prevent them from detection by plant chitin receptors.

Signalling During Fungal–Plant Pathogen Interaction

The most critical step in fungal–plant interactions is the recognition of appropriate plant host. The process begins prior to direct contact between partners. Fungi detect chemical and physical signals and respond through differentiation, movement to an appropriate infection site, and/or formation of invasion-related structures (Kumamoto 2008; Bonfante and Genre 2010). The following section summarizes the current knowledge on the signals as well as signalling pathways involved in plant–fungal interactions with focus on fungal partner.

Signalling Mechanism

Root Exudates

Plant roots release both low and high molecular weight substances into rhizosphere. Amino acids, ion-free oxygen, sugars, phenolics and secondary metabolites are low molecular weight substances, whereas mucilage and proteins are high molecular weight substances (Bais et al. 2006). Root exudates can be produced both constitutively (so-called phytoanticipins) and in response to pathogen attack (so-called phytoalexins) (Baetz and Martinoia 2014). When soil-borne pathogen *F.graminearum* attacks barley, phenylpropanoids are released by its roots (Boddu et al. 2006). Similarly, the production of terpenes in barley roots is triggered by *Cochliobolus sativus* and *Fusarium culmorum* (Fiers et al. 2013).

Flavonoids also contribute towards signalling in plant–fungus interaction. They exert both positive and negative effect on fungal phytopathogens. On one hand, flavonoid inhibits spore germination and hyphal growth in several fungal pathogens, whereas on the other have a stimulatory effect. In case of *F. solani* f.sp. *pisi*, the isoflavonoid pisatin induces expression of *pda*1 encoding a pisatin demethylase, a virulence factor of this fungus (Khan et al. 2003). Recent studies revealed that class III peroxidase (POX) secreted by tomato roots function in chemotrophic

sensing by *F. oxysporum* via a pheromone receptor homologue and MAPK signalling (Turrà et al. 2015).

Oxylipins

Oxylipins are an oxygenated lipid secondary metabolites produced by plant and fungi. They are implicated in pathogenicity and promote disease progression. Fungal oxylipins act as endogenous signalling molecule that manipulates host lipid metabolism and alter its defence response (Tsitsigiannis and Keller 2007; Brodhagen et al. 2008). In contrast, plant oxylipins (jasmonates, JA) directly influence survival of invasive structures (Calvo et al. 1999), reproduction and production of secondary metabolites (Burow et al. 1997) in fungi.

Oxylipins act by inducing JA-responsive genes (Thatcher et al. 2009). JA signalling mediated by protein, coronatine insensitive (COL1) is responsible for susceptibility of *Arabidopsis thaliana* to *Fo* wilt. Oxylipins bind to G protein-coupled receptors (GPCRs) which induce cAMP signalling. Recently, it has been reported that cAMP signalling stimulated by plant oxylipin was absent in Gpr D (GPCR-encoding) mutant *Aspergillus nidulans* (Affedt et al. 2012). It was also reported that in the soil-borne plant pathogen *Aspergillus flavus*, endogenous oxylipins mediate, spore and sclerotia production and the biosynthesis of aflatoxin, which are regulated by Gpr C and Gpr D; Gpr C and Gpr D could be thus important for fungal–plant interactions.

Reactive Oxygen Species

Oxypilin-mediated signalling is linked to ROS-stimulated cell signalling during plant–fungus interaction, ROS interacts with phosphorylation cascades and controls transcription factors. Thus, mediating defence gene expression or oxypilins are generated through non-enzymatic oxygenation by ROS (Reverberi et al. 2012). In invading fungi nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes mediate production of superoxide. ROS accumulates at the plant–fungus interface and acts as signals for triggering attack and counterattack responses. In *M. grisea,* NOX1 and NOX2, NADPH oxidases trigger a local oxidative burst during plant infection (Egan et al. 2007). NOX1 and NOX2 are associated with appressorium formation. To establish infection, fungal pathogen must overcome plant's oxidative defense by employing ROS scavenging enzymes and modifying ROS accumulation in plant host, e.g. DES1 (defence suppressor 1) in *M. oryzae* (Chi et al. 2009), Leucine zipper (bzip) transcription factor and yes-associated protein (YAP1) in *U. maydis* (Molina and Kahmann 2007).

Plant Surface Signals

Plant signals, cutin monomers and leaf waxes trigger appresssorium formation in foliar rice pathogen *M.oryzae* (Liu et al. 2011; Perez-Nadales et al. 2014). Appressorium formation is mediated through multicopy suppression of budding defect2 (Msb2) signalling mucin and synthetic high osmolarity sensitive 1 (Sho1) tetraspanin protein (Lanver et al. 2014) present on fungus. Msb2 also plays an

important role in non-appressorium forming root-infecting, *F. oxysporum* by regulating plant infection and invasive growth through phosphorylating the Fmk1 MAPK in response to plant surface signals (Perez-Nadales and Di Pietro 2011). In pathogenic fungi, MAPK regulates the mechanical and enzymatic penetration of the host plant while the plant uses MAPK signalling for activation of immunity. In *M. oryzae*, cAMP-PKA signalling pathway controls plant surface recognition (Zhao and Xu 2007; Li et al. 2012) while Pmk1 (pathogenicity MAPK) stimulates appressorium formation and fungal growth in plant tissues (Xu and Hamer 1996). The membrane protein pth11 (aGPCR), that recognizes surface hydrophobicity, functions upstream of the cAMP-PKA pathway. It has been shown that although appressorium formation continues in PTH11 gene deletion mutant, they have reduced virulence (DeZwaan et al. 1999). This gives clear evidence of the overlapping roles of the Pth11 receptor and the signalling mucin Msb2 (which acts upstream of the Pmk1 MAPK cascade) in sensing surface hydrophobicity and regulation of appressorium formation (Xu and Hamer 1996; Liu et al. 2011).

In *U.maydis*, Kpp2 MAPK mediates virulence-related processes as filamentation and appressorium formation (Mendoza-Mendoza et al. 2009). Hence, there is a complex cross-talk of MAPK signalling with cAMP pathway. The two pathways appear to be connected at the Gpa3G protein subunit and the perforin 1 (Prf1) transcription factor. The Prf1 carries sequence motifs specific for PKA and MAPK-dependent phosphorylation, essential for its function (Bolker 2001). Similarly, proteins involved in calcium signalling are required for appressorium formation, turgor generation and host penetration in *M.oryzae* (Liu and Kolattukudy 1999). Hence, rice blast fungus is a model for deciphering the interplay of various signalling pathways in development of pathogenic potential.

Fungal Metabolic Diversity

The metabolic diversity of fungus determines whether fungus-plant interaction is beneficial or harmful (Zeilinger et al. 2015). Secondary metabolites enable fungus to colonize plant host systematically, survive in its niche and determines its virulence (Keller et al. 2005). Fungal species produce plant–specific secondary metabolites. The environmental changes affect the production of such secondary metabolites. Secondary metabolites associated with iron uptake govern the virulence potential of *A. brassicicola*, *C. heterostrophus, C. miyabeanus* and *F. graminearum* on their specific host plants (Oide et al. 2006).

Signalling Pathways

Plant distinguishes whether fungus is friend or a foe at multiple levels (Fig. 10.4). The first level is regulated by the receptor protein, pattern recognition receptors (PRRs) located in the plasma membrane. PRRs recognise microbial-associated molecular patterns (MAMPs) and pathogen-associated molecular patterns (PAMPs). These lead to activation of PAMP-triggered immunity (PT1) via calcium signalling and mitogen-activated protein kinase (MAPK) cascades. MAPK is

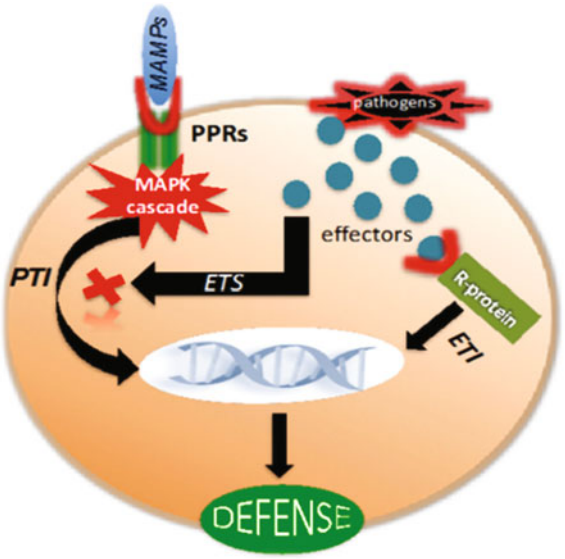

Fig. 10.4 Signalling in plant–fungal pathogen interactions. (reproduced from Zeilinger et al. 2015)

two-component system that regulates pathogenicity in fungal pathogens. It comprises of a membrane-bound histidine kinase which sense specific environmental stimuli and a response regulator that transmits the signal to a downstream pathway (Catlet et al. 2003). MAPK regulates stress responses and virulence in *C. heterotrophus* and *F. graminearum* (Oide et al. 2010) and in *Alternaria brassicicola* (Cho et al. 2009). Pathogens induce effector-triggered susceptibility (ETS) by blocking PTI response through effector proteins (Kazan and Lyons 2014). Calcium signalling is common cascade for plants to open a dialogue with their fungal partners because intracellular calcium levels are elevated during pathogenic as well as beneficial interactions (Navazio et al. 2007).

MAPK Cascade and Its Role in Virulence

The MAPK cascades of both partners help establish a molecular dialogue between plant and fungus (Hamel et al. 2012). MAPKs are organised as cascades consisting of three interlinked protein kinases, MAPK kinase (MAP3K), MAPK kinase (MAP2K) and MAPK, sequentially activated by phosphorylation (Widmann et al. 1999). The MAPK FmK1, an orthologue of the yeast Fus3/KSSI MAPKs, is essential for virulence of *F. oxysporum* on tamoto plants (Di Pietro et al. 2001). It is widely conserved and determines pathogenicity in all plant pathogenic fungi (Rispail et al. 2009). It is essentially required for all infection-related processes, invasive growth, fusion of vegetative hyphae and root adhesion (Di Pietro et al. 2001; Prados Rosales and Di Pietro 2008). Upstream and downstream components of this signalling cascade have been elucidated. The transcription factor Ste12 functions downstream of Fmk1 and regulates invasive growth of pathogen during plant infection (Rispail and Di Pietro 2009), and Msb2, a transmembrane protein is

an upstream component of this cascade (Perez-Nadales and Di Pietro 2011). In addition, *Saccharomyces cerevisae* high osmolarity (Hog 1) and cell integrity Mpk1 gene orthologues have been identified in *F. oxysporum* also. The GTPase Rho1, which function upstream of Mpk1, was essential for morphogenesis and pathogenicity (Martinez-Rocha et al. 2008).

Fusarium Genomics

The genomes of three economically important and phylogenetically diverse species, *Fusarium graminearum* (Fg) strain PH-1, *Fusarium verticilloides* (Fv) strain 7600 and *Fusarium oxysporum* f.sp. *lycopersici* (Fol) strain 4287 were compared and analysed. Fg strain H-1 causes head scab disease of small grain cereals, Fv strain 7600 is a maize pathogen-producing mycotoxin, fumonisin that contaminate grain and Fol strain 4287 is a tomato pathogen. The fully completed genome of *F. graminearum* PH-1 and its manually curated annotation is available at ensemble databank (King et al. 2015). Whole shot gun genome of Fol strain 4287 and Fv strain 7600 is available at BROAD Institute Website (Ma et al. 2010). The Fol genome (60 megabase) is about 44% larger to Fv (42 Mb), and 65% larger to Fg (36 Mb). Fol genome has a greater number of protein-encoding genes. 28% of the *F. oxysporum* genome corresponds to short interspersed elements (SINES) and class II transposable elements (Table 10.2). *Fusarium* genome is compartmentalized into core and accessory genomes. Core genome is identical in all *Fusarium* species and encodes for growth and survival, whereas accessory genome varies amongst formae speciales and characterizes host specialization, virulence and production of secondary metabolites. In *Fusarium solani* f. sp. *pisi*, the three

Table 10.2 Genome comparison amongst different *Fusarium species*

Species	*F. oxysporum*	*F. verticillioides*	*F. graminearum*
Strain	4287	7600	PH-1
Sequence coverage (fold)	6	8	10
Genome size (Mb)	59.9	41.7	36.2
Number of chromosomes	15	11[a]	4
Total scaffolds	114	31	36
N_{50} scaffold length (Mb)	1.98	1.96	5.35
Coding genes	17,735	14,179	13,332
Median gene length (bp)	1,292	1,397	1,355
Repetitive sequence (Mb)	16.83	0.36	0.24
Transposable elements (%)	3.98	0.14	0.03
NCBI accession	AAXH01000000	AAIM02000000	AACM00000000

N_{50} represents the size N such that 50% of the nucleotides is contained in scaffolds of size N or greater. Reprinted by permission from Macmillan Publishers Ltd: [Nature] (Ma et al. 2010), copyright (2010)

dispensible chromosomes have been linked to habitat specialization (Coleman et al. 2009) and pathogenicity towards pea (Han et al. 2001). In *Fusarium oxysporum* f. sp. *lycopersici*, chromosome 14 has been shown to convert a non-virulent strain into a virulent towards tomato via acquisition of the entire chromosome (Ma et al. 2010). In *F. graminearum*, regions of high single nucleotide polymorphism (SNP) density were found at the ends of chromosomes and in interstitial regions on three of the four chromosomes (Cuomo et al. 2007).

A total of over 9000 conserved syntenic orthologues were identified amongst Fol, Fv and Fg genomes (Ma et al. 2010). Fol and Fv orthologues display 91% nucleotide sequence identity within themselves and 85% with Fg orthologues. The orthologues of three species, Fol, Fv and Fg are enriched for predicted transcription factors ($P = 2.6 \times 10^{-6}$), lytic enzymes ($p = 001$) and transmembrane transporters ($p = 7 \times 10^{-9}$) when compared to other ascomycete genomes. In all three genomes, a total of 46 secondary metabolite synthesis (SMB) gene clusters have been identified. Microarray analysis confirmed that the genes in 14 of 18 Fg and 10 of 16 Fv SMB gene clusters were co-expressed. Ten out 14 Fg and eight out of the 10 Fv SMB gene clusters are co-expressed and novel (Ma et al. 2010).

Lineage-Specific (LS) Genomic Region

Lineage-specific (LS) genomic region, also known as supernumerary chromosome, constitutes accessory genome. They are usually small (<2 MB) and specific for forma specialis. They acquire foreign genes (i.e. xenologs) through horizontal transfer of an entire plasmid or chromosome from other *Fusarium* species and subsequent integration into the core chromosome. LS chromosomes are characterized by, (a) lack of housekeeping genes, (b) G + C content different than core chromosomal complement, (c) varying within related species and (d) 95% of transposable elements (TEs) present in an entire genome. The LS region harbours genes putatively related to host–pathogen interaction or pathogenicity (Ma et al. 2010). In all, 20% LS genes have been identified functionally. They encode for secreted effectors, transcription factors and virulence factors, involved in signal transduction. Analysis of genome sequence data suggests that *F. oxysporum* LS region differs considerably in strains with varying host specificities.

Comparisons amongst Fol, Fg and Fv genomes revealed the presence of four lineage-specific (LS) chromosomes. The genome assembly of Fol, Fv and Fg has 15, 11 and 4 chromosomes, respectively (Table 10.2).The number of chromosomes in Fg are less as compared to Fv and Fo due to chromosome fusion. The fusion occurs in high diversity regions (Cuomo et al. 2007). The genomic region in Fol is larger due to the presence of additional and unique sequences in extra chromosomes. All 11-mapped chromosomes, except for their telomere-proximal regions in the Fv assembly (41.1 Mb), correspond to 11 chromosomes in Fol (41.8 Mb). Syntenic region in Fol, Fg and Fv are 'core' region of genome. The core region of Fol has 80% similarity with that of Fg and 90% with that of Fv. About 40% of the Fol genome assembly is designated as Fol lineage-specific (Fol LS) region.

The Fol LS regions include four entire chromosome (3, 6, 14, and 15), parts of chromosome 1 and 2 (scaffold 27 and scaffold 31, respectively), and most of the small scaffolds not adhered to the optical map. The Fol LS region is 19 Mb, 28% of which is transposable elements (TEs). These are long interspersed nuclear elements (LINEs), retro elements copia-like and gypsy-like LTR retrotransposons, short interspersed nuclear elements (SINEs) and DNA transposons. DNA transposon classes like Pogo, hAT-like elements and MITEs are well represented in Fol. All in, about 74% TEs in Fol LS region are identifiable.

Fol genome has one intra-chromosomal and two inter-chromosomal segmental duplications, totalling approximately 7 Mb. Overall, these regions share 99% sequence identity indicating recent duplication events. Proteins encoded by 20% of Fol LS region are known. These are related to pathogenicity and include secreted effectors, transcription factors and virulence factors, involved in signal transduction and ethylene induction (Qutob et al. 2006). The enzymes that degrade or modify plant or fungal cell walls (Ma et al. 2010) are related to pathogenicity. Many of these enzymes have been reported to be expressed during early stages of infection on tomato root. It also harbours genes that encode for lipid metabolism and lipid-derived secondary messengers (Ma et al. 2010). These genes play important role in fungal pathogenicity. Fol LS region also has transcription factor sequences related to FTF 1 and specifically involved during early stages of *F. oxysporum* f. sp. *phaseoli* infection to its host (Ramos et al. 2007). The core genome in all *F. solani* isolates is well conserved (Coleman et al. 2009). Its accessory genome contains three LS regions distinct from its 'core' genome. *F. solani* LS region is distinct from that of Fol.

Therefore in conclusion, *Fusarium* species have similar core region and distinct LS regions. The LS regions are distinct in genes related to host–pathogen interactions.

Secondary Metabolite Gene Clusters

Fusarium species produce an array of bioactive secondary metabolites of which polyketides and non-ribosomal peptides are most abundant (Table 10.3). The comparative analyses of non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) from ten different *Fusarium* species, such as *F. avenaceum*, *F. acuminatum*, *F. culmorum*, *F. equiseti*, *F. fujikuroi*, *F. graminearum* (two strains), *F. oxysporum* (12 strains), *F. pseudograminearum*, *F. solani* and *F. verticillioides*, led to the identification of 52 NRPS and 52 PKSs orthology groups (Hansen et al. 2015). All NRPS and PKS were not functional in the strains analysed. A total of eight NRPSs (NRPS2-4, 6, 10-13) and two PKSs (PKS3, PKS7) were conserved in all the strains analysed. However, the products of the majority of PKSs and NRPSs are unknown. For example, in *F. graminearum*, the products of, only 8/15 PKSs and 3/19 NRPSs are known (Hansen et al. 2012b; Jørgensen et al. 2014; Sørensen et al. 2014). Some of the RPSs and PKSs genes have not been linked to metabolite production and may possibly represent pseudogenes.

Table 10.3 Different protein families including secondary metabolites produced in pathogenic and saprophytic fungi

Protein family	*Cochliobolus*	*Fusarium*	*Botrytis*	*Neurospora*	*Ashbya*	*Saccharomyces*
Peptide synthetases	30	37	29	7	0	0
Polyketide synthases	40	35	42	7	0	0
ABC transporters	51	54	46	39	17	29
Cytochrome P450s	63	40	33	44	ND	4
Protein kinases	112	94	70	120	ND	117

Source Yoder and Turgeon (2001)

Polyketide Synthases

Fungal pathogens synthesize polyketides from carboxylic acid derivatives (acetyl-CoA and malonyl-CoA) (Hopwood and Khosla 1992). Fungal PKSs contain five to eight functional domains and form two major groups based on their domain content: non-reducing PKSs (NR-PKSs) in which carbonyl groups are not reduced and reducing PKSs (R-PKS) in which carbonyl groups are partially or fully reduced. Fumonisin B, bikaverin, fusarubin and aurofusarin are examples of NR-PKS whereas fusaric acid and fusarielin are those of R-PKS. Fusarin C is NR-PKS with NRPS module. Zearalenone is both NR-PKS and R-PKS. Hansen et al. (2015) analysed the sequenced *Fusarium* strains through Blast P. The KS domains of PKS were extracted, phylogenetically analysed to identify PKS orthology group. Out of 52 different PKS genes, PKS3 and PKS7 were present in all the strains. In addition, PKS8 was also highly conserved amongst Fusarium strains although a part of KS domain was absent in *F. culmorum*, entire gene was absent in *F. oxysporum* f. sp. *melonis*. *F. graminearum* strain CS3005 shares 15 PKSs with Fg strain PH-1 which includes PKSs for biosynthesis of aurofusarin (PKS12), fusarubins PKS3, fusarins (PKS10), fusaristatin (PKS6 + NRPS7, and zearalenone (PKS4 + 13), (Sørensen et al. 2014) and a unique PKS (PKS52) which might have been acquired from *Colletotrichum* strains through horizontal gene transfer (HGT). *F. graminearum* and *F. culmorum* were closely related as there is overlap of 13 PKSs. PKS2 and PKS9 were absent in *F. culmorum*. Out of 14 putative PKSs identified in *F. pseudograminearum*, 13 have orthologue in *F. graminearum*, PKS40 was specific to *F. pseudograminearum* and encodes for W493A and B (a polyketide non-ribosomal peptide). Recently, PKSs and NRPS identified in *F. fujikuroi* earlier by Wiemann et al. (2013) have been renumbered (Hansen et al. 2015). Of the 17 PKSs identified in *F. fujikuroi*, 13 have orthologues in *F. verticillioides* and *F. oxysporum*, including bikaverin synthase (PKS16=BIKI) and hybrid PKS-NRPS (PKS18). PKSs ranging from 10 to 14 have been identified amongst completely sequenced 12 strains of different formae speciales. Six PKs were present in all 12 strains (PKS3, 7, 18, 20, 21 and 27), although the AT, KR, KS and MET domains are missing in *F. oxysporum* strain (F05176). The analysis

shows that *F. graminearum* had the highest number of species-specific PKSs followed by *F. solani, F. verticillioides* and *F. oxysporum*, respectively (Hansen et al. 2015). The polyketide synthase gene clusters in Fol, Fv, Fg and Fs have been characterized (Ma et al. 2010).

Non-ribosomal Peptide Synthetases (NRPSs)

These play a role in fungal pathogenesis because the products of several NRPSs are proven virulence factors, e.g. enniatin is essential for virulence of *Fusarium avenaceum* on potatoes (Herrmann et al. 1996), AM-toxin for *Alternaria alternata* on apple (Johnson et al. 2000) and HC-toxin for *Cochliobolus carbonum* race1 on corn (Walton 1996). The NRPS genes are up to 63 kb in size and form products in conjunction with other mega synthetases (Straight et al. 2007).

These are large multi-modular enzyme assembly lines (NRPSs) that synthesize non-ribosomal peptides (NRPs). The NRPSs consist of modules, each possessing catalytic domains in a specific order facilitating the sequential initiation and modification of the growing peptide chain. It has the adenylation domain (A) which recognizes the specific amino acid substrate, which is then transferred by the peptide acyl carrier domain (T or PCP) to the condensation domain (C) where the formation of the peptide bond takes place. These core domains are often supported by tailoring domains such as: thioesterase domains (TE) for cleavage or cyclization of the final peptide, reductase domain (R) for reducing the final peptide, epimerization domains (E) which can change the epimeric form of the amino acid substrate, cyclization domain (Cy) for modification of serines, and threonines and cysteines and N-methylation domains (NM) (Strieker et al. 2010). NRPSs were identified through Blast P analysis using a selected panel of variable A domains. The A domains were extracted from each NRPS and used for phylogenetic analyses. In all, six NRPS (NRPS2, 3, 6, 10, 11 and 12) were detected in all sequenced strains. NRPS 2 and NRPS6 are responsible for production of the siderophores ferricrocin and fusarinine, respectively (Oide et al. 2006; Tobiasen et al. 2007). NRPS6-produced fusarinine acts as an extracellular siderophore and is important for plant infection. NRPS4 was identified in all strains except *F. oxysporum* (Fo5176) and is reported to be involved in surface hydrophobicity in *F. graminearum* (Hansen et al. 2012a). In *F. graminearum*, a total of 16 NRPS genes were identified, of which NRPS32 was species-specific. In *F. culmorum*, 18 NRPS were identified. In *F. fujikuroi*, all except one (NRPS31) was common to those present in other sequenced *Fusarium* species. Out of a total of the 12 *F. oxysporum* strains, two *F. oxysporum* f. sp. *cubense* strain NRRL54006 and *F. oxysporum* f. sp. *pisi* strain HDV247 had identical distribution of NRPS genes. All *F. oxysporum* strains possess nine common NRPSs. These included two siderophore synthetases, NRPS2 and NRPS6 and the enniantin/beauvericin synthetase NRPS22. NRPS1 which encodes malonichrome, a type of siderophore is absent in *F. oxysporum* f. sp. *raphanin* and *F. oxysporum* Fo5156 and *F. fujikuroi*. Further, NRPS39, an orthologue of ferrirhodin synthatase, FNR1 was present in seven *F. oxysporum* species. Malonichrome (NRPS1), ferricrocin (NRPS2) and ferrirhodin (NRPS39) has similar domain structure (ATC-ATC-ATCTCTC).

Molecular Basis of Pathogenicity

Our understanding of molecular mechanisms involved in pathogenicity has improved through the genome sequencing and application of forward and reverse genetics. Michielse et al. (2009) identified more than 100 potential pathogenicity genes in *Fusarium oxysporum* f. sp. *lycopersici* upon analysis of 10,000 transformants for pathogenicity. With sequencing of more Fusarium genomes, similar genes have also been identified in *Fusarium* species other than f. sp. *lycopersici* (Kazan et al. 2012; Walter et al. 2010). A few with known functions have been listed (Table 10.4; Michielse et al. 2009; Sutherland et al. 2013). Functional characterization of putative genes indicates that those encoding for cell wall integrity, cell wall degrading enzymes, transcriptional regulators for carbon and nitrogen metabolism, cellular processes, such as amino acid and lipid metabolism, cell wall remodelling, protein translocation and degradation, seem to be important for complete pathogenicity of *F. oxysporum*. MAPK and cyclic AMP-protein kinase A (CAMP-PKA) cascade regulate virulence in Fo (Delgado-Jarana et al. 2005; Di Pietro et al. 2001; Jain et al. 2002, 2003, 2005). Cell wall integrity is necessary for invasive growth and resistance to plant defence compounds (Caracuel et al. 2005; Madrid et al. 2003; Martinez-Rocha et al. 2008; Martin-Udiroz et al. 2004, 2008). Cell wall degrading enzymes have been implicated in root penetration and colonization, but their role in infection process is not yet completely known. *F. oxysporum* f. sp. *lycopersici* virulence remains unaffected upon inactivation of individual genes, e.g. pectate lyase gene *plt*1, xylanase genes *xyl*3, *xyl*4 and *xyl*5, polygalacturase genes, *pg*1, *pg*5, *pgx*4 and subtilase gene *prt*1. Similarly, deletion of *xln*R did not affect virulence. However, targeted disruption of SNF1 reduced virulence as well as expression of various CWDEs indicating that central carbon metabolism plays key role in pathogenicity (Ospina-Giraldo et al. 2003). It has been reported that inactivation of Fnr1 (global nitrogen regulator) abolished the expression of nutrition genes, normally induced during early phase of infection, leading to reduction in pathogenicity. In addition, several plant degrading genes, pH-responsive transcription factors and regulators also play important role in pathogenicity. Moreover, the genes for peroxisome biosynthesis, ion homeostasis and toxin biosynthesis are also related to virulence. All the pathogenicity genes are categorized into basic and specialized pathogenicity genes.

Basic Pathogenicity Genes

The genes are common in *Fusarium* and other pathogenic fungi. These genes encode essential components of conserved pathways involved in sensing exogenous or endogenous signals. For example, mitogen-activated protein kinase (MAPK) signalling pathways in pathogens (Di Pietro et al. 2001; Hou et al. 2002; Urban et al. 2003), Ras protein (small GTPase) (Bluhm et al. 2007), G-protein signalling component and their downstream pathway (Jain et al. 2002; Park et al. 2012),

Table 10.4 Potential pathogenicity genes in *Fusarium oxysporum* f. sp. *lycopersici*

Category	Gene ID	Feature	Specific function
Chitin synthases	*chs*2, *chs*7, *chs*v, *chs*Vb	Cell wall integrity	Protect pathogen against host defences
GTPase	*rho*1	Cell wall integrity	Protects pathogen against host defences
β1,3-glucanosyltransferase	*gas*1	Cell wall integrity	
Pectate lyases	*plt*1	CWDEs	Pathogen entry into host
Xylanase genes	*xyl* 3, *xyl* 4 and *xyl* 5	CWDEs	Pathogen entry into host
Polygalacturonase Endo-polygalacturonase Exo-polygalacturonase	*pg*1, *pg*5, *pgx*4	CWDEs	Pathogen entry into host
Subtilase gene	*prt* 1		
FOL Frp1 gene	*frp1*	F-box protein	Assist pathogen to enter host xylem
FOX sucrose non-fermenting (SNF) gene	*snf1*	Expression of CWDEs through carbon catabolite repression	Assist pathogen to enter host xylem
Serine/threonine protein kinases	*ste*12		Regulate genes involved in MAPK cascade
FOX ste12 homologue	*fost*12		
FOL mitogen-activated protein kinase gene	*fmk*1		
Transcriptional regulator	*xlnR*		Expression of xylanolytic and cellulolytic genes
Global nitrogen regulator	*fnr*1		Expression of nutrition genes
FOX transcription factor	*fow*2	Zn(11) 2 Cys6 transcription factor	Rapid invasion of pathogen
FOX argininosuccinate lyase	*arg1*		
FOX plasmid pWB60S1 mitochondrial carrier protein gene	*fow1*		Mitochondrial carrier protein
FOX cyp55A1 gene	*cyp*55	*Cytochrome P450*	Regulate nitrogen respose pathway
CLC- voltage- gated Chloride channel gene	*clc1*		Regulate expression of laccase activity
Chloride conductance regulatory gene	*fpd 1*		Regulate expression of laccase activity
Cellular biosynthesis Mannose-6-phosphate isomerase			Mannose biosynthesis

(continued)

Table 10.4 (continued)

Category	Gene ID	Feature	Specific function
L-threo-3-deoxyhexlosonate aldolase			Catabolism of galacturonate
Catechol dehydrogenase			Catabolism of aromatic compounds
3-carboxy-cis,cis-muconate cyclase			
Succinate-semi aldehyde dehydrogenase (NADP+)			Enzyme involved in GABA-shunt
Peroxisome biogenesis	*pex* 1, *pex*10, *pex*12, *pex* 26		
Protein Translocation genes	*sec*61β, *sec*61α, *sec*62		
Major facilitator superfamily (MFS) multidrug transporter			Translocation of sugars, Kreb's cycle metabolites, aminoacids, osmolites, siderophores
Manganese superoxide dismutase	*mn* SOD		
Ion homeostatis		P type ATPase	
Redox balance NADH-ubiquinone oxido reductase			
G protein α subunit G protein β subunit	*fga*1 *fgb*1		

FOX, *Fusarium oxysporum*; FOL, *Fusarium oxysporum* f. sp. *lycopersici*

components of the velvet (LacA/veA/VelB) complex (Lee et al. 2012; Weimann et al. 2010) and cAMP pathway (Garcia-Martinez et al. 2012).

Specialized Pathogenicity Genes

These genes determine the pathogenicity of individual *Fusarium* species on specific plant host. These include *avr* genes and *six* (secreted in xylem) genes, mycotoxin and gibberellins encoding genes. Of these, *six* are directly involved in host pathogen interaction and encode for hundreds of small secreted proteins that play significant role in determining host specificity. Mycotoxins are additional virulence factors and act in a host or pathogenic-specific manner. A few *Fusarium* species produce mycotoxin, trichothecene which promotes virulence towards wheat (*Triticuma estivum*) and maize (*Zea mays*) but not barley (*Hordeum vulgare*)

(Jansen et al. 2005). The complete gibberellin gene cluster is present in all species within *F. fujikuroi* species complex but expressed/detected in only three, *F. fujikuroi*, *F. sacchari* and *F. konzum*. During disease development, gibberellins alter host tissues. The genes that encode for CWDEs and hydrolytic enzymes and are significant in gaining access to nutrition during infection are present in all *Fusarium* genomes. However, very few of these genes have been directly connected to pathogenicity. One exception is FGLI, a secreted lipase gene, which is responsible for virulence of *F. graminearum* of barley, maize and wheat (Ilgen et al. 2008, Voigt et al. 2005). Further, if FGL1 gene is overexpressed, the virulence of non-pathogenic MAPK mutant on wheat is restored (Salomon et al. 2012).

Six Genes

Host specificity between different races of *F.oxysporum* f. sp. *lycopersici* and tomato cultivar is determined by genes encoding small cysteine-rich effector protein termed *six* (secreted in xylem) (Rep et al. 2004). One of these proteins Avr1 triggers a resistance response in tomato plant carrying resistance (R) gene I-1. Interestingly, Avr1 also functions as a virulence effector by suppressing disease resistance conferred by two other R gene, I-2 and I-3 (Houterman et al. 2007). In Fo, all *six* genes are located on lineage-specific chromosome 14, also called the pathogenicity chromosome (Ma et al. 2010). Expression of the *six* genes requires a transcription factor Sge1 located on a core chromosome (Michielse and Rep 2009). Most of six genes are induced *in planta*.

Fusarium Oxysporum as a Model for Fungal Trans-Kingdom Pathogenicity

F.oxysporum f sp *lycopersici* isolate FGSC 9935 was the first fungal strain reported to cause disease both on plant (tomato) and mammalian host (immuno-depressed mice) (Ortoneda et al. 2004). The fungal genes encoding Fmk1, MAPK or small G protein Rho1 are essential for its pathogenicity on tomato but not on mice (Di Pietro et al. 2001; Martinez-Rocha et al. 2008), whereas pH response factor Pac C (Caracuel et al. 2003) or the secreted pathogenesis-related 1(PR1) like protein Fpr1 (Prados Rosales et al. 2012) are required for virulence in mice but not on plants. Recently, Hapx, a transcription factor that governs iron homeostasis, was identified in the fungal strain virulent on both plant and animal (Lopez-Berges et al. 2012). Similarly, the velvet protein complex contributes to infection of plants and mammals, in part by promoting the biosynthesis of beauverin, a mycotoxin (Lopez-Berges et al. 2013). The fungal pathogenicity on plants and animals has fundamentally distinct evolutionary origins despite involvement of common virulence proteins.

Evolution of Pathogenicity

The genetic and evolutionary relationships within and amongst formae speciales of *F. oxysporum* are revealed by sequence analysis of DNA-directed RNA polymerase II subunit and elongation factor-1α (EF-1α) genes (O'Donnell et al. 1998; Baayen et al. 2000; Mbofung et al. 2007). It was revealed that *F. oxysporum* formae speciales viz.,*lili* and *tulipae* were monophyletic (Baayen et al. 2000) while f.sp. *asparagi*, *cubense*, *dianthi, lycopersici* and *vasinfectum* were polyphyletic (O'Donnell et al. 2000; Baayen et al. 2000; Skovgaard et al. 2001; Cai et al. 2003). The evolution of pathogenesis in FOX isolates can be traced by analysing formae speciales of closely related plant species assuming that pathogens of closely related plant species are also closely related. The phenomenon of gene duplication (GD) and horizontal gene transfer (HGT) are responsible for their constant/continuous diversification. Virulence genes are acquired through HGT and further diversify due to GD and gene loss (Joaramillo et al. 2015; Steindorff et al. 2015). *Fusarium* genome very well exemplifies the role of HGT in acquiring diversity. A non-pathogenic strain of *F. oxysporum* f.sp. *lycopersici* was transformed into a tomato pathogen subsequent to transfer of a pathogenicity chromosome to it (Ma et al. 2013).

Origin of LS Regions

LS region in Fol might have originated in three ways: (i) Fol LS region was present in the last common ancestor of four *Fusarium* species but lost in Fg, Fv and Fs during vertical transmission, (ii) LS regions arose from the core genome by duplication and divergence of Fol lineage and (iii) LS regions acquired as a result of horizontal transfer. In all, 90% of the Fol genes in core regions have homologues in Fg and Fv. About 50% of the genes on FOL LS regions lack homologues in either Fg or Fv. sequence divergence between Fol and Fv orthologues in core regions was less as compared to Fol and Fg orthologues. The LS genes that have homologues in the other *Fusarium* species are roughly equally distinct from both Fv and Fg genes indicating that the phylogenetic history of the LS genes differs from genes in the core region of the genome. The distinct evolutionary trend of the core and LS region is supported by the distinct codon usage in LS encoding genes compared to core/conserved genes. The most significant differences were observed for amino acids Ala, Cys, Gln, Glu, Gly, Thr and Val, with a preference for G and C over A and T amongst Fol LS regions. Their third codon positions have higher GC content. Nearly 93% of 1285 LS-encoded proteins are homologous to other ascomycetous fungi. Phylogenetic analysis of 362 proteins sharing homologues in seven ascomycete genomes—Fg, Fv, Fol, Fs, *Magnaporthe grisea* (Dean et al. 2005), *Aspergillus nidulans* (Galagan et al. 2005) and *Neurospora crassa* (Galagan et al. 2003) indicates that they originated within the genus *Fusarium* but were placed basal to the three most closely related *Fusarium* species Fg, Fol and Fv. Thus, it is

concluded that Fol LS regions originated through horizontal acquisition of genes or gene regions from other *Fusarium* species.

Host Specificity: Variations in LS Regions

F. oxysporum is a species complex, comprising of several different asexual lineages that are non-pathogenic or pathogenic towards different hosts. Fo strains with varying host specificities have different LS regions which has been determined by comparing sequences of Fo strain 5176, pathogen of *Arabidopsis* (Thatcher et al. 2009) and Fo f.sp *vasinfectum* (Dowd et al. 2004), a pathogen of cotton. Although overall sequence divergence between common sequences of Fol and Fo5176 is less than 2%, sequences in the Fo LS region do not have homologues in Fo5176. Fov EST sequences (Dowd et al. 2004) have 99% sequence identity to the Fol genome in core region only. These are large-scale genome polymorphism within Fo as karyotypes between strains vary (Teunissen et al. 2003). Small polymorphic and conditionally dispensable chromosomes confer host-specific virulence in the fungi *Nectria haematococca* (Miao et al. 1991) and *Alternaria alternata* (Harimoto et al. 2007). Small (<2.3 Mb) and variable chromosomes are absent in non-pathogenic *F. oxysporum* isolates indicating that Fol LS chromosomes are responsible for pathogenicity towards specific host. Chromosome 14 of Fol is probably responsible for its pathogenicity towards tomato; its transfer rates could increase the overall pathogenesis. Proteome analysis revealed that small proteins Six1 (Avr3), Six3 (Avr2) and in-planta oxidoreductase (Oxi1) are secreted during colonization of Fol in tomato xylem system (Houterman et al. 2007; van der Does et al. 2008) and are involved in virulence (Houterman et al. 2009; Rep et al. 2004). It was further revealed that genes encoding above proteins are present on chromosome 14 present in strains causing tomato wilt, but are generally not present in other strains (van der Does et al. 2008). Further, genome analysis identified that *six*5, *six*6, *six*7 are also present on chromosome 14. It has been demonstrated that entire LS chromosome 14 could be transferred through simple co-incubation between two, otherwise genetically separated members of Fo leading to the emergence of new pathogenic lineages. Horizontal transfer of host specificity factors between otherwise distant and genetically separated lineages of Fo explains the host specialization originated polyphyletically (Gale et al. 2003). Fol LS regions are enriched for genes regulating host-pathogenic interactions. These chromosomes could transfer an entire set of genes required for host compatibility to a new genetic lineage in a single event. If transferred to the recipient lineage with an environmental adaptation different from the donor, the overall incidence of disease in a host increases because pathogenicity is introduced in the genetic background pre-adapted to a local environment.

Conclusion and Future Prospectives

The establishment of *F. oxysporum* as plant and animal infection model, the use of molecular genetics approaches in this species, and the genomic characterization of different *Fusarium* f. sp. has advanced our understanding of several key aspects related to fungal pathogenicity and its evolutionary origins.

The availability of sequenced genomes, gene annotations and genome expression data of various *Fusarium* spp., has conclusively shown that pathogens harbour conserved as well as specialized pathogenicity genes. With the analysis of genome expression data, several conserved pathogenicity genes, such as those encoding MAPKs have been characterized (Ma et al. 2013), whereas specialised pathogenicity genes linked to host adaptation or evasion have remained largely undefined except, secreted in xylem (SIX) effectors in *F. oxysporum* f. sp. *lycopersici* and several mycotoxins in other *Fusarium* species. The multiple processes, e.g. population diversity in specific genomic regions, horizontal acquisition of whole pathogenicity chromosomes (Ma et al. 2010) or a few pathogenicity-related genes (Gardiner et al. 2014) have been involved in evolution of pathogenicity and host specificity in *Fusarium*. Diversifying selection studies in the three *Fusarium* pathogens Fg, Fv and Fol with distinct pathogenicity profiles have revealed that all *Fusarium* species have core group of genes under purifying selection to preserve their function and specialised group of genes as those encoding proteins with a N-terminal [SG]-P-C-[KR]-P sequence motif and pathogen-associated proteins evolve at a faster rate. These rapidly evolving gene groups are functionally associated with pathogenicity (Sperschneider et al. 2015). Further, diversifying selection acts strongly on accessory-/lineage-specific chromosomes. Moreover, diversifying selection studies combined with *in planta* expression data are useful for identifying pathogenicity genes involved in competition between pathogen and host. Future developments will be in the form of improved gene annotations, greater sequencing depth in the genus so that genes that show weak signal for diversifying selection during pathogen–host interactions are identified and generation of *in planta* expression data for other *Fusarium* species to detect effector production at early stages during infection. In future, *F. oxysporum* is likely to provide valuable new insights into molecular mechanisms of host specificity and pathogenicity in evolutionarily distant hosts.

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Yeast Genome Sequencing: Basic Biology, Human Biology, and Biotechnology

11

Krishna Kant Sharma

Abstract

Whole-genome sequencing of *Saccharomyces cerevisiae* is one of the milestones of genome research which complemented the understanding in basic biology and expanded the field of yeast biotechnology. It was the first fungus as well as first eukaryote used for reverse genetic experiments, which created consensus among research community to sequence the whole genome, followed by the creation of species-specific database, thus further sustaining and strengthening the status of model organism. Thereafter, the successive completion of genome sequencing of fission yeast, i.e., *Schizosaccharomyces pombe* and *Neurospora crassa*, a genetic model, created a revolution in the genome biology of non-filamentous fungi. Further, in very less time, a significant number of yeast genomes have been sequenced, annotated, and released in public domain. Genome-sequencing program provides enormous data on genes, proteins, and metabolic pathways within the fungal kingdom, thereby helping in solving the long awaited problems of the medical science, ecological science, bioenergy, and several fermentation and enzyme-based biotech industries. Interestingly, yeast genomics have great potential in understanding human health, developing novel drugs, and harvesting stored energy from lignocellulosic substrate. With the significant rise in sequenced yeast genomes, the knowledge of genes encoding proteins, antibiotics, enzymes, metabolites, and their pathways has increased exponentially. Till date, over hundreds of genome sequences of several strains of domesticated and wild yeast are available in public domain; however, not a single review inclusively on the basic and applied aspects of yeast genome has appeared. This chapter is an attempt to review the significance of yeast genome-sequencing information in basic biology and biotechnology.

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Next-generation sequencing · Database

Introduction

The era of -omics, more specifically eukaryotic genomics, began with the sequencing of the complete genome of *Saccharomyces cerevisiae* (Goffeau et al. 1996) in the year 1996. But the milestone was achieved by the collaborative effort of several laboratories from 19 countries, which revolutionized the work on yeasts and enabled the comprehensive studies of eukaryotic gene expression and function. The subsequent completion of the genome sequencing of *Schizosaccharomyces pombe* (Wood et al. 2002) revealed the importance of yeast and answered some of the evolution-based queries. Further, using a comparative genomic approach in *S. cerevisiae*, Wang and co-workers identified and characterized the novel open reading frame (ORF) and have shown its probable role in the evolution of yeast species (Cai et al. 2008). One step ahead, Duan and co-workers have proposed a method to study chromosomal interactions and further to generate a three-dimensional model of the haploid genome of *S. cerevisiae* to provide an interface between the form of a eukaryotic genome and its functions (Duan et al. 2010). Further, to generate and expand the available genomic resources by linking variation with function, the pan-genome study of *S. cerevisiae* has been initiated (Song et al. 2015). For the initiation of the yeast pan-genome, Song and co-workers re-sequenced the genomes of 25 strains of the commonly used yeast in the both basic science and applied science, using next-generation sequencing (NGS) technology. The comparative genomics of the different yeast strains had helped to identify the modified genome fragments during recent course of evolution. This also gives us an idea of the evolution due to recombination and selection processes that are currently re-wiring the metabolic pathways. The exponential growth, evolution, and the inclusion of NGS technologies have created an enormous increase in the volume of genome and transcriptome data (Gattiker et al. 2007). Furthermore, the use of DNA-seq, RNA-seq, and ChIP-seq, which are more targeted approaches in yeast genome sequencing, has also increased the diversity of available data. This development in model yeast has provided significant information in the field of population genetics, evolutionary biology, and comparative analysis of the high-resolution genome architecture (Duan et al. 2010).

Despite the need for sequencing fungal genomes, progress had been slow in the beginning. But later, the need was realized, and in the last decade, the sequencing work gained the pace by the joint venture of a consortium of mycologists and Broad Institute, Center for Genome Research at MIT. The Broad Institute has launched the Fungal Genome Initiative (FGI) (http://www.broad.mit.edu/annotation/fgi/) and set

a goal to sequence the fungal genomes throughout the kingdom. Eventually, 'The Genome Online Database' (GOLD) appeared as an outcome of the global initiative, which provides additional support to the different aspects of fungal biology (http://www.genomeonline.org) (Sharma 2015).

However, the biotechnological applications create huge amount of challenges and opportunities, which can be addressed with the tools of -omics. Fortunately, the advancement in genomic data has yielded the basic mechanism in understanding the genetic pathways of eukaryotic systems. Genomic data has potential to bring revolution in food, pharma, and bioethanol industry. The present chapter is focused on the importance of yeast genome-sequencing programs and its importance in basic biology, yeast pathology, and biotechnology.

Yeast Genetics and Yeast Biotechnology in Pre-genomic Era

The pioneering research findings of Carl Lindegren and Øjvind Winge during the 1940s initiated the field yeast genetics (Engel et al. 2014), but the systematic analyses of a eukaryotic genome and proteome become feasible only after the complete genome sequencing of the budding yeast *S. cerevisiae*. Furthermore, the recent studies involving genome-wide and transcriptomic approaches have helped in understanding basic mechanism of biochemical reactions, gene mutation phenotypes, protein abundance, protein–protein interactions and transcriptional regulation and have taken a analyzing messenger RNA abundance and stability.

Traditionally, *S. cerevisiae* has been used in the production of fermented foods and alcoholic beverages, but today it is also used in pharmaceutical industry. *S. cerevisiae* serves as a widely used biotechnological cell factories as well as a eukaryotic model system. Due to its non-pathogenic nature and long history of application in the production of consumable products such as ethanol and baker's yeast, it is a suitable and attractive organism to work upon. Furthermore, its GRAS organism (generally regarded as safe) status, well-established fermentation, and downstream process technology for large-scale production with *S. cerevisiae* make this organism of choice for several biotechnologists. *S. cerevisiae* are susceptible to genetic modifications by recombinant DNA technology and gene transformation, which help them to achieve a status of model in the field of basic biology as well as biotechnology (Hinnen et al. 1978; Gietz et al. 1992), which has been further sustained by the availability of the complete genome sequences.

Strains of baker's and brewer's yeasts have been improved by the conventional methods like, random mutagenesis or classical genetic crossing of two strains followed by screening for improved traits in the mutants (Ostergaard et al. 2000). Earlier, transposon-mediated random epitope tagging and plasmid-based over-expression of epitope-tagged proteins have also been used for the protein localization studies in *S. cerevisiae* (Huh et al. 2003). Significant developments in high-throughput instrumentation and novel methods in the field of genomics, proteomics, and genetic engineering have enabled us to manipulate the metabolic

pathways to achieve the desired output in enzyme biotechnology and biofuel development. Further, it can be achieved by metabolic engineering, which deals with the analysis of the cells for the identification of the desired target(s) for genetic manipulation and genetic modification of the cells (Ostergaard et al. 2000).

Comparative Yeast Genome Sequencing

Budding yeast, *S. cerevisiae* is an established model organism in molecular biology work and microbial genetic research; therefore, it has been in the vanguard for many systematic and theoretical advances. In the year 1996, it became the first privileged eukaryotic organism with information of complete genome sequence (Goffeau et al. 1996). Its experimental manageability and the simplicity of genetic modification have made it to reach the status of completely characterized eukaryotic organism. Therefore, considering its versatility, well-defined features and small genome size ($\sim$13 Mb) has made *S. cerevisiae* one of the best-suited systems for mass sequencing and comparative genomics.

Several reports of new strains, like *S. cerevisiae* strain RM11-1a, a haploid strain of *S. cerevisiae* Bb32(3), an isolate collected from wild habitat, whereas *S. cerevisiae* strain YJM789 is also haploid form of an opportunistic pathogen isolated from the lung of an immuno-compromised patient in 1989 (Wei et al. 2007; Engel and Cherry 2013). Strain YJM789 was capable for flocculence, heat tolerance, and deadly virulence; therefore, it can be used for infection studies and quantitative genetic analysis (Wei et al. 2007). With these consecutive reports on the strains of *S. cerevisiae*, comparative yeast genomics was born. Thereafter, researchers began to investigate the functional significance of genetic polymorphism or variation at a genomic scale (Engel and Cherry 2013).

In 2009, *S. cerevisiae* became one of the first organisms to enter the population genomics era because multiple isolates ($\sim$70) of evolutionally related *S. cerevisiae* and *S. paradoxus* were sequenced (Table 11.1) (Liti et al. 2009; Engel and Cherry 2013). Recently, six industrial yeast strains of *S. cerevisiae* were selected for genomic analysis, comprising an average of 20-fold coverage with a combination of paired-end method and shotgun method (Borneman et al. 2011). These comprehensive genomic study revealed variation in gene content, single-nucleotide polymorphism (SNPs), copy numbers, and transposable elements. Therefore, it was concluded that variation in phenotype correlates with global genome-wide phylogenetic relationships.

Moreover, population ecology with comparative genomics of more than 200 natural isolates has been used in resolving the phylogeny by establishing *S. cerevisiae* and *Saccharomyces eubayanus*, a novel cryotolerant and progenitors of *Saccharomyces pastorianus*, therefore enlightening on the evolution and domestication of yeasts (Libkind et al. 2011). Comparative genomics has also been used to study the hybridization events of *Saccharomyces*, finding genome

Table 11.1 Whole-genome sequencing of economically important strains of *Saccharomyces*

S. No.	*Saccharomyces cerevisiae* strain	Year of sequencing	Culture source	BioProject accession
1.	S288C	1996	Laboratory strains of *Saccharomyces*	PRJNA128
2.	RM11-1a	2005	Haploid (vineyard isolate of *Saccharomyces*)	PRJNA13674
3.	YJM789	2007	Haploid (opportunistic human pathogen)	PRJNA13304
4.	M22	2008	Italian vineyard isolate	PRJNA28815
5.	AWRI1631	2008	African commercial wine	PRJNA30553
6.	YPS163	2008	Woodland isolate	PRJNA28813
7.	JAY291	2009	Brazilian industrial bioethanol strain	PRJNA32809
8.	EC1118	2009	Commercial wine strain	PRJEA37863
9.	Foster's B	2010	Commercial strain of *Saccharomyces*	PRJNA48569
10.	AWRI796	2010	Red wine strain of *Saccharomyces*	PRJNA48559
11.	VIN13	2010	White wine strain of *Saccharomyces*	PRJNA48563
12.	CLIB215	2010	Bakery isolate of *Saccharomyces*	PRJNA60143
13.	CBS7960	2011	Bioethanol (Brazilian isolate)	PRJNA60391
14.	FL100	2011	Laboratory strain of *Saccharomyces*	PRJNA60147
15.	CLIB324	2011	Bakery isolate of *Saccharomyces*	PRJNA60415
16.	EC9-8	2011	Haploid (Israeli isolate)	PRJNA73985
17.	CLIB382	2011	Beer - Irish isolate of *Saccharomyces*	PRJNA60145
18.	QA23	2011	Portuguese white wine strain	PRJNA48561
19.	UC5	2011	Sake yeast (Japanese strain)	PRJNA60197
20.	PW5	2011	Palm wine isolate of *Saccharomyces*	PRJNA60181
21.	T73	2011	Red wine (Spanish strain)	PRJNA60195
22.	Y10	2011	Coconut (Philippine isolate)	PRJNA60201
23.	W303	2011	Laboratory strain of *Saccharomyces*	PRJNA167645
24.	VL3	2011	white wine strain (French)	PRJNA48565
25.	YJM269	2011	*Blauer Portugieser* wine grapes (Austrian strain)	PRJNA60389
26.	ZTW1	2012	Corn mash bioethanol (Chinese isolate)	PRJNA174065

mosaicism and patterns of introgression between *Saccharomyces bayanus*, *Saccharomyces uvarum,* and *S. cerevisiae* (Nguyen et al. 2011).

Broad Institute (http://www.broadinstitute.org/) is using CBS7001, and the Washington University School of Medicine (http://genomeold.wustl.edu/projects/yeast/index.php) is using the spore clone of CBS7001, strain 623-6c ura3-1, and Génolevures (http://www.genolevures.org/) sequenced the genomes of the representative strains of *S. uvarum* (Nguyen et al. 2011).

The report on comparative genome sequencing of *C. neoformans* reveals that their multiple lineages had evolved parallely from a recent common ancestor during *C. neoformans* infection (Ormerod et al. 2013). In another example, the genome sequence of *S. uvarum* CBS7001 has been used in the upgradation by the *Saccharomyces* Sensu Stricto consortium (www.SaccharomycesSensuStricto.org) (Scannell et al. 2011). The annotation of genome sequence confirmed that the *S. uvarum* genome is free from *S. cerevisiae* sequences. In 2005, based on sequence data, *S. uvarum* was proposed to be a distinct species, eventually modifying its taxonomic status and distancing with *S. bayanus* (Nguyen and Gaillardin 2005; Nguyen et al. 2011). Therefore, it can be concluded that comparative genome sequencing can be used to solve some of the taxonomic and evolutionary conflicts.

The fermentation of xylose is necessary for the efficient conversion of lignocellulose biomass into bioethanol, but traditionally the strains of *S cerevisiae* are not capable to metabolize xylose; therefore, the xylose metabolism pathway (Fig. 11.1) has been engineered to develop a recombinant strain. Normally, the glucose

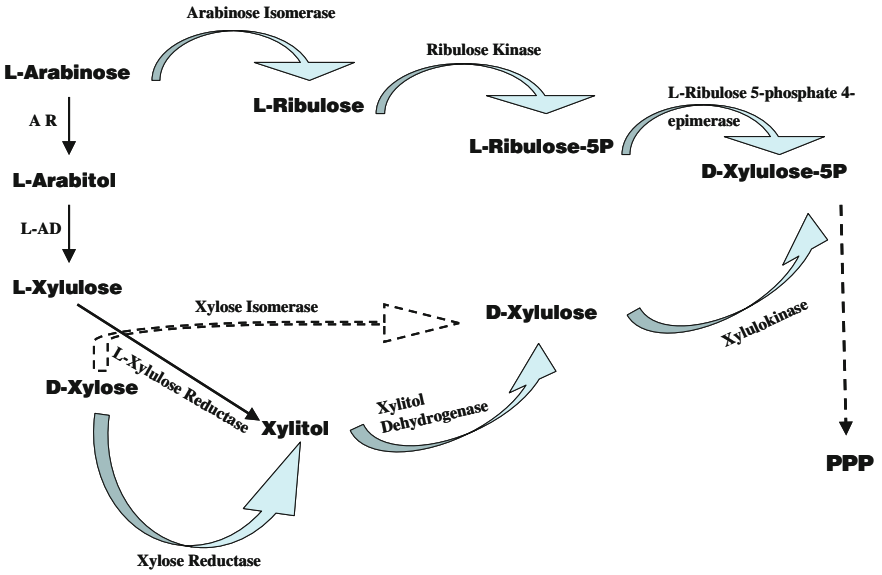

Fig. 11.1 Arabinose and xylose metabolism pathway in yeast. Dotted arrow represents xylose metabolism pathway in bacteria. *Abbreviations* AR arabinose reductase; AD arabitol dehydrogenase

transporters have been reported to mediate xylose uptake, but no specific transporter for xylose has been reported till date. In some anaerobic fungi and bacteria, xylose isomerase is responsible for direct conversion of xylose to xylulose (Fig. 11.1) and xylulose is ultimately phosphorylated to form xylulose-5-phosphate by an enzyme xylulokinase (XK) (Hahn-Hägerdal et al. 2007). However, in pentose-growing yeasts, xylose is reduced by xylose reductase (XR) to xylitol, which is eventually oxidized to xylulose by xylitol dehydrogenase (XDH).

To explore the ability to ferment xylose by *S. cerevisiae*, Wenger and co-workers have reported high-throughput sequencing of more than 600 strains in concurrence with mass segregant analysis in, and found that this 'xylose-positive' phenotype, which was present in approx 5% of the tested strains, clustered within wine yeasts (Wenger et al. 2010). Further, with the help of comparative yeast genome sequencing, a novel xylitol dehydrogenase gene *XDH1* was found in the Simi White strain, in the same sub-telomeric 65-kb insert on chromosome XV (Engel and Cherry 2013).

Yeast Genome Sequencing and Gene Clustering

For comparative genomics studies, yeast has been used as a powerful model system. The increasing trend of sequencing multiple complete genome sequences of several strains or groups has enabled the researchers to develop a broad outlook on the evolution of genome (Scazzocchio 2014). One of the most interesting outcomes is in the upcoming areas, such as gene losses, gene displacements, and gene relocations that can be attributed to the action of natural selection. The genes for secondary metabolites in yeast are usually clustered with a common chromatin organization (Gacek and Strauss 2012).

Several gene clusters with codes for secondary metabolism are located in sub-telomeric positions (Palmer and Keller 2010), but till date there sub-telomeric heterochromatic silencing is unknown, as described for *S. pombe* (Palmer and Keller 2010). If the cluster of gene is lost, the complete catabolic pathway is lost and therefore no toxic intermediate is produced (McGary et al. 2013; Slot and Rokas 2010). Contrary to this, if the genes are clustered, the probability of loosing single gene encoding the protein responsible for the catalysis of the detoxification of a toxic intermediate gets diminished. The clustering of the genes involved in primary metabolism of fungi occurs in the case of galactose utilization, where Galactose-1-phsophate is toxic, and clustering of three enzymes of galactose metabolism has occurred independently within the Saccharomycotina and *Candida* species due to horizontal transfer (Slot and Rokas 2010).

In the genus *Saccharomyces,* clustering of six genes involved in 5-ureidohydantoin (allantoin) utilizations is reported to be a relatively recent event appearing at specific stage of the genome evolution. This novelty coincides with the ability to grow in anaerobic conditions and with the limitation to utilize urate as a nitrogen source (Wong and Wolfe 2005; Wolfe 2006). The nitrate assimilation

pathway has been studied in detail, which are completely clustered in a member of Saccharomycotina, i.e., *Pichia angusta* (*Hansenula polymorpha*). This cluster comprises of two Cys6Zn2 transcription factors (*Yna1* and *Yna2*) (Siverio 2002), addition to the three earlier reported genes. The horizontal transmission of genes can be explained by sequencing the whole genome of the closely related species of yeast for the ancestral presence of the cluster. In *Arxula* (*Blastobotrys*) *adeninivorans,* the gene cluster has been functionally characterized, which includes two transporter genes but not the transcription factor genes (Böer et al. 2009) and nitrate utilizing member of the Saccharomycotina, e.g., *Kuraishia capsulata* (Morales et al. 2013). Interestingly, in the genome scanning of *A. adeninivorans,* no possible orthologues of *Yna1*, *Yna2* (regulatory genes from *K. capsulate*) could be reported (Morales et al. 2013).

Database for Yeast Genome Sequencing

Over the past two decades, the mycologists and molecular biologists have together taken up a comprehensive Fungal Genome Initiative (FGI) by prioritizing a set of fungi for their genome sequencing. The initial priority was given to the species of those fungi which has potential to negatively affect threat to human health or has been used as an important model for basic and applied biomedical research. Grants from the US Department of Energy (DoE), European Commission (STREP Fung Wall grant), and French Ministry of Research have also invested in the genome-sequencing programs of economically important fungi (http://www.broad.mit.edu/annotation/fgi/). Maximum number of genome projects is funded to National Institute of Health (NIH) followed by DoE. In February 2002, the first white paper was developed and submitted by FGI to the National Human Genome Research Institute to determine priority for genome sequencing of the 15 fungal species, out of which 7 were declared to be high-priority organisms for genome sequencing.

The first whole genome of eukaryotes, *S. cerevisiae,* has been sequenced by a global effort involving almost 600 scientists from North America, European Union, UK, and Japan; therefore, considered as a landmark in the eukaryotic genomic era (http://genome-www.stanford.edu/saccharomyces/) (Table 11.2) (Goffeau et al. 1996).

Two of the genome-sequencing programs, i.e., Genolevures I and II, were devoted to the exploration of hemiascomycetous yeast. Fungal genomic data is available, and it can be accessed through a number of online resources. The resources are (1) Broad Institute Fungal Genome Initiative Web site (http://www.broad.mit.edu/FGI/); (2) The Joint Genome Initiative (JGI) Integrated Microbial Resource database (http://img.jgi.doe.gov/pub/main.cgi/); (3) National Center for Biotechnology Information Entrez (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj); (4) The TIGR fungal database (www.tigr.org.tbd/fungal); (5) The Munich Information Center for Protein Sequences (MIPS-http://mips.gsf.de/

Table 11.2 Comprehensive list of Yeast Genome Databases and its operational application

Genome	Database (URL)	Functions/applications	References
Yeast and other fungi	http://www.mips.biochem.mpg.de	The Munich Information Center for Protein Sequences (MIPS) develops and maintains high-quality curated genome databases, e.g., ORF, classification of protein sequence (ProtFam) and collection of protein sequence database	Mewes et al. (2000)
Yeast genome	http://mips.gsf.de/genre/proj/yeast/	CYGD provides information of exploring yeast genomes with the help of the available genome of *S. cerevisiae*	Güldener et al. (2005)
TIGR fungal database	www.tigr.org.tbd/fungal	Provides an overview of available fungal genomes and highlights biological insights and protocol to analyze EST sequences	Galagan et al. (2005)
Saccharomyces cerevisiae	http://www.thebiogrid.org/	The Biological General Repository for Interaction Datasets (BioGRID) database provides interaction data with *S. cerevisiae* and other model organisms	Breitkreutz et al. (2008)
Comparative fungal genomics platform	http://cfgp.riceblast.snu.ac.kr/main.php	Standardized pipeline for annotating transcription factor (TFs) in fungal genomes and termed it Fungal Transcription Factor Database (FTFD)	Park et al. (2008)
Candida albicans	http://www.candidagenome.org/	Functional information about genes/proteins from human pathogen, *Candida albicans*; Biochemical Pathways and tool for full-text literature search on clinical and epidemiological studies	Skrzypek et al. (2010)

(continued)

Table 11.2 (continued)

Genome	Database (URL)	Functions/applications	References
Saccharomyces cerevisiae	www.stanford.edu/Saccharomyces/ http://www.yeastgenome.org http://pathway.yeastgenome.org	Collections and organizations of key biological data about genes and proteins and also used for genome-wide computational analyses of *S. cerevisiae*; Data for genetics, basic science, human health, and fermentation industry	Cherry et al. (1998, 2012), Christie et al. (2004), Engel et al. (2010), Engel and Cherry (2013)
Fungal kingdom	http://mips.gsf.de/projects/fungi/	A novel literature-mining tool gives access to structured information on relations between genes, proteins, phenotypes, and diseases. All databases can be accessed through the MIPS WWW server (http://mips.helmholtz-muenchen.de)	Mewes et al. (2010)
Schizosaccharomyces pombe	http://www.pombase.org/	PomBase is a comprehensive database for the fission yeast *S. pombe*. It provides a central hub for the fission yeast community, supporting both exploratory and hypothesis-driven research	McDowall et al. (2015)
Fungal kingdom	http://www.broad.mit.edu/FGI/	Genome sequence of fungi viz. S. *pombe*, *S. octosporus*, *S. cryophilus*, and *S. japonicus*	Rhind et al. (2011)
Fungal kingdom	http://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/fungal-genome-initiative	Describe tools and methods leveraged for eukaryotic genome annotation with a focus on the annotation of fungal nuclear and mitochondrial genomes	Haas et al. (2011)

(continued)

Table 11.2 (continued)

Genome	Database (URL)	Functions/applications	References
	http://genome.jgi-psf.org/programs/fungi/index.jsf http://fungi.ensembl.org/index.html		
Fungal genome	http://www.genomesonline.org	The Genomes OnLine Database (GOLD, http://www.genomesonline.org/) is a comprehensive resource for centralized monitoring of genome and metagenome projects. Information on complete and ongoing projects, along with their associated metadata	Pagani et al. (2012)
Fungal genome	http://FungiDB.org	Provides interface for comparative and functional genomic data mining of fungal species that differs from other fungal databases, e.g., SGD, CGD, and AspGD. It is also a functional genomics resource for pan-fungal genomes	Stajich et al. (2012)
Fungal genome	http://jgi.doe.gov/fungi	Fungal genome portal, developed by the US Department of Energy-JGI, for analysis, support integration, and dissemination of fungal genome data by interactive web-based tools. JGI Genome Portal automatically generates and monitors BioSample and BioProject submissions to NCBI	Nordberg et al. (2014), Grigoriev et al. (2014)

projects/fungi/); (6) MetaDB (http://www.neurotransmitter.net/metadb/); and (7) The Genomes Online Database (GOLD) (http://www.genomesonline.org/) (Table 11.2).

These databases generated by a European consortium, serves as a common resource, is freely available in the public domain. There are provision of providing sequence information and functional annotations on complete set of genes and proteins. Further, it also provides information on the biochemical interactions among different biomolecules, e.g., genes, proteins, carbohydrates, and lipids (Güldener et al. 2005). Additionally, the Comprehensive Yeast Genome Database (CYGD) had compiled huge amount of data resource on the cellular functions of *S. cerevisiae* and other yeast model organism

Need to Sequence Yeast Genome

The access to yeast genome sequence database is paramount to upgrade our knowledge of key genes and pathways of industrial relevance, fungal infection as well as the host–pathogen interaction. Genomics influences multiple areas of microbiology and thus affects key microbiological concepts. The sequence data also provides crucial information on how these pathogenic organisms multiply and survive persist in the living and non-living environment. Completion of the genome sequences has yielded greater insights into the general functions required for eukaryotic life and empowered the researchers to address a number of fundamental questions. Moreover, many of the sequenced yeast species form diverse sources have enabled to do comparative studies and explore an exceptional options to explore the basic biology and evolution of this medically and industrially significant kingdom (Sharma 2015).

Thousands of genome sequences have been completed, and with the use of next-generation sequencing technologies, many more genome sequences of different strains of industrial yeast are likely to be elucidated in coming years. Such sequences are pivotal for understanding the functional component of the genome, as well as determining the expression time, evolution of genes, and their regulation to mediate cellular metabolism and developmental processes (Nagalakshmi et al. 2008)

Yeast Genome Sequencing and Its Role in Biotechnological Advancement

In Ethanol Fermentation

Conventional yeasts, i.e., *S. bayanus*, *S. cerevisiae*, and *S. carlsbergensis*, are known to ferment ethanol from hexose sugars, but they do not have the machinery

to ferment pentose sugars (e.g., arabinose and xylose). Because of these limitations, a substantive amount of research has been done on metabolic pathway engineering of *S. cerevisiae* for improved pentose (e.g., xylose) metabolism (Galazka et al. 2010; Hahn-Hägerdal et al. 2006; Jeffries 2006). To solve this problem using fungal genomics information, researchers have explored a number of unconventional yeasts (Table 11.3) (Stephanopoulos 2007), e.g., native xylose fermenting species, *Pachysolen tannophilus* (monotypic genus) (Schneider et al. 1981), *Candida shehatae* (Dupree and Vanderwalt 1983), and *Pichia stipitis* (Jeffries 2006; Smith et al. 2008; Kurtzman and Robnett 2010). Several probable genes had also been identified by the genome-sequencing projects on yeast, which was unknown for long time. Comparative genome study of the large number of *S. cerevisiae* strains (Table 11.1) also helped in the study of gene clusters that have integrated into the genomes of bioethanol strains (Borneman et al. 2011).

Another important model organism, *Pichia pastoris,* has been used for the study of peroxisomal functions and methanol assimilation. Under tightly controlled methanol-inducible transgene expression, *P. pastoris* grows to high cell density, provides and efficiently secretes heterologous proteins in defined media. Therefore, *P. pastoris* expression technology has also been used for research and commercial purposes for several years (Cregg et al. 2000). Several *P. pastoris* strains have been developed for biopharmaceuticals product for which glycosylation is needed for proper folding, e.g., several vaccines are already on the market (Hamilton et al. 2006; Jacobs et al. 2009). Unfortunately, a few genetic tools or engineered strains have been generated for *P. pastoris* despite its biotechnological importance. Therefore, to facilitate development in the area of basic biology and biotechnology, Callewaert and co-workers have presented the 9.43 Mbp genomic sequence of the GS115 strain of *P. pastoris*. They also provided manually curated annotation for its 5313 protein-coding genes (De Schutter et al. 2009).

Later, *P. pastoris* (*Komagataella phaffii*) CBS7435, which was the parental strain of commonly used *P. pastoris* recombinant protein production hosts was sequenced for further improving the understanding of associated genomic features (Küberl et al. 2011). The whole-genome sequencing of *P. pastoris* CBS7435 has revealed high-quality genome sequence (9.35 Mbp) by using NGS technologies (Küberl et al. 2011).

Genome sequences of these model fungi could help scientists to produce 'designer strains' for specific usage in substrate saccharification and ethanol fermentation (Pain and Hertz-Fowler 2008; Annaluru et al. 2014).

In Food and Beverages

Pichia anomala strains have the ability to grow under high osmotic pressure and low pH due to which it is considered as a spoilage yeast in dairy and beer industry (Bonjean and Guillaume 2003). Furthermore, the yeast is also applied as a food-flavouring agent and food bioemulsifiers. *P. anomala* also facilitate to the aroma of wine by the production of ethyl acetate. Enzyme system, e.g.,

Table 11.3 Yeast Genome sequencing and its economic importance

Organism	Genome size (Mb)	GenBank accession/release date	Center/consortium	Uses/significance	References
S. cerevisiae	12	1996	International effort	Used in wine-making, baking, and brewing since pre-historic period times; exhaustively studied eukaryotic model organisms	Goffeau et al. (1996)
P. pastoris DSMZ 70382	9.4	04/14/2009	Natural Resources and Applied Life Sciences, Vienna, Austria	Used in biofuel, biochemical engineering, and genetic engineering research; also used in biotech industry	Blandin et al. (2000)
C. neoformans var. neoformans JEC21	19.05	07/13/2004	Stanford University	Causes meningitis in immuno-compromised hosts	Loftus et al. (2005)
Candida albicans WO1	14	03/28/2006	Broad Institute	Causes systemic disease in immuno-compromised patient post-surgery	Moore and Meškauskas (2006)
P. stipitis CBS 6054	15.4	02/23/2007	DOE- Joint Genome Institute	Ferments pentose sugar (xylose) to ethanol, used in lignocellulosic biofuel industry	Jeffries (2006), Wohlbach et al. (2011)
Wickerhamomyces anomalus (formerly *Pichia anomala*)	25.47	2012		Bio-control agent; wine fermentation; phytate digestion; source of vitamins and proteins	Passoth et al. (2006), Schneider et al. (2012)
Paracoccidioides brasiliensis Pb03	29	ABHV01000000	NIAID	Causes paracoccidioidomycosis—a systemic mycosis geographically restricted to South and Central America	Almeida et al. (2007)

(continued)

Table 11.3 (continued)

Organism	Genome size (Mb)	GenBank accession/release date	Center/consortium	Uses/significance	References
Wangiella (Exophilia) dermatitidis NIH/UT8656 (Black Yeast)	30	AFPA01000000	Broad Institute, NHGRI	Emerging human mycosis (phaeohyphomycosis) in immuno-compromised patient infects skin, brain, lung, eye, joints, and endocardium	Martinez et al. (2012)
Kuraishia capsulata CBS1993	11.4	CBUD020000001-CBUD020000057 HG793125-HG793131	JGI	Nitrate-assimilating Saccharomycetales, isolated from insect larvae, known for the production of extracellular polysaccharides	Morales et al. (2013)
Blastobotrys (Arxula) adeninivorans	11.8	PRJEB4557	Joint effort	Dimorphic thermotolerant, xerotolerant, and osmotolerant yeast uses wide range of carbon and nitrogen sources	Kunze et al. (2014)

glycosidases and xylosidases, is also reported to help in the development of aroma. Killer toxins of *P. anomala* also act against several undesirable molds and yeasts (Passoth et al. 2006).

Another fission yeast *Schizosaccharomyces pombe* is well known as a model organism in molecular and cell biology, as well as for traditional brewing. It is a unicellular eukaryote, whose cells are rod-shaped, 3–4 mm in diameter and 7–14 mm in length. Its genome has been reported to be approximately 14.1 million base pairs and is estimated to contain 450 non-coding RNAs and 4970 protein-coding genes (Wilhelm et al. 2008). The 13.8-Mb genome of *S. pombe* is distributed between chromosomes I (5.7 Mb), II (4.6 Mb), III (3.5 Mb) and mitochondrial (20-kb) genome (Wood et al. 2002). A fragment of 10.4-kb tandem arrays of 100–120 repeats containing the 5.8S, 18S, and 25S ribosomal RNA genes account for around 1.1 Mb. The three centromeres were found to be of 35, 65, and 110 kb length for chromosomes I, II, and III, respectively. Overall this contributes to approximately 12.5 Mb of unique sequence, similar in size to that of yeast *S. cerevisiae,* and smaller than few other sequenced model eukaryotic organisms (Wood et al. 2002).

The genome of *Arxula adeninivorans*, yeast of biotechnological interest, has been recently sequenced with 11.8 Mb assembly of the nuclear genome emerged in four different contigs (Kunze et al. 2014). This species is widely used as biocatalyst for the production of various biotechnologically important products such as tannase (Böer et al. 2011), 1-(S)-phenylethanol (Giersberg et al. 2012) or β-D-galactopyranoside (Rauter et al. 2013), for the detection of estrogenic activity (Hahn et al. 2006; Kaiser et al. 2010) and for the production of low purine food content (Jankowska et al. 2013). Due to the production of an extracellular redox molecule, *A. adeninivorans* have a higher power output, after the construction of microbial fuel cell in comparison with other yeast (Haslett et al. 2011). This organism is also used as a host for the production of recombinant proteins, and as a donor for genes encoding valuable products (Wartmann and Kunze 2003; Gellissen et al. 2005).

In the development of new traits, i.e., antimicrobial activities, fungal metabolites, biocatalysts, and strains, many be addressed by a more targeted approach if the genome sequence of multiple yeast is available in the public domain. Few of them are, i.e., search for novel enzymes understanding of the killer system, or identification of potential virulence factors. The JGI initiative to sequence yeast strain has improved our understanding of some important species (Passoth et al. 2011).

Yeast Genome Sequencing and Human Health

The priority of genome sequencing was those fungi that have the ability to act as a model organism for biomedical research or disturb human health significantly (Table 11.3). *Candida albicans* is one of the preferred eukaryotic pathogens which was selected for genome sequencing, because in healthy individuals and in severely immune-compromised humans it causes life-threatening infections, e.g., mucosal

and skin infections. In *C. albicans,* the extensive allelic differences help to increase the genetic diversity which eventually helps in aquiring drug resistance (Cowen et al. 2002). Further, comparative analyses of genome reveal important indication about the evolution of *C. albicans* and its mechanisms of pathogenesis (Jones et al. 2004). Comparison of *C. albicans* with other fungal genomes also provides information on numerous fungus-specific genes which can be targeted for anti-fungal therapy.

Cryptococcus neoformans, a basidiomycetous model yeast, is encapsulated and an obligate aerobe which causes life-threatening infections in the lungs and central nervous system of human. It can live in both plants and animals as teleomorph, i.e., *Filobasidiella neoformans*, belonging to the class Tremellomycetes. It is often found in bird excreta and can cause disease in both immune-competent as well as immune-compromised human. Complete genome (approx 20 Mb) of *C. neoformans* has been sequenced by Loftus and co-workers for fungal pathogenesis. They reported approximately 6500 intron-rich gene and a transcriptome with alternately spliced sequences (Loftus et al. 2005). The whole-genome sequencing revealed that *C. neoformans* genome is rich in transposons responsible for phenotypic variation (Loftus et al. 2005).

Further role in-host microevolution, Ormerod and co-workers had analyzed the isolates from a patient with acquired immunodeficiency syndrome of *cryptococcal meningoencephalitis* (Loftus et al. 2005). In contrast to *C. neoformans* and *A. fumigates*, which are acquired from the environmental sources, some of the *Candida* spp. are commensal in human gut and therefore form an integral component of the normal microflora (Fidel et al. 1999). The species of *Candida,* i.e., *Candida albicans* and *Candida glabrata,* are clinically most important species (Li et al. 2007). Interestingly, *C. glabrata* is the second most important pathogenic species of *Candida*; which has emerged as a leading organism of nosocomial infections. Its close relationship to *S. cerevisiae* and reduced susceptibility to antifungal drugs makes it a preferred material for genome research.

Recently, Linde and co-workers have provided a detailed RNA-seq-based analysis of the transcriptomic landscape of *C. glabratain* nutrient-rich media, as well as under nitrosative stress and during pH shift (Linde et al. 2015). Using RNA-seq data together with state-of-the-art gene prediction tools, they refined the annotation of the *C. glabrata* genome and predicted 49 novel protein-coding genes. Among all these novel genes, 14 have homologs in *S. cerevisiae* and 6 are shared with other *Candida* species (Linde et al. 2015).

Future Perspectives Yeast Genome Sequencing

The high-throughput technology of next-generation sequencing (NGS) machines has increased significantly in last one decade. In less than €15 per sample, approximately 384 libraries can be prepared from the yeast genomic DNA in less than one week using NGS technology (Wilkening et al. 2013). Wilkening and

co-workers have demonstrated a robust protocol by sequencing over 1000 yeast genomes (~30x coverage) at a very low price (Wilkening et al. 2013). NGS has been used to study recombination and aneuploidy in different model fungi, which can serve as a foundation for future studying linkage, crossing over (recombination), and chromosomal aberrations.

Transcriptome analysis using NGS technologies if yeast and other important fungi has generated some important data on differential gene expression and has also complimented the data of real-time PCR. It has successively replaced microarray technologies in the term of cost and reliability. Microarrays often cannot easily distinguish between closely related gene sequences due to cross-hybridization. The important aspect of transcriptome study of yeast is generation of large number of expressed gene sequences with overlapping 3′ ends. Snyder and co-worker have suggested that the yeast avoid degradation due to the presence of overlapping transcripts and absence of dicer homologs (Nagalakshmi et al. 2008).

For strain engineering, a better understanding of all aspects of the yeast's protein production machinery and the secretory system *P. pastoris* and engineered promoters is needed. Humanized glycosylation of *P. pastoris* strains has further increased the importance of *P. pastoris* for biopharmaceutical production. Moreover, monoclonal antibodies can be made at higher scale (g/L) in the humanized glycosylation-homogenous strains (Hamilton et al. 2006; Jacobs et al. 2009). To facilitate the investigation of *P. pastoris* and other methylotrophic yeasts, Callewaert and co-workers from Belgium have reported the 9.43 Mbp genomic sequence of the GS115 strain of *P. pastoris* (De Schutter et al. 2009). Subsequently, another group has also published high-quality genome sequences of other economically important yeast, e.g., 9.35 Mbp genome of *P. pastoris* CBS7435 using both Roche 454 and Illumina NGS (Küberl et al. 2011).

The comparative analysis of yeast genome and transcriptome reveals the rearrangement of chromosomal features while retaining function and taxonomic position (Rhind et al. 2011). Comparative yeast genomic analyses also support the cellular hypothesis proposed for several model species, for example, pathogenicity arose several times in the *Nakaseomyces*, a group more closely related to *S. cerevisiae*. Furthermore, this group represents a true genus, with similar ancestral traits that may favor human adaption. With the advancement of genome-sequencing techniques, more genera will be chosen for comparative genomics, and by considering all these data, one can depict the origin of pathogenesis. Extraordinary opportunities for comparative genetic analyses are possible due to the availability of yeast genome sequences (http://www.tigr.org/tdb/mdb/mdbinprogress.html) and several planned genome projects (http://www.genome.wi.mit.edu/seq/fgi).

The new age in yeast biology has potential to provide insights into basic understanding of the cellular processes identical to all eukaryotes and fungi per se. The construction of designer chromosome III (Annaluru et al. 2014) and synthetic genome (Gibson et al. 2008), of budding yeast *S. cerevisiae*, indicates the feasibility to develop a customized genome for all economically important yeast in future with the help of genome sequences (Fig. 11.2). Further, applying genome-scale

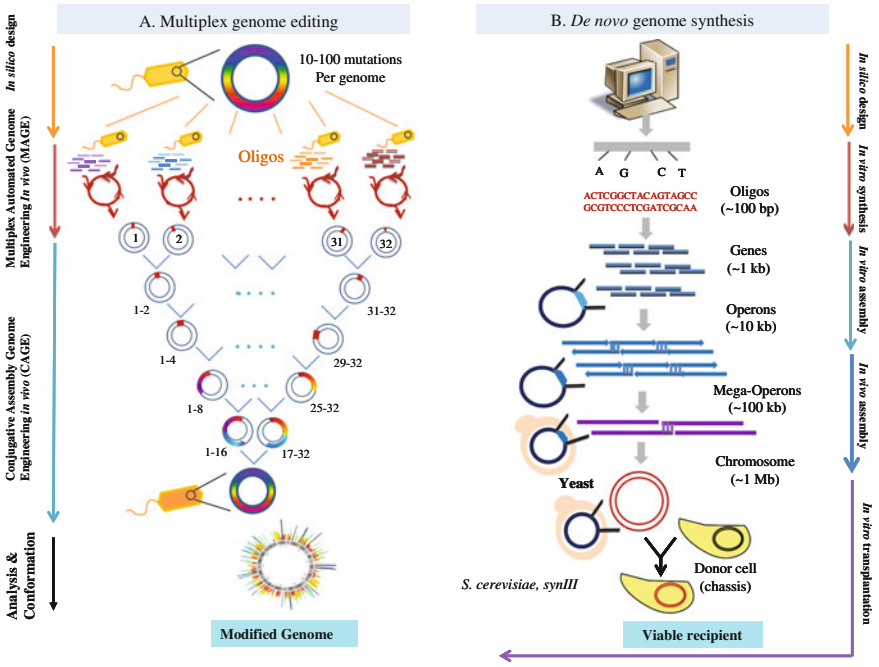

Fig. 11.2 Strategy for the construction of synthetic genome of *S. cerevisiae*

information to metabolic engineering provides a platform to predict logical design, computational models, and multiplexed pathway construction to address the genuine pathway engineering challenges (Esvelt and Wang 2013).

Efficient automatic methods for protein structure prediction are becoming increasingly relevant as a result of the influx of huge amount of genomic data arising from sequencing projects. A new fold recognition method, GenTHREADER, can be applied to either whole, translated yeast genomic sequences (Jones 1999). This method can be used particularly for detecting super-family relationships and exploits a conventional sequence profile-based alignment algorithm to generate sequence-structure alignments, which are then analyzed by a set of statistical potentials.

Conclusions

The magnificent power of yeast genomics has improved our knowledge of industrially relevant genes and gene products which have been in the service of human being since 6000 years. Information on the gene expression, metabolic pathways, protein levels, subcellular localization yields a 'genomic understanding' of how yeast metabolizes, grows, and reproduces. The yeast genome sequence along with

transcriptome sequence analysis of infected plants and humans will lead to a more exhaustive understanding of the pathogens and pathogenesis, which may further help in the formulation of disease management program. Results of comparative genome data of diverse strains may improve our knowledge of evolution processes in eukaryotes and eukaryotic genome, using yeast models. The comparative genome analysis would likely shed light on the regulatory divergence that has accompanied the ecological diversification in several strains of yeast species. With the availability of complete genomic sequences, and the numerous molecular tools for yeast genetics and genomics, no other species in fungal kingdom offers similar potential. To conclude, the high-throughput technologies will extend the wisdom enjoyed by the study of model yeast, *S. cerevisiae* to other eukaryotic organisms, initiating genome engineering and metabolic re-wiring of genes for desired outcome in basic biology and biotechnology.

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Fungal Differentiation: A Model Phenomenon to Screen Antifungal Drugs

12

E. K. Pathan, S. G. Tupe and M. V. Deshpande

If we can solve the same problem equally well with yeasts or with human cells, common sense tells us to stick with the simpler and less expensive system.

— James Watson
Molecular Biology of the Gene Vol. I, 1987

Abstract

Pathogenic fungi change the morphology to a suitable vegetative form such as unicellular yeast or filamentous mycelium for the survival and proliferation in the host as well as to defeat cellular and physiological defences of the host. The microenvironment of the pathogen triggers different biochemical processes finally leading to differentiation. These biochemical processes, which correlate with morphological change from saprophytic/less virulent to pathogenic form, can be targeted for the development of novel antifungal agents. The present chapter deals with the role of differentiation in fungal pathogenesis and discusses different biochemical events as target for the antifungal strategies.

Keywords

Antifungal · *Benjaminiella poitrasii* · Dimorphism · Fungal pathogenesis
Yeast-Hypha reversible transition

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Introduction

Over the past few decades, fungal infections in humans have become more prevalent due to mainly immune compromization in the hosts. Furthermore, the capacity of the pathogens to change the morphology for the survival and proliferation in the host makes them uncontrollable even with broad spectrum antibiotics. Most of the fungi differentiate generally in three phases, vegetative, asexual and sexual. Moreover, to defeat the physiological and cellular defences of the host, most of the pathogenic fungi change to a convenient vegetative morphological form such as unicellular yeast or filamentous mycelium (Doiphode et al. 2009a; Ghormade et al. 2012). Under form-specific favourable conditions, the asexual and sexual spores too germinate into either unicellular yeast or filamentous mycelium. In case of *Histoplasma capsulatum*, the sexual ascospores germinated into yeast-like cells at 37 °C (Kwon-Chung 1971). Thus, a sexual spore that has the ability to germinate into yeast cells may have a potential for invading a susceptible host. In case of *Cokeromyces recurvatus*, a dimorphic zygomycete, the sporangiospores were reported to germinate into the unicellular yeast which is an infective propagule for pathogenesis (Kemna et al. 1994). In case of *Benjaminiella poitrasii*, a non-pathogenic zygomycete, under yeast form favouring conditions (presence of glucose in yeast extract peptone medium and 37 °C), the sporangiospores and zygospores germinated into yeast cells, while under hypha favouring conditions (no glucose and 28 °C), both the asexual and sexual spores germinated by producing germ tubes (Ghormade and Deshpande 2000). These two forms change reversibly in response to the host environment too. In short, halting the germination of the asexual or sexual spores can be one of the important approaches to develop a new antifungal strategy. The following sections describe the significance of the morphological changes in fungal pathogenesis and different biochemical correlates of morphogenesis as potential targets for the development of new antifungal drugs.

Role of Differentiation in Fungal Pathogenesis

Brown et al. (2012) reported that more than 90% of deaths due to fungal infections are because of *Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus* and *Pneumocystis jirovecii*. Candidiasis is the most common fungal infection caused by *Candida* species due to which 40% mortality rates were reported (Pukkila-Worley et al 2009). Systemic and localized candidiasis is a significant health issue. Other major dreadful fungal pathogens are: *Rhizopus oryzae, Blastomyces dermatitidis, Coccidioides immitis, H. capsulatum, Paracoccidioides brasiliensis* and *Penicillium marneffei* (Brown et al. 2012).

In most of the cases, the process of pathogenesis can be divided into stages such as infection, adhesion, invasion, dissemination, pathogenesis/damage to host cells and escape from the phagocytes by the pathogen. Most of these stages are related to physiological make-up leading to morphological outcome and therefore can be

target for the antifungal agents. Table 12.1 describes morphology-associated events in different fungal pathogens.

Infection

In majority of fungi, the infective propagules are either spores, asexual/sexual, or unicellular yeast cells. As mentioned above, theses infective propagules respond to the host environment and either produce germ tubes for penetration in the host tissue or unicellular form for further dissemination in the host body (Ghormade and Deshpande 2000; Kwon-Chung 1971).

Adhesion

In *P. brasiliensis*, adherence was correlated with the virulence as in an in vitro experiment more virulent strains in animals showed enhanced adhesion (Hahn et al. 2003). The fungal cell wall, a crucial component in adhesion, is mostly composed of chitin, glucan, mannan and carbohydrate binding proteins. Adhesion of fungi to host surface involves interaction between the adhesins, mainly mannoproteins in fungal cell wall and glycosides on host epithelial cells surface (Fukazawa and Kagaya 1997; Kanbe and Cutler 1998; Lima et al. 2001; Ruiz-Herrera et al. 2006). In most of the human pathogens, the cell walls of yeast-form cells have more mannan contents as compared to hyphal-form cell. The similar trend was observed in non-pathogenic *B. poitrasii* (Khale and Deshpande 1992). Joshi et al. (2010) reported that cell wall mannan content in *B. poitrasii* can be manipulated by using NAD-glutamate dehydrogenase (NAD-GDH) modulating substances such as α-ketoglutarate, glutamate and isophthallic acid, an inhibitor of NAD-GDH. The same approach can be used to develop antifungal compounds which can hinder adhesion of the pathogen. As yeast-form cells are involved in attachment to host, inhibition of hypha to yeast transition (and reverse) can be an alternate strategy to control the adhesion.

Invasion

In general, one of the forms, either yeast or hypha, is considered as saprophytic/natural form, while other is pathogenic one. For instance, Y-H morphogenesis is most crucial step to promote the invasion in host tissue (Gow et al. 2012; Jacobsen et al. 2012; Klein and Tebbets 2007; Nadal et al. 2008; Nemecek et al. 2006; Phan et al. 2007; Thompson et al. 2011). The mutants of respective fungal pathogens defective in such transitions were reported to be non-pathogenic

Table 12.1 Morphology associated events in host–fungal pathogen interaction

Organism	Morphology associated events in host–pathogen interaction					Remarks
	Infective propagule	Adhesion	Invasion	Dissemination	Pathogenesis/damage	
B. dermatitidis (telemorph *Ajellomyces dermatitidis*)	Conidia	Yeast form	Yeast form	Yeast-form cells	Yeast form	Adhesin WI-1, also called as BAD-1, is dispensable for virulence
C. albicans	Commensalic forms (H and Y)	Hyphal form	Hyphal form	Yeast form in blood stream: H form across epithelial barrier	Higher with hyphal form	H form can escape from phagocytosis
C. neoformans	Basidiospores or desiccated yeast cells	Yeast form	Yeast form	Yeast form penetrates blood-brain barrier	Capsulated yeast cells of different forms	Enlarged yeast form (titan cells) can escape from phagocytosis. Melanin present in the cell wall acts as antioxidant and also helps in evading phagocytosis
H. capsulatum	Conidia	Yeast form	Yeast form	Yeast form throughout reticuloendothelial system	Yeast-form cells	Calcium binding proteins and sulfhydryl group are important in virulence
P. brasiliensis	Spores	Yeast form	Hyphal form	Yeast form in blood stream	Crescent bodies, balloon-like yeasts	Glycoprotein gp43 is essential for virulence (adhesion and invasion). Yeast cells induce apoptosis in host for survival. After adhesion, it induces the host epithelial cells to produce pseudopodia that engulfs the organism and pull into cell. It is important in virulence
Mucor, *Rhizopus*,	Sporangiospores		Hyphal form	Yeast form in blood	Aseptate hyphae in the tissue	Large sporangiospores are more virulent; calcineurin is important in producing large

(continued)

Table 12.1 (continued)

Organism	Morphology associated events in host–pathogen interaction					Remarks
Absidia and *Cunninghamella*						spores; proteolytic, lipolytic and glycosidic enzymes as well as metabolites like alkaloids or mycotoxins as agroclavine are virulence factor; possible involvement of endosymbiotic bacteria in the pathogenesis of the disease
Sporothrix schenckii	Conidia	Both conidia and yeast cells	Yeast form	Yeast form	Cigar shaped and budding yeast cells/asteroid bodies	Acid phosphatase and proteases are important in virulence; both morphological stages have the ability to synthesize melanin, important in virulence. Thermotolerance is an important virulence factor
Penicillium marneffei	Conidia	Conidia can recognize fibronectin and bind to laminin via a sialic acid-specific lectin	Fission yeast form	Yeast form	Fission arthroconidia or unicellular round to oval cells, which may divide by cross wall formation in macrophages or histiocytes	Virulence factors—both mycelia and yeast expressed alkaline phosphatase, acid phosphatise phosphohydrolase esterases, lipases and galactosidases were found in some isolates
Aspergillus fumigatus	Conidia	Conidia	Hyphal form	Hyphal form	Hyphal cells	Virulence factors include thermotolerance, production of adhesins, pigment-like melanin, toxic metabolites and extracellular hydrolytic enzymes. Hyphal form synthesizes melanin that protects the organism from UV radiation, enzymatic lysis, extreme temperatures, as well as against reactive oxygen species during infection

Compiled from Barros et al. (2011), Bradsher et al. (1985), Jacobsen et al. (2012), Karkowska-Kuleta et al. (2009), Klein and Chang (2002), Lee et al. (2015), Mendes-Giannini et al. (2008), Montenegro (1995), Morace and Borghi (2012), Okagaki et al. (2010), Porta et al. (2012), Restrepo (2000), Vanittanakom et al. (2006)

(Jacobsen et al. 2012; Lo et al. 1997; Murad et al. 2001; Saville et al. 2008). Therefore, targeting the morphological transition could serve as unique process to design antifungal drugs. The small molecules such as farnesol, fatty acids, rapamycin, geldanamycin and inhibitors of chitin synthases (CSs), glutamate dehydrogenases (GDHs), ornithine decarboxylase (ODC) and cell cycle were reported to modulate the yeast-to-hypha transition (Chaudhary et al. 2009; Ghormade et al. 2005; Joshi et al. 2013; Shareck and Belhumeur 2011). For rapid screening of such "antidimorphism", molecules fungal model system needs to be established.

Dissemination

The fungi use different dissemination processes depending on the environment in the vicinity of the specific tissues (Casadevall and Pirofski 2001; Latge and Calderone 2002). For instance, yeast morphology is convenient in the blood stream. In case of *C. albicans*, however, serum induces Y-H transition and it was reported that more than 40% of yeast cells in blood produced germ tubes within 30 min (Fradin et al. 2005). But the complete hyphal formation was hindered by neutrophils. There was no sufficient evidence that neutrophils stimulate yeast growth in the presence of serum which is an effective trigger for hyphal formation (Jacobsen et al. 2012). It is, therefore, unlikely that in *C. albicans* H-Y transition in the blood is possible as a dissemination process. Nevertheless, yeast cells are convenient form for pathogenic fungi, in general for dissemination through blood stream.

Virulence Factors

In case of most of the human pathogenic fungi, virulence factors are growth at higher temperature, viz. human host temperature, morphological transitions such as dimorphism, capsule formation, cell wall modulation and production of a variety of enzymes. Indeed, all fungal metabolites, per se, are not considered as virulence factors.

Rhodes (1988) reported that the isolates of *C. neoformans* which could not grow in the human host (i.e. at 37 °C) did not cause fatal infection in mice. Similarly, in case of *H. capsulatum*, more virulent strains were reported to withstand human body temperature as well as could exhibit rapid H-Y transition (Maresca and Kobayashi 1993; Maresca et al. 1994). Most importantly, in case of non-pathogenic *Saccharomyces cerevisiae* the strains which could grow normally at 37 °C were reported to be potential pathogens due to their transition ability to produce pseudo-hyphae at 39–42 °C (McCusker et al. 1994). Heat shock proteins such as HSP17, HSP70 and HSP82 were reported to be essential for survival at higher temperature as well as for H-Y transition for pathogenesis (Goldani et al. 1994; Porta et al. 2012).

The capsule formation by different types of yeast-form cells of *C. neoformans* was reported to be important to combat against phagocytosis (Kwon-Chung and Rhodes 1986; Rhodes et al. 1982). The formation of enlarged yeast (titan) cells and pseudo-hyphae with capsule were found to be important in fighting against phagocytosis (Lee et al. 2012; Zaragoza and Nielsen 2013). Primarily, two polysaccharides, namely glucuronoxylomannan and galactoxylomannan, in addition to a smaller proportion of mannoproteins form the capsule. Therefore, the molecules which can hinder the synthesis of these polysaccharides probably be useful as antifungal agents. Though earlier it was suggested that hyphal development was associated with decreased virulence, Feretzaki et al. (2014) reported in their study that the serotype D highly filamentous strain of *C. neoformans* was found to be hyper-virulent in an animal model. Therefore, it would be necessary to understand the biochemical correlation among capsule formation, Y-H transition and virulence.

The quantitative differences in the cell wall polymers and in turn their deposition patterns in yeast- and hyphal-form cells contribute significantly in the pathogenesis. For instance, in *Blastomyces dermatitidis* H-Y transition was correlated with increase in α-(1,3)-glucan and decrease in *β*-(1,3)-glucan contents in cell wall (San-Blas and San-Blas 1984). Usually, the level of α-(1,3)-glucan in the cell wall significantly correlated with the virulence (Hogan and Klein 1994). Therefore, targeting the synthesis of α-(1,3)-glucan appears promising route for antifungal drug development. As chitin, a *β*-1,4-linked *N*-acetylglucosamine polymer, is main structural component of the fungal cell walls and is absent in mammals, targeting its synthesis or hydrolysis can be a useful approach to control fungal pathogens (Chaudhary et al. 2013; Ghormade et al. 2000). It has been reported earlier that chitin synthesis is the main event during protoplast regeneration as well as growth and Y-H transition in *B. poitrasii* (Chitnis and Deshpande 2002; Chitnis et al. 2002; Ghormade et al. 2012). In *C. albicans*, hyphal cells contain approximately 3–5 times more chitin than yeast cells (Díaz-Jiménez et al. 2012). The presence of chitin in cell wall was reported to play an important role in interaction with host immune system too (Bates et al. 2006).

One of the relatively less explored targets is melanin synthesis which was reported to be important as a virulence factor in *C. neoformans* (Plonka and Grabacka 2006; Perfect 2006).

Fungi secrete several hydrolytic enzymes such as chitinases, lipases, proteinases and phospholipases that play an important role in pathogenesis, damaging the host cells and releasing nutrients in a restricted environment (Ghormade et al. 2000; Ogawa and Tsuboi 1997; Rhodes 1988). In addition to the above enzymes, dermatophytic fungi also produce extracellular enzymes such as keratinase, collagenase and gelatinase involved in establishing the infection.

The secretion of proteinases by certain pathogens, viz. *C. albicans*, *C. neoformans*, *C. immitis,* is considered as an important virulence factor (Kwon-Chung et al. 1985; Resnick et al. 1987). Aspartyl proteinases were found to be involved in hydrolysis of host proteins such as collagen, laminin, fibronectin, immunoglobulins, α2-macroglobulin and precursors of several blood coagulation factors

(Naglik et al. 2004; Schaller et al. 2005). In case of *C. albicans*, for instance, 8 aspartyl proteinases (SAPs) were reported to be involved in virulence by selective expression in different morphological forms. For example, SAPs 1–3 were found to be yeast-form-specific, whereas SAPs 4–6 were specific for hyphal form (Naglik et al. 2004; White and Agabian 1995). Phospholipases (PLs) are involved in tissue invasion and therefore were reported to be significantly higher in hypha (Ghannoum 2000). The spores and hyphae of *C. immitis* produced serine proteinases which contribute significantly in pathogenesis (Resnick et al. 1987). While in case of *Sporothrix schenckii*, in addition to proteinases, acid phosphatases produced by conidia, yeast and hyphal-form cells were necessary for interaction with macrophages and skin components (Garrison and Arnold 1983; Tsuboi et al. 1987, 1988).

Biofilms are principal microbial growth forms and are critical for infections of many pathogenic fungi, including *Candida*, *Aspergillus*, *Cryptococcus*, *Trichosporon* and *Coccidioides* (Fanning and Mitchell 2012). In general, biofilm formation is associated with hyphal morphology; however, *C. albicans* yeast form was also found to contribute in the biofilm formation. Fanning and Mitchell (2012) suggested that in the biofilm the resistance of the pathogen to antifungal drugs is due to its structural complexity, presence of extracellular matrix (ECM) and/or over-expression of efflux pump genes. Since fungal cell wall polysaccharides were found to be involved in formation of biofilm, the combinational therapy with cell wall polymer inhibitors, inhibitors of efflux pumps responsible for multidrug resistance and other molecules targeting different mechanisms can be used for treatment.

Evasion of Host Immune System

C. albicans evades the phagocytosis by shielding cell wall β-glucan with cell surface mannan. The oxidative stress of phagocytic cells was reduced by superoxide dismutase produced in hyphae protecting the pathogen from the attack of host monocytes, macrophages and neutrophils (Hostetter 1994; Leng et al. 2000; da Silva et al. 2010). Therefore, Y-H transition is a critical step for evading phagocytes. *H. capsulatum* fights against phagocytosis by modulating the phagolysosomal pH to 6.5 that leads to inactivation of fungicidal activity and thereby supports its survival inside the macrophages (Eissenberg et al. 1988; Howard 1965).

As mentioned earlier, capsule of *C. neoformans* helps the pathogen to establish in host cells (Murphy and Cozad 1972; Sundstron and Cherniack 1993). Additionally, *C. neoformans* activates the alternate pathway of suppressing the cytokine production by macrophages to evade the host immune system (Masih et al. 1991; Rubinstein et al. 1989; Vecchiarelli et al. 1994, 1995). Similar mechanism of stimulation of suppressor cells and cytokine production was also seen in *P. brasiliensis* (Coelho et al. 1994).

Different Biochemical Events to Target for Inhibition of Fungal Morphogenesis

Since morphological transition is important for fungal pathogenesis, understanding the biochemical events involved in this process is important to design new antifungal drugs. The changes in the cell wall architecture accompanied with the increased or decreased levels of the carbon and nitrogen metabolizing enzymes involved in the cell wall synthesis and hydrolysis facilitate the morphological change in fungi. These enzymes have been studied in various fungi as biochemical correlates of the morphological transition which could be useful antifungal drug targets (Chaudhary et al. 2013; Deshpande 1996, 1998; Deshpande et al. 1997; Ghormade et al. 2012; Joshi et al. 2013).

Significant differences in the chemical composition of cell wall between yeast and hypha were observed for *B. poitrasii*, *M. rouxii*, *C. albicans*, *S. schenckii*, *B. dermatitidis*, *H. capsulatum* and *P. brasiliensis* (Khale and Deshpande 1992; Orlowski 1991; San-Blas and San-Blas 1985). Fungal cell walls are composed of β-1,3-glucans covalently linked to β-1,6-glucans as well as chitin, mannans and cell wall proteins. The enzymes involved in cell wall synthesis and hydrolysis, like chitin synthase, glucan synthase, chitinase and *N*-acetylglucosaminidase, play an important role in Y-H reversible transition. Chitin synthase is one such promising target as it is absent in plants and mammals. Polyoxin and nikkomycin were the first reported CS inhibitors isolated from culture filtrates of *Streptomyces* sp.; thereafter, a number of CS inhibitors were isolated from natural sources like plants, microorganisms. Synthetic chitin synthase inhibitors have also been designed and prepared mainly based on diversity-oriented synthesis of UDP-GlcNAc analogues. Nikkomycin Z, a chitin synthase inhibitor, is under clinical development (Chaudhary et al. 2013). The echinocandins act as non-competitive inhibitors of β-1,3-glucan synthase and thereby cause a loss of cell wall integrity and severe cell wall stress (Eschenauer et al. 2007).

The ammonia-assimilating enzymes, viz. NAD- and NADP-dependent glutamate dehydrogenases (NAD- and NADP-GDH) and/or glutamate synthase (GOGAT) and glutamine synthetase (GS), play significant role in nitrogen metabolism which is necessary for chitin synthesis. It was for the first time reported that relative proportion of NAD- and NADP-GDHs showed correlation with Y-H transition in *B. poitrasii* (Joshi et al. 2013; Khale-Kumar and Deshpande 1993; Khale et al. 1992). Thykaer et al. (2009) reported that the disruption of NADP-GDH gene was found to affect morphology in *Penicillium chrysogenum*. Therefore, GDH inhibitors could be used as antifungal drugs to control fungal pathogenesis (Choudhury and Punekar 2007; Cunliffe et al. 1983; Joshi et al. 2013). Most of them were competitive inhibitors and analogues of the substrates (2-ketoglutarate and L-glutamate). Isophthalate was one of the most studied inhibitor of both NAD-GDH (Cunliffe et al. 1983; Stevens et al. 1989; Veronese et al. 1974) and NAD (P)-GDH (Caughey et al. 1956, Rogers et al. 1972) of fungi. Rogers (1971) reported the use of structural analogues of L-glutamate such as glutaric acid, thiodiglycolic acid, oxydiglycolic acid and iminodiacetic acid as inhibitors of bovine GDH. Analogues of

2-ketoglutarate such as 2-oxoglutarate, 2-iminoglutarate, 2-methyleneglutarate, 2,4-pyridinedicarboxylate, 3,5-pyrazoledicarboxylate and others were reported to be screened for the inhibition of NADP-GDH from *Aspergillus niger* (Noor and Punekar 2005). In *B. poitrasii*, 1,2,3 triazole-linked β-lactam–bile acid conjugates were found to be potent inhibitors of purified NAD-GDH, which also significantly affected Y-H transition in *B. poitrasii*. Furthermore, these compounds also inhibited dimorphic transition in *C. albicans* strains (Joshi et al. 2013).

In fungi, cAMP plays an important role in a variety of morphological processes, such as conidiation, dimorphism, phototropism, hyphal branching, spore germination. (Bahn et al. 2007; Deshpande et al. 1997; Robson et al. 1991). The cAMP levels were suggested to be more in the yeast form than hyphal cells and exogenous addition of cAMP or dibutyryl derivative of cAMP (dbcAMP) favoured H-Y transition in *B. poitrasii* (Khale-Kumar and Deshpande 1993). Ocampo et al. (2012) reported that cAMP-dependent protein kinase A regulatory subunit isoform R4 is essential for differentiation in *Mucor circinelloides*. The other enzymes involved in this regulation include adenylate cyclase and ammonium ion permease. These enzymes can be targeted for inhibition of fungal morphogenesis. The compounds such as phenylaminopyrimidines (PAP)-pyridines, gleevec, BIRB796, rapamycin, triazolo [1, 5-α] pyrimidine could be used to inhibit the cAMP dependent protein kinases (Brunn et al. 1996; Pillonel 2005; Richardson et al. 2006). In *B. Poitrasii,* the cAMP-dependent phosphorylation of NAD- and NADP-GDHs was reported, which influenced Y-H transition (Joshi et al. 2013; Khale-Kumar and Deshpande 1993). Furthermore, in the presence of protein kinase inhibitors, (genistein and H-7) and a Ca^{++} channel blocker (verapamil), both chitin synthase activity and germ tube formation during Y-H transition in *B. poitrasii* was found to be affected (Deshpande et al. 1997). Therefore, chitin synthase, GDH and others as a correlate of Y-H transition in *B. poitrasii* can be an ideal choice for antifungal drugs. Another such enzyme is trehalase, which was shown to be activated by cAMP-dependent phosphorylation, which caused trehalose breakdown and breaks the spore dormancy and induced germination in fungi, in general (cited in Thevelein 1984).

Another second messenger molecule, Ca^{++}, plays an important role in growth and differentiation in several yeasts and fungi (Muthukumar et al. 1987; Shapiro et al. 2007). Effect of Ca^{++} is mediated by calcium–calmodulin (Ca–CaM), which affects the phosphorylation and dephosphorylation of enzymes including protein kinase, phospholipase A2, NAD^{+} kinase, adenylate cyclase and phosphodiesterase and glutamate dehydrogenase (Anraku et al. 1991; Deshpande et al. 1997; Ghormade et al. 2005). Muthukumar and Nickerson (1984) reported that Ca–CaM interaction was necessary for the hyphal growth in *Ceratocystis ulmi,* and its absence led to the yeast development. With the help of Ca–calmodulin-specific inhibitors like trifluoroperazine (TFP), it was demonstrated that Ca–CaM interaction was necessary for Y-H transition in *C. albicans* (Holmes et al. 1991; Paranjape et al. 1990) and *S. schenckii* (Rivera-Rodriguez and Rodriguez-del Valle 1992).

The polyamine metabolism also plays an important role in fungal morphogenesis. The polyamines, putrescine, spermidine and spermine are low-molecular weight substances, synthesized in the cells from their immediate precursor,

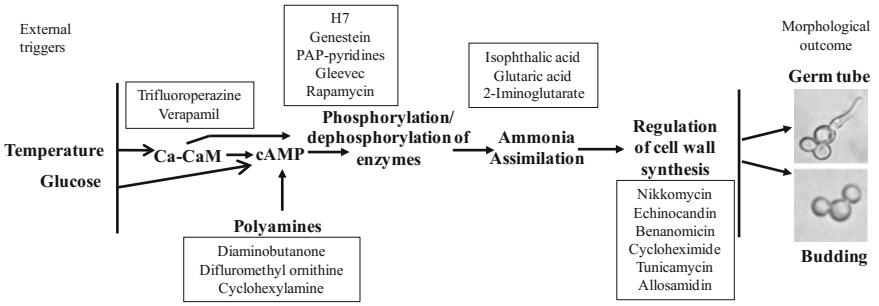

Fig. 12.1 Possible biochemical events linking environmental changes and morphological outcome in *Benjaminiella poitrasii*. The specific respective inhibitors are mentioned in the boxes

ornithine. They are essential for cell growth, differentiation and transformation (Calvo-Mendez et al. 1987; Garcia et al. 1980; Ruiz-Herrera et al. 1983; Tabor and Tabor 1985). The ornithine decarboxylase is an important enzyme for the synthesis of the polyamines (Ruiz-Herrera and Martinez-Espinoza 1998; Ruiz-Herrera 1994). Ruiz-Herrera (1994) showed that addition of diaminobutanone (DAB), a competitive inhibitor of ODC, caused inhibition of sporulation, spore germination and Y-H transition in *Mucor* and *Phycomyces*. The other inhibitors of ODC, viz. difluromethyl ornithine (DFMO) and dehydromonofluromethyl ornithine, also showed similar effect on morphogenesis.

The sulphur metabolism is known to play an important role in Y-H transition of *H. capsulatum* (Kumar et al. 1983). The enzymes involved are cysteine reductase, cysteine oxidase and sulphite reductase. The differential regulation of cysteine oxidase, cysteine reductase and sulphite reductase in yeast- and hyphal-form cells of *H. capsulatum* illustrated the importance of sulphur metabolism in a dimorphic transition (Maresca and Kobayashi 1989, 2000).

Different biochemical correlates of morphogenesis described above could be targeted for prevention of morphological transitions important in fungal pathogenesis and development of novel and effective antifungal agents (Fig. 12.1).

Need for New Antifungal Drugs

Worldwide, ~1.2 billion people suffer from various fungal infections and estimated mortality due to invasive mycosis is around 1.5–2 million/year. The mortality is higher than the deaths caused by malaria or tuberculosis (Denning and Bromley 2015). Presently, five classes of drugs are used for the treatment of invasive fungal infections. The drugs, their class and targets are given in Table 12.2. Among these antifungal agents, azoles are the most frequently employed antifungal drugs, due to their high therapeutic index and broad spectrum of activity. However, over the years, many fungal pathogens have developed

Table 12.2 Currently available antifungal agents and their targets

Class	Target	Inhibition of pathogenesis	Currently available agents
Polyenes	Binding to ergosterol and disruption of cell membrane	– Inhibit adherence of *Candida* and *Cryptococcus* – Inhibit biofilm formation	Amphotericin B, Nystatin
Azoles	Ergosterol biosynthesis (inhibits lanosterol 14-α demethylase)	– Inhibit host tissue damage during pathogenesis – Inhibits yeast-to-hypha transition in *C. albicans*	– Imidazole (Clotrimazole, ketoconazole, miconazole) – Triazoles (Fluconazole, itraconazole, voriconazole, posaconazole, etc.)
Allylamines	Ergosterol biosynthesis (inhibits squalene epoxidase)	– Inhibit budding of yeast cells of *Candida*	Terbinafine
Echinocandins	Glucan synthesis (inhibits *β*-glucan synthase)	– Inhibit adherence of *Candida* and *Cryptococus* – Inhibit biofilm formation	Caspofungin, Micafungin
Fluorinated pyrimidine	Nucleic acid synthesis	– Inhibits budding of yeast cells of *Candida*	5-fluorocytosine

resistance against fluconazole and other azoles due to their wide use as first line drugs in treatment (Kanafani and Perfect 2008) Fluconazole is ineffective against filamentous fungi like *Aspergillus.* Other drawbacks associated with azoles include mainly low oral bioavailability and hepatic toxicity (Sheng et al. 2011). Posaconazole is the most effective amongst azoles with extended antifungal spectrum, significant activity against the zygomycetes and optimal safety profile. Amphotericin B is the most effective wide spectrum antifungal agent. Amphotericin B is known to inhibit adherence of *Candida* and *Cryptococcus* to host epithelia during initial stage, thereby inhibiting biofilm formation (Dorocka-Bobkowska et al. 2009; Martinez and Casadevall 2006). However, its use is limited because of infusion-related toxicities, the frequent association of renal dysfunction and the intravenous formulation (White et al. 1998). Use of nystatin is also restricted to topical application due to host toxicity. The use of 5-fluorocytosine is limited because of hematological toxicity, effect on non-target cells and rapid development of resistance. Echinocandin, e.g. caspofungin, exhibits in vitro activity against *Aspergillus* and *Pneumocystis carinii* but it is reported to be inactive against zygomycetes, *Fusarium solani*, *Scedosporium prolificans*, *Cryptococcus* sp and *Trichosporon* spp. (Ito et al. 2000; Pacetti and Gelone 2003; Zaas and Alexander 2005) Caspofungin is a viable option for treatment of refractory aspergillosis. However, its use narrows down because of higher incidence of side effects,

potential drug–drug interactions and resistance towards some of the *Candida* spp. (Eschenauer et al. 2007).

The current status of fungal infections and the drawbacks of existing drugs enlisted above including acute and chronic side effects, less clinical efficiency and effect on non-targeted cells demand for the development of more effective, safe antifungal agents with novel targets.

Benjaminiella poitrasii as a Model to Screen Antifungal Compounds

To screen antifungal compound, researchers have used different model systems. Kneifel et al. (1974) reported the use of zygospore formation inhibition test using *Mucor hiemalis* to screen antifungal antibiotics. Nucleoside antibiotic thrautomycin, produced by *Streptomyces exfoliates*, was identified using this test. It was observed that (+) and (−) hyphae which came together for zygospore formation had different sensitivities towards thrautomycin. Several pathogenic fungi require 24–48 h for complete yeast-hypha transition (Domer 1985). The transition of yeast cells into hypha in *P. brasiliensis* was within 18 h. *Wangiella dermatitidis*, a causative agent of phaeohyphomycosis in humans, in addition to the long incubation time, displayed polymorphic forms during transition from thin-walled and thick-walled yeast, multicellular form, moniliform hyphae and true hyphae (Kester and Garrett 1995). Therefore, the use of *W. dermatitidis* as a model is not suitable. The dimorphic zygomycete *B. poitrasii* has been extensively studied for the biochemical correlates of Y-H and reversible transitions (Khale 1990; Khale et al. 1990). It has many advantages as model for dimorphism over other fungi, viz: (i) it is non-pathogenic, (ii) transition in either direction (Y-H or H-Y) is relatively rapid and without any intermediate forms, (iii) simple dimorphism triggering factors (pH, temperature and glucose), (iv) it shows temperature-dependent dimorphism similar to pathogens like *H. capsulatum*, *P. brasiliensis* and (vi) the asexual and sexual spores of *B. poitrasii* also respond to the dimorphism triggering conditions similar to the vegetative cells. With the help of stable, monomorphic (yeast-form; Y-2 and Y-5) mutants of *B. Poitrasii*, the extensive studies on biochemical enzyme correlates (GDH, CS, chitinase, *N*-acetylglucosaminidase, ODC, chitin deacetylase) of Y-H transition and role of intracellular effectors (Ca–CaM and cAMP) in the morphogenesis, have been reported (Doiphode 2007; Doiphode et al. 2009a, b; Ghormade 2000; Ghormade et al. 2012). These advantages establish *B. poitrasii* as a complete and ideal system for the screening of inhibitors of differentiation and identification of promising antifungal agents. Various potent antifungal agents have been identified using *B. poitrasii* as a model (Bavikar et al. 2008; Chaudhary et al. 2009; Joshi et al. 2013; Salunke et al. 2004; Tupe et al. 2015; Vatmurge et al. 2008a, b).

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Candida Albicans Biofilm as a Clinical Challenge

13

Ashwini Jadhav and Sankunny Mohan Karuppayil

Abstract

Candida albicans is a unicellular eukaryote commonly found in humans as a part of the microbiome, but it may cause infections under immune-suppressed conditions. *C. albicans* is seen in different parts of the body like gastrointestinal tract, urinogenital tract, and oral cavity. The infections caused by *C. albicans,* collectively referred as candidiasis, may vary from superficial skin infections to life-threatening systemic infections. In women, vulvovaginal candidiasis is caused by *Candida albicans* and other species in the vagina and it may affect the quality of life. Virulence factors of *C. albicans* include morphological switching, biofilm formation, secretion of hydrolytic enzymes, stress adaptation during infection, and formation of invasive filaments. Biofilm-related infections are a major threat to human health because the biofilms are highly resistant to antifungals. *Candida albicans* can develop biofilms on various medical prosthetic devices like heart valves, pacemakers, dentures, prosthetic joints, intrauterine devices, and catheters. Biofilms are complex three-dimensional structures consisting of yeast and hyphal form enclosed in a self-produced extracellular polymeric matrix making them inaccessible to antibiotics and host defense. Biofilm eradication is a major challenge for drug developers.

Keywords

Candida albicans · Yeast · Drug resistance · Biofilm · Virulence
Antifungal antibiotics

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Introduction

There are millions of fungal species on earth, but fortunately, only few of them are pathogenic to humans. *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* are the major pathogenic fungi which can cause deadly infections in immune-compromised patients (Shapiro et al. 2011). *Candida albicans* is one of the common species of genus *Candida* and is the fourth most common cause of bloodstream infections in India and the USA (Chander et al. 2013). *Candida* infections also constitute the most common fungal infections in HIV patients (Fidel 2006; Hasan et al. 2009). This yeast normally lives on the skin and mucous membranes without causing infection. *C. albicans* is a member of normal microbiome in humans. It is present in various locations in the body like skin, oral cavity, urinogenital, and gastrointestinal tracts (Kennedy and Volz 1985; Achkar and Fries 2010; Ganguly and Mitchell 2011; Kumamoto 2011). In the USA, treatment for invasive fungal infections costs about $2.6 billion annually and mortality rate is 40% (Wilson et al. 2002; Horn et al. 2009). *Candida albicans* causes invasive infections in immune-compromised patients known as candidiasis or systemic infections called candidemia. *Candida* causes high mortality in immune-compromised patients like HIV patients and those on immunosuppressive drug therapy (Pfaller and Diekema 2007; Kim and Sudbery 2011).

Candida albicans also causes vulvovaginal candidiasis (VVC), a common infection affecting the quality of life in women. Vulvovaginal candidiasis is caused by the growth of *Candida* species in the vagina and is characterized by curd-like vaginal discharge, itching, and erythema (Achkar and Fries 2010). About 90% of the VVC infections are caused by *Candida albicans* (Paulitsch et al. 2006); 75% of the women suffer from *Candida* infection at least once in their lifetime. Recurrent episodes of vaginal infections associated with significant treatment cost and morbidity are a major feature of *Candida* infection (Powell and Nyirjesy 2014). The *C. albicans* isolates from VVC patients have shown drug resistance to various antifungal drugs like miconazole, caspofungin, fluconazole, itraconazole, voriconazole, ketoconazole, and terbinafine (Shi et al. 2015). The high incidence and associated healthcare cost of VVC highlight the need for the development of effective agents for its prevention. There is a limited availability of effective antifungal drugs which are free from side effects. Emergence of drug resistance and intrinsic drug resistance of biofilm forms of *Candida albicans* pose a challenge to drug developers as well as physicians.

Virulence Factors in *C. albicans*

Candida pathogenicity is mainly due to various virulence factors like adhesion to epithelial cell surfaces (Zhu and Filler 2010), phenotypic switching (Soll 2004; Peters et al. 2014), biofilm formation (d'Enfert 2006), secretion of hydrolytic enzymes that induce host cell damage (Naglik et al. 2004), stress adaptation during

infection (Brown et al. 2007; Hube 2006), and the ability to transition between yeast and filamentous growth (Kumamoto and Vinces 2005; Whiteway and Oberholzer 2004).

Candida hyphae have invasiveness property so they can colonize and invade epithelial cell surfaces, solid tissues, and cause infection (Gow et al. 2012). Yeast to hyphae transition is required for invasion of epithelial cell layer causing inflammatory response in vaginitis (Peters et al. 2014). Along with invasion, it has the property to form biofilm on implanted medical devices and is hard to treat. Biofilms may be responsible for systemic infection in large population of immune-compromised patients (Douglas 2003; Andes et al. 2004). Biofilms are highly resistant to various antifungal drugs; hence, there is a need to find novel drugs from natural sources (Sardi et al. 2013).

Candida has the ability to secrete proteolytic enzymes like aspartyl proteases (SAP) responsible for enhancement of virulence and pathogenicity (Naglik et al. 2004; Kumar et al. 2015). SAPs are responsible for degradation of a number of important host proteins such as immunoglobulins and complements. Protease production helps *Candida* cells to colonize and penetrate host tissues and to overcome the host immune response. Other enzymes like phospholipase, lysophospholipase transacetylase and lysophospholipase produced by *Candida* enhance the membrane damage, adhesion, and penetration (Cutler 1991; Calderone and Fonzi 2001). *C. albicans*is also reported to produce acid phosphatase, trehalase, glucoamylase, esterase, lipase, hyaluronidase, chondroitin sulfatase, and metallopeptidase in specific environmental conditions (Chaffin et al. 1998; Cutler 1991; Tsuboi et al. 1989). Hemolytic activity is one of the virulence factors of *Candida*. Hemolysis of erythrocytes in blood may provide iron for *Candida* (Chaffin et al. 1998). Targeting of selective virulence factors is considered as a good strategy for chemotherapy.

Biofilm Formation: A Major Virulence Factor

C. albicans biofilm is a highly heterogeneous structure which is made up of yeast, hyphae, pseudohyphae, elongated, and cylindrical hyphal cells surrounded by an extracellular matrix (Chandra et al. 2001; Fox and Nobile 2012; Ramage et al. 2009, 2012). Biofilms are the major cause of nosocomial infections (Privett et al. 2010). Development of biofilm involves different stages like adherence of yeast cells to the substrate, proliferation of yeast cells, formation of hyphal cells, accumulation of extracellular matrix, and dispersion of yeast cells from the biofilm complex (Uppuluri et al. 2010). Dispersal of cells from biofilm is mainly responsible for spread of infection to various parts of the body. These free cells can be eliminated by antifungals, but biofilms are hard to remove (LaFleur et al. 2006). Biofilm-associated infections are responsible for high rate of mortality and morbidity, and treatment costs may increase (Pfaller and Diekema 2007). *Candida* can form biofilm on various prosthetic devices like artificial heart valves,

urinary catheters, intrauterine devices, central venous catheters, joint prostheses, pacemakers, dentures, and soft contact lenses (Cauda 2009; Donlan and Costerton 2002; Seddiki et al. 2013; Pierce et al. 2015). It is the fourth most leading pathogen in the world causing hospital-acquired blood stream infections and medical device infections (Dominic et al. 2007; Pfaller and Diekema 2007; Wenzel 1995; Wisplinghoff et al. 2004). Improved clinical facilities are also responsible for biofilm infection in 50% of the catheters and estimated 100,000 deaths and $6.5 billion in excess expenditure annually in the USA alone (Nobile and Johnson 2015).

In Vitro and In Vivo Development of *C. albicans* Biofilm

In Vitro Model

The 96-well polystyrene microtitre plate model is mostly used to study biofilm formation and its inhibition (Pierce et al. 2008; Ramage et al. 2001). Kinetics of formation of biofilm has been studied using 96-well tissue culture-treated microtitre plates (Shinde et al. 2012). Usually, 200 µl of cell suspension (1×10^7 cell/ml) is added in well. Cells are allowed to adhere for 90 min at 100 rpm. Non-adhered cells are washed out, and after addition of fresh medium, plates are incubated for 48 h at 37 °C to form biofilm (Chandra et al. 2005). In vitro models are easy to manipulate, and one can study all the developmental stages during biofilm formation by observing microscopically (Nett and Andes 2006; Blankenship and Mitchell 2006).

In Vivo Model

Andes et al. (2004) developed rat central venous catheter model for in vivo studies. In this model, viable cells are grown on implanted catheter in vivo and biofilm development is observed. Intraoral denture system for rodent is also used to study in vivo biofilm development (Johnson et al. 2012). Rodent acrylic denture model is efficient for in vivo study of biofilm formation, architecture, and drug resistance (Nett et al. 2010). Urinary catheter in a rat model shows similarity in colonization of *Candida* on urinary catheter in patients. The model gives specific anatomic location, urine flow, and it shows symptoms of candiduria and inflammation so it is useful for investigation of host response (Nett et al. 2014). For persistent candiduria, candiduria model in mice with indwelling catheters is established (Wang and Fries 2011).

In vivo and ex vivo model of rat shows biofilm formation on vaginal mucosa layer (Harriott et al. 2010). In vivo model for biofilm includes rodent subcutaneous, oropharyngeal, burn wound, and oral mucosal models (Cole et al. 1995;

Dongari-Bagtzoglou et al. 2009). Biofilm development in vulvogenital candidiasis can be studied by using bioluminescence imaging (Doyle et al. 2006). Also, bioluminescent biofilm formation can be studied in animal models like implanted catheter models, oropharyngeal, subcutaneous, and cutaneous (Enjalbert et al. 2009; Mosci et al. 2013; Pietrella et al. 2012; Vande et al. 2014).

Antifungal Drugs and Their Targets

Several antifungal drugs are available for treatment of candidiasis; major molecules are listed in Table 13.1.

Azoles

The azoles are the most widely used antifungal agents (Cowen et al. 2002). Fluconazole, voriconazole, posaconazole, and itraconazole are the widely used antifungals. The target of azoles is biosynthesis of ergosterol. For example, fluconazole inhibits 14α-demethylase cytochrome P-450 which is required for conversion of lanosterol to ergosterol during cell membrane synthesis (Hitchcock et al. 1990; Becher and Wirsel 2012; Ghannoum and Rice 1999). Ergosterol provides cell integrity and membrane fluidity. Inhibition of 14α-demethylase is responsible for reduction of ergosterol content and accumulation of sterol precursors like lanosterol, 4,14-dimethylzymosterol, and 24-methylenedihydrolanosterol hence change in structural integrity of cell membrane.

Table 13.1 Different sites of action of antifungal agents

Antifungal drugs	Site of action	References
Polyenes, amphotericin	Membrane polymerization	Ghannoum and Rice (1999); Akins (2005)
Azoles, allylamines, thiocarbamates, morpholines	Ergosterol synthesis	Carrillo-Muñoz et al. (2004)
Griseofulvin, benomyl, benzimidazoles	Nuclear division	Richmond and Phillips (1975)
Polyoxins, nikkomycins, echinocandins	Cell wall synthesis	Georgopapadakou (2001)
Cispentacin, difluoromethyl, 5-fluorocytosine	Metabolic inhibitors	Groll et al. (1998)
5-fluorocytosine, trimethoprim, sulfamethoxazole, pentamidine	Nucleic acid function and Synthesis	Ghannoum and Rice (1999)
Blasticidin, sinefungin	Protein synthesis	Lacal et al. (1980)

Polyenes

Because of the amphipathic nature of polyenes, it binds to ergosterol and this complex forms membrane-spanning channels (Ostrosky-Zeichner et al. 2010; Gray et al. 2012) and causes leakage of ions (Gruszecki et al. 2003; Ostrosky-Zeichner et al. 2010). It is also reported that amphotericin B produces reactive oxygen species which is responsible for fungicidal action (Mesa-Arango et al. 2014; Delattin et al. 2014). Amphotericin B is effective against systemic fungal infections and has in vitro and in vivo activities against several *Candida* species as well as *C. neoformans* and *Aspergillus* species. Although polyenes are widely used as antifungals, their major side effects include toxicity and dysfunction of kidney, because they have structural similarities between ergosterol and cholesterol in the mammalian cell membrane.

Echinocandins

Echinocandin group of drugs includes caspofungin, micafungin, and anidulafungin. These are inhibitors of enzyme (1, 3)-β-D-glucan synthase involved in fungal cell wall synthesis. The disruption of this polysaccharide results in the loss of cell wall integrity and induces cell wall stress. Echinocandins are generally fungicidal in nature. This class of antifungals shows specificity toward fungal targets, and these targets are not conserved in mammals (Ostrosky-Zeichner et al. 2010). Studies of long-term mechanisms of echinocandin resistance are lacking because of their relatively short duration use in the clinics.

Need for Novel Antifungal Drugs

All the antifungal agents have limitations for use due to various side effects, and prevalence of drug resistance is reported against these drugs. Intrinsic drug resistance is major reason for looking for effective antifungals. Various side effects of echinocandins include headache, heart failure, liver toxicity, phlebitis, fever, histamine release, hamolysis, rashes (Laniado-Laborín and Cabrales-Vargas 2009; Wasko et al. 2012). Fluconazole has harmful side effects in pregnant women. Such cases have been reported only in women who took large doses for most of the first trimester (Mastroiacovo et al. 1996). Fluconazole is secreted in human milk at a concentration similar to plasma. Therefore, the use of fluconazole in lactating mothers is not recommended (Amichai and Grunwald 1998).

Mechanisms of Drug Resistance in *C. albicans* Biofilm

C. albicans biofilm is resistant to most of the antifungal drugs, and this is a characteristic feature of the biofilm which may be up to 2000 times more resistant to antifungal drugs than the free cells (Baillie and Douglas 2000; Chandra et al. 2001; Jabra-Rizk et al. 2004). Biofilms may act as reservoirs of cells which may cause spreading of infection. Cells in biofilm remain unaffected by antibiotic treatment, but free cells are sensitive and can cause reinfection (LaFleur et al. 2006; Khot et al. 2006).

Antifungal drug resistance is one of the important issues for antifungal drug therapy. There are many reasons behind drug resistance. Biofilms are very compact having high cell density, extracellular matrix, enhanced drug efflux pump, and stress resistant persister cells (Mathe and Van Dijck 2013; Taff et al. 2013). Extracellular matrix consists of different polymers like nucleic acids, proteins, carbohydrate. Extracellular DNA present in extracellular matrix helps to enhance structural integrity and strength and contribute to antifungal drug resistance (Martins et al. 2010; Rajendran et al. 2010). Treatment of DNAase on biofilm causes increased susceptibility to antifungals like caspofungin and amphotericin B (Martins et al. 2012).

The drug efflux pumps CaCDR1 (Prasad et al. 1995) and CaMDR are distributed on the plasma membrane (Pasrija et al. 2005). When biosynthesis of ergosterol and sphingolipid is disrupted, ABC transporters accumulate in the cell plasma (Mukhopadhyay et al. 2004; Pasrija et al. 2005). ATP binding cassette (ABC) transporter gene family (e.g., *CDR1* and *CDR2*) and the major facilitator (MF) class (e.g., *MDR1*) are the major types of efflux pump proteins in *C. albicans* (Akins 2005; Cowen et al. 2014; Prasad et al. 2015). Over expression of these transporter proteins in in vitro and in vivo biofilms are observed. Efflux pumps are upregulated during biofilm development (Ramage et al. 2002a, b). Expression of drug efflux proteins after exposure to antifungal drugs is one of the mechanisms of resistance in planktonic cells (Xie et al. 2014; Ren et al. 2014). Adhesion of *C. albicans* to a solid surface is sufficient to activate expression of the genes encoding the efflux pumps (Mateus et al. 2004). The efflux of drugs remains active in mature biofilms too and continues to be a cause of biofilm-related drug resistance (Nobile et al. 2012). On prolonged exposure to fluconazole, 10-fold increase in CDR1 expression is reported which gives resistance against fluconazole, itraconazole, and ketoconazole and especially BENr gene is overexpressed in fluconazole resistant *C. albicans* (Sanglard et al. 2009).

The biofilm matrix contains higher levels of β-1,3-glucans in their cell wall than that of planktonic cells. The glucan can bind to drug more efficiently so biofilm cannot be affected by the drug (Nett et al. 2007; Taff et al. 2012). Disruption of β-1,3-glucans by glucanase treatment results in increased drug susceptibility of biofilms. Further evidence comes from the observation where down regulation of glucan synthase gene was found to increase the susceptibility of biofilm to anidulafungin, flucytosine, and amphotericin B (Nett et al. 2010). *C. albicans* cell wall is primarily made of carbohydrates and glycoproteins. Carbohydrates such as

β-glucan and chitin form an inner core of cell wall, responsible for its mechanical strength, and mannoproteins that include adhesions that form an outer fibrillar layer (Gow and Hube 2012).

High cell density in compact biofilm shows antifungal drug resistance against fluconazole, ketoconazole, caspofungin, and amphotericin B (Perumal et al. 2007; Mathe and Van Dijck 2013). Farnesol is a signaling molecule of *Candida* which helps to enhance drug susceptibility (Hornby et al. 2001; Cao et al. 2005; García-Sánchez et al. 2004).

Persister cells are another contributor to the drug resistance in biofilms. They are dormant metabolically and variant phenotypically in biofilm. Persister cells are extremely resistant to antifungal drugs (LaFleur et al. 2006). Drug resistance of persister cells is due to metabolically dormant nature but not efflux pump over expression (Khot et al. 2006; LaFleur et al. 2006).

Biofilm development starts with adherence of cells to surfaces, and various pathways are activated like protein kinase C (Kumamoto 2005). Mkc 1 is a terminal MAP kinase protein in PKC cascade which is required for biofilm development and these biofilms are resistant to Azole drugs (Kumamoto 2005). Activation of a heat shock protein Hsp90 also contributes to azole and echinocandin resistance. This is through calcineurin pathway for stress responses (Cowen 2009; Cruz et al. 2002). Inhibition of the protein phosphatase, i.e., calcineurin or intervention of Hsp90 results in sensitization of *C. albicans* biofilm to various antifungal drugs (Uppuluri et al. 2008). Overall, drug resistance exhibited by yeast biofilms is governed by a complex network of multiple factors.

Novel Therapeutic Options

Modified Surfaces and Antimicrobial Coatings

Different types of biomaterials or biomedical implants give opportunity for adhesion, colonization, biofilm formation, and dispersal of cells of *Candida albicans* (Ramage et al. 2006). There is a need to develop biomaterials in such a manner that there is no adhesion of microbes nor it exhibits antimicrobial or antiadhesive properties. A recent study tested the effect of surface and serum on biofilm development by different *Candida* clinical isolates (Frade and Arthington-Skaggs 2011). Various types of materials included polycarbonate, polystyrene, stainless steel, Teflon, polyvinyl chloride, and hydroxyapatite used to make prosthetics. Results indicated that a serum conditioning increases the adherence to both metallic and nonmetallic materials and that roughness and hydrophobicity can modulate *C. albicans* biofilm formation. Another study reported that surface modifications of polyetherurethane, polycarbonate-urethane, and polyethylene terephthalate influenced fungal biofilm formation in vitro (Chandra et al. 2005). Impregnation of prosthetics with different concentrations of caspofungin displayed an inhibitory role on subsequent biofilm formation of *C. albicans* on polystyrene surfaces

(Bachmann et al. 2002). Similar inhibitory effect was demonstrated for voriconazole. Surfaces previously coated with this new-generation azole (which is not active against preformed biofilms) showed reduced formation of biofilms by different *Candida sp*. (Valentin et al. 2012). Thin-film coating of polymethylmethacrylate incorporating different antifungals, including nystatin, amphotericin B, and chlorhexidine, effectively inhibited *C. albicans* biofilm formation as a potential preventive therapy for denture stomatitis. Novel formulations allow slow release of drugs over time (Redding et al. 2009). Precoating the surface of acrylic disks with chlorhexidine or histatin 5 (a naturally occurring antimicrobial peptide in saliva) significantly inhibited *C. albicans* biofilm formation (Pusateri et al. 2009). Silver-coated catheters have been used in clinics to inhibit microbial growth, and silver nanoparticles show activity against fungal biofilms (Monteiro et al. 2011; Silva et al. 2013).

Combination Therapy

A combination of two different classes of antifungals provides new strategy against different targets of fungal biofilm (Pierce et al. 2013). However, an antagonistic trend has been described for combinations of azoles and echinocandins against biofilms, both when used concomitantly and in a sequential therapy regimen (azole, first followed by an echinocandin) (Bachmann et al. 2003; Sarkar et al. 2014). Biofilms formed by multiple clinical isolates of some *C. albicans* show resistance toward caspofungin after exposure to fluconazole (Sarkar et al. 2014). Uppuluri et al. (2008) demonstrated that *C. albicans* cells in biofilms were resistant to individually delivered fluconazole or calcineurin inhibitors, but they were susceptible when the azole and the calcineurin inhibitor were used in combination. Some antifungals like amphotericin B are observed to show synergism with calcineurin inhibitors FK506 (Srinivasan et al. 2013). Hsp90, one of the key regulators of *C. albicans* biofilm growth and maturation, reduced glucan levels in the biofilm matrix and abrogated resistance of *C. albicans* biofilms to the azole antifungal agents both in vitro and in vivo (Robbins et al. 2011). Geldanamycin, one of the Hsp90 inhibitors, increased the susceptibility of *A. fumigatus* biofilms in vitro toward azoles and echinocandins (Robbins et al. 2011). The combinations of antifungal drug with cyclosporine A has shown synergisitic effect on biofilm formation. The MIC of antifungal drugs against biofilm was reduced hence biofilm became sensitive to antifungal drugs (Shinde et al. 2012).

Natural Products as Anti-*Candida* Agents

Natural products like plant molecules or small molecules of bacterial origin influence growth of *Candida*. Plant extracts, essential oils, and their constituent molecules exhibit novel antimicrobial and antifungal properties (Raut et al. 2013, 2014; Raut and Karuppayil 2014). Most importantly, phytochemicals have been found to possess inhibitory potential against drug resistant biofilms of bacterial and fungal

pathogens (Raut and Karuppayil 2014). Efforts are being done to identify molecules with antibiofilm potential through random screening of small molecules of natural origin including phytochemicals. It includes search for plant molecules which can prevent biofilm development as well as those which disrupt mature biofilms (Raut et al. 2013). Phytochemicals or other synthetic molecules can be used in combination with existing drugs to potentiate the activity of available antifungal agents. β-asarone like plant molecules are tested against morphogenesis of *C. albicans* (Rajput and Karuppayil 2013). Plant oils act as inhibitors of *Candida albicans* growth (Devkatte et al. 2005). Terpenoids can inhibit the *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle (Zore et al. 2011). Alkaloids like berberine show anti-*Candida* activity (Dhamgaye et al. 2014). Anti-*Candida* activities of anticancer drugs are also reported (Routh et al. 2011). In vitro activity of terpenoid derivatives (carvacrol, farnesol, different terpenoids, and phenyl propanoids have shown antibiofilm activity against *Candida albicans* Raut et al. 2013). Curcumin is known to inhibit *Candida albicans* growth and also shows synergism with CWPs. Curcumin affects calcineurin and mitogen-activated protein (MAP) kinase pathway and inhibits cell wall synthesis (Kumar et al. 2014). Quercetin is another plant molecule having strong anti-*Candida* activity which also sensitizes the biofilm against fluconazole (Singh et al. 2015).

Along with plant metabolites, various bacterial metabolites also show anti-*Candida* activity. For example, *Pseudomonas* phenazines and their derivatives influence the *C. albicans* growth, morphology, cell to cell interaction, and biofilm formation (Morales et al. 2010). Some phenazines like phenazine-1-carboxylate (PCA) and pyocyanin (PYO) inhibit the growth of fungi (Thomashow et al. 1990; Kerr et al. 1999). 5-methyl-phenazinium-1-carboxylate (5MPCA) is more toxic than PCA or PYO (Gibson et al. 2009). Phenazine methosulphate (PMS) is a phenazine derivative which inhibits *Candida* growth and biofilm formation (Morales et al. 2010). The influence of ethyl alcohol and acetaldehyde on *C. albicans* morphogenesis is also studied by Chauhan et al. (2011). Indole is a signaling molecules produced by bacteria which inhibit the growth of *C. albicans*.

Future Perspectives

Combination of different compounds with antifungals may successfully remove and kill the biofilm and also prevent recurrent infection (Fox et al. 2015). A number of genes involved in adherence, morphogenesis, quorum sensing, matrix production, cell wall biosynthesis, and metabolism may have important roles in biofilm regulation (García-Sánchez et al. 2004; Nobile et al. 2012; Desai et al. 2014). Targeting of the different genes in metabolic pathways of biofilms may be helpful for the development of potential antifungal therapy. Hence, there is need to find out novel antifungal drugs. Various natural products are antifungal and have the ability to kill biofilm (Raut et al. 2013). Plant extracts, essential oils, and their constituent molecules exhibit novel antimicrobial and antifungal properties (Raut et al. 2014).

Most importantly, phytochemicals have been found to possess inhibitory potential against drug resistant biofilms of bacterial and fungal pathogens (Raut and Karuppayil 2014). Phytochemicals or other synthetic molecules can be used in combination with existing drugs to potentiate the activity of available antifungal agents. Combinatorial approach may be useful to mitigate the drug resistance associated with biofilm communities. Drug efflux inhibitors or cell sensitizer molecules may be used to overcome the problem of biofilm-mediated resistance (Shinde et al. 2013; Doke et al. 2014). Other effective approaches include combination of biofilm disruptive agents with drugs so that EPM surrounding the biofilm is disturbed. For example, combinatorial therapy of AMB and CSP with DNase significantly disrupted EPM and sensitized *C. albicans* biofilm to antifungal drugs (Martins et al. 2012). Use of broad-spectrum antimicrobial metal ions like silver or nanoparticles of silver is another interesting way. It can be used for coating a catheter surface or medical device to prevent adhesion and biofilm formation by yeasts. Its combination with antifungal drugs can be very effective. Silver nanoparticles have been shown to inhibit *C. albicans* and *C. glabrata* biofilms at various stages of development. These have been utilized in hydrogels used in chronic wounds and also in denture acrylic (Monteiro et al. 2011, 2012). Molecules that interfere with the quorum sensing involved in biofilm formation and regulation are also an attractive alternative for biofilm mitigation (Nickerson et al. 2006; Kalia 2013). Screening of clinical and preclinical non-antifungal drugs, drug compound libraries, and repurposing of them against fungal biofilms is a recent approach being investigated (Routh et al. 2011; Shinde et al. 2013; Pierce et al. 2013). To improve antifungal therapy, there is a necessity to find out novel efficient approaches against biofilm-related infections.

Conclusions

Most of the fungal infections in humans are biofilm related and highly resistant to antifungal and host defenses. Uses of drug combinations, multi-targeting, chemo sensitization, inhibition of virulence factors, and drug repositioning are some of the strategies which are being explored. Drugs targeting fungal specific targets without causing any side effects to humans are still elusive. Molecules of natural origin targeting drug resistant biofilm and other virulance factors in Candida albicans have shown potential as antifungals in laboratory studies. Clinical studies and animal experiments are lacking. It is hoped that these molecules may find their way to clinics in the future.

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Characteristics and Multifarious Potential Applications of HAP Phytase of the Unconventional Yeast *Pichia anomala*

14

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Abstract

Most of cereal and legume seeds and their products contain 1–2% phytic acid that represents around 60% of the total phosphorus content. A large portion of phytic acid in seeds is in the form of salts known as phytates. The phytic acid-bound phosphorus (*myo*inositol 1,2,3,4,5, 6-hexakis dihydrogen phosphate) is poorly available to monogastrics. Therefore, inorganic phosphorus (Pi), a non-renewable mineral, is supplemented in diets for swine, poultry and fish to meet their Pi requirement. Furthermore, the unutilized phytate P from plant-based feeds is excreted, which becomes an environmental pollutant in the areas of intensive animal rearing. The excess P in soils flows into lakes and the sea that causes eutrophication, leading to water blooms and death of aquatic animals. The high negative charge on phytic acid results in the chelation of positively charged divalent metal ions (e.g. Fe^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}) of nutritional significance, rendering a poor absorption and thus unavailable. This is partly attributed to the widespread human nutritional deficiencies of calcium, iron and zinc in developing countries where plant-based diets are predominantly consumed. The challenges in three areas of animal nutrition, environmental protection and human health justify research on phytases from

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different microbial sources for minimizing anti-nutritional effects of phytates and to enhance growth by improving phosphorus assimilation. This chapter reviews the developments on the production, characteristics and multifarious potential applications of phytase of the unconventional yeast *Pichia anomala*.

Keywords
Pichia anomala · Phytates · Phytase · Dephytinization · Cell permeabilization Feed additive

Introduction

The popularly used term phytase (*myo*inositol hexakisphosphate phosphohydrolase) refers to a group of phosphatases which can liberate at least one phosphate molecule from *myo*inositol hexakisphosphate. Despite remarkable developments on phytases, *myo*inositol pentakisphosphate (IP5) has not yet been identified as the end product of IP6 hydrolysis. Normally, the IP6 hydrolysis ends with less phosphorylated myoinositol phosphates such as IP3 or IP (Hara et al. 1985; Wyss et al. 1999; Kerovuo et al. 2000; Quan et al. 2004; Casey and Walsh 2004). According to the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC–IUB), there are two classes of enzymes that hydrolyze phytate, viz. 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.28), initiating the dephosphorylation of IP6 at the third and sixth positions, respectively. Phytases are widely distributed in environment as they are present in animals, plants and microbes. For instance, phytate-hydrolyzing enzymes were found in calves (McCollum and Hart 1908), fishes, reptiles and birds Rapoport et al. 1941) and in plants like maize (Huebel and Beck 1996), rice (Hayakawa et al. 1989) and wheat (Nagai and Funahashi 1962; Nakano et al. 1999). However, microbial phytases have been explored extensively, especially those from fungi such as *Aspergillus ficuum* (Gibson 1987), *A. fumigatus* (Pasamontes et al. 1997), *Rhizopus oligosporus* (Casey and Walsh 2004), *Mucor piriformis* (Howson and Davis 1983), *Cladosporium* sp. (Quan et al. 2004) and the thermophilic mould *Sporotrichum thermophile* (Singh and Satyanarayana 2008). In the last decade, phytate-hydrolyzing enzymes of bacteria such as *Escherichia coli* (Greiner et al. 1993), *Pseudomonas* spp. (Cho et al. 2003; Kim et al. 2003), *Klebsiella* spp. (Tambe et al. 1994; Sajidan et al. 2004) and various *Bacillus* spp. (*B. amyloliquefaciens*, *B. amyloliquefaciens*, *B. licheniformis*, *B. mycoides*, *B. pseudomycoides*, *B. subtilis*, *B. thuringiensis*, *B. Velezensis* (Kumar et al. 2017) and yeasts (Nakamura et al. 2000) such as *Schwanniomyce occidentalis* (Segueilha et al. 1992), *P. anomala* (Vohra and Satyanarayana 2001, 2002a), *Arxula adeninivorans* (Sano et al. 1999) have also been identified and characterized. The distribution and the biochemical properties of phytases have been reviewed from time to time (Kumar et al. 2017; Oh et al. 2004;

Konietzny and Greiner 2002). Physicochemical properties and potential applications of phytases have also been reviewed in detail by Rao et al. (2009). *P. anomala* is among the most important unconventional yeasts as it has been found associated with food and beverages and is useful in different biotechnological fields such as biopreservation (Ingvar and Petter 2011) and in production of low molecular weight metabolites (Van Eck et al. 1993; Fredlund et al. 2004). This chapter reviews developments on various aspects of phytase of this yeast.

Isolation of Phytase-Producing Strain of *P. anomala*

Flower buds of two angiosperm plant species *Maduka latifolia* (Mahua) and *Woodfordia fruiticosa* (Dhataki) were collected from different geographical locations of India, and subsequently, many yeast strains were isolated from dried flower buds of these plants (Vohra 2002). It has been well documented that yeast species can survive in nectar of flowers with high sugar content because of their osmophilic nature (Spencer and Spencer 1997). Vohra screened these isolates for extracellular, intracellular and cell-bound phytase activities. As cell-bound fractions showed high titres of phytase, further studies on phytase producers were focused on isolates with high phytase activity. A very high titre of phosphohydrolase was recorded in a yeast strain which was identified as *P. anomala* . Identification of this yeast was based on several parameters including morphological, physiological and biochemical features including the analysis of fatty acid methyl ester [FAME] (Vohra and Satyanarayana 2001). The yeast culture of confirmed identity was later deposited at the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh (India) [MTCC-4133].

Optimization of the Phytase Production Medium

The reduction of cost of production and time is critical factors in order to use any microbial product. Efforts have been made to achieve higher phytase production in minimum fermentation time. Both medium components and fermentation parameters were optimized by using various statistical approaches. By one parameter/variable at a time (OVAT) approach, it was seen that concentrations of glucose, beef extract and Fe^{2+} were found to affect phytase production. OVAT approach enhanced phytase production from 2.5 to 68 U g^{-1} DYB (Dry Yeast Biomass), when *P. anomala* was cultivated at 20 °C for 24 h. The optimized medium composed of 4.0% glucose, 1.0% beef extract and 0.15 mM Fe^{2+} (Vohra and Satyanarayana 2001). A statistical approach known as response surface methodology (RSM) further increased phytase titres and minimized the production cost. In contrast to other parameters tested, inoculum density was observed to be insignificant (Vohra and Satyanarayana 2002a). Cultivation in the laboratory

fermenter permitted improvement in production over that in flasks due to efficient control of fermentation parameters such as maintenance of pH, aeration and mixing, and homogenous distribution of nutrients, oxygen and heat transfer. Upon cultivation in a 22 L laboratory fermenter, the *P. anomala* biomass increased twofold, increased from 4.2 to 8 g L^{-1}. Usually, a lag phase is observed during fermentation, but lag phase was eliminated in fermenter and highest phytase titres were attained in 16 h instead of 24 h in shake flask (Vohra et al. 2006). Fed-batch fermentation in an airlift bioreactor further improved phytase production levels. To reduce the production cost, cane molasses was used as one of the media components, which is a low-cost carbohydrate source. Besides sugar (~50%), molasses contains nitrogenous substances, vitamins and trace elements (Huang and Tang 2007). Statistical approach RSM was used for optimization of fermentation conditions using cane molasses that led to high enzyme yields in a medium containing glucose and beef extract (Vohra and Satyanarayana 2004). The optimum concentrations of various media components (80.0 mL/L cane molasses, 4.0 g/L urea and 20.0 mL/L inoculum density) increased phytase production by fivefold (Kaur and Satyanarayana 2005). Further improvement in phytase production was achieved by cyclic fed-batch fermentation in comparison with batch and fixed-volume fed-batch fermentation (Verma and Satyanarayana 2012). In cyclic fed-batch fermentation, phytase production was sustainable over seven days in cane molasses medium (Verma and Satyanarayana 2012). When *P. anomala* cells were subjected to permeabilization by employing different detergents and solvents, the phytase activity increased. Among permeabilization agents, Triton X-100 was the most effective as 30-min treatment with 5.0% Triton X-100 led to 15.0% increase in PPHY activity. Cell permeabilization caused shrinkage of cell protoplasm as revealed by electron microscopy. The shelf life of the PPHY in the form of permeabilized cells was increased as these cells retained phytase activity for two months and one month when stored at 4–60 °C, respectively. Immobilization studies were carried with permeabilized *P. anomala* cells in alginate, which allowed reuse of biomass with sustained phytase activity (Kaur and Satyanarayana 2010).

Purification and N-Terminal Sequence of Phytase

The phytase of *P. anomala* is a cell-bound phosphohydrolase. Disintegration of yeast cells in French Press liberated 78.0% of total cellular phytase. The use of protein purification methods such as acetone precipitation and column chromatography with anion exchanger DEAE-Sephadex matrix resulted in homogenous preparation of phytase. The purified protein was of ~64 kDa molecular mass on SDS-PAGE which formed a homohexamer under native conditions and exhibited an isoelectric point (pI) of 4.5. Values of kinetic parameters, Michaelis–Menten constant (K_m) and maximum velocity (V_{max}), for phytic acid as substrate were 0.20 mM and 6.34 μmol mg^{-1} protein min^{-1}, respectively (Vohra and Satyanarayana 2002b).

The N-terminal amino acid sequence of the purified PPHY is'VAIQKALVPG', which shows similarity with N-terminus sequences of phytases from the yeasts *S. occidentalis* and *Debaryomyces castellii*. Based on the sequence similarity of N-terminal amino acid of the native phytase, the *PPHY* of *P. anomala* along with its 5′–3′ untranslated regions (UTRs) was amplified and sequenced (Kaur et al. 2010). It was seen that *PPHY* gene was 1386 bp long, which encoded a protein of 462 aa. Upon BLAST analysis, it was observed that PPHY sequence displays similarity with various other yeast phytases including those from *S. occidentalis* and *D. castellii*. The presence of the highly conserved seven amino acid long active site heptapeptide motif (RHGXRXP) and the catalytically active dipeptide (HD) at C-terminus confirmed PPHY to be a phosphohydrolase belonging to histidine acid phosphatase (HAP) family of phytases. Southern hybridization confirmed that *P. anomala* genome harbours one copy of *PPHY* gene (Kaur et al. 2010).

Biochemical Characteristics of Native Phytase

The endogenous phytase of *P. anomala* is capable of hydrolyzing various organic phosphates such as *p*-nitrophenyl phosphate, ATP, ADP, glucose-6-Pi in addition to phytic acid, thus exhibiting broad substrate specificity. Among different metal–phytate complexes, insoluble calcium and magnesium phytates get hydrolyzed very efficiently by this phytase, but iron phytate largely remains intact. The optimum pH and temperature for the activity of purified phytase were recorded as 4.0–60 °C, respectively. While the cell-bound phytase exhibited optimal activity at same pH 4.0 but at 75 °C. As half-life of any commercial enzyme is a deciding factor for storage conditions of the product, half-life of the purified phytase were calculated. It was found that purified native phytase exhibits a half-life of 7 days, 48 h and 5 min at 60, 70 and 80 °C, respectively. In contrast to cell-free purified endogenous phytase, the cell-bound form exhibited higher thermostability. As a prerequisite during feed pelleting, enzymes applied as feed supplements must be thermostable and should tolerate temperature as high as 60–90º (Wyss et al. 1998). The presence of phytic acid exerted a positive effect on thermostability of phytase and increased the $T_{1/2}$ from 5 to 45 min at 80 °C (Vohra and Satyanarayana 2002b). Phytase activity was reduced to half when various metal ions [Cu^{2+}, Zn^{2+}, Hg^{2+} (1 mM)] were added to the reaction mixture. In the presence of Fe^{3+}, phytase activity was drastically reduced ($\sim$90%). None of the tested cations exhibited a stimulatory effect on phytase activity.

Heterologous Expression of PPHY

For heterologous expression of proteins of eukaryotic origin, eukaryotic expression systems are preferred over prokaryotic systems, as the former have similar post-translational machinery as that of eukaryotes. *P. anomala* phytase is an ideal

candidate to be used as a feed and food additive. For certain applications such as selective removal of phosphate from IP6 in immobilized enzyme reactors, dephytinization of soya milk and a bread additive, soluble enzyme is preferable. Hence, to express PPHY extracellularly, Kaur et al. (2010) cloned and expressed *P. anomala* phytase gene in yeast hosts, which included *Saccharomyces cerevisiae* Cl3ABYS86, *A. adeninivorans* G1212 and *Hansenula polymorpha* RB11. Phytase activity could be detected in cell-bound fractions of the recombinant strains. Among the three yeast strains used, *H. polymoropha* produced the highest titres of recombinant phytase. Phytase thus produced by transgenic *H. polymorpha* RB11/Xplor1-URA3-SwARS-FMD-PPHY and wild-type *P. anomala* was purified and characterized. In this study, *PPHY* gene was kept under the regulation of an inducible formate dehydrogenase (FMD) promoter. Induction was done using glycerol in *S. cerevisiae* Cl3ABY86/pYES2-PPHY in YMM-galactose medium and in *A. adeninivorans* G1212/YRC102-PPHY in YMM-glucose medium. It was observed that both the wild-type and recombinant phytases exhibited a molecular mass of ~380 kDa which corresponds to a hexameric form. Values of optimum pH and temperature for the phytase activity were 4.0–60 °C, respectively. Similar to endogenous phytase, recombinant phytase also exhibited a broad substrate spectrum and hydrolyzed a number of organic compounds including *p*-nitrophenylphosphate, glucose-6-phosphate, ADP, AMP, 1-naphthylphosphate, ATP and sodium pyrophosphate (Kaur et al. 2010). After unsuccessful attempts of extracellular expression of PPHY in *S. cerevisiae*, *A. adeninivorans* and *H. polymorpha,* further efforts were made for extracellular expression of *PPHY* in methylotrophic host *P. pastoris*. In two protein expression studies, different promoters (i.e. AOX and GAP) were used to control the expression of *PPHY* gene. Most exploited alcohol oxidase gene promoter (AOX) offers the tight regulation of gene expression upon the addition of methanol, while GAP promoter is constitutive in nature and is known to allow expression of foreign genes at equivalent levels to that of attained under AOX promoter (Latiffi et al. 2013; Waterham et al. 1997). *PPHY* was cloned in *Escherichia coli–P. pastoris* shuttle vectors pPICZαA and pGAPZαA, and recombinant *P. pastoris* harbouring *PPHY* gene were generated by electrocompetent host cells. Once the phytase-producing recombinant *P. pastoris* clones was generated, the clone secreting a high enzyme titre was selected and used in media optimization studies.

Optimization of fermentation variables is known to improve recombinant protein production in *P. pastoris* (Wang et al. 2009). Employing Plackett–Burman (PB) design and RSM, ~22-fold higher recombinant phytase production was attained than that by the endogenous yeast (Joshi and Satyanarayana 2014). In case of *PPHY* expression under GAP promoter, OVAT and two factorial designs were used to optimize the enzyme secretion that led to ~44-fold improvement in PPHY production as compared to that of the native host (Joshi and Satyanarayana 2015a).

Biochemical Characteristics of Recombinant Phytase

The recombinant phytase is a hexameric glycoprotein of ~420 kDa (monomeric protein is of ~70 kDa), where N-linked glycan represents 24.3% of the recombinant phytase. The temperature and pH optima of rPPHY are 60 °C and 4.0, which are similar to the endogenous enzyme. The kinetic characteristics K_m, V_{max}, k_{cat} and k_{cat}/K_m of rPPHY are 0.2 ± 0.03 mM, 78.2 ± 1.43 nmol mg^{-1} s^{-1}, 65,655 ± 10.92 s^{-1} and 328.3 ± 3.12 μM^{-1} s^{-1}, respectively (Joshi and Satyanarayana 2014). $T_{1/2}$ and activation energy (E_a) for rPPHY are 4.0 min (80 °C) and 27.72 kJ mol^{-1}, respectively. The temperature quotient (Q_{10}) and activation energy of thermal inactivation (E_d) for enzyme are 2.1 and 410.62 kJ mol^{-1}, respectively, while the value of enthalpy (ΔH_d^0) is ~407.8 kJ mol^{-1} (65–80°C). With rise in temperature, the free energy of the hydrolysis process (ΔG_d^o) increases from 49.56 to 71.58 kJ mol^{-1}, while the value of entropy of inactivation (ΔS_d^0) remains unchanged at ~1.36 kJ mol^{-1} K^{-1} (Joshi and Satyanarayana 2015b). Competitive inhibition of rPPHY by vanadate ions has been observed, as maximum velocity (V_{max}) remained almost constant (~78.13 η kat mg^{-1} s^{-1}) at all the concentrations of the inhibitor used. When the concentration of vanadate was increased from 0 to 500 μM, the values of apparent K_m ranged between 200 and 1000 μM. At 50, 100 and 500 μM concentration of the inhibitor, K_i values for metavanadate were 333, 200 and 1.2 μM, respectively. rPPHY exhibited haloperoxidase activity in the presence of metavanadate ions (Joshi and Satyanarayana 2015b). It has been reported that 5 mM of Ag^{2+}, Al^{2+}, Pb^{3+} and Sn^{2+} completely inhibited rPPHY activity, while a lesser inhibition in the presence of Ba^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}. The enzyme activity was not affected by Hg^{2+}, Mn^{2+} and Na^{+}. In contrast to the aforementioned metal ions, Ca^{2+} and Ni^{2+}slightly stimulated rPPHY activity. As compared to the native phytase, equilibrium between the folded and unfolded fractions for rPPHY was attained early as suggested by the values of melting temperature (T_m) of the native and recombinant phytase, which are 73–70 °C, respectively. High values of melting temperatures of endogenous and recombinant phytases suggest higher thermostability, since high T_m is an indicator of protein thermostability (Kumar et al. 2000).

Applications of *P. anomala* Phytase

There are multifarious applications of phytases. They are majorly being used in phytate reduction and enhancing nutrient availability in the feed and food industries, and in preparation of lower myoinositol phosphates for biochemical investigations and therapeutic applications. An emphasis is also being laid on their use in aquaculture, as an agent in soil amendment, in the environmental phosphorus pollution management and in the semisynthesis of peroxidase. About 66% phosphorus in plant-based feedstuffs is present as phytic acid or in the form of metal

complexes (Harland and Morris 1995). Ruminant animals sustain the microflora that enzymatically release Pi from phytic acid. Monogastrics like humans, chicks, fish and pigs have very low levels of phytase activity in their guts and therefore cannot utilize the phytic acid P and suffer from low P availability, besides anti-nutritional effects of phytic acid (Vohra et al. 2006). Exogenous addition of phosphate in soya bean and other meals is a regular practice to meet this requirement of phosphorus. The unutilized phytic acid phosphorus accumulates in the environment through faecal matter (Mullaney et al. 2000), which gets enzymatically hydrolyzed by the phytase activities of soil and waterborne microorganisms. The excess P thus released ultimately reaches water bodies leading to eutrophication (Vohra and Satyanarayana 2003). This surplus phosphorous results in excessive algal growth, leading to oxygen depletion in the water body. This problem can be addressed by adding microbial phytases to the feed or by using phytate-rich cereal diets that will improve not only the availability of phytic acid-bound phosphorous but also will reduce the release of unutilized P in the environment (Nelson et al. 1971). The addition of yeast biomass containing phytase to the feed of broiler chicks improved growth of the broilers and increased phosphorus retention. A significant decline in the phosphorus excretion in the faeces of chicks fed with phytase-supplemented diets was observed. The feed conversion ratio (FCR), which is a parameter to know the efficiency by which the livestock body converts the feed into the desired output, was also measured, and it was found that FCR was reduced from 2.272 (control) to 1.949 in biomass-fed broilers (Vohra and Satyanarayana 2003; Vohra et al. 2006). Owing to assured supply, economical market price and biochemical composition, the products derived from soya beans are reported to be the most promising alternative ingredients to fishmeal (Swick 2002). In soya bean-based fish feed, about 60–80% of the P is found in the form of phytate that remains unavailable to monogastric fishes (Pallauf and Rimbach 1997; Raboy 1997). Upon investigation, it was found that endogenous cell-bound phytase of *P. anomala* is very useful supplement to plant-based feed for both marine and freshwater fishes. During fish-feeding trials on mass cultivation of marine milkfish Chanos chanos, the performance of fishes reared on phytase-supplemented soya bean-based feed (1000 U kg^{-1}feed) was better than that of fishes reared on unsupplemented feed, as the excretion of phosphorus was higher in the latter, indicating that *P. anomala* phytase can be used for environment-friendly mass cultivation of marine milkfish (Hassan et al. 2009). In a similar study on the freshwater fishes *Labeo rohita* and *Clarias batrachus*, survival and growth was better because of ameliorated P and protein assimilation, besides diminished excretion of phosphorus and inorganic form of nitrogen, i.e. ammonia in the water bodies. The overall growth of freshwater fish *L. Rohita* and phosphate and protein utilization by the fish was also improved upon supplementation of 1000 U phytase per kg of fishmeal supplemented with soya bean meal. The endogenous cell-bound phytase of *P. anomala* has been shown to efficiently dephytinize wheat flour, rice flour, soya bean flour and wheat bran. In another important study, it has been shown

that the endogenous phytase is capable of dephytinizing soya milk. The Triton X-100 permeabilized cells exhibit higher potential over the unpermeabilized cells. The permeabilized cells had an added advantage, as they could be immobilized and reused for dephytinization of soya milk, indicating their potential in designing continuous bioreactor systems (Kaur and Satyanarayana 2009). Phytases are known for their valuable environmental role in lowering the phosphorus levels in animal excreta and reducing the need to supplement diets of monogastrics with exogenous phosphorus. Owing to its environment-friendly effects, application of phytases as animal feed supplement is growing rapidly. Similarly, the utilization of phytase in fish cultivation is also rising, since it permits the use of economical plant-based fish diets.

Applicability of recombinant phytase was also tested. The treatment of soya protein with rPPHY for 3 h led to the separation of glycinin and liberation of phytate-bound phosphorous (Joshi and Satyanarayana 2014). Upon the treatment with rPPHY, glycinin was precipitated due to the hydrolysis of phytate leading to the dissociation of glycinin–phytate complex. The phytate hydrolysis reduced the solubility of glycinin that led to its precipitation, while β-conglycinin fraction remained soluble in the supernatant (Fig. 14.1) due to its glycoprotein nature (Saito et al. 2001).This finding may pave the way for the use of PPHY in producing soya products with improved quality as well as special foods for people who are allergic to the specific components of soya proteins. The treatment of poultry feeds with rPPHY for 2 h led to the liberation of inorganic phosphate (Joshi and Satyanarayana 2015a). Among the feeds tested, broiler starter feed exhibited speedy hydrolysis, while the hydrolysis of prestarter and finisher feeds was slower (Fig. 14.1). This indicates that ingredients of prestarter and finisher feeds are not resilient to dephytinization by rPPHY, but may require a slight change or pre-treatment for effective hydrolysis of phytate. It has been reported that wheat flour contains up to 4.0 mg of phytic acid per gram (Garcia-Estepa et al. 1999) that lowers the bioavailability of minerals. Upon addition rPPHY to the dough, an increase in the Pi, reducing sugars and soluble protein was recorded in breads as compared to those prepared with the help of industrial enzymes, without any change in the texture of the bread (Fig. 14.1). The supplementation of recombinant phytase resulted in 72.5% drop in phytic acid content (Joshi and Satyanarayana 2015a). Apart from the applications of rPPHY as food and feed additive, the recombinant enzyme exhibits haloperoxidase activity upon inhibition with vanadate ions; therefore, its potential as vanadium-dependent haloperoxidase can be explored further.

Both endogenous yeast phytase and its recombinant counterpart are of industrial importance due to its applicability as an additive in chick and fish feeds, in decreasing phytate levels of wheat flour and soya milk, in fractionation of allergenic soya protein and in the preparation of both whole wheat and unleavened flat Indian breads.

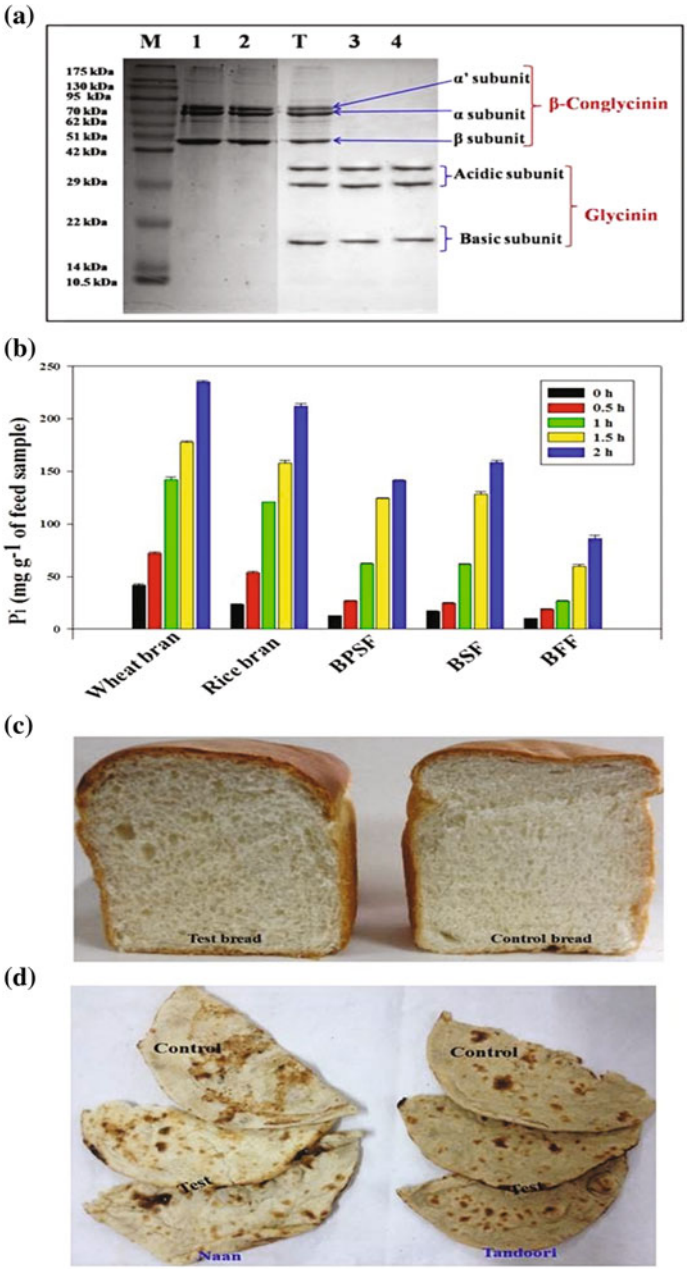

Fig. 14.1 Applications of recombinant phytase: **a** fractionation of allergenic soya proteins, viz. β-conglycinin and glycinin. SDS-PAGE profile of separated glycinin and β-conglycinin by the action of rPPHY; M: molecular weight markers, L1-L2: β-conglycinin fraction, LT: total soya protein, L3-4: glycinin fraction; **b** the liberation of phytate phosphorous by the hydrolysis of different poultry feeds by rPPHY under simulated gut conditions; **c** texture of whole wheat breads prepared with (test bread) and without the addition (control bread) of rPPHY; **d** texture of unleavened flat Indian breads prepared with (test bread) and without the addition (control bread) of rPPHY

Conclusions

The unconventional yeast *P. anomala* produces a cell-bound phytase that displays desirable biochemical characteristics such as acid stability, thermostability, resistance to digestive proteases and broad substrate spectrum. Optimization of production parameters and use of low-cost media resulted in cost-effective production of phytase. Heterologous expression of PPHY in methylotrophic yeast *P. pastoris* followed by process optimization led to high secretion of recombinant phytase that simplified downstream processing of the enzyme. Both native and recombinant phytases could be used as an additive to different feeds and foods. Novel applications of recombinant phytase in the fractionation of allergenic soya proteins and in generating vanadate-dependent virtual haloperoxidase open new research avenues for this enzyme.

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Fungal Inulinolytic Enzymes: A Current Appraisal

15

Hemant Kumar Rawat, Hemant Soni and Naveen Kango

Abstract

Inulinolytic enzymes produced by molds and yeasts have many applications. Inulin is being looked upon as an abundant and renewable source of fructose, a low-calorie sweetener, and a readily fermentable substrate. Inulin can be exploited at industrial scale for generation of high-fructose syrup (HFS) using fungal exoinulinases and may also be selectively hydrolyzed using endoinulinase for generation of prebiotic inulooligosaccharides (IOS). Some members of Aspergilli, Penicillia, and a yeast, *Kluyveromyces marxianus*, are known as potential producers of inulin-hydrolyzing enzymes; however, recently, it has been characterized from extremophilic and marine-derived microorganisms as well. Inulinases find applications in nutraceutical, feed, pharmaceutical, and biofuel industries. This chapter discusses production, molecular aspects, and biotechnological applications of inulinases.

Keywords

Exoinulinase · Endoinulinase · Fungi · Yeast · Inulin · Fructose

Introduction

Inulin is non-structural polysaccharide, used as energy-rich compounds, and also has role in plant metabolism and energy storage. After starch, inulin is one such storage polysaccharide found widely dispersed in many plants. Inulin is made of β-(2 → 1) linked linear poly-fructose units (2–60) terminated by a sucrose residue

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(Fig. 15.1) (Kango and Jain 2011; Rawat et al. 2015a). Plants that store and synthesize inulin usually do not store other materials as energy reserve. Several temperate and tropical plants, such as dandelion (*Taraxacum officinale*), chicory (*Cichorium intybus*), Jerusalem artichoke (*Helianthus tuberosus*), dahlia (*Dahlia pinnata*), and asparagus (*Asparagus officinale*) (Table 15.1), reserve this polymer (Kango 2008; Chi et al. 2011; Rawat et al. 2016).

Commercial production of fructose by inulin hydrolysis is more effective than other conventional method which required starch hydrolysis by the action of group of enzymes (α-amylase, amyloglucosidase, and glucose isomerase) liberating less fructose (∼45%) in the end product. Inulin hydrolysis using microbial inulinase yields 90–95% fructose solution. Fructose syrup production from inulin-rich material is a major area of inulinase application (Kango 2008; Vijayaraghavan et al. 2009; Liu et al. 2013).

Inulin is utilized by a variety of fungi, bacteria, and yeasts which degrade and modify inulin by enzymes such as endoinulinase, exoinulinase, and invertase. Exoinulinase (EC 3.8.1.80; β-D-fructohydrolase) hydrolyzes the terminal unit of fructose which is linked by β-fructofuranosidic bonds and liberates fructose.

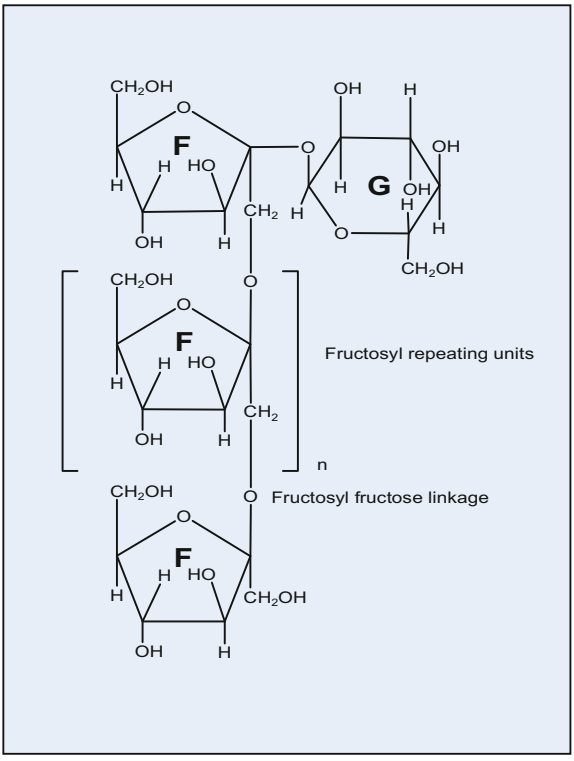

Fig. 15.1 Structure of inulin (F—fructose, G—glucose)

Table 15.1 Inulin content of some plants

Source of inulin	Scientific name	Storage organ	Inulin content[a]
Asparagus	*Asparagus racemosus*	Root	10–15
Banana	*Musa cavendishii*	Fruit	0.3–0.7
Camas	*Camassia quamash*	Bulb	12–22
Chicory	*Cichorium intybus*	Root	15–20
Dandelion	*Taraxacum officinale*	Root/leaves	12–15
Dahlia	*Dahlia pinnata*	Tuber	9–13
Garlic	*Allium sativum*	Bulb	9–16
Jarusalem artichoke	*Helianthus tuberosus*	Tuber	14–19
Onion	*Allium cepa*	Bulb	2–6
Yacon	*Smallanthus sonchifolius*	Root	3–10

[a]% of fresh weight (Modified from Kango and Jain 2011)

Endoinulinase (EC 3.2.1.7; β-D-fructan fructanohydrolase) breaks the internal glycosidic bonds of inulin and generates inulobiose (F2), inulotriose (F3), inulotetraose (F4), and inulopentaose (F5) as important end products (Fig. 15.2). β-D-fructofuranoside fructohydrolase (EC 3.2.1.26; invertase) hydrolyzes β-2, 1-fructosidic bond of sucrose to release fructose and glucose. Inulin is also degraded by yeast non-specific β-fructosidases which release fructose units from its reducing end. Relative activities toward sucrose and inulin are represented as inulinase/sucrase (*I/S*) ratio to differentiate between inulinase and invertase. High *I/S* ratio indicates predominant inulinase activity (Chi et al. 2009; Kango and Jain 2011; Rawat et al. 2015b; Singh et al. 2016).

Properties and Molecular Biology of Fungal Inulinases

Protein sequence of fungal inulinases has revealed several conserved motifs. Six highly conserved motifs of inulinases are WMND(E)PNGL, EC(V)P, SVEVF, FS(T), RDP, and Q that played an important role in substrate binding and inulin catalysis (Fig. 15.3). SVEVFV and Q are segments common in fungal exo- and endoinulinases, while SVEVFV amino acid segment was not noticed in yeast exoinulinases (Liu et al. 2013; Rawat et al. 2016). Fungal inulinases have been expressed in *Yarrowia lipolytica* (Liu et al. 2010), *S. cerevisiae* (Yuan et al. 2013), *P. pastoris* (Cao et al. 2013; Ma et al. 2015), *Kluyveromyces lactis* (Yu et al. 2010), and *E. coli* (Zhou et al. 2015). Such yeasts hydrolyze inulin and ferment fructose into ethanol simultaneously paving the way for consolidated bioprocessing (CBP) (Yuan et al. 2012).

Inulin rich substrates (Dahlia, Chicory, Dandelion)

Extraction

Inulin

Reducing end

β,2-1 fructosyl linkage

Exoinulinase

Endoinulinase

Fructose

(Low calorie sweetener, Feed stock)

Inulooligosaccharides

(Prebiotics, Nutraceutical)

Fig. 15.2 Schematic showing generation of fructose syrup and inulooligosaccharides from inulin-rich plant extract

Among fungal strains, *Aspergillus* spp. (Kango 2008; Rawat et al. 2015a) and *Penicillium* spp. (Rawat et al. 2015b), are common sources of exo- and endoinulinase (Table 15.2). Characteristics of some fungal inulinases are described in Table 15.3. Incidence of both exo- and endo-acting inulinolytic enzymes has been reported in fungi. For instance, exoinulinase gene from *A. niger* (*inuE*) encoded

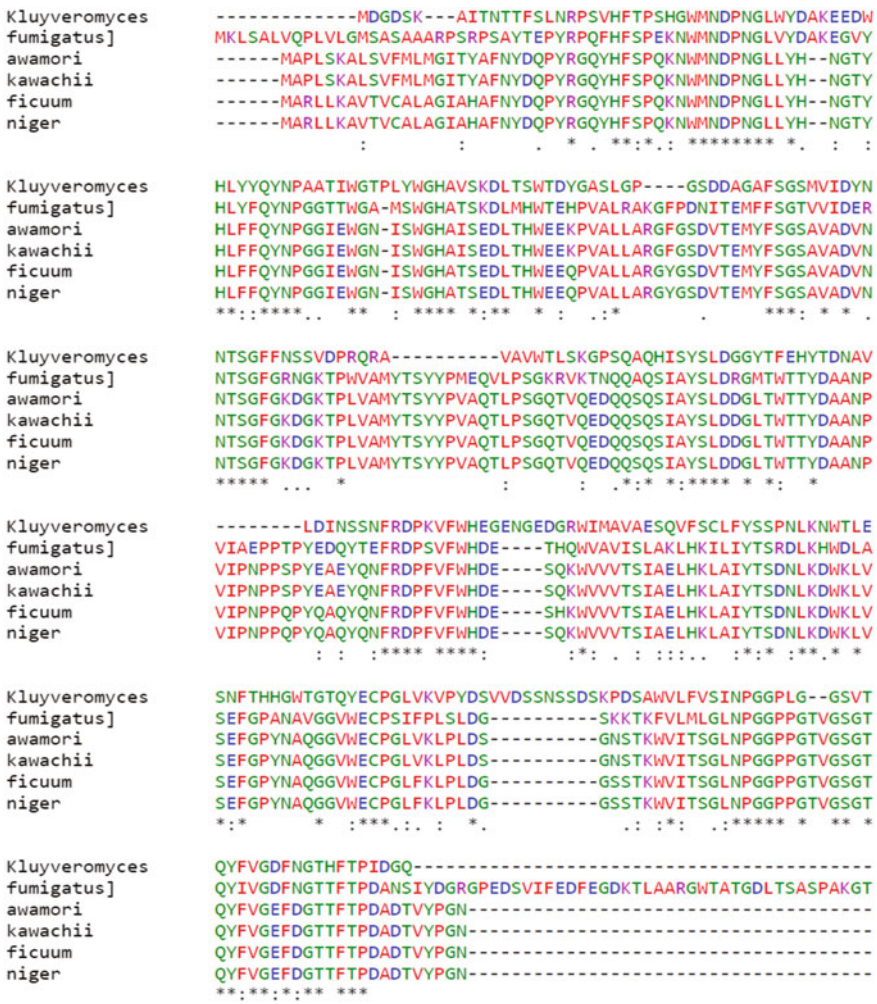

Fig. 15.3 Amino acid sequence alignment of various fungal exoinulinase using the Clustal Omega software

protein of 57 kDa (Goosen et al. 2008), while endoinulinase gene from the same strain encoded 54 kDa (Ohta et al. 1998). Another inulinase gene, *Inu*2 from *A. ficuum* ATCC 1688L encoded 55.1 kDa protein, while *endo1* gene from *A. ficuum* JNSP5-06 consisted of 1482 bp and was 98% identical to *A. niger* CBS513.88 and 60% identical to *A. fumigatus* AF293 inulinase (Chen et al. 2012). Exoinulinase genes, (*inu*D) and (*inu*A1), from *Penicillium* sp. (TN-88) and *P. janthinellum* (B01) have been isolated and cloned (Moriyama et al. 2002; Wang et al. 2011). Details of various inulinase genes cloned from molds and yeasts are summarized in Table 15.4.

Table 15.2 Fungal sources of inulinases

Source	Microorganism	Type[a]	References
Molds	*Aspergillus niger* NK 126	Exo, and endo	Kango (2008)
	Aspergillus ficuum JNSP5-06	Exo and endo	Chen et al. (2011)
	Aspergillus tubingensis CR-16	Exo	Trivedi et al. (2012)
	Penicillium subrubescens FBCC 1632	Exo	Mansouri et al. (2013)
	A. niger ATCC 26011	Exo and endo	Dinarvand et al. (2013)
	A. niger AUMC 9375	Exo, and endo	Housseiny (2014)
	A. niger CICIM F0620	Endo	He et al. (2014)
	Aspergillus fumigatus Cl1	Endo	Chen et al. (2015)
	A. niger 20 OSM	Exo, and endo	Trytek et al. (2015)
	Aspergillus awamori MTCC 2879	Exo	Rawat et al. (2015a)
	Penicillium citrinum MTCC 1256	Endo	Rawat et al. (2015a)
	Penicillium sp. NFCCI 2768	Exo, and endo	Rawat et al. (2015b)
Yeasts	*Pichia guilliermondii* M-30	Exo	Yu et al. (2009)
	Kluyveromyces marxianus MTCC 188	Exo	Dilipkumar et al. (2013)
	K. marxianus NRRLY-7571	Exo	Treichel et al. (2009)
	P. guilliermondii	Exo	Zhang et al. (2009)
	K. marxianus NRRL Y-7571	Exo	Mazutti et al. (2010)
	Candida guilliermondii TISTR 5844	Exo	Songpim et al. (2011)
	K. marxianus MTCC 3995	Exo	Jain et al. (2012)

[a]Exo–Exoinulinase; Endo–Endoinulinase

Heterologous Expression of Inulinase Genes of Fungi

Several exo- and endoinulinases encoding genes of filamentous fungi have been cloned in yeasts and characterized (Chi et al. 2011; Liu et al. 2013). The inulinase gene from *K. marxianus* CBS6556 was expressed in *Y. lipolytica* ACA-DC50109, and inulinase activity up to 41 U/ml was obtained (Zhao et al. 2010b). Recombinant yeast containing inulinase gene was used in inulin hydrolysis, with production of citric acid and SCP. The *K. marxianus* (*INU* 1) gene was expressed in *S. cerevisiae,* and the recombinant enzyme showed improved thermostability due to hyperglycosylation (Kim et al. 1997).

The endoinulinase gene (*inu* B) of *A. ficuum* was expressed in the mutant (Suc Z) *S. cerevisiae*. The recombinant inulinase was free from sucrase and exoinulinase activity, and the endoinulinase yield was up to 83 U/ml (Park et al. 2001). Yu et al. (2011) isolated inulinase gene from *Kluyveromyces cicerisporus* and expressed in a hexokinase muted *S. cerevisiae* strain. The yield of inulinase reached up to 31 U/ml, and recombinant yeast accumulated glucose-free fructose in fermentation broth containing Jesrusalem artichoke tubers. Workers have also expressed inulinase genes in high ethanol producing yeast for direct processing of inulin into ethanol.

Table 15.3 Characteristics of fungal inulinases

Microorganism	M (r) kDa	Optimum		K_m	V_{max}	Action	References
		pH	T (°C)				
Molds							
Aspergillus fumigatus CL1	58	6.0	55	2.18 mM	1590 μmol min^{-1} mg^{-1}	Endo	Chen et al. (2015)
Aspergillus ficuum JNSP5-06	63	4.0	60	7.1 mM	1000 μmol min^{-1} mg^{-1}	Exo	Chen et al. (2013)
A. ficuum JNSP5-06	70	4.5	45	43.1 mg ml^{-1}	32.7 mg min^{-1} ml^{-1}	Exo	Chen et al. (2009)
A. ficuum	63	5.4	50	4.75 mM	833.3 μmol min^{-1} ml^{-1}	Exo	Mutanda et al. (2009)
Aspergillus niger	68.1	6.0	50	3.53 mM	666.7 μmol min^{-1} ml^{-1}	Endo	Mutanda et al. (2008)
Penicillium janczewskii	80	4.0–5.5	60	6.3×10^{-2} M	2.09×10^{-2} μmol min^{-1} ml^{-1}	Exo	Pessoni et al. (2007)
A. fumigatus	62	6.0	60	1.25 mM	526 μmol min^{-1} mg^{-1}	Exo	Gill et al. (2006)
Chaetomium sp. C34	66	6.0	55	0.199 mM	115 μmol min^{-1} mg^{-1}	Endo	Zhang et al. (2004)
Aspergillus awamori var. 2250	69	4.5	–	0.003 mM	–	Exo	Arand et al. (2002)
Yeasts							
Kluyveromyces cicerisporus	90	4.5	55	0.32 mM	4317 μmol min^{-1} ml^{-1}	Exo	Ma et al. (2015)
Cryptococcus aureus G7a	60	5.0	50	20.06 mg ml^{-1}	0.0085 mg min^{-1}	Exo	Sheng et al. (2008)
Kluyveromyces marxianus Y1	–	5.5	55	–	–	Exo	Yuan and Bai (2008)
Pichia guilliermondii	–	6.0	60	–	–	Exo	Gong et al. (2007)

Table 15.4 Characteristics of some inulinase genes from molds and yeasts

Molds/yeast	Gene type	Size (bp)	Accession no.	References
Kluyeromyces cicerisporus	Exo *kcINU1*	1665	AF178979	Ma et al. (2015)
K. marxianus	Exo *rKmINU*	3223	X68479	Zhang et al. (2012)
Meyerozyma guilliermondii	Exo *INU1*	1732	EU195799	Liu et al. (2014)
Aspergillus ficuum	*Endo 1* gene	1482	FJ 984582	Chen et al. (2012)
A. fumigatus CL1	*Endo*	1561	EAL86248.1	Chen et al. (2015)
A. niger CICIM FO620	*En Inu*	1614	XM_001395842	He et al. (2014)
A. ficuum JNSP5-06	*Exo I* gene	1600	HM587130	Chen et al. (2012)
Penicillium janthinellum B01	*Exo inu A1*	2115	JF961344	Wang et al. (2011)
P. citrinum ESS	*Exo*	1608	KM364035	Flores-Gallegos et al. (2015)

For instance, inulinase (*INU* 1) gene was isolated from marine *Pichia guilliermondii* strain 1 and expressed in *Saccharomyces* sp. W0 (Zhang et al. 2010). Wang et al. (2011) noticed that *INU* 1 gene integration into rDNA in *Saccharomyces* sp. W0 leads to production of more inulinase and ethanol from inulin in less time as compared to *Saccharomyces* sp. W0 strain carrying *INU* 1 gene in plasmid. Codon-optimized inulinase gene (*INU1Y*) from yeast *Meyerozyma guilliermondii* was expressed in *Saccharomyces* sp. W0, and recombinant strain (W0 Y13) produced 43 U/ml inulinase which was higher than native gene *INU1* containing recombinant yeast (Liu et al. 2014).

Moriyama et al. (2002) have expressed *inu* E gene from *A. niger* strain 12 in *P. pastoris* yielding 16 U/ml inulinase having larger molecular mass (86 kDa) than inulinase produced by wild-type *A. niger* strain 12. Similarly, Wang et al. (2004) have expressed endoinulinase gene from *A. niger* 9891 (CGMCC 0991) in *P. pastoris* and obtained 291 U/ml yield in inulin-containing medium. Wang et al. (2011) obtained 11-fold high exoinulinase production (272 U/ml) when *inu* A gene from *P. janthinellum* strain B01 was expressed in *P. pastoris* X-33. Recombinant *P. pastoris* containing gene *KmInu* of *K. marxianus* produced 6667 U/ml inulinase (Zhang et al. 2012). The recombinant inulinase showed good stability up to 50 °C and 5.0 pH as compared to native enzyme. Zhou et al. (2014) disrupted *MIG1* gene in *K. marxianus* and developed a derepressed mutant producing high inulinase (133 U/ml).

Several inulinase genes have been expressed in *Kluyvermyces lactis* and *E. coli* (Liu et al. 2013; Rawat et al. 2016). Yu et al. (2010) have isolated inulinase gene (*Kcinu*) from *K. cicerisporus* and expressed in mutant *K. lactis*. They have noticed

twofold increase in inulinase activity (391 U/ml) than wild-type strain. Kwon et al. (2000) expressed inulinase gene (*inuZ*) of *Pichia mucidolen* in *E. coli,* and recombinant inulinase was a monomeric protein with MW of 55 kDa. Endoinulinase of *A. ficuum* was expressed in *E. coli* expression system (Chen et al. 2012). This endoinulinase was used in IOS production from inulin. Chen et al. (2013) have expressed exoinulinase gene from *A. ficuum* in *E. coli* and characterized recombinant enzyme.

Application of Fungal Inulinases

Hydrolysis of inulin can be selectively directed using microbial inulinases (exoinulinases and endoinulinases) for production of fructose-rich syrup and fructooligosaccharides (FOS), and this preparation can also be used as feedstock for production of single-cell protein (SCP), citric acid, ethanol, and other useful products (Chi et al. 2011; Kango and Jain 2011). Hydrolysis of inulin for the fructose bioconversion to ethanol by utilizing fructose from engineered yeast which has a prominent ability of consolidated bioprocessing (CBP) of inulin (Yuan et al. 2012).

Inulin is a renewable and commonly occurring polysaccharide that can be used as fructose feedstock generation. *S. cerevisiae* can easily utilize and convert fructose into ethanol (Chi et al. 2009; Nevoigt 2008). The same yeast strain was used for SCO production using Jerusalem artichoke (JA) tuber extract. This yeast strain accumulated 48.8% (w/w) and 52.2% (w/w) oil while growing on hydrolyzates of inulin (Zhao et al. 2010a). *Y. lipolytica*, an oleaginous yeast, accumulated 0.44-0.54 g lipid/g of biomass and produced 9-12 g/l dry biomass (Papanikolaou et al. 2002). An inulin utilizing mutant of this strain, *Y. lipolytica* ACA-DC 50109 (uracil mutant) containing inulinase gene of *K. marxianus* CBS 6556, was developed (Papanikolaou and Aggelis 2003).

Inulinase enzyme secreted by this engineered yeast was used to hydrolyze JA juice followed by SCO production from inulin (Zhao et al. 2010b) (Table 15.5).

Inulinase preparations, therefore, can be used in feed, pharmaceutical, biofuel, and nutraceutical industries. Endoinulinase of *Aspergillus niger* was immobilized on chitosan and prepared for continuous generation of inulooligosaccharides (IOS) syrup from artichoke juice. This syrup contained IOS with DP 3–7 (Nguyen et al. 2011). Inulinase of *A. niger* was immobilized on Concanavalin-A (lectins) for the generation of syrup of fructose (Altunbas et al. 2013). IOS are also applicable in animal nutrition for significant change in colonic bacterial populations (Kelly, 2009). IOS also have various applications in food industries like chocolate, ice cream, milk desserts, confectionary, and sauces (Kuntz et al. 2013). Inulin and IOS contribute in improvement of the mineral balance of Ca, Mg, and Fe and show anti-carcinogenic effect by enhancing the bifidogenic flora which improves immunity of the system (Kango and Jain 2011). Endoinulinase *inuA* gene of *A. niger* was cloned and expressed in *S. cerevisiae.* The resultant recombinant enzyme

Table 15.5 Potential uses of inulinase in generation of industrially and nutritionally important end products using fungal inulinases

SN	Fungal strain used	End product[a]	Enzyme type	Fermentable substrate	References
1.	*Aspergillus niger* NK 126	F, IOS	Exo, endo	Chicory inulin	Kango (2008)
2.	*Saccharomyces* sp. W0	Ethanol	Inu	JA	Zhang et al. (2010)
3.	*Yarrowia lipolytica*	Citric acid	Inu	Inulin	Liu et al. (2010)
4.	*Cryptococcus aureus* G7a	SCP	Inu	Yacon	Zhao et al. (2010a, b)
5.	*Yarrowia lipolytica*	SCP	Inu	Inulin	Cui et al. (2011)
6.	*A. niger* (Megazyme)	Oligofructose syrup	Endo	JA	Nguyen et al. (2011)
7.	*Rhodotorula mucilaginosa* TJY15a	SCO	Inu	JA	Zhao et al. (2011)
8.	*A. niger* (Fructozyme L)	Tequila	Exo, endo	*Agave tequilana*	Waleckx et al. (2011)
9.	*Kluyveromyces marxianus*		Inu	JA	Yuan et al. (2012)
10.	*Aspergillus niger* (Fructozyme)	FOS	Inu	Inulin, Sucrose	Kuhn et al. (2012)
11.	*Kluyveromyces marxianus*, *S. cerevisiae*	Ethanol	Inu	JA	Hu et al. (2012)
12.	*Pichia guillermondii* Pcla22	SCO	Inu	Inulin	Wang et al. (2012)
13.	*Saccharomyces* W0	Ethanol	Inv	Inulin	Li et al. (2013)
14.	*Saccharomyces cerevisiae*	Ethanol	Inu	JA	Yuan et al. (2013)
15.	*Pichia pastoris*	IOS	Endo	Inulin	He et al. (2014)
16.	*Meyerozyma guillirmondii, Saccharomyces* W0	Ethanol	Exo	Inulin	Liu et al. (2014)
17.	*Rhodosporidium toruloides* 2F5	SCO	Inu	Inulin	Wang et al. (2014)
18.	*Kluyveromyces marxianus*	Ethanol	Inu	JA	Gao et al. (2015)
19.	*Aspergillus* spp., *Penicillium* spp., *Fusarium oxysporum*, *Kluyveromyces*	F, IOS, FOS	Exo, endo	Chicory inulin, Sucrose	Rawat et al. (2015a)
20.	*Penicillium* sp. NFCCI 2768	F, IOS	Exo, endo	Dahlia inulin	Rawat et al. (2015b)

[a] *F* fructose; *IOS* inulooligosaccharides; *FOS* fructooligosaccharides; *SCP* single cell protein; *LA* lactic acid; *SCO* single cell oil; *CA* citric acid; *Exo* exoinulinase; *Endo* endoinulinase; *Inu* inulinase; *Inv* invertase; *JA* Jerusalem artichoke

showed maximum activity 3.1 U/ml and ethanol concentrations 55.3 g/L (Yuan et al. 2013).

Conclusions and Future Perspectives

Inulin hydrolysis for generation of fructose and fructooligosaccharides has recently received considerable interest. Some yeasts and fungi have been noticed to produce exoinulinases, while endoinulinase production is limited to few fungal and bacterial strains. Unconventional raw materials with high inulin content are being explored for optimized inulinase production. Cloning and expression of novel inulinases in suitable hosts, mostly yeasts such as *Pichia*, *Saccharomyces*, has been useful in consolidated bioprocessing of inulin to bioethanol. Thermostable inulinases from *Bacillus smithii* T7 and *Sphingomonas* sp. JB13 indicate possibility of finding robust inulinases among extremophiles. Inulin can be obtained from horticultural crops such as Jerusalem artichoke, chicory and utilized for generation of high-fructose syrup or oligosaccharides. Search for novel inulinase producers, parametric optimization for production and application, enzyme immobilization, and cloning of inulinase gene in suitable hosts are some of the challenges in this area. Efforts for the development of cost-effective bioprocesses suiting to food and nutraceutical industries using inulooligosachrides and fructose in commercial preparations would be helpful in realizing the applications.

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Fungal Tannase: Recent Advances and Industrial Applications

16

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Abstract

Tannin acyl hydrolase (E.C.3.1.1.20) universally known as tannase is an inducible enzyme that predominantly acts on tannins. Tannins have been documented as high molecular weight polyphenolic compounds possessing molecular weight in the range (500–3000 kDa). Tannins are the second most abundant polyphenolic compounds existing in nature after lignin. Tannins are water-soluble secondary metabolites existing in abundance in plants. Apparently, tannins exist in plants as the fourth most bountiful constituents behind cellulose, hemicellulose, and lignin. Tannins possess acrid properties and have the inherent capability of binding with proteins, cellulose, gelatin, and pectin thereby forming insoluble complexes. Tannases derived from microbial sources have enormous applications in various industries. This tremendous biocatalytic potential of tannase is attributed to their higher stability and feasibility. Tannases have a vast range of applications in various industrial bioprocesses ranging from food, feed to chemical as well as pharma sector. Furthermore, tannery effluents

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are loaded with surplus amount of tannins, predominantly polyphenols, which are life-threatening pollutants and pose severe environmental and health hazards. As a matter of fact, tannases can be substantially utilized for degrading the tannins that predominantly exist in the tannery effluents, thus offering a much cheaper treatment for the eviction of these compounds. The enzyme also finds significant utilization in cosmetic industries to so as to lessen the extensive turbidity of plant extracts. Tannase can also be predominantly used for high-grade leather tannins preparation in the leather industry. The present chapter is an attempt to emphasize on microbial sources, substrates for maximal tannase production, factors governing tannase production, mechanism of action, purification, immobilization, inhibitors as well as widespread applications of tannases. The advancements in molecular tools and techniques have enabled a better understanding of tannase structure, underlying mechanism of its action as well as the more precise understanding of various process parameters governing tannase production. Over the years, tannases have witnessed a significant rise in their utilization in commercial sector; thus, there is always an opportunity for researchers to search out novel tannases with better and improved characteristics.

Keywords
Polyphenolics · Tannase · Purification · Characterization · Immobilization

Introduction

Enzymes or biocatalysts are exclusively synthesized by the living cell and no life can exist without enzymes. They are basically proteins and catalyze certain chemical reactions involving naturally occurring organic materials such as carbohydrates, protein, fats. Enzymes are important because of their extraordinary specificity and catalytic power which are greater than those of artificial catalysts. Tannin acyl hydrolase (E.C.3.1.1.20) universally known as tannase is an inducible enzyme that has been widely utilized in the biotransformation of hydrolysable tannins to simple phenolic molecules like gallic acid. Tannases precisely act upon ester and depside linkages in hydrolysable tannins particularly the gallotannins. Filamentous fungi have been documented as the prominent tannase producers amongst all tannase-producing microorganisms.

History

Van Teighem (1867) accidentally explored tannase enzyme and he reported gallic acid production after treating an aqueous solution of tannins with two fungal species. He was the pioneer who revealed that fungal activity is responsible for gallic acid production. Loraque documented the gallic acid production from tannic acid is either due an organism's action or due to the oxidation process. In this context, he further reported numerous toxic substances inhibiting the gallic acid production from tannic acid in gallnut (Knudson 1913). Fenbach in 1901 cultivated *Aspergillus niger* in Raulin's solution that consisted of tannic acid as a carbon source in place of sugar thereafter isolating tannase from the cultured organism (Knudson 1913).

Libuchi and his coworkers in (1967) proposed a spectrophotometric assay for the quantitation of tannase activity. A vast range of assay methods is available for estimation of tannase activity. Presently, two methods are predominantly used by researchers to evaluate the tannase activity. The first method as described by Mondal et al. (2001) relies on the Hydrolysis of tannic acid and measuring the leftover tannic acid using tannic acid standard curve. However, the second method as described by Sharma et al. (2000) relies on measurement of gallic acid given off post-enzymatic reaction using the gallic acid standard curve. As a matter of fact, apart from filamentous fungi tannase production by bacteria was also documented (Deschamps et al. 1983).

Tannins: Natural Substrates for Tannase

Tannins belong to phenolics are one of the major class of plant secondary metabolites that serve as a defense machinery against attack by herbivores. These phenolics are synthesized in plants as a counter response to invasion by microbes, herbivores, pathogens, cold, and UV light as well as nutrient limitation. Tannins universally exist in angiosperms, gymnosperms as well as pteridophytes. Tannins predominantly accumulate in plant parts such as bark, roots, fruits, and leaves (Scalbert 1991). Frutos et al. (2004) documented the abundance of tannins in plant leaves and flowers.

Occurrence of Tannins

Belmares et al. (2004) documented the localization and distribution of tannins in various plant parts like flowers, needles, bark, seeds, leaves. Most common sources of hydrolysable tannins used universally in commercial sector are Turkish gall (*Quercus infectoria*), myrobalan nuts (*Terminalia chebula*), sumac (*Rhus coriaria*), Chinese gall (*Rhus semialata*), chestnut (*Castanea sativa*, Bhat et al. 1998),

tara (*Caesalpinia spinosa*), chestnut gum arabic tree (*Acacia nilotica*, Lal et al. 2012), red gram (*Cajanus cajan*, Kuppusamy et al. 2015), and Cashew testa (*Anacardium occidentales*, Lokeshwari 2016). Hydrolysable tannins can be hydrolyzed to gallic acid and glucose molecule by through chemical treatment either by acidic hydrolysis or by alkaline treatment. However, in the past few years, enzymatic hydrolysis of tannins by tannases has attained ample importance. Tannins are principally localized in vacuoles or surface wax of plants. These localization sites keep tannins in their active state to counter the invasion by herbivores and pathogens. Only after cell breakdown and death, they can act and have metabolic effects. The concentration as well as their chemical attributes vary significantly amongst different plant species. Frutos et al. (2004) documented that tannin content in plants is boosted by factors such as water stress, poor soil quality, high temperature, extreme light intensity. Such variability in tannin concentration amongst different plant species is governed by several environmental parameters such as temperature, water availability, light, CO_2 as well as nutrient availability and limitation. Long-lived trees have been documented to have higher tannin content in comparison to short-lived trees.

Importance of Tannins

Bhat et al. (1998) documented tannins as the most bountiful class of polyphenolics right behind lignins. Tannins have been reported as the products of plant secondary metabolism. The term tannin refers to tanna, an "Old High German" word for oak or fir tree. The term "tannin" may also refer to "tanning" or preservation of skins to create leather. Tannins have been reported to possess molecular weights ranging from 500 to 3000 kDa (Aguilar et al. 2007). Chemical basis of tannins defense mechanism has been credited to their inherent capability of precipitating the proteins. This tannin–protein complex was documented to render the microbial attack ineffective and simultaneously limiting the accessibility of metal ions obligatory for microbial metabolism (Scalbert 1991). The defense mechanism of tannin–protein complex was documented to be because of their acrid property and masculine sensation.

Classification of Tannins

Tannins have been categorized into three subgroups: hydrolysable tannins, condensed tannins, and complex tannins. Condensed tannins differ from hydrolysable tannins in not having any sugar molecule in their structure. Hydrolysable tannins are subdivided into two types: gallotannins and ellagitannins which are esterified to a sugar molecule most commonly the glucose molecule. Hydrolysable tannins can

undergo easy hydrolysis upon acidic treatment or mild alkaline treatment as well as by treatment with hot water and more efficiently with enzymatic treatment.

Gallotannins are composed of gallic acid units esterified to a glucose molecule. Examples of gallotannins are Turkish galls (*Quercus infectoria*), Chinese gall (*Rhus semilata*) and sumac tannin (*Rhus coriaria*), Tara pods (*Caesalpinia spinosa*). Gallotannins represent the simplest form of hydrolysable tannins. Tannic acid has been documented as commercial form of gallotannins. Gallotannins give off gallic acid and glucose molecule upon hydrolysis. Gallotannins can undergo easy hydrolysis to yield gallic acid and glucose molecule upon acidic treatment or mild alkaline treatment—treatment with hot water and more effectively with enzymatic treatment by tannase.

Ellagitannins are composed of ellagic acid units bonded to glucosides. Molecules carrying a quinic acid core rather than glucose also represent ellagitannins. These cannot undergo easy hydrolysis because of their complex structure including C–C bonds.

Condensed tannins represent the compounds composed of building blocks of flavonoid units usually (from 2 over 50). They predominantly exist in woods and tree bark. Examples of condensed tannins include wattle (*Acacia mollisima*) tannin, quebracho (*Schinopsis lorentzii*) tannin and from tree bark (Bhat et al. 1998).

Complex tannins are an intermediate group that shares the features of both hydrolysable tannins as well as condensed tannins. They are composed of catechin or epicatechin units linked via glycosidic linkage to a gallotannin or an ellagitannin unit. Acutissimin A, Acutissimin B, Epicutissimin A, Mongolicain, Mongolicain A, and Mongolicain B are common examples of complex tannins. They give off catechin or epicatechin and gallic acid or ellagic acid on hydrolysis (Mingshu et al. 2006). The catechin tannins principally occur in tea leaves and tropical shrub legumes (Bhat et al. 1998).

Various sources of Tannase

Plants as a Tannase Source

Tannase has been documented in hydrolysable tannin-containing plants such as Turkish gall (*Quercusinfectoria*), sumac (*Rhus coriaria*), Chinese gall (*Rhus semialata*), tara (*Caesalpinia spinosa*), chestnut (*Castanea sativa*, Bhat et al. 1998), gum arabic tree (*Acacia nilotica*, Lal et al. 2012), red gram (*Cajanus cajan*, Kuppusamy et al. 2015) and waste testa (*Anacardium occidentales*, Lenin et al. 2015), divi divi (*Caesalpinia coriaria*) pods, English oak (*Quercus robur*), Pendunculate oak (*Quercus rubra*), Karee tree (*Rhus typhina*) leaves. Plants having condensed tannins are babul (*Acacia arabica*), konnam (*Cassia fistula*), avaram (*Cassia auriculata*) and others. Physiological worthiness of tannase in plants has been demonstrated in the synthesis of tannins. Plants synthesize gallic acid, hexahydroxyphenic acid, and chebulinic acid in addition to significant amount of sugar.

These acids possibly undergo esterification with glucose molecule during the ripening process with the aid from tannase ultimately resulting in synthesis of tannins.

Animals as a Tannase Source

Animal sources of tannase include bovine intestine and ruminal mucus. Apart from this certain insects have been reported to produce tannase in their larval state. Several gastrointestinal bacteria of animal origin have also been documented to be the effective tannase producers. Many species of these bacteria have been from explored from feces of cows, humans, goats, etc. Tannase-producing bacteria *Streptococcus pneumonia* and *Streptococcus bovis* strains isolated from fecal samples of native sheep and goats which can hydrolyse acorn tannin in rumen and reduce negative effects of tannin on animals (Mosleh et al. 2014).

Microbial Sources

Microbial route for tannase production has gained worldwide importance over other sources since the microbial enzymes offer several advantages over other sources since the microbial enzymes are much more stable in comparison to similar enzymes from other sources. Furthermore, microorganisms possess the ability to produce higher titers of tannase. Microorganisms can undergo genetic modification, thus, they are easy to manipulate genetically. This property results in a significant uplift in the enzymatic activity. Most of the reported tannase-producing organisms are fungi, only a few bacteria, and yeast. Tannases vary in their degree of specificity as well as activity toward different tannin substrates. Fungal tannases have been documented to possess higher activity titer in the degradation of hydrolysable tannins. However, as a matter of fact, yeast tannases relatively disintegrate tannic acid easily and flaunt a relatively lesser affinity on the other hand in the degradation of natural tannins (Deschamps et al. 1983).

Bacteria

Several bacterial strains have been documented for their tannase production capability. Deschamps et al. (1983) were the pioneer to document tannase production from bacteria. The main tannase-producing genera among bacteria are: *Bacillus* (Belur et al. 2012), *Lactobacillus* (Rodriguez et al. 2008), *Pesudomonas* (Selwal et al. 2010), *Erwinia carotovora* (Sahira et al. 2015), *Bacillus gottheilii M2S2* Subbulaxmi and Murty (2016).

Yeast

Over the years, couple of tannin degrading yeasts have been documented viz *Candida* sp., *Debaromyces hansenii*, *Pichia adeyshi*, *P. monospora*, and *P. pseudopolymer.* Aoki et al. (1976a, b) documented the tannin degradation by *Candida* spp., which possessed the capability to synthesize both extracellular as well as intracellular tannase by utilizing tannic acid as a substrate.

Fungi

Hadi et al. (1994) documented the potential of filamentous fungi in tannin degradation. Filmenteous fungi belonging to *Aspergillus* genus have been documented as one of the most potent tannse producers worldwide (Banerjee et al. 2001). *Aspergillus* spp. possess the capability of tannase production even in the scarcity of tannic acid. However, as a matter of fact, these fungi can tolerate high tannic acid concentration up to 20% without causing any negative effect on fungal growth as well as enzyme production. Fungi have an edge in having much pronounced growth and ease of separation from fermentation broth (Belmares et al. 2004). A few examples of tannase-producing fungi are: *A. fumigatus* (Manjit et al. 2008), *R. oryzae* (Mukherjee et al. 2006), *A. awamori* (Beena et al. 2010), *P. purpurogenum* (Reddy and Rathod 2012), *A. aculeatus* (Bagga et al. 2015), and others.

Mode of Action of Tannase

Tannase predominantly acts on ester and depside linkages in hydrolysable tannins preferably the gallotannins. Lekha and Lonsane (1997) documented the hydrolytic action of tannase on tannic acid giving off gallic acid and glucose. Lekha and Lonsane (1997) further documented that tannases show their catalytic action on complex tannins such as in similar fashion but do not show their catalytic action on condensed tannins. Tannase also precisely acts upon the ester linkage of methyl gallate and the depside linkage of m-digallic acid. Aguilera-Carbo et al. (2008) documented a few reports on the biocatalytic action of tannse on elligitannins. However, as a matter of fact, none of the reports demonstrated a clear cut mechanism of tannase action on ellagitannins.

Induction, Synthesis and Regulation of Tannase

Tannase has been reported as an inducible enzyme. Enzyme induction, expression, and production occur to varied levels credited to the strain used and the culture conditions employed. Tannase synthesis is efficiently induced by various phenolic compounds viz tannic acid, pyrogallol, gallic acid, and methyl gallate (Costa et al. 2008). Gallic acid, the major structural component of gallotannins, has been documented to efficiently induce tannase synthesis in submerged fermentation; however, it has been reported as a repressor of tannase synthesis in solid-state fermentation (Bajpai and Patil 1997). Belmares et al. (2004) documented enhanced tannase production by *Aspergillus niger* upon the addition of carbon sources like sucrose, fructose, glucose to the fermentation media in the concentration range 10–30 g/L.

Production Aspects of Tannase Through Fermentation

Tannase production in fungi has been documented via liquid-surface, submerged fermentation, liquid-surface and solid-state fermentation processes, respectively. However, submerged fermentation has been the preferred method for tannase production in bacteria and yeasts (Belur and Mugeraya 2011). Submerged fermentation has been the most preferred method for tannase production worldwide; however, as a matter of fact, few studies on tannase production through solid-state fermentation have also been documented. Selection of an effective production technique is governed by several parameters such as strain to be used, nutrient availability, type and nature of substrate being used.

Tannase Production Through Submerged Fermentation (SmF)

Submerged fermentation principally involves growing the microbial culture as a suspension in the fermentation medium having all the nutrients to sustain and support its growth (Aguilar et al. 2001). In the present day scenario, submerged fermentation is the most preferred process for commercial production of several valuable enzymes including tannase credited to ease of sterilization and better process control. Bajpai and Patil (1997) documented enhanced tannase production by *Aspergillus* spp. at higher aeration rates. Murugan et al. (2007) documented tannase production by *Aspergillus xavus*, *Aspergillus niger*, *Fusarium* spp., *Penicillium* spp., and *Trichoderma* spp. in submerged fermentation under controlled conditions. *A. niger* was documented as the most potent tannase producer amongst all isolates with an apparent enzyme activity of 16.77 U/mL. Paranthaman et al. (2009) optimized a temperature of 35 °C, 96 h of incubation time, and 2% tannic acid substrate concentration for maximal tannase production from *A. flavus* through

submerged fermentation. Srivastava and Kar (2009) optimized extracellular tannase and gallic acid production by *A. niger* isolate through submerged fermentation at 37 °C, 72 h and 4% (w/v) pomegranate rind powder as a tannin-containing substrate. (Ahmed and Rahman 2014) optimized various process parameters (pH, temperature, and incubation period) for tannase production by an *Aspergillus niger* strain.

Tannase Production Through Solid-State Fermentation

Solid-state fermentation (SSF) principally commences in the scarcity of free water and essentially requires a solid support which could be either natural support or an inert support material. However, as a matter of fact, the substrate being used ought to have moisture content good enough to sustain the growth and metabolism of the strain used in the process. The low moisture content renders the free water unavailable to other microorganisms thereby limiting the chances of their growth and making the fermentation process feasible only by a constricted number of microorganisms particularly yeasts and fungi and few bacteria. Most of the reports have reported fungi as highly acclimatized to SSF conditions credited to their hyphae growth on particle surfaces and colonization on solid substrate. SSF principally involves two types of process that can be differentiated from each other on the basis of characteristics of the solid phase being utilized. The first one and most widely used process principally involves a solid phase that essentially serves as a support material as well as a source of nutrition. The substrates used are predominantly heterogenous, water-insoluble, and includes major byproducts from agriculture and food industry such as tamarind seed powder, pomegranate rind powder, potato, palm kernel cake, cassava, sugar beet pulp, coffee husk, and others (Sabu et al. 2005). The second process principally consists of an inert support like polyurethane foam, sugarcane bagasse, vermiculite, resins impregnated with liquid medium consisting of all essential nutrients. Deepa et al. (2015) documented tannase production from *Aspergillus niger* by utilizing wood chips as a solid substrate.

Purification of Tannases

Bhardwaj et al. (2003) reported that cell-deprived fermentation broth could be directly utilized as a source of extracellular tannase. However, as a matter of fact, the intracellular enzyme needs to be released out of the cells through their lysis. This could be achieved either by grinding of cellular fraction in sand or by crushing of cells by using homogenizer and subsequently extracting the cell lysate in suitable buffer. Naidu et al. (2008) utilized ammonium sulfate precipitation as preliminary purification step which resulted in partial purification as well as concentration of the crude enzyme. Mahapatra et al. (2005) described a purification procedure of tannase

produced extracellularly by *A. awamori nakazawa*. The properties of the purified enzyme including pH and temperature optima and effects of urea, surfactants, and chelators were investigated.

Mukherjee et al. (2006) documented tannase production by a co-culture of the two filamentous fungi, *R. oryzae* and *A. foetidus*, by modified solid-state fermentation of tannin-rich substrates, and extracellular enzyme was purified through solvent precipitation and thereafter using DEAE-Sepadex column chromatography. They also conducted studies concerned with the effects of various process parameters on the activity of enzyme. The process parameters mainly studied were pH, temperature, effect of K_m and V_{max} along with the thermal stability of the enzyme at different temperatures. Chhokar et al. (2009) obtained a purification fold of 19.5 with 13.5% yield upon purification of tannase from *A. awamori* MTCC 9299 using ammonium sulfate precipitation followed by ion-exchange chromatography.

Naidu et al. (2008) reported tannase purification from *A. foetidus* through aqueous two-phase extraction using Polyethylene glycol (PEG) and documented a purification fold of 2.7 with an apparent yield of 82%. Ramirez-Coronel et al. (2003) documented purification of tannase from *A. niger* using a different protocol involving preparative isoelectric focusing for initial purification and ion-exchange chromatography thereafter for complete purification of enzyme. SDS-PAGE analysis of purified enzyme flaunted two protein bands with apparent molecular weights of 90 and 180 kDa, respectively. Deepa et al. (2015) documented purification of tannase from *A. niger* using DEAE-Sephadex gel filtration chromatography followed by SDS-PAGE analysis.

Tannase Characterization

Tannase has been documented as an inducible enzyme having high molecular mass ranging from 59 to 320 kDa (Table 16.1). The molecular mass of *A. niger* MTCC 2425 tannase was reported to be 185 kDa which consisted of two units of apparent molecular weights 102 and 83 kDa, respectively (Bhardwaj et al. 2003). Tannase from *A. niger* ATCC 16620 has a single monomeric unit of 168 kDa (Sabu et al. 2005). Kasieczka-Burnecka et al. (2007) reported that tannases from *Verticillium* spp. were oligomeric enzymes which consisted of two kinds of subunits with molecular masses of 39.9 and 45.6 kDa, respectively. Sharma et al. (2008) documented a molecular mass of 310 kDa for tannase from *Penicillium variabile*. The tannase from *A. niger* GH1 is composed of three subunits of molecular masses of 50, 75, and 100 kDa (Mata-Gomez et al. 2009). Beena et al. (2010) documented six identical subunits of 37.8 kDa from tannase of *Aspergillus awamori.* Goncalves et al. (2011) reported a heteromeric tannase of *Emericella nidulans* with three copies of each polypeptide. This enzyme has two polypeptide bands which correspond to 45.8 and 52 kDa as observed after 12% SDS-PAGE.

Table 16.1 Molecular mass of tannase from various fungal sources

Source	Molecular weight (kDa)	References
Selenomonas ruminantium	59	Skene and Brooker (1995)
Aspergillus oryzae	63	Hatamoto et al. (1996)
Aspergillus awamori MTCC 9299	101	Chhokar et al. (2009)
Paecilomyces variotii	149.8	Mahendran et al. (2006)
Fusarium subglutinans	150	Hamdy (2008)
Aspergillus niger	168	Sabu et al. (2005)
Aspergillus tamarii	180	Costa et al. (2012)
Aspergillus niger MTCC 2425	185	Bhardwaj et al. (2003)
Aspergillus awamori BTMF032	230	Beena et al. (2010)
Cryphonectria parasitica	240	Farias et al. (1994)
Candida spp.	250	Aoki et al. (1976a, b)
Emericella nidulans	302	Goncalves et al. (2011)
Penicillium variabile	310	Sharma et al. (2008)
Arxula adeninivorans	320	Boer et al. (2009)

pH Optima and Stability of Tannase

Battestin and Macedo (2007) in their research study demonstrated that pH alters enzyme activity by determining the nature of the amino acids at active site which undergo protonation and deprotonation since change in pH alters their protonation pattern. Reddy and Rathod (2012) demonstrated tannase as a protein having acidic nature with an optimum pH around 5.0–6.0 and is unstable above pH 6.0. The tannases obtained from *A. aculeatus* DBF9, *A. niger*, *A. awamori nakazawa*, and *Erwinia carotovora* had a pH optima of 5.0 (Mahapatra et al. 2005). Chhokar et al. (2010) documented pH 5.5 to be optimum for tannase of *A. awamori* MTCC 9299. However, an optimal pH of 6.0 was recorded for *A. niger* tannase (Ramirez-Coronel et al. 2003; Mata-Gomez et al. 2009). Battestin and Macedo (2007) documented more than 80% stability of tannase in the narrow pH range between 4.5 and 6.5. Mahendran et al. (2006) in their research study documented a pH optimum in the range of 5.0–7.0 for the activity of tannase of *P. variotii*.

Temperature Optima and Stability of Tannase

The temperature optima for fungal tannases fall between 30 and 50 °C. Battestin and Macedo (2007) demonstrated optimum temperature as that maximizes the velocity of enzymatic reaction beyond which the rate of reaction declines as an effect of thermal denaturation thus rendering the enzyme inactive. Any increase in temperature beyond optima causes decline in catalytic rate of tannase due to

denaturation (Mukherjee et al. 2006). The optimal temperature for tannase from *Rhizopus oryzae* for the free and immobilized enzyme was 40 and 55 °C, respectively. Sharma et al. (2008) reported that temperature optima for both free and immobilized enzyme of *P. variabile* were similar.

pI of Tannase

The pI of tannases has been documented in the range of 3.5–8.0. Tannase from *A. niger* GH1 had a pI value of 3.5 (Mata-Gomez et al. 2009) and for *A. niger* Aa20 tannase a pI value of 3.8 (Ramirez-Coronel et al. 2003). Isoelectric point of another tannase from *A. awamori* is 4.4 (Beena et al. 2010).

Kinetic Constants (K_m and V_{max})

K_m of tannase has been documented most preferably for methyl gallate and tannic acid substrates. However, as a matter of fact, a few researchers also documented K_m values for other substrates including glucose-1-gallate, propyl gallate and others. Bhardwaj et al. (2003) documented propyl gallate to have lowest affinity for tannase from *A. niger* with an apparent K_m value of 2.05 mM. For tannic acid, highest affinity for the same enzyme with a K_m value of 0.28 mM and V_{max}/K_m of 2.53.

Enzyme Inhibitors

Barthomeuf et al. (1994) documented inactivation of tannase from *A. niger* by 0-phenanthroline and phenyl methyl sulfonyl fluoride (PMSF). Kar et al. (2003) reported 1,10-O-phenathrolein as an inhibitor for *R. oryzae* tannase. Beena et al. (2010) observed that amongst the different inhibitors tested, PMSF showed highest enzyme inhibition (4.5% residual activity), followed by sodium deoxycholate (26.4%) and phenanthroline (61.04% R). Sharma et al. (2008) reported that N-ethylmaleimide exhibited strong inhibition of tannase activity while working with that of *Penicillium variabile*.

Metal Ions

The inhibitory effects of Fe^{3+}, Cu^{2+}, Zn^{2+} ions on tannase have been reported (Kar et al. 2003; Sabu et al. 2005; Kasieczka-Burnecka et al. 2007; Chhokar et al. 2010). Tannase from the strains of *A. niger* is strongly inhibited by Mg^{2+} and Mn^{2+}

(Bhardwaj et al. 2003). However in the separate case, Mg^{2+}, Mn^{2+}, Ca^{2+}, Na^{+}, K^{+} were documented to boost the activity of tannase from *A. awamori* MTCC 9299. Cu^{2+}, Fe^{3+} and Co^{2+} showed strong inhibition of tannase activity, whereas Zn^{2+} does not show any pronounced effect on tannase activity (Chhokar et al. 2010). Enzyme tannase was strongly inhibited by Fe^{3+}, whereas Cu^{2+} and Zn^{2+} showed only a mild inhibition, while Co^{2+} showed stimulatory effect on the activity of tannase from *A. niger* GH1 (Mata-Gomez et al. 2009). Goncalves et al. (2011) documented an spike in the activity of tannase with increase in Zn^{2+}, Hg^{2+}, Co^{2+}, Mg^{2+} (33–39%), and NH^{4+} (15%) and showed inhibition in presence of Fe^{3+}, Al^{3+}, and Ag^{+}.

Tannase Immobilization

Immobilization of enzymes involves physical confinement on inert/insoluble polymers, such as membranes or particles, which act as carriers/support of the enzyme during a continuous catalytic process. This permits recovery or removal of enzymes from a reaction mixture and their reusage (Sanderson and Coggon 1974) as well as use in non-aqueous environment, thereby improving the economy of the process. Retention of the enzyme is favored by coating alginate matrices with high or low molecular weight chitosan (Abdel-Nabey et al. 1999) or by cross-linking with glutaraldehyde, while storage under low temperatures favors stability of activity for longer periods. Sharma et al. (2008) documented Amberlite IR 1204 as the most suitable support for immobilizing *P. variabile* tannase with 69% immobilization credited to its reusability up to six times without any significant decline in the activity of enzyme. Yu et al. (2004) reported microencapsulation of tannase by chitosan-alginate complex coacervate membrane. Its kinetic properties revealed a slight shift in optimum pH toward alkaline from acidic and an increase in thermal stability.

Abdel-Nabey et al. (1999) documented another method of immobilization of tannase from *Aspergillus oryzae* on carriers like chitosan, chitin, Dowex 50 W, DEAE-Sephadex A-25. Maximum activity was observed on chitosan with bifunctional agent glutaraldehyde. This immobilized enzyme is widely used for the production of gallic acid. Mahendran et al. (2006) and Sharma et al. (2008) recorded that immobilized tannase is widely used for gallic acid production and for gallic acid esters (Yu et al. 2004). Hota et al. (2007) and Yu et al. (2004) reported a significant increase in K_m value of tannase upon immobilizing preferably with tannic acid as substrate in the fermentation process.

Applications of Tannase

Tannases have a vast range of applications in various industrial bioprocesses ranging from food, feed to chemical as well as pharma sector.

Gallic Acid (3,4,5-Trihydroxy Benzoic Acid) Production

The most significant application of tannase is the gallic acid production from hydrolysable tannins (Kar et al. 2002). Gallic acid is a versatile precursor for the manufacture of a variety of chemicals used in food and pharmaceutical industries. Gallic acid is utilized worldwide as a precursor for manufacturing of trimethoprim, a broad spectrum antibacterial agent which is bacteriostatic, since it inhibits folic acid metabolism in pathogenic bacteria (Mukherjee and Banerjee 2003). In combination with sulfonamide, trimethoprim exerts antibacterial effect at very low concentrations against Streptococci, Staphylococci, *Shigella*, *Corynebacterium diptheriae*, *E. coli*, *Vibrio cholerae*, Gonococci, *Bacillus pertussis*, and *Clostridium welchii*. Prasad et al. (2006) documented emergence of gallic acid as a highly valuable molecule credited to its worldwide use and demand as an antioxidant, antiviral, radio protective agent, anticancer, and antitumor agent. Choi et al. (2010) documented the potential of gallic acid as an antiviral agent.

Instant Tea Production

Boadi and Neufeld (2001) documented the utilization of tannase in instant tea production. The conventional process of instant tea preparation principally involves low-temperature treatment of hot water extract of tea with continuous agitation and centrifugation of tea cream thereafter. Tenco Brooke Bond Ltd. has solubilized tea cream using tannase. The product obtained at 5 °C was hazy and had an undissolved solid content of 13.5%, while an untreated sample at low temperature was visually opaque and had an undissolved solid content of 7.5%. British Patents GB-B-1, 413,351 and GB-B-1, 380,135 (Unilever) described the method of removal of tea cream and dissolution of the cold water-insoluble constituents of a hot water extract of tea by treating the tea with free or immobilized tannase.

Beer and Juices Clarification

Tannase is utlitized worldwide in juice clarification in order to remove the undesirable bitterness present in various fruits. Tannase also lowers the haze without deteriorating the quality of juices (Rout and Banerjee 2006).

Effluent Treatment

Tannery effluent wastes are ranked as high pollutants among all industrial wastes. Awareness on environmental issues among the world community especially in developing countries has reached a level which was not seen till now. Murugan and Al-Sohaibani Saleh (2010) documented eviction of tannin from tannery effluents by using biomass and enzyme from *A. candidus* MTCC 9628.

Future Prospectives

The industrial importance of tannase is well established. Amongst different hydrolases, tannase is gaining commercial importance due to its potential applications in food, feed, chemical, and pharmaceutical industries. Furthermore, the advancements in molecular tools and techniques have facilitated a better understanding of tannase structure, its induction, synthesis, regulation, and underlying mechanism of action. Due to surplus availability of tannin-rich agro-industrial residues and toxic tannery effluent waste, there is always an opportunity for researchers to search out novel tannases with improved activity and stability. The prospects of tannin hydrolysis by tannase from filamentous fungi are quite promising. Furthermore, tannase-based treatment of toxic tannery effluent offers a cheaper and much reliable way for bioremediation of toxic tannery effluent. In conclusion, tannase is an industrially important enzyme loaded with huge potentials for use in various bioprocessing applications. Over the years, tannase has witnessed a significant rise in its utilization in commercial sector in various industrial applications. Thus, further research related to increasing the tannin hydrolysis rate, tannin tolerance as well as to assure better process control for increased tannase production would be envisaged.

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Mycoremediation: An Alternative Treatment Strategy for Heavy Metal-Laden Wastewater

17

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Abstract

Industrial wastewater containing heavy metals constitutes a major source of contamination in the environment. Remediation of toxic metals from wastewater has been a challenge since long. Several physicochemical techniques are used to detoxify metal polluted sites. However, these traditional techniques are cost prohibitive due to use of chemical compounds, expensive and release of secondary toxic solid waste. Biosorption is a metabolism-independent and cost-effective method for removal of toxic metals from discharged liquid waste. Application of fungal biomass as biosorbent for toxic metal remediation has gained interest because of high surface to volume ratio, enough availability, rapid biosorption/desorption efficiency, and cost competitiveness. This chapter presents an overview of heavy metal biosorption studies performed on few potential fungal sorbents.

Keywords

Heavy metals · Bioremediation · Biosorption · Biosorbent · Fungi

Introduction

Toxic heavy metals are prevalent environmental contaminants of immense concern. Such pollutants are non-biodegradable and therefore persist and subsequently bioaccumulate in the food chain. Pollution caused due to metals is the major

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problem of the twenty-first century. Heavy metal pollution occurs directly by discharged effluent from industries and also by the pollutants that find entry into the water supply from various water systems (Vijayaraghavan and Yun 2008). In trace amounts, certain metals are considered essential for many organisms, but at elevated levels they are carcinogenic, toxic, mutagenic, and teratogenic (Munoz et al. 2006; Rehman and Anjum 2010). They cause a serious hazard to the environment which ultimately leads to ecological and health issues in humans, animals, and plants (Chisti 2004; Hogan 2010). Few highly toxic heavy metals such as mercury (Hg) are very toxic at very low concentration of 0.001–0.1 mg L^{-1} (Vieira and Volesky 2000; Bayramoglu and Arica 2008). Garg et al. (2012) have compiled the scattered information on various aspects of chromium bioremediation employing various conventional physicochemical, biological, and modern microbial strategies.

During last twenty years, environmental management has greatly aimed at the control of toxic metals leading to necessity of a cost-effective technology for decontaminating metal-containing industrial wastewater. Figure 17.1 illustrates various physicochemical and biological strategies for the remediation of heavy metals from the effluents. The conventional techniques to detoxify heavy metals from environment involve reduction of chemicals followed by ion exchange, precipitation, electrochemical, and adsorption on various adsorbents (Kadirvelu et al. 2001; Bishnoi and Garima 2005). These techniques are very costly due to their operational complexity and produce large amount of toxic secondary sludge which requires further remediation before final disposal (Das et al. 2008; Iram et al. 2015). Further, these techniques are dependent on the metals concentrations which are not effective in diluted solutions.

The microbial bioremediation has appeared as a promising method for the treatment of metal species from waste (Kulshreshtha et al. 2014). Bioremediation is a technique by which microorganisms are employed to transform toxic contaminants to environmentally safe levels in groundwater, soil, materials, and sediments. Microbes of metal-contaminated sites acclimatize to toxic metal concentrations and become resistant to metals (Congeevaram et al. 2007). Fungi have been proved to be very efficient in remediating toxic metals from industrial wastes (Park et al. 2005; Shazia et al. 2013). Among all microorganisms, fungal biomass, particularly species of *Aspergillus*, *Rhizopus*, *Phanerochaete*, *Streptoverticillium Saccharomyces*, etc., have high percentage of cell wall content, which reveals metal-binding characteristics through biosorption mechanism.

Heavy Metals

Heavy metal is a group of certain metals and metalloids with atomic density greater than 4000 kg m^{-1} or specific gravity more than water. They are natural components of the earth's crust. Some well-known toxic metallic elements are chromium, arsenic, cadmium, iron, lead, and mercury. The heavy metals can be classified based on their toxicity and physiological effects (Thakur 2006). At high

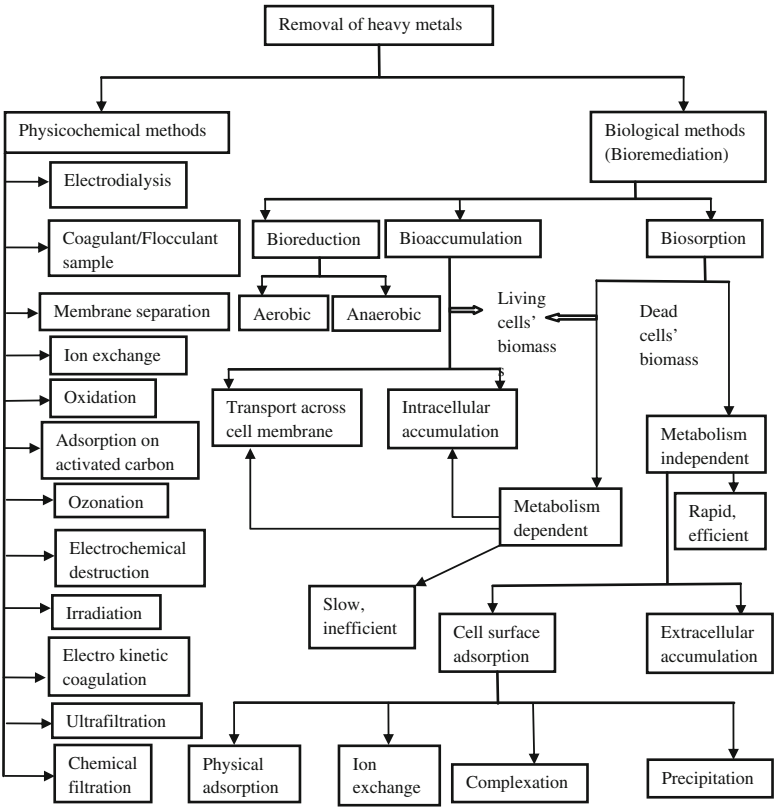

Fig. 17.1 Flowchart of various physicochemical and biological techniques for remediation of heavy metals from industrial effluents

concentrations, heavy metals cause various toxic effects, and they are categorized as average and low toxicity groups. Few metals that gained attention are Hg, Cd, Cr, Ni, Zn, As, and Pb, due to their extremely hazardous nature and impact on the environment as well as on the living organisms (Wang and Chen 2006; Barakat 2011).

Sources of Heavy Metal Pollution and Toxicity

The toxic heavy metals present in the earth's crust are naturally solubilized in water. Moreover, groundwater gets contaminated with heavy metals from various sources (Świątek and Krzywonos 2014). Several industrial processes such as manufacturing of metallic alloys, fabric dyes, paints, wood preservatives, fungicides, corrosion inhibitors, chrome plating, steel production, leather tanning, thermal power plants,

electroplating, wood preservation. employ various metallic compounds (Verma et al. 2004) and discharge their metal-laden waste into the sewerage drains, ponds, and open agricultural lands without any proper treatment (Maurya and Verma 2014). Heavy metals can undergo a number of transformations reactions, such as oxidation, reduction, sorption, precipitation, and dissolution in the soil or sediment. Also, the heavy metals available in the solid wastes of dumping sites are percolated to the subsoil and terrestrial water sources during the monsoon. Non-occupational contact to heavy metals occurs through ingestion of metal-containing edibles, however occupational exposure results mainly by skin absorption and inhalation. It is well established that there is a safe limit of every metal above which they are highly hazardous (Gadd 1992). Several metal ions are required in trace quantities, but at higher levels they are highly toxic for all forms of life. The toxicity appears due to metals' rapid permeability via biomembranes followed by binding with intracellular proteins and nucleic acids (Verma et al. 2009). In humans and animals, heavy metals produce several health disorders (Carmona et al. 2011). Metal toxicity can be classified into two major groups (i) blocking the necessary functional groups of biomolecules and (ii) replacing the essential metallic ions in biological molecules. Toxicity effects of toxicants greatly depend on their bioavailability that can be assimilated by the organism.

Microbial Metal Resistance

Certain heavy metals are essential micronutrients for all living organisms. At higher levels, metallic ions form non-specific complexes within the cell, which leads to hazardous effects. Therefore, the intracellular level of metal ions has to be strongly organized, and metal resistance is a general demand of every viable microbes for few metal homeostasis system in cells. Microbes have developed many types of resistance mechanisms to survive under metal-stressed condition. The suggested mechanisms of heavy metal resistance in microbes are (i) exclusion of metal, (ii) intracellular metal sequestration by protein binding, (iii) active transport of metal away from the microbial cell, (iv) extracellular accumulation, (v) enzymatic metal detoxification to innocuous form, and (vi) decrease in metal sensitivity of cellular targets (Benazir et al. 2010). Moreover, the largest group of metal resistance system works by active efflux (energy dependent) of toxic ions.

Microbial Remediation of Heavy Metals

Discovery of microorganisms capable of detoxifying toxic heavy metals to non-toxic or less toxic forms has important role in development of on-site treatment techniques. *Pseudomonas* strains were the first reported bacterial isolates obtained from chromate contaminated waste by the Russian scientists (Romanenko and

Korenkov 1977). The microorganisms employed in remediation may be autochthonous to a polluted site or from other sources and carried to a polluted site. When microbes are used to enhance bioremediation onto a contaminated site, the method is known as bioaugmentation (Vidali 2001). The bioremediation process is a cost-effective treatment strategy, minimizes the accumulation of secondary chemical and biological toxic sludge, highly efficient, environment friendly, and has a possibility of metal recovery. Among the several microorganisms, fungal biomass is effective because of more cell wall material with efficient metal-binding potential. Many fungi and yeasts, such as species of *Rhizopus, Aspergillus, Streptoverticillum,* and *Saccharomyces,* have excellent biosorption efficiency (Bishnoi and Garima 2005). The biotransformation of metals depends on the microbial cell metabolism and based on that they are categorized as metabolism dependent and metabolism independent (Fig. 17.2). Further, the bioremediation may be due to extracellular accumulation, cell surface sorption, intracellular accumulation, and enzymatic reduction. Lately, genetic engineering techniques resulted in alteration of morphological and physiological features of microbial cells that enhanced their bioremediation efficacy (Abbas et al. 2014; Garg et al. 2012).

The use of cheap waste biomass, less biosorbent immobilization cost, and biosorbent reusability are the main features for selecting bioremediation for treating toxic metals from wastewater (Chen et al. 2014). The factors affecting microbial metal bioremediation are pH, temperature, metal load, interaction time, dose of inoculums, presence of other metals, nutrients, and other kinetic factors, and therefore, these factors must be evaluated before selecting a particular

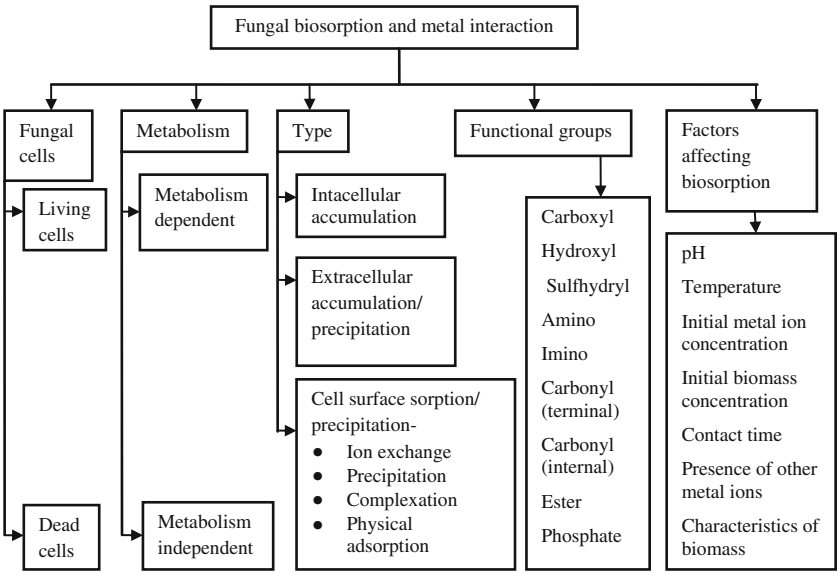

Fig. 17.2 Schematic representation of fungal biosorption and metal interaction process

bioremediation process. Such parameters control the efficiency of bioremediation techniques in environment that are contaminated with heavy metals (Abbas et al. 2014; Świątek and Krzywonos 2014). Microorganisms can interact with metallic ions in various ways. Bioreduction, biosorption, and bioaccumulation are the three major bioremediation mechanisms used to treat metal-contaminated wastes (Fig. 17.1).

Bioreduction

Microbes are efficient in changing the chemical state of hazardous heavy metals. Metals are actively transported across the biological membranes of the microbes. Once inside the microbial cells, the mechanisms by which microbes reduce toxic metals species to less toxic forms are variable and are species specific (Maurya and Verma 2014). Detoxification of toxic metal occurs in two steps; first, the reduction of toxic metal that occurs in microbial cells. Second, some species use the metallic ions as an ultimate electron acceptor in the respiratory chain, whereas in few strains some specific enzymes (soluble) are accountable for its conversion into non-toxic or less toxic form. Such types of biotransformation of metals reduce the level of metal toxicity on the viable organisms and also precipitate the metals nearly neutral pH for further removal, and therefore offer a promising biotreatment process.

Bioaccumulation

Living organisms absorb and retain several types of elements from their environment. Bioaccumulation allows binding of toxic metals intracellularly. Biological activity of bacterial cell is essential in metal bioaccumulation and its removal also requires cellular metabolic activity. Thus, bioaccumulation could occur only when cells are alive. During bioaccumulation, the metal permeates inside the microbial cells through the same route by which nutrients are supplied (Malik 2004).

Microbial heavy metal accumulation often occurs in two steps. The first step involves the physical adsorption of heavy metal ions or ion exchange at the cell surface. This process is passive and metabolism independent which is similar to the biosorption process. The second step involves active metabolism-dependent energy-driven transport into bacterial cells. The metal uptake rate is also affected by the physiological condition of cells and the medium components, and can also be influenced by the initial metal concentration (Chapman et al. 1996). Further, analysis revealed that specific uptake system does not exist for each metallic ion. Also, remobilization of the accumulated metal can be a significant problem during in situ bioremediation through bioaccumulation.

Biosorption

Remediation of toxic heavy metals by live microbes is problematic because metals exert toxic effect on the live cells. Contrary to that, the dead biomass of microorganisms is also able to bind metallic ions on the surface of their cell wall through various physicochemical interactions (Shumate and Strandberg 1985). This finding attracted considerable attention toward the biosorption strategy. Figure 17.2 depicts the overall process of fungal biosorption for heavy metal remediation. The biosorption mechanism has been reported by many researchers (Abbas et al. 2014; Iram et al. 2015). The chemical functional groups present on the fungal cell wall are negatively charged and abundantly available. They include carboxyl, phosphate, imidazole, sulfhydryl, sulfate, thioether, phenol, carbonyl, amine, and hydroxyl groups (Fig. 17.2) among which carboxyl groups are actively involved in the binding of metal cations (Abbas et al. 2014). Also, amines are very effective functional groups at remediating heavy metal ions, as they chelate cationic metal ions and also adsorb anionic metal ions through different interactions such as electrostatic and hydrogen bond. Kang et al. (2007) also reported the role of amine groups in chromate ions biosorption.

Biosorption is the most suitable method for heavy metal remediation as few biosorbents are capable of binding and concentrating metallic species from extremely dilute solutions (Abbas et al. 2014). The microbial materials can be effective biosorbents for metals remediation. The sorption has several advantages such as specific metals remediation over a wide pH and temperature range, its rapid kinetics of adsorption/desorption, low operational cost, no secondary sludge production, reusability due to biosorbent regeneration, and metal recovery (Gautam et al. 2014). Also, biosorbent is unaffected by other toxicants, heavy metal ions, and adverse unfavorable conditions that are normally present at metal-contaminated sites. Biosorbent can easily be produced employing low-cost growth media. It is advantageous to grow microbial sorbents that can remediate diverse metal ions. On the other hand, a mixture of dead biosorbent containing more than one type of microorganisms can be used as biosorbents (Tomko et al. 2006). The sorbate is adsorbed and interact to sorbent by several mechanisms (Świątek and Krzywonos 2014).

Various biomaterials such as dead cells of bacteria, molds, yeasts, and algae have been employed as effective metal-removing agents (Ahluwalia and Goyal 2007; Rahim et al. 2012). Among all, fungal biomass has emerged as an excellent biosorbent because of large cell size, high percentage of cell wall material, simple medium/nutrient requirements, high biomass yield, and easy genetic and morphological manipulations.

Bioremediation by Fungi (Mycoremediation)

Fungi are efficient and economical sorbents for sequestering toxic heavy metals by biosorption from polluted sites and even dilute aqueous solutions (Das et al. 2008). Filamentous molds and yeasts are mostly used in wastewater treatments to bind metallic elements. Fermentation process can be employed for their mass production; for example, *Aspergillus caespitosus* can be easily isolated from soils and grown in cheap medium to synthesize higher quantity of biosorbent; alternatively, such species are also produced as industrial waste products of many enzymes such as invertase, alkaline phosphatase, and xylanase production systems (Aftab et al. 2014). Besides, such fungi have high heavy metal resistance and metal-binding ability (Ahluwalia and Goyal 2010).

In general, fungal cell walls have a very high polysaccharide content (80–90%). Some other components such as inorganic ions, proteins, lipids, and polyphosphates are also present on fungal cell wall. Differences in cell wall composition can cause significant variation in the type and amount of metal ion-binding capacity. In filamentous fungi, the outer layers of cell wall mainly contain neutral polysaccharides (glucans and mannans), while the inner layers contain more of glucosamines (chitin and chitosan) in a microfibrillar structure. Ligands within these matrices contain carboxylate, amine phosphate, hydroxyl, sulfhydryl groups, etc. Different functional groups and proteins are associated with metal binding (Iskandar et al. 2011).

A wide variety of fungi have been employed for sorption of toxic metals (Table 17.1). These include *Lentinus edodes*, *Phanerochaete chrysosporium,* and *Neurospora crassa* for sorption of several toxic metals (Bayramoglu and Arıca 2008; Çeribasi and Yetis 2004; Kiran et al. 2005). The strong biosorbent behavior of microorganisms for metal ions is a function of their chemical makeup. Several researchers have employed various fungi for sorption of toxic heavy metals from aqueous solutions (Iskandar et al. 2011; Morley and Gadd 1995; Mullen et al. 1992). The techniques employing tools, such as electron microscopy with the energy-dispersive X-ray analysis and atomic force microscopy, have been often used in studying the fungal biosorption mechanism and also for the characterization of a microbial cell. Fungi can be used in filamentous, pelletized, or powder form. Fungal biomass separation from liquid phase is slightly difficult when fungal sorbent is employed in powdered forms (Järup 2003), whereas the above problem can be solved if fungal biomass is used in its pelletized form, as pellets can be easily separated from the medium broth (Gomez et al. 1988).

Biosorption by Live and Dead Fungi

The binding of heavy metal ions on the surface of fungal cell wall involves interaction between the metal and the functional groups on the surface of fungal biomass followed by inorganic deposition of metal ions to the extracellular

Table 17.1 Remediation of various metals by different fungal strains

Fungi	Heavy metals	References
Acremonium strictum	Cr	Ahluwalia and Goyal (2010)
Agaricus macrospores	Cd, Cu, Hg,	Garcia et al. (2005)
Amanita rubescens	Pb	Sarı and Tuzen (2009)
Aspergillus fumigatus	Cr, Pb	Iram et al. (2013)
Aspergillus flavus	Cr, Hg, Pb	Iram et al. (2013), Kurniati et al. (2014)
Aspergillus foetidus	Cr	Ahluwalia and Goyal (2010)
Aspergillus niger	Cd, Cu, Cr, Ni, Pb, Zn	Kapoor et al. (1999), Ahluwalia and Goyal (2007), Thakur and Srivastava (2011), Ali (2013), Iram et al. (2013), Sallau et al. (2014)
Aspergillus oryzae	Cr	Sepehr et al. (2005)
Aspergillus terreus	Fe	Dias et al. (2002)
Aspergillus terricola	Cr	Ahluwalia and Goyal (2010)
Aspergillus versicolor	Pb	Cabuk et al. (2005)
Aureobasidium pullulans	Cr	Ahluwalia and Goyal (2010)
Auricularia polytricha	Cd, Cu, Pb	Zhang et al. (2011)
Candida spp.	Cd, Cr, Cu, Fe, Hg, Ni, Pb, Zn	Anaemene (2012), Ali (2013)
Cladosporium resinae	Cr	Ahluwalia and Goyal (2010)
Claviceps paspali	Zn	Luef et al. (1991)
Cyberlindnera fabianii	Cr	Bahafid et al. (2013)
Ganoderma lucidum	Cu	Muraleedharan and Venkobachar (1990)
Hirsutella sp.	Cr	Srivastava and Thakur (2006)
Metarhizium anisopilae	Pb	Cabuk et al. (2005)
Mucor rouxii	Cd, Ni, Pb, Zn	Yan and Viraraghavan (2003)
Paecilomyces lilacinus	Cd, Cr	Sharma and Adholeya (2011), Zeng et al. (2013)
Paecilomyces sp.	Cr	Cárdenas-Gonz´alez and Acosta-Rodríguez (2010)

(continued)

Table 17.1 (continued)

Fungi	Heavy metals	References
Paecilomyces variotii	Cr	Ahluwalia and Goyal (2010)
Penicillium chrysogenum	Cd, Cr, Cu, Ni, Pb, Zn	Ahluwalia and Goyal (2007)
Penicillium citrinum	Cu	Verma and Singh (2013)
Penicillium lanosumcoeruleum	Cu, Ni, Pb	İlhan et al. (2004)
Penicillium sp.	Cr	Arévalo-Rangel et al. (2013)
Penicillium verrucosum	Pb	Cabuk et al. (2005)
Phanerochaete chrysosporium	Cd, Cr, Cu, Ni, Pb, Zn	Say et al. (2001), Gopal et al. (2002), Çeribasi and Yetis (2004), Li et al. (2004), Marandi et al. (2010), Murugavelh and Mohanty (2012), Xu et al. (2013)
Rhizopus arrhizus	Zn	Preetha and Viruthagiri (2005)
Rhizopus nigricans	Cd, Cr, Cu, Ni, Pb, Zn	Kogez and Pavko (2001), Ahluwalia and Goyal (2007)
Saccharomyces cerevisiae	Al, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Sr, Zn	Avery and Tobin (1992), Mapolelo and Torto (2004), Benazir et al. (2010), Kumar and Rao (2011), Gohari et al. (2013)
Trichosporon cutaneum	Cr	Bajgai et al. (2012)
Volvariella volvacea	Cd, Co, Cu, Pb	Purkayastha and Mitra (1992)

material. The biosorptive capacity of dead fungal cells biomass has been studied extensively as compared to living cells biomass. The main reasons for better biosorption efficiency by dead cells biomass include the high surface area associated with the dead cells, as adsorption of metals on fungal biomass is the physical adhesion of adsorbate onto the two-dimensional adsorbent due to interaction between them (Cabuk et al. 2005). There are reasons for using dead cells biomass which includes (i) the absence of metal toxicity on the dead biosorbent, (ii) cost-effective fungal dead biomass, (iii) reusability of dead biomass after regeneration, (iv) the biosorptive capacity of dead cell biomass is much higher because in order to prepare dead cells, the live cells are ruptured after heat treatment which yield cells with high number of attachment sites.

The greater biosorption efficiency of dead cells over viable cells biomass was observed with *Rhizopus arrhizus*, *Aspergillus niger,* and *Saccharomyces cerevisiae* for several toxic metals (Omar et al. 1997; Bayramoglu and Arıca 2008). The authors reported that dead cells of *S. cerevisiae* biosorbed more chromate compared to live cells. Difference in performance between live and dead cells is due to change

in pH which is an important factor affecting sorption process (Paknikar et al. 1999). Application of dead cells biomass at commercial scale offers benefits over viable cells in that it can be obtained as waste product from various industrial fermentation processes. The uptake of some heavy metal ions such as chromate is governed by an "acid adsorption" mechanism (Sharma and Forster 1993; Kratochvil et al. 1998). Other researchers also reported the effect of pH on biosorption process (Park et al. 2005; Srinath et al. 2002).

Biosorbent Modification for Enhancement in Biosorption Efficiency

Cell walls of microorganisms play a pivotal role in biosorption process. Obviously, modification of fungal cell wall can significantly influence the capacity of metal ion binding. The physical and chemical treatment methods may be employed for cell wall modification. Physical methods include heating/boiling, freezing, drying, and freeze drying. The heated *Chlorella* species had significantly higher ability to remediate manganese (5100 mg g^{-1}) as compared to living cells (Singh and Stapleton 2002) which was probably due to disruption of cell wall resulting in increasing number of additional binding sites for adsorption of manganese.

Fungal biomass can also be subjected to alkali or acid treatments. Ross and Townsely (1986) observed efficient sorption of copper by detergent-treated *Penicillium spinulosum* biomass. Similar findings were reported by Gadd et al. (1988) for the biomass of *S. cerevisiae* and *R. arrhizus*. Contrary to that, acid treatment of biomass caused a decrease in metals adsorption. This was attributed to relatively more competition between protons and dissolved metals (Chojnacka 2010; Sugasini et al. 2014).

Factors Affecting Metal Biosorption

Many environmental parameters such as hydrogen ion concentration, temperature, interaction time, presence of other ions, and load of metals and biosorbent concentration also influence the biosorption of heavy metal ions (Fig. 17.2). Several workers have studied the mycoremediation of various toxic heavy metals through biosorption under different cultural and environmental conditions (Table 17.2).

Medium pH

The pH of the metal-containing solution is the most important factor for metal speciation on biomass (Paknikar et al. 1999). Determination of the optimum pH value is very important for maximum metal sorption efficiency. Several researchers reported that the dead cells biomass of *Penicillium sanguineus* absorbed significant

Table 17.2 Biosorption of heavy metals using fungal biosorbents under specific operating conditions

Biomass	Heavy metals	pH	Temperature (°C)	C_0 (mg L^{-1})	W_t (g L^{-1})	N_s (rpm)	Time (h)	Q_e (mg g^{-1})	References
Trichoderma longibrachiatum	Pb	7.0	25	100 (25–100)	–	–	0.33	71.0	Osman and Bandyopadhyay (1999)
Trichoderma viride	Cr	6		175	3.75	150	0.75	4.66	Eneida et al. (2002)
Rhizopus delemar	Cu Co Fe	4.5	30	5 mM 4 mM 4 mM	30	220	1	34.3 38.9 23.1	Tsekova and Petrov (2002)
Pleurotus ostreatus	Pb	5.5	25	1–25	2	–	3	4.84	Barros et al. (2003)
Hydrilla verticillata	Cd	5.0	25	1–100	3–9	150	0.33	15	Ismael et al. (2003)
Candida spp.	Hg Zn Cd Pb Fe Cu Cr	5	28	0.008 0.091 0.006 0.003 0.075 0.82 0.027	0.2	150	2	2.74 31.16 2.05 1.03 25.68 28.08 9.28	Mapolelo and Torto (2004)
Pleurotus ostreatus	Cr	4.5	25	14.35 4–20	2	150	3	1.97	Park et al. (2005)
Aspergillus niger	Cr	2	22–28	50 25–200	10	200	158	2.2	Kujan et al. (2006)
Aspergillus flavus I–V, *Aspergillus fumigatus* I–II	Hg	5.5	30	100 100–500	10	100	8	95.3%	Murugesan et al. (2006)
Agaricus campestris	Al Sb Cu	5.2 2.8 5.5	20	10 7.5 15	2	–	10 min 10 min 10 min	90% 95% 78%	Tomko et al. (2006)

(continued)

Table 17.2 (continued)

Biomass	Heavy metals	pH	Temperature (°C)	C_0 (mg L^{-1})	W_t (g L^{-1})	N_s (rpm)	Time (h)	Q_e (mg g^{-1})	References
Amanita muscaria	Al Sb Cu	5.2 2.8 5.5	20	10 7.5 15	2	–	10 min 10 min 10 min	80% 70% 85%	Tomko et al. (2006)
Trametes gibbosa	Al Sb Cu	5.2 2.8 5.5	20	10 7.5 15	2	–	10 min 10 min 10 min	90% 90% 90%	Tomko et al. (2006)
Aspergillus parasiticus	Pb	5.0	20	–	–	–	70 min	4.02×10^{-4} mol g^{-1}	Akar et al. (2007)
R. arrhizus	Cr	2.0	–	100	–	–	72	78	Aksu and Balibek (2007)
Aspergillus sp.	Cr Ni	5.0	30–35	100 50	–	–	48	92% 90%	Congeevaram et al. (2007)
Aspergillus niger	Cr	4.5	28	300	10	150	1	16.39	Javaid and Bajwa (2007)
P. chrysogenum	As	3–4	25	1–300	1	190		24.52	Mamisahebei et al. (2007)
Aspergillus niger	Cd	4.75	25	5–10	0.7	125	6	13	Sushera et al. (2007)
Lentinus edodes (live biomass)	Hg Cd Zn	6.0	15–45	–	–	–	–	336.3 ± 3.7, 78.6 ± 2.6 33.7 ± 1.6	Bayramoglu and Arica (2008)
Lentinus edodes (heat inactivated)	Hg Cd Zn	6.0	15–45	–	–	–	–	403.0 ± 2.9, 274.3 ± 3.6 57.7 ± 1.1 mg/g	Bayramoglu and Arica (2008)
Mucor sp.	Cr	5.5	35	50 50–400	0.0165	–	–	–	El-Kassas and El-Taher (2009)
Penicillium canescens	Cr	6	20	100 10–750	2	100	4	34.8	Chhikara et al. (2010)

(continued)

Table 17.2 (continued)

Biomass	Heavy metals	pH	Temperature (°C)	C_0 (mg L^{-1})	W_t (g L^{-1})	N_s (rpm)	Time (h)	Q_e (mg g^{-1})	References
Aspergillus niger	Cd Zn	7	30	–	0.1	–	48	50% 58%	Kumar et al. (2010)
Phanerochaete chrysosporium	Cr Cu	27	100 20–500	–	–	100	2	48.6 90.6	Sethi et al. (2010)
A. lentulus	Cu	6	35	100	4	180	0.41	–	Sutherland and Venkobachar (2010)
Fusarium solani	Cr	4.0	30	500	4.5	150	24	60	Sen and Dastidar 2011
Saccharomyces cerevisiae	Hg Cu Ni Zn Cd Co Cr Fe Al	7	25	25–200	2	100	2	76.2 29.9 14.1 11.8 12.3 8.2 6.2 5 4.1	Anaemene (2012)
Aspergillus flavus	Pb Ni Cr	–	27	50 ppm	0.22 0.32	150	4 days	0.70 0.63 0.66	Dwivedi et al. (2012)
Aspergillus niger	Pb Ni Cr	–	27	50 ppm	0.46 0.36	150	4 days	0.67 0.64 0.74	Dwivedi et al. (2012)
Pleurotus ostreatus	Cu	5	25	50	2	125	0.16	4.0	Jha et al. (2012)
Aspergillus cristatus	Cd	6.0	25	100	0.4	120	2	23.26	Martínez-Juárez et al. (2012)

(continued)

Table 17.2 (continued)

Biomass	Heavy metals	pH	Temperature (°C)	C_0 (mg L^{-1})	W_t (g L^{-1})	N_s (rpm)	Time (h)	Q_e (mg g^{-1})	References
Rhizopus nigricans	Pb	5.5	25	300 (10–300)	25 25–200	225	–	80.8	Hassan and El-Kassas (2012)
Aspergillus niger	Ni	4.5	25	25–100	1	150	3	7.69	Tay et al. (2012)
A. oryzae *A. niger* *Fusarium oxysporum* *Polyporus squamosus*	Cu	6.24	30	50 25 50 50	13.33 8.88 11.23 11.80	150	8 days	3.31 2.35 3.90 3.59	Simonesco and Ferdes (2012)
Aspergillus flavus	Zn	5.0	30	600	–	–	–	287.8 ± 11.1	Aftab et al. (2013)
Aspergillus fumigatus	Pb Cd Cr Cu Ni Zn	5	30	200–800 ppm	10	150	4	76.07 40.0 69.6 25 20	Shazia et al. (2013)
A. niger *A. terreus* *A. tamari* *A. flavus*	Cr	7	30	1–3 mM	1	125	18	17.3 42.65 21.8 19.01	Sugasini et al. (2014)

where
C_0 is initial concentration of heavy metal in solution (mg L^{-1})
W_t is the weight of used adsorbent (g L^{-1})
Q_e is the uptake removal of pollutant (mg g^{-1})
N_s is the agitation speed of shaker (rpm), and T is the temperature of the experiment (°C)

Pb, Cd, and Cu at optimal pH of 0.5–0.7 (Dursun 2006; Simonesco and Ferdes 2012).

The pH 4.0–8.0 is optimal range for biosorption of cationic species on surface of microbes (Tobin et al. 1984; Volesky and Holan 1995; Kratochvil et al. 1998). However, chromate ions are adsorbed more favorably at acidic pH (Srinath et al. 2002; Tripathi et al. 2011). The chemical functional groups present on microbial cell walls have high affinity for metallic species (Collins and Stotzky 1992). Garg et al. (2012) reported that the surfaces of adsorbents become highly protonated that favors the uptake of chromate anions under acidic conditions. Parvathi et al. (2007) reported neutral pH for the highest chromate biosorption.

Temperature

The process of metal biosorption by dead cells' biomass is non-enzymatic and passive process. Since the biosorption process responsible for metal remediation is mainly physicochemical, it is less likely to be influenced by temperature (Gulay et al. 2003). Several researchers studied the effect of temperature on biosorption efficiency (Marques et al. 1991; Mashitah et al. 2008; Sag and Kustal 1996).

Contact Time

Biosorption is also affected by interaction time between biosorbent and the metal present in aqueous solution. At the very beginning, biosorption proceeds fast, and most metals are biosorbed. But for achieving equilibrium, knowing the interaction time between biosorbent and metal is important to ascertain the biosorption behavior. It is very important for cost-effective commercial exploitation. If equilibrium is optimally achieved within less time period, biosorption occurs rapidly. Many researchers evaluated the effect of contact time on biosorption of heavy metals (Merrin et al. 1998; Prakasham et al. 1999; Srinath et al. 2003). The transfer of metal from solution to attachment sites of the biosorbent involves many steps (Gadd et al. 1988; Weber 1985). Because of mixing and smooth flow of metal to the binding sites of the biosorbent, the first step is rapid, second step is diffusion of the metal on surface of biomass, and third step is real adsorption of the metal ions on the biosorbent (Weber 1985).

Presence of Other Metal Ions

Wastewaters are polluted with various types of pollutants including toxic heavy metals which have an effect on biosorption kinetics. Natural habitats generally coexist with several toxic and non-toxic cationic and anionic species (Verma et al. 2008). Therefore, it is important to evaluate the effect of other metals available in waste on the microbes' growth while studying the bioremediation processes

(Verma et al. 2001). Depending on the degree of competition between metallic ions for binding sites on surface of the biomass and other metal toxicity, the presence of such cationic or anionic species can complicate the biosorption process (Volesky and Holan 1995). Sag et al. (1998) reported that the biosorption of Cr^{6+} and Fe^{3+} was antagonistic for the cells of *R. arrhizus*. Other researchers also reported the effect of presence of other metal ions on biosorption process (Gadd et al. 1988; Antuner et al. 2001).

Initial Metal Ion Concentration

The initial metal concentration is an important factor to evaluate the rate of absorption by dead cells biomass. Metal adsorbed by the biomass is increased with increasing initial concentration of metal. However, at higher metal concentration, the availability of metal-binding sites at the biomass cell surface is limited, which ultimately leads to decreased metal adsorption (Javaid and Bajwa 2008). The initial ratio of the metal ions to biosorbent level plays an important role during optimization of biosorption process (Paknikar et al. 1999; Puranik and Paknikar 1999; Tripathi et al. 2011).

Initial Biomass Concentration

The initial biosorbent concentration is a key factor in ascertaining the efficiency of biosorption process. Srinath et al. (2003) observed a decline in Cr^{6+} uptake, with an increase in biosorbent concentration (from 2 to 10 g L^{-1}). They reported a curvilinear adsorption isotherm for Cr^{6+} biosorption, which revealed that Cr^{6+} uptake was chemically equilibrated in a pattern that involved a saturable process. Thus, there was an increase in uptake of metal so long as the binding sites were freely available. Other researchers also study the role of biosorbent concentration in biosorption of heavy metals (Fourest and Roux 1992; Puranik and Paknikar 1999). Some other researchers reported that cell-to-cell distance increases at lower biomass dose that favors the metal adsorption by increasing the surface area (Mashitah et al. 2008; Shakya et al. 2015).

Characteristics of Biomass

The type of the biomass is one of the important factors, such as freely suspended cells, immobilized preparations, living biofilms, that may influence the biosorption process. There are other factors such as age of cells and the overall quality of biomass which can also influence the biosorption process. Depending on the type of organisms used for biosorption, the older cultures can exhibit better efficiency for removal of metals (Świątek and Krzywonos 2014). Physical treatment methods (boiling, drying, autoclaving, mechanical disruption, etc.) also influence the binding

properties. The alkali treatment often improves biosorption ability due to acetylation of chitin which forms chitosan-glycan complexes with higher metal affinities, especially in some fungal systems (Avery and Tobin 1993).

Immobilization of Fungal Biomass

The important aspect of successful bioremediation is recovery of metals from the biomass. The biomass remains loaded with metal ions during bioremediation. Therefore, heavy metals will remain in the environment only without the recovery step. Moreover, regenerated biomass as well as process can be more economically viable if biomass is reused for subsequent biosorption cycles. However, if biomass is to be regenerated and reused, there must be cost-effective viable recovery process for it. To achieve the specific goal of microbial remediation and recovery of metal, one alternative is to immobilize the biomass on a suitable inert and insoluble support material by attaching or entrapment of microbial cells onto it (matrix). Immobilization imparts mechanical strength and stability to the microbial biomass. Several researchers used immobilized fungal biomass employing different support materials such as agar, alginates, cellulose, glycol, cross-linked ethyl acrylate-ethylene dimethylacrylate, silica gel, and polyacrylamide for removal of toxic heavy metals (Chen et al. 2014; Jha et al. 2012; Poopal and Laxman 2008; Tsekova et al. 2010; Verma and Singh 2013; Xu et al. 2013).

Furthermore, there may be problems when free-cell biomass is used in commercial processes, which are associated with the physical characteristics of the cells, such as small size, low density, poor mechanical strength/rigidity, and solid–liquid separation. These problems can be overcome by immobilizing microbial cells on suitable natural or synthetic polymer matrices. Therefore, selection of the appropriate immobilization support is a key factor in building an optimal immobilized biocatalyst (Poopal and Laxman 2008). Further, by using whole-cell immobilization, improvements over free cells are achieved in increased stability, ease of biomass regeneration, easier solid–liquid separations, and minimum clogging in continuous systems (Tsekova et al. 2010; Jha et al. 2012).

Recovery of Heavy Metals and Biomass Regeneration

Another aspect for the remediation of heavy metals is the possibility of metal recovery via desorption. Biomass is regenerated and reused for another cycle of biosorption that enhances metal bioremediation at industrial level. The adsorption and desorption steps concentrate the pollutants in less volume which can be recovered in cost-effective mode. Research on biosorbent regeneration is still important task in biosorption studies (Gautam et al. 2014). Several researchers

employed fungal biomass for biosorption of chromium (Akthar and Mohan 1995; Saifuddin and Raziah 2007).

Conclusion

Bioremediation is a powerful cleanup technology for heavy-metal-contaminated sites. Among the various bioremediation processes, biosorption is the most promising one for remediation of heavy metals from polluted sites. Interestingly, the biosorption could be mediated by both living and dead biomass. Fungi are highly preferred biosorbents for toxic heavy metals sequestration as they can be grown easily, produce enormous quantity of biomass, bear large surface area for metal interaction, more metal tolerant, permit easy metal separation and simple genetic and morphological manipulations. Biosorption has many advantageous features including separation of metals over wide range of pH/temperature and rapid kinetics of adsorption and desorption processes. The biosorption process is influenced by several parameters which have been addressed by several researchers. Regeneration of fungal biosorbent increases the possibility of metal bioremediation at industrial level because biomass can be employed several times for biosorption process.

Future Prospects

Industrialization and technological developments may sometimes have adverse effects on the environment. Microbial activities play a pivotal function in renewal of environment. Toxic metals, synthetic compounds, and chemicals can be transformed/degraded by a variety of microorganisms. However, bioremediation process has limitations pertaining to heavy metals; microbes are not only unable to break toxic metals into innocuous form, but they exert inhibitory effects on microbial cells. Molecular biology studies in future might enhance the biodegradative capabilities of microorganisms. The need for tailoring, via genetic manipulation, microbial strains capable of remediating wide variety of pollutants on multiple heavy-metal-contaminated sites under all the possible adverse/stress conditions would be another possible area of research. Therefore, the use of genetic engineering techniques for bioremediation of heavy metals has also gained great interest. Genetically engineered microbes (with higher enzymatic abilities) degrade/alter any environmental pollutants into innocuous form. Chemical alteration of biosorbent surfaces has been employed effectively in few cases to enhance the adsorption capacity and removal efficiency of fungal strains. Biosensors may be easy, rapid, and cost-effective tools for metal detection compared to conventional techniques. Many fungal species are useful biosensors for environmental metal pollution. The future studies are focused on sensitivity, accuracy, stability, and storability of these biosensors.

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Treatment of Landfill Leachate Using Fungi: An Efficient and Cost-Effective Strategy

18

Pooja Ghosh and Indu Shekhar Thakur

Abstract

Landfill leachate contains hazardous xenobiotic organic compounds and heavy metals which have emerged as a major threat to receiving waterbodies and human health even at trace levels. In the absence of proper treatment and safe disposal, leachate is a source of water contamination. Commonly, various types of physicochemical treatment methods are used to treat leachate. However, they have the limitations of being expensive and less versatile compared to the biological treatment methods. Fungi have considerable application in wastewater treatment due to production of powerful oxidative and non-specific extracellular enzymes, peroxidases, and laccases. This article reviews the application of fungi in leachate treatment as a sustainable technology and discusses the mechanism involved in degradation and detoxification.

Keywords

Landfill leachate · Fungi · Organic contaminants · Heavy metals
Biodegradation · Biosorption · Detoxification

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Introduction

Rapid urbanization and population growth has resulted in pressing environmental concerns. Thus, municipal solid waste (MSW) management is of paramount importance in modern urban settlements. MSW includes household waste, industrial and commercial non-hazardous solid waste, market waste, yard waste, and street filth collected by municipal authorities (Jain 2007). The situation has worsened due to inappropriate selection of technology and public indifference toward MSW. Unrestrained dumping of waste near urban habitats has generated severe public health nuances. Land disposal is the most common method adopted for municipal solid waste management. Engineered landfills are constructed and maintained in developed countries (DOE/EIA 1999). But in emerging economies like India, engineered landfills are rarely found. The waste is disposed off in open dumps that results in severe environmental degradation and vital loss of natural resources. After being dumped, the waste undergoes decomposition through combined physicochemical and biological processes and generates highly contaminated wastewater called leachate which contaminates the environment (Ghosh et al. 2014; Mor et al. 2006).

Conventionally, landfill leachate is treated using various physicochemical and biological methods. Physicochemical treatments are typically used to reduce colloidal particles, suspended solids, color, and some toxic compounds. However, the major disadvantage of these treatment methods is the high cost. Therefore, biological treatment methods are gaining more popularity as they have the benefit of being cost-effective and sustainable. Among the various microbial treatment methods used, bacteria are the most commonly used. Lately, fungi, due to their high tolerance to toxicity, are being considered an excellent option for treating leachate.

Scenario of Landfilling in India

India stands second in terms of economic growth along with being the second most populated country in the world. Its urbanization has increased from 17.6 to 28% in the past 50 years and is expected to reach 38 by 2026 (Talyan et al. 2008). Urbanization has led to increased waste generation and unscientific management of the waste to deterioration of environment and effects on human health. This is a critical issue in budding economies like India. In 1996, globally, urban population had generated about 114,576 tonnes day^{-1} of MSW, which is predicted to reach 440,460 tonnes day^{-1} by 2026 (Hoornweg and Laura 1999). Changing lifestyles, food habits, and living standards would lead to such exponential increase. In addition to that, paucity of sufficient financial investments, infrastructural constraints, lack of technically skilled personnel, lack of available land for safe waste disposal, little public awareness and motivation inhibit progress in efficient, and safe management of urban solid waste (Asnani 2004).

Landfills can be classified into three categories—sanitary or engineered landfills, semi-controlled landfills, and crude or non-engineered landfills. Developed countries have sanitary landfills for MSW and engineered facilities for disposing wastes by spreading them on land in thin layers and squeezing them to smallest volume possible. The lowest component of these landfills comprises of drains with impermeable barriers to minimize leachate migration to groundwater so as to curtail any hazardous impact on public health and environment (Tchobanoglous et al. 1993). The principal components of an engineered landfill are the containment liner, liner protection layer, leachate drainage layer, and top cover. At semi-controlled landfill sites, dumped refuse is compressed and a topsoil cover is provided everyday to avert nuisances. In these landfills, all types of wastes (municipal, industrial, hospital) are dumped without segregation. These landfills are unengineered and are unable to control the leachate drainage into groundwater or emissions of landfill gases into the atmosphere. In developing countries such as India, 90% of MSW is disposed in crude landfills which lack base liners, leachate treatment ponds, and gas ventilation system. At those sites, the refuse is dumped indiscriminately on open land, posing environmental hazards, thereby causing ecological imbalances (Narayana 2009). These refuse are subjected to physical, chemical, and microbial processes leading to transferring of the pollutants from the waste to the water passing through the waste layers leading to the formation of highly contagious wastewater called leachate (Christensen and Kjeldsen 1989). Therefore, absence of containment liner and collection system for leachate leads to contamination of soil, ground, and surface water.

Only nine cities (Pune, Ahmedabad, Surat, Jodhpur, Chandigarh, Navi Mumbai, Mangalore, Nashik, and Delhi) have sanitary landfills in India. For most of the landfill sites in India, no proper environmental impact assessment (EIA) has been done. They have not been designed systematically before use and therefore are a threat to the environment; even after closure as they continue to produce leachate which contaminates the ground and surface water.

Leachate: Why Is It a Cause of Concern?

Leachate composition not only varies within a landfill but also among the landfills depending on various parameters such as waste composition, degradation stage of the waste, and landfill technology (Kjeldsen et al. 2002). However, most leachates have the similar basal composition consisting of four groups of contaminants: dissolved organic matter expressed as chemical oxygen demand (COD) or total organic C (TOC) including CH_4, volatile fatty acids, and more refractory compounds like fulvic-like and humic-like compounds; inorganic macro components like Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, NH_4^{+}, Cl^{-}, SO_4^{2-}, HCO_3^{-}; heavy metals and xenobiotic organic compounds (XOCs) (Christensen et al. 2001). Reports indicate that even trace amounts of leachate could contaminate groundwater, making it unfit for usage (Bakare et al. 2000).

In recent decades, there has been a drastic change in the kind of waste dumped in the landfills. Dumping of consumer products like batteries, paints, electrical appliances as well as pharmaceuticals has increased with time. They are known to contain additives that have a negative impact on environmental and human health. Majority of the earlier reports on landfill leachate have only evaluated physico-chemical parameters like organic matter, COD, BOD, and metal ions (Eggen et al. 2010). However, a large number of emerging contaminants (ECs) can be anticipated to be present in the waste disposed and consequently in the leachate produced. These ECs, though are present in trace amounts in leachate (<1 mg L^{-1}), may either individually or synergistically lead to health issues in humans and animals ingesting the contaminated water (Andrews et al. 2012). Baderna et al. (2011) carried out an extensive monitoring campaign in landfill of northern Italy over a period of 11 years combined with toxicity assays. They reported that bisphenol A, benzyl alcohol and its derivatives, hexanoic acid, and benzothiazolone were the most frequently detected compounds in landfill leachate. Results from their study suggested toxic effects on fresh water fish and small rodents, mainly due to ammonia and inorganics. Öman and Junestedt (2008) studied samples from 12 MSW sites of Sweden and evaluated 400 different parameters and compounds in the landfill leachates. Greater than 90 organic (halogenated aliphatic compounds, benzene and its derivatives, phenols and its derivatives, ethoxylates, PAHs, phthalic esters, PCBs, chlorinated dioxins and chlorinated furans, bromated flame-retardants, pesticides) and metal organic compounds (organic tin, methyl mercury) and 50 inorganic elements were detected in the samples. Eggen et al. (2010) also showed the prevalence of various new and emerging contaminants in leachate, many of which were persistent compounds used as additives in products of daily use. With increasing concern on chemicals in household products, the potential effects to the environment from the disposal of MSW are becoming important. It is therefore essential to determine the level of risk posed by disposal of MSW and the leachate generated.

Hazardous XOCs and inorganics present in the leachate can be carcinogenic, estrogenic, teratogenic, and mutagenic along with being bioaccumulative and/or persistent (Cozzarelli et al. 2011; Matejczyk et al. 2011). The properties of these compounds can be amplified by bioaccumulation in the food chain. Gajski et al. (2012) assessed the genotoxicity of landfill leachate in Croatia and reported increased frequency of micronuclei, nuclear buds, and nucleoplasmic bridges in human lymphocytes. The genotoxicity of landfill leachate has also been shown using microbial bioassays, namely the *Bacillus subtilis* DNA repair bioassay, and the diploid *Aspergillus nidulans* chromosome damage bioassay (Schrab et al. 1993). Srivastava et al. (2005) reported leachate toxicity using subacute toxicity assay of *Allium cepa*, reporting a reduced bulb weight in *A. cepa* exposed to leachate. The cytotoxic and genotoxic effects caused by landfill leachate mean that humans consuming water contaminated by leachate are at higher risk of developing adverse health effects (Ghosh et al. 2015). Consequently, monitoring leachate toxicity is of utmost importance.

Leachate Treatment Options

An integral part of MSW management is treatment of landfill leachate. Sadly, MSW in India is mostly disposed off in open dumps and most dumping sites do not even have a leachate collection as well as treatment system. Luckily even if present, majority of the treatment processes are not properly deliberated based on the variable characteristics of the leachate generated in the landfill site. As, leachate varies widely both in quantity and in composition from one place to another, so the treatment process should be designed specific to the leachate characteristics on-site. Various leachate treatment methods are described in detail below to provide an insight into their advantages and disadvantages.

Physicochemical Methods

It includes methods such as coagulation-flocculation, adsorption, air stripping, membrane filtration, chemical precipitation, and chemical oxidation. These mainly reduce the concentration of suspended solids, colloidal particles, floating material, color, and toxic compounds.

Coagulation-Flocculation

These are comparatively simple techniques, but lead to moderate removal of COD (Rivas et al. 2004). In addition, this method has some disadvantages such as production of sludge and an increased concentration of aluminum or iron, in the liquid phase (Silva et al. 2004).

Chemical Precipitation

This is commonly a pretreatment step for removing high concentrations of ammonium nitrogen from leachate. Li et al. (1999) used this method for precipitating ammonium ions into magnesium ammonium phosphate (MAP) by adding $MgCl_2{\cdot}6H_2O$ and $Na_2HPO_4{\cdot}12H_2O$ and successfully reduced the concentration of ammonium ions by 50-folds.

Adsorption

The adsorption of leachate contaminants onto activated carbon in columns (Foo and Hameed 2009) or in powder form (Zamora et al. 2000) leads to higher COD reduction than the chemical methods. However, the major shortcoming of adsorption is the frequent need of regenerating the columns along with high utilization of powdered activated carbon (PAC). Park et al. (2001) effectively used activated carbon along with biological treatment for landfill leachate treatment. Rodríguez et al. (2004) studied the efficiency of PAC and other resins for reduction of COD from landfill leachate. Activated carbon was found to be the most efficient adsorbent having a capacity of reducing 85% COD.

Chemical Oxidation

Advanced oxidation processes (AOP) have recently been gaining interests for treating leachate. It uses a combination of strong oxidants such as ozone, chlorine, and hydrogen peroxide, along with ultraviolet (UV), ultrasound (US), electron beam (EB), or photo-catalysis irradiation for increasing the degradation of pollutants (Renoua et al. 2008). However, the major drawback of this method is formation of chlorine or hypochlorite, leading to poor economic suitability for large-scale applications (Murray and Parsons 2004).

Air Stripping

It is one of the commonly used methods for removing high concentrations of NH_4^+–N present in leachate. Marttinen et al. (2002) reported 89% reduction in ammonia at pH 11 and 20 °C within 24 h using air stripping. A 99.5% reduction in ammonia has also been reported by Silva et al. (2004). However, a major concern with this method is the discharge of ammonia into the atmosphere. Other disadvantage includes problem of foaming and $CaCO_3$ scaling of the stripping tower (Li et al. 1999).

Membrane Filtration

Microfiltration, ultrafiltration, and nanofiltration are the membrane processes applied for leachate treatment (Trebouet et al. 2001; Wiszniowski et al. 2006). High-efficiency removal of both organic and inorganic contaminants can be achieved by modern membranes. However, one of the shortcomings of this method is fouling or biofouling of the membrane. This results in short lifetime and decreased permeate flux reducing the economic efficiency of the method (Schlichter et al. 2003).

Limitations of Conventional Physicochemical Methods

The disadvantages associated with the conventional physicochemical methods (high cost and generation of sludge) are big issues (Kapoor and Viraraghavan 1995). Also, the sludge that is generated needs to be landfilled. Sometimes, the sludge is burnt to reduce the cost of disposal, which leads to polluting the environment with huge quantities of toxic volatile organic compounds (VOCs) like dioxins, furfurals, and others. There is an urgent need to improve methods not only to reduce the cost of treatment residues but also to fulfill the ecological and economical requirements.

Biological Treatment Methods

Microbes have the unique ability to convert natural as well as synthetic chemicals into energy and raw materials for their own growth. Also, biological processes have the advantage of being more cost-effective and environmentally friendly to

compared physical remediation processes. Therefore, microorganisms are an untapped resource for bioremediation (Kumar et al. 2011). Biological treatment methods can be categorized into aerobic and anaerobic based on the presence or absence of oxygen. Aerobic treatment includes activated sludge treatment, aerated lagoons, and aerobic biological reactors. Anaerobic ones used to treat landfill leachate includes anaerobic filter, upflow sludge blanket (UASB), fluidized bed, anaerobic lagoon and, anaerobic contact reactors (Stegmann et al. 2005). All these treatments make use of microbes. A number of bacteria, fungi, and algae have been reported to have leachate treatment capabilities.

Use of Fungi

Using white rot fungi (WRF) and their enzymes for detoxification of wastewaters and a wide range of xenobiotic contaminants shows potential by offering several advantages and compensating for the shortcomings of the other processes (Kaushik and Malik 2009). In particular, WRF have shown the ability to not only degrade contaminants but also to remain active even under adverse conditions. Both these characteristics delineate their potential for application in wastewater treatment (Tigini et al. 2013). Unlike bacteria, the filamentous growth of basidiomycetes via mycelia represents a significant advantage through patches limited in nutrients (Baldrian 2008). Also, they are known to produce extracellular oxidative enzymes which are non-specific and can act on an array of contaminants structurally similar to lignin, including organic compounds like PAHs, pesticides, polychlorinated biphenyls (PCBs), and a range of other compounds (Baldrian et al. 2000; Bending et al. 2002; Takagia et al. 2007; Wesemberg et al. 2003). The extracellular enzymes, laccase (Lac), lignin peroxidise (LiP), and manganese peroxidase (MnP) are involved in degradation of lignin and related substrates (Wesemberg et al. 2003). WRF have been applied for the treatment of textile wastewater (Abadulla et al. 2000) and industrial dye degradation (Anastasi et al. 2010) due to their excellent ability of decolorization and detoxification of dyes. The results of application of fungi for leachate treatment are presented in Table 18.1 and briefly discussed in the paragraphs below.

Ellouze et al. (2008) have reported successful treatment of a young landfill leachate from Tunisia by fungal strains of *Trametes trogii*, *Phanerochaete chrysosporium*, *Lentinus tigrinus*, and *Aspergillus niger*. They reported that *P. chrysosporium*, *T. trogii*, and *L. tigrinus* were of able to remove COD by 68, 79, and 90%, respectively, after the leachate was diluted by twofolds. Reduction in COD was found to be along with enzyme secretion and toxicity reduction. In a subsequent study, Ellouze et al. (2009) studied the detoxification ability of two strains of white rot fungi (*T. trogii* and *P. chrysosporium*) for treating landfill leachates having elevated concentrations of phenols, hydrocarbons, NH_4–N exhibited high COD and high toxicity. Results obtained suggested that ammonium chloride was problematic above 5 g L^{-1} as growth and enzyme secretion were inhibited at this concentration. There was 79% and 68% COD removal with *T. trogii* and *P. chrysosporium,* respectively, in 50 v/v % of the leachate. This was

Table 18.1 Fungi used for treatment of landfill leachate

Fungi	Landfills	Removal	Treatment method	Reference
Trametes trogii	DjebelChekir, Tunisia	79% COD	Shake flask culture	Ellouze et al. (2008)
Phanerochaete chrysosporium	DjebelChekir, Tunisia	68% COD	Shake flask culture	Ellouze et al. (2008)
Lentinus tigrinus	DjebelChekir, Tunisia	90% COD	Shake flask culture	Ellouze et al. (2008)
Eleven fungi (basidiomycetes and ascomycetes)	Italy	Up to 38% color removal	Multiwell plate	Tigini et al. (2014)
Dichomitus squalens	Slovenia	60% COD	Shake flask culture	Kalčíková et al. (2014)
Trametes versicolor	Nonthaburi, Thailand	78% color removal	Immobilization	Saetang and Babel (2009)
Trametes versicolor	Nonthaburi, Thailand	57% COD, 69% BOD	Immobilization	Saetang and Babel (2010)
Ganoderma australe	Malaysia	51% COD, 46% NH_4^+-N	Immobilization	Abdullah et al. (2013)
Fungus (Phanerochaete sp.*) and Bacteria (Pseudomonas* sp.*)*	Delhi, India	76.9% COD, 45.4% color	Sequential bioreactor	Ghosh and Thakur (2014)
Phanerochaete chrysosporium	Chunchon, Korea	21.5% Soluble COD, 16.6% NH_3, 31.2% color	Bioreactor	Kim et al. (2003)

associated with secretion of high amounts of enzyme by each strain and a reduction in phenols and hydrocarbons concentration along with reduction in toxicity.

Tigini et al. (2014) conducted ecotoxicological assays to assess landfill leachate toxicity before and after treatment with autochthonous fungi. Eleven fungi (basidiomycetes and ascomycetes) showed promising remediation potential achieving up to 38% decolorization yields.

Treatment potential of WRF *Dichomitus squalens* and its extracellular lignolytic enzymes for mature municipal landfill leachate was studied by Kalčíková et al. (2014). Leachates were obtained from a functional and a closed MSW landfill of Slovenia. The WRF were capable of growing in the mature leachate from the closed landfill. However, the fungal growth was inhibited in the leachate from the active landfill. Nonetheless, by using crude enzyme filtrate for leachate treatment, a 61 and 44% removal of COD and DOC, respectively, was achieved. The study clearly showed the efficiency of fungal and enzymatic treatment for mature landfill leachates.

Saetang and Babel (2009) immobilized the fungus *Trametes versicolor* on polyurethane foam (PUF) for decolorization of landfill leachate. After determining optimized conditions, the reusability of the fungus was tested. Results indicated that the immobilized fungus could be reused for at least four cycles and by adding 3 g L^{-1} glucose, there was an enhanced (about 50%) removal of color, BOD, and COD. In another study by Saetang and Babel (2010), two fungal strains *Trametes versicolor* and *Flavodon flavus* were immobilized on PUF cubes for treatment of landfill leachate. They studied the efficiency of removal of color, BOD, and COD by the fungal strains. Under optimum conditions, removal of 60–78% of color and reduction in 52–69% of BOD and COD was observed.

Abdullah et al. (2013) immobilized fungus *Ganoderma australe* on Ecomat for leachate treatment. 51% COD removal was observed in case of diluted leachate, whereas in case of raw leachate, only 23% COD removal was observed after the tenth cycle of recycling. Ammonium nitrogen was also found to reduce after eight cycles for the diluted (46%) and the raw leachate (31%).

A sequential fungal (*Phanerochaete* sp.) and bacterial (*Pseudomonas* sp.) treatment was studied by Ghosh and Thakur (2014) in a sequential bioreactor under optimized conditions. They found a removal of 76.9% of COD and 45.4% of color. In addition, no statistically significant genotoxicity was observed at the end of the treatment, making the treated leachate safe for disposal.

A combined process using the WRF *Phanerochaete chrysosporium* and the natural zeolite Clinoptilolite was used by Kim et al. (2003). It involved a pretreatment step by clinoptilolite for removing ammonia nitrogen. This was followed by fungal treatment. The combined process could remove 81.5% ammonia nitrogen, 65% soluble COD (SCOD), and 59% color.

Mechanism Involved in Biodegradation and Decolorization of Leachate by Fungi

Two major mechanisms are involved in wastewater remediation by fungi, namely enzymatic process and biosorption.

Enzymatic Process Involved in Degradation of Organic Contaminants

Lignin Peroxidise (LiP)

LiP (E.C. 1.11.1.14), a heme-containing glycoprotein, catalyses the oxidation of phenolic aromatic substrates, a variety of non-phenolic lignin model compounds as well as a range of organic compounds in the presence of hydrogen peroxide (endogenously generated) as an oxidant, leading to the formation of aryl cation radicals which oxidizes a variety of lignin-related aromatic structures such as aromatic amines, phenols, ethers, PAHs (Wong 2009).

LiPs are very good pollutant degraders because of their non-specificity and high oxidation-reduction potential (Gianfreda and Rao 2004). These are secreted during secondary metabolism primarily as a response to nitrogen limitation (Mishra and Thakur 2012). The production of lignolytic enzymes depends on both the carbon and nitrogen source and its relative concentration. Several fungal species such as *Phanerochaete chrysosporium* produce LiP and MnP under nutrient-limited conditions and their production is suppressed by high nitrogen concentrations in the medium. In contrast, other species such as *Pleurotus ostreatus* and *Trametes trogii* produce high amounts of laccase and MnP in the presence of high concentration of nutrient nitrogen (Janusz et al. 2013).

Manganese Peroxidase (MnP)

MnP (EC 1.11.1.13) is also a heme-containing glycoprotein which requires H_2O_2 as an oxidant. MnPs catalyze the oxidation of Mn(II) to Mn(III), and this latter mediates the oxidation of a variety of phenolic substrates to phenoxy radicals leading to the degradation of compounds like PAHs, humic acids, synthetic dyes, and polychlorinated biphenyls (PCBs) (Wariishi et al. 1992).

It is reported in *Phanerochaete* sp., *Pleurotus* sp., *Trametes* sp., *Phlebia* sp., *Bjerkandera* sp., and *Ganoderma* sp. (Hofrichter 2002). MnP produced by *Pleurotus ostreatus* has been used for the degradation of 80% of Bisphenol A, an endocrine-disturbing chemical in 12 days (Hirano et al. 2000). MnP from *Bjerkandera adusta* and *Pleurotus eryngii* has been applied for degradation of industrial azo- and phthalocyanine dyes (Heinfling et al. 1998).

Laccase (Lac)

Laccase (E.C. 1.10.3.2) is a copper-containing oxidoreductase enzyme that catalyses the oxidation of various aromatic compounds (particularly phenols) with concomitant reduction of oxygen to water (Thurston 1994).

Laccase is widely found to be produced in higher plants, fungi and in some bacterial strains (Margot et al. 2013). The higher redox potential (+800 mV) of fungal laccases in comparison to plants or bacterial laccases makes them suitable for the degradation of lignin and other related organic compounds (Brijwani et al. 2010). Laccases are produced by the majority of lignin-degrading fungi, the best-studied being those of *Trametes* (Han et al. 2005), *Pycnoporus* (Eggert et al. 1996), and *Phanerochaete* (Srinivasan et al. 1995). Laccases have been used for detoxification of paper and pulp, textile effluents as well as a bioremediation agent to clean up herbicides, pesticides, and certain explosives in soil (Couto and Herrera 2006).

Role of Biosorption in Removal of Contaminants

There are two main processes involved in biological decolorization include enzymatic action and biosorption. Biosorption is a metabolically passive process, involving removal of materials by inactive, non-living biomass (materials of biological origin) due to "high attractive forces" present between the two (Volesky and Holan 1995). The process involves a solid phase (biosorbent) and a liquid phase containing a dissolved species to be sorbed (sorbate). Owing to the high affinity of the sorbate to the sorbent, the sorbate gets bound to the sorbent till equilibrium is established between the two (Chojnacka, 2010).

Mechanism of Biosorption

For long, the mechanism of biosorption was not fully understood due to the complex nature and structure of microorganisms. Several processes were thought to play a role in biosorption. But only recently, the process of biosorption has been described as biological ion exchange. Various functional groups (carboxyl, sulfonate, phosphoryl, amido, amino, imidazole) on the cell wall having distinguishable pKa are responsible for the binding properties of a given group (Volesky 2007).

Factors Affecting Biosorption

The factors which influence the process of biosorption include:

(1) pH is the most important factor influencing the process (Schiewer and Volesky 2000). It determines protonation or deprotonation of metal ion binding sites determining the availability of the sites to the sorbate (Ofomaja and Ho 2007).
(2) Type and concentration of biomass, i.e., sorbent: Increased concentration of the biomass generally increases the amount of solute biosorbed due to the increase in the number of binding sites (Esposito et al. 2001). Also, small size of the

biosorbent favors biosorption due to larger surface area and results in shorter equilibration time.

(3) Type and concentration of the sorbate: At very high concentrations of the sorbate, the sites available for sorption become fewer compared to the moles of the sorbate present.
(4) The mode of biosorption—whether it is carried out in suspension or by immobilized biomass.
(5) Presence of other competing ions (both cations and anions).
(6) Within the range of 20–35 °C, temperature does not affect biosorption to much extent (Vegliò and Beolchini 1997). Temperatures above 35 °C usually augment sorption due to the increased surface activity and kinetic energy of the solute (Sağ and Kutsal 2000). However, physical damage can also be expected to the biosorbent at very high temperatures.

Fungi as a Biosorbent: Potential Applications

The sorption properties fungal biomass is mainly due to the unique properties of their cell wall. They have the ability to bind to both cations and anions as their cell wall contains chitin and chitosan having amino, amido, and hydroxyl groups. The chemical and structural characteristics may vary in different fungi. For example, the cell wall of *Aspergillus niger* lacks chitosan which may be one of the reasons of its inferiority as a metal biosorbent to that of *Rhizopus arrhizus* which belongs to the Mucorales family possessing usually higher chitin content in the cell wall (Sağ 2001).

Fungi are of interest for biosorption because:

1. They are able to remove heavy metals in relatively large quantities. In certain cases, fungi have outperformed the conventional adsorbents for heavy metal removal (Tsezos and Volesky 1981; Williams et al. 1998).
2. They are available in large amounts as waste from fermentation industries.
3. Many fungal species have low nutritional requirements.
4. Separation of fungal biomass from the growth medium is a simple operation.
5. Dead biomass can be treated physically and chemically for enhancing its sorption performance.

Future Perspectives

Treatment of landfill leachate using fungi and their extracellular enzymes seems to be a promising method for the removal of organic contaminants and heavy metals. However, before scaling-up of the treatment process, more research is needed specially taking into account the detoxification aspect after fungal treatment. Monitoring detoxification during the course of the bioremediation is often neglected

but is of great importance to conclude the efficiency of the treatment method. Also, there exists a gap regarding mathematical modeling of the leachate treatment process. With mathematical modeling, behavior of the process can be predicted and controlled and improved by appropriate changes in the model.

Conclusions

Landfill leachate could be a major source of environmental pollution unless it is treated and disposed properly. Therefore, finding a sustainable option to treat leachate effectively is of utmost importance. Over the years, various sustainable treatment techniques have been proposed and tested for treating highly polluted leachate. Fungi have been found to be a potent microorganism for removal of recalcitrant compounds due to their extracellular non-specific enzymes. In spite of their great potential, they still have not been applied for leachate treatment on a pilot scale. Further research in this direction is needed to improve the treatment process by means of mathematical modeling as well as taking into account their detoxification potential.

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Studies on Mycorrhiza in *Pinus gerardiana* Wall. ex D. Don, a Threatened Pine of the NW Himalaya

19

Sunil Kumar, Vaneet Jishtu, J. S. Thakur and T. N. Lakhanpal

Abstract

Chilgoza pine, *Pinus gerardiana*, inhabits cold desert areas of Kinnaur in Himachal Pradesh. It is a multiutility tree with much value attached to the seeds, which are edible and a commercial crop, therefore due to the over-exploitation, the pine is facing great threat and proceeding towards extinction. Various methods have been tried to restore the regeneration status but most of these have been of no avail. This paper reports the studies carried out on the use of mycorrhizal biotechnology in the regeneration and establishment of the seedlings. The mycobionts were isolated from the roots, cultured and multiplied, and then the seedlings were inoculated artificially. There was considerable increase in the performance of seedlings, and all the growth parameters were significantly improved. The transplanting period was also greatly reduced, economizing on time, money and energy.

Keywords

Mycorrhizae · *Pinus gerardiana* · Chilgoza · Hartig net

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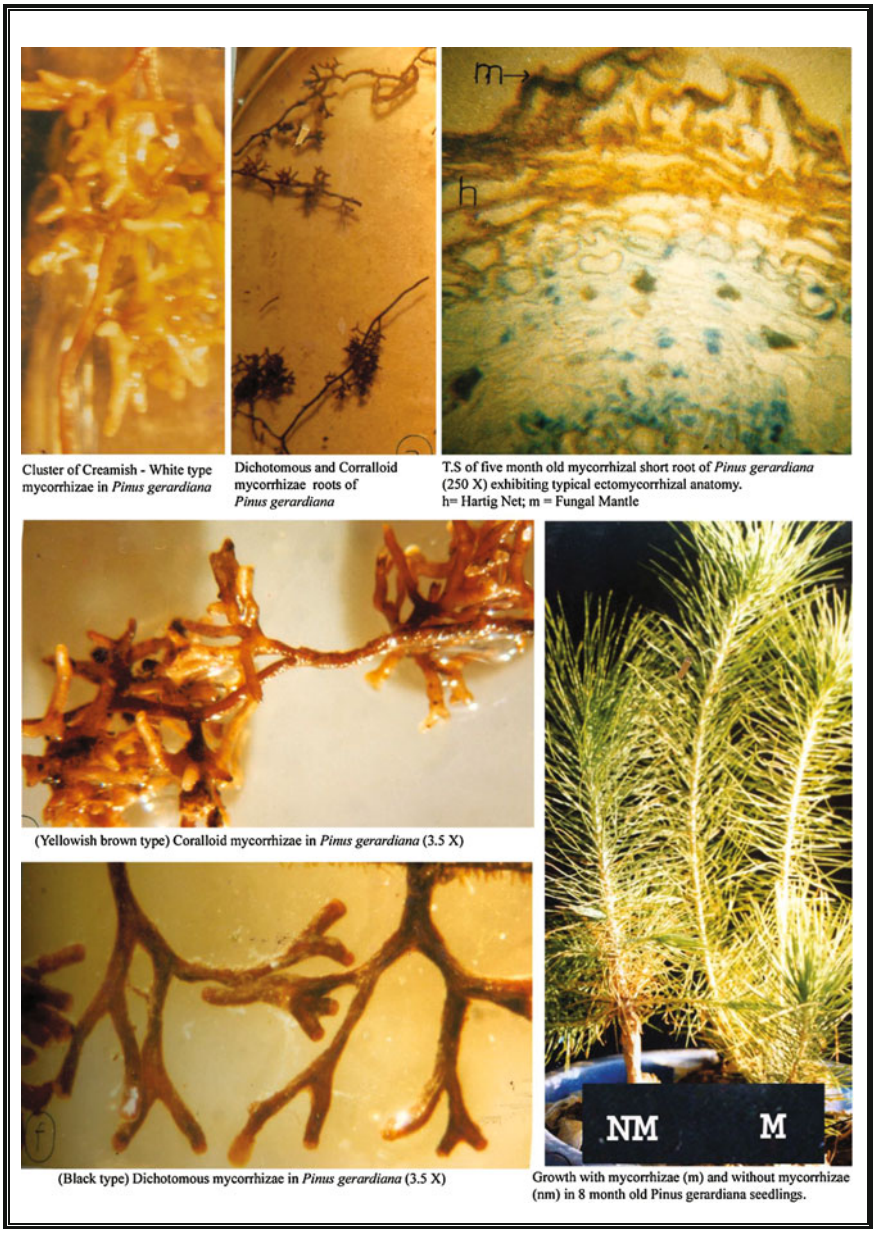
Introduction

Pinus gerardiana Wall. ex D. Don is a multipurpose pine distributed in fragments in eastern Afghanistan, China, S Xizang (Tibet), India, (Jammu and Kashmir and Himachal Pradesh) and northern Pakistan. It is a threatened plant with listing of Near Threatened (NT) in the version 3.1 of the IUCN Red List. The total area of distribution is stated to be around 2000 Km^2 and the altitudinal range between 2000 and 3350 m. This pine is cultivated for forest edible seeds in Afghanistan, and the seeds are evidently dispersed by birds but studies on disposal mechanism are lacking (Farjon 2013).

Habit and Habitat Ecology

Pinus gerardiana in India is commonly and commercially known as Chilgoza or Neoza pine. It has been named after its discoverer "Captain Gerard". In India, it is restricted to the dry inner valleys of North-West Himalayas. It is found growing at altitudes of 2200–3000 m, forming forests of a somewhat open type, though moderately dense pole crops are also met with. Its distribution in India is limited to the Sutlej river valley in Kinnaur district and the Ravi and Chenab catchments in Chamba District of Himachal Pradesh. Thereafter, it extends westwards to Kashmir, and then to Afghanistan and northern Baluchistan. It can be found growing in admixture with deodar in varying propositions in the region outside the influence of monsoons in the demarcated forests of Kalpa and Kilba, Kailash Range of Kinnaur district. The annual precipitation, which varies between about 250 and 270 mm, is received mainly in the form of snow during winter and can endure extreme winters. The summer temperature within its habitat, however, seldom exceeds 39 °C. The tree makes little demand on the fertility of the soil and is capable of growing on very dry hillsides with shallow soils. It is a slow-growing evergreen tree, moderate-sized somewhat branchy tree, attaining ordinarily a girth of 1.5–2.5 m and a height of 15–25 m, and occasionally a girth up to 3.6 m or more and a height up to 25 m. The branches usually bear ascending leaves in bundles of three, dark green, somewhat stiff, with basal sheath about 1 cm long. Bark thin, grey, smooth, with a mottled appearance, exfoliating in irregular thin flakes which leave shallow depressions. Wood is hard, tough, and very resinous, not much used except in regions where other timber is not available (Troup 1921) (Plate 19.1).

The tree bears male or female cones separately on the same plant. They are wind pollinated, and pollination generally takes place during May–June. The young slow-growing female cone rapidly gains growth and size during the second year and ripens to full size by September–October. The cones are harvested green and unripe, while the old cones if left remain on the tree after shedding the seeds. The trees with broad spreading cones are considered superior in seed quantity and quality. The number of cones depends upon the size of the crown and diameter of the trunk. The Chilgoza of commerce is extracted from the mature tree, still green



Cluster of Creamish - White type mycorrhizae in *Pinus gerardiana*

Dichotomous and Corralloid mycorrhizae roots of *Pinus gerardiana*

T.S of five month old mycorrhizal short root of *Pinus gerardiana* (250 X) exhibiting typical ectomycorrhizal anatomy. h= Hartig Net; m = Fungal Mantle

(Yellowish brown type) Coralloid mycorrhizae in *Pinus gerardiana* (3.5 X)

(Black type) Dichotomous mycorrhizae in *Pinus gerardiana* (3.5 X)

Growth with mycorrhizae (m) and without mycorrhizae (nm) in 8 month old Pinus gerardiana seedlings.

Plate 19.1 .

cones are usually gathered, heaped up and burnt. The burning makes the cones open, and then the seeds are easily extracted. The cone harvesting is not a scientific exercise, but brutal in approach. In most instances, the branches bearing the cones

are lopped, while in other cases, the cones are removed from the branches by breaking them with metal hooks. The whole operation is very wasteful, and a lot of damage is incident to the plant because of this exercise. The tree becomes disfigured, whereas otherwise, it is very elegant and majestic tree. The quality and quantity of seed are variable with site location.

Economic Importance

Pinus gerardiana is highly valued for its dry fruits, the edible seed or chilgoza seed. Seed is pain relieving and stimulant. It is also valued for its hard, tough and resinous wood. The seed is rich in starch, oil and albuminoids and is sold in the Indian market at a price of about Rs. 1000.00–1500.00 per kg. The seed is large; it can be as much as 25 cm long and is considered a delicacy. Besides, the edible seed, the oil obtained from the seed is also used for dressing wounds and ulcers and in headache. The pulpwood is also a source of vanillin flavouring material which is obtained as a by-product of other resins. The needles too yield a green dye containing terpene.

Regeneration Status

The natural regeneration of this pine is almost negligible because of a number of factors. Firstly, "Chilgoza" being a commercial cash crop, the right holders remove almost all the cones for seed collection; secondly, the seeds which are left behind are damaged by rodents, birds and reptiles; thirdly, there is a lot of mortality of seeds due to drought; fourthly, the seed being big in size does not embed properly into the loose sandy soil with poor soil moisture; and fifthly, heavy and unrestricted sheep and goat grazing causes lot of damage to young seedlings (Chauhan 1986). All these factors hinder the natural regeneration of this pine. It has been pointed out by Kumar (1986) that severe biotic interference and lack of regeneration in this pine may result in the extinction of this species sooner or later (also see Sehgal and Chauhan 1989; Kumar 1989).

For obtaining natural regeneration, it has been suggested that areas bearing chilgoza pine should be closed for a period of 30 years for right holders. Artificial regeneration has been tried at a number of places in Kinnaur, by both sowing and planting of nursery-raised plants at Kalpa, Ralli (Kilba Range), Akpa (Moorang Range), Shongtong and Purbani in KaIpa Range (Chauhan 1986); but, the performance of seedlings has been reported to be very poor. Grafting experiments on *P. roxburghii* have also not produced the desired results. The nursery-raised seedlings are usually planted after they attain a height of about 5–10 cm and when they are 3–4 years old.

It is surprising that so far no attention has been paid to the use of mycorrhiza in artificial regeneration, which is such an important component of the pines. This paper studies the effect of artificial mycorrhizal inoculation on *P. gerardiana* seedlings and also reports results on characterization and identification of mycorrhiza, response to water stress and the effect of soil fumigants, the first ever studies on the mycorrhiza of this pine.

Methodology

For morphological and anatomical studies, the roots from the top 12 to 15 cm of soil were taken from different plants of *P. gerardiana*. For morphological studies, the colour, shape, presence or absence of root hairs, presence of hyphae or rhizomorphs on the surface of short roots (roots of limited growth) and branching pattern were considered. For ascertaining the formation of ectomycorrhizae, short roots were removed, thoroughly washed in water and fixed in formalin, acetic acid and alcohol (FAA) for 24 h and then finally preserved in 70% alcohol for future investigations. Both stained (cotton blue in lactophenol) and unstained sections were prepared and examined microscopically. The presence of "Hartig net" or fungus mantle was considered evidence of ectomycorrhizal development. Plants grown in glasshouse at 3, 4, 5 and 6 months were also assessed for the presence of mycorrhiza in the same manner.

Characterization and identification of *P. gerardiana* mycorrhizae were done according to Zak (1971) and Agerer (1986). Mycorrhizosphere analysis was done by dilution plate method (Warcup 1960). Isolation and culturing of fungal symbiont were carried out following the procedure of Marx et al. (1982). Pure mycelial cultures of selected fungi were raised following Mikola (1973). The media used were those of Martins (1950), White's modified (Vasil 1959) and Potato Dextrose Peptone-Agar (Rawlings 1933).

Pure mycelial cultures were isolated from the ectomycorrhiza itself. Important criteria considered when selecting fungus for inoculation were those standardized by Molina (1977). After inoculation, roots were sampled periodically at regular intervals for estimation of mycorrhiza.

Estimation of Mycorrhiza

For multiplication of the mycorrhizal fungus, test tube agar stands containing either Norkans as modified by Marx (1969) or PDA were used.

Four-week-old seedlings after they crossed the seedlings stage were picked up from the experimental beds and planted in sterilized plastic pots containing sterilized soil. The requisite inoculum was taken from the culture tubes and mixed with sterilized soil. A thin layer of inoculation was also put on the planting hole as an

additional safeguard to ensure that every seedling receives the inoculation as suggested by Mikola (1969).

The health of seedlings and characters, particularly as regards green lustre on the foliage, better height, growth and root development, indicatives of mycorrhizal development were noted during the course of experiments. On termination of experiments shoot height was recorded. The seedlings were harvested taking care that all root ends remained intact. Data on root lengths, number of laterals, total short roots (including both uninfected and mycorrhizal) and total mycorrhizal roots were recorded for individual seedlings. In *P. gerardiana* where mycorrhizal roots exhibit repeated dichotomy, the ultimate branch was counted as one mycorrhiza. The short roots were placed in either of the two categories, viz. mycorrhizal or uninfected and examined under microscope.

For estimating the physical status and chemical status of mycorrhizal and non-mycorrhizal seedlings, seeds of *P. gerardiana* were sown separately in wooden boxes (60 × 45 cm) filled up to 15 cm with forest soil. For control, seeds were sown in thermally sterilized glasshouse conditions, after washing with 0.01% mercuric chloride followed by distilled water. The controlled and experimental seedlings were assessed for the presence of ectomycorrhiza at regular intervals. Anatomically, the presence of "Hartig net" and "fungal mantle" was considered as evidence of ectomycorrhizal development. While comparing the physical characteristics of mycorrhizal and non-mycorrhizal seedlings, shoot height and shoot/root ratio (fresh weight and dry weight) per plant were compared when the seedlings were eight months old. For comparing the nutrients (needle nutrient percentage and total percentage of nutrients, N, P, K, Ca and Mg), six-month-old mycorrhizal and non-mycorrhizal plants were taken. The analysis was done with AAS at Directorate of Horticulture, Nav Bahar, Shimla. For comparison of water stress tolerance in mycorrhizal and non-mycorrhizal seedlings, four-month-old seedlings were subjected to increasing level of water stress using PEG (6000) following Zur (1966). Control medium comprised of distilled water and nutrient solutions.

The methodology adopted for evaluating the effect of soil fumigants on damping-off disease and mycorrhizal development was the split-plot design using three replicates each, containing (12' × 6') plots. Main plot was divided into six plots (2′ × 6′) each for six treatments under trial at random. Each plot was further divided into three subplots of 2′ × 2′ size. Two hundred seeds per subplot were sown after soaking them for 24 h in water and giving repeated washings. Formalin* and methyl bromide** each in six different doses were applied 15 days before sowing to reduce their phototoxic effect on germination and seedlings growth.

*Formalin (ml) in 41 of water: 170, 190, 210, 230, 250, 270.
**Methyl bromide (ml) in 41 of water: 110–120, 130, 140, 150, 160.

The germinated seedlings were counted on alternate days, and seedlings' mortality due to damping off was recorded up to 3 months. Seedlings were fertilized every three weeks with NPK fertilizer diluted in water to 2500 ppm. Seedlings of

different treatments were lifted monthly for studies. Shoot height, root length, fresh weight and dry weight were recorded for each seedling after washing carefully. Percentage of ectomycorrhizal development was estimated visually. Estimation of mycorrhizosphere fungi was done by dilution plate method.

Observations

Morphology and Anatomy of Mycorrhizal Roots

Ectomycorrhizal roots of *P. gerardiana* are bifurcate or dichotomous and occasionally multiforked (coralloid). Such roots have swollen root tips, covered with hyphal sheath. The mycorrhizal dichotomies are creamish white, brown-black and black in colour. Short hyphae radiating from the hyphal sheath envelope the mycorrhizal root. Average diameter of mycorrhizal root at the base of dichotomy varies from 0.6 to 1.5 mm, whereas at the tips, it is 0.4 to 0.7 mm. The mycorrhizal short roots possess rounded apices. The length of distal part of each mycorrhizal dichotomy varies from 0.3 to 0.5 mm.

Anatomically, the L.S. and T.S. of mycorrhizal short roots exhibits well-developed mantle and Hartig net. T.S. of mycorrhizal short root of three- to four-month-old seedlings reveals initial stages of the development of mantle and Hartig net; mantle being 4–6 μm and Hartig net formation being restricted to 2–3 layers of cortical cells. T.S. of five- to six-month-old seedlings reveals well-developed Hartig net and mantle, being 8–16 μm thick. The fungal mantle is smooth to woolly and velvety to warty with sparsely radiating hyphae. The intercellular spaces of the cortex are invaded by fungal hyphae, and the middle lamella is partly or completely replaced by hyphae. Hartig net is well developed as is characteristics of the ectomycorrhizal roots (Plate 19.2).

Longitudinal sections of the mycorrhizal roots show that the hyphae are in close contact with each other in the intercellular spaces, running parallel to each other. Growing point of mycorrhizal short root is seen covered with the fungal hyphal sheath, and the apex is rounded. There is no penetration of stele or meristem region, and the intensity of infection increases with the increase in time, and there is a progressive development of mycorrhiza.

Three types of mycorrhizae have been observed in *P. gerardiana:* white creamish, yellowish brown and black (Table 19.1). The three types differ in colour, thickness and texture of the mantle. There is no significant difference in odour and taste in white and brown types of mycorrhizae, but the black type has mushroom-like odour and taste. Hartig net is very well developed, and intercellular hyphae are present in between all the cortical cells. The hyphae have occasional clamps in brown type, but they are more frequent in black type.

In all the three types of mycorrhizae identified in *P. gerardiana*, mantle texture was observed to be most variable. It ranged from smooth in white to smooth velvety in brown and warty granular in black type. Thickness of mantle varied with types of


(a)
(c)
(d)
(e)
A. Destructive harvest of cones B. Green cones on branches
C. Bark of young tree D. Seed "NEOZA" E. *Pinus gerardiana* forest

Plate 19.2 .

Table 19.1 Characterization of three distinct, ectomycorrhizae in *Pinus gerardiana* Wall

Character	White creamish	Yellow-brown	Black-brown
Macroscopic characteristics			
Form of roots	Forked dichotomous	Forked dichotomously	Exhibited repeated dichotomy
Colour of mantle	Pseudoparenchymatous mantle sheath transparent (colourless)	Mantle sheath yellowish brown	Outer mantle layers brown to black and inner hyaline to pale
Thickness of mantle	5–10 μm	8–10 μm	12–20 μm, mantle two layered, outer layer 8–18 μm, inner layer 5–10 μm
Texture	Smooth	Smooth to velvety	Thick granular to warty
Surrounding mycelium present or absent	Surrounding mycelium present, white hyphae radiating outwards	Mycelium loose many long hyphae irregularly radiating outwards	Mycelium loose with brown mycelial strands, short brown hyphae projecting outwards
Odour and taste	Not distinct	Not distinct	Mushroom-like
Microscopic characteristics			
Degree of the development of Hartig net	Poorly developed hyphae found only in two to three outer cortical layers of mycorrhizal roots	Fairly well-developed hyphae present in intercellular spaces of all the cortical cells	Well-developed hyphae present in intercellular spaces of nearly all the cortical cells
Hyphal characteristics	Hyphae with clamp connections	Hyphae with clamps occasionally absent	Hyphae with numerous clamp connections

mycorrhizae. In case of white type, it was 5–10 μm thick; in yellowish brown, 8–10 μm thick; and in black-brown, it was 12–20 μm thick.

Mycorrhizosphere of *Pinus gerardiana*

In *P. gerardiana*, fourteen fungi have been isolated in all. Ten of these belong to Deuteromycotina, three to Zygomycotina and one represented the sterile Basidiomycetous mycelium (Table 19.2a). Among the member of Deuteromycotina, *Fusarium oxysporum, Penicillium nigricans* and *Trichoderma viride* were the most predominant forms with percentage frequency occurrence of 100, 100 and 83%, respectively. Among the abundant forms were species like *Aspergillus flavus* (66.6%) and *Rhizoctonia* sp. (66. 6%). In Zygomycotina, *Circinella simplex* (66. 6%), *Mucor bacilliformis* (66.6%) and *Rhizopus nigricans* (66. 6%) were quite abundant. The Basidiomycetous mycelium was also equally well distributed with

Table 19.2 a List of fungi inhabiting mycorrhizosphere and mycorrhizoplane of *Pinus gerardiana* Wall and their appearance in the sample taken from November 1983 to September 1984

S. No.	Name of fungus	November–December 1983	January–February 1984	March–April 1984	May–June 1984	July–August 1984	September–October 1984
1.	*Alternaria alternata*	−	−	+	+	−	+
2.	*Aspergillus flavus*	+	+	−	−	+	+
3.	*Aspergillus niger*	+	+	+	−	−	−
4.	*Circinella simplex*	+	+	−	+	−	+
5.	*Cladosporium herbarum*	−	+	−	+	+	−
6.	*Colletotricum gloeosporiodes*	+	−	−	−	−	+
7.	*Fusarium oxysporum*	+	+	+	+	+	+
8.	*Helminthosporium tetramera*	−	−	−	+	−	+
9.	*Mucor bacilliformis*	−	+	+	+	+	−
10.	*Penicillium nigricans*	+	+	+	+	+	+
11.	*Rhizopus nigricans*	+	+	+	−	+	−
12.	*Rhizoctonia* sp.	−	+	+	−	+	+
13.	*Trichoderma viride*	+	−	+	+	+	+
14.	Basidiomycetous mycelium Bo	+	−	+	+	+	+

frequency occurrence of 66. 6%. *Fusarium oxysporum* and *Rhizoctonia sp*. represent the fungi causing damping off of the seedlings. The sterile Basidiomycetous mycelium was primarily isolated from the rhizoplane. Other fungal forms which were represented less in the rhizoplane, and the mycorrhizospheres are *Penicìllinm nìgricans, Trichoderma viride, Mucor bacilliformis* and *Alternaria alternata.* The monthly distribution of species isolated is represented in Table 19.2b. It is clear that the number of species isolated from rhizoplane is much less than those isolated from the mycorrhizosphere. Except for the sterile mycelium, the fungi isolated from both rhizoplane and mycorrhizosphere are almost the same.

So far as the seasonal distribution of these fungi is concerned, it is clear from Table 19.2c that during winter, *Aspergillus niger, Fusarium oxysporum, Penicillium nigricans, Mucor bacilliformis, Rhizopus nigricans* and *Rhizoctonia* sp. were most predominant.

In the spring season, *Alternaria alternata, Circinella simplex, Mucor bacilliformis, Penìcillium nigricans, Trichoderma viride, Cladosporium herbarum* and *Helminthosporium tetramera* were most predominant. In the rainy season, the species which appeared to be predominant were *Aspergillus niger, Fusarium oxysporum, Penicillium nigricans Rhizoctonia* sp., *Trichoderma viride, Cladosporium herbarum, Mucor bacilliformis, Rhizopus nigricans* and the Basidiomycetous mycelium. The autumn season yielded the following predominant forms: *Aspergillus flavus, Circinella simplex, Colletotricum gloeosporiodes, Fusarium oxysporum, Penicillium nigricans, Trichoderma viride, Aspergillus niger, Rhizopus nigricans* and the Basidiomycetous mycelium.

Isolation, Inoculation and Estimation of Mycorrhizal Development in *Pinus gerardiana*

In *P. gerardiana,* one mycorrhizal fungus was isolated and inoculated into the seedlings. The results of inoculation, on the development and estimation of mycorrhiza, are presented in Table 19.3. The seedlings inoculated with (B_o) mycorrhizal fungus attained better shoot height, root length, stem diameter, total shoot −root fresh weight and high shoot/root fresh weight ratio. The shoot height, root length and fresh weight shoot/root ratio are highly significant (at 0.01% level of probability) in the inoculated seedlings, whereas the stem diameter is significantly higher (at 0.05% level of probability). The development of mycorrhiza in inoculated seedlings results in green lustre on the foliage, and, therefore, these are easily distinguished from un-inoculated control seedling which remained pale green.

There is a significant difference (at 0.05% level of probability) in the mycorrhizal counts between inoculated and un-inoculated control seedlings. The mycorrhizal counts show that all the seedlings which were inoculated developed 100% ectomycorrhizal infection. None of the plants in control developed any mycorrhizal short root (zero %). Seedlings inoculated with B_o have 67.6% of mycorrhizal short roots and 32.4% of un-inoculated shoot roots. The total number of short roots (144 maximum) is higher in inoculated seedlings than in the un-inoculated seedlings (96 maximum).

Table 19.2 **b** Showing monthly distribution frequency of fungi inhabiting the mycorrhizosphere and mycorrhizoplane of *Pinus gerardiana* Wall

Fungal species	November	December	January	February	March	April	May	June	July	August	September	October	(%)
Alternaria alternata	−	−	−	−	+	+	+	+	−	−	+	+	50.0
Aspergillus flavus	+	+	+	+	−	−	−	−	+	+	+	+	66.6
Aspergillus niger	+	+	+	+	+	+	−	−	−	−	−	−	50.0
Circinella simplex	+	+	+	+	−	−	+	+	−	−	+	+	66.6
Cladosporium herbarum	−	−	+	+	−	−	+	+	+	+	−	−	50.0
Colletotricum gloeosporoides	+	+	−	−	−	−	−	−	−	−	+	+	33.3
Fusarium oxysporum	+	+	+	+	+	+	+	+	+	+	+	+	100
Helminthosporium tetramera	−	−	−	−	−	−	+	+	−	−	+	+	33.3
Mucor bacilliformis	−	−	+	+	+	+	+	+	+	+	−	−	66.6
Penicillium nigricans	+	+	+	+	+	+	+	+	+	+	+	+	100
Rhizopus nigricans	+	+	+	+	+	+	−	−	+	+	−	−	66.6
Rhizoctonia sp.	−	−	+	+	+	+	−	−	+	+	+	+	66.6
Trichoderma viride	+	+	−	−	+	+	+	+	+	+	+	+	83.3
Basidiomycetous mycelium Bo	+	+	−	−	+	+	−	−	+	+	+	+	66.6

Table 19.2 c Presenting the seasonal distribution frequency of fungi inhabiting mycorrhizosphere and mycorrhizoplane of *Pinus gerardiana* Wall

Name of fungus	Winter (%)	Spring (%)	Rainy (%)	Autumn (%)
Alternaria alternata	33.3	100	33.3	33.3
Aspergillus flavus	66.6	0.00	100	100
Aspergillus niger	100	33.3	0.00	66.6
Circinella simplex	66.6	66.6	33.3	100
Cladosporium herbarum	66.6	66.6	66.6	100
Colletotricum gloeosporiodes	0.00	0.00	33.3	100
Fusarium oxysporum	100	100	100	100
Helminthosporium tetramera	0.00	66.6	33.3	33.3
Mucor bacilliformis	100	100	66.6	0.00
Penicillium nigricans	100	100	100	100
Rhizopus nigricans	100	33.3	66.6	66.6
Rhizoctonia sp.	100	33.3	100	33.3
Trichoderma viride	33.3	100	100	100
Basidiomycetous mycelium Bo	33.3	33.3	100	100

Comparison of Water Stress Tolerance in Mycorrhizal and Non-mycorrhizal Roots of *Pinus gerardiana*

A gradual decrease in the fresh weight moisture percentage of both mycorrhizal and non-mycorrhizal seedlings with increasing stress level occurred. The decrease was highly significant in the non-mycorrhizal seedlings (Table 19.4). It is also clear from this table that with increasing stress level, there was decline in the accumulation of dry weight in both types of seedlings, but again this decline was highly significant at 0.01% level of probability in non-mycorrhizal seedlings as compared to non-mycorrhizal plants. The decrease in dry matter content was less significant in mycorrhizal seedlings as compared to non-mycorrhizal plants.

Visual observation made with regard to the beginning of defoliation revealed that defoliation starts in non-mycorrhizal seedlings at a much earlier stage, (fifth day) at −4 Atm, of stress level, whereas in mycorrhizal seedlings, the defoliation started at −6 Atm, on eighth day (Table 19.5). The extent of damage to the mycorrhizal symbiont was not observed in the present studies. The fungal mantle was darker in the seedlings subjected to stress. It is clear from Tables 19.5 to 19.6 that WSD values increased gradually in both mycorrhizal and non-mycorrhizal seedlings. WSD values were highly significant in non-mycorrhizal seedlings. Mycorrhizal seedlings tolerated water stress of −6 Atm, while non-mycorrhizal seedlings only up to −4 Atm. After eighth day of treatment, they showed complete wilting and defoliation.

Table 19.3 Effect on growth and development of *Pinus gerardiana* Wall. ex D. Don seedlings after six months of inoculation with ectomycorrhizal fungus

Soil infestation treatment	Shoot height (cm)	Root length (cm)	Stem diameter (mm)	Shoot fresh weight (gm)	Root fresh weight (gm)	Total	Shoot/root ratio	Foliage lustre
Control	12.5	8.6	4.2	12.0	8.1	20.1	1.48	
	11.6	8.3	4.6	13.5	7.9	21.4	1.70	Pale
	13.0	8.5	4.5	13.0	7.8	20.8	1.54	
Basidiomycetous mycelium Bo	21.2**	14.0**	4.8*	18.0*	11.0*	29.0*	1.63*	
	20.3*	13.6*	4.7*	19.5**	10.8**	30.3**	1.80**	Green
	20.6*	13.2*	5.0*	19.8**	10.5*	30.3**	1.88**	

Mycorrhizal counts of *Pinus gerardiana* Wall seedlings after six months of inoculation

Soil infestation treatment	Un-infected short roots	Mycorrhizal short roots	Total number of short roots	Percentage of mycorrhizal development
Control	80	0	80	0
	96	0	96	0
	92	0	92	0
Basidiomycetous mycelium Bo	48 (37%)	82 (63)	130	100
	46 (32.40%)	9667.69%)	142	100
	54 (39.89%)	8861.11%)	144	100

Each figure represents the mean of five readings
Bo Basidiomycetous mycelium
*$P < 0.05$ = significant; **$P < 0.01$ = highly significant

Table 19.4 Moisture percentage and water saturation deficit (WSD) changes under different osmotic levels of water stress with increase in time in mycorrhizal and non-mycorrhizal seedlings of *Pinus gerardiana* Wall

No. of days	Moisture percentage							WSD percentage						
	Stress level (Atms)							Stress level (Atms)						
	0	−1	−2	−4	−6	−8	−10	0	−1	−2	−4	−6	−8	−10
1.	89 (89)	87 (85)	85 (83)	83 (79)	80 (78)	80 (76)	77 (68)	4 (4)	5 (5)	6 (5)	15 (9)	30 (24)	32 (30)	39 (33)
2.	89 (89)	86 (84)	84 (82)	82 (79)	76 (76)	72 (71)	71 (68)	4 (4)	6 (5)	7 (5)	16 (6)	31 (27)	37 (32)	43 (33)
3.	90 (89)	85 (82)	84 (82)	81 (80)	79 (79)	77 (71)	70 (70)	4 (4)	7 (5)	8 (5)	17 (10)	31 (29)	34 (32)	48 (32)
4.	88 (88)	87 (86)	83 (84)	79 (78)	70 (71)	75 (70)	72 (60)	2 (4)	6 (6)	9 (6)	18 (10)	33 (30)	42 (42)	52 (12)
5.	88 (87)	82 (79)	80 (79)	80 (78)	68 (69)	70 (64)	69 (57)	6 (5)	4 (6)	8 (6)	22 (10)	34 (20)	40 (30)	59 (41)
6.	88 (86)	82 (78)	78 (79)	80 (78)	75 (76)	72 (72)	71 (65)	3 (4)	6 (5)	10 (6)	20 (12)	31 (28)	44 (34)	62 (38)
7.	88 (88)	81 (79)	78 (75)	79 (70)	72 (76)	64 (64)	65 (62)	4 (4)	8 (6)	9 (7)	21 (15)	29 (29)	47 (37)	59 (42)
8.	89 (88)	79 (82)	76 (80)	78 (69)	72 (73)	71 (59)	70 (56)	4 (4)	8 (6)	9 (8)	20 (17)	36 (31)	46 (30)	51 (54)
9.	87 (87)	82 (81)	79 (70)	74 (75)	72 (71)	60 (62)	69 (60)	4 (5)	8 (6)	10 (8)	20 (14)	38 (35)	50 (37)	61 (51)
10.	87 (87)	80 (79)	74 (72)	72 (72)	71 (69)	70 (62)	68 (53)	4 (4)	7 (5)	12 (8)	12 (14)	42 (28)	60 (44)	64 (40)

Each value is the mean of five replicates
Figure in parenthesis is for non-mycorrhizal seedlings

Effect of Soil Fumigants on Mycorrhizal and Non-mycorrhizal Development and Damping off of the Seedlings of *Pinus gerardiana*

Eight fungi belonging to the Deuteromycotina, two to the Zygomycotina, one to the Diplomastigomycotina and one representing the sterile Basidiomycetous hyphae were isolated from the untreated nursery beds of *P. gerardiana*. Among the eleven species isolated, the most common species were those of *Fusarium* sp., *Rhizoctonia* sp., *Pythium* sp., *Circinella* sp., *Cladosporium* sp., *Mucor* sp., *Penicillium* sp., *Rhizopus* sp. and *Trichoderma* sp. Out of these, the three species of fungi, i.e. *Fusarium, Rhizoctonia and Pythium,* are known casual organisms of damping off of the seedlings in nursery soils.

Table 19.7 also tabulates the changes in microflora of nursery soil after treatment with different doses of formalin (130, 150, 170, 190, 210, 230, 250 and 270 ml/4 l of water) and methyl bromide (80, 90, 100, 110, 120, 130, 140 and 150 ml/4 l of

Table 19.5 Observations on the tap root length in *Pinus gerardiana* seedlings

S. No.	Number of months	Experimental seedlings (cm)	Control (cm)
1.	Three months	48.1	51.2
2.	Four months	53.3	57.3
3.	Five months	58.5	62.0
4.	Six months	62.8	71.4

Observations on the number of primary laterals in *Pinus gerardiana* seedlings

S. No.	Number of months	Experimental seedlings	Control
1.	Three months	3	2
2.	Four months	4	3
3.	Five months	7	5
4.	Six months	8	6

Table 19.6 Moisture percentage and water saturation deficit (WSD) upon rehydration with increase in time in mycorrhizal and non-mycorrhizal seedlings of *Pinus gerardiana* Wall

No. of days	Moisture percentage					WSD percentage				
	Stress level (Atms)					Stress level (Atms)				
	0	−1	−2	−4	−6	0	−1	−2	−4	−6
3.	87 (88)	86 (80)	80 (75)	75 (75)	74 (70)	4 (4)	29 (23)	24 (21)	13 (22)	4 (17)
4.	87 (87)	85 (80)	82 (75)	80 (75)	77 (67)	4 (4)	27 (22)	21 (22)	11 (18)	9 (18)
5.	88 (88)	85 (75)	85 (90)	82 (80)	75 (68)	4 (5)	28 (20)	18 (20)	10 (23)	8 (19)
6.	90 (89)	85 (80)	85 (75)	82 (80)	77 (67)	4 (5)	20 (22)	19 (21)	9 (22)	7 (16)
7.	89 (82)	88 (81)	87 (75)	87 (70)	75 (68)	4 (5)	13 (21)	18 (22)	10 (22)	7 (18)
8.	88 (89)	89 (85)	90 (80)	90 (79)	78 (67)	4 (5)	15 (29)	13 (28)	7 (23)	6 (20)

Each value is the mean of five replicates
Figure in parenthesis is for non-mycorrhizal seedlings

Table 19.7 Effect of different dosages of formalin and methyl bromide on fungi isolated from mycorrhizosphere of *Pinus gerardiana* Wall. ex D. Don

Name of the fungi	Initial microflora	Alternation in microflora after treatment															
		Formalin (ml/4 l)								Formalin (ml/4 l)							
	Control	130	150	170	190	210	230	250	270	80	90	100	110	120	130	140	150
Aspergillus flavus	+	+	+	−	+	−	−	−	−	+	+	−	+	−	−	−	−
Aspergillus niger	+	+	−	+	−	−	−	−	−	+	+	−	−	+	−	−	−
Circinella simplex	+	+	+	−	−	−	−	+	−	−	−	+	−	−	−	−	−
Cladosporium herbarum	+	+	+	−	−	−	−	−	−	+	+	−	+	−	−	−	−
Fusarium oxysporum	+	+	+	+	−	−	−	−	−	+	+	−	−	−	−	−	−
Mucor bacilliformis	+	−	+	−	+	−	−	−	−	+	+	−	−	−	−	−	−
Penicillium nigricans	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
Pythium sp.	+	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−
Rhizopus nigricans	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−
Rhizoctonia sp.	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
Trichoderma viride	+	+	+	+	−	−	−	−	−	+	−	−	−	+	−	−	−
Basidiomycetous mycelium Bo	+	+	+	+	+	−	−	−	−	+	+	−	+	+	−	−	−

water). From the table, it is clear that the incidence of damping-off fungi remained almost the same as in the control with doses 130, 150 and 170 ml/4 l of water of formalin. With dose 210, 230, 250 and 270 ml/4 l of water, there was suppression of the mycorrhizal development.

At dose 190, the damping-off fungi as well as other fungi were almost completely eliminated from the nursery beds, but the mycorrhizal development in the seedlings was unhindered and normal. Therefore, this dose was considered to be the best for eliminating damping-off fungi and for establishing the normal mycorrhizal association.

Table 19.8 reveals the effect of different doses of formalin and methyl bromide on germination percentage and seedling biomass of *P. gerardiana*. It reveals higher germination percentage, increased survival percentage of seedlings, higher shoot/root ratio (fresh weight and dry weight) and maximum development of mycorrhizae in 190 ml/l of water of formalin and 110 ml/4 l of water of methyl bromide. It is clear from Table 19.6 that survival percentage, average shoot height, average fresh shoot/root ratio and mycorrhizal short roots per plant were highly significant ($P < 0.01$). Whereas survival percentage, average roots length and average dry weight root/shoot ratio were significantly high. Similarly, doses of 80, 90 and 100 ml/4 l of water had little effect on the mycorrhizosphere fungi, whereas doses 120, 130, 140 and 150 ml/4 l of water of methyl bromide had depressing effect on the damping-off fungi and other members of soil microflora and also on the mycorrhizal development. It was only the dose of 110 ml/4 l of water which has so far been good for suppressing damping-off fungi and for mycorrhizal development.

Estimation of mycorrhizal short roots per plant is also significantly higher (126) per plant at 110 ml/4 l of water of methyl bromide and 146 per plant at 190 ml/4 l of formalin as compared to control and other treatment on *P. gerardiana*. The total percentage accumulation of K, Ca and Mg in the shoots and roots of mycorrhizal plants is significantly higher than non-mycorrhizal plants.

Comparison of Physical and Chemical Status of Inoculated and Un-inoculated Seedlings of *Pinus gerardiana*

Table 19.9 shows that there is significant increase in the shoot height of mycorrhizal seedlings as compared to non-mycorrhizal seedlings. The sample numbers 5, 7, 8 and 9 were highly significant at 0.019% level of probability, rest of all the samples were significant at 0.05% level of probability. Table 19.10 shows the fresh weight and oven-dry weight of both shoots and roots per plant of mycorrhizal and non-mycorrhizal plants. The fresh weight as well as dry weight of shoots and roots of mycorrhizal plants is higher as compared to the non-mycorrhizal ones. Dry weight production was greatest for all the mycorrhizal seedlings. It is clear from Table 19.11 that the fresh weight shoot/root ratio and dry weight shoot/root ratio are significantly higher in mycorrhizal plants at 0.05 and 0.01% levels of probability.

Table 19.8 Effect of different dosages of formalin and methyl bromide added to soil on germination percentage, survival percentage, biomass and development of mycorrhizal short roots in *Pinus gerardiana* Wall. ex D. Don seedlings

Germination and seedling characteristics	Control	Formalin ml/4 l of water								Formalin ml/4 l of water							
		130	150	170	190	210	230	250	270	80	90	100	110	120	130	140	150
Seedling germination percentage	68*	64*	74*	64*	74**	58*	54*	48*	61*	58*	69*	58*	72*	63*	69*	68*	64*
Survival percentage	58*	72*	80*	92*	94*	88	79*	80*	70	80*	86*	74*	96**	78*	78	79*	71
Average shoot height (cm)	8.5*	7.8*	7.6*	7.8	8.8**	7.6	7.2*	6.9*	7.1	7.4*	6.5*	8.1*	7.2*	6.9	7.1*	6.2	6.1*
Average shoot length (cm)	9.6**	9.1*	8.8*	8.2*	9.6*	8.1*	8.1*	7.4	8.8*	8.1	8.2*	8.4*	9.2**	7.4*	8.4*	7.4*	6.3*
Average fresh weight (g) S/R ratio	1.1*	1.0*	1.2*	1.1*	1.2**	1.0*	1.1*	1.1	1.2*	1.1*	1.2	1.0*	1.3**	1.1*	1.2	1.2*	1.0
Average dry weight (mg) S/R ratio	1.7*	1.6*	1.6*	1.7**	1.8*	1.7*	1.7*	1.6*	1.4*	1.6	1.7*	1.5*	1.8**	1.6*	1.6*	1.5	1.4
Mycorrhizal short roots per plant	88*	114*	89*	108	146**	78*	68*	118*	62	98*	96*	74*	126**	114*	48	68*	62

S/R = shoot/root ratio

*$P < 0.05$ = significant; **$P < 0.01$ = highly significant

Table 19.9 Physical characteristics of 8-month-old mycorrhizal and non-mycorrhizal seedlings of *Pinus gerardiana* Wall. ex D. Don

Shoot height (cm)			
S. No.	Mycorrhizal	Non-mycorrhizal	'*t*' value *df* = 8
	Mean ± SE	Mean ± SE	
1.	17.2 ± 0.28	12.1 ± 0.28	2.65*
2.	16.8 ± 0.32	11.2 ± 0.32	2.65*
3.	16.4 ± 0.31	10.2 ± 0.31	3.00*
4.	14.7 ± 0.32	8.2 ± 0.32	2.25*
5.	17.4 ± 0.36	8.4 ± 0.36	3.00**
6.	16.8 ± 0.28	9.2 ± 0.28	2.65*
7.	16.2 ± 0.30	8.8 ± 0.30	2.65**
8.	17.2 ± 0.31	8.6 ± 0.31	1.75**
9.	17.4 ± 0.28	9.1 ± 0.28	3.00**
10.	16.6 ± 0.34	8.5 ± 0.34	2.25*

SE standard error; *df* degree of freedom
*$P < 0.05$ = significant; **$P < 0.01$ = highly significant

Table 19.10 Fresh weight and oven-dry weight (gm) of 8-month-old mycorrhizal and non-mycorrhizal seedlings of *Pinus gerardiana* Wall. ex D. Don

S. No.	Mycorrhizal				Non-mycorrhizal			
	Fresh weight (g)		Dry weight (g)		Fresh weight (g)		Dry weight (g)	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
1.	1.76	1.15	0.66	0.46	1.62	0.96	0.36	0.21
2.	1.84	0.98	0.47	0.37	1.71	0.72	0.29	0.19
3.	1.90	0.86	0.61	0.42	1.58	0.64	0.40	0.24
4.	1.86	0.95	0.86	0.38	1.38	0.82	0.32	0.26
5.	1.56	1.20	0.94	0.46	1.48	0.86	0.32	0.23
6.	1.64	1.16	0.96	0.51	1.62	0.78	0.36	0.31
7.	1.75	0.88	0.90	0.32	1.70	0.98	0.32	0.27
8.	1.82	0.96	0.65	0.46	1.56	0.96	0.31	0.26
9.	1.58	1.05	0.70	0.42	1.35	0.94	0.28	0.21
10.	1.72	1.22	0.98	0.42	1.68	0.72	0.36	0.24

S. No. sample number

Table 19.12 reveals the analysis of sterilized and unsterilized soils which were used to raise non-mycorrhizal and mycorrhizal plants, respectively. There were no obvious differences in soil nutrients (organic carbon percentage, total nitrogen percentage, available phosphorus lbs/acre and available potassium lbs/acre) and pH of the soils. The soils were low in nitrogen content, available phosphorus and available potassium. The pH of unsterilized soil was 6.4 and of sterilized soil, it was 6.2, both the soils being acidic. The sterilized soil was more acidic as compared to unsterilized soil. Table 7.10 clearly indicates that there were no significant differences in the nutrients and pH of the soils in which mycorrhizal and non-mycorrhizal seedlings were raised.

Table 19.11 Shoot/root ratio (fresh weight and dry weight) per plant of 8-month-old mycorrhizal and non-mycorrhizal *Pinus gerardiana* Wall. ex D. Don seedlings

S/R ratio	Mycorrhizal	Non-mycorrhizal	'*t*' value *df* = 8
S. No.	Mean ± SE	Mean ± SE	
Fresh weight			
1.	6.28 ± 0.21	4.54 ± 0.21	2.17*
2.	9.51 ± 0.26	5.21 ± 0.26	2.65**
3.	6.28 ± 0.24	5.16 ± 0.24	2.365**
4.	5.82 ± 0.18	3.86 ± 0.18	1.75
5.	6.78 ± 0.22	4.42 ± 0.22	3.00*
6.	6.36 ± 0.24	4.36 ± 0.24	1.65*
7.	6.14 ± 0.23	4.08 ± 0.23	2.35**
8.	6.36 ± 0.24	4.62 ± 0.24	1.75*
9.	6.52 ± 0.25	4.15 ± 0.25	1.65
10.	6.64 ± 0.24	4.36 ± 0.24	2.60*
Dry weight			
1.	3.98 ± 0.20	2.82 ± 0.20	2.25**
2.	4.38 ± 0.17	3.16 ± 0.17	2.65**
3.	4.32 ± 0.18	3.26 ± 0.18	2.40*
4.	4.28 ± 0.21	2.17 ± 0.21	1.50
5.	4.36 ± 0.24	3.67 ± 0.24	3.25*
6.	4.08 ± 0.22	2.87 ± 0.22	3.00*
7.	3.68 ± 0.21	3.67 ± 0.21	2.54*
8.	4.28 ± 0.24	3.64 ± 0.24	3.75**
9.	4.26 ± 0.21	3.07 ± 0.21	2.65*
10.	4.17 ± 0.18	2.94 ± 0.18	3.00*

SE standard error; *df* degree of freedom
*$P < 0.05$ = significant; **$P < 0.01$ = highly significant

Table 19.12 Nutrient concentrations of sterilized and unsterilized soils in which non-mycorrhizal and mycorrhizal seedlings of *Pinus gerardiana* Wall were raised

Treatment	Soil pH	Organic carbon (%)	Total nitrogen content (%)	Available	
				P_2O_5 1 bs/Acre	K_2O 1 bs/Acre
Unsterilized soil	6.4	0.54	0.32	48	128
Sterilized soil	6.2	0.57	0.34	49	132

Each figure represents mean of five readings

Significant differences were obtained in the percentage of N, P, K, Ca and Mg accumulated in needles of the mycorrhizal and non-mycorrhizal seedlings. Needles of the mycorrhizal seedlings generally showed the higher concentration of N, P, K, Ca and Mg (Table 19.13). The percentage of N accumulation in the needles varied from 0.98, 0.96 and 0.95 in mycorrhizal seedlings and from 0.74, 0.72 and 0.76, in the non-mycorrhizal seedlings. The difference was significant at 0.057% level of probability.

The gain in P by the needles of mycorrhizal seedlings was three times than that of non-mycorrhizal seedlings. In the mycorrhizal needles, it varied from 1.28, 1.27 and 1.28, whereas in non-mycorrhizal ones, it varied from 0.41, 0.43 and 0.39. The difference was significant at 0.01% level of probability. Except one (mean) reading in Table 19.11, K was significantly higher in needles of mycorrhizal seedlings. The difference was significant at 0.01% level of probability. The level of K, Ca and Mg in the needles of mycorrhizal seedlings was significantly higher as compared to non-mycorrhizal seedlings.

It is clear from Table 19.14 that the total nutrient percentage in shoots and roots per seedlings was higher as compared to non-mycorrhizal seedlings. The difference in accumulation of P in mycorrhizal and non-mycorrhizal seedlings was three fold, and the difference was significant at 0.01% level of significance, whereas the N, K, Ca and Mg are significantly higher in the roots and shoots of mycorrhizal plants at 0.05% level of probability.

Table 19.13 Elemental composition (N, P, K, Ca and Mg) of needles of 6-month-old mycorrhizal and non-mycorrhizal seedlings of *Pinus gerardiana* Wall. ex D. Don

Nutrient Element (%)	Mycorrhizal	Non-mycorrhizal	'*t*' value *df* = 8
	Mean ± SE	Mean ± SE	
Nitrogen	0.98 ± 0.04	0.74 ± 0.04	0.80*
	0.96 ± 0.06	0.72 ± 0.06	0.85*
	0.95 ± 0.03	0.76 ± 0.03	1.00*
Phosphorus	1.28 ± 0.02	0.41 ± 0.02	3.25**
	1.27 ± 0.05	0.43 ± 0.05	2.80*
	1.28 ± 0.03	0.39 ± 0.03	3.00**
Potassium	0.63 ± 0.04	0.43 ± 0.04	2.58
	0.67 ± 0.04	0.44 ± 0.04	2.25**
	0.72 ± 0.05	0.49 ± 0.05	2.65**
Calcium	0.36 ± 0.06	0.32 ± 0.06	2.60*
	0.39 ± 0.03	0.36 ± 0.3	2.48**
	0.37 ± 0.04	0.38 ± 0.04	1.90*
Magnesium	0.30 ± 0.02	0.21 ± 0.02	3.25*
	0.28 ± 0.02	0.22 ± 0.02	3.00*
	0.28 ± 0.04	0.26 ± 0.04	3.20*

Each figure represents mean of five readings
SE standard error; *df* degree of freedom
*$P < 0.05$ = significant; **$P < 0.01$ = highly significant

Table 19.14 Nutrient content N, P, K, Ca and Mg of shoots and roots of 6-month-old mycorrhizal and non-mycorrhizal seedlings of *Pinus gerardiana* Wall. ex D. Don

Nutrient Element (%)	Mycorrhizal			Non-mycorrhizal			't' value *df* = 8
	Shoot	Root	Total (SE)	Shoot	Root	Total (SE)	
Nitrogen	0.98	0.76	1.74 ± 0.02	0.68	0.50	1.18 ± 0.02	1.65*
	0.96	0.62	1.78 ± 0.03	0.72	0.53	1.25 ± 0.03	2.65*
	0.98	0.81	1.17 ± 0.02	0.64	0.54	1.18 ± 0.02	1.75*
Phosphorus	1.17	0.98	2.15 ± 0.06	0.39	0.26	0.65 ± 0.06	2.00**
	1.18	0.93	2.11 ± 0.05	0.32	0.27	0.59 ± 0.05	3.65**
	1.11	0.89	2.00 ± 0.07	0.36	0.30	0.66 ± 0.07	3.25*
Potassium	0.54	0.46	1.00 ± 0.01	0.24	0.18	0.42 ± 0.01	2.30*
	0.52	0.50	1.02 ± 0.2	0.26	0.21	0.47 ± 0.02	3.25**
	0.57	0.54	1.11 ± 0.01	0.25	0.20	0.25 ± 0.01	2.65*
Calcium	0.32	0.30	0.62 ± 0.03	0.24	0.22	0.46 ± 0.03	2.80*
	0.34	0.32	0.66 ± 0.04	0.21	0.18	0.39 ± 0.04	2.65*
	0.31	0.33	0.64 ± 0.01	0.23	0.21	0.44 ± 0.01	3.00**
Magnesium	0.28	0.26	0.54 ± 0.02	0.18	0.16	0.34 ± 0.02	1.65
	0.24	0.23	0.47 ± 0.02	0.20	0.17	0.37 ± 0.02	2.65*
	0.23	0.21	0.44 ± 0.03	0.18	0.13	0.31 ± 0.03	2.65*

Each figure represents mean of five readings
SE standard error; *df* degree of freedom
*$P < 0.05$ = significant; **$P < 0.01$ = highly significant

Discussion

Zak (1971) emphasized that the mycorrhizal research is hampered by the lack of practical system to characterize and identify natural mycorrhizae of forest trees. Ideally, identification of mycorrhizae should include both tree and fungus species. He discussed characterization and identification of Douglas-fir mycorrhizae and pointed out that the best method to identify a fungal symbiont is to link the sporocarp to underlying mycorrhizae by rigidly comparing the respective mycelia. Aegerer (1986) refined the criteria significantly for characterization and identification of mycorrhizae. He simultaneously explained that this type of characterization is limited by the production of sporocarps. Alternatively, he listed some key characters for the identification of mycorrhizae. The same characters have been used for characterizing and identifying the mycorrhizae of *P. gerardiana* during the present studies. It was not possible to identify and name the fungal symbiont though its mycelium has been isolated in pure culture.

In the three different types of mycorrhizae in *P. gerardiana,* i.e. white creamish, yellowish brown and blackish brown, it was not known whether they were formed by different fungal symbionts or by single fungal symbionts. Marx (1975, 1977) observed that many species of fungi are involved in ectomycorrhizal association

under natural forest conditions. Laiho (1970) and Trappe (1962) reported that many ectomycorrhizal fungi can form mycorrhizae with diverse hosts. Marx (1975) suggested that a single fungus species may also enter into ectomycorrhizal association with numerous trees species. Zak (1969) described two distinct Douglas-fir mycorrhizae.

Mycorrhizal inoculation is practised as normal routine in a new nursery to ensure the development of mycorrhizae and healthy growth in the seedlings. In *P. gerardiana* seedlings inoculated with fungal symbionts, there was increase in shoot height, length of root, stem diameter and growth as presented in Table 19.3, compared to non-inoculated control seedlings. The inoculated seedlings produced abundant roots and side branches. The percentage of the development of ectomycorrhiza in inoculated seedlings was significantly higher. The inoculated seedlings were also stouter than control and produced branches more profusely. Shoulders (1972) had reported that plants inoculated with mycorrhizal fungi normally show striking increase in growth.

Ability of plants to grow normally under conditions of water stress has been termed drought resistance (May and Milthorpe 1962). Water stress is known to affect almost every aspect of plant growth and development (Gates 1968). Since the mycorrhizal seedlings tolerated more water stress than the non-mycorrhizal seedlings, they also sustained better growth under water deficient conditions than the control. Water stress reduces turgor pressure affecting cell membrane, cell enlargement and cell division (Hsiao, cell membrane 1973; Whitmore and Zahner 1967; Joyce et al. 1983). These cellular aberrations occurring due to induced moisture tension may account for defoliation, serving as biological adaptations for conserving moisture (Hall et al. 1977). A progressive and continuous decline in turgor of plants has been observed which results from an initial increase in transpiration rate than absorption rate under stress (Slatyer 1956).

Results from the needle nutrient percentage and total nutrient percentage in shoots and roots per seedling for chilgoza pine reveal an overall enhanced ability to absorb nutrients in plants with mycorrhiza. The percentage of N as well as of P, K, Ca and Mg is significantly higher in the needles and shoots and roots of mycorrhizal seedlings compared to the non-mycorrhizal ones. The uptake of nutrients N, P, K, Ca and Mg in chilgoza pine is one and a half, three, two and a half, two and again two times than that of non-mycorrhizal plants, respectively.

The ectomycorrhizal fungi, as has been shown, are all able to use ammonium and some amino acids and other simple organic nitrogen compounds for their growth in culture, but different species and even strains vary in their ability to use nitrate. Harley and Smith (1983) stated that the sources of nitrogen which might be available to them are ammonium, simple soluble organic nitrogenous substances and complex, often insoluble, nitrogen compounds. In the coniferous forests, the quantity of nitrate in soil is low. This may be due to the low pH and the presence of phenolic compounds which inhibit organisms which might oxidize ammonium in nitrate in their surface horizons where ectomycorrhizal roots are concentrated. Carrodus (1967) found that the mycorrhiza of *Fagus* that he used did not take up nitrate significantly and did not reduce it. Their inability to absorb nitrate rapidly

was confirmed by Smith (1972) who showed that the rate of entry of nitrate in *Fagus* mycorrhiza was much lower than that recorded for ammonium. This corroborates the results of Harley et al. (1954) who showed that nitrate had a much lesser effect on the uptake of oxygen than ammonium or phosphate.

The uptake of P in the mycorrhizal seedlings of chilgoza pine is significantly higher as compared to non-mycorrhizal seedlings. It has been established by Morrison (1957) and Mejstrik (1970) that the rate of phosphate uptake of mycorrhizal roots is greater than non-mycorrhizal roots of radiata pine, and it has been suggested by McComb and Griffith (1946) that same is true for the roots of Douglas-fir. Gadgil (1971) by using P^{32} found that plants with well-developed mycorrhiza absorbed more phosphate than plants with poorly developed mycorrhiza. In the physiological studies of mycorrhizae, uptake of phosphate has received more attention than that of all other major and minor elements combined. Kormainik et al. (1977) observed that high levels of soluble phosphate in the soil seemingly depress mycorrhizal development, and low levels adversely affect plant growth. Thus, mycorrhizal plants grown in soils with low quantities of available phosphates show heavy mycorrhizal development and grow much better than non-mycorrhizal ones. The soil in which the seedlings of chilgoza pine are raised has generally low content of P, and, therefore, the development of mycorrhiza is profuse. Little or no growth differences, however, are often observed between mycorrhizal and non-mycorrhizal plants when high levels of soluble phosphates are added to the growing medium. Under these conditions, mycorrhizal development may even be quite sporadic.

The problem of damping-off disease in conifer nurseries in India *vis-a-vis* assessment of damage, identity of pathogens and their pathogenicity was discussed by Reddy and Mishra (1970). They screened nine chemical [viz., formalin (250 ml/4 l of water), sulphuric acid (250 ml/4 l of water), captan (20.5 g of 83% dust), thiride (22.5 g of 75% dust), Zineb (26 g of 65.0% dust), Cuman (22.5 g of 80% dust), Brassicol (22.5 g of 75% dust), Blitox (19.3 g of 50% dust) and zinc oxide (17.0 g of 100% dust)] for fungicidal soil treatment to control damping-off diseases in three species of pines. They observed that except Brassicol and zinc oxide, all other chemicals effectively checked the disease. However, only Blitox, Zineb and Cuman controlled the disease, significantly increasing the number of seedlings. Formalin, sulphuric acid, captan and thiram were found to control the disease effectively. However, these compounds decreased the germination, possibly due to the phytotoxic effect of dosages of chemicals and thus resulted in reduced number of seedlings. Bakshi (1974) also reported the effect of eight biocides on growth and mycorrhizal development of *Pinus patula*. Two of these were fungicides, Zineb (19.5 and 13.0 g) and Blitox (14.5 and 9.0 g); one partial soil sterilant, formalin (250 ml); one nematicide, methyl bromide (150 ml); one insecticide, gemmexane (3 g); and one herbicide, fernoxone (3.1 g) per square yard of seedbed. He concluded from the experiments conducted that biocides of these dosages retarded the development of mycorrhiza initially though in course of time, mycorrhiza develops in seedlings.

In the present studies, only two of these chemicals, i.e. formalin and methyl bromide, were used in the experimentation. These two, especially the first one, are being presently used in fumigating the nursery soil before raising seedlings even though these are reported to be phytotoxic but still these are cheap and effective. The dosages being used at present by nursery men are 250 ml/4 l of water of these chemicals. Studies were conducted even earlier (Reddy and Mishra 1970; Bakshi 1974) with these chemicals, but observations in those studies were made primarily as to find out how far these chemicals control the damping off of the seedlings. In the present studies, however, the emphasis was twofold; firstly to control the damping off of the seedlings and secondly to see at which dose the mycorrhizal development is normal.

Therefore, during the present studies, dosages higher and lower than the one being presently practised were used in the experimentation to see as to which dose can be most suitable for controlling damping off and for allowing good development of mycorrhiza. Out of the six doses used in each case, it was found that 210 ml of formalin in 4 l water and 130 ml of methyl bromide in 4 l of water are the doses which control damping off of the seedling of *Picea smithiana* without suppressing the mycorrhizal development. Similarly in *P. gerardiana*, out of the eight doses used in each case, i.e. formalin and methyl bromide, it were dosages of 190 ml/4 l of water and 110 ml/4 l of water, respectively, which controlled the damping-off fungi but did not suppress the mycorrhizae development. These doses are much less than the doses which are being presently used. The doses worked out during present studies are therefore cheaper and economical.

Ectomycorrhiza is also known to function as biological deterrents to infection of the feeder roots by pathogens, such as species of *Pythium* and *Phytophthora*. But even before seedlings develop ectomycorrhiza, they may be killed by damping-off fungi (Marx 1976). Pawuk et al. (1980) estimated the effect of seven fungicides in ectomycorrhizal development of container grown *Pinus palustris* seedlings. It was observed that the degree of ectomycorrhizal development differed significantly among fungicide treatments.

Greater soil exploitation by mycorrhizal roots as a means of increasing phosphate uptake is well established. The main question, which is still unresolved, is whether the mycorrhizae are able to exploit phosphates in the soil not available to non-mycorrhizal roots or whether mycorrhizae are just more efficient in capturing the available soluble phosphates. Sanders and Tinker (1971) and Hayman and Mosse (1972) have demonstrated that the insoluble phosphate sources were not exploited for absorption by mycorrhizal plants. Smith (1974) reported that mycorrhizal plants absorb and accumulate more phosphates than non-mycorrhizal plants, especially when grown in soils low in this nutrient. He further observed that this increased uptake by mycorrhizal roots may be due to a more efficient translocation of phosphates by external hyphae. Marx (1980) observed that the results from studies on increased uptake of the other major and minor elements by mycorrhizal plants have been even more variable than those concerning phosphate. With regard to the absorption of K and Ca, Harley and Wilson (1959) observed that K is absorbed readily and accumulated in the sheath, whereas Ca was found in

polyphosphate granule (Strullu 1982; Strullu et al. 1983). Clement et al. (1977) have shown that on calcareous soils, the excessive uptake of K and Ca is reduced by the effect of mycorrhizae. Investigations of Mention and Plassard (1983), Rygiewicz et al. (1984) and Bledsoe and Rygiewicz (1986) pointed out that the high rates of ammonium uptake decrease uptake of K, Ca and Mg in several mycorrhizal mycelia. If this can be proven for mycorrhizal roots too, it may give an additional explanation for the observed Mg deficiency in damaged trees with yellowing of needles (Kenk et al. 1984).

Hatch (1937) observed that mycorrhizal seedlings contained higher proportions of N, P and K than non-mycorrhizal seedlings, and McComb (1938) concluded that P uptake especially was promoted. A number of other researchers have confirmed that mycorrhizae increase phosphorus concentrations in the host. By using radioisotope technique, Mcially was promoted by infection; innumerable researchers have confirmed that Elin et al. (1958) demonstrated that the fungal symbionts are able to transfer carbon (C^{14}), nitrogen (N^{15}), phosphorus (P^{32}), sodium (Na^{22}) and calcium (Ca^{45}) from nutrient solution into the plant by considerably larger quantities as compared to non-mycorrhizal plants. Increased nutrient uptake was related to the increased solubility of nutrients in soil in the presence of mycorrhizae which may result from higher metabolic activity of mycorrhizal roots. This correlation has been made in studies of McComb and Griffith (1946) and Kramer and Wilbur (1949). Absorption of nutrients by the mycorrhizal roots greatly depends upon fungus sheath around mycorrhizae and the "Hartig net". Considerable variation in mantle thickness occurs between the fungi, which accompanies mycorrhizal transformation and increases volume of the root over that of uninfected laterals, and this alone will increase uptake of nutrients. Another general feature of uptake of nutrients is that it is more closely related to volume than to the length of surface area (Russell and Newbould 1969). In general, it can be concluded from the results that growth and survival of chilgoza pine seedlings were significantly improved by ectomycorrhiza by modifying elemental composition of the seedlings. Seedlings with ectomycorrhizal fungus had root system that was capable of increased absorption of nutrients. The higher percentage of these nutrients in mycorrhizal seedlings apparently seems to be due to the ectomycorrhizal fungi only.

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Role of Phosphate-Solubilizing Fungi in Sustainable Agriculture

20

Gurdeep Kaur and M. Sudhakara Reddy

Abstract

A variety of nutrients such as nitrogen (N) and phosphorus (P) are generally used by crop plants extensively. Available P is one of the prominent limiting and decisive factors that determine the productivity of many agricultural soils. Mineral resources such as rock phosphate (RP) have potential to restore soil phosphorus content. The main drawback associated with the use of RP directly to the soil is that the release of phosphate is often not enough for uptake by crop plants. Phosphate-solubilizing microorganisms (PSM), especially plant growth-promoting fungal stains (PGPF), have the potential to solubilize insoluble P to soluble forms through chelation and ion exchange processes, organic acid production, thus making phosphorous available to plants. Therefore, the use of phosphate-solubilizing fungi (PSF) along with RP is considered to be a cost-effective means for facilitating the P availability. Application of biofertilizers reduces the adverse effects of chemical fertilizers on the health of plant vis-à-vis the fertility of the soil. Microorganisms as inoculants serve as integrated solutions to agro-environmental problems as they play a significant role in plant growth promotion, increase nutrient availability and support the health of plants. Hence, biofertilizers along with RP are used as an alternate source, which are both economic and eco-friendly. This chapter thus focuses on the role of PSM on P solubilization, plant growth promotion, PSF along with RP in managing a sustainable agricultural system for crop production.

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Chemical fertilizers · Phosphorous · Crop yield · Plant nutrition

Introduction

In order to meet the increasing demand of food supply, a great deal of efforts focusing on the agro-ecosystem requires a better understanding of the process and associated interactions that govern the stability of agricultural lands. Phosphorus (P) serves as the most important macronutrient after nitrogen required for the plants. Phosphorus is reported to be a limited factor due to its non-availability in soluble forms in soils of many crop production systems (Xiao et al. 2011). It plays a significant role in important metabolic processes including energy transfer, respiration, photosynthesis, signal transduction, macromolecular biosynthesis and fixation of nitrogen in legumes (Saber et al. 2005; Khan et al. 2010). Phosphorus limits the agricultural production in the next millennium due to lack of atmospheric sources like nitrogen which can be made biologically available (Ezawa et al. 2002).

The soluble form of P available for plant uptake is only 0.1% of the total P because of its presence in soil as an unavailable form (Zhou et al. 1992). Generally, the P is taken up by plants in diverse forms of orthophosphate ions (HPO_4^{-2}, HPO_4^{-1} and PO_4^{-3}) and these are available in the order of $H_2PO_4^{-1} > HPO_4^{-2} > PO_4^{-3}$. Availability of these ions is mainly dependent on soil reactions. At lower pH (pH of 4–5), usually $H_2PO_4^{-1}$ ions exist, and with rise in pH, first HPO_4^{-2} ions are formed which will then be converted to PO_4^{-3} as the soil becomes alkaline. Large amount of P applied in the form of fertilizer is converted into the immobile forms by precipitation reaction with Fe^{3+} and Al^{3+} in acidic soil and Ca^{2+} in normal or calcareous soils (Gyaneshwar et al. 2002). Because of this, most of the P is fixed in the soil and requires supplementation of P through chemical fertilizers. This will not only incur a major cost of agricultural production but also affects soil health (Tilman et al. 2002). Continuous and uncontrolled applications of chemical P fertilizers affect the fertility of the soil (Gyaneshwar et al. 2002) which in turn influence the microbial diversity and crop yields. Some of the microbial activities affected due to chemical fertilizers are inhibition of substrate-induced respiration by constituents such as streptomycin sulphate and actidione and microbial carbon (Bolan et al. 1996). Chandini and Dennis (2002) reported significant reduction in respiration and metabolic quotient of microbial systems due to the application of triple superphosphate. It has been reported that the efficiency of P applied in the chemical form do not exceed above 30% because of its fixation as calcium phosphate in neutral to alkaline soils or iron/aluminium phosphate in acidic soils (Norrish and Rosser 1983; Lindsay et al. 1989).

The availability of P in the soluble state is of high agronomic value, and application of chemical phosphatic fertilizers is expensively increasing the need for alternative sources (Relwani et al. 2008). The reserves of rock phosphates and other deposits were considered as promising sources for P fertilizer (Reddy et al. 2002). In recent years, the use of rock phosphates as fertilizer received a significant interest. In India, significant amounts of phosphatic rock deposits are available and this phosphate source should provide a cheap and alternative source of P fertilizer (Halder et al. 1990). It has been reported that in soils with a pH above 5.5–6.0, rock phosphate is generally not bioavailable, even conditions are optimal, and crop yields are lower compared with soluble P (Khasawneh and Doll 1978). In recent years, attention has been paid to the microbial products, which have significant agricultural value. Microorganisms produce secondary metabolites and some of which have phosphate-solubilizing capabilities. Phosphate-solubilizing microorganisms use diverse variety of processes such as acidification, chelation and exchange reactions (Gerke 1992), for facilitating the P solubilization.

Although microbial inoculants are in use for improving soil fertility during last century, not much work has been reported on phosphate-solubilizing microorganisms as compared to nitrogen fixation (Anand et al. 2016). The P in the soil has been managed by manipulating soil and rhizosphere processes, improving P recycling efficiency and developing P-efficient crops (Sharma et al. 2013). Phosphate-solubilizing microorganisms are capable of converting inorganic and organic soil P by solubilization and mineralization processes and make bioavailable form uptake by plant roots (Khan et al. 2009).

Filamentous fungi such as black *Aspergilli* and some *Penicillium* species are widely used as organic acid producers (Mattey 1992) and were capable of solubilizing rock phosphates (Asea and Kucey 1988; Gaur 1990; Vassilev et al. 1996; Goenadi et al. 2000; Narsian and Patel 2000; Reddy et al. 2002; Singh and Reddy 2012). As a result of this activity, P-solubilizing fungi play an important role in supplementing P to plants and allowing the sustainable use of P fertilizers. It has been reported that inoculation of P-solubilizing microorganisms in soil amended with RP to increase P availability (Reyes et al. 2002) and improve the physicochemical, biochemical and biological properties of RP-amended soil (Caravaca et al. 2004; Kaur and Reddy 2014).

Rock Phosphate

It has been estimated that P fertility levels in majority of the Indian soils (80%) fall under low or medium, which reinforces application of external supply of P through fertilizers (Subba Rao et al. 2015). This creates an alarming situation, where about 90% of the P consumption is met through imports in the forms of either fertilizers or raw materials/intermediaries. In the year 2008, the prices of RP escalated by 800% which resulted in the central fertilizer subsidy of 65,550 crore rupees on fertilizers (Fertiliser Statistics 2011). RP is the only source of P for the production of

phosphate fertilizers. In India, the resources of RP are about 296.3 mt (Indian Minerals Yearbook 2011) and these resources have potential to reduce the cost of fertilizers. Rock phosphate is recommended for direct application in many states of India. Some of the constraints are limiting its use which mainly includes the following: (i) variation in composition of the available RP is variable, (ii) non-availability of RP in the open market, (iii) limited understanding of the soils on which RP is effective and (iv) non-support from the extension services (Dev 1998).

The use of P-solubilizing microorganisms such as *Pseudomonas striata*, *Aspergillus awamori* and arbuscular mycorrhizae is taken for effective dissolution of RP. Understanding of natural physicochemical processes associated with P sequestration by the plants can initiate and promote agents that are target-specific. It is essential to develop efficient P-solubilizing microorganisms by recombinant DNA technology for effective utilization of P in RP (Subba Rao et al. 2015). Production of organic acids and subsequent solubilization of rock phosphates by microbes can therefore be an effective alternative to harsh chemical fertilizers (Kang et al. 2002; Maliha et al. 2004; Zaidi 1999; Gull et al. 2004). RP can be sourced from biogenic, igneous, sedimentary, metamorphic and other similar rock matrices. Fluorapatite, hydroxylapatite, carbonated-hydroxylapatite and francolite are the P-bearing minerals in RP (Van Straaten 2002). Carbonate-substituted forms of such as francolite solubilize and facilitate release of P for plant use (Anderson et al. 1985), more readily than other forms of apatite. Chien and Menon (1995) indicated that other factors such as crop varieties and associated management practices also play a role in RP solubility (Chien and Menon 1995).

Engelstad et al. (1974) reported that available P from RP increases with lower pH of the soil. Addition of RP is expected to replenish plant-available P as well as exchangeable pool of P providing alternative for chemical P fertilization. The use of RP application is not feasible in soils with high in pH and P sorption; and low in rainfall, cation exchange capacity, organic matter content as well as microbial activity. Keeping this in view, there is increasing emphasis towards manipulating RP, i.e. biosolubilization of RP using phosphate-solubilizing microorganisms for enriching the value of RP (Vassilev and Vassileva 2003).

Phosphate-Solubilizing Microorganisms

Phosphorus fertilizers added to the soil, part of which is utilized by the plant and other will be fixed in insoluble form (Mehrvarz and Chaichi 2008). Phosphate-solubilizing microbes dissolving bound phosphates improve the P availability, since it is scarce/negligible from RP under neutral and alkaline conditions. Microbial involvement of inorganic phosphate solubilization was early found in 1903 by incubating tri-calcium phosphate with bacteria from milk and soil infusions. Subsequently, Sackett et al. (1948) using the agar plate technique provided conclusive evidence to show that soil bacteria dissolve bone meal and rock

phosphates in addition to di-calcium phosphate, tri-calcium phosphate, etc. Further, Pikovskaya (1948) reported the solubilization of P by microbes. Increase in yield and phosphate uptake by tomato and wheat due to *Bacillus megaterium* var. *Phosphaticum* were reported by Sundra Rao and Sinha (1963).

The knowledge on P-solubilizing microorganisms (PSM) has significantly increased during the last two decades (Richardson 2001; Rodriguez and Fraga 1999). PSM include different groups of microorganisms, bacteria and fungi in particular which have been reported to solubilize insoluble phosphatic compounds (Fig. 20.1). Numerous rhizosphere microorganisms possess P-solubilizing ability (Pandey et al. 2008) which include the P-solubilizing bacteria such as *Bacillus* and *Pseudomonas* (Illmer and Schinner 1992) and fungi such as *Aspergillus* and *Penicillium* (Wakelin et al. 2004).

The most efficient P-solubilizing strains among bacteria are *Pseudomonas striata* and *Bacillus polymyxa* and among fungi are *Aspergillus awamori*, *A. niger* and *Penicillium digitatum* (Ostwal and Bhide 1972; Reddy et al. 2002). Dave and Patel (2003) while comparing the solubilization of various insoluble inorganic phosphates by *Pseudomonas* isolates observed the following trends of solubilization of different P sources: bone meal > TCP > DCP > iron phosphate > Senegal rock phosphate > aluminium phosphate. Reyes et al. (2006) studied the biodiversity of PSM of rock phosphate mines and observed more diversity in the rhizosphere than in the bulk soil. P-solubilizing organisms are ubiquitous in different ecosystems and vary in their density and ability to solubilize from one soil to another. About 1–50% of bacteria and 0.1–0.5% of fungi contribute to P solubilization in total microflora population in a given soil (Wakelin et al. 2004). These strains are isolated from rhizosphere and non-rhizosphere soils, rhizoplane, phyllosphere as well as RP deposit areas using serial dilution method or enrichment culture technique (Zaidi et al. 2009). Kucey (1983) reported that P-solubilizing ability is not lost upon repeated sub-culturing under laboratory conditions in fungi as it happens with bacteria.

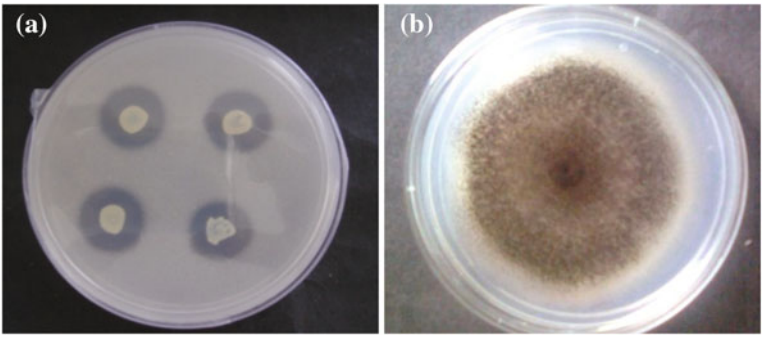

Fig. 20.1 **a** Phosphate-solubilizing bacteria and **b** phosphate-solubilizing fungi showing zone of solubilization on Pikovskaya's agar plates

Aspergilli and *Penicillium* are organic acid producers (Mattey 1992) and therefore have been widely tested for rock phosphate solubilization (Narsian and Patel 2000), though *Trichoderma* (Altomare et al. 1999) and *Rhizoctonia solani* (Jacobs et al. 2002) have also been reported as P solubilizers. The physicochemical and biological properties of RP-treated soils have shown improvement due to inoculation of P-solubilizing and mycorrhizal fungi. Such fungal treatments also resulted in higher available P, organic carbon, lowering of soil pH and significantly high enzymatic activity in soils (Singh and Reddy 2011). Among P-solubilizing microorganisms, fungi perform better in acidic soils than bacteria (Ahmad and Jha 1968; Nahas 1996). *Aspergillus*, *Penicillium* and *Fusarium* species showed higher P-solubilizing potential than *Bacillus subtilis* and *B. megaterium* (Rajankar et al. 2007).

Generally, more acid production is observed in P-solubilizing fungi than bacteria exhibiting higher P-solubilizing activity (Venkateswarlu et al. 1984). The fungal hyphae form larger matrix in soil making better nutrient availability to plant including phosphates (Marmeisse et al. 1998). Crop productivity is increased by inoculating P-solubilizing fungi, *Aspergillus niger*, *Curvularia lunata*, *Rhizoctonia solani* and *Fusarium oxysporum*. In another study, two isolates of *A. niger* and *A. tubingensis* have shown the highest RP solubilization ability (Reddy et al. 2002). Rudresh et al. (2005) investigated P solubilization abilities of nine *Trichoderma* isolates and compared with *B. megaterium* var. *phosphaticum*. The nematofungus *Arthrobotrys oligospora* solubilized various types of phosphate rocks (Duponnois et al. 2006). Achal et al. (2007) developed mutants (phenotypic) of *A. tubingensis* by UV irradiation exhibiting higher P solubilization when rock phosphate was used as P source compared to its wild type. The mutant strains also showed higher enzymatic (acid phosphatase and phytase) activity. The number of P-solubilizing microorganisms varies with soil type and their nutrient status along with other environmental factors. Few studies have been reported to assess the P solubilization abilities of yeasts which include *Yarrowia lipolytica* (Vassilev et al. 2001), *Schizosaccharomyces pombe* and *Pichia fermentans.*

P Solubilization Mechanisms

The prominent mechanisms applied by soil microorganisms for P solubilization are: (i) release of organic acids, protons, hydroxyl ions, CO_2 and siderophores that dissolve or complex with compounds for release of P; and (ii) secretion of extracellular enzymes (McGill and Cole 1981). Microbial communities play important role through processes that facilitate dissolution to precipitation, sorption to desorption and mineralization to immobilization, the three major components of the soil P cycle.

Inorganic P Solubilization

P-solubilizing microorganisms solubilize inorganic P forms by exudating the organic acids which (i) lower the pH, (ii) enhance cation chelation, (iii) compete with P for adsorption sites on the soil or (iv) form metal complexes with insoluble P (Ca, Al, Fe), and thus, P is released.

Organic Acid Production

Solubilization of phosphate is a result of acidification of the periplasmic space followed by the region surrounding the cell/colony due to glucose oxidation. It results gluconic acid production through activity of quinoprotein glucose dehydrogenase. Depending upon the organism, gluconic acid undergoes additional oxidation resulting in 2-ketogluconic acid production. Organic acids produced by P-solubilizing microorganisms are summarized in Table 20.1.

Acidification

Organic acids generally dissociate into anion(s) and proton(s) in a pH-dependent equilibrium. By shifting the equilibrium of the dissolution, the H^+ ions favour P solubilization resulting in release of more P into solution. Organic acids also buffer the pH and continue to dissociate as protons (Welch et al. 2002). Microorganisms export organic acids in the form of anions, which are actively transported through the plasma membrane. In fungi, organic acid anions are transported via H^+-transport system, resulting in acidification (Netik et al. 1997). The major organic acids released by P-solubilizing microorganisms are gluconic, oxalic, citric, lactic, tartaric and aspartic acids (Kim et al. 1997; Bar-Yosef et al. 1999; Venkateswarlu et al. 1984). Organic acids secreted by P-solubilizing microorganisms can be determined by HPLC and enzymatic methods (Whitelaw, 2000). Subba Rao (1982) reported that acidification is not only the mechanism of solubilization and suggested operation of other mechanisms too. Altomare et al. (1999) while investigating the plant growth-promoting activity of *Trichoderma harzianum* T-22 did not detect the exudation of organic acids in the culture filtrates. The P solubilization by inorganic acids such as HCl has also been reported, which was observably lesser than citric acid or oxalic acid at same pH (Kim et al. 1997). The bacterial genera such as *Nitrosomonas* and *Thiobacillus* produce nitric and sulphuric acids to dissolve phosphate compounds (Azam and Memon 1996). P-solubilizing organisms remove and assimilate P from the liquid medium and activate solubilization of calcium phosphate compounds by removing P from culture medium. H_2S production is the other mechanism of P solubilization in which H_2S reacts with ferric phosphate to

Table 20.1 Production of organic acids by different phosphate-solubilizing microorganisms

Microorganisms	Predominant acid produced	References
Aspergillus fumigatus, *Bacillus firmus*, *Micrococcus* spp.	Oxalic acid, tartaric acid, citric acid	Banik and Dey (1982)
Aspergillus sp., *Penicillium* sp., *Bacillus* spp.	Oxalic acid, succinic acid, citric acid, 2-ketogluconic acid	Banik and Dey (1983)
A. niger	Succinic acid	Venkateswarlu et al. (1984)
A. awamori, *P. digitatum*	Succinic acid, citric acid, tartaric acid	Gaur (1990)
A. niger, *P. simplicissimum.*	Citric acid	Burgstaller et al. (1992)
P. bilaji	Citric acid, oxalic acid	Cunningham and Kuiack (1992)
A. niger	Citric acid, oxalic acid, Gluconic acid	Illmer and Schinner (1995)
Pseudomonas cepacia	Gluconic acid, 2-ketogluconic acid	Bar-Yosef et al. (1999)
Aspergillus sp., *Bacillus* spp., *Arthrobacter* sp., *Enterobacter* spp., *Pseudomonas* spp.	Succinic acid	Vazquez et al. (2000)
E. intermedium	2-ketogluconic acid	Hwangbo et al. (2003)
Burkholderia cepacia	Gluconic acid	Lin et al. (2006)
A. niger	Citric acid, oxalic acid, gluconic acid	Illmer and Schinner (1995)
P. cepacia	Gluconic acid, 2-ketogluconic acid	Bar-Yosef et al. (1999)
Aspergillus sp., *Bacillus* spp., *Arthrobacter* sp., *Enterobacter* spp., *Pseudomonas* spp.	Succinic acid	Vazquez et al. (2000)
E. intermedium	2-ketogluconic acid	Hwangbo et al. (2003)

produce ferrous sulphate with concomitant release of P. Sperber (1957) reported the formation of inorganic acids such as sulphuric acid leads to P solubilization.

H^+ Excretion

The excretion of H^+ in microbial systems occurs due to assimilation of cations, primarily related to nitrogen source. In fungi, H^+ is excreted in exchange for NH_4^+ (Asea and Kucey 1988). Rock phosphate is solubilized more in presence of NH_4^+ than NO_3^- as source of nitrogen (Whitelaw et al. 1999). It was also found that pH

was lower when NH_4^+ was used as nitrogen source. In some microorganisms, assimilation of NH_4^+ due to release of H^+ ions seems to be the sole mechanism involved in P dissolution. Phosphate solubilization without the production of acids is due to protons release or ammonium assimilation (Kucey 1983). Illmer and Schinner (1995) reported that release of protons accompanying respiration or assimilation of NH_4^+ might be the reasons for P solubilization without acid production. Krishnaraj et al. (1998) reported that the protons that are pumped out of the cell are responsible for P solubilization. Asea and Kucey (1988) tested RP solubilization of *Penicillium bilaii* and *P. fuscum* in the presence of NH_4^+ or without nitrogen and showed that only *P. bilaii* decreased the pH of the medium and mobilized P without nitrogen. The involvement of H^+ pump in P solubilization is also reported in *P. rugulosum* (Reyes et al. 1999).

Chelation

Chelation is the formation of bonding ions and molecules to metal ions (Whitelaw 2000). The hydroxyl and carboxyl groups of organic acids form stable complexes with cations such as Ca^{2+}, Fe^{2+}, Fe^{3+} and Al^{3+} that are bound with phosphate (Kucey 1988). By forming complex with cations on the mineral surface, anions of organic acid lose cation–oxygen bonds of the mineral structure and catalyse the release of cations into solution. Anions of organic acids alter the dissolution equilibrium reaction by complexing with cations in solution and lower the saturation point of the solution (Welch et al. 2002). The solubility of RP is affected by organic acids based on their chemical structure and the type and position of functional groups of the organic ligand (Kpomblekou and Tabatabai 1994). Narsian and Patel (2000) studied the effect of chelating agents such as EDTA, DTPA, NTA, aluminon and oxine on RP solubilization and reported that RP solubilization is influenced by chelator functional groups particularly carboxylic and phenolic hydroxyls. Gerke et al. (2000) reported that due to ligand exchange between the citrate and the phosphate adsorbed to the Fe and Al sites, P was mobilized than solubilization from Ca–P precipitates.

Organic P Solubilization

Soil organic P mineralization plays an important role in phosphorus cycling in agriculture systems. Organic P constitutes about 4–90% of the total soil P (Khan et al. 2009). Phosphorus is released from organic P compounds into the soil by the action of different enzymes which include:

(a) **Phosphatases**: These enzymes dephosphorylate phosphoester or phosphoanhydride bonds of organic matter. Among different classes of phosphatase

enzymes released by P-solubilizing microorganisms, phosphomonoesterases (known as phosphatases) are the predominant and widely studied enzymes (Nannipieri et al. 2011). These enzymes are categorized as acid phosphatases and alkaline phosphatases based on their pH optima, and both these enzymes are produced by P-solubilizing microorganisms (Kim et al. 1998). Acid phosphatases are more frequent in acidic soils and alkaline phosphatases in neutral or alkaline soils (Renella et al. 2006). Plant roots are also known to produce acid phosphatases but not alkaline phosphatases indicating a potential niche for P-solubilizing microorganisms (Criquet et al. 2004). Though it is difficult to differentiate phosphatases produced by microbes or roots, it has been suggested that microbial phosphatases show high affinity for organic P compounds than plant roots (Tarafdar et al. 2001; Richardson et al. 2009a, b). *Aspergillus* and *Penicillium* species are known to produce phosphatases and phytases (Aseri et al. 2009). High production of acid phosphatase and phytase enzyme by filamentous fungi in culture filtrate was reported by Relwani et al. (2008) and Singh and Reddy (2012). Aseri et al. (2009) reported more phytase activity than phosphatase activity in fungal systems. Richardson et al. (2005) reported that at low soil pH phytase becomes less effective.

(b) **Phytases**: Release of P from phytate degradation is mainly mediated by phytases enzyme. Phytate is the major component of organic P in soil, major stored form of P in plant seeds and pollen and primary source of inositol (Richardson 1994). Uptake of P directly from phytate is very limited in plants, but the growth and P nutrition of *Arabidopsis* plants significantly improved when they were genetically transformed with the phytase gene (phyA) derived from *Aspergillus niger* and grown in the presence of phytate (Richardson 2001). Mineralization of phytate in soil is regulated by microorganisms and makes it available to plants (Richardson and Simpson 2011).

(c) **Phosphonatases and C–P lyases**: These enzymes break the C–P bond of organophosphonates (Rodriguez et al. 2006). Considerable variation exists among the organisms in relation to P solubilization. Mechanisms involved solubilization and mineralization of insoluble phosphates by microorganisms in the soils are shown in Fig. 20.2.

Role of Siderophores in P Solubilization

Siderophores are a group of agents with high affinity for iron. Siderophores are produced by majority of microorganisms due to iron deficiency. These compounds act as solubilizing agents by mineralizing iron from minerals or organic compounds in iron-limiting conditions. Among different siderophores known, most of them are produced by microorganisms and plants and some of the siderophores are produced exclusively by some species of microorganisms (Crowley 2007). Many researchers

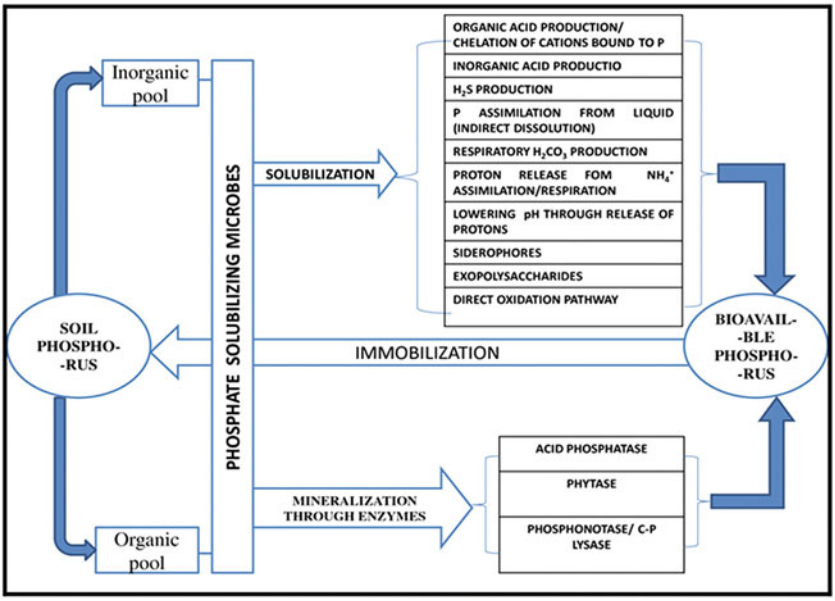

Fig. 20.2 Schematic representation of mechanism of phosphate solubilization/mineralization and immobilization by phosphate-solubilizing microorganisms (Sharma et al. 2013)

have reported the production of siderophores by P-solubilizing microorganisms (Vassilev et al. 2006; Caballero-Mellado et al. 2007; Hamdali et al. 2008).

Plant Growth Promotion by P-Solubilizing Fungi

Plant productivity and immunity are enhanced by plant growth-promoting microorganisms which are associated with plant roots. Mechanisms of plant growth promotion can be grouped into direct and indirect ways. In direct method, microorganisms play an important role in fixation of atmospheric nitrogen, production of plant growth regulators such as auxins, cytokinin, gibberellins and ethylene and solubilization of phosphates. The indirect mechanisms include production of HCN, siderophores, synthesis of cell wall-degrading enzymes, antibiotics and competitions with pathogenic microorganisms for sites on plant roots (Ahemad et al. 2008). The interaction among rhizospheric microorganisms and plant roots has been largely investigated by Rosso et al. (2010), and it is well known that they can improve plant nutrition, water efficiency, bioprotection against pathogens and crop productivity.

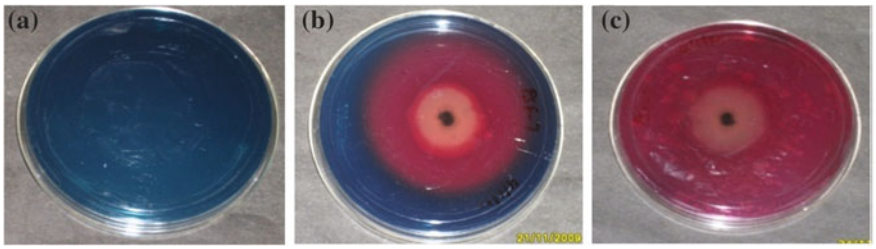

Fig. 20.3 **a** Control (Chrome Azurol 'S' agar plate) and **b–c** point inoculation of P-solubilizing fungi (*Aspergillus*) forming pink-coloured zone surrounding the colony showing siderophore production

Siderophore Production

Siderophores produced by microorganisms promote plant growth directly by enhancing the iron availability in the soil or indirectly by competing with pathogens for iron uptake (Marek-Kozaczuk et al. 1996). Majority of siderophores produced by fungi are hydroxamates except rhizoferrin (carboxylate-type siderophore) produced by Zygomycetes. Majority of the *Aspergillus* species are known to produce siderophores which are hydroxamate type (Fig. 20.3) and were characterized by several researchers (Dube et al. 2000). Rhizospheric population of phytopathogenic fungi and bacteria significantly reduced due to the production of siderophores by many fungi. Siderophores also play an important role in clinical applications as well as in agriculture as chelators (Machuca and Milagres 2003).

Phytohormone Production

Several microorganisms including bacteria and fungi have the ability to synthesize phytohormones apart from the plants. Indole-3-acetic acid (IAA) production was detected in culture filtrates for the first time in *Rhizopus suinus* (Tudzymski and Sharon 1994) before IAA was identified in plants. Many fungi are known to produce different secondary metabolites such as IAA, cytokines and other substances involved in plant growth promotion. Gibberellin production by many plant growth-promoting fungi was reported by several researchers showing the importance of this compound in plant growth and development (Muhammad et al. 2010). Phytohormones influence biochemical, physiological and morphological processes in plants even at extreme low concentrations (Fuentes-Ramírez and Caballero-Mellado 2006).

Fungi as Biocontrol Agents

Fungal-based biological control agents are gaining much acceptance after bacteria due to their broader spectrum of disease control and yield improvement. The genus *Trichoderma* is considered as biocontrol agent acting against many soil-borne plant pathogens (Benítez et al. 2004). Different mechanisms proposed to control the phytopathogens by *Trichoderma* are mycoparasitism, secretion of antibiotics, competition for nutrients, space and cell wall-degrading fungal enzymes (Harman et al. 2004). Apart from these, *Trichoderma* is also known to stimulate plant growth due to modification of soil conditions (Naseby et al. 2000). Non-pathogenic fungi and soil-inhabiting, saprophytic fungi are beneficial to many crop plants as they not only promote the growth but also protect them from diseases (Pandya and Saraf 2010). Many of these fungi include the genera of *Trichoderma*, *Verticillium*, *Aspergillus*, *Penicillium* and others. Isolates of *Penicillium*, *Paecilomyces* and *Aspergillus* are known to have antagonistic effect on soil-borne pathogens in the rhizosphere of black pepper (Noveriza and Quimio 2004). It has been reported that *T. hamatum* 382 is capable of reducing diseases of vegetable crops by altering the genes involved in stress and protein metabolism (Khan et al. 2004). *Trichoderma* species also known to help plants to tolerate against abiotic stresses by increasing the secondary root length deep into the ground and provide protection against drought by improving the water-holding capacity (Mastouri et al. 2010).

Synergistic Effect of Phosphate-Solubilizing Fungi and Nitrogen-Fixing Microorganisms

Inoculation of phosphate-solubilizing microorganisms along with nitrogen fixers benefits the plants more than individual group of organisms (Sarojini et al. 1989). Revathi et al. (2013) reported that under normal soil conditions, inoculation of *Rhizobium* and arbuscular mycorrhizal fungi improved the growth and biomass of Shisham while in alkaline soil blending micronutrients along with *Rhizobium* arbuscular mycorrhizal fungi. Hence, mixed cultures or co-inoculation with other microorganisms are beneficial to plants over individual inoculation. Gull et al. (2004) reported that nitrogen fixers and P-solubilizing microorganisms enhanced the growth of legumes by increasing nitrogen and phosphate in the soil. The nitrogen status of soil can be enhanced when nitrogen-fixing organisms are inoculated in combination with PSM. Under field conditions, the chickpea yield significantly increased due to *Rhizobium* and *A. awamori* inoculation (Dudeja et al. 1981). Inoculation of *A. niger*, *A. fumigatus*, *P. pinophilum* and *Rhizobium leguminosarum* improved the growth, yield, uptake of phosphorus and nitrogen of *Vicia faba* under field conditions (Mehana and Wahid 2002). In contrast, Kucey (1987) reported that inoculation of beans with *Penicillum bilaji* and *R. phaseoli* in sterilized soil did not increase the growth and P uptake. Further, inoculation of *Penicillium bilaji* and *Rhizobium leguminosarum* showed decreased levels of total

nitrogen fixation in field peas suggesting the adverse effect of *Penicillium bilaji* on nitrogen fixation (Downey and Van Kessel 1990). Phosphate-solubilizing fungi secrete organic acids which enhance the solubilization of phosphates, while for nodulation, rhizobia prefer neutral or alkaline conditions (Venkateswarlu et al. 1984). These results suggest that the compatibility between the two associates needs to be tested in vitro before in vivo studies.

P-Solubilizing Fungi in Field Trials

The most important constraint which limits the crop yields in developing nations worldwide is soil fertility. It is essential to increase the soil fertility by using improved varieties and productive cultural practices. In general, crop yields can be enhanced with increase in available P by the application of P fertilizers. However, the cost of chemical fertilizers is increasing due to dwindling resources and global energy crisis. To ensure competitive yields of crops, agronomists are looking for an alternative source of P fertilizers to replace the chemical fertilizers. Microorganisms are actively engaged in P transformation, and the use of P-solubilizing microorganisms in agronomic practices improves soil fertility. They also increase crop yield, soil heath and conditioning, protect plants against some soil-borne pathogens and do not cause environmental pollution (Khan et al. 2007).

The effects of PSM inoculation for the crop growth and yield have been reported (Zaidi and Khan 2005). Jisha and Mathur (2005) reported high yield and nutrient status of *Triticum aestivum* due to *A. niger* and *P. vermiculosum inoculation*. Dual inoculation of *A. niger* and *P. italicum* showed significant increase in growth and yield of soybean plants (El-Azouni 2008). Mittal et al. (2008) reported the stimulatory effect of *A. awamori* and *P. citrinum* on the yield of chickpea. The primary role of soil fungi is to degrade organic matter and help in aggregation of soil. Apart from this, some species of *Alternaria*, *Aspergillus*, *Cladosporium*, *Dematium*, *Gliocladium*, *Helminthosporium*, *Humicola* and *Metarhizium* were able to produce humic substances in soil to maintain soil organic matter (Pandya and Saraf 2010).

P-Solubilizing Fungi and Rock Phosphate

Biofertilizers are considered the most effective plant growth promoters to supply phosphorus in P-deficient soil. It has been shown that the use of P-solubilizing fungi and bacteria increases the efficiency of the soil in providing available phosphorus to the plants (Mehrvarz and Chaichi 2008). Compared with chemical treatments, microbial solubilization of rock phosphate is an environmentally sustainable approach (Vassilev and Vassileva 2003). Among the sources of P, rock phosphate and pyrite provide best sources to enhance the crop yield. This basic idea has forced

the scientists to see the effect of phosphate-solubilizing microorganisms, when applied in conjunction with low-grade rock phosphates, and the results obtained were overwhelming. Inoculation of P-solubilizing bacteria, *B. megaterium* and *P. striata* and fungus *A. awamori* improved the yield of legumes when soil is amended with low-grade rock phosphate in both neutral and alkaline soils (Dubey and Billore (1992). *Aspergillus tubingensis* and *A. niger* were able to solubilize rock phosphate and improves the growth of maize in RP-amended soils (Richa et al. 2007; Pandya and Saraf 2010; Reddy et al. 2002).

To increase the availability of P from RP, most experiments using P-solubilizing microorganisms employ two major strategies: (i) optimize soil microbial populations to mobilize P and (ii) specific microbial inoculum development (Richardson 2001). Babana and Antoun (2006) demonstrated that inoculation of P-solubilizing microorganisms in RP-amended soils improved the wheat grain yields and these results are comparable with those produced by the expensive di-ammonium phosphate fertilizer. Singh and Reddy (2012) reported a significant increase in yield and total P in maize and wheat crop when *A. niger* and *A. tubingensis* were inoculated along with rock phosphate solubilization compared to bio-inoculation treatment alone. RP-fertilized soil inoculated with these isolates also showed higher organic carbon than non-inoculated soils, which may be due to the production of microbial organic colloidal materials and solubilization of P.

P-Solubilizing Fungi and Chemical Fertilizers

Excessive application of chemical fertilizers to increase the yield per unit area is one of the severe problems the modern agriculture is facing which is causing pollution of environmental as well as disgrace of the land resources (Abbasdokht and Gholami 2010). Huge amounts of synthetic fertilizers are applied to soil to reload nitrogen and phosphorus in the soil causing harmful effects on environment. Biofertilizers substitute the synthetic fertilizers and help in restoration of environment (Whitelaw 2000). Increasing cost of chemical fertilizers and negative environmental effects is the major reason to search for an alternative P sources. Phosphate rocks have been considered as alternative valuable sources for P fertilizers. Inoculation of P-solubilizing microorganisms along with RP significantly increased P uptake, and the results are comparable with that of chemical fertilizers (Rodriguez and Fraga 1999; Singh and Reddy 2012; Swarnalakshmi et al. 2013). Plants fertilized with biofertilizers increased the yield and total P uptake, indicating its potential as a low-cost alternative to expensive soluble fertilizers. Kaushik et al. (2004) reported that 30 kg P_2O_5 as single superphosphate can safely be replaced with 30 kg P_2O_5 RP in the presence of phosphorus-solubilizing bacteria. The yield of paddy and wheat significantly increased along with the soil health in terms of carbon build-up and available phosphorus. Kaur and Reddy (2015) reported the P-solubilizing fungi in RP-amended soils and the economics involved with the chemical fertilizers. Economic analysis of the maize crop in the first year showed

highest returns due to inoculation. The net returns were also more in the second year due to RP fertilization and inoculation, and the expenditure of chemical fertilizer was more than fungal treatments. RP fertilization along with fungal inoculation yielded better economic returns due to their low cost as compared with DAP (Kaur and Reddy 2015). Several other authors have also reported that inoculation and RP fertilization showed promising results compared to chemical fertilizers (Sharma and Prasad 2003; Swarnalakshmi et al. 2013).

Conclusion

The requirement of P in agriculture can be met by the application of chemical fertilizers, but excessive application of fertilizers not only poses environmental problems but also affects soil health. Phosphate rocks (RP) have been considered as an alternative for P fertilizers. Normally, in soils with a pH more than 5.5–6.0, RP is not plant available. Application of P-solubilizing microorganisms to these soils solubilizes RP and makes them plant available to increase the crop productivity apart from sustainable soil health. Therefore, isolation of efficient P-solubilizing microorganisms and application of these microorganisms under field conditions are needed to increase the crop productivity. The use of P-solubilizing microorganisms can eliminate or lower the use of chemical fertilizers, decrease the environmental pollution and improve soil health.

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Biotechnological Advancements in Industrial Production of Arbuscular Mycorrhizal Fungi: Achievements, Challenges, and Future Prospects

21

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Abstract

The recent technological advancements in arbuscular mycorrhizal (AM)–plant symbiosis have helped improve the potential applications of mycorrhizal biotechnology in agriculture, horticulture, landscaping, phytoremediation, and other areas of plant market. The most common conventional methods used for large-scale production of AM fungi include cultivation in pots with sterilized soil, aeroponics, hydroponics, or greenhouse-based in vivo methods. However, these techniques suffer from severe problems of cross-contamination in the inoculum production; therefore, production of high-quality inoculum remains a major challenge. The most advanced method is transformed root organ culture (ROC) to produce AM propagules without adulterated microbial contaminants under strictly controlled sterilized conditions after pure AM fungi are inoculated into the transformed root organ. The scientific breakthroughs and advancements in the field of mycorrhizal research during past two-to-three decades have resulted in new technological developments with different types of products and diverse modes of their applications. For example, mycorrhizal formulations are available in the market for seed coating, liquid applications, or biostimulants. An established symbiosis in the plant roots confirms the adaptation even under unsuitable soil or unfavorable climatic conditions. These advantages have led to an increasing demand for mycorrhiza products in the last few years. There is a growing interest among the enterprises in the developed as well as developing world for the production of mycorrhizae-based inoculum given the fact presented by the emerging market trends in developing economies. However, even today it is not possible to trace out the absolute origin of the fungal

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species/strain used in commercial inoculum. Despite the regulatory challenges imposed by the regulatory bodies to maintain the highest quality standards, a significant number of commercialized products may still be found in the market which claims for extensive and effective mycorrhizal colonization even though they lack the necessary potential for this. This review provides an updated overview of the recent developments in the technology adoption and commercial production of mycorrhizae-based quality inoculum.

Keywords
AM fungi · AM symbiosis · Biofertilizers · *Rhizophagus irregularis* *Glomus* · Mycorrhization · Phytoremediation

Introduction

Mycorrhiza represents a mutually beneficial symbiotic association between roots of higher plant and soil fungi in which the fungal mycelium developed acts as a connecting link between plant roots and the surrounding microhabitats in the vicinity of soil. More than 80% of the land plants are estimated to have a most common type of mycorrhizal association, the arbuscular mycorrhiza (AM) association, and are found in almost all the ecosystems worldwide (Barea et al. 2005). AM fungi are ubiquitous soil-borne fungi and are believed to have been evolved more than 450 million years ago which has led many to suggest that possibly AM fungi had played a significant role in colonization of land by plants (Redecker et al. 2000). The hypothesis is supported by some reports in the recent which describe that some AM species are capable of infecting the most ancient plant lineages (e.g., liverworts) and improve their ecological fitness (Humphreys et al. 2010). The plant–AM association is mutually beneficial with bidirectional flow of nutrients; carbon flow from plant host to the fungus, and flow of mineral nutrients from fungal partner to the host plant. AM fungi may be considered as the mother of plant root endosymbiosis that establishes symbiotic relationships with plants and subsequently plays an essential role in plant growth, protection from pathogens, and overall improvement of the soil quality. In addition to this, AM fungi also impart other important benefits to the host including enhanced drought tolerance (Jayne and Quigley 2014) and unfavorable soil conditions especially salinity (Porcel et al. 2012), deficiency of nutrients, heavy metal stress (Garg and Chandel 2010), and inimical pH level of soil (Rouphael et al. 2015). These plant growth promotory traits of the AM fungi make them potential candidate to be used as biofertilizers.

A major limitation for mass production of AM fungi is their absolute biotrophic nature; i.e., they grow only in the presence of living host plants and are absolutely dependent on them for growth and multiplication. From an evolutionary perspective, the ecological success of AM fungi clearly indicates the advantages of such an

obligate association with plants over the risks arising from the loss of saprotrophic capabilities (Bonfante and Genre 2010). However, still they are globally used in agriculture as biofertilizers, particularly *Rhizophagus irregularis/Rhizophagus intraradices* (formerly known as *Glomus intraradices*) and *Funneliformis mosseae* (formerly known as *Glomus mosseae*) (Kruger et al. 2012). The plant–AMF symbiosis is mutually beneficial and is very important particularly for enhancing the uptake of phosphate ions present in soil which otherwise are either insoluble or relatively immobile because of their interactions with bi- or trivalent cations, especially Ca^{2+}, Fe^{3+}, and Al^{3+} (Fitter et al. 2011). The AM fungi have a unique capacity to develop a network of extraradical hyphae which increase the effective surface area of absorption several folds (up to 40 times) and contribute to the increased uptake of micronutrients also such as Cu^{2+}, Zn^{2+}, and other mineral cations present in the soil such as Fe^{3+}, K^{+}, Mg^{2+}, and Ca^{2+} (Smith and Read 2008; Rouphael et al. 2015). The nutrients' exchange and other benefits in plant–AM fungi are in the purview of contamination-free preservation and propagation of AM fungi for commercial cultivation in vitro.

Ecology of Tripartite Interactions in the Rhizosphere

The interactions between AM fungi, plant roots, and other soil microbes are of crucial importance within sustainable and low-input agricultural cropping systems which rely upon biological processes to maintain soil fertility and crop productivity. The interaction between AM fungi and bacteria may be synergistic that stimulates plant growth through varied mechanisms (improved nutrient acquisition and/or inhibition of pathogens) (Artursson et al. 2006). Given the bacteria living in close association with mycorrhizal roots/sporocarps/spores/extraradical hyphae, it may influence the efficiency and establishment of AM fungi and some species of bacteria have been found to promote the spore germination and asymbiotic hyphal growth of AM fungi. For example, the presence of *Streptomyces orientalis* promoted the spore germination in *Funneliformis mosseae* (formerly known as *Glomus mosseae*) (Mugnier and Mosse 1987). Several bacterial groups live in intimate association with AM fungal spores, more commonly in the outer spore wall layers or in microniches produced by interwoven peridial hyphae around the spores of few *Glomus* species (Filippi et al. 1998). Using denaturing gradient gel electrophoresis (DGGE), it was revealed that species from several bacteria taxa including *Cellvibrio*, *Chondromyces*, *Flexibacter*, *Lysobacter*, and *Pseudomonas* were able to degrade biopolymers such as chitin (main component of outer walls of AMF spores) and thus helps to promote germination of spores (Roesti et al. 2005; Long et al. 2008).

The AM fungi-associated bacteria were also shown to exhibit other plant growth promotory activities, viz. indole acetic acid (IAA) production, phosphate solubilization, N_2 fixation, suggesting the joint application of AM fungi and associated bacteria as bio-inoculants (Bharadwaj et al. 2008; Richardson et al. 2009).

Recently, phosphate-solubilizing bacteria living in the hyphosphere (the zone of soil surrounding individual AM fungal hyphae) of *R. irregularis* have been reported to promote soil phytate mineralization and uptake of plant phosphorus uptake (Zhang et al. 2014). In another study, Agnolucci et al. (2015) evaluated the diversity of bacterial communities found in association with the spores of six AMF isolates from different genera/species. These isolates were maintained under identical conditions for several generations. However, the PCR-DGGE profiles exhibited considerable diversity among the bacterial communities, thereby indicating the presence of different microbial entity harbored on the spores of each isolate. It appears that certain groups of bacteria may be associated with a more intricate manner in the mycorrhizosphere as compared to other groups based on the group specific interactions between AM fungi and PGPR. Further, the work of Artursson et al. (2005) also indicates that AM fungi themselves may have significant impact on the composition of bacterial communities. However, this effect may be relayed through the plant roots because mycorrhizal association may alter root exudates composition, the source of nutrients for bacteria in the mycorrhizosphere (Artursson et al. 2006).

The mycorrhizae occurrence is affected by host plants, environmental factors, and soil conditions (Chakraborty and De 2013). Crop management practices also impact AM association (Rouphael et al. 2015). For example, long periods of monoculture have been found to affect the microbial diversity and community structure of soil (Hijri et al. 2006; Jiao et al. 2011), suggesting that crop rotation in a diversified manner is better for AM fungi. Application of organic fertilizers, viz. manure and compost, and other slow release mineral fertilizers, such as rock phosphate, although do not appear to suppress AMF but in some cases have been reported to stimulate them (Douds et al. 1997; Singh et al. 2011; Fernandez-Gomez et al. 2012; Cavagnaro 2014). Therefore, selection of appropriate organic amendments is recommended before application (Ustuner et al. 2009).

Root Organ Culture: Biotechnological Advancements

The most common conventional methods used for large-scale production of AM include cultivation in pots with sterilized substrates. Greenhouse-based pot cultivation is the cheapest way for AM propagation wherein host plants inoculated with AM fungi are cultivated in inert substrates to maintain and propagate the AM inoculum. At the end of the growing cycle, plant roots colonized with AM and/or soil-containing propagules are harvested and dried and are used as inoculum. The technique, although being simpler in terms of propagation method suffers from serious limitations, such as higher risks of contamination in the inoculum, difficulty in harvesting of spores, and inconsistent production, thus seems unlikely to be adopted as per the industrial requirement. Other tested methods of AM cultivation are hydroponics (where host plants are inoculated with AM fungi in a specialized hydroponic device with nutrient liquid) and aeroponics (where host plants are

inoculated with AM fungi in a specialized aeroponic container where vaporized nutrient liquid is provided). However, in both the methods, the requirement of huge amount of nutrient liquid (in hydroponics) or vaporized nutrient liquid (in aeroponics) with necessary work to monitor and refresh them, and failure to avoid the problem of adulterated microbial and pathogen contaminants are the constraints for their industrial application.

The monoxenic cultivation (in vitro culture system) system for AM production offers pure, sterile, and bulk contamination-free propagules which otherwise is practically difficult to achieve using conventional methods. The cutting edge of this technique allows several folds of increase in spore/propagule production over conventional modes of mass production in lesser time and space. Two types of in vitro systems have been developed for the production of sterile mycorrhizal inoculum (Rouphael et al. 2015): (1) AM fungi is grown and produced on transformed plant roots (induced by Ri-TDA of *Agrobacterium rhizogenes*), popularly known as root organ culture (ROC) (Fortin et al. 2002; Declerck et al. 2005); and (2) AM fungi are produced on autotrophic plants which are grown such that the aerial part of the plant grows outside the Petri dish (Voets et al. 2005) or grows in a sterile tube vertically connected to the Petri dish (Dupre de Boulois et al. 2006). Figure 21.1 provides an overview of the various techniques used for ex vitro and in vitro production of mycorrhizae and the possible outcome of the techniques followed in terms of the quality of the product. ROC-based AM production systems have been successfully adapted for large-scale commercial production of mycorrhizal inoculum (Adholeya et al. 2005). The in vitro production method involves isolation of potential viable propagules from the monosporal cultures (pot cultures raised from single spore) followed by surface sterilization and optimization of growth conditions for spore germination under aseptic conditions. This follows the association of propagules with suitable excised transformed host plant root for production and recovery of propagules. The mass-produced mycorrhizal propagules can be formulated as per the requirement and stored for 1–2 years or more depending upon the shelf life of the product and the storage conditions before application. A comparative account on the mycorrhizal inoculum production between the monoxenic culture systems versus conventional techniques for large-scale production has confirmed the effectiveness of monoxenic system of cultivation (Verma and Adholeya 1996; Douds et al. 2000). The advantages of in vitro culture system include pure and contamination-free concentrated product, are easy to trace out, and can be produced throughout the year. The in vitro product also meets cross-boundary regulations.

Mosse and Hepper (1975) first proposed to use excised roots as host for AM symbiosis. Since then several attempts have been successfully made for culturing the AM fungi (axenic or monoaxenic) (Diop 2003). However, the production yield had been a serious limitation as the recovery of spores (no. of spores/ml of media) was very low. For example, Chabot et al. (1992) established the production of 750 spores in 30 ml of the medium (25 spores/ml) after nearly 120 days of incubation. Similarly, Diop et al. (1994) were able to produce about 890 spores only after an incubation period of 90 days. A significant breakthrough came after an advanced

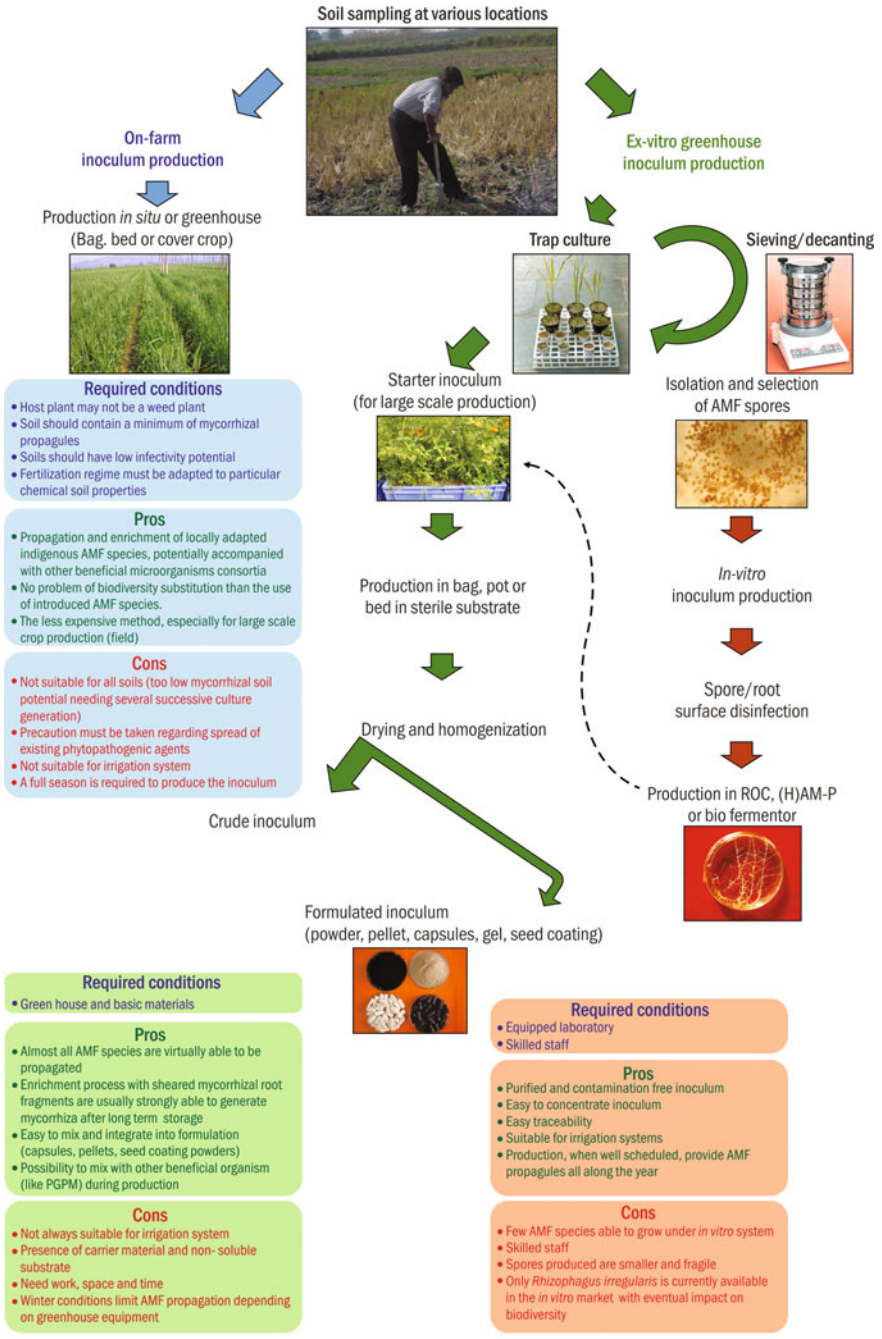

Fig. 21.1 A schematic view of the on-farm, ex-vitro in greenhouse, or in vitro production of AMF inocula in root organ culture or biofermentors. Adapted from Rouphael et al. (2015)

mode of airlift bioreactor-based production system was introduced which resulted in significant increase in production yield, i.e., 12,400 spores/liter of the medium (Jolicoeur et al. 1999). Another study proclaimed of 15,000 spores produced in a bicompartment Petri plate during an incubation of 90–120 days (St-Arnaud et al. 1996). This technique was further improved by replacing the medium with fresh medium at regular intervals of time and spore production increased to 65,000 spores in 210 days (7 months) (Douds 2002). The Centre for Mycorrhizal Research, The Energy and Resources Institute (TERI), New Delhi, India, has successfully translated the nutrient tapping potential of mycorrhizae and developed a technology that produces 250,000–300,000 highly infective propagules from 100 ml of the nutrient medium in 90 days. The evolution of this technology is based on optimization at various levels including identification of several rate limiting steps and their optimization as well as efficient root colonization (70–80%) attained after field application (Tiwari and Adholeya 2003). The technology is less dependent on personnel, therefore reduced the number of man-days, and achieves higher productivity. These improvements have culminated into the higher spore and propagules recovery per unit volume of media as compared to those obtained in previous studies (Adholeya et al. 2005). The technology has proven to be very efficient in terms of time, space, and energy utilization. Further, optimization at various process levels has led to the significant reduction in the contamination levels <5% which otherwise is somewhat 10–15% (common in tropical areas). The advancements achieved in the technology have made a significant impact on mycorrhizae production and transformed into a technologically viable venture for the industries to assimilate and adopt. Till date, the technology has been successfully transferred to some leading companies in the field of agriculture and pharmaceutical in India.

Formulation Development

Formulation development is an important aspect for maintaining the shelf life, uniform distribution, and characteristics of microbial inoculants and therefore determines the success of a biological agent. The characteristics of a good carrier material include: It provides an appropriate microenvironment for the bio-inoculants to thrive and establish and ensures a considerable shelf life of the commercial product (minimum 1 year) under ambient conditions. Moreover, the formulated product should be easily dispersed in the soil in the vicinity of root system. Thus, a good quality carrier material accommodates, if not all, most of the following desired requirements: easily available in adequate amounts, low cost, easy to process, and free-flowing, i.e., no lump formation, sterile as such, or easily sterilizable either under superheated steam (autoclave) or by irradiation (γ-irradiation), good moisture absorption capacity and good pH buffering capacity Malusa et al. 2012). It is clear in light of above requirements that a single universal carrier is not available that fulfills all the desirable requirements; however, one should select a carrier with as many as possible traits. Some carriers that have been used include peat, lignite and coal (Dube

et al. 1980), clays and inorganic soils (Chao and Alexander 1984), compost, farm yard manure, soybean meal (Iswaran et al. 1972), wheat bran (Jackson et al. 1991), press mud (Philip and Jauhri 1984), agricultural waste material (Sadasivam et al. 1986), and spent mushroom compost (Bahl and Jauhri 1986). Besides, several other carriers such as vermiculite (Paau et al. 1991), perlite, rock phosphate, calcium sulfate, polyacrylamide gels (Dommergues et al. 1979), and alginate (Bashan 1986) have also been evaluated. A majority of the carriers studied have been either naturally abundant resources or waste materials.

Usage of sterile carrier has its own advantages such as reduction in contamination level or decontaminated product; however, there are some disadvantages, viz. increase in the production cost, labor cost, requirement of a sterilizing unit, and the packaging of the product under aseptic conditions. Given the fact that in commercial production where addition of each step adds to the cost of product, selection of carrier material is of paramount importance in the process development. Several types of mycorrhizae inoculum formulations have been developed, such as vermiculite (Kuppusamy and Kumutha 2012), glass beads (Redecker et al. 1995), sheared root inocula (Sylvia and Jarstfer 1992), or expanded clay (Plenchette et al. 1983). The formulated product helps to entrap the mycorrhizal spores and roots in a natural manner; for example, the beads which possess highly porous surface bear innumerable air spaces wherein AM fungal propagules can thrive and establish. Redecker et al. (1995) reported that alginate beads could be used to entrap the intraradical spores/vesicles of *Glomus* spp. and subsequent application. The intraradical vesicles from isolated beads possessed the capability to regenerate and recolonize the new plant roots under optimum growth conditions (Strullu and Plenchette 1991).

A good quality biofertilizers normally contain sufficient population of desired bio-inoculants, minimum desired level of viability, and can be stored for longer period without any in-built contamination. The carriers used in general are nearly inert material, form clumps upon drying, and thereby may result in significant reduction of viability. Further, seed is not a favorable environment for many bio-inoculants, as many of them are soil borne, yet seed inoculation is a common practice for microbial inoculation. Hence, it is important for a high-quality biofertilizer to maintain viability of bio-inoculants on seed upon inoculation. The usual carrier-based biofertilizers have some drawbacks for seed inoculation such as seed coat damage, seed coat toxicity, death of cells due to desiccation, or possible contact of microorganisms with agro-chemicals. Currently, advancements in technologies for inoculum development target the major impediments such as improvement of the quality, to extend the useful shelf life and develop new formulations for applications under varied environmental conditions. With regard to this, it seems appropriate to use certain additives in the mycorrhizae inoculum which may have stimulatory effects on mycorrhizae growth or root colonization, for example, flavonoids (Scervino et al. 2009), and addition of mycorrhizal helper bacteria (MHB) or bacteria with synergistic interactions with mycorrhizae (Miransari 2011).

In addition to formulation, it is important to define the application modes. The application modes of inoculum most suitable as per the local cultivation system need to be addressed. For example, in wide range of crops (cereals), there is requirement for developing seed-coating formulations, and in some cases, where drip irrigation is in place, liquid formulations are being used. Innovation of products leading to use of highly concentrated, high quality, and high efficacy which can integrate with the propagation package of practices can significantly increase applicability of mycorrhiza in plant production.

The storage practices of the product should not only be simple and inexpensive but also able to consistently maintain the high quality of the product. The viability and infectivity of AM inoculum can be maintained for several months and years at room temperature (20–25 °C); however, it is recommended that the inoculum be partially dry and stored intact in the original packing. The most common problem encountered during the storage is reduction in percentage germination of spores because some of the spores may become dormant (although the viability is not affected), which can be overcome by giving a brief cold storage treatment (Juge et al. 2002; Oehl et al. 2010). For long-term storage of liquid/dry inoculum produced either in vitro or in vivo, it is recommended that products be stored at 5 °C (Dalpe and Monreal 2004). Regular monitoring and conformation for QC is a crucial aspect.

Industrial Production: Commercial Aspects, Market Analysis, and Challenges

Market research trends have indicated that the global biofertilizer demand, which stood at USD 440.0 million in 2012, is expected to reach USD 1028.7 million by 2019 at a CAGR of 13.0% from 2013 to 2019 (http://www.transparencymarketresearch.com/pressrelease/global-biofertilizers-market.h-tm). The report has hinted the global dominance of biofertilizer market from North America in 2012, owing to the growing demand of organic food in agriculture. However, the highest growth in this sector is expected to occur in the Asia Pacific region because of increasing awareness among the population owing to the new government policies. The production level of biofertilizers in this particular region is expected to significantly increase as a result of favorable policies formulated by the government and rising demand of organic produce by the growing population. Overall, the biofertilizers demand in Asia Pacific is estimated to grow rapidly at a CAGR of 13.3% during 2013–2019.

Among the biofertilizers, in past few years, production of mycorrhizae-based inoculum has increasingly been accepted as a biotechnological advancement (Vosatka et al. 2008a, b). There are numerous examples suggesting the positive impacts of mycorrhizae-based fertilizers in improving the crop production and plant health (reviewed in Rouphael et al. 2015). Nowadays, owing to the increasing interests supported by extensive applied and commercial interests, the production

technology of AM inoculum has been successfully translated to the industrial scale and relies on eco-sustainable aspects of the mycorrhizae (Vosatka and Albrechtova 2009; Gianinazzi et al. 2010; Adholeya et al. 2005). Commercial production of mycorrhizal inoculum began during the early 1980s and flourished in the 1990s. Consequently, several companies nowadays are involved in production and sale of AM-based products indicating the market potential of AM-based products. During the last decade, the numbers of manufacturers of mycorrhiza-based inocula have been increased significantly. According to a study, there are number of small/medium enterprises in Europe alone involved in mycorrhizae production; however, current regulatory norms do not allow the differentiation of the primary producers and from the re-sellers or distributors. Some of companies/enterprises have been taken over by multinational companies which market the product under their trade name all over the world. Although some manufacturers have chosen the single formulation approach others produce a range of products as per the requirement of end users who want the formulation to be applied under varied environmental conditions and to different host-plant groups.

Most commonly, the species of *Glomeraceae* are used as inoculum; however, species from other genera including *Gigasporaceae*, *Scutellosporaceae*, and *Acaulosporaceae* families are also being exploited for inoculum production. These species are also found in symbiotic association with a range of host plants, widely distributed across the globe, and therefore can be used for inoculum production as much efficiently as members of *Glomeraceae*. Although many inoculum-producing companies claim about the wide applicability of their product across a range of host plants and varied environmental conditions, they fail to keep their promises under field performance of the products. The inconsistent performance of the products in the field has imparts radical generalizations, about the efficacy of the products available in the market. The most important is that success of the product, in terms of root colonization and plant response, is unpredictable given the fact that generally no plant species is known to perform best with the same AM inoculum under all the climate, soil conditions, and agricultural practices (Berruti et al. 2014).

The quality control of the AM inoculum is a challenging task that needs to be duly taken care of by the manufacturer in compliance with the regulatory agencies. Therefore, the inoculum manufacturers are expected to fulfill certain criteria: (1) recommending use of the product that forms association with plants after inoculation; (2) the product should be free of undesirable entities (pathogens, contaminants, etc.) which could otherwise cause negative impact on normal growth and development of the plants; (3) while the production costs have to be kept to a minimum, the shelf life of the product should meet the quality standards as per the requirement of end user; and last; (4) free of chemical additives. Among these, the two important aspects are vital: One is to maintain a minimum level of viable cells per unit, and second is the level of contaminant. In most of the countries around the world, there are no regulations regarding the level of contaminants.

The above-discussed issues must be resolved by the inoculum producers to ensure that the product after used properly as per the manufacturer's guidelines should result in mycorrhization of the treated plants as an outcome of the price paid for the mycorrhizae product. Further, the improved quality standards will help to ensure the delivery of quality products of highest standards to the end user and are also in the best interest of the inoculum-producing industry. Prevention of low-quality bio-inoculants to reach to the market will help thwart a bad public image of the inoculum-manufacturing industry and may facilitate the acceptability of bio-inoculants among the users (Olsen et al. 1994).

New Potentials in Mycorrhizal Research and Future Prospects

The recent advancements in scientific knowledge on AM–plant symbiosis have helped improve the potential applications of mycorrhizal biotechnology in agriculture, horticulture, landscaping, phytoremediation, and other areas of plant market. The advancements developed include novel molecular techniques in order to detect the fungus inoculated under field conditions; explore the mechanisms adopted by various fungi for coexistence in the single root system; understand the mechanisms of interactions between mycorrhizae and other microorganisms including PGPRs or other saprophytic fungi; and discover new compounds with mycorrhizae supportive potential such as strigolactones and new findings about the physiology of in vitro-grown AM fungi in root organ culture (Vosatka et al. 2012). Further, the existing global economic scenario suggests for reduced input of phosphorous-based chemical inputs and increased application of mycorrhizae-based inoculum to exploit the natural potential of AM for sustainable agricultural systems. Applications of mycorrhizae for phytoremediation (phytoextraction, phytostabilization) have opened up a new window with newer potentials (Miransari 2011; Bhargava et al. 2012; Meier et al. 2012). The available reports suggest that some AM fungi can help reduce the translocation of heavy metals into the shoot, leaves, and other aboveground plant parts and thus may contribute to the enhanced quality of edible parts of plants (Rivera-Becerril et al. 2002). Similarly, there are some species of fungi known which can increase phytoextraction and subsequently help in remediation of polluted soils (Cabral et al. 2015). Another exciting area of mycorrhizal applications is improving the food quality of crops. In past few years, it has been shown that application of mycorrhizae can increase the sugar content, essential elements, such as Zn and Mg, antioxidants, and some other beneficial elements in crop plants (Table 21.1) (Perner et al. 2008; Gianinazzi et al. 2010; Albrechtova et al. 2012).

Another aspect for successful development of the product can be the combination of AM with other beneficial microorganisms. The application of inoculants with synergistic modes of action seems more beneficial for the plant production; therefore, some have already been chosen. Some studies have shown positive

Table 21.1 Effects of mycorrhizal inoculation on the growth and physiology of host plants

Sr. no.	Mycorrhizal species	Host plant	Effect(s) on host plant	Type of effect	References
1	*F. mosseae* and *R. intraradices*	*Pistacia vera*	Increased conc. of P, K, Zn, and Mn in the leaf as compared to control	Drought tolerance	Bagheri et al. 2012
2	*F. mosseae*, *G. versiforme*, *R. intraradices*	*Cucumis melo*	Inoculation with *G. mosseae* resulted in improved tolerance to drought as indicated by their enhanced growth parameters and physiological studies	Drought tolerance	Huang et al. 2011
3	*F. mosseae*, *G. versiforme*	*Solanum lycopersicum*	Increased growth responses and yield (19–32%) under various water stress conditions	Drought tolerance	Wang et al. 2014
4	*F. mosseae*, *F. geosporus* and *mixed inoculation*	*Fragaria* × *ananassa*	Improved strawberry growth, yield, and water use efficiency (WUE) under water stress conditions	Drought tolerance	Boyer et al. 2015
5	*R. intraradices*	*Tagetes erecta*	Improvement in the activities of antioxidant enzymes and reduced translocation of Cd to shoots leading to a higher biomass production	Decontamination of heavy metal (Cd)	Liu et al. 2011
6	*F. mosseae*	*Chrysanthemum maximum*	Less accumulation of Pb and Cu in the shoot while no exclusion effect was recorded for Zn	Decontamination of heavy metals (Pb and Cu)	Gonzalez-Chavez and Carillo-Gonzalez 2013
7	*R. intraradices*	*Ocimum basilicum*	Increased heavy metal conc. (Cd, Pb, and Ni) in shoots thereby reduced yield; high conc. of inoculum reduced metal concentration in shoot with beneficial effect on yield	Decontamination of heavy metals (Cd, Pb, and Cu)	Prasad et al. 2011
8	*G. gigaspora* and *Scutellospora spp.*	*Petunia* hybrid, *Callistephus chinensis*, *Impatiens balsamina*	Improved both vegetative and reproductive parameters of the three ornamentals; the expenses of phosphorus fertilization could be reduced up to 70%	Enhanced nutrient uptake	Gaur and Adholeya 2000

(continued)

Table 21.1 (continued)

Sr. no.	Mycorrhizal species	Host plant	Effect(s) on host plant	Type of effect	References
9	*F. mosseae*	*Asparagus officinalis*	Increased efficiency of phosphate utilization, the soil P concentration required for maximum yield growth of asparagus seedlings lowered by inoculation with *F. mosseae*	Enhanced nutrient uptake	Xu et al. 2014
10	*Glomus iranicum* var. *tenuihypharum*	*Euonymus japonica*	Increased plant growth parameters under reclaimed wastewater; increased P, Ca, and K concentration in leaves	Salinity stress tolerance	Gomez-Bellot et al. 2015

results with dual inoculations consisting of AM fungi and rhizobacteria which enhanced the plant growth in a synergistic way (Vazquez et al. 2000; Gryndler et al. 2002; Miransari 2011). In another study, application of AM coupled with saprotrophic fungi, which decompose the organic plant matter supplied, exhibited positive result on the growth of plants and quality of food (Albrechtova et al. 2012). The application of mycorrhizae-based inoculum for sustainable agriculture is growing rapidly and requires value addition by the manufacturers to ensure the end users about its potential. Given there are only a handful of natural biotechnologies available to aid, the risks for their inefficient applications include little or no availability of funds for applied research which should be made available for research. Therefore, collaboration between science and business is imperative to develop this market, as much further research will be needed to tune the products for the markets.

Conclusions

It is estimated that by 2050, global agriculture production needs to be doubled to meet the growing demand of human population (Ray et al. 2013). Further, human population explosion coupled with ongoing challenges of climate change, degradation of soil, and the escalating costs of chemical fertilizers calls for a need of absolute "New Green Revolution" which necessitates for the better exploitation of available natural resources (Lynch 2007). To achieve these goals, significant advancements in AM research are required for their applications in sustainable agriculture.

Future of inoculant technology and its benefits for sustainable agriculture depends on improving inoculant quality and effectiveness. Hence, the challenge is to develop and popularize an inoculant formulation with long shelf life and effective in its response once inoculated, be it seed or soil. There is a need for extensive research to synthesize a new inoculant formulation like liquid, freeze-dried, and fluid bed-dried inoculants. The failure of commercial inoculum to realize the actual benefits which are generally publicized as applicable for a wide range of plants and environmental conditions is also one of the reasons for lack of confidence of the farmers on biofertilizers (Corkidi et al. 2004; Faye et al. 2013). Moreover, the lack of availability of sensitive and universal bioassay(s) to rapidly assess the AM colonization of host plant, complicated procedures for species identification of AM, and management of quantity as well as quality of inoculum owing to its high demand are some of the other challenges which also contribute to the drawbacks of inoculum applicability in sustainable agriculture.

For these reasons, scientific expertise is expected to strengthen the bond between research and manufacturers for the promotion of market development and improvement of AM inoculum and develop a series of "best practices" to resolve issues pertaining to the functionality of commercial inoculum production and applications. Both in the developed and in developing world, the number of

enterprises involved in the production of AM-based inoculum is continuously increasing, especially in the emerging markets indicating a growing interest of private sector which is a sign of improved collaboration between the scientific community and the corporate world. Even though there are several bottlenecks which are yet to be resolved, the achievements of mycorrhizal industry are indeed very promising and further research is required to commercially exploit the full potential of mycorrhizal fungi.

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Role of Fungicides in Crop Health Management: Prospects and Challenges

22

T. S. Thind

Abstract

Fungicides are essential components of crop protection and have played a significant role in managing several devastating crop diseases and realizing optimum crop yields. Their use has assumed importance in the control of more damaging plant pathogens against which host resistance is not easily available or is unstable, such as polycyclic oomycete pathogens. In some cases, the benefit gained through fungicide use is more critical to the extent that certain crops, such as potato, melons, and grapes, to name a few which cannot be cultivated in the absence of disease control that remains heavily dependent on the use of fungicides. About 150 different chemicals belonging to diverse classes are used as fungicides the world over. The need to produce more food per unit area necessitates the rationale behind producing novel fungicides to be developed to protect precious cultivars, which lack genetic disease resistance. This equilibrium between genetic resistance and disease control metabolites of chemical, biochemical, or biological nature will persist and is not likely to alter dramatically in the nearest future.

Keywords

Fungicides · Food production · Fungicide resistance · New-generation fungicides · Plant disease management

Agricultural scenario is fast changing the world over with an urgent need to produce more food per unit area for feeding the ever-increasing human population. The challenge before agricultural scientists is to ensure not only required quantity but also better quality food materials. Agriculture thus has to find ways to feed the

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world while being environmentally sustainable, economically viable, and socially acceptable. Managing proper crop health from the ravages of biotic and abiotic stresses is highly important to get desirable yields. Pests and diseases take a heavy toll on our crop produce every year, and their scenario keeps on changing with the introduction of new agricultural technologies. With climate change showing its impact on agriculture the world over, several less-known crop diseases have become more important. Plant pathologists have an important role to play in managing crop diseases through development of effective and economical technologies. About 20% of the gross production of food and cash crops worldwide is reduced due to plant diseases. Out of over 100,000 mentioned species of fungi in the world, nearly 20,000 produce different diseases in various plants. In last 150 years, a lot has been studied with regard to control measures to get rid of plant diseases and several latest approaches such as biological, chemical, and cultural, and regulatory approaches were developed for their control. Depending on the cultivar, the disease and the convenience of control approaches, a different set of methods, are implemented.

Role in Sustainable Food Production

Although losses due to plant diseases possibly will be reduced by the practice of disease resistance varieties, crop rotation, or sanitation practices, fungicides are often important to increase crop yields. Fungicides can play an important role in safeguarding crop health safety by supervision of shattering diseases in agricultural cultivars. The estimated increase in world's population from 7 billion at current (1.27 billion in India alone) to 8.3 billion by 2030 presents a major global task to encounter necessary food demand. At the same time, per capita farmland is decreasing drastically. This will result in increased demand for food, feed, and fiber. India has produced 264 million tonnes of food grains during 2013–2014, and with the increase in rate of population growth, it would require about 400 million tonnes by 2050. Yield improvements are attainable only if farm inputs are optimized, including fungicides. Fungicides also play an important role in improving food value. They add to food safety aspects by checking many fungi which produce toxins. About one quarter of food crops across the world is affected by mycotoxins such as aflatoxins, ergot toxins, *Fusarium* toxins, patulin, and tenuazonic acid (Knight et al. 1997). In the USA alone, growers apply fungicides for controlling more than 200 diseases of 50 crops in the field. Fungicides are now well deliberated to be the second line of defense in plant infection control programs after disease resistance.

The use of any chemical to a cultivar or food raises the demand of related risks and advantages. The risk is basically concerned with toxicity to the user in the crop field and the consumer (Frazer 1963) to a broader aspect that comprises the whole environment and the ecosystem in which the cultivars are growing (Schlundt 2002). As a result, detail studies are required before applying fungicide, leading to gigantic

development costs. In the USA, the IR-4 program has been proven to provide safe and cost-effective pest management solutions for specialty crop growers.

Largely, most studies come to the conclusion that the advantages of fungicides far overshadow the risks, if they are used safely and within recommended limits. Presently, over 80% of the fruit and vegetable crops grown in the USA get a fungicide every season. The significance of fungicide application in the US agriculture is reported to enhance farm income by approximately $13 billion annually (Gianessi and Reigner 2006). In general, the cost–benefit ratio of fungicide spray application in managing foliar diseases comes out to be 1:3 in most of the cases, which is still much higher in case of seed treatment. The alternatives suggested by organic farmers, who are opposed to intensive farming overall, exclude the use of artificial fungicides, but allow the use of copper and sulfur-based inorganic fungicides. Still, there are concerns regarding the traditionally and organically grown crops as being safe for consumers. For instance, a large number of investigations are being steered to estimate the risk of mycotoxins in the two farming systems.

Development of Fungicides—Past and Present

Like many other inventions, good observation led to the development of first fungicide. The first use of treating wheat grain with salt water followed by liming took place in the middle of the seventeenth century to get rid of bunt and led to the observation that wheat grains salvaged from the sea were free of bunt contamination. This had happened long before Tillet (1755) recognized that seed-borne fungi (*Tilletia tritici, T. laevis)* were the cause of bunt of wheat and that it could be checked by treating seed with lime or lime and salt. Starting with initial 1800, researchers and chemists have traveled long way to discover and find out fungicides that had striking effect on disease management and would reduce disease losses (Klittich 2008; Morton and Staub 2008).

In 1882, Millardet from France made a remarkable discovery, who observed that grapevine that had been treated with a bluish-white solution of copper sulfate and lime to deter pilferers retained their leaves through the season, whereas the unsprayed vines lost their leaves. The discovery of Bordeaux mixture in 1885 is considered as the first important landmark in the era of chemical control of disease. Bordeaux mixture belongs to first generation of fungicides along with other inorganic chemicals. Till the 1940s, chemical disease control depended upon inorganic chemical formulations, most frequently prepared by the user.

From 1940 to 1960, a large number of new chemistry classes of fungicides were introduced. The dithiocarbamates and later the phthalimides signified a prominent advancement over the laterly used inorganic fungicides in that they were more active, less phytotoxic, and convenient to formulate by the user. The fungicide research domain of that time was described by Horsfall (1975), who was vital in the discovery and the starter of the dithiocarbamates, the most largely used group of

organic fungicides. The organical chemicals which are the second generation of fungicides, dates from 1934 with the increase in dithiocarbamates along with quinines, organotins, low-soluble copper compounds, captan and related compounds, chlorothalonil, and pentachloronitrobenzene. Compounds like inorganic fungicides were used as surface protectants.

The decade from 1960 to 1970 saw a consistent increase in research and development along with a rapid change of the fungicide markets. In this decade, mancozeb and chlorothalonil were the most widely used protectant fungicides. The decade also provides us the first systemic seed treatment compounds carboxin and oxycarboxin for controlling seed-borne smuts, foliar rusts in cereals, and assorted *Rhizoctonia* infections and also the first broad-spectrum systemic fungicides group benzimidazoles (benomyl, thiabendazole, carbendazim).

The more important modern fungicides were introduced since 1970. These third-generation fungicides were also organic but could infiltrate the plant tissue (systemic) and control established infections. These included 2-aminopyrimidnes (ethirimol, dimethirimol), dicarboximides (iprodione, vinclozolin, procymidone), organophosphorous compounds (iprobenphos, edifenphos), triazoles (triadimefon, propiconazole, flusilazole, myclobutanil), piperazines (triforine), imidazoles (imazalil, prochloraz), phenylamides (metalaxyl, ofurace, benalaxyl, oxadixyl, mefenoxam), alkylphosphonates (fosetyl-Al), cyanoacetamideoximes (cymoxanil), cinnamic acid amides (dimethomorpph), and morpholines (tridemorph, fenpropimorph), piperidines (fenpropidine). There was a marked decrease in quantity per ha (acre) as more operational and discriminating fungicides were introduced over this time. For example, the present use rates of well below 100 g/ha for many triazoles in contradiction of the same pathogen is a 200-fold reduction.

The fourth generation of fungicides comprises chemicals that are ecologically safe, have still new modes of action, somewhat broad spectrum, and applied at quite low dose rates. Examples of these are strobilurins (azoxystrobin), phenoxyquinolines (quinoxyfen), oxazolidinediones (famoxadone), anilinopyrimidines (cyprodinil, pyrimethanil), phenylpyrroles (fenpiclonil, fludioxonil), spiroketalamines (spiroxamine), benzamides (fluopicolide, zoxamide), valinamides (iprovalicarb, benthiocarb), cyanoimidazoles (cyazofamid), thiocarbamates (ethaboxam), mandelamides (mandipropamid), and amidoximes (cyflufenamid) showing different chemistries and mechanism. A large number of these have been established for use against oomycete pathogens thus indicating the necessity in managing these pathogens. The latest phenylamide to be registered in 2003 is boscalid from BASF. Boscalid is listed for foliar use on a varied range of fruits, vegetables, and nut crops, either alone or in combination with pyraclostrobin.

Strobilurins, introduced in 1996, are the class of fungicides that are widely used on cereals and more importantly now on soybeans. Companies have also endorsed the plant health benefits of this class of fungicides on corn and soybeans. While broad-spectrum fungicides such as strobilurin are highly effective and are appropriate for a wide variety of crops. Some issues with disease resistance are affecting markets (e.g., *Septoria* in wheat in Europe, and the US turf market). As a consequence, companies are minimizing the use by developing mixtures and other uses,

incorporating seed treatments. More commonly used strobilurins after azoxystrobin are kresoxim-methyl, trifloxystrobin, pyraclostrobin, picoxystrobin, and more recent fluoxasrobin.

Benthiavalicarb (from Kumiai) and mandipropamid (Syngenta) are the novel active ingredients newly developed, from the carboxylic acid amide (CAA) fungicide group, metrafenone (BASF), fluopicolide (Bayer), proquinazid (DuPont), and zoxamide (Dow Agro) for managing various diseases in different crops. For a more in-depth description of contemporary fungicides, containing chemistry, the reader may refer to the treatise edited by Krämer and Schirmer (2007). More recently during 2013 and 2014, new fungicides with novel modes of have been launched by agrochemical companies for use against different diseases. These include fluopyram (pyridinylethylbenzamides) by Bayer against gray mold, powdery mildew, *Sclerotinia* and *Monilia*, penthiopyrad (carboxamide) by Du Pont against rust, *Rhizoctonia*, gray mold, powdery mildew, apple scab and fluxapyroxad (carboxamide) by Agrow-BASF for seed treatment/protectant for different crops.

Fungicides—Indian Scenario

A number of fungicides have been registered in India belonging to different chemical groups, and these are being applied to combat pathogens in fruits, plantation crops, vegetables, and some field crops. Fungicides such as sulfur, dithiocarbamates, copper-based, phthalimides, benzimidazoles, phenylamides, triazoles are now regularly used in Indian agriculture, with the use of costly site-specific fungicides on high value crops only. As on January 20, 2012, a total of 69 antifungal compounds have been registered in India (CIBRC 2014a) including four antifungal/antibacterial antibiotics and four bio-control agents.

Among various fungicides used in India, consumption of mancozeb is the highest followed by sulfur compounds, copper oxychloride, copper sulfate, carbendazim, and thiram as per the demand pattern (Agnihotri 2000). These six fungicides constitute more than 85% of the total fungicides used with mancozeb alone accounting for 25% of the total. Hexaconazole, propiconazole, metalaxyl-M + mancozeb, cymoxanil + mancozeb, edifenphos, flusilazole, triadimefon, tricyclazole, and azoxystrobin are few other fungicides that account for substantial fungicide market in India. Agrochemicals domain in India is valued at Rs. 4800 crore, out of which, fungicides contribute for closely 12% of the market. Crop-wise consumption of fungicides in India is maximum on pome fruits, followed by potatoes, rice, tea, coffee, chillies, grapevines, other fruits, vegetables, and field crops (Thind 2005).

Apart from contact compounds like sulfur, dithiocarbamates, copper-based, mercurials, phthalimides several of the site-specific fungicides of the groups like benzimidazoles, oxathiins, thiophanates, organophosphorus, triazoles and related sterol inhibitors, phenylamides, cyanoacetamide oximes, cinnamic acid derivatives,

and some other modern fungicides are also being used in India for controlling diseases on a number of crops.

Some of the new-generation fungicides have also been registered in India for use against different diseases. These include Strobilurins (azoxystrobin, kresoxim-methyl, trifloxistrobin, pyrachlostribin against powdery mildew, downy mildew in grapevine, cucurbits, rice sheath blight), Valinamides (iprovalicarb, benhiovalicarb against Oomycete diseases in grapevine, potato, tomato, and cucurbits), Oxazolidinediones (famoxadone, against potato late blight). Imidazoles (fenamidone against potato late blight and grape downy mildew), Phenyl-ureas (pencycuron, against rice sheath blight, black scurf of potato) Mandelamides (mandipropamid, against late blight of potato, downy mildew of grapevine), Melanin biosysnthesis inhibitors (carpropamid, against rice blast), Triazolinthiones (prothioconazole against rice blast). A few recently developed compounds such as zoxamide and fluopicolide (Bezamides), and cyazofamid (Cyanoimidazoles) are under evaluation against Oomycete diseases.

Few of the old fungicides have either been abandone or withdrawn in India by Central Insecticides Board or their use has been made restricted because of few side effects on environment and ecology. Mercury compounds ethyl mercury chloride and phenyl mercury acetate are banned because of their longer persistence in the environment and food chain, while there is a restricted use for methoxyethyl mercury chloride. Quintozene (PCNB) and pentachlorophenol are also banned due to adverse effects on ecology. Captafol is now allowed to be used as seed treatment only and its use as spray is banned. Likewise, ferbam and nickel chloride have been withdrawn (CIBRC 2014b).

Changes in Fungicide Research and Development

The last half-century has seen new developments in pesticide research, yet last 25 years have recorded numerous discoveries of unique and highly efficient products (Leroux 1996, 2003; Gullino et al. 2000). Developments in chemistry in the formulation and synthesis of novel molecules, in specific combinatorial and robotic chemistry, and enormous increase in the understanding of genetic and genomic information have led to the design of novel selection methods for assessing more than a hundred thousand novel compounds per year (Corran et al. 1998). In addition, biochemists have designed tools to find out compounds having a novel mechanism and initiated research for screening. Substantial development has also been made to detect the biochemical targets of these compounds (Beffa 2004). The discovery of new mechanism that results in enhanced efficacy of fungicides on commercially significant targets, combined with assured environmental and public safety, is a critical step in safeguarding food security. By the 1990s, there were total 113 active ingredients registered as fungicides worldwide (Knight et al. 1997).

The shift of manufacturers from in vitro screens to in vivo screens has led to the beginning of this new chemistry. In these in vivo cultures, young canned plants were treated with the test fungicides and inoculated with a main pathogen later. The plants were further studied for their phytotoxicity, systemic efficacy by checking its morphological features in growth chamber or in greenhouse, and the same process was repeated for several test compounds.

Presently, the discovery platform for most chemical companies across the world for crop protection includes combinational approaches comprising high-volume chemical screens, chemistry-based and biological target-centered formulations programs, and natural product development (Wenhua 2009). Key elements of research and discovery of MNCs include high-throughput screening, combinatorial chemistry, advanced formulations, and molecular and environmental studies which operate in combination to develop new products and technologies. The focus areas of research in fungicide discovery are combinatorial chemistry, high-throughput screening, advanced formulation, molecular toxicology, and environmental safety.

In order to develop potential leads in the area, high-throughput-automated screening can be utilized to save time, and it can tremendously result in better testing and development of chemicals. Advances in miniaturized screens have helped research groups to develop millions of tests a year on targeted sites as well as on whole organisms. The advancement in this huge high-throughput screening is the adoption of a small micro-titer plate. These plates help to test multiple compounds in small wells at a time with precise results. A major benefit is that these new leads only need a few micrograms of a test compound. With the development in automation linked high-throughput screening, a large number of new leads is being identified with better efficacy. For high-throughput screening, many chemical compounds must be tested to find initial points called leads, which aid in emphasizing new research into targeted areas of chemistry. Still, a major challenge that remains is to identify and detect new leads using high-throughput screening and developing it to a level that it could be commercialized. However, a number of approaches are used by MNCs equipped with in-house chemical synthesis, collaborations with third parties, the testing of chemical collections called libraries, and robotic synthesis of developed libraries of chemicals.

Although several novel products had been discovered by the high-volume screening approaches, the difficulties in obtaining reliable sources for novel chemicals for biological screening system make this approach unsustainable. The target-based approach is the key discovery rationale for the pharmaceutical industry; however, the significant output of this rationale to pesticide discovery has still to be accomplished. Another approach is the natural product platform. The discoveries of crop protection chemicals from natural products are well documented (e.g., pyrethroids, abamectins, spinosyns), and the availability of unique biological samples from new natural sources is limited and unreliable. Finally, the chemical-based approach is the most productive pesticide discovery platform today. This platform requires experienced and insightful chemistry teams and the support of rapid biological screening system (Oerke et al. 1994; Wenhua 2009).

Remarkable progress has been made in the last few years in the formulation chemistry and delivery techniques used to apply new chemicals. For example, water-soluble bags have been developed having new micro-encapsulated formulations. These and other technologies have benefits such as checking the release of the chemicals, lower toxicity, mixture compatibility, and enhanced bio-performance of the product.

Role of Seed Treatment

Good seed should be pure and free of weed seeds, seed-borne pathogens, insects, and others. Seed treatment with chemical, biological, or physical agents provides protection to seed and helps in the establishment of healthy crops. It provides good protection to the seed from seed and soil-borne pathogens during the germination period and supports early plant development. A good range of seed treatment fungicides are now available ranging from the traditional chemicals like thiram, captan, mancozeb, carbendazim, and carboxin to the more effective and safer compounds such as mefenoxam + fludioxanil and azoxystrobin (Syngenta), pyraclostrobin + metconazole (BASF) developed during 2008–2010. More recently, two new seed treatment fungicides sedaxane (Syngenta) and fluxapyroxad (Agrow-BASF) have been introduced in 2013. The seed treatment market on a global scale is assumed at over $2.5 billion, with USA accounting for more than 50%, and it will consistently increase as it is considered to be cost-effective for safeguarding important cultivars.

As per the estimates of the Directorate of Plant Protection, Quarantine and Storage, Faridabad, nationwide 70% of the seed is utilized by farmers own stock and most of it is sown without the pre-treatment of seeds. Even the private and public agencies provide untreated seeds to the farmers resulting in lower yields. The assessments further disclose that on an average, 80% of the seed sown in the country is untreated, as compared to 100% treated seed practice in the developed nations. This may be one of the several reasons for our lower productivity in many crops compared to developed nations. There is thus an ample scope for improving our crop yields through appropriate seed treatment practices. The future seed treatments may move toward multiple compound products for complete spectrum of pest and disease control as also abiotic stress tolerance.

Fungicide Mixtures

Fungicide mixtures, containing two or more fungicides with diverse mechanism of action, have been developed with the twin objectives of broadening the activity spectrum against diverse plant diseases and to check the development of resistance in the target pathogens. Often, these compounds have synergistic effects and

enhance the efficacy levels of the individual compounds together. Some of the recently developed and commonly used fungicide mixtures are Ridomil Gold (mefenoxam + ancozeb) against downy mildews, *Phytophthora* and *Pytyium* diseases, Prosaro (prothioconazole + tebuconazole) against *Fusarium* head scab in cereals, Input (prothioconazole + spiroxamine) against powdery mildew, *Fusarium, Septoria*, rusts in cereals, Twinline (yraclostrobin + metconazole) against cereal rusts, Amistar Top (azoxystrobin + difenoconazole) against various diseases on diverse crops, and Luna Sensation (fluopyram + trifloxystrobin) for controlling gray mold, powdery mildew, *Sclerotinia,* and *Monilia* in fruits.

Decision Support Systems

Reaching an informed and rational resolution for infection management is a tedious task based on disease epidemiological parameters. Decision support systems (DSS) integrate all relevant information to generate spray recommendations that could help lessen fungicide usage and as a result preserve the robustness of fungicides efficacy. An important factor related to disease management decision is dependent on how potential damage can be caused to crops. Some diseases are very infectious and can lead to mass devastation under favorable growth conditions that eventually lead to complete destruction of crops, for example, potato late blight, caused by *Phytophthora infestans* or apple scab caused by *Venturia inaequalis.* Thus, the use of fungicide is dependent upon value of the crop, the type of disease, and the extent of damage it causes and the cost of control measures (Carisse et al. 2010). Many DSS such as Wheat Disease Manager for wheat diseases (UK), More Crop for rusts and other diseases of wheat (Pacific Northwest USA), Crop Protection Online for wheat diseases (Den mark), Blight Management and NegFry for potato late blight (UK, USA), RIMPro for apple scab (the Netherland), and many more are now available which help the growers reduce fungicide applications up to 50%. A Web-based DSS has also been developed for deciding fungicide applications against late blight of potato in Punjab (Thind 2011a).

Fungicide Residues

The residue levels of certain fungicides in the soil or edible plant parts may sometimes be well above the acceptable limit, and this varies with the dose of the fungicides used and with total number of sprays done. With the overuse of systemic fungicides for managing diverse fungal diseases in agricultural crops, there are ample chances of their residue build up in harvested produce. Not adhering to the recommended pre-harvest interval may further compound the problem. This not only poses health risks to consumers, but may also affect the export potential of certain commodities to the developed countries. Identification of residues of

carbendazim and isoprothiolane above MRLs in basmati rice shipments sent from India to Germany in 2010 and that of tricyclazole in the shipments shipped to USA in 2011 has referred cautionary signals among the agriculturists and traders in India. Modern HPLC technology makes it possible to detect minute amounts (in ppb) of the fungicides in the food products. Farmers need to be educated to follow the recommended guidelines while using fungicides and other pesticides.

Non-Target Effects

Though fungicides are primarily used to manage plant diseases caused by different kinds of fungal pathogens, these may, sometimes, exert adverse effects on other microbial flora and fauna some of which may be beneficial to the crop plants. Farmers often use overdose of fungicides, regardless of their environmental impact. Many organic fungicide products have minimal ecological impact which is an effective way to control disease. However, inappropriately applying any fungicide (organic or otherwise) will lead to poor control and may affect non-target organisms.

Besides affecting plants, some fungicides have adverse effect on native soil and native soil microflora. The fungicides, triadimefon, and propiconazole, on frequent use, could alter the soil microbial ecology over a longer period of time (Jui-Hung et al. 2009). Soil microbial populations comprising various fungi, denitrifying bacteria, and aerobic diazotrophs are also greatly affected by the application of mancozeb (Pozo et al. 1994). Similarly, fungicides like benomyl, fenpropimorph, fenhexamid, mepronil, furalaxyl, thiram also affect mutual relationship between the plant and mycorhhizae. Elemental Sulfur, benomyl, and preparations of difenoconazole are reported to be toxic to predatory arthropods and non-target parasitic. A transient negative effect of propiconazole, a widely used fungicide, on the overall abundance of higher collembolan taxa such as springtails has also been reported. Fungicides may also pose potential environmental and human health hazards (Goswami et al. 2015).

Problem of Fungicide Resistance and its Management

Although fungicides play a vital role in crop production, their rigorous and faulty use can have detrimental effects on crops, useful unfriendly organisms, and environment. However, the most serious problem is the progress of fungicide resistance strains leading to failure of disease control. Fungicide resistance is now a serious problem in global agriculture (Thind 2008).

Fungicide treatment builds resistance caused by the survival and spread of initially rare mutants. The development is either of discrete type caused by single gene mutation or gradual type which is supposed to be polygenic. There are various

resistance mechanisms although the mechanism mainly involves modification of action site of fungicide at its prime location within the fungal pathogen. Fungicide resistance is often regarded as a problem for the agrochemical industry rather than growers but in fact, resistance has wider implications for sustainable farming. Fungicide resistance is harm to individual users of fungicides leading to loss of yield and poor farm economy, a result of poor disease control. Hence, it is in everyone's interest to ensure the continued efficacy of the best available products in commercial use. Fungicide resistance problems have been studied for more than 100 diseases for pathogen to crop combinations and for almost half of the known fungicide groups (Brent and Hollomon 2007). All these studies are documented, while the rest suspected cases are yet to be considered. The study concludes to implement a management strategy before resistance becomes a problem. Mostly strategies for fungicide management aim at delaying development. There are specific resistance management strategies for different groups of fungicides, target pathogens, and crops (Thind 2011b). Monitoring of these strategies is necessary, keeping a record for those with lack of disease control and to check if they are working or not. It must start early, to gain valuable baseline data before commercial use begins. In order to avoid any misleading conclusion, results should be interpreted in a careful manner. Resistance management should be based on information gathered from cultural practices and patterns of optimum fungicide use. Expected results are achieved as a result of minimization of selection pressure with reduction in exposure time or population size for the at-risk fungicide. Fungicide use and resistance strategies are developed to fight against the threats of fungicide resistance. Industry is making regular efforts to conduct research in areas of mode of action, risk at resistance, field monitoring at baseline sensitivity, and sensitivity variations in treated fields.

The results recorded build up strategies to be effective in reducing the risk of fungicide resistance as well as the loss of efficacy of fungicide classes. Collaboration has to be established between Fungicide Resistance Action Committee (FRAC) and different manufacturers. The collaboration is based on the threat developed by fungicide resistance and cross-resistance to related products. Strategies thus build up targeting a minimum risk. FRAC is working in this field by contributing to the production of monographs related to various aspects of fungicide resistance. FRAC had also grouped almost all the available fungicides based on various criteria related to resistance risk of the different fungicide groups, although these assessments about the resistance risk are made difficult by the unpredictability of cross-resistance. Among different fungicide groups, some like benzimidazoles, dicarboximides, phenylamides, and strobilurins carry high level of resistance risk. The FRAC working groups and fora for fungicide risk assessment must meet from time to time and suggest ways and means for managing risk of resistance build up in important pathogens. It is emphasized to follow a pro-active approach at the very beginning of the introduction of a new fungicide compound.

Fungicides—the Way Ahead

It is assumed that in disease management domain, fungicides will have a crucial role to play particularly in intensive management systems. In order to reduce the side effect on human health and maintaining ecological balance, sustainable approach must be followed. A more rational use of fungicides must be made for better results.

An insight into the past will give us a better understanding of what to anticipate in the future. The widespread modifications in fungicide practice have usually been linked with differences in the spectrum of harmful microbes as well as in practices, prices, or cultivar intensities. The spread of soybean rust into the Americas or tobacco blue mold into Europe had a significant impact on the application of fungicides on the diseased crops. The rust affected a wide area of North and South America which decreased the yield of soybean. Such changes in pathogen ranges could not be predicted and will continue to happen in the future due to enhanced global trade with plant varieties and trans-boundary transportation. More apparently, ever-increasing world demand will lead to increased areas of intensive cropping, and hence, most likely, fungicide application will also rise (Collins 2007).

The trend toward sustainable use of fungicides in conjunction with disease forecast systems can be expected to follow in the future. The efforts of breeding and role of genetic engineering will possibly help in combating disease resistance. The equilibrium between genetic and chemical control will most probably continue because research in both areas will support each other.

Fungicides as Inducers of Host Resistance

Growing concern for environmental and regulatory hurdles promotes awareness on fungicides or chemicals that act indirectly by interfering with processes involved in fungal infiltration into crop or by enhancing host resistance rather than the fungus such as carpropamid (Morton and Staub 2008). Probenazole can control the infection of rice blast and some bacterial rice diseases by stimulating the storage of toxins and enzyme linked with systemic acquired resistance, but is less effective in other cereals. Acibenzolar-S-methyl (Actigard, Bion) has the widest spectrum of function among non-fungal toxic compounds introduced to date. It is active against several fungi, bacteria, and viruses in several crops and stimulates plant's natural defense system when applied for a week after infection (Leadbeater and Staub 2007). Actigard has delivered best when amalgamated into a suite of chemical sprays, as the natural level of pathogen control has rarely been adequate when used alone. The product has initiated new avenues in research for using peptide molecules to mitigate diseases, and other ways of inducing SAR and the jasmonic acid pathway with biological agents and chemicals in plants. Because no selection pressure is applied directly on the pathogen population, these are unlikely to

encounter resistance. Such compounds with indirect action against the pathogens are likely to gain prominence in crop protection in future.

Concern for Environment and Public Safety

The critical steps in food safety have major challenges in discovering new modes of action that provide better activity of fungicides against important targets, along with secure environmental and public safety. Fungicides in future will be characterized by reduced application rates and reduced toxicity to other organisms (Hewitt 2000). Such products must also add to our potential to accomplish the development of fungicide resistance, thereby extending the credibility of existing fungicides. An important aspect of the present fungicide development has been identification of accurate and new target sites. Fungicides that interrupt fungal development before the host penetration are very important. In the last few years, many of novel products had been shown to be active before the host dissemination (e.g., carpropamid against rice blast, Quinoxyfen against powdery mildew). In addition, naturally derived bioactive compounds from bacteria, fungi, and higher plants have shown to be effective against fungal pathogens. They may possibly be established either as products per se or used as preliminary points for synthesis. Already many natural antibiotics have been successfully developed like blasticidin S, kasugamycin, and validamycin for protection against sheath blight and rice blast. Polyoxins are other class of antibiotics used to safeguard fungal pathogens of trees, fruits, and vegetables.

Novel Modes of Action

With rationale to efficiently safeguard plants from diverse fungal pathogens, new antifungal compounds need to be developed with higher efficacy and activity, i.e., specificity, low dose rate application, and low environmental effect. Moreover, new modes of action are prerequisite to overcome resistance to existing products. In addition, public education and awareness are necessary to amend the negative perception of chemical control that meets the toxicological and ecological standards. An emerging new domain of research is the application of antimicrobial peptides (AMP) for combating resistance to microbes using plants as bio-factories for fungicides or bactericides production.

Role of Modern Disease Diagnostic Tools

Future success in chemical control will be determined by research and development. Sustainable development is presently a vital idea of global arena to mitigate our needs. For IPM to lead a sustainable way to crop safety, it must incorporate the rational and

environmentally harmless use of chemical control of crop diseases. Contemporary analytical tools will play a crucial role in developing rational use of fungicides.

The need to produce more food per unit area necessitates that novel fungicides must be developed to safeguard our crops, where genetic disease resistance is not a constraint. This equilibrium between disease control products of biochemical, chemical, or biological nature and genetic resistance will persist and is not expected to change all of a sudden in the nearest future. For sustainable development and disease control, wise integration of newer technologies with cultural and sanitation approaches will be required.

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Bioherbicides: Strategies, Challenges and Prospects

23

K. R. Aneja, S. A. Khan and A. Aneja

Abstract

Availability of enough food for ever-growing population is a serious task faced by human race due to pests. Weeds are major kind of pests; their competitive effects on the production and yield of various crops are well documented in India and the world. An increased and indiscriminate use of chemical pesticides, in order to control pests, has resulted in resistance, immunity and resurgence in them. The negative effects of chemical herbicides on nature and natural resources have forced scientists to shift focus on the reliable, sustainable and environment-friendly agents of weed control, the bioherbicides. Microbial-based pesticides, called bioherbicides, are the formulations of host-specific plant pathogens that are applied at high inoculum rates in a similar way as chemical herbicides for the management of weeds. In the majority of the bioherbicides, the fungal organisms are the active ingredients; therefore, the term mycoherbicide has often been used interchangeably with bioherbicide. There has been a great number of naturally occurring fungal plant pathogens worked out for possible use as potential biocontrol agents, but a small proportion has been developed to commercial products. Among the bacterial plant pathogens, *Xanthomonas campestris* and *Pseudomonas fluorescens* have shown the potential to be developed as bioherbicides. Some viruses also have been investigated for the potential to control invasive or undesirable weeds (e.g. tobacco mild green mosaic tobamovirus (TMGMV) for control of tropical soda apple in Florida).

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Twenty-four bioherbicides, based on fungi, bacteria and viruses have been registered around the globe, with several other microbial candidates in various stages of evaluation as bioherbicides. Most of the fungal biocontrol agents used as mycoherbicides have been found to be hemibiotrophs. To make mycoherbicides economical and popular among farmers, there is a strong need to develop mycoherbicides composed of consortia of compatible plant pathogens, each specific to a different weed species that could simultaneously control several weeds at a time. Scientist should lay emphasis on enhancing the pathogenesis of the biocontrol agents for overcoming the dependence on dew, one of the major constraints to make them potential bioherbicide. The pace of development of available bioherbicides including their success and failure, constraints, commercialization as well as current status has been discussed in this paper.

Keywords

Weeds · Bioherbicides · Mycoherbicides · Biocontrol agents · Hemibiotrophs DeVine^{R} · Collego™ · ABG-5003 · Dr. Biosedge® · Gibbatrianth *Xanthomonas campestris*

Introduction

Weeds are the most severe and widespread biological constraint to crop production and cause invisible damage till the crop is harvested. Most of the weeds causing losses are of alien origin. They have been introduced either negligently or accidentally and have become problematic in the absence of host-specific natural enemies in the new environment. Weeds are a problem in crop production associated with declines in crop yields and quality, as a source of allergenic pollen and as an aesthetic nuisance (Stewart-Wade et al. 2002; Oerke 2006; Gadermaier et al. 2014). They continue to cause major problems in agriculture by quality of crops, competing with neighbouring plants for space, moisture, nutrients and light (Aneja 2009). The competitive effect of weeds on the production of several crops is well documented not only in India but also in many countries of the world.

Unlike other pests, weeds are ubiquitous and affect almost all the crops. Presence of weeds in general reduces crop yield by 31.5%. Weed problems are likely to increase due to increased emphasis on high-input agriculture. Further, globalization would also result in new weed problems notwithstanding strict quarantine regulations. The worldwide losses of attainable production of eight principle food and cash crops like rice, wheat, barley, maize, potato, soybean, cotton and coffee due to pests have been estimated to be $243.7 billion annually in the years 1988–1990. Out of this total loss, $76.3 billion is attributed to weeds only (Oerke et al. 1994; Oerke 2006).

According to Chattopadhyay et al. (2011), the current annual losses due to insects, pests and diseases in the agriculture sector in India is around Rs. 15,000 million. The management of these pests in agriculture consumes nearly Rs. 3150 million worth of pesticides annually. Furthermore, a report released by an industry body—Assocham and Yes Bank on annual crop losses due to pests reveals that pest and disease infestation resulted in crop losses worth Rs. 50,000 millions annually in India (Mathur 2014). Keeping in view the nuisance caused by weeds, an increasing public concern with the harmful effects of pesticide residues; there is an urgent need to discover and develop alternative methods of controlling weeds and to improve existing weed control technologies that are economically and environmentally sustainable. Chemical weed control is not an ideal option in organic cropping systems. Bioherbicides, the formulations of host-specific plant pathogens applied similarly to the chemical pesticides to weeds, are the ideal options because of their several advantages over chemical pesticides.

Biocontrol of Weeds

Management of weeds is necessary but an expensive challenge. Herbicides, chemicals that kill or suppress plants, annually account for over $14 billion (Kiely et al. 2004). Recent trends in environmental awareness concerning the side effects of chemical pesticides have prompted weed scientists, botanists, plant pathologists, microbiologists and geneticists to develop novel, sustainable and protective weed control strategies. Biological control agents (BCAs), particularly fungal plant pathogens offer a tremendous opportunity to provide agriculture with effective tools for abundant crop production while minimizing impacts on health and the environment (Panetta 1992; Aneja 2009).

Interest in the development of biological control agents as biohebicides is favoured for a number of reasons. The development of tolerance and resistance to herbicides leading to a shift in weed flora towards species more difficult to control is one of the reasons. In addition, certain herbicides have been banned in a number of countries, and the use of others is severely restricted. Similarly, the high cost involved in the development, screening and marketing of novel herbicides limits their potential. On the other hand, there are some facts which favour the use of microbes as biocontrol agents. Chemicals are purified by concentration and microbes by dilution. Chemicals cannot multiply; microbes invariably do so. Chemicals do not respond to a changing environment or a resistant target, but microbes do so by virtue of their genetic system (Sands and Miller 1993).

The biological control agents including fungi, bacteria, viruses, mycoplasmas, protozoa and insect parasitoids (parasites thriving on insects) are available in nature abundantly and have been exploited to control a range of agricultural pests—arthropods, nematodes, weeds and plant pathogens, in addition to human pathogens/vectors (Watson et al. 1999; Aneja 2000). The major characteristics of potential biocontrol agents to control weeds should: be enormous biodiversity, easy

to produce and store, inexpensive to use, reliable at a high predictable level of control and eco-friendly for the user and environment. Many of these characteristics are exhibited by weed infecting fungi that infect plants (TeBeest and Templeton 1985), and they are easy to identify than bacteria and viruses; their taxonomic position is well defined and is of high virulence (Watson 1989).

On the basis of the mode of nutrition, the phytopathogenic fungi are distinguished into three different forms as, necrotrophs/perthotrophs (those derive their energy from killed cells); biotrophs (those derive their energy from living cells through haustoria); and hemibiotrophs (the organism having an initial biotrophic phase followed by a necrotrophic phase). Most of the mycoherbicides have been found to be hemibiotrophs, in which the biotrophic phase provides high host—specificity and the necrotrophic phase causes extensive tissue death (Goodwin 2001).

Biological Control Strategies

Biological weed control with live plant pathogens is adopted and classified as: inoculative or classical approach or strategy and inundative or bioherbicidal approach or strategy (Daniel et al. 1973; Templeton et al. 1979).

Inoculative or Classical Strategy

This strategy involves the import and release of one or more natural enemies which attack the target weed in its native ranges, into areas where the weed is introduced and is troublesome; and where natural enemies are absent. The main objective of this approach is generally not the eradication of weed species as such, but it rather aims at self-perpetuating regulation of the weed population at acceptable level (Watson 1991; Evans 1995).

The plant pathogens used in this strategy are generally self-disseminating pathogens (e.g. rusts, smuts and certain dry spore-producing foliar fungi). The fungal pathogen is simply released into a target weed population of an alien species to establish well in due time, and no further manipulations are required. In severe epidemics, the weed is killed or reduced to economically most acceptable level. The control of the target weed is dependent upon self-perpetuation and natural dispersal of the pathogen.

The classical strategy is comprised of three phases: (i) selection of target weed; (ii) search for the control organisms with the native distribution area of the target weed; and (iii) release of selected biocontrol agents and follow-up studies (Schroeder and Muller-Scharer 1995). Introduction of *Puccinia chondrillina* from Mediterranean (South Europe) to control *Chondrilla juncea* (skeleton weed) in Australia is the first successful example of classical strategy (Quimby 1982; Aneja

and Mehrotra 1996). Generally, classical (inoculative) strategy has not been used as a component of integrated weed management system.

Inundative or Bioherbicidal Strategy

According to Watson (1989), bioherbicides are living entities (natural enemies) used deliberately to suppress the growth or reduce the population of a weed species. This may include an insect, a microbe (bacterium, fungus or virus) or parasitic nematode. These organisms operate directly or indirectly (by production of toxin) and their mode of action proves deleterious to weed species. These toxins are termed bio-control phytotoxins or natural herbicides and are not intended to be included in the definition of herbicide but some investigators use the term loosely to include both of these. Mycoherbicides are simply plant pathogenic fungi developed and used in the inundative strategies to control weeds the way chemical herbicides are used. They are highly specific disease inducing fungi which are isolated from weeds, cultivated in fermentation tanks and sprayed in fields to control biologically a specific weed without harm to the crop or any non-target species in the environment (TeBeest and Templeton 1985). Mycoherbicide research to control agricultural and environmental weeds began in the 1940s. The earliest experiments simply involved moving indigenous fungi between populations of target weeds (e.g. *Fusarium oxysporum*, a fungus, used against prickly pear cactus, *Opuntia ficus-indica* in Hawaii, before the release of the *Cactoblastis cactorum* moth). Bioherbicides are being preferred over chemical herbicides due to the following reasons.

- They are inherently less harmful as compared to the conventional chemical herbicides;
- They are effective in very small quantities and often die with the host;
- BCAs used as bioherbicides are safe and non-toxic to the environment;
- Less cost is involved in their production.

The inundative biological control strategy is more relevant to the needs of agriculture and turf management, as it can generally be implemented through the application of inoculum as liquid sprays or solid granules in a similar manner to conventional herbicides (Auld et al. 2003; Caldwell et al. 2012).

Phases of Mycoherbicide Development

According to Templeton (1982, 1992), the development of a mycoherbicide involves basic three phases: discovery, development and deployment

- Discovery phase includes the collection of diseased plant material, isolation and identification of the suspected pathogen on nutrient media, demonstration of

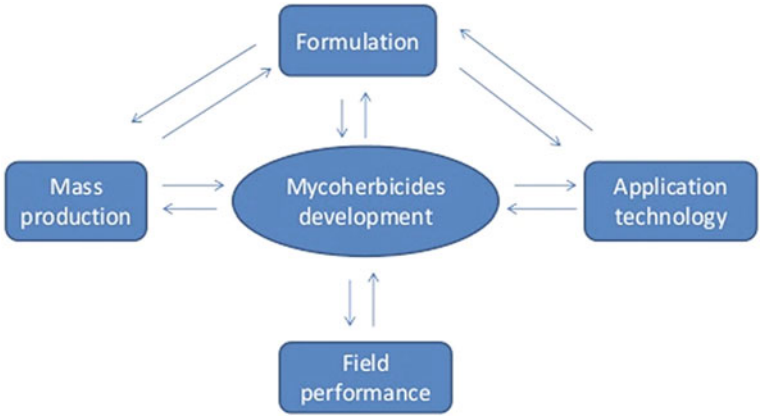

Fig. 23.1 Protocol for evaluation, development and commercialization of a mycoherbicide

Koch's postulates and maintenance of the pathogen cultures in short-term and long-term storage.

- Development phase includes the determination of optimum conditions for production of inoculums required for infection and disease development, determination of host range and elucidation of mechanism of action of the pathogen. The search and identification of an efficacious BCA (nature's best) is the most important step in the development of a mycoherbicidal product.
- Deployment/commercialization phase often involves close collaboration between non-industrial and industrial sectors through the formulation, scale-up, field evaluation and marketing stages of commercialization process of a new mycoherbicide (Fig. 23.1).

Factors Affecting Infections of BCAs

The biocontrol efficacy of a plant pathogen to control a weed depends on the establishment of the disease during the primary infection by the formulation and on the complete control of the weed via its secondary infection.

Primary and Secondary Infection

Researches carried out on different weed pathogen systems related to primary infection (Boyette et al. 1979; Auld 1993; Aneja et al. 2000; Babu et al. 2003) and secondary infection (Boyette et al. 1979; Shabana 1997; El-Morsy 2004) have found Dew and temperature as a major environmental constraints on the biocontrol efficacy.

Surfactants

Surfactants are often necessary to disperse fungal spores within a formulation to enhance its effectiveness in the way to aid spread and adherence of spores on plant surfaces (Mitchell 1988). The surfactants/adjuvants are defined as a class of compounds designed to modify and facilitate the effectiveness of the active ingredients. The major adjuvants used in a formulation are ammonium sulphate, Enhance, Ethokem, Frigate, LT-700, Trion-XR, Tween-80 and Tween-20 (Prasad 1992). According to Womack and Burge (1993), various functions assigned to adjuvants includes "Adhesion", "Provision of nutrients", "Inhibition of resistance" and "Protection from desiccation".

Approaches to Overcome Challenges in Bioherbicide Development

The challenges or constraints that have limited the advancement of bioherbicides have been categorized by Boyetchko and Peng (2004) into the following four types: biological factors, environmental factors, technological factors and commercial factors.

For foliar pathogens, the challenges are biological and environmental factors like temperature, free moisture and protection from ultraviolet irradiation; all these are essential for infection, inconsistent efficacy and limited host range. For any mycoherbicide to be successful, the host pathogen relationship must be tipped in favour of the pathogen. The strategies to overcome these challenges are described below.

Selection and Improvement of Biocontrol Agents

The selection of nature's best biocontrol agent should be done with low number of screening attempts because a relatively smaller range of variations exist among naturally occurring fungal population. This diversity provides excellent opportunities in the search for potential biocontrol agent (Avis et al. 2001). Findings of Yang and TeBeest (1992, 1993) reveal that pathogens showing high virulence along with essential epidemiological traits like rapid infection rates and dispersal are considered as potential biocontrol agent of the various strategies used for improving biocontrol agents; mutagenesis is apparently a quick way of creating new fungal strains with variable traits.

Mass Production

According to Auld (1993), the mass production of mycoherbicide agent is carried out by three potential fermentation systems: submerged liquid culture, solid substrate fermentation and two-phase system. Of the three systems, submerged fermentation system is often used for microbial inoculum production at the industrial level, since it is the most economical method. Fungal propagules (e.g. conidia, mycelia) require moisture (dew) for their germination and infection of the host. Dew dependence in fungi limits their infection efficacy under dry conditions. Collego and DeVine, the first two commercially produced mycoherbicides, are manufactured this way (Churchill 1982; Stowell 1991).

Except for the mushroom spawn production, solid substrate fermentation method is not widely used in bioherbicide production due to the following reasons: higher labour costs, more chances of contamination and recovery of spores from the substrate (Rosskopf et al. 1999).

The fungal sporulation, spore yield and spore viability are the three important aspects to increase the efficacy of a liquid mycoherbicidal formulation. The carbon concentrations and C/N ratios are the main factors to enhance sporulation of a biocontrol agent (BCA). In order to overcome problems with storage, establishment and efficacy in the field, the fungal spores are dried; however, the drying process is not very easy. Frequently used drying methods are freezing, air, spray fluid-bed and a combination of these methods (Churchill 1982).

Application Technology

The most challenging aspect in the application of a mycoherbicide is delivery and retention of sufficient number of fungal propagules on weeds. Initially, the mycoherbicidal agent should be sprayed to the weed foliage till runoff by using aerosol sprayers. This method generally applies excessive volumes (up to 3000 l/ha) that can maximize the retention and exaggerate the potential of biocontrol agents (Greaves et al. 2000). According to Peng et al. (2001), use of high concentration of spores may potentially reduce application volumes without compromising the efficacy of weed control.

Commercialization

Considerable efforts have been made during the last four decades in the commercialization of nature's best biocontrol agents against weeds. The pace of development and commercialization is slow due to an array of biological, economical and regulatory constraints. Several workers have pointed out that despite intensive research and numerous apparently successful biological control agents, very few have reached the marketplace (Aneja 2014).

According to Stowell (1991), successful development of a bioherbicide requires the optimization of nutrients in the fermentation medium, the culture environment and economic aspects. In addition to above factors, pathogen's virulence and its self-life to survive in adverse environmental conditions are the other constraints in the development of bioherbicide. The use of alginate gel technology, microencapsulation, invert emulsions and various additives to enhance germination, virulence and efficacy of a biocontrol agent is important and has been used to overcome the above constraints in the formulation and application of bioherbicide products (Boyette et al. 1979; Connick et al. 1991).

Current Status of Bioherbicides

The number of reports on bioherbicide research has increased tremendously since the early 1980s. Both the number of weeds targeted for control and the number of candidate pathogens studied have increased. Practical registered or unregistered uses of bioherbicides have also increased worldwide. Likewise, the number of US patents issued for the bioherbicidal use of fungi and the technologies have increased, perhaps foretelling an increased reliance on bioherbicides in the future (El-Sayed 2005). The most commercial biological weed control products researched and registered around the globe are based on formulations of fungal species (Table 23.1). The majority of the bioherbicides are based on three fungal texa namely *Colletotrichum*, *Chondrostereum* and *Fusarium* (Aneja 2014; Bailey 2014).

Xanthomonas campestris and *Pseudomonas fluorescens* have been found to be the promising bacterial herbicides (Imaizumi et al. 1997; Tateno 2000). The production of extracellular metabolites with phytotoxic effects has also been observed in a strain of *P. fluorescens*, referred to as BRG100, an extracellular metabolites of *P. fluorescens* with phytotoxic activity have been found to be good to control *Setaria viridis* grassy weed (green foxtail) (Quail et al. 2002; Caldwell et al. 2012).

Viruses, on the other hand, have been suggested to be inappropriate candidates for inundative biological control due to their genetic variability and lack of host specificity (Kazinczi et al. 2006).

Twenty-four bioherbicides have been registered around the globe. Out of these ten are registered in the USA, five in Canada, two in South Africa and one each in Japan, the Netherlands, Florida and China (Aneja 2009, 2014; Aneja et al. 2013; Dagno et al. 2012; Harding and Raizada 2015). A summary of commercial bioherbicides and type of formulation used is presented in Table 23.1.

Interestingly, maximum commercially produced formulations are in liquid state. Liquid formulations have the potential to produce infections soon after application provided. They remain moist on the target plant surface. Several attempts have been made to improve the water-holding capacity in liquid formulations.

Table 23.1 Examples of commercial bioherbicides and type of formulation used

S. No.	Year of registration and country	Target weed	Biocontrol agent	Product name	Formulation type
1.	1960 Canada	Persimmon (*Diospyros virginiana*) trees in rangelands	*Acremonium diospyri*	*Acremonium diospyri*	Conidial suspension
2.	1963 China	Dodder (*Cuscuta chinensis* and *C. australis*) in soybeans	*Colletotrichum gloeosporioides* f. sp. *cuscutae*	Lubao	Conidial suspension
3.	1981 USA	Milkweed vine (*Morrenia odorata*)	*Phytophthora palmivora* (*P. citrophthora*)	DeVine^{R}	Liquid spores suspension
4.	1982 USA	Northern joint vetch (*Aeschynomene virginica*)	*Colletotrichum gloeosporioides* f. sp. *aeschynomene*	Collego™ (Lockdown^{R})	Wettable powder
5.	1983 USA	Sickle-pod and coffee senna (*Cassia* spp.)	*Alternaria cassiae*	Casst™	Solid
6.	1984 USA	Water hyacinth (*Eichhornia crassipes*)	*Cercospora rodmanii*	ABG-5003	Wettable powder
7.	1987 USA	Yellow nutsedge (*Cyperus esculentus*)	*Puccinia canaliculate*	Dr. Biosedge®	Emulsified suspension
8.	1987 Canada	Velvet leaf (*Abutilon theophrastus*)	*Colletotrichum coccodes*	Velgo^{R}	Wettable powder
9.	1992 Canada	Round-leaved mallow (*Malva pusilla*)	*Colletotrichum gloeosporioides* f. sp. *malvae*	BioMal^{R}	wettable powder in silica gel
10.	1997 South Africa	Turf grass (*Poa annua*) in golf courses, *Acacia* sp.	*Cylindrobasidium leave*	Stumpout™	Liquid (oil) suspension
11.	1997 Netherlands	Woody plants blackberry weed (*Prunus serotina*)	*Chondrostereum purpureum*	BioChon™	Mycelial suspension in water
12.	1997 Japan	Turfgrass (*Poa annua*) in Golf courses	*Xanthomonas campestris* pv. *poae*	Camperico™	Bacterial strain cell suspension
13.	1999 South Africa	*Hakea gummosis* and *H. sericea* in native vegetation	*Colletotrichum acutatum*	Hakatak®	Conidial suspension

(continued)

Table 23.1 (continued)

S. No.	Year of registration and country	Target weed	Biocontrol agent	Product name	Formulation type
14.	2002 USA	Dyer's woad (*Isatis tinctoria*) in farms, rangeland, waste areas and roadsides	*Puccinia thlaspeos*	Woad Warrior	Powder
15.	2002/2005 Canada	Deciduous tree spp.	*Chondrostereum purpureum*	MycoTech™ paste	Paste
16.	2004/2007 Canada	Alder, aspen and other hardwoods	*Chondrostereum purpureum*	Chontrol™ (EcoClear™)	Spray emulsion and paste
17.	2005 USA	Dodder species	*Alternaria destruens*	Smolder^{R}	Conidial suspension
18.	2007 Canada	Dandelion (*Taraxacum officinale*) in lawns/turf	*Sclerotinia minor*	Sarritor	Granular
19.	2008 Africa	*Striga hermonthica* and *S. asiatica*	*Fusarium oxysporum* f sp. *stigae*	Striga	Solid, dried chlamydospores + Arabic gum
20.	2009 Florida, USA	Soda apple (*Solanum viarum*)	Tobacco mild green mosaic Tobamovirus (TMGMV)	SolviNix™	Wettable powder/Foliar spray suspension
21.	2010 Canada	Broadleaved weeds	*Lactobacillus* spp. + *Lactococcus* spp.	Organo-sol	Liquid
22.	2011 Canada/USA	Broadleaved weeds	*Phoma macrostoma*	Formulation product name not specified	Granules composed of mycelial fragments and flour
23.	2012 USA/Japan	Broadleaved weeds	*Streptomyces* spp.	MBI-005 EP	Killed, non viable *S. acidiscabies* strains RL-110^{T}
24.	2014 India	Horse purslane (*Trianthema portulacastrum*) in agricultural crops	*Gibbago trianthemae*	Gibbatrianth	Conidial suspension

Modified from Aneja (2014)

In Canada, a significant number of biological agents for control of insects, plant pathogens and weeds have been approved by the Pest Management Regulatory Agency (PMRA), with 24 such products registered between 1972 and 2008 (Bailey et al. 2010). An even greater number of microbes and microbe-derived chemicals have been registered with the United States Environmental Protection Agency (EPA) for crop, forest or ecological management, with 53 such products registered between 1996 and 2010. In 2014, a total of 47 different microbial strains were approved in the EU for the purpose of controlling fungi or insects (Harding and Raizada 2015). Surprisingly, there are no microbes approved for the control of weed species in the European Union (European Parliament 2014). Unfortunately in India, the pace of development of mycoherbicides is still slow. There are many more bioproducts currently under development targeted against specific weeds in several countries. Unfortunately, biological, environmental and technological constraints specially relating to formulation chemistry have been attributed as the main reasons why so many promising biocontrol agents have failed to reach the commercial phase of development.

Brief descriptions of the commercially available bioherbicides are discussed here:

Acremonium diospyri

Acremonium diospyri, commonly called the persimmon wilt fungus, is a member of *Deuteromycetes* (now a member of Ascomycotina). It is characterized by single-celled hyaline catenate conidia produced on simple conidiogenous cells. It had been used as a mycoherbicide since 1960 to control persimmon trees (*Diospyros virginiana*) in rangeland of south central Oklahoma. By 1968, only 5% of the persimmons population in the infected stand were alive. Due to certain limitations, the commercialization of this formulation has been stopped (Schroers et al. 2005).

Lubao

Lubao is a mycoherbicide based on the anthracnose fungus *Colletotrichum gloeosporioides* f. sp. *cuscutae.* It is being used since 1963 to control this parasitic weed in People's Republic of China to control dodder (*Cuscuta chinensis* and *C. australis*) parasitic on broadcast-planted soybeans. Conidial concentrations of 2×10^7 conidia/ml are applied with a hand sprayer until runoff and provide greater than 80% control of this parasitic weed, resulting in yield loss reductions of 30–80% (Templeton 1992). An improved formulation of Lubao 2 is in use today (Wan and Wang 2001).

DeVine^{R}

DeVine^{R} is the first registered mycoherbicide in the world in the year 1981. It is a liquid formulation of chlamydospores of *Phytophthora palmivora* (*Phytophthora citrophthora*) and is used as a post-emergent directed spray to control milkweed vine (*Morrenia odorata*) in Florida citrus groves. The formulation was commercially registered as DeVine^{R} with the EPA in 1981 and re-registered in 2006, however is not commercially available now.

Collego™

Collego™ (Lock Down^{R}) was registered in 1982 for the selective control of a leguminous weed-northern jointvetch (*Aeschynomene virginica*), whose black seeds contaminate harvests of rice and soybeans in the USA. It is a dry powdered formulation of the fungus *C. gloeosporioides* f. sp. *aeschynomene*. The fungus is temperature sensitive; hence, the formulation is stored in air-conditioned area at a temperature of 40–80 °F. Spore germination reduces by exposure to temperature below 32 °F or above 105 °F (Bowers 1986). The commercial production of Collego™ was stopped due to lack of commercial demand and was reintroduced in the market as Lock Down^{R} in 2006 due to the revival of the demand.

Casst™

Casst™ a solid formulation of the dematiaceous hyphomycetous plant parasitic leaf blight fungus *Alternaria cassiae* characterized by dark muriform conidia with long thin beaks. It is an effective mycoherbicide for three weeds namely sicklepod (*Cassia obtusifolia*), coffee senna (*Cassia occidentalis*) and showy crotalaria (*Crotalaria spectablis*). This mycoherbicide was developed by Charudattan and co-workers and was registered in 1983 in the USA (Charudattan et al. 1986). This mycoherbicide is preferred over others due to two reasons: it controls three weeds simultaneously and has the potential to control weed alone as well as with integration of chemical herbicides.

ABG-5003

ABG-5003 mycoherbicide is the wettable powder formulation of hyphomycetous, leaf blight fungus *Cercospora rodmanii*, for controlling aquatic weed-water hyacinth (*Eichhornia crassipes*). First isolation of this biocontrol agent was made by Conway in 1973 from the Rodman reservoir, Florida, USA and the second from Kurukshetra, India, by Aneja between 1988 and 1991. The experiments carried out at Kurukshetra University, Kurukshetra, Haryana (India), of this fungus with the insect *Neochetina eichhorniae* showed good potential to control this notorious

number one aquatic weed in India (Aneja et al. 1990). This formulation was developed in 1984 by Abbott laboratories. ABG-5003 consisted of mycelium and conidia of the biocontrol agent (Charudattan 1986; Aneja 2014).

Dr. Biosedge®

Dr. Biosedge® is a formulation of *Puccinia canaliculata*, a rust fungus to control yellow nutsedge (*Cyperus esculentus*). It was registered in 1987 in the USA. *P. canaliculata* is an endemic rust that completely parasitizes yellow nutsedge. However, this product is not commercially available now due to uneconomic production system and resistance in some weed biotypes (Phatak et al. 1983).

Velgo^{R}

Velgo^{R} a wettable powder formulation of an anthracnose fungus *Colletrotrichum coccodes* was developed as a mycoherbicide in 1987 to control velvet leaf (*Abutilon theophrasti*) in corn (*Zea mays*) and soybeans (*Glycine max*) in the USA corn belt and southern Ontario, Canada. The formulation at the rate of 1×10^{9} spores m^{-2} kills 40% of plants, when inoculated at the two to three leaf stages (Mortensen 1988).

BioMal®

BioMal® is a wettable powder formulation of *C. gloeosporioides* f. sp. *malvae*. It was registered in 1992 in Canada to control an annual weed, *Malva pusilla* (round-leaved mallow). The pathogen attacks stems, leaves and flowers causing lesions on them. On germination, conidia germinates to form black appresoria with pegs within 24 h post spraying of the inoculums. Infection is followed by wilting and necrotic zones and finally death of the plants. The pathogen requires a period of 12–15 h dew for the first 48 h after its application for its biocontrol efficacy. A cool temperature of 20 °C favours the disease development. This mycoherbicide is compatible with many chemical herbicides (Aneja 2014).

Stumpout™

Stumpout™ was registered in South Africa in 1997 to control wattle stumps, turf grass (*Poa annua*) in golf courses and *Acacia* spp.: black wattle (*A. mearnsii*) and golden wattle (*A. pycnantha*). Stumpout™ is a liquid (oil) formulation of the basidiospores of the *Cylindrobasidium leave*, a rust fungus which is applied on the cut stumps like paint (Morris et al. 1999). Recently, this formulation has shown a great potential for controlling *Rohododendron*, an ericaceous shrub commonly

found in riparian areas of the Appalachian Mountains of North America. *Rohododendron* commonly called rose tree, a member of the heath family Arecaceae, is characterized by bright coloured roseous flowers. Stumpout™ is being used for controlling weedy hardwoods trees in South Africa, Canada, the Netherlands and North America (Green 2003).

BioChon™

BioChon™ is the fourth formulation made up of the mycelia water suspension of the fungus *Chondrostereum purpureum*. It was developed in the Netherlands and Canada in 1997 to control broadleaved weedy trees like black/wild cherry (*Prunus serotina*) in the forests. It was developed in 1997. This myeroherbicide is currently being marketed by Koppert Biological systems as an environmentally solution to undesirable tree re-growth. The use of this pathogen for management of weedy, endemic, deciduous trees in conifer plantations and amenity areas is also being evaluated in Canada (Aneja 2014).

Hakatak®

Hakatak® is a formulation of conidial suspension of the fungus C*olletotrichum acutatum* developed for controlling *Hakea gummosis* and *H. sericea* in native vegetation. It was developed in 1990 in South Africa. It is applied as aerially broadcasted pellets or by wound inoculation containing dry spores of the fungus. Hakatak® is currently available only to the interested users (Morris et al. 1999).

Woad Warrior

Woad warrior is a formulation of an autoecious and microcyclic rust *Puccinia thlaspeos*. It was registered in the USA in 2002 to control dyer's woad (*Isatis tinctoria*), a noxious weed in Eight Western States. *P. thlaspeos* infects dyer's woad rosettes in the first year of growth followed by the production of telia (teliospores) and spermatia in the second year resulting in suppressing the production of flowers and seeds on the infected plants. This product is no longer commercially available (Bailey 2014).

MycoTech™

MycoTech™ gel is a formulation of viable mycelium of the saprophytic basidiomycetous fungus *C. purpureum* strain HQ1. It was registered as a mycoherbicide for controlling deciduous trees spp. with the PMRA in 2002 (PMRA Reg. No. 27019) and the EPA in 2005 (EPA Reg. No. 74128-2). This formulation is

applied as a thin layer over the surface area of freshly cut stumps of deciduous trees within 30 min of cutting. Extensive spread of the hyphae takes place throughout the xylem after application of MycoTech that inhibits resprouting, re-growth and causes brown staining and wood decay (Barton 2005).

Chontrol™

Chontrol™ or EcoClear is the formulation of PFC 2139 strain of the basidiomycetous fungus *C. purpureum.* It was registered under the product name Chontrol Paste with the EPA in 2004 (EPA Reg. No. 74200-E/R) and with the PMRA in 2007 (PMRA Reg. No. 27823 and 29293). This mycoherbicide is used to control alder, aspen and other hardwoods deciduous trees and shrubs (Aneja 2014). Both these registrations are currently active, and this product remains commercially available.

SMOLDER®

SMOLDER® is a bioherbicide, based on a dematiaceous, hyphomycetous fungus *Alternaria destruens* (strain 059) characterized by muriform, dark coloured, beaked conidia almost similar in morphology to *Alternaria alternata*. It was registered on May 5, 2005, in the USA to control dodder (*Cuscuta* spp.) in agriculture, dry bogs and ornamental nurseries. It is being manufactured by Loveland Products Inc USA and is available in two forms: **Smolder G**, a soil-applied granular products applied to the moist soil surface at 50 lb per acre immediately prior to dodder emergence; **Smolder WP**, a liquid formulation, is applied to *Cuscuta* vines reaching to the top of crop canopy (Chutia et al. 2007; Aneja and Mehrotra 2011). Currently, Smolder is not commercially available (Bailey 2014).

Sarritor™

Sarritor™ is a granular formulation of the fungus *Sclerotinia minor* to control Dandelion (*Taraxacum officeinale*) weed in lawns/turf. This formulation was registered in Canada in the year 2007. After application to dandelion weed in lawns/turf, the fungus grows into the weed and absorbs the plant tissues until the complete death of the plants. The fungus also disappears with the disappearance of the weed (Abu-Dieyeh and Watson 2007). Hence, it is a very safe biocontrol agent.

Gibbatrianth

Gibbatrianth is a formulation of an indigenous leaf blight, hyphomycetous fungal pathogen, *Gibbago trianthemae*, characterized by muriform non-beaked dark coloured multicellular conidia produced singly on conidiogenous cells with this

ability to germinate by several germ tubes. Each germ tube has the ability to produce another conidium. It has been developed as a mycoherbicide in the laboratory for controlling horse purslane (*Trianthema portulacastrum*), an important weed of agricultural crops in India and the USA. The conidial formulation with the surfactant has the ability to control the weed plants within a few days of its application. Once the weed is gone, *G. trianthemae* also disappears (Aneja and Kaushal 1998; Aneja et al. 2000, Aneja 2014).

Phoma macrostoma

Phoma macrostoma strain 94-44B has been registered for control of broadleaf weeds in turf systems in Canada and the USA (Evans et al. 2013). The name of the product has not been specified by the Scotts Company. This mycoherbicide was conditionally registered in 2011 in Canada but fully registered in 2012 in the USA. *Phoma macrostoma* is a deuteromycetous fungus characterized by dark coloured pycnidia which can tolerate adverse environmental conditions. This formulation is composed of granules of mycelial fragments with flour (Bailey et al. 2011, 2013). An investigation of 64 strains of *P. macrostoma*, including 94-44B, found that the bioherbicidal activity of this species was limited to a genetically homogeneous group of strains, all of which were isolated from Canada thistle (*Cirsium arvense*) (Pitt et al. 2012).

Camperico®

Camperico® is the bacterial formulation of *X. campestris* pv. *poae* strain JTP-482. It was commercially developed as Camperico® by Japan Tobacco Inc., Yokohama for controlling annual bluegrass (*P. annua*). The pathogen enters the host through wounds and migrates to the xylem which was blocked by bacterial exudates resulting in the wilting of the host plant. Eighty-nine isolates of *X. campestris* were evaluated for their biocontrol potential (Imaizumi et al. 1997). This biocontrol agent is temperature sensitive and required low temperature for its efficacy.

MBI-005 EP

MBI-005EP, a bacterial formulation of *Streptomyces acidiscabies* Strain RL-110, was registered as bioherbicide to control annual grasses, broadleaf and sedge weeds in turf, ornamentals and crops such as corn, rice and wheat in 2012 in the USA. It is the first bioherbicidal product that is fermented and then heat-treated to kill bacterial cells before application. The natural compound thaxtonin-A, present in the biocontrol agent, is a known fast-acting phytotoxin that causes necrosis and inhibition in cell biosynthesis and division, resulting in suppression of the growth of the plant (Harding and Raizada 2015).

SolviNix™

SolviNix™, a viral formulation of Tobacco mild green mosaic Tobamovirus (TMGMV), was developed against tropical soda apple (*Solanum viarum*), an invasive weed in Florida. This bioherbicide is available in two forms: liquid concentrate (SolviNix LC) and a wettable powder (SolviNix WP) for controlling the weed on fenced-in pasture areas in the USA (EPA 2015).

Future Prospects

The main hurdles in the development and commercialization of any bioherbicide are slow effect, lack persistence and wide spectrum activity, rapid degradation by UV lights, poor water solubility and their availability. Virulence, efficacy, fermentation, formulation and application are aspects of prime importance. Prospects for the development and utilization of bioherbicide technology for major weeds are very bright. Industry must become more involved in small niche markets, and techniques must be developed for subsistence farmers as well as modern ones. Although there are so many challenges inherent in the development and commercialization of bioherbicides, the excess prevalence of both herbicides-resistant weeds and public concern with chemical pesticides use creates a strong impetus for continued investigations in this field. The progress made in the area of biocontrol of weeds among developing countries has been found almost nil. Government and non-government organizations (NGOs) should come forward to combat this problem and to help biocontrol workers. Result oriented and long term research on the development of bioherbicides, especially in the developing countries is need of the hour.

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Characterization of Lamellate Mushrooms—An Appraisal

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Abstract

Ever since the work on mushroom systematics started, it remained centred around conventional morphology-based parameters. Over a period of time with the advancement of microscopic techniques, the use of internal anatomical details and other microscopic features including SEM details strengthened the taxonomic conclusions leading to the discovery of newer characters for supplementing the morphological features. Simultaneously, the importance of macrochemical and microchemical reactions in mushroom systematics has been emphasized along with the utility of chemotaxonomic and numerical taxonomic methods, although all such characters play a supplementary role in their taxonomic categorization. All such studies enabled the mushroom mycologists to reach right taxonomic conclusions. Towards the end of 1990s, there has been a rapid transformation from conventional morphology-based system to contemporary molecular phylogenetic system based on sequence analysis employing next-generation sequencing technology. Presently, the scientists are trying to understand the higher level of relationship among lamellate mushrooms and their non-lamellate relatives by sequencing gene coding for several specific non-coding ribosomal DNA regions such as *ITS*, *nLSU*, *nSSU*, mitochondrial

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non-coding gene like *mtSSU* and protein coding genes such as *rpb1*, *rpb2*, *atp6*, *tef1*. Of late the use of newer techniques to study mushroom taxonomy and to understand their phylogeny has started slowly picking up in India as well, which is a welcome step. Mushrooms exhibit immense variation of shape, texture, colour, smell, taste and have varied ecological preferences. In the present paper, the importance of morphological, anatomical, chemical, numerical and molecular aspects in the characterization of mushrooms on modern lines has been discussed.

Keywords
Agarics · Taxonomic categorization · Hymenophore · Phylogeny · Field key

Introduction

Lamellate mushrooms, commonly referred as agarics, are a group of basidiomycetous fungi belonging to Class Agaricomycetes (Kirk et al. 2008). They produce fleshy sporophores which are primarily concerned with the production and dissemination of spores. Their origin probably dates back to the mid-Cretaceous period (Hibbett et al. 1997a). In nature, these come up on the forest floor, barren places, road-side verges, and variety of substrates including leaf litter, wooden logs, dung and even living trees during and just after the rains especially representing monsoon season. They are highly fascinating to look at and exhibit immense variation of shape, texture, colour, smell, taste and ecological preferences. The science of collection and identification of mushrooms is age old since earlier these were collected primarily for culinary purposes. To begin with the taxonomy of fungi has traditionally relied on their morphological characters which have evolved through parallel evolution. The phenotypic plasticity in the above-ground reproductive structures among these microbes has resulted in the artificial classification of mushrooms where many of the totally unrelated taxa have been assembled together resulting in lot of confusion. Some such works are those of Mattioli (1560), Clausius (1601), Bauhin (1623, 1651), Ray (1686), Magnol (1689) and Micheli (1729).

On living tree trunk

On dung

On leaf litter

On fallen twigs

Besides their culinary relevance, now many of the mushrooms have become the objects of curiosity and newer researches concerning human welfare, especially in the field of nutrition, as a source of nutraceutics and in ecosystem sustenance and replenishment. There are a variety of aspects including morphological, anatomical, chemical, numerical and molecular, which are being used in the characterization of mushrooms on modern lines.

Morphological Characterization

Traditionally, mushrooms were characterized based upon their gross morphological features and spore print colour. Linnaeus (1753) was the pioneer who applied binomial system of nomenclature to name mushrooms. Persoon (1801) was the first to introduce the term 'hymenium' and use the hymenial characters for characterization of mushrooms. Fries (1821–32) employed macromorphological features including the features of the hymenophore for their grouping into higher level of taxonomic entities. Friesian system employed spore deposit colour to categorize mushrooms into five series, namely Leucosporie (Spores white), Hyporhodii (Spores rosy red), Derminii (Spores rusty red, brown), Pratellae (Spores purplish

black or dark in colour) and Coprinarii (Spores black). Despite being artificial, Friesian system influenced the work carried out on mushroom characterization for a long time. Some such contributors who added bit by bit to the characterization of mushrooms after Fries include Léveillé (1846), Bresadola (1881), Patouillard (1887, 1900), Murrill (1906, 1915), Kauffman (1922), Kühner (1938), Heim (1931, 1955), Gilbert (1931), Malençon (1931), Singer (1932, 1956, 1958, 1962, 1973, 1974, 1975, 1977, 1986), Smith (1949, 1951, 1964, 1972, 1973), Møller (1950, 1952); Romagnesi (1951, 1967), Horak (1968), Rayner (1968, 1969, 1970), Hesler (1969), Pegler (1969, 1972, 1977, 1983), Watling (1970, 1982), Hibbett et al (2014), Hibbett (2016), etc. Significant contributions by these mycologists during the nineteenth and twentieth centuries resulted in substantial progress in the methodology for investigating mushrooms. This has led in the establishment of large number of new genera and species of agarics.

It is an established fact now that macromorphological features of the mushroom sporophores, which can be recorded without the aid of microscope, are important in the systematic study of mushrooms. These should be recorded immediately after their collection in the field itself as they are lost with the passage of time. As suggested by Smith (1949), all members of the *Coprinus* group should be worked out on priority basis followed by fragile specimens like those of *Mycena, Marasmius* followed by fleshy and viscid mushrooms like species of *Russula* and *Lactarius*. Tough and leathery fungi should be taken up afterwards. All such features of the sporophore with respect to pileus, lamellae, stipe, spore deposit, colour change, veil characters, etc., are quite variable and of immense significance in the overall understanding of the mushroom architecture and their categorization. A Field Key for mushroom collector incorporating all such features has been prepared so as to facilitate fieldwork in mushroom systematics. It was published elsewhere as well (Atri and Saini 2000; Atri et al. 2005); however, it is being published again after updating (Appendix).

The Sporophore

The overall sporophore morphology and its different segments are of fundamental importance in mushroom systematics. To draw comparison and to describe the sporophore size, the terms large, medium, small and minute are frequently used. Overall texture is also important which may be soft, watery, tough or even leathery. The size ratio of pileus vs stipe, whether equal to or greater than 1 (Campestroid) or less than 1 (Placomycetoid), is also important in the taxonomic treatment of genus *Agaricus*. The use of this feature was emphasized by Freeman (1979) while working on this genus.

Placomycetoid

Campestroid

The Pileus

The various pileus features which are of fundamental importance in the taxonomy of agarics include its size, shape, colour, surface features, zonation, cuticle and context.

Size and shape of the pileus: Size of the pileus in individual specimens is quite variable and is used along with other characters for taxonomic segregation of agarics. In most of the cases, the young pilei are convex. At maturity, they usually attain different shapes such as convex, conical, hemispherical, ovoid, planoconvex, infundibuliform, campanulate, flabelliform, applanate, reflexed, petaloid, umbonate, involute, depressed, cuspidate, and reniform.

Convex

Infundibuliform

Conical

Umbrella shaped

Flattened depressed

Ovoid

The presence or absence of umbo and variation in its outward appearance is also of taxonomic importance. The cap is said to be umbilicate, if the umbo is reduced and placed in the depression of the pileus. In some species, the margin of the pileus is raised resulting in depressed centre. In some species, the margin becomes uplifted to the extent that the shape of the pileus resembles the funnel as in *Russula*. The shape of the cap is umbonate when there is a rounded or sometimes pointed central hump as in *Termitomyces*. In this way, varied shapes are attained by the pileus.

Obtuse umbo in depressed centre

Broad obtuse raised umbo

Umbo acute

Spiniform perforatorium

Colour: In agarics, the colour of the pileus may be white, creamy, yellowish, orange, pinkish, reddish, greyish, brownish or even the mixture of any of these. The colour of the pileus may remain same throughout its life or may change markedly from the young-to-old condition. Some species exhibit striking colour changes on bruising or cutting. This change may be quick or slow as in *Russula nigricans, Russula albonigra* and *Russula densifolia*. Colour also changes when washed out or on drying, and this feature in some cases is also of taxonomic significance. Textured pilei may exhibit complex colouring. Sometimes the background colour of the cap is different from the colour of the fibrils or squamules. For recording the pileus colour tone, standard colour dictionaries are available. One such dictionary is 'Methuen Handbook of Colour' by Kornerup and Wanscher (1978). Other such standard colour notations were given by Ridgway (1912) and Kelly and Judd (1955). All these features need careful recording in the field itself for their taxonomic utility.

Surface zonation and cuticle: The term zonate is used to describe the condition when the pileus surface bears the concentric markings of alternating regions of light and dark colours. These markings may be entirely absent in some species, while in others, the dark-coloured zones are present alternating with light-coloured zones, but not purely in concentric rings.

The arrangement of hyphae on the pileus surface is quite variable, and due to this, the surface may appear altogether smooth, glabrous or may bear cuticular elements in the form of erect, decurrent or repent outgrowths on its surface. In such situations, the pileus surface is described as pruinose, furfuraceous, granulose, silky, fibrillose, tomentose, floccose, pubescent, squamulose, scaly, echinate, etc., depending upon the relative abundance and nature of outgrowths on the surface. The surface may also be leathery, rugulose, corrugated or may even develop crackings in some cases. Further, it may be dry, moist or viscid. In some pilei

surface, bear pits and in such a situation, it may be described as scrobiculate, alreolate or even lacunose.


Zonate


Radially splitting


Pruinose


Pyramidal


Echinate


Coloured marginal band


Squamulose


Tomentose


Hairy

Margin of the pileus may be smooth, regular, irregular (sulcate, crenate, pectinate, tuberculate, dentate, etc.) or even striate. If the lines on the pileus surface are not the part of the image of the lamellae underneath, but are part of the cap itself, the margin is called striate. If the lines form groove, the margin is described as sulcate, and if small bumps are apparent on the striae, the margin is described as tuberculate-striate. The margin is usually incurved in the young basidiocarps but becomes flat or reflexed at maturity. In some thin fleshed species, the lamellae are apparent through the pileus in moist condition, which is referred to as translucent striate or pellucid. The edge of the pileus may be naked, pubescent or bearded. The cuticle may be peeling partially or not peeling at all. The flesh may be coloured or colourless under the cuticle. This is also a taxonomically important feature and is of great interest.

Pileus context: Some significant characters of pileus context are colour, taste, odour and thickness of the pileus flesh, when fresh or dried. The taste may either be mild, bitter, peppery or even acrid. Similarly, odour may also vary from nil to fragrant, fruity, heavily aromatic, disagreeable, farinaceous, etc. Although these characters are more subjective due to the great varying abilities of different persons in detecting taste and odour, even then it is always desirable to record these features as they may be of some help in certain difficult situation especially taste. The thickness of the flesh is another important feature which must be recorded when the pileus is fresh. In russulaceous mushrooms, taste of flesh is one of the most important features in taxonomic categorization of the species of *Russula*, *Lactarius* and *Lactifluus*.

The Lamellae

Taxonomically important characters of lamellae are their colour, shape, width, depth, attachment, spacing, presence or absence of lamellulae, forking, taste, etc.

Colour: The colour of the lamellae in agarics may be pure white, pale, pinkish, purple, greenish, greyish, dark brown to sepia and even black. It is taxonomically significant to note the colour of gills at all its stages of development. Generally, as in *Agaricus,* the lamellae are whitish to light salmon or even light greyish in young sporophores which become darker at maturity. The lamellae colour may or may not change on bruising or where injured and this is of taxonomic importance.

Shape and width: The relative width of the lamellae is an important character. These may be narrow, moderately broad, broad or even ventricose.

Lamellae attachment: The lamellae may be free or even attached variously to the stipe. The free lamellae may be remote or seceding from the stipe. When attached, these are described as emarginate (abruptly adnexed), adnexed, adnate, broadly adnate, sinuate (notched), arcuate (subdecurrent or decurrent).

Lamellae spacing and depth: The spacing among the adjacent lamellae is fairly reliable character in mushroom. The lamellae are described as equal, unequal, distant, subdistant, close or crowded depending upon the spacing. Besides spacing, the depth of lamellae is another important feature which primarily depends upon the width of the adjacent lamellae.

Lamellae face: The features of the lamellar face may vary depending upon the presence of cystidia and appear pruinose or even pubescent. Sometimes lamellae appear as if waxed.

Lamellae edge: Lamellar edge may be smooth, serrate, dentate, fimbriate, wavy, lacerate, crenate, eroded, crisped or even undulating. Colourwise also, edges may be concolorous with the rest of the lamellae or differently coloured from the rest of the lamellae (*Russula aurata*).

Lamellulae: The term lamellulae is applied to those lamellae which do not reach the stipe, i.e. the short lamellae. These may be absent or present. The lamellae have been described as equal, where the lamellulae are altogether absent and unequal

where these are present. The degree and level to which these lamellulae develop and extend from margin towards the pileus centre have an important bearing on the spacing of lamellae. The frequency of occurrence of short lamellae and the number of series in which these appear on the undersurface of the pileus holds taxonomic relevance.

Lamellae edge smooth

Lamellae edge serrate

Lamellae edge crisped

Lamellae taste: Just like flesh taste, the taste of lamellae is also an important taxonomic feature. The character needs to be noted independent of the flesh taste.

Lamellae forking: The lamellae may be branched or unbranched. The level at which the branching occurs and the number of times a lamellae branches is of taxonomic value. Besides this, whether the lamellulae or lamellae are connected with each other by veins (intervenose) is taxonomically significant and needs to be recorded.

Forking towards the centre

Forking towards the margin

Lamellae forked all over

The Stipe

The various characters of the stipe such as colour, shape, size, surface, firmness, veil, context play an important role in the taxonomy of various taxa of agarics.

Attachment of stipe to the substrate: Various aspects in this regard like whether directly growing in association with the living host, dead substrate or directly on the soil, presence or absence of basal tomentum, rhizomorph, pseudorrhiza, etc., are of significance in the characterization of mushrooms.

Position, shape and size: The stipe may be central or excentrically positioned as in *Pleurotus.* As far as the shape is concerned, the diameter of the stipe may be almost equal throughout (cylindrical) or it may be broad above (clavate) or below (obclavate or bulbous) or even swollen in the middle (ventricose). Rarely it may taper downwards to form a pseudorrhiza as in *Termitomyces*. The width of the stipe is usually variable at different levels. As for the size of the stipe is concerned, it may be comparable to the width of the pileus or it may be longer or even shorter in comparison.

Surface: The stipe surface may be dry, viscid to slimy. If dry, it may be smooth, pruinose, fibrillose, squamulose, rugulose, scaly, scrobiculate, reticulate, ridged or wrinkled, veined, longitudinally striate and even longitudinally grooved. In those taxa, where annulus is present, there may be marked difference in the stipe surface above and below the annulus. As in some species of *Agaricus*, the surface is smooth above the annulus and fibrillose below or vice versa. In some taxa, there may also be a dense growth of mycelium at the base of the stipe.

Turnip like base **Scurfy bulbous base** **Abruptly bulbous base** **Rooting base**

Firmness: The stipe may be fragile or hard, fleshy or cartilaginous. Internally, it may be solid with pith in the centre. The pith may remain persistent in many mushroom taxa, but often developing a hollow with age in others.

Colour: Just like pileus, the stipe colour varies from white, cream, yellowish, orange, pink, red, grey to even brown. Sometimes stipe may be concolorous with the pileus or gills or it may be of different colour. Especially when annulus is present, the stipe may have different colour above and below the annulus. As in case of pileus and lamellae, the colour of the stipe may also change with age and on bruising.

Ratios: The size ratio of pileus vs stipe, whether equal to or greater than 1 (Campestroid) or less than 1 (Placomycetoid), is of significance in the taxonomic treatment of genus *Agaricus*. The use of this feature was emphasized by Freeman (1979) while working on this genus. Similarly, Tullose and Yang (2005) stressed upon recording the ratio of length of the striations on the pileus margins to the radius of the pileus and the width of the central cylinder of the stipe to the overall width of the stipe at least for the largest and the smallest carpophore in a collection for characterizing the mushrooms at species and varietal level.

Latex: Colour of the latex, its subsequent colour change on exposure to air and taste of latex are important aspects for characterization of members of family Russulaceae, especially *Lactarius* and *Lactifluus* at subgeneric, species and varietal level.

Veil: Veil is a layer of specialized tissue that initially protects the developing mushroom and its fertile portion and then breaks up or collapses so as to release the spores. Two basic types of veils, i.e. partial veil (extending from the margin of the cap to the stalk, covering the lamellae when young and towards maturity breaking up to form an annulus around the stipe and cortina along the margin of the pileus as in genus *Agaricus*) and the universal veil (covering the complete sporophore when it is young and then while maturing breaking up into volva at the base, annulus around the stipe, cortina along the margin of the pileus and scales on the pileus and the stipe surface as in genus *Amanita*). In some taxa, both the universal and partial veils may be present, while in others, any one of these may be present. Both may be altogether absent in other taxa.

Annulus: Attachment, architecture, colour, texture, shape and position of the annulus are important in characterization of mushrooms. Annulus ring, when present may be attached or movable, single (*Agaricus*) or double (*Macrolepiota*), white or coloured. In some cases, the colour changes with maturity, while in others, the colour of upper and lower surface of the annulus may be different. Annulus ring may appear collar-like, skirt-like, sheathed above or peronate. The surface of the annulus may be smooth, pruinose or scaly. The scales may be present either on the upper surface or lower surface or sometimes on both the surfaces. Further structurally it may be compact, fibrillose, peppery, persistent, lacerate or even evanescent. The position of annulus is also an important feature. It may be superior, median or inferior.

Volva: It refers to the portion of the visible remains of the universal veil at the base of the stalk in the form of sack or free flaring collar-like structure which may be sometimes in the form of series of zoned concentric scales arranged around the base of the stipe or even powdery. The presence or absence of volva and variation in volval features including its attachment, organization, colour are important in mushroom systematics. It varies greatly with regard to its texture in some of the mushroom genera like *Amanita* and *Volvariella*. Usually, it is membranous with its surface smooth, glabrous, pruinose, areolate or even scaly. Sometimes in case of species of *Amanita,* small pieces of volva are carried on to the pileus surface in the form of warts or even larger volval patches.

Cortina: It refers to the left out portion of the universal and partial veils hanging in the form of short scales and fibrils from the margin of the pileus in mature sporophores. It is quite common in the species of genus *Agaricus* and *Amanita*.

Spore deposit: Historically, the spore deposit colour has played an important role in the classification of mushrooms. Friesian system is one such example in which based upon the colour of the spores, different groups were proposed. Even within the species of the same genus as in case of genus *Russula*, *Lactarius* and *Lactifluus*, the spore deposit colour varies to the extent that it can be relied upon for the taxonomic segregation at infrageneric level and infraspecific level. Romagnesi (1967) has provided separate colour notations depicting the possible range of available colour variation among the *Russula* species. In mushrooms, the spores in print may be white, pale, yellow, green, pink, grey, orange brown to sepia or even black. Under the microscope, the spores may appear white or pigmented. A thick spore deposit is always needed for recording the deposit colour correctly.

Colour change: The change in the flesh colour on exposure is also a taxonomically reliable character. The change may be rapid or slow. The flesh may change to yellow, orange, pink, reddish brown or even to black. Møller (1950) used this character for segregating the genus *Agaricus* into two groups, namely *Rubescentes* (flesh turns red on exposure) and *Flavescentes* (flesh turns yellow on exposure). In some of the taxa like *Russula nigricans, R. adusta, R. densifolia*, there is a change in colour of both the pileus and stipe flesh on exposure. In some cases, the change in stipe flesh may be different near the pileus surface from that near the stipe base.

Macrochemical colour reactions: Macrochemical or metachromatic reactions with pileus surface, stipe surface, lamellae, spores and flesh play an important role in the taxonomy of mushrooms. Detailed information is this regard is available in Singer (1986). Møller (1950) considers Shaeffer's reaction along with other chemical characters to segregate the genus *Agaricus* into two groups. Reaction of spores with Melzer's reagent, cresyl blue, cotton blue, is also very important from taxonomy point of view. Reactions of spores of *Coprinus*, *Psathyrella* and *Panaeolus* with concentrated sulphuric acid play an important role in segregating these from each other. Reaction of stipe with ferrous sulphate in case of *Russula* species is quite important in segregation of *Russula cyanoxantha* and *Russula xerampelina* from other species of the genus. Cresyl blue is another important dye which gives metachromatic reaction with the spore wall of number of mushrooms which is of immense utility in their systematic categorization. The importance of variation in metachromatic colour reactions and metachromatic stains by different parts of the sporophore has been successfully evaluated and documented by Kühner (1934, 1938), Singer (1986) and number of other workers in their treatise.

The Latex

In some of the agarics, latex is exuded when fresh basidiocarps are cut, bruised or broken as in case of genus *Lactarius* and *Lactifluus*. It may be of different colour, and it may also undergo a change in colour, when exposed to air. Just like the flesh,

the latex may be mild, acidic, bitter, acrid or tardily acrid. Latex characters are important in taxonomic segregation in *Lactarius* and *Lactifluus*.


Orange latex changing to green


White milky latex


White latex changing to blue

Microchemical Reaction

Reactions of sporophore parts with iodine provide characters that are of immense significance in mushroom taxonomy. In this regard, the reaction of Melzer's reagent with walls of light-coloured basidiospores, hyphae and epicuticular elements is worth mentioning. In russulaceous mushrooms, a mixture of aldehyde with strong acids leads to darkening of the contents of macrocystidia, dermatopseudocystidia, oleifers and lactifers. The reaction with sulfovanillin, sulfoformol, sulfobenzaldehyde and chlorovanillin is worth mentioning (Singer 1986).

Microscopic Characterization

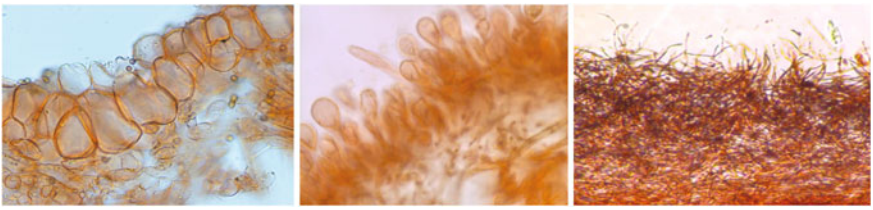
Patouillard (1900) was the pioneer to employ anatomical features of mushrooms for their taxonomic categorization. Peck (1872, 1884, 1907) observed spores, lamellar faces and other mushroom tissues to describe mushrooms. It was the beginning of the use of both macroscopic and microscopic features in mushroom systematics. During the same time, Fayod (1889) also laid much emphasis on the anatomical and microscopic features of mushrooms for their characterization. Murrill (1922, 1924, 1973) was another pioneer in this regard. Subsequently, Kauffman (1905, 1922, 1926), Singer (1932, 1952, 1957, 1962, 1973, 1974, 1975, 1977, 1986), Smith (1949, 1972, 1973), Smith and Singer (1964) and many other mushroom mycologists using better and better microscopes re-described the mushrooms described by the earlier workers in the light of the information obtained through microscopic studies. During the later part of the twentieth century, Friesian genera were critically evaluated in the light of microscopic details resulting in the discovery of large number of interesting agaricoid taxa. With the advancement of facilities, many new anatomical features of mushrooms became handy in their categorization as is apparent from the detailed descriptions available in number of monographs on

individual genera by different mushroom mycologists including *Entoloma* by Horak (1980), *Agaricus* and *Micropsalliota* by Heinemann (1980), *Lactarius* by Hesler and Smith (1979), *Russula* by Romagnesi (1967), *Psathyrella* by Smith (1972), *Lentinus* by Pegler (1983). All such contributions took the understanding of mushroom systematics to a level where it is today. Monumental works of Singer (1975, 1986), Pegler (1977, 1983), Kühner (1984) also played significant role towards the development of present concept of mushroom systematics. In all these treatments, much emphasis has been laid on external and internal sporophore features, basidiospore morphology and cytological characteristics because of which many artificial assemblages have resulted. Numbers of unrelated taxa were classified under the families and orders to which they do not belong thereby resulting in lot of undesired phylogenetic confusion. The availability of molecular techniques is proving handy in solving this confusion and streamlining the classification and placement of various genera and species. Hibbet et al. (1997b), Garnica et al. (2007) and Hibbett (2007) have made significant contributions in this direction. The internal details of the sporophores with respect to pileus, stipe, hymenophore, spores, etc., which are of relevance in mushroom systematics are detailed in the ongoing account.

The Pileus

The internal details of the pileus cuticle and trama have an important bearing on the taxonomy of agarics.

Pileus cuticle: The pileus cuticle is a specialized surface layer which is quite variable in different taxa and hence is of immense taxonomic significance. It is called 'cutis', when made up of repent hyphae oriented mostly in a radiating pattern from the centre of the pileus towards its margin. It may be present as a thin pellicle, as a 'hymeniform palisade' of cells, as a 'pseudoparenchymatous' cellular layer or as an interwoven layer of filamentous hyphae from which other elements arise. The cuticle can also be ill differentiated. In such a case, the hyphae of the pileus surface and flesh are of the same size and are interwoven in the same manner except that there is a narrow zone extending from the surface to some distance in the flesh, in which the hyphae are more compactly interwoven and more densely pigmented. Such a cap is described as 'homogenous' and is found in *Tricholoma, Clitocybe*, etc.



Cellular cuticle Turf of pedicellate cells Trichoderm

In many species, a thin layer of very narrow hyphae more or less radially arranged is found to cover an otherwise homogenous pileus. In such cases, the diameter of the hyphae forming pellicle is almost half in contrast to the hyphae immediately beneath them. The pilei possessing such a pellicle are typically glabrous and polished in appearance. Another variation in the agarics with hyphal cuticle is that the hyphae walls gelatinize causing the cap surface to be sticky or slimy to touch. In such cases, cuticle is called as an 'ixocutis'. In some agarics, the cuticle may be 'cellular' also, when it is composed of palisade of more or less pear shaped to clavate cells. In certain species, the cuticular cells are similar to those found in a palisade except that they are not arranged regularly. In some members, the cuticle may be divisible into 'epicutis' and 'subcutis'. Cuticle surface may be smooth without any structure over it or may bear projecting hyphae and other sterile structures. The structures on the cuticle may be sparsely spaced or closely aggregated so as to form a 'turf'. The cuticle of this type is referred as a 'trichoderm'. The projecting elements of the trichoderm may be purely hyphal in nature or mixed with pileocystidia of different shapes and sizes. Sometime the trichoderm is gelatinized and in such a case, it is described as 'ixotrichoderm'. In the trichoderm when there are thick-walled, clearly differentiated, ascending anticlinal terminal elements, the cuticle is called lamprotrichoderm.

Pileus trama: The pileus trama or context is the region of the pileus below the cuticle. It may be 'homoiomerous' depending upon whether it is composed of hyphae exclusively or 'heteromerous', if it possesses nests of spherocysts intermixed with connective hyphae (Russulaceae). Lot of variations are there in context with respect to the size and pigmentation of connective hyphae. Gelatinous layers may occur in a few species and when present are useful in identification. In some cases, conducting elements may also be present in the trama.

The Stipe

In stipe, the microscopic detail of the cuticle, context, veil and annulus is important in mushroom characterization.

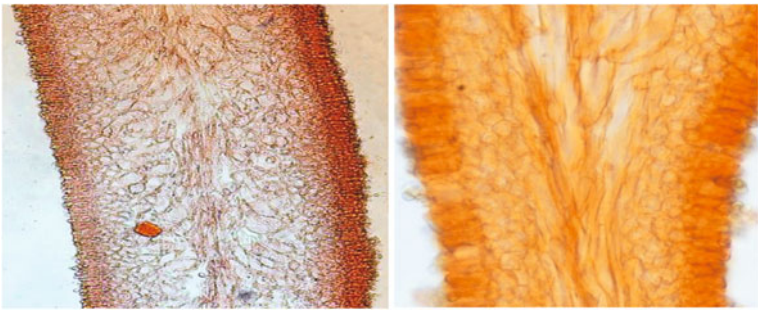
Stipe cuticle: The stipe cuticle shows little variability as compared to pileus cuticle. Generally, the stipe cuticle is composed of a layer of narrow hyphae running parallel to the long axis of the stipe. The stipe surface may be smooth representing a typical 'cutis' or beset with appendages which may either be in the form of hyphal projections typically forming a 'trichoderm' or in some cases varied types of sterile elements called 'caulocystidia' are also present interspersed among the cuticular elements. Some times as in pileus, stipe cuticular elements may be gelanized forming an 'ixocutis'.

Stipe Context: The stipe context may be homoiomerous (*Agaricus*) or heteromerous (*Russula*). In contrast to pileus context, it is more compact, denser and cartilaginous. Generally, the hyphae of the stipe core disintegrate with maturity, thus developing a hollow in the centre. Sometimes the pith may be persistent also. The stipe context hyphae may be hyaline or pigmented.

Universal veil: The nature of universal veil, whether hyphal or cellular, is of great taxonomic relevance. Further the width of hyphae or cells, i.e. whether narrow or broad and whether inflated or uninflated, is helpful in segregating different taxa especially in the genus *Agaricus.* Just like the universal veil character, the microscopic details of annulus and volva are also of taxonomic significance.

Hymenophore and Its Associated Sterile Structures

Hymenium: Hymenophore is associated with spore formation in agarics. It is restricted to the lamellar region. The central tissue of the hymenophore constitutes the hymenophoral trama. On the periphery of the trama lies the subhymenium which may be distinct or indistinct. The hymenium arises from the subhymenium. As in the pileus, the lamellae trama may be 'heteromerous' or 'homoiomerous'. The manner in which the tramal hyphae are arranged in homoiomerous members is of immense importance in taxonomy. Depending upon the arrangement of hyphae, trama may be 'unilateral' or 'bilateral'. When unilateral, the hyphae may be parallel, subparallel or interwoven as is found in large number of agarics. In regular trama, the hyphae are arranged in a parallel manner while in subregular trama, they are rather intricately tangled, and in this type, the hyphae cells are often relatively short and curved. Bilateral trama can be 'divergent' or 'convergent'. In case of bilateral divergent gill trama, the hyphae project downwards as in parallel type but then branch or curve (diverge) towards either subhymenium as in *Amanita*. In *Volvariella*, the hymenophoral trama is bilateral inverse type or convergent type in which the hyphae project downwards and towards the centre of trama so that the impression of 'V' is created, if one observes them progressively from the gill edge towards the pileus. The hyphal cells in this type are quite conspicuous due to their length and width.

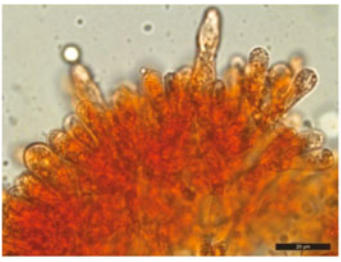


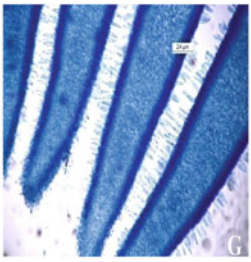
Bilateral divergent trama Bilateral convergent trama

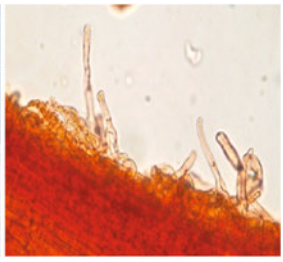
Subhymenium: Subhymenium may be hyphal or pseudoparenchymatous or sometimes altogether indistinct. When pseudoparenchymatous, the cells are variable in their shape and size.

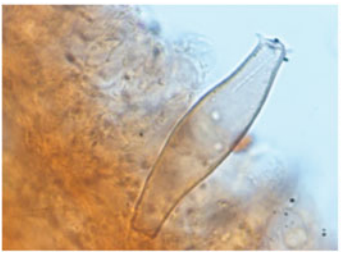
Basidia: Hymenium is chiefly made up of basidia and basidioles but in some species, cystidia are present along with all the structures in the hymenium. In mushrooms the basidia are clavate autobasidia which are 2–4 spored and always chiastic, with mostly half sickle-shaped sterigmata. Generally, basidia are as broad as spores and about 2–5 times as long as longer axis of the spores. Any variation from this constitutes taxonomically important character.

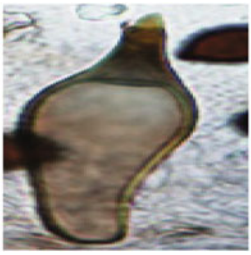
Cystidia: The sterile structures that are found interspersed with basidia in any part of the hymenium are termed as cystidia. Romagnesi (1944) and Singer (1975, 1986) independently stressed that different types of cystidia can be distinguished both morphologically and chemically. In mushrooms these may be cylindrical, fusiform, clavate, ventricose, lanceolate, lagniform, pyriform, metuloidal or even hyphoid. Hesler and Smith (1979) in their monograph on 'North American species of *Lactarius*' recognized 'macrocystidia' and 'pseudocystidia' depending upon their origin and overall shape. Macrocystidia are thin walled to thick walled which arises deep in the hymenium and are normally fusiform to conical in shape in comparison to pseudocystidia which are thin walled contorted to hyphoid in shape without a basal septum. Thick-walled macrocystidia are normally referred as 'lamprocystidia' while thin-walled macrocystidia are called 'leptocystidia'.

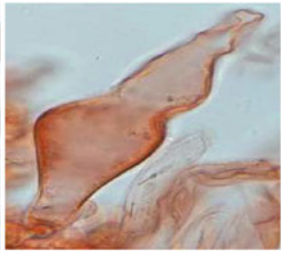

Cheilocystidia


Pleurocystidia


Caulocystidia


Cystidium with frongs


Metulloid


Fusiod ventricose cystidium

Based upon their distribution on various parts of the sporophore following types have been recognized:

A. **On the hymenophore**

(a) On the sides of lamellae Pleurocystidia
(b) On the edge of lamellae Cheilocystidia.

B. **On the surface of the pileus or stipe** Dermatocystidia

(c) On the pileus cuticle Pilocystidia
(d) On the stipe cuticle Caulocystidia.

The cystidial details with respect to their shape, size, abundance, location, pigmentation, reaction with chemicals, etc., are important from taxonomic point of view. Nakamori and Suzuki (2007) emphasized the defensive role of cystidia in basidiomycetous mushrooms. Depending upon the presence or absence of cheilocystidia on the lamellae edge, these are described as fertile when the edges are occupied by basidia only, heteromorphous when both basidia and cystidia are present on the lamellae edges and sterile when edges possess cystidia only. The reaction of cystidia with Sulfovanillin, Sulfoformol and Chlorovanillin in *Russula* and with Ammonia in *Psathyrella* is quite unique and important in mushroom characterization (Singer 1986).

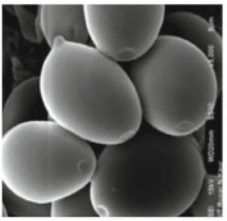
Clamp Connections

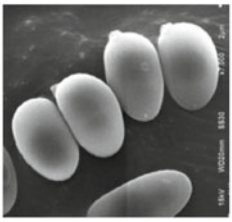
The presence or absence of clamp connection in the basal mycelium, hyphae of the stipe, pileus context or at the base of the basidium is also of some taxonomic value. In the members of family *Russulaceae* (*Russula*, *Lactarius*, *Lactifluus*), normally clamps are absent.

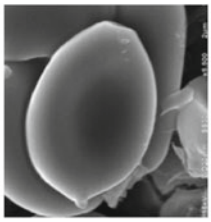
Spores

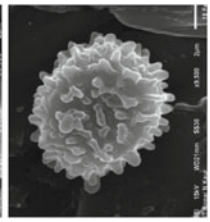
In agarics, the spores are produced on the sickle-shaped sterigmata born on the basidia. Their shape, size and pigmentation are fairly constant characters. These are unicellular single- or double-walled and may be small to gigantic, smooth or ornamented, exhibiting various shapes as evenly rounded nodulose, nodose-stellate, cruciform, angular, ellipsoid, elongated, lentiform, amygdaliform, etc. These may be apiculate or non-apiculate, porate or non-porate, when porate, apical pore may be truncate or lens-shaped. Vellinga (1988) has framed guidelines for measuring spore size based on the ratio of spore length to width (Q) and designated spores as globose (Q = 1.00–1.05), subglobose (Q = 1.05–1.15), broadly ellipsoidal (Q = 1.15–1.3), ellipsoidal (Q = 1.3–1.60) and elongate (Q = 1.60–2.00). These may be smooth or variously ornamented. Singer (1986) has recognized 12 types of exosporial ornamentation in agarics. Key provided by Singer (1986) is quite handy for categorization of spore types in different mushroom genera. Electron microscopic details of ornamented spores provide additional features of taxonomic importance.

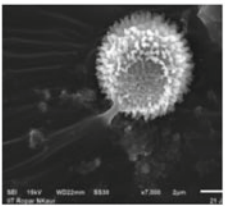
Spore plage is the differentiated zone at the adaxial side of the spore above the hilar appendix (Pegler and Young, 1971). Plage needs to be measured for size and characterized for the level of amyloidy (Clémençon, 1970). Based upon amyloidy, 4 types of plage, namely non-amyloid, centrally amyloid, distally amyloid and totally amyloid, have been recognized by Verbeken (1997).

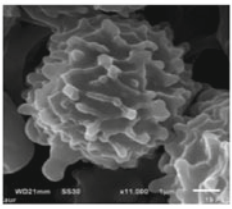

Spores with apical pore

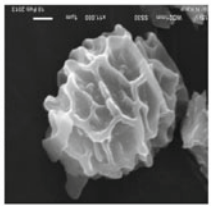

Elongate spores

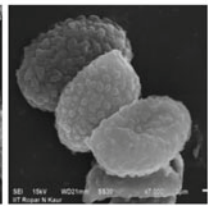

Truncated apex


Few warts connected


Plage


Incomplete reticulum


Complete reticulum


Isolated warts

Molecular Characterization

Molecular techniques are now becoming an important tool for studying taxonomy and phylogenetic relationships among fungi. The techniques for analysing the structures of nucleic acids, i.e. DNA and RNA are additional to the traditional methods of identification in systematic mycology. Molecular markers of rDNA sequencing, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), microsatellite and mitochondrial genotypes have been used in taxonomic studies of various mushroom species. The comparative studies of coding and non-coding regions of ribosomal DNA are widely in use for construction of phylogenetic trees of various organisms including mushrooms. The increased sophistication in molecular biology techniques and advancement in computer hardware and software have lead to an exponential growth of molecular systematic applications now a days.

Internal transcribed space (ITS) region is the most extensively sequenced DNA region in fungal molecular systematics at the species and within the species level. This is primarily because of higher degree of variation, than other genetic regions of rDNA, in this region and its polymorphic nature which provide sequence variability which is of immense help in making distinction among different species or strains of mushrooms.

For molecular taxonomy of mushrooms, firstly DNA is extracted from the material using standard protocol or by using commercial kits. By the use of polymerase chain reaction (PCR), small amounts of total genomic DNA are amplified to large amounts of specific DNA. For this, PCR primers (commonly

ITS1 and ITS4 for fungi) are used that anneal to specific regions in the genomic DNA as a template. Then, the resulting fragments are separated and visualized by electrophoresis (SDS-PAGE). Unique DNA fragments are excised and purified from the gel, and the amplified product is used for nucleotide sequencing. The DNA sequence data is analysed by comparing to DNA database to locate analogous sequences like Basic Local Alignment Search Tool (BLAST) searches of GenBank. Evolutionary distances among isolates and related taxa are calculated, and phylogenetic trees are constructed.

Towards the end of 1990s, phylogenetic studies with broad taxonomic sampling across agaricomycetes began to appear (Hibbett and Donoghue 1995; Larsson et al. 2004). Lentinoid fungi were among the first agaricomycetes to be studied with molecular approaches (Hibbet and Vigalys 1991). Molecular phylogenetic analysis of ribosomal RNA sequence has transfigured the circumscription of the *Agaricales* sensu lato in the past decade as is apparent from the studies of Hibbett et al. (1997a, b, 2007), Larsson et al. (2014), Moncalvo et al. (2002), etc. The major revelation of these studies showed that the classification based on the fruiting body form and the hymenophore type are reported to be phylogenetically misleading (Hibbett et al. 1997a, b). In the earlier treatment of mushrooms, there has been over emphasis on the morphological characters because of which many genera and families of agarics are not monophyletic (Moncalvo et al. 2002). Some of the broad molecular studies (Bodensteiner et al. 2004; Binder et al. 2005) have demonstrated evolutionary relationship among non-lamellate resupinate and cyphelloid forms of agaricomycetous fungi, and there are instances where molecular investigations have united non-lamellate and gasteroid representatives in various clades with lamellate relatives (Matheny and Bougher 2006). Justo et al (2014) gave the molecular phylogeny and phylogeography of Holarctic species of *Pluteus* section *Pluteus*. Seelan et al. (2015) detailed the phylogenetic relationships and morphological evolution in *Lentinus*, *Polyporellus* and *Neofavolus* while emphasizing Southeast Asian taxa. Yang et al. (2015) took morphological and molecular evidence while describing a new species of *Russula.*

Presently, the scientists are trying to understand the higher level of relationship among the major groups of agarics and their non-lamellate relatives by sequencing the genes coding for nuclear large and small subunits, ribosomal RNA, mitochondrial rDNA genes and nuclear and mitochondrial protein encoding genes. Hibbett (2007) described the current state of systematic and evolutionary studies in mushroom forming agaricomycetous fungi. The revision of classification of higher ranks including mushrooms within fungal kingdom, largely based on the results from the Assembling the Fungal Tree of Life (AFTOL) project, is primarily dedicated to enhancing our understanding of the prevailing evolutionary trends in this major clade of life.

Chemical Characterization

Mushroom scientists have been working on the chemical characterization of mushrooms after realizing their relevance in human health. Robbers et al. (1964) while working on the chemotaxonomic importance of the chemical constituents of 39 species of *Inocybe* emphasized its taxonomic importance at the infrageneric level. In the literature, it has been emphasized that high percentage of allantoic acid present in *Coprinus* and *Leucocoprinus*, as against low percentage in *Macrolepiota*, shows a certain chemical affinity between *Coprinus* and *Leucocoprinus* and increases the hiatus between *Leucocoprinus* and *Macrolepiota* on the other hand. The presence or absence of psychotropic alkaloids also has some taxonomic importance in dark spored agarics (*Strophariaceae*, *Coprinaceae* and *Bolbitiaceae*), where it is linked with the activity of certain enzymes which contribute to the oxidative transformation of psilocin to blue product.

Rapior et al. (1990) by using thin-layer chromatography emphasized the importance of polyols, amino acids and phenolic acid and microscopic characters of basidiospores in the classification of species of *Cortinarius*. Some other works which have emphasized the possible utility of chemical constituents in mushroom systematics include Arpin and Fiasson (1971), Frisvad et al. (1998), Pizzaferiato et al. (2000), Petrini et al. (2009), etc.

Numerical Taxonomy

Numerical taxonomy helps in constructing the classification by grouping taxa that share similar characteristics (Sneath and Sokal 1973). In agaricomycetous fungi, number of workers including Kendrick and Weresub (1966), Demoulin and Schumacker (1972), Machol and Singer (1971, 1977), Clémençon (1970), etc., have demonstrated the utility of numerical taxonomic methods in the systematic categorization at the supra-generic level. Objective classification scheme for *Laccaria* has been developed in North America on the basis of numerical taxonomic analysis of the data obtained through the detailed examination of basidiocarp and somatic cultures of this mushroom. Numerical taxonomic analysis of cultural characters of mushrooms has also been reported to be of utility for examining the interrelationships between the taxonomic entities as has been demonstrated between boletes and some gasteromycetous fungi and also in the generic circumscription of *Laccaria* and *Lactarius* (Dickinson and Hutchinson 1977).

Appendix: Field Key to Mushroom Collector

1. Date of Collection: 2. Collection No. 3. Field Photograph No.
4. Habit type: Agaricoid/ Amanitoid/ Lepiotoid/ Coprinoid/ Boletoid/ Tricholomatioid/ Pleurotoid/ Volverielloid/ Pluteoid/ Inocybeoid/ Armillarioid/ Pholiotoid/ Strophariod/ Paneoloid/ Clitocybeoid/ Collybioid/ Mycenoid/ Omphalioid/ Naucoroid
5. Locality: State: Altitude:
6. Habitat: On wood/ On humus/ On pasture/ On dung/ On compost/ In woods
7. Type of Soil:
8. Forest type……..Coniferous / Broad leaved/ Mixed
9. Forest Composition:
10. Size of the fructification:
11. Deliquescing at maturity or not:
12. Stature of the carpophores: Campestroid/ Placomycetoid
13. Colour: When young…… at maturity…….
14. Growth type: Solitary/ In groups/ Caespitose/ Gregarious/ Scattered
15. Sporophore morphology:

I. THE PILEUS

(a) Colour: When young……at maturity…….

(b) Size……..

(c) Shape:

Infundibuliform/ Convex/ Campanulate/ Flabelliform/ Umbonate/ Involute/ Umbilicate/ Applanate/ Conical/ Reflexed/ Petaloid/ Depressed/ Cuspidate/ Hemispherical/ Reniform

(d) Umbo: Present/ Absent: Broad/ Acute

(e) Margin: Regular/ Irregular: Splitting at maturity or not: Whether transulscent striations visible (Pellucid) or not/ Notched/ Lobed/ Normal/ Involute/ Reflexed

(f) Surface: Dry/ Moist/ Viscid (Hygrophanous/ Non- hygrophanous)

Moist Pilei: Atomate/ Glabrous

(g) Scales: Present/ Absent. If present whether Appressed fibrillose/ Recurved fibrillose/ Squarrose/ Squamulose/ Innate/ Surperfical/ Powdery; Whether aggregated along the margin/ Centre or cover the entire pileus surface. Colour of scales.....

(h) Latex: Present/ Absent. If present whether Watery/ Milky/ Coloured. Taste of latex.....Any colour change in latex colour on exposure.....

(i) Any colour change where pileus is cut/ bruised, if any; whether colour change is quick (take less than 1 minute)/ slow (take more than 1 minute).

(j) Apex: Plane/ Umbonate/ Pointed/ Nippled/ Depressed. Detailed description of umbo----------

(k) Cuticle: Not peeling/ Half peeling/ Fully peeling. Flesh colour under the cuticle.....

(l) Flesh: Original flesh colour.....Whether flesh colour changing/ unchanging on exposure. Flesh thickness and colour change, if any.....

(m) Texture: Fleshy/ Coriaceous/ Membranous/ Brittle/ Leathery. Pileal Veil: Present/ Absent, if present whether Patchy/ Scaly/ Warty/ Powdery/ Appendiculate/ Areolate

II. THE LAMELLAE

Free/ Adnexed/ Adnate/ Broadly adnate/ Sinuate/ Decurrent; Equal/ Unequal; Crowded/ Subdistant/ Distant; whether in Series or not.

(a) Width of lamellae: Narrow/ Moderately broad/ Broad/ Ventricose

(b) Colour of the lamellae: When young..... At maturity.....

(c) Any colour change where bruised.....

(d) Lamellae edges: Smooth/ Serrate/ Dentate/ Fimbriate/ Wavy/ Lacerate

(e) Nature: Deliquescent/ Fragile/ Waxy/ Normal

III. THE STIPE

Central/ Lateral/ Excentric

Length.....Breadth.....Colour.....Whether conclorous with the pileus or differently coloured.

(a) Any colour change on cutting/ bruising (particularly near the base) Whether Fleshy/ Cartilaginous/ Stiff hair like.

(b) Shape: Equal in diameter throughout/ distinctly bulbous/ Tapering downward so as to form pseudorrhiza/ Tapering at both ends and broad in the middle/ Clavate/ Obclavate/ Tubular/ Mycelioid at the base or not.

(c) Whether latex is exuded on cutting the stipe: Yes/No

If yes whether Milky/ Watery/ Coloured; Change in the latex colour on exposure.

(d) Type of Veil: Universal/ Partial

Volva: Yes/No, If yes its description: Friable/ Limbate/ Saccate/ Floccose/ Evanescent/ Membranous/ Lobed

Annulus: Yes/No.....Single/ Double

Decidous/ Patchy/ Arachnoid/ Evanescent/ Persistant/ Funnel shaped/ Pendulus, whether attached above or below (Peronate), movable or not.

(i) Position: Superior/ Median/ Inferior

(ii) Colour: Outer surface.....Inner surface

(e) Stipe surface; Smooth/ Pruinose/ Fibrillose/ Pubescent/ Viscid/ Slimy

16. Taste: Yes/No, if yes: Peppery/ Acrid/ Tardily acrid/ Spicy/ Sour/ Disagreeable/ Agreeable/ Mild.
17. Odour: Yes/ No, if yes: Spicy/ Fragrant/ Fruity/ Aromatic/ Farinaceous/ Mild
18. Colour of the Spore Print:
19. Colour Reaction of Spores with Melzer's Reagent: Dextrinoid/ Amyloid/ Inamyloid/ Pseudoamyloid. (Particularly if spore print is white, creamy or yellow):
20. Macrochemical Tests
21. Shaeffer's Reaction (For genus *Agaricus*): Positive/ Negative
22. Identification: Genus.....Species.....
23. Information about edibility, if any (Recipe detail if edible):
24. Medicinal utility, if any
25. Ethnomycological Information
26. Any other field information
27. Field Sketch

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Occurrence and Distribution of Mushrooms in Semi-evergreen Sal (*Shorea robusta*) Forest Chhattisgarh, Central India

25

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Abstract

Chhattisgarh is an important state covering largest semi-evergreen Sal (*Shorea robusta*) forest area in the country, where the Achanakmar–Amarkantak Biosphere Reserve (ABR) harbours a rich mushroom wealth in association with the varieties of tree species. Mushrooms, an important component of the forest ecosystem, grow on a variety of habitats open or shady grounds. Some grow under certain kinds of plant litter, and some of these grow only with one kind of tree species showing their host specificity. The mushroom species like *Amanita, Boletus, Cantharellus, Russula and Lactarius* are usually associated with certain types of trees. There are number of ectomycorrhizal mushrooms (EM) many of which have narrow host range, facing greater threat due to transformation of forests land. The mushrooms such as *Agaricus, Amanita, Cantharellus, Calocybe, Geastrum, Ganoderma, Lentinus, Pleurotus, Russula, Termitomyces, Volvariella, Scleroderma* appear widely in different forests; however, local people lack knowledge of in situ conservation of these species, thus could not maintain their regular accessibility. Therefore, distribution, biology, taxonomy, chemistry, cultivation and conservation of mushroom have great significance and relevance, particularly among the tribal groups many of which prefers to live in the forests.

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Pine · Rhizosphere · Ectomycorrhiza · Litter

Introduction

In Central India, the Gondwana landscape is one of the richest tracts of wildlife and tribal population in India. This is an old land made up of ancient, geological sedimentary rocks occupied by variety of tree species, which represents maximum number of varieties of tree species. The tree species occur in soil type derived from highly weathered parent rocks exhibit exclusive characteristics as they are adapted to survive in nutrient-deficient soils (Maguire 1970; Berry et al. 1995). Moreover, the land area of tropical world identified a global hotspot of biodiversity which is characterized for varieties of indigenous tree species.

Chhattisgarh a third largest undulated hilly state representing maximum varieties of tree species in the country. The state is lying between 17.46′N to 24.6′ latitude and 80.15E to 84.51E longitude, which is characterized for a marked variation in climate, vegetation and topography due to its geographical bedrock types and the ranges over altitudinal belts. The forest ranges of northern and southern parts in uplands (500–1050 m) are defined for semi-evergreen true Sal forests (Shukla et al. 2014) and the central part at low elevation gradients (250–400 m) is defined for mixed Sal forest. Thus, state determines a Sal tree due to its dominating character occurring at 35–40% in mixed and 90–95% in true Sal forests. No matter if Teak tree (*Tectona grandis*) has been originated as an exception in a single confined area of Bastar.

Although in reference to anthropogenic impacts, a large part of primary forests have been converted into secondary forests reflecting structural differentiation in between native and other tree types assemblages, but still the state's forests representing numerous genera of native deciduous and semi-evergreen tree species, which are comparatively more selective to support herbaceous ground flora rather than shrubby species. The forests harbour much more decomposer groups particularly mushrooms, because leaf litter of varieties tree species maintains an adequate temperature and soil moisture content to the ground surface.

This is true for all types of the forest where varieties of mushroom species and other members of fungal community occur mostly either on living or on dead organic mass (Richard et al. 2004; Dighton et al. 1986). Richard et al. (2004) observed some old-grown forests for higher numbers of mycorrhizal fungi (MF) than the saprotrophic fungi. In case of productive undisturbed forest, the occurrence of MF (often >50% of total macro-fungi) seems to be an indication of forest health (Arnolds 1988). In this context, the forests having high proportion of

MF considered better conserved as compared to the forests comprising of lower proportion of such fungi (Ortega and Lorite 2007).

The study on documentation of mushrooms has been conducted by several workers from northern and southern parts of India, but from Central India particularly in Chhattisgarh, no concerted effort has been made for any detailed study on Agaric flora available in a vast area of Sal forests. A large number of workers (Verma et al. 1995; Saini and Atri 1993; Lakhanpal 1991, 1995; Natarajan 1995; Khaund and Joshi 2011) from different parts of the country have reported mushrooms. The 181 taxa of 20 genera have been worked in the Himalaya (Thind 1961; Kaul 1992). The edible mushrooms, viz. *Volvariella diplasia, Pleurotus ostreatus, Boletus crocatus, Agaricus arvensis* and some puff-balls have been identified from Baroda Gujarat (Moses 1948). Two new tropical species each belonging to *Russula* and *Lactarius* have been reported from Madhya Pradesh and were taxonomically described (Rahi et al. 2003). Krishnamoorthy et al. (1998, 2000) experimented techniques for commercial production of a high yielding strain of *Calocybe indica*.

Miles and Chang (1987) have suggested a procedure for collecting and conserving the germ plasm of *Lentinus edodes*, while Suharban and Natarajan (1999) suggested some preservation methods of oyster mushrooms. Shukla et al. (2005) worked on spawn production of *Lentinus cladopus*, and Pandey et al. (2000) worked on *C. indica* spawn production. Rahi et al. (2002) studied nutritional potential of *Termitomyces heimii* as well as its nutraceutical importance (Rahi et al. 2005). Deshmukh (2004) emphasized the tropical basidiomycetes macro-fungi as they have novel secondary metabolites of pharmaceutical importance, while Joshi and Khaund (2014) showed the importance of DNA bar coding of wild edible mushrooms. Miller et al. (1989), Neal et al. (1973), Rovira (1965) have studied the rooting systems of plants, which are responsible for different types of root exudates and micro-flora. Melin (1963) has shown that root exudates and extracts of certain plants contain substances that stimulate fruiting of certain mushrooms. Romell (1938) stated that some members produce fruit bodies only when attached to host plant. Moreover, the study on biodiversity, distribution and conservation of mushroom is more important (Manoharachary et al. 2005) due to their economic importance.

The richness of Sal tree and its wild relative tree species, viz. *Anogeissus latifolia, Buchanania lanzan, Lagerstroemia parviflora, Pterocarpus marsupium, Terminalia tomentosa, T. bellerica, T. chebula* on geographical scale in Chhattisgarh as whole acquires particular relevance to investigate mushroom diversity because the plants (tree species) and mushrooms ecology of one forest stand will not be the same in the other stand. During an ecological study in Puerto Rico, a higher percentage (20–30) of Agarics has been found as new records (Lodge 1988). Dennis (1970) has listed 2412 species of macro-fungi in his fungal biota of Venezuela, but believed it to be a 20-fold underestimated of the probable species total for the region. However, in our experience of different forest ranges, the species of *Amanita, Agaricus, Cantharellus, Lentinus, Pleurotus, Termitomyces, Russula, Volvariella* are more important as per the edibility and number of species.

The Agarics as a group occurs in a varieties of habitat. Some species exist in areas that are geographically separated, while some are known only from restricted areas and many species do seem to show preference for a certain type of natural habitats as well as for a particular substrate. So far bulk of excellent edible mushrooms more than requirement met by the tribals found in soil, dung, plant derbies, independently or in association with particular plant species has never been conducted for documentation and germ plasm collection. Therefore, study on identification of unexplored novel varieties of mushrooms as food supplements, medicinal importance and characterization of toxic mushroom to prevent their consumption by tribals inhabiting in and around the forests seems to be more important.

Materials and Methods

Collection Site

The Achanakmar–Amarkantak Biosphere Reserve (ABR)

The biosphere named after Achanakmar (ACH) tribal village in Bilaspur, Chhattisgarh, and Amarkantak (AMK) is located on an edge of Anuppur district in Madhya Pradesh. The area has a special place in the tribal psyche as they are experiencing healthy livelihood. The topography of the biosphere fall under biogeographical zone —Deccan Peninsula—a central high lands (6A) of the country. Physiographically, ABR can be divided into three ranges; (i) a narrow elevation in ACH, (ii) a wide elevation gradient in Lamni and (iii) slightly narrow elevation in AMK.

ABR biosphere differs with the neighbouring northern state of the country due to habitat specificity of metalliferous rocks, the soil composition, elevational gradients and occurrence of natural primary and man-made secondary forests. The hot and moist geographical area explains numbers of analytical and synthetic parameters relating variability and richness of tree species. The medium-height mountain (400–800 m) consisting environmental heterogeneity offers to both the deciduous and semi-evergreen tree species. Nevertheless, the reserve is a very specific habitat to understand unusual longevity of relic mixed tree species and endemicity of Sal tree which monopolizes at increasing elevation up to 1150 m.

Achanakmar (ACH)

The Achanakmar tiger reserve a core zone of 551.15 km^2 of biosphere located in Bilaspur district (Shukla 2003; Tiwari et al. 2009) is selected to record the tree diversity, as its one hectare area represents for many more variety of tree species denoting. The forest is important in respect to its one hectare area representing for maximum tree diversity, stem density and the degree of dominating local tree species. The area divided into lower and upper elevational ranges has an average rainfall 146 cm in general, and the average relative humidity remains found to be 60% in the morning and 52% in the evening hours. The soil is acidic reddish brown

and dark yellowish brown. During present course of study, a long-term monitoring of ACH, an area of 500 × 5 m was designed at three sites, S-1, S-2, S-3, to record the mushroom flora (Shukla 2002a, b, 2003; Shukla 2006; Tiwari et al. 2009).

Amarkantak (AMK)

Besides ACH (56 km), Chhaparwa (62 km), Lamni (70 km) and the Amarkantak (90 km) are the areas where the higher temperature in summer (April–June) at altitudinal ranges (400–1100 m) is not similar. The Amarkantak of Madhya Pradesh state, a pristine habitat separates the river Narmada, Johilla and Sone, to drain into the Arabian Sea by way of Bengal. The area is situated in 22° 40′N, 81° 45′ E/22.67°N 81.75°E/22.67; 81.75 at the elevation of 1048 m. The plateau of this area is made up of bauxite rocks that were mined out for commercial use. The topography of the area varies from the rice fields below in Anuppur and from the wheat fields in Dindori district.

The Vegetation

The state's forests representing primary true Sal, mixed Sal, Teak forests and secondary Sal, Teak, Pine, Eucalyptus forests in highly dissected hills put a bio-climatic map for the occurrence of ground flora and wild life. Within the defor-estation and plantation practices, the vegetational area is divided into the natural and man-made types which greatly differ in macro- and microclimatic factors such as temperature, potential evapotranspiration, soil moisture and below-ground diversity in respect to non-forest areas.

Primary Forests

The primary forests of the state represent unique combination of Dipterocarp, Caesalpinoid, Mimosoid and a higher percentage of Combretaceae. The dominating Sal tree occupies every possible habitat and widely distributed in both the true and mixed forests as it grows well in humid acidic soil. In mid elevations, the floristic composition of mixed forest is largely composed of semi-evergreen tree types and deciduous tree types, viz. *Acasia arabica, Butea monosperma, Diospyros melanoxylon, Eagle marmelos*, while up-land plateaus offer Sal tree for its fairly homogeneous status.

Secondary Teak and Pine Forests

Presently, the secondary forests after tree felling, the nutrient represents larger area than that of primary forests seems to be an ephemeral structure in respect to eco-logical sustenance and population dynamics. In plantation sites (Teak, Pine, etc.),

the large tree canopy gap allows penetration of too much radiation to cause stony soil surface layer depleting bioconversion of leaf litter. The increased soil surface temperature enhances vertical movement of water and energy altering physical soil conditions, where the planted tree types fail to achieve a considerable plant girth and height. Overall, the tree felling and planting teak and pine tree cut down a better shelter to many symbiont groups like bacteria, mycoflora and insects, which prefer to grow only in rhizomorphic net work of their own host.

Tribals

The state represents for the largest population of primitive tribal in the country (Tiwari 1994). The tribal groups who prefer to live in and around the forest learn their bioresources through experience and compulsions (Kaul 1993; Harsh et al. 1993a, b; Kumar and Shukla 1995; Rajak et al. 2002; Shukla 2002a, b). The Baiga tribal remains greatly depend on non-traditional food like tubers, fruits and mushrooms which avoid agricultural practices; therefore, they are better in knowledge and experience on wild mushrooms using potential dietary supplements, prevention and treatment of various human diseases. Due to lack of proper education, the critical information regarding these varieties of mushroom that the tribal communities possess and utilize in their daily-living practices are far from reach to common people. These limitations hinder large-scale use of ethnic value of the mushroom by the Indian pharmaceutical and food industry (Tables 25.1).

Ectomycorrhizal Mushrooms (EM)

Ectomycorrhizae exhibit specific characteristics in a type of host plants, as several ectomycorrhizal mushrooms established a symbiotic relationship with Sal tree found in a kind of habitat of the biosphere (Table 25.2). The rhizosphere of Sal tree harbours specific mushrooms denoting role of its own exudation that promotes

Table 25.1 Potential utilization of tribal mushrooms of Chhattisgarh by tribals

S. no.	Mushrooms sp.	Local name	Potential utilization
1.	*Agaricus* spp.	Gobari pihari	For treatment of goitres/edible purpose
2.	*Cantharellus* sp.	Baans pihari	Tonic for easy delivery for women/eating purpose
3.	*Russula* spp.	Sarai pihari	Healing wounds/eating purpose
4.	*Termitomyces microcarpus*	Bhaat pihari	Tonic for weakness/eating purpose
5.	*Calvatia cyathiformis*	Dharti phool	Used for healing wounds
6.	*Lycoperdon* spp.	Ghundi	Used for coagulation of blood

Table 25.2 Edible mushrooms and their habitats

Living plant spp. rhizosphere, bark	*Cantharellus, Geastrum, Russula*, *Clitocybe, Armillaria*
Fresh plant residues	*Lentinus, Pholiota, Pleurotus, Agrocybe*
Compost materials	*Volvariella, Coprinus, Agaricus*
Soil and humps	*Lepiota, Termitmyces*
Megalithic lateritic monument	*Psilocybe, Amanita*
Wood logs	*Psilocybe, Lentinus*
Paddy straw heaps	*Volvareilla*

growth of the fungi. Since a large variety of tree species had been reared over the mixed Sal forest of ACH thousands thousand years back. Therefore, some other species like Sal tree can be identified possibly facilitate EM to establish symbiotic relationship as a number of tree species have been reported (Beenken 2004; Smith and Bonito 2012) from tropical world. The endemicity of Dipterocarp, Caesalpinoid, Mimosoid and of *Terminalia* species and abundance of EM found in phosphate deficient forest (Högberg and Pierce 1986) increases expectations to obtain more mushroom symbionts, which may have evolved with the ancient tree plant(s).

The abundance of varieties of tree species under nutrient-deficient thick forest corridor in ABR is an indication to understand tree dependency on specific mushroom types. The study reveals that many Dipterocarp tree plants found associated with EM to improve plant survival against elevated temperature, drought, poisonous compounds and high acidic soil conditions (Islam et al. 2007). Therefore, in order to determine occurrence and distribution of EM mushrooms in association with host tree species, one has to make keen observation on rhizosphere of corresponding tree plant(s). This is important as per the tree felling and other forms of disturbances such as agricultural practices (Thompson 1987), crop rotation, mining (Jasper et al. 1987; Gardner and Malajczuk 1988; Bellgard 1993), as the above activities markedly reducing populations of mycorrhizal fungi (Table 25.3).

The Collection of Mushrooms

Mushrooms were collected in the morning hours, and the essential study of the collected specimens was done at the spot itself, which includes the recording of necessary general information and morphological characteristics regarding the specimen. Habitat photographs of the mushrooms were also taken. The preservation of the specimens was done by drying the specimens in the specially designed cabinet over hot air convector.

Table 25.3 Common mushrooms of ABR

S. No.	Name of the species	Site-I + 2	Site-3	Site-4
1	*Amanita emilii*	+	−	−
2	*Amanita pantherina*	+	−	−
3	*Amanita vaginata*	+	−	+
4	*Amanita fulva*	+	−	−
5	*Amanita hemibapha*	+	−	−
6	*Amanita caesaria*	+	−	−
7	*Amanita Verna*	+	−	−
8	*Amanita gemmata*	+	−	−
9	*Amanita pachycolea*	+	−	−
10	*Camarophyllus* sp.	+	−	−
11	*Cantharellus minor*	−	+	−
12	*C. tropicallis*	−	+	−
13	*Chlorophyllum* sp.	+	−	−
14	*Coprinus* sp.	+	−	−
15	*Coprinus* sp.	+	−	+
16	*Cortinarius* sp.	+	−	−
17	*Ganoderma* sp. I	+	−	−
18	*Ganoderma* sp. II	+	−	−
19	*Ganoderma* sp. III	+	−	−
20	*Geastrum* sp. I	+	−	−
21	*Geastrum* sp. II	+	−	−
22	*Inocybe* sp. I	+	−	−
23	*Inocybe* sp. II	+	+	+
24	*Laccaria* sp.	+	−	−
25	*Lactarius eccentrica*	+	−	−
26	*L. angustifolius*	+	−	−
27	*L. deliciosus*	+	−	−
28	*Lentinus cladpus*	+	−	−
29	*Lentinus* sp. I	−	+	−
30	*Lentinus* sp. II	−	+	−
31	*Lepiota* sp. I	+	−	−
32	*Lepiota* sp. II	−	+	−
33	*Lepiota* sp. III	+	−	−
34	*Leucoagaricus* sp.	+	−	−
35	*L.cepaestipes*	+	−	−
36	*Macrolepiota* sp.	+	−	−
37	*Marasmius* sp. I	+	−	+
38	*Marasmius* sp. II	+	+	+
39	*Mycena* sp. I	+	+	−
40	*Mycena* sp. II	+	+	+

(continued)

Table 25.3 (continued)

S. No.	Name of the species	Site-I + 2	Site-3	Site-4
41	*Mycena* sp. III	+	−	+
42	*Pleurotus* sp. I	−	+	−
43	*Pleurotus* sp. I	+	−	−
44	*Rhizopogon* sp.	+	−	−
45	*Russula delica*	+	−	+
46	*R. pseudodelica*	+	−	+
47	*R. emetica*	+	+	−
48	*R. nigrainitialis*	+	−	−
49	*Russula* sp. I	+	−	+
50	*Russula* sp. II	+	−	−
51	*Schizophyllum* sp. I	+	−	−
52	*Schizophyllum* sp. II	−	+	−
53	*Scleroderma* sp.	+	−	−
54	*Sinotermitomyces* sp.	+	+	+
55	*Termitomyces clypeatus*	+	−	−
56	*T. microcarpus*	+	+	−
57	*T. rabuorii*	−	+	−
58	*Termitomyces* sp. I	+	−	−
59	*Termitomyces* sp. II	+	−	−
60	*Termitomyces* sp. III	+	−	−
61	*Termitomyces* sp. IV	+	−	−
62	*Volvariella volvacea*	+	+	
63	*Volvariella* sp.*	+	−	−

+ Present, − absent

*Species found paddy straw stock in tribal village

The completely dried specimens were packed in 6″ × 4″-sized polythene bags containing crystalline silica gel to moisture absorber and naphthalene balls to prevent the insect-pest infestation. The packets containing the dried samples were then labelled in brief. The spore prints were obtained by removing the pileus and placing over white paper with gills facing the paper and covered with a large Petri dish. After preservation of all samples, microscopic observation was done for the identification of particular mushroom.

Sections of dried fruit bodies were taken and revived in 10% KOH. The sectioned materials were then stained in phloxine, and camera lucida diagrams made. Other reagents, which were used, include Melzer's (for testing amyloidity) and cresyl blue (for testing metachromatism). Photomicrographs were also taken. After microscopic study of collected specimens, they were keyed out to their respective families, genera and species level mainly consulting (Pegler 1977, 1986). The descriptions and illustrations of some of the specimens also carried out with the help of the literature. This being a preliminary study, more field trips had been

undertaken to the four sites during the rainy period to get to know the mushrooms diversity of the ABR and to compare quantitatively the agaric assemblage in different sites.

Site-1 (Mixed Sal Forest)

During investigation, a number of species of *Amanita, Boletus, Cantharellus*, *Lentinus, Russula* and *Termitomyces* were collected from ABR and various other neighbouring forests. A total of more than 500 collections of mushrooms were recorded which includes 80 species belonging to 26 genera, out of which 20 specimen of Agaricales were described fully with their microscopic and macroscopic details. Several genera of mushroom including *Agaricus, Amanita, Agrocybe, Boletus, Coprinus, Lentinus, Lepiota, Pleurotus, Russula, Termitomyces, Tricholoma, Volvariella* have been collected from decaying wood, leaf litter and the ground surface. Thus, primary forests were found rich in diversity of EM fungi which were greater in this mixed forest site than the true Sal forests (Site-2) (Table 25.4).

Table 25.4 Distribution of Agaricales in ABR

Name of the species	Site-1 + 2 Sal forests	Site-3 Bamboo tree	Site-4 Teak tree
Amanita emilii		−	−
A. fulva	++	−	−
A. hemibapha	+	−	−
A. pantherina	+	−	−
A. vaginata	+++	−	+
Cantharellus sp.	−	++	−
Coprinus spp.	+++	+	+
Inocybe sp. 1	+	−	−
Laccaria sp. 1	++	−	−
Lentinus cladopus	++	+	−
Lepiota spp.	++	+	−
Marasmius spp.	+++	+	+
Mycena spp.	+++	++	+
Russula spp.	+++	+	+
Termitomyces clypeatus	+	−	−
T. microcarpus	+++	++	−
T. rabuorii	−	++	−
Termitomyces sp. 1	+	−	−
Termitomyces sp. 2	++	−	−
Termitomyces sp. 3	+	−	−
Termitomyces sp. 4	+	−	−
V. volvacea	+	+	−

Abundant = +++, common = ++, rare = +, absent = −

Site-2 (True Sal Forest)

Habitat and Host Specificity of Mushrooms

Many more micro-fungi and mushrooms are cosmopolitan in distribution, but certain species are found in association with a particular tree type and a kind of habitats. The present work is more relevant in reference to uniformity of Sal tree in different elevational gradients and the abundance of Bamboo plant in mixed forest. The ectomycorrhizal mushrooms, *Amanita*, *Boletus*, *Russula, Scleroderma*, are specialized to occur with Sal tree only and are thus not found within the uniform vegetation of Teak and Pine trees in secondary forests. The species, viz. *Amanita vaginata, A. pantherina, A. emilii, A. fulva, Russula* spp., *Lactarius* spp. *Scleroderma* sp. were observed to be mycorrhizal while associated with Sal tree. The *Amanita hemibapha* was found closely associated with the root of *T. tomentosa* and observed to be mycorrhizal with this tree. The *Inocybe* sp. was recorded from the root of *L. parviflora* tree, whereas a *Laccaria* sp. suspected for its symbiotic relationship with the Sal tree. The *Cantharellus* was only mycorrhizal genera recorded from the bamboo forest.

Some of the EM grows symbiotically with the roots of certain plants also found capable to grow saprophytically forming fruiting bodies (Mandadori 1985). Such fungi have been termed as facultative ectomycorrhizal group (Batton and Chalot 1995; Marks and Foster 1973; Mandadori 1985), for example *Lactarius* sp. is found to grow under the same condition in association with Sal tree (Rahi et al. 2008). Many other mycorrhizal mushroom, viz. *A. vaginata, Russula* spp. and *Lactarius* spp. are found in the secondary forests (Teak, Pine), but they fail to established their symbiotic relationship with the tree species. In this direction, investigation on economically important EM is essential, which may or may not be capable of their symbiotic relationship with other tree types (Teak, Pine, etc.) of secondary forests.

Amanita

Mushrooms are important for both human beings and for maintenance of forest ecosystem. Many *Amanita* valued edible species and some others are extremely poisonous. In India, a large number of new *Amanita* species have been found to establish their ectomycorrhizal relationship with Sal tree (Bhatt et al. 2003). The ectomycorrhizal genus *Amanita* found in association with ancient tree species explains its autonomy and the primitiveness of an old forest. Therefore, study on Amanita is important to relate origin and evolution of relic mixed tree species which exclusively survive with unusual longevity under environmental distress conditions. Among *Amanita* spp. (*A. pantherina, A. emili, A. fulva, A. vaginata, A. gemmata, A. verna, A. pachycolea*), only *Amanita caesaria* was found to be edible.

Termitomyces

T. heimii is one of the most popular wild mushrooms, which is under immense collection pressure in the state. The species of *Termitomyces* growing in association with termites are known to originate from tropical forests (Nobre and Aanen 2010)

like Sal forest. The Sal tree provides shelter for many more species of termites to build their nests between ridges and furrow at various heights of tree trunks. The large tree canopy and its litter found most suitable to the home of various species of Termites and *Termitomyces*. The fruiting bodies of weather oriented *Termitomyces* species appear in corresponding termites mounds during rainy season. The mutualistic association of *Termitomyces species* with an animal group keep the genus in advance position rather than EM denoting symbiosis primitive feature of a forest ecosystem. So far, in present knowledge, not a single species of *Termitomyces* could be grown independently (Kuja et al. 2014). A total of nine species of *Termitomyces, T. heimii, T. clypeatus, T. microcarpus, T. rabuorii, T. radicatus, T. erruhizus, Termitomyces* sp.-1*, Termitomyces* sp.-2*, Termitomyces* sp-3*, Termitomyces* sp-4, were found edible.

Cantharellus

Cantharellus species is somehow host-specific as it more prominently obtained in bamboo corridor. The mushroom can be easily recognized by their fleshy and firm fruit bodies, often trumpet- or funnel-shaped. They are in bright yellow, orange, grey colour and have usually fruity pleasant smell. This genus has been extensively studied by many workers including Manjula (1983), Purkayastha and Chandra (1985). Bilgrami et al. (1991) listed four species of *Cantharellus* from India, and Lakhanpal (1997) reported three Indian species. During investigation, *C. minor* has been collected. The species was found associated with bamboo culms showing symbiotic mycorrhizal plant.

Volvariella

The mushroom called as straw mushroom which is a more common edible tropical mushroom and very popular among the tribal community. This is an adapted mushroom of hot and humid habitat-specific Chhattisgarh state. The mushroom showed thermotolerant tendency to grow under the high-temperature conditions (32–38 °C). The cultivation of mushroom is the easiest as it grows quickly within a little period of four days in naturally composting paddy straw stored in farmer house-yard. Therefore, under eco-climatic conditions and availability of enormous paddy straw as a substrate, the rural community practised mushroom cultivation by their own ways. However, in Chhattisgarh, two species, namely *V. volvacea* and *V. pseudovolvacea* have been reported, and many other important species still to investigate are unknown. In Central India, the genus *Volvariella* is represented by three species (*V. volvacea, V. media* and *V. speciosa*); only *V. volvacea* is edible.

Russula

Singer (1986) retained the family Russulaceae in Agaricales and included two genera—*Russula* and *Lactarius.* The species of *Russula* could be obligatorily symbiotic association forming ectomycorrhizal relationships with various tree species (Beenken 2004). Some species could survive 1–3 years establishing new individuals (Smith and Bonito 2012). Since the species of *Russula* occur in a wide range of climatic conditions (Romagnesi 1967; Singer et al. 1983; Buyck 2007;

Beenken 2004), therefore, estimation of its various degrees of host specificity among the variety of tree species is more important particularly in a mixed forest of ACH.

Topographically, the richness of varieties of tree species over metalliferous rocks and altitudinal gradients between Achanakmar and Amarkantak accounts more harbouring a rich diversity of Russula than other forests of the country. The genus *Russula* showed higher diversity, which might be due to the richness of Sal trees, a most important partner of ectomycorrhizal mushrooms species. This is very much true in reference to the temperature and moisture variables as these are found influencing the colour and morphological characters of *Russula* species (Busuioc and Elekes 2013).

Although many species of *Russula* possess excellent edibility as per the experience of local tribals, many of them are not advisable as they absorb higher concentrations of toxic elements (Busuioc and Elekes 2013), rather it depends on metallic concentration of soil type (Kalac and Svoboda 2000; Stijve et al. 2004). However, *Russula nigricans* indicates capacity as a bioremedial agent in heavy metal-polluted soils (Busuioc et al. 2011). Moreover, *R. nigricans* an edible mushroom found in ectomycorrhizal relationship with Sal tree described as new species from Chhattisgarh (Rahi et al. 2003). The present study revealed more than 25 species of *Russula*, out of these more than 15 were found edible among the tribals and many were used for treatment of various ailments and diseases (Rahi et al 2002; Shukla 2002a, b; Tiwari et al. 2009; Tiwari 2011).

Lactarius

During our investigation, a number of *Lactarius* species were collected many of which seem to be ectomycorrhizal. Among the *Lactarius* species, *L. piperatus. L. controversus, L. stramineous, L. volemus, L. corrugis, L. angustifolius, L. deliciosus*, the edible one *Lactarius eccentric was* found in ectomycorrhizal relationship with Sal tree described as new species (Rahi et al. 2003). All these species were found edible among tribals and local inhabitants of the state found with ectomycorrhizal association.

Lentinus

Lentinus species are also very common as they are more confined to tropical and subtropical regions. The moist and hot low land area of ABR has been found more suitable to this genus. This is found abundantly in dead wood log stand of Sal tree. The *L. cladopus*, which is called Lakri pihari in local language, is very important edible mushroom, and tribals used it in their routine meal. The mushroom had been grown in artificial conditions (Shukla et al. 2005). During investigation, six species have been collected and described as unknown species.

Pleurotus

A total of 6 species of *Pleurotus* have been discovered out of which 3 species, viz. *P. ostreatus, P. florida, P. sajo-caju* were identified. Fruiting body, of most of the species are found solitary on decaying Sal wood log. A different *Pleurotus* sp. of which pileus 1.5 cm in diameter, plane, pale yellow, dry, smooth, found growing gregarious in Bamboo wood stumps and another one species was found grown over the living tree *Butea superba*. All the recovered species were edible and consumed by the tribals. Some of the species were grown and obtained better spawn in mushroom cultivation.

Ganoderma

The mushroom grows exclusively on tree species of the family Mimosoideae, Caesalpiniaceae, Euphorbiaceae. The powder of mushroom is used to treat cough and asthma. Presently, the mushroom has its commercial applications, as dried specimen is often collected and sold to traders.

The Sal forests occur in undulated hills of this state quite different possessing enormous number of deciduous and semi-evergreen tree species. There are different story of tree species with intact foliar canopy maintaining temperature and moisture. The substantial amount of leaf litter fall protecting soil by water run-off and enhancing population of decomposer groups. Thus, a habitat of mixed Sal forest returns back its own leaf litter conserving soil moisture, activating biotic interactions for rapid decomposition and mineralization maintaining soil nutrients to ground flora. This is a fate and specificity of renewable of organic waste under moist warm habitat of mixed forest in low land area ACH (Site-1), where a short circuited energy consistently flows between the soil and old tree species. Despite a due course of nutrition supply, the uniformity of Sal tree (Site-2) also explains its habitat betterment providing shelter free from harsh atmospheric temperature to large herbivore animal to urinate and make bulk of dung to enrich the soil and ground flora. Nevertheless, the old forest maintains both the macro-scale (temperature and moisture) and micro-scale factors (micro-biota) in way to bioconversion of organic substrate and availability of nutrients to the mushrooms most of which exclusively found in association with Sal tree.

The precipitation is one of the most important macro-scale factors to determine the mushrooms which are typically weather dependent. The occurrence, abundance, association and the extent of presence or absence of the mushrooms like *A. vaginata, Mycena, Marasmius, Coprinus* and *Rhizopogon* which appeared soon after the first rain. The *A. pantherina, A. hemibapha, T. clypeatus, A. emilii*, *A. fulva, Laccaria* sp. 1, *Termitomyces* sp. 1, *Termitomyces* sp. 3, *Termitomyces* sp. 4, *Chlorophyllum, Leucoagaricus, Lentinus* and *Pleurotus*, obtained in middle of the season, whereas *Termitomyces rabuorii, T. microcarpus* and *Geastrum*, appeared from mid-to-late rainy season, and *Inocybe* sp. 1, *Termitomyces* sp. 2 were found in

late period of rainy season. However, *A. vaginata, Volvariella volvacea, Mycena, Marasmius, Russula, Coprinus* and *Schizophyllum* were collected around the rainy season (mid-June to mid-October).

The beginning and duration of field seasons of mushrooms are typically dependent on precipitation, but the temperature, humidity, plant litter, also equally leading broad effects in different eco-climatic zones (Odum 1953). A thick leaf litter in ground surface maintains both the temperature and moisture (Fernández-Toirán et al. 2006) to stimulate spore germination as members of Agaricales appears at different times of the year due to their different temperature and humidity requirements (Moyersoen 2006). Therefore, from an ecological view point and occurrence of mushrooms, the uniformity of Sal tree and its higher percentage in mixed forest is more meaningful than other tree types (Teak, Pine, Eucalyptus, etc.). In a forest floor, the leaf litter is a chief source that counts lesser or greater to microbial invasion and their growth availing nutrients to the tree plants (Christensen et al. 1989).

The litter complexity or its removal depleting growth of mushrooms and other fungi (Eaton et al. 2004; Sayer 2005) as in the case of Teak and Pine forests where the tree plants also fight back due to dissimilar nutrient deficient conditions. Anyhow the forests sites representing mostly saprotrophic mushroom types such as the species of *Marasmius, Mycena, Agaricus, Coprinus, Termitomyces* and others occasionally occur lesser than the mushrooms found in leaf litter of Sal and Bamboo tree types. This explains that Teak tree leaf litter supports lesser than that of Sal and bamboo trees in a way to enhance activities of decomposer group and to regulate nutrients flow to the tree plants.

The Sal tree (Site-1 Site-2) ensures woody debris and rhizomorphic network in soil to activate saprotrophic mushrooms and other fungi and EM which ultimately help in retention or development of a large tree community in nutrient-deficient metalliferous rocks. Evidently, a dense canopy cover in undisturbed primary forest ecosystem produces more EM species fruiting bodies as compared to the disturbed forest with low canopy density. However, the saprotrophic mushrooms did not vary significantly. Many species of *Russula*, *Geastrum*, *Lactarius*, etc., characteristically found associated with Sal tree, whereas *Cantharellus* species obtained associated with Bamboo plant, but could not be traced in open and deforested land areas. Although few of *Russula* species found in Teak and Pinus forests, but they are not capable to establish their symbiotic relationship with the tree plants. However, it is assumed that basidiospores and sclerotia have considerable longevity than mycelia strands separated from roots of host plant Fries (1987).

A *Ganoderma* sp. in Site-1

Rhizopogon sp. in Site -1

A termite mound with *Termitomyces*

In fact, the relic mixed Sal forest in which the tree species exclusively grow in geo-climatic limitations showed their endemicity and unusual longevity in association with many mycorrhizal fungi (Dwivedi et al. 2012; Shukla 2002a, b; Tiwari et al. 2009; Tiwari 2011). The gilled-fungi such as *Lentinus clavata*, *Russula delica*, etc., that are characteristically present in an area of Sal tree develop abundant mycelium over decomposing litter where fruit bodies easily develop after overturning partly decomposed leaf litter. The EM species of *Russula* and *Lactarius* showed wide acceptability of climatic condition as these are found associated with *Pinus* and other conifers in temperate climate also occur in tropics with association of Sal tree. The abundance of *Scleroderma texense* and *Inocybe* sp. near seedlings of Sal tree suggesting that both the above mushroom types might be specific among the mycorrhizal fungi to occur in 'early stage'.

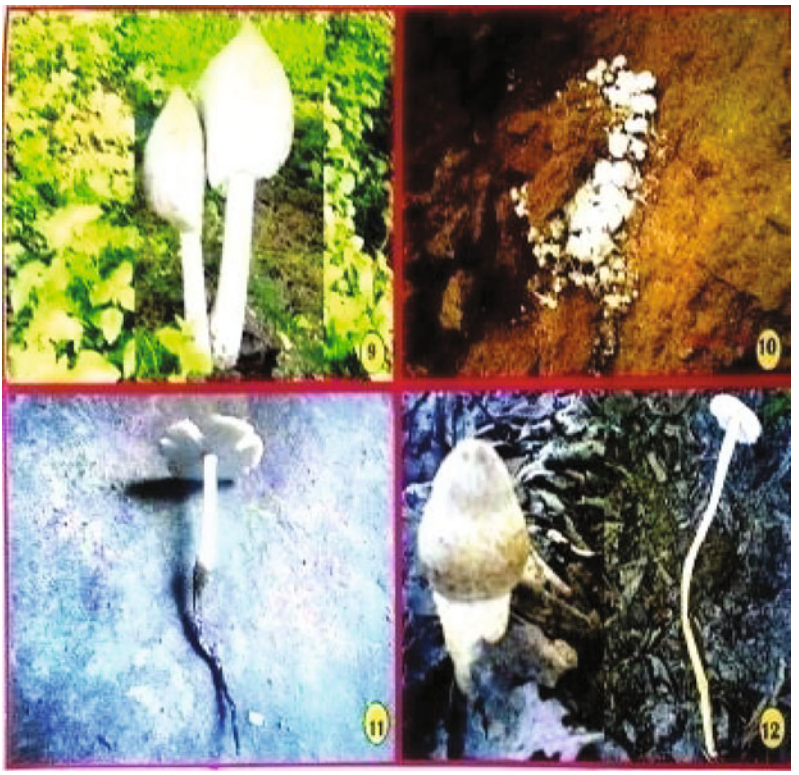
The Sal tree in association with Terminalia group (*T. arjuna, T. tomentosa, T. billerica, T. chebula*) denoting rhizomorphic interaction for recurrence of many higher fungi such as *Geastrum fimbriatum, R. delica*, *S. texense* and *Termitomyces* spp. (Shukla 2003; Tiwari et al. 2009, 2010). But induction of other tree types, viz. Teak and Pine in absolute uniformity over the habitat of Sal tree attributes adverse macroclimatic conditions (temperature, humidity, light intensity) where EM could not establish symbiotic relationship with the tree plants. This explains that land-use changes through plantation of Pinus and Teak trees have not only affected the abundance of mushrooms, but also affected micro-biota responsible to rejuvenate the forests' ecosystem. Thus, transformation of forest land under man-made management has a greater impact on germination and growth of mushrooms (Lakhanpal

1993), as in case of Teak tree (Site-3) has already been pointed out that conversion of natural forest to Teak alters the physical soil conditions (Jose and Koshy 1972).

Many edible and medicinal mushrooms such as *Amanita, Lacterius, Cantharellus,* and *Termitomyces* do not develop their fruiting bodies in the cultural practices due to their obligatory nature. *T. heimii* is one of the wild mushrooms, which is often collected and sold in the market during the rainy season when other minor forest products are not available. Certain species of *Amanita, Cantharellus, Rhizopogon, S. texense, G. fimbriatum*, *R. delica* found are intimately associated with the Sal, a dominating tree species in Chhattisgarh. The compounds are extracted from *Agaricus bisporus, L. edodes, Coprinus comatus* reported antitumor, anti-fungal and antibacterial properties.

Conclusion

The Sal forests in Central India are characterized due to richness of varieties of tree species and varieties of mushrooms, where macro- and micro-scale factors are found in support to mushrooms diversity in different ways. The variability and type of tree species in the forests suggest some governing forces for having enormous macro-fungi of economic importance. The Sal tree with consistent association with EM suggests the tree for ancestral status. Indeed, over-harvesting of wild mushrooms in successive years has created great loss of mushrooms (ectomycorrhiza) seed bank. The EM *Amanita, Boletus, Lactarius* with narrow host range face greater threat due to habitat destruction. Therefore, mycorrhizal association of individual specific tree partner and documentation of mushroom in general is essential to understand the importance of native tree plants in afforestation program.



9- ***Termitomyces clypeatus*****, 10 –** ***T. microcarpus*****, 11,** ***T. rabuorii*****, 12-** ***T. striatus*** **var.** ***annulatus***

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Fungal Pigments: An Overview

26

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Abstract

Since ages, colors have been an integral part of humankind whether it belongs to foodstuff, clothing, or day-to-day living. Long back in history, various pigments are used by all the races. Earlier the colors that were in use were natural in origin, but due to rise in demand mankind shifted to manufacturing of synthetic colors. With the passage of time, it has been now proved that these synthetic colors have many side effects like being immunosuppressive, carcinogenic. Due to deleterious health effects, the need for some alternative has emerged that can be used as a color. Plants, insects, and other microorganisms have started taken place of synthetic colors. As there are many factors that limit the usage of plants and insects, research turned toward the microorganism. There are many fungi whose pigments are now considered as safe and economical. Fungi like *Aspergillus, Fusarium, Penicillum, Monascus, Trichoderma, and Laetiporus* are reported to produce quinones, anthraquinones, Rubropuntamine, Rubropuntatin, Ankaflavin, Monascin, β-carotene, and many other pigments responsible for various colors, viz. red, purple, yellow, brown, orange, and green. In addition to providing natural colors, these pigments possess many therapeutic applications like immune modulators, anticancer, antioxidant, antiproliferative. These pigments are produced as secondary metabolites by utilizing one of the pathways: polyketide, mevalonate, and shikimate pathways. The pigments are fermentative products so are affected by temperature, pH, carbon source, aeration, and type of fermentation (solid or submerged). There are many

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agencies that approve the usage of pigments for humankind. Fungi can work as cell factories for color production that is economical and human friendly.

Keywords
Pigments · Polyketide · Food colorant · Fermentation

Introduction

Since time, immemorial colors have always fascinated the humankind. Usage of colors dates back to the Bronze Age in European culture. In the world, the earliest written record for usage of natural colors found in Chinese culture that dates back to 2600 BC. In India, it records back to 2500 BC in Indus valley period and has been substantiated by the colored garments of Mohenjo-Daro and Harappa civilization. In central and North America, cochineal dye was used by the Aztec and Maya culture (Aberoumand 2011; Gokhale et al. 2004). The usage of biocolor in food was done by Japanese people shown in shosoin text of Nara period (eighth century). Color also decides the appeal to food. Food colors are divided into four categories: (1) natural colors, (2) naturally identical colors, (3) synthetic colors, and (4) inorganic colors. Natural colors or pigments are the metabolites produced by the living organisms; it includes carotenoids, anthocyanins, etc. Natural identical colors are manmade pigments that are found in nature like β-carotene, canthaxanthin. Synthetic colors are also manmade colors that are not found in nature, and it usually includes azo dyes while the inorganic colors are also manmade colors and includes titanium dioxide, gold, silver, etc.

With the passage of time, and due to increase in population and thus the rise in requirements, it was realized that it is not possible to be completely dependent on the natural colors. This promoted the formulation and usage of synthetic colors. There are many synthetic colors that are used in foodstuff, dyestuff, cosmetics, and in pharmaceutical industry. But over the period of time and advancement in technology, it was conferred that synthetic colors have many harmful effects. The precursors that are used for the production of synthetic colors are carcinogenic, some are immunosuppressive and imparting many non-environment friendly and non-biodegradable impact. Due to negative impact of synthetic colorants, the research has turned toward the production of natural pigments and has become significant worldwide. Over a decade, many metabolites have been discovered from diverse sources of nature including plants, animals, insects, and microorganisms. Among these, the pigments produced by the microorganisms are holding special place (Dufosse 2006).

Among the many microorganisms, fungi play an important role in pigment production that can be used safely; moreover, fungi are reported to produce larger amount of pigments (Kirti et al. 2014). Fungi produce many primary metabolites (require for its own metabolism) and secondary metabolites (not required for its own maintenance), organic acids, enzymes, pigments, and other food additives. These products of fungi possess many therapeutic applications like immune modulators, anticancer, antioxidant, antiproliferative. In addition to this, natural colorants possess antimicrobial activity and lesser chance of being allergenic. They are even more stable than that of synthetic colorants and are more eco-friendly (Velmurugan et al. 2010).

Various Pigments

Pigments from natural sources have been obtained since longtime and with time, interest in production of natural colorants have been increased due to toxic effects of synthetic colorants. Natural pigments like carotenoids, flavonoids (anthocyanins), chlorophylls, phycobiliproteins, betalains, and quinones are common pigments that are in use. Among these, due to the ease of cultivation, extraction, and genetic diversity, microorganism are most promising. Microorganism such as *Bacillus*, *Achromobacter*, *Yarrowia*, *Rhodotorula*, *Phaffia*, *Monascus* produces a large number of pigments. Carotenoids that are yellow, red, and orange are widely used as food and feed supplements and as antioxidants in pharmaceutical industry. *Phaffia rhodozyma*, *Haematococcus pluvalis*, *Agrobacterium aurantiacum* are widely known for the production of astaxanthin, which is added to poultry feed. Astaxanthin consumption has also been found to be beneficial in case of cardiovascular diseases prevention, immune enhancer, and cataract prevention (Ciapara et al. 2006). Prodigiosin-like pigment which is red in color is found to be produced by *Serratia rubidaed* (Moss 2002). Similarly, Cyanobacterium *Nostoc muscorum* produces Phycoerythrin pigment (Ranjitha and Kaushik 2005). The red mold *Monascus purpureus* that is traditionally used for the production of red rice is a promising source of red pigment (Mukherjee and Singh 2011). But as mentioned earlier, fungi produce higher amount of pigment and due to ever-rising demand by the consumers, natural colorants from fungi have replaced the use of synthetic dyes in food industry as well.

Fungal pigments are secondary metabolites that are sometimes produced due to scarcity in the nutritional value. When the nutritional supply of essential nutrients decreases or there is some disfavoring environmental condition, mycelium produces secondary metabolites (Gupta and Aggarwal 2014). There are some fungi including *Aspergillus*, *Fusarium, Penicillum,* and *Trichoderma* that produce various pigments as intermediate metabolites during their growth (Atalla et al. 2011). Fungal pigments are classified as carotenoids and polyketides. Fungal polyketides are made up of tetraketides and octaketides having eight C_2 units forming polyketides chain.

Anthraquinone is most common class that is proved to be potentially safe (Mapari et al. 2010). Pigment anthraquinone is widely used in dyestuff industry and most commonly produced by *Trichoderma*, *Aspergillus,* and *Fusarium* (Duran et al. 2002). It is now known that single fungal species can produce mixture of different pigments, having various biological properties. Production of theses pigments plays an important role in fungi. Like melanin production helps the fungi to survive in severe environmental stress, helps to cope up with UV light. Study conducted by Kunwar et al. (2012) showed that its consumption by BALB/c mice increases the survival time in radiation-exposed mice. *Monascus* produce six various pigments that are polyketide in origin and imparts yellow, orange, and red colors. Monascin and ankaflavin are yellow pigment, while monascorubrin and rubropunctatin are orange pigment and monascorubramine and rubropuntamine are red pigment (Feng et al. 2012). There are four species of *Monascus,* namely *M. pilosus*, *M. purpureus*, *M. rubber,* and *M. froridanus* that account for majority of pigments isolated so far. *Monascus* pigments are believed to be sensitive to heat and fade with light, unstable at low pH and also have low water solubility although upon reacting with amino containing compounds their stability increases (Dufosse 2009). In addition to this, even the higher fungi, mushrooms, have also been reported for production of various pigments. Gupta et al. (2013) utilized *Trichoderma* sp. for dyeing of silk and wool as it imparts yellow color to silk and wool. Wood-rotting edible mushroom, *Laetiporus sulphurous*, contains non-isoprenoid polyene known as laetiporic acid A and 2-dehydro-3-deoxylaetiporic acid A as the main pigments imparting yellowish or orange color in the fruiting bodies (Davoli et al. 2005).

Similarly, there are many other species of mushrooms that are produce various pigments imparting different colors. There are more than 100 pigments that have been reported in fungi, and it holds place after the plants. Some of the fungal pigments are summarized in Table 26.1.

Various colors of the fungi are one of the very important characteristics that help in their identification. Green color of *Penicillin*, violet color of *Cortinarius*, yellow (Chen et al. 1969), orange, and red color of *Monascus* (Feng et al. 2012) are their distinct feature. Their pigments provide them protection against UV light and may also from the bacterial attack. The pigments of fungi differ greatly from higher plants being not possessing chlorophyll or the anthocyanins that impart various colors to flowers. Many of the fungal pigments are quinones or similar conjugated structures. The pigmentation in fungus sometimes varies with its age. As observed in *Penicillium chrysogenum* that initially, their colonies appear white in color and later that changes to blue-green (Tiwari et al. 2011).

Quinones are very common polyketide fungal pigments that are produced by following polyketide pathway. As its reduction product usually accompanies quinone, this is not necessary that it will show the color of the fungus from which it has been isolated (Feng et al. 2015).

Fumigatin (1) (Fig. 26.1) is isolated from *Aspergillus fumigatus* (Anslow and Raistrick 1938). It was observed that solution in which *Aspergillus fumigatus* grown was initially yellowish-brown and later changed its color to purple when treated with alkali (Hanson 2008). Auroglaucin (2) and Flavoglaucin (3) (Fig. 26.1)

Table 26.1 Various fungal pigments and their sources

Fungi	Pigment	Color	Reference
Penicillium herquei	Atronenetin	Yellow	Takahashi and Carvalho (2010)
Penicillium purpurogenum	Purpurogenone	Orange to yellow	King et al. (1970)
	Mitorubrinol	Red	Teixeria et al. (2012)
	Mitorubrin	Yellow to orange	Martinkova et al. (1995)
Penicillium oxalicum	Anthraquinone	Red	Atalla et al. (2011)
Trichoderma virens	Viridol	Yellow	Mukherjee and Kenerley (2010)
Monascus sp.	Ankaflavin	Yellow	Mostafa and Abbady (2014)
	Monascin	Yellow	Juzlova and Martinkova (1996)
	Rubropuntamine	Red	Yang et al. (2014)
	Monascorubramine	Red	Babula et al. (2009)
	Rubropuntatin New pigment	Orange Red	Moharram et al. (2012) Mukherjee and Singh (2011)
Fusarium oxysporum	Anthraquinone	Pink/violet	Gessler et al. (2013)
Fusarium verticillioides	Naphthoquinone	Yellow	Boonyapranai et al. (2008)
Aspergillus sclerotiorum	Neoaspergillic acid	Yellow	Teixeria et al. (2012)
Aspergillus niger		Brown	Atalla et al. (2011)
Aspergillus versicolor	Asperversin	Yellow	Miao et al. (2012)
Phycomyces blakesleeanus	β-carotene	Yellow-orange	Malik et al. (2012)

are the pigments first studied in 1930s and 1940s in *Aspergillus, Penicillia,* and *Helminthosporium* species (Raistrick 1940; Quilico et al. 1949). Species of *Aspergillus glaucus* series was characterized by green conidial heads and hyphae with varying colors of bright yellow to red. These organisms are found as spoilage organism. Dried form of these organisms gave various pigments like auroglaucin (orange-red needles), flavoglaucin (lemon-yellow needles), and rubroglaucin (ruby-red needles) (Gould and Raistrick 1934). Studies on pigments from Rubroglaucin was eventually shown to be a mixture of hydroxyanthraquinones physcion (4) and erythroglaucin (5) (Fig. 26.1). *Helminthosporium germineum* a causative organism of leaf stripe diseases of barley yielded deep red color when grown in Czapek medium. Main constituent was trihydroxy anthoquinone helminthosporin (6) (Fig. 26.1) (Charles et al. 1933; Hanson 2008).

Later, 40 various species of *Helminthosporium* were studied and various pigments and their isomers were established, it includes catenarin (7) (Raistrick et al. 1934) (Fig. 26.1). An isomer of helminthosporin, Islandicin (8) (Fig. 26.1) was isolated from *Penicillium islandicum* (Howard and Raistrick 1949). Studies reveled that all the red pigments were not anthraquinones. Another pigment Xanthone,

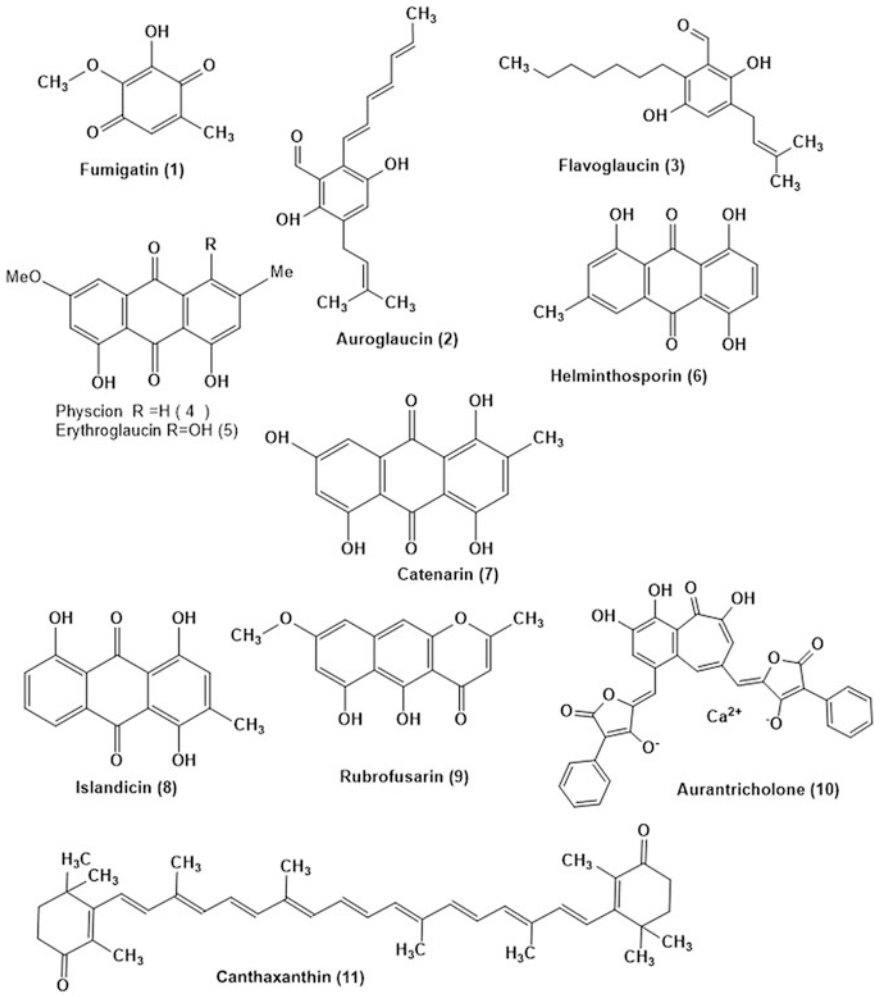

Fig. 26.1 Structure of various pigments

rubrofusarin (9) (Fig. 26.1) was isolated from genus *Fusarium graminearum* (Ashley et al. 1937; Tanaka and Tamura 1962). In 1960s, extended quinone structures were established which were formed by the dimerization process. Fungus *Chlorociboria aeruginosa* imparts green color to the wood, which is due to the extended quinone xylindein. Studies in the field were continued, and later findings suggested that there are many more pigments that are dimerized and occur as complex (Saikawa et al. 2000). Like orange-red cups of *Tricholoma aurantium* contain a pigment aurantricholone (10) (Fig. 26.1) in which pyragallole ring is attached to pulvinic acid and oxidatively dimerized (Klostermeyer 2000). It occurs as calcium complex. Since fungi are non-photosynthetic organism, still there are

some species that have been reported to have carotene hydrocarbons. It includes *Blakeslea trispora, Phycomyces,* and *Neurospora crassa.* Yellow pigment canthaxanthin (11) (Fig. 26.1) is isolated from *Cantharellus* sp. And this pigment might have arisen from the carotenoid (Haxo 1950). Studies reveled that fungi due to various environmental conditions produce various pigments also like naphthoquinone pigments are released as a response of fungi under stress conditions. *Monascus purpureus* was selected for the production of food colorant (Lin 1973). Its studies done under submerged conditions reveled that improvement of the O_2 supply increases the production of secondary metabolite (red pigment) and low O_2 transfer coefficient was required to improve the red pigment. Before preferring any strain for production of food colorant, it is important to check the pigment productivity and production of mycotoxins (Hanson 2008).

Genetic Basis of Pigment Production

Polyketides are most commonly found fungal secondary metabolites. Genetically, yellow *A. nidulans* spore-pigment intermediate naphthopyrone (WA), the carcinogen aflatoxin and the commercially important cholesterol-lowering compound lovastatin are best described. Type I polyketide synthases (PKSs) plays an important role for the synthesis of fungal polyketides. These PKS's are multidomain proteins and are related to eukaryotic fatty acid syntheses. For the synthesis, usually acetyl coenzyme A (acetyl CoA) and malonyl CoA condensed to form carbon chains of varying lengths and reduction of the β-carbon is optional. In addition, ketoacyl CoA synthase, acyltransferase, and acyl carrier domains are also essential for polyketide synthesis, while the ketoreductase, dehydratase, and enoyl reductase domains which are required for ketone reduction in fatty acids are not present in all fungal PKS enzymes. Due to the restriction to one module, they can carry out recurring biosynthetic reactions, and are, therefore, called 'iterative PKSs.' Aromatic polyketide naphthopyrone are formed due to claisen-type cyclization which is performed by the C-terminal region of the enzyme, having thioesterase domain motif. Fungal polyketides structures can perform iteration reactions which results into its diversity. Further, variety is achieved by the introduction of many different post-polyketide synthesis steps (Fujii et al. 2001; Keller et al. 2005). Non-ribosomal peptides are derived from both proteinogenic amino acids and non-proteinogenic amino acids by multidomain, multi-modular enzymes named non-ribosomal peptide synthases (Keller et al. 2005). Fungi are capable of synthesizing many important terpenes, like aristolochenes, carotenoids, gibberellins, indole-diterpenes, and trichothecenes. Terpenes are composed of several isoprene units that can be linear or cyclic, saturated or unsaturated. Utilizing different diphosphates and enzyme terpene cyclase various terpenes are produced. Although terpene cyclases have structural homology, they have little primary sequence similarity and seem to have diverged relatively rapidly from a common ancestor. Several fungal terpene cyclases have been characterized, including a bifunctional terpene cyclase from

Gibberella fujikuroi (Tudzynski et al. 2001), a trichodiene synthase from *Fusarium sporotrichioides* (Rynkiewicz et al. 2001) and an aristolochene cyclase from *Aspergillus terreus* and *Penicillium roquefortii* (Keller et al. 2005). Gene cluster and the arrangement in various domains are one of the major causes of diversity among the fungi and the production of various secondary metabolites.

Factors Affecting Pigment Production

Fungal pigments are secondary metabolites known as polyketides. Although these come under the nonessential metabolites for growth and reproduction, the developmental stage of fungus greatly influence the production of these pigments, and the developmental stages are influenced by the extrinsic as well as intrinsic factors, including pH, substrate, oxygen, temperature, water activity, and light availability. Like any other fermentative production medium composition, aeration rate, agitation rate, nutritional limitation, status of carbon supply affects the production of secondary metabolites. pH of the medium affects the growth and thus the production of secondary metabolites by the fungus. At pH 5 *M. purpureus* produces red pigment, while at the same pH *Penicillum sclerotiorum* produces orange pigment (Mukherjee and Singh 2011; Lucase et al. 2010). The pigments of *Monascus* vary from red-yellow to orange depending upon the culture conditions. Similarly, other growth conditions like aeration and nitrogen source for *Monascus sp.* result production of extra cellular water-soluble pigment (Hajjaj et al. 1998). Temperature is another important factor that affects the pigment production. The optimum temperature for the *Monascus sp.* was found to be within 28–30 °C and pH range of 4.5–8.5. Maximum pigment production has been reported at pH 4.5, which is yellow in color (Dikshit and Tallapragada 2011). The *Penicillium* sp. was found to produce extracellular pigment at pH 9.0 and 30 °C. In the higher fungi, usually, incubation temperature 25 °C is preferred as they require longer time period for mycelial growth. Other sources like carbon, nitrogen, oxygen, phosphorous also play an important role. The higher level of oxygen and lower level of carbon dioxide result in decreased ratio of biomass and thus the pigment production (Han and Mudgett 1992). Even the light conditions also influence the pigment production. It has been reported that incubation of *Monascus* sp. in darkness results in effective production of red pigment while illumination results in loss of pigment and thus postulated the photoreceptor response of fungus. Even the illumination with red and blue light affected the pigment yield (Babitha 2009).

Musaalbakri et al. (2006) explained that *M. purpureus* FTC 5391 was able to produce red pigment with different carbon sources, viz. glucose, potato starch, and rice starch. And the addition of tryptophan or 6-furfuryl aminopurine as nitrogen source was helpful in production of extra cellular pigment (Zhang et al. 2013). The addition of gibberlic acid, vitamin B2, and other amino acids (L-leucin and glycin) to the liquid medium also enhanced the pigment production (Baneshi et al. 2014). The pigment production was found to increase in *Fusarium verticillioides* with the

proportional rise carbon source, glucose, and yeast extract (Boonyapranai et al. 2008). In *Fusarium moniliforme,* KUMBF1201 as compare to others peptone and yeast extract showed the best result for higher pigment production (Pradeep et al. 2013).

Fermentation for Pigment Synthesis

The production of microbial pigments by fermentation is a fascinating area, and nowadays, due to lot of advancements, this biotechnological approach has attracted lot of attention. Based upon the requirement, solid-state fermentation or submerged fermentation is in the practice. Vegetative cells or spore suspension are used as inoculum for the fungal fermentation. The spore suspension is more advantageous over the vegetative cells due to ease of handling, high viability, longer storage, easy maintenance, and preservation (Ajdari et al. 2011). In submerged fermentation, there is utilization of free-flowing substrates (broth, molasses, etc.). The required product is secreted into the fermentation broth. In this method, the continuous supply of substrate and nutrients is required as their utilization is fast. The product is easily purified from the fermentation broth. Although solid-state fermentation is a traditional method used for pigment production (Vendruscolo et al. 2010), for some species submerged fermentation has been developed. Submerged fermentation has been explored for *Monascus* pigments so that the demerits like problems of space, scale-up, and development control of solid culture can be achieved. The submerged fermentation is also helpful in production of many secondary metabolites; it also decreases the cost of production as compared to solid-state fermentation. For the large-scale production of new red pigment from *Monascus purpureus* submerged fermentation would be more economical and beneficial (Mukherjee and Singh 2011). *Penicillium funiculosum* IBT3954 found to yield (0.13 g/L) greater amount of red pigment under the submerged condition (Jens et al. 2012). Similarly, *Paecilomyces sinclairii* showed good yield of 4.40 g/L under the submerged fermentation (Cho et al. 2002). Addition to this, carotenoid production from *Aspergillus sp*. is also favorable through submerged fermentation. *Penicillium purpurogenum* produce color both in solid as well as liquid media while *P. purpurogenum* DUPA 1275 showed good results in submerged culture (Mendez et al. 2011; Santos-ebinuma et al. 2013). Many other pigments with higher commercial value including prodigiosin, monascorubramie, astaxanthin, canthaxanthin, β-carotene, etc. are produced by utilizing submerged fermentation. Solid-state fermentation is the process where fermentation process is performed on a non-soluble substance which acts as support source for growth ad also a nutrient supplier. There are many physical supports and nutrient supplier like rice bran, wheat bran, coconut oil cake, jackfruit seed powder that are used for solid-state fermentation. The main advantage of this type of fermentation is this that it utilizes nutrient-rich waste material. The utilization of nutrients is very slow, and same substrate can be used for longer time period. This is best suited for those fermentation processes where requirement for

moisture content is very low. Among so many fungal sp. *Monascus sp.* holds a special place, as since ages, it is utilized in this for production of red pigment. *M. purpureus* KACC 42430 gave the higher yield under the solid-state fermentation when corncob powder is used as a substrate. Corncob supplies higher amount of cellulose and hemicellulose that promotes the pigment production (Babitha et al. 2007). Rice is considered as one of the best substrates for *Monascus* under the solid substrate fermentation, at optimum fermentation time it produces considerable amount of pigment (Singh et al. 2015).

Mycotoxins and Their Replacement

Study on the fungal secondary metabolites is not complete, if we do not discuss the mycotoxins. Mycotoxins are the secondary metabolites produced by many fungi which have the capacity to damage health, productivity and sometimes lead to death also.

Monascus sp. produces yellow and red pigments that are commercially and legally used as food colorant and used in the form of red rice powder in Southeast Asia. But, *Monascus* pigments are not approved in European Union (EU) and the USA (US), mainly due to the risk of the possible contamination by the nephrotoxic, and hepatotoxic metabolite citrinin. Another example is Quorn TM produced by *Fusarium venenatum* which also produces cytotoxic metabolite 4,15-diacetoxyscirpenol. But fungal producers are generally categorized as GRAS, which implies that with continuous monitoring their mycotoxins can be controlled. But the controversy has triggered the scientific community to find out some of the alternatives, either by manipulating culture condition, developing strains incapable of synthesizing citrinin by metabolic engineering or by screening some genera other than *Monascus* that produce polyketide pigments (Dufosse et al. 2014). Rigorous search for some potential strain has resulted in discovery of some strains of *Talaromyces species* (*Talaromyces aculeatus, T. funiculosum, T. pinophilus, T. purpurogenum*) that are producing *Monascus*-like polyketide azaphilone pigments without co-producing citrinin or any other known mycotoxins (Mapari et al. 2009). Attention is now moving toward the marine fungi also; studies have shown that marine fungi can produce more brilliant color with more stability and lesser or no mycotoxins.

Relevance of Pigments in Various Fields

With the advancement in the biotechnological tools, interest in search of natural pigments has also been augmented. The inclination of society for the search of natural ingredients has hard-pressed the scientist to work in the field for the production of more economical products with health benefits. Moreover, nowadays food industry is facing lot of challenges in replacing the synthetic colors with natural colors having antimicrobial and antioxidant properties (Vendruscolo et al. 2013). The red color

pigment derived from *Monascus sp.* is very well documented as one of the oldest pigments used in Chinese culture. The red pigment produced by *Monascus purpureus* possesses antimicrobial activity as compared to ciprofloxin (Kumar et al. 2012). *Monascus* sp. producing rubropuntatin (orange pigment) as pigment is found to possess anticancer activity (Moharram et al. 2012). Another pigments Monascorubramine and Rubropuntamine forming red color showed antioxidant activity (Babula et al. 2009; Yang et al. 2014). Similarly, pigment virone produced by *Trichoderma virens* showed antifungal activity (Kamala et al. 2015). In the last decade, much research has been focused to limit the production of citrinin that has limited the use of *Monascus* in food directly. Many strains have been developed showing low or no production of citrinin. Although the gene responsible for pigment production in *Monascus* is still under controversy, only few genes are reported. It has been reported that *MpigE* had great impact on pigment production and its overexpression has led to low concentration of citrinin in fermentation media (Liu et al. 2014). *Penicillium* sp. has been reported to produce many pigments with significant therapeutic value and can be used in agriculture also. *P. oxalicum* var. Armeniaca CCM 8242 produces Ar pink red pigment that possesses anticancer activity.

Mitorubrin pigment (yellow to orange in color) produced by *P. Purpurogenum* plays important role in pharmaceutical and food industry (Mapari et al. 2005). *Penicillium herquei* producing yellow color pigment, Atronenetin showed antioxidant activity and is used in food industry (Takahashi and Carvalho 2010). *Aspergillus sp.* showed the production of secondary metabolite that are useful in agrochemical industry like *Aspergillus sclertiorum* DPUA 585 produces neoaspergillic acid that showed antibacterial activity against *E. coli, Mycobacterium smegmatis*, and *S. aureus* and antifungal activity against *C. albicans* (Teixeria et al. 2012). Astaxanthin produced from *Phaffia rohodozoa* and *H. pluvalis* is a red pigment and is used in feed, pharmaceutical, and aquaculture industries. Textile is another such sector where coloring agents are required and if they are from natural resources they are more preferred, due to better biodegradability and higher compatibility with environment. *Trichoderma virens*, *Alternaria atternata, and Curvularia lunata* have been utilized for pigment production for textile industry (Shrama et al. 2012). Many species of *Aspergillus* are being studied in this field. *A. niger* NRC 95 produces brown pigment that is used for the dying of wool (Atalla et al. 2011). *Fusarium oxysporum* produces anthraquinone compound, which is also used for the dyeing of wool and silk (Nagia and EL-Mohamedy 2007). Poorniammal et al. (2013) purified pigments from *P. purpurogenum* that are helpful in dyeing of cotton fabrics and additionally possess antibacterial activity. Likewise, there are many more fungal pigments that are helpful to us in many ways. The world of fungi is very colorful and still needs rigorous work to isolate more pigments that are friendly to us.

Future Prospects

As already discussed that use of natural colors as food colorants in the form of red rice, wine, etc., dates back to Bronze Age. The place of natural colors was taken by chemically synthesized colors in the nineteenth century. But with the reports of health hazards imposed by these chemically synthesized colors, various regulations throughout the world thus resulted in resurgence of demand for natural colors. Nowadays consumer awareness between diet and health is increasing, that has promoted the food colorant market, and even the demand of natural dyes has also increased. Fungi specifically ascomycetous, basidiomycetous and lichens are known to produce various pigments. Out of these, ascomycetous has taken most of the attention as they are easy to grow under laboratory conditions and thus provides ease for large industrial production. There are many reports showing the large-scale pigment production from ascomycetous fungi in a bioreactor under controlled conditions. Moreover, the usage of fungi does not make producer season dependent. The usage of natural food colorant was earlier confined to the semi-fermentative production of riboflavin, which is a natural yellow food colorant, produced by *Eremothecium ashbyii* and *Ashbya gossypi* (Wickerham et al. 1946). But it has some limitations, as it is light sensitive and readily gets fade away. The production of β-carotene from the *Blakeslea trispora* by DSMTM in the Netherlands was a breakthrough in the world of food colors. Earlier, tomato was the only source of carotenoid lycopene, but now EU legislation has approved *B. trispora* as a potential source of lcopene (Commission regulation 2006). With the production of many natural colors, many synthetic colors have been banned. Import of red colorants of the Sudan series is banned, as there are reports showing the carcinogenic effect of red color.

In the European parliament, it is clearly mentioned that any food article having synthetic color (sunset yellow carmoisine, ponceau 4R, etc.) will require a proper safety label. There is wide range of Polyketide pigments including anthraquinones, hydroxyanthraquinones, naphthoquinones, and azaphilone structure, each of which exhibits a wide range of colors. Polyketide pigments of *Monascus* sp. is traditionally used in Southern China, Japan, and Southeast Asia for making red rice wine, red soybean cheese, and Anka. *Monascus* sp. produces various pigments including ankaflavin and monascin producing yellow color, monascorubrin and rubropunctatin producing orange color, and monascorubramine and rubropunctamine producing purple-red color. Additionally, it produces mycotoxin citrinin, a hepato-nephrotoxic compound, which limits its usage.

Although literature did not show any reports showing death due to consumption of red rice wine, red soybean cheese or Anka. Apart from *Monascus, Penicillium* has also been reported for the production and further utilization of human-friendly pigment. A pink red, anthraquinone-based colorant, derived from *Penicillium oxalicum* has been reported as a safe biocolor. In addition, there are other species of *Penicillium,* viz. *Penicillium aculeatum, P. pinophilum* that are reported to produce azaphilone *Monascus*-like pigment. These strains do not produce any other

mycotoxins and are safe for the human use (Mapari et al. 2008). *Penicillium herquei, Cordyceps unilateralis* show a promise for a future, having similar structure like that of plant-derived red pigments shikonin and alkanin.

Food colors from plants are already in the market to a greater extent. But due to their unavailability throughout the year and lesser production per cycle has moved the trend toward the microorganisms. Exploration of fungal diversity for biocolor production and lesser or no mycotoxin production is still going on, with an emphasis on production of water-soluble pigments (Mapari et al. 2010). We can conclude that fungal pigments have quite good future prospects for robust industrial production of various colors. The natural pigments from ascomycetous fungi can serve as a sustainable natural color. Available data indicate that filamentous fungi can be used as cell factories for pigment production. Despite production of mycotoxins by some of the species, studies are still going on to find out other fungal cell factories that are economical and human-friendly.

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Ex situ Conservation of Fungi: A Review

27

Sanjay K. Singh

Abstract

Present chapter deals with conservation of fungi which are considered as essential genetic material for advancement in fungal biodiversity, biology and biotechnology research and development, beyond depletion of natural resources. Loss of natural resources causes social and ecological impacts, therefore, need sound strategies of long-term preservation and maintenance, which has become a topic of great concern to almost all the countries possessing rich fungal diversity for their utilization on sustained basis and help in generating knowledge-based bio-economy. Subsequent to the Convention on Biological Diversity (CBD) which came into force in 1993, many member countries have acted in conformity with the spirit of the CBD by respecting biological habitats and the rights of indigenous people. Considering fungi are also threatened by climate change, habitat destruction, invasion, pollution, over-exploitation, etc., their conservation has been recognized as important as animal and plants, and convention has emphasized member countries to pay serious attention to conservation and documentation of fungi. It is for these reasons various short and long-term conventional and non-conventional methods, protocols for set up and culture recovery are provided. In addition, important tips are provided regarding care to be taken while applying a particular protocol for preserving and maintaining target fungi.

Keywords

Long-term · Short-term preservation · Maintenance · Storage · Ex situ conservation

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Introduction

In principle, biodiversity includes diversity of the living organisms, their genes and the biomes, which is the result of millions of years of evolution (Koh et al. 2004). Biodiversity on Earth is increasingly challenged by human interference, and among the critical factors are the destruction of habitats, climate change, as well as the introduction of invasive species and environmental pollution. Global changes and human population growth have attracted global attention. Conservation of biodiversity has become a common concern to all the nations. Preamble to the CBD (1992) aptly emphasizes to sovereign rights of countries over their biological resources and is responsible for conserving and using their biological resources in sustainable manner. Microorganisms and their derivatives represent an untapped resource and serve as essential raw materials to the advancement of microbial technology, which offers future beyond depletion of natural resources. Bio-resources, in general, are associated with traditional knowledge, and loss of natural resources and habitats causes social and ecological concerns; hence, traditional knowledge is an important component of daily life of millions of people and help finding useful solutions to ample of problems through microbe-based innovation. Due to spiraling demands of the bio-products, commercialization of traditional knowledge associated with bio-resources has been on the rise globally and therefore protection of bio-resources has become a topic of great concern of the nations.

Fungi are ubiquitous and their adaptability to various environmental conditions make them model organism for research in terms of biological and biotechnological perspectives. Conservation of fungi is important due to on-going destruction of natural habitats, which necessitates development of alternative strategies for conserving natural fungal genetic resource. Ex situ conservation of fungi in laboratory conditions can compliment to conservation strategy and can be achieved by preserving live cultures in germplasm banks and dried/exsiccate materials in herbaria. Subsequent to the Convention on Biological Diversity (CBD), many member countries have progressively acted in conformity with the convention by respecting biological habitats and the rights of local peoples. It has been accepted since 1970 that fungi belong in their own separate biological kingdom and contain far more species than the plant kingdom. Where plants produce, and animals consume, fungi are the recyclers. In a recent development, fungi have received special consideration as the largest group of organisms in number on Earth, next only to insects, and treated differently from other microbes. They are also threatened as animals and plants due to climate change, habitat destruction, pollution, over-exploitation, etc. Fungi are known to provide important ecosystem services like soil fertility, mutualism, crop protection, litter decomposition, checks, and balances. These services are invaluable and need serious attention. Fungi are also a very important source of unusual chemicals of great value in industry and medicine. Therefore, fungal conservation and their sustainable utilization have been recognized by International Union for Conservation of Nature, just as important as animal and plant conservation. All the signatory countries on Convention on Biological Diversity have been

asked to pay serious attention and compile substantial information on status of fungal conservation in fifth national report (NR 5) submitted to the convention.

Prolonged periods of growth in artificial cultural conditions lead to undesirable morphogenetic changes in fungi. Knowledge on conditions necessary for optimum growth, nutritional requirements for vegetative growth, sporulation, and sensitivity to cold storage is considered important for successful preservation and storage. Review of the literature reveals various methods/techniques used in short-term and long-term preservations and maintenance of fungi with numerous modifications in recipes. Some allow growth and reproduction to some extent, while others suspend or reduce metabolism to almost zero, and maintenance under sub-zero temperature is generally regarded as safe, but for some exceptions. However, selection of appropriate method is crucial for long-term preservation and maintenance. This chapter deals with a review on various conventional and non-conventional methods used in conservation of different groups of fungi. In addition, simplified protocols as ready reckoner are intended to provide.

Preservation and Maintenance of Fungal Cultures

In principle, fungi grow best on media formulated from the natural materials from which they were originally isolated. Major factors that affect growth of fungi are nutrient medium, temperature, light, aeration, pH and water activity. As a general practice, laboratories use available standard media. However, optimization of growth conditions of a particular fungus is an important step. Even individual laboratory or specialized culture collection follows similar procedures in maintaining germplasm on long-term basis and an extension of taxonomic study which is extremely important due to declining interest, allowing to species and genetic variability to be maintained for longer periods. Though numbers of methods are available for maintaining pure cultures, it is advised to take into account various considerations before applying a method to a particular group of fungi, viz. type and number of cultures to be maintained, manpower, time and facility available, climate and suitability of the method to particular group of fungi. Therefore, various conventional and non-conventional methods and their protocols, recovery steps, and important tips which are provided here.

Serial Transfer/Sub-Culturing

This method is simple, inexpensive, and widely used in laboratories as well as in collections. It is laborious and time-consuming method and suitable for maintaining for relatively short-term studies (a few weeks to a couple of months). It is suggested in the literature that this method can serve to maintain small numbers of collections for a few years, but there are several drawbacks to rely on this method.

Disadvantage of this method is that cultures dry out rapidly, hence need subculturing every three to four months. Repeated subculturing, sometimes leads to deleterious changes in culture like loss of pathogenicity, virulence, or sporulation. Fennell (1960) and Onions (1971) discussed in detail about what fungal structures (hyphae, spores, etc.) constitute appropriate inocula for serial transfers. As a general practice, both hyphae and, if available, spores are preferred as inocula for periodic/serial transfer.

Protocol

- Grow the culture on suitable agar medium in Petri plate.
- Check for the contamination by other fungi/bacteria/mites.
- Transfer a loop-full of inoculum to the test tubes containing suitable media and incubate at optimal temperature.
- After proper growth, transfer tube into a refrigerator/cold room as per requirement.

Recovery of Culture

- A small mass of mycelium/spores is taken out.
- Streak aseptically on a suitable agar medium and incubate at suitable temperature and time.
- Check for viability and contamination.
- Check for growth characteristics.

Important Tips

- Continuous monitoring for contamination and growth pattern is required.
- Phenotypic character of the culture is required to be checked periodically, as it may change after sometimes.

Oil Overlay/Storage in Oil

Preservation of cultures under paraffin oil is one of the oldest, simple, and effective methods of long-term preservation. Originally described by Lumiere and Chevrotier (1914), these techniques have been modified to suit the specific requirements and recognized as an effective technique for preserving different bacteria, algae, fungi, and yeast strains. Simplicity, effectiveness to very diverse group of microbes and economics were studied and reaffirmed by Ajello et al. (1951), Schulze (1951), Hartsell (1953), and Annear (1956). In principle, sterile mineral oil prevents desiccation and is reported to diminish gas exchange which substantially reduces the

metabolism of fungal strains stored. Beerstecher (1954) presented relation of microbes to the oil industry and emphasized importance of microbes in exploration, production, and manufacturing processes of oil. It has been stated that microbes may be responsible for various types of deterioration leading to color changes in it. By correctly applying this method, culture (s) can be maintained for years together with or without an intervening transfer. Longevity of culture storage varies from a few weeks to about 14 years, and in exceptional cases, up to 32 years, at room temperature or at 15–20 °C (Cavalcanti 1991; Silva et al. 1994); they also reported that this method facilitates to survive unusual conditions of temperature variations. As commercial interest, retention of important biochemical reactions as well as the longevity is the important aspects, and in many cases, cultures/strains stored under oil retain this characteristic, e.g., several chromogenic strains of yeasts were reported to retain this characteristic for more than seven years of storage. Schulze (1951) reports beer produced from yeasts had remained two and half years under oil behaves quite normal. Though this method is one of the oldest one, it is still widely accepted and considered appropriate for mycelial or non-sporulating cultures, especially basidiomycetous culture not amenable to freezing or freeze-drying. The growth rate of the culture slowed with the increase in storage time. As such longevity and maintenance of cultural characteristic largely depend upon the strains, the storage medium, the temperature, the subculturing medium, and the frequency of transfer. As an advantage, this technique is low-cost and technologically simple, reduces mite infestations, while as a disadvantage fungi continue to grow, and thus, selection for mutants that can grow under adverse conditions may occur. This method is space intensive and needs periodic inspection for contaminations.

Protocol

- Use vigorously growing culture in glass test tube.
- Autoclave medical grade white mineral oil to two consecutive days, to kill any bacterial spores activated after first autoclave.
- Aseptically cover the culture slant with sterile oil to the depth of one cm.
- Cover tube with caps/cotton plugs and then apply couple of layers of parafilm.
- Store overlaid tube in upright position either in refrigerator or at room temperature.

Recovery of Culture

- A small mass of mycelium with spores is taken out with needle/loop.
- Excess oil is drained off and then streaked on a suitable agar medium.
- Initial growth of the culture is slow due to the presence of oil, but in two to three transfers, the original growth rate can be restored.

- Oil can be removed either by washing the culture mass in sterile water or by inoculating an agar slant and incubating in upright position so that the oil can drain to the bottom.

Important Tips

- It is recommended not to use tube/bottles with rubber gaskets, as oil soluble components in the rubber may be toxic to the fungal cultures (Onions 1971).
- Medical grade neutral, white, mineral oil/paraffin oil with specific gravity of 0.86–0.89 is recommended for use.
- Mineral oil to be used need to be autoclaved on two consecutive days, to kill any bacterial spores.
- Mineral oil becomes cloudy due to moisture gets released into it during autoclaving.
- Moisture in oil may be removed by drying for 1–2 h at 170 °C in oven.
- Oil from container is poured slowly to prevent spores blow from culture, which otherwise leads to contamination of stock of oil in container.
- Cover the culture slant with sterile oil to the depth of one cm; otherwise, culture dries out quickly.
- Oil overlaid culture may be stored at ambient temperature or in refrigerator.
- Oil level in the tubes or vials must be checked periodically, and more oil should be added, if necessary.

Sterile Water Storage

This method is easy for application and low-cost technique described way back in 1930s in mycological literature. The principle lies in extending the life of an agar culture (Burdsall and Dorworth 1994). This technique is extremely successful, and many filamentous molds, yeasts, and yeast-like fungi have been reported to survive for decade or more in sterile water. Zygomycetous fungi are reported to be less stable in water storage, surviving only for a few months, but fungi commonly encountered in clinical and plant pathology laboratories remain viable for years and become important to those group of fungi that generally fail to survive freeze-drying, e.g., Oomycota (Oomycetes) (Clark and Dick 1974). In general, this method has been used successfully with a wide range of fungi including human or plant pathogens (Castellani 1967; Figueiredo and Pimentel 1975). Figueredo and Pimental (1975) successfully stored phytopathogenic fungi for ten years. Onions and Smith (1984) reported 58% viability in water after five years of preservation of *Pythium* and *Phytophthora* strains. Qiangqiang et al. (1998) found more than 89% viability of cultures in water after 12 years of preservation. Burdsall and Dorworth (1994) reported proper growth rate, viability, and genetic stability of cultures of Basidiomycota after seven

years. Ectomycorrhizal fungi have been stored by this method (Marx and Daniel 1976), while the storage of ectomycorrhizal basidiospore slurries in water was reported to be unsuccessful (Torres and Honrubia 1994). The advantages of storage in water are the low-cost and easy application. However, the length of storage is often variable. Some fungi are reported to remain viable even up to 20 years (Hartung de Capriles et al. 1989) while others lose viability much sooner. In nutshell, this method is considered suitable storage technique for most of the fungal pathogens.

Protocol

- Use young and vigorously growing culture for inocula.
- Grow on an agar slope or in a cryovial/universal bottle/test tube.
- Autoclave distilled water in separate bottle/tubes.
- Pour sterile distilled water to tube containing agar culture to cover completely the agar surface.
- Alternatively cut agar blocks (5-mm^3) from the growing edge.
- Transfer agar blocks (10–12) to 10 ml of sterile distilled water in bottles/tubes.
- Alternatively, sporulating or non-filamentous organisms can be harvested without agar and can be suspended in pre-sterilized distilled water.
- Label one bottle/tube as reserve stock and the other as working stock.
- Screw the cap of the bottle/tube tightly and store between 20–25 °C (Boeswinkel 1976).

Recovery of Culture

- Remove an agar block from the working stock and inoculate on a suitable agar medium.
- Alternatively, organism can be recovered by transferring a small amount of the suspension on to suitable agar medium.
- Incubate under optimum growth conditions.
- Monitor for viability and contamination.
- Monitor for growth characteristics.
- Reseal the tube and return to storage.

Important Tips

- It is advised to maintain a reserve stock for use when re-preservation is necessary.
- It is advised to avoid preservation of excess inoculum which may reduce the ability of the fungus to withstand long-term storage.
- It is advised to maintain volume of sterile water about ≥ 40 times greater than that of the inoculum in a test tube.
- A single vial can be used as a continuous source of active fungal culture.

- Several fungi do not lose sporulation or virulence for more than two years.
- Tap water or physiological saline (0.85% NaCl) can be used, if distilled or deionized water is unavailable.
- Poorly sealed storage tubes can lead to loss of water in tubes by evaporation results in death of the stored cells.
- Periodic addition of sterile water can be done to overcome this problem.

Silica Gel Storage

This method is inexpensive, and reliable for many fungi (Smith 1993) including entomopathogens (Bell and Hamalle 1974), and is recommended for long-term storage of fungi. This method is limited to fungi and aerobic bacteria. However, fungi with high ratios of vacuolar to cytoplasmic volumes (e.g., Entomophthorales and Oomycota), microsporidia, anaerobes, and viruses are considered not suitable for storage on silica gel. Viability of *Fusarium* spores on silica gel is reported after 10 years (Windels et al. 1993). Anhydrous silica gel crystals are used as a carrier of culture propagules, and it is advised to use relatively large and uncolored material (with no indicator dye). Because the uptake of water by anhydrous silica gel is a strongly exothermic reaction, it has been recommended to put the tubes containing silica gel in ice bath or may be chilled in a freezer (−20 °C) to dissipate heat of hydration during their inoculation. As a disadvantage of this method, success depends on security of screw-top seal (Humber 1997).

Protocol

- Sporulating cultures of fungi may be used for preparing inoculum.
- Fill one-third of the tubes/universal bottle (20–30 ml) with medium grain plain 6–22 mesh (grade 40) non-indicating anhydrous silica gel.
- Sterilize the tubes and silica gel by dry heat in an oven at 160–180 °C for 3–6 h.
- Place tubes/bottles in a tray containing water up to the level of the silica gel and leave overnight in a −20 °C freezer.
- Prepare spore suspensions in cooled 5–7% (w/v) skim milk by dispensing 1–5 ml sterile water or an autoclaved solution of 5–7% (v/v) skim milk into a well-sporulating culture.
- Cap the tubes/bottle and agitate properly for proper mixing.
- Dispense about one ml of suspension by drops at least in two bottles of the silica gel crystal (in the frozen water).
- Remove the tube/bottle after 20 min from the ice and shake them to disperse the suspension.
- Label both reserve and working stocks properly at ambient temperature.

- Place the tube/bottle containing inoculum at ambient temperature (25 °C) for a few days or week, agitating them periodically.
- Store at −20 °C, alternatively tubes may be kept at room temperature.
- Check viability and sterility of the stored preparation after about 2–3 weeks.

Recovery of Culture

- Sprinkle a few granules from stored working stock on to a suitable agar medium and incubate under appropriate growth conditions.
- In case, if the organism fails to grow, re-attempt it.
- Alternatively, streaking silica gel granules over agar medium to dislodge the cells may lead to successful revival.
- Reseal tightly and return tubes/bottles to storage place.

Important Tips

- It is advised to use coarse non-indicator silica gel.
- Assure that silica gel is sterile and fully anhydrous during sterilization.
- Shake/rotate/agitate tubes/bottles periodically until water gets absorbed and the crystals/granules are separated.

Soil Storage

This method is simple and applicable to different groups of fungi. *Fusarium* species are very successfully stored with this method (Booth 1971). The shelf-life of the cultures varies from one to twenty years depending upon the species (Smith and Onions 1994).

Protocol

- Collect about five-gram garden soil and fill to one-third in a tubes/bottles (20–30 ml).
- Autoclave the tubes/bottle containing soil twice on consecutive days at 121 °C for 20 min.
- Prepare a mycelial/spore suspension from pre-grown culture in sterile water.
- Add about one ml of suspension to two tube/bottle of sterile soil for each strain.
- Label both reserve and working stocks.

- Incubate the inoculated tube/bottle at suitable temperature and time depending on the type of the fungus to be stored.
- Screw the tube/bottle caps down tightly and store in a refrigerator (4–7 °C).

Recovery of Culture

- Take out little particles of soil from the working stock.
- Sprinkle on to a suitable agar medium and incubate under appropriate growth conditions.
- Monitor for viability and contamination.
- Monitor for growth characteristics.

Important Tips

- It is advised to autoclave the soil twice or thrice on consecutive days at 121 °C for 20 min.
- After inoculation, initially, fungus use available moisture and gradually become dormant.

Liquid Nitrogen Storage (Cryopreservation)

Cryopreservation in liquid nitrogen (LN) is most reliable method for long-term storage of microorganisms. Though, no single method is ideal for preserving all groups of microorganisms including fungi, their maintenance under sub-zero temperature is generally regarded as safe and reliable, but with some exceptions. Most sporulating and non-sporulating fungi that grow well in culture survive cryopreservation. However, success of this method depends on several factors. The critical periods in cryopreservation are during freezing and thawing processes. Principally, two types of freezing protocols are recognized; a slow or controlled one and fast or uncontrolled one. Both have been used, and their positive and negative impacts have been reported from time to time. Generally, too low freezing rates are reported to cause excessive dehydration ultimately leading to cell damage; on the contrary, too fast freezing leads to insufficient dehydration and formation of abundant ice crystals with lethal consequences. Freezing damage has been a matter of great concern (Lovelock 1953; Polge et al. 1949) led to develop modern cryotechnology. However, freezing damage can be avoided by using some compounds termed as cryoprotectant also referred to as cryoprotective agent (CPA). Generally, three categories of CPA/additive are distinguished (Tao and Li 1986), viz. a. Cryoprotectants penetrating cell wall and cell membrane, b. Cryoprotectants penetrating cell wall but not cell membrane, c. Cryoprotectants neither penetrating cell wall nor cell membrane. Preservation of filamentous fungi at the ultra-low

temperature of –196 °C in liquid or vapor phase has been regarded as safe and one of the best methods (Smith et al. 2001; Kirsop and Doyale 1991). With adequate care of freezing and thawing, any phenotypic or genotypic changes can be avoided. Optimization of the method for target fungal strain (s) has enabled successful freezing of many recalcitrant fungi too (Morris et al.1988).

Cryopreservation of fungi is in practice in many leading culture collections in the world as one of the most reliable methods (Homolka 2013). Literature indicates that in beginning researchers tried different options to cryopreserve spore suspension, mycelial/vegetative culture, separately as well as in combinations, with or without a CPA, and from time to time required improvements have been made in the method/recipe (Hwang 1968; Stalpers et al. 1987; Chandler 1994). Recently, Homolka et al. (2001, 2006) devised a method using perlite (aluminosilicate volcanic mineral) as a carrier for fungal mycelia and successfully tested for preserving basidiomycete strains. As most preferred condition, a cooling rate of −1 °C min^{-1} with use of 10% (v/v) glycerol as a CPA is applied and reported to have good impact on major groups of fungi. The same protocol is followed in the author's laboratory (NFCCI-WDCM 932) for cryopreservation of fungi (Singh and Baghela 2017). However, there have been reports on some members of the Basidiomycota and Oomycota that they do not survive cryopreservation well compared to sporulating fungi. Storage of cultures in liquid nitrogen is although simple, involve relatively high-running costs because of the necessity of regular filling of the containers. However, for most workers, this method is convenient, well tried, and tested.

Protocol

- Grow the culture (s) on suitable agar medium-like PDA/MEA in Petri plate (s).
- Check for contamination by slide preparation and microscopy (quality check).
- Cut the agar plugs with flame sterilized cork borer.
- Aseptically transfer to cryovial filled with 10% glycerol and label using cryomarker.
- Place the tightly capped cryovials in Nalgene freeze containers filled with isopropanol.
- Nalgene® freeze containers are kept in −70 °C deep freezer for 4 h.
- Transfer the frozen cryovials to pre-cooled (at −70 °C) cryobox and store at −70 °C until the box is filled to max capacity.
- Alternatively, cryocane can be used instead of cryobox.
- Transfer the labeled cryoboxes and or cryocanes to the respective racks and cryocanister then place in the cryocan filled with liquid nitrogen.
- Maintain the inventory of cryo-preserved strains/cultures of fungi.

Recovery of Culture

- Thaw vial in a water bath at +37 °C and remove the ampoules when the last ice has melted (do not allow the suspension to reach the temperature of the water bath).
- Open the ampoule aseptically in a laminar flow bench/cabinet.
- Inoculate onto a suitable agar medium.
- If agar blocks have been preserved, remove a few blocks with an inoculating needle leaving behind cryoprotectant solution.
- Place mycelium side down onto a suitable agar medium and incubate at suitable temperature.
- Monitor for viability and contamination.
- Monitor for growth characteristics.

Important Tips

- It is essential to follow safety procedures strictly in all activities involving use of liquid nitrogen.
- It is essential that protective clothing is worn, as the major risks are the intense cold, which causes injury similar to burn.
- Areas, where liquid nitrogen is used or stored, must be well ventilated.
- It is advised to use polystyrene foam box which provides insulation for 1 °C/minute cooling.
- Use polypropylene cryovials which are resistant to cracking at ultra-low temperature.
- Cryovials can be protected from infiltration of nitrogen (in case of emerged storage) by sheathing vials in plastic tubing (heat-shrunk) onto the vials.
- This heat-shrunk tubing may be sealed either ends by heat crimping before freezing (Humber 1997).
- Some repositories prefer glass cryovials too.
- It is advised to use an alcohol resistant cryomarker for proper labeling.
- It is advised to use standard cryolabel.
- Prior to opening for revival of preserved strain (s), disinfect the surface of cryovials by dipping in alcohol.

Lyophilization/Freeze-Drying

This method is technologically sophisticated long-term storage method for effective preservation of nearly most of the conidial fungi, ascomycetes, and basidiomycetes. However, this method is not useful to the fungi with very “watery” cells having large vacuolar volumes like Oomycetes (Oomycota) and the Entomophthorales

(Mycota) (Humber 1997). In lyophilization, the water content is reduced to about 2–3% by high vacuum drying and the fungus is stored in the absence of oxygen and water vapor. The spores/mycelial fragments can be freeze-dried. Several modified protocols are available, and the shelf-life of stored organisms have been reported from 17–24 years (Simione and Brown 1991; Smith and Onions 1994); it is considered as the primary technique used at most of the general service culture collections and individual laboratory. Users mostly prefer manifold or centrifugal freeze-dryers and lyophil ampoules or glass tubing sealed at one end to make small tubes. As an advantage, the standard method can be used for many fungi. However, this method has been most advantageous for sending cultures by post. As a disadvantage, equipment is expensive and freeze-dried ampoules are refrigerated and many fungi do not survive this method.

Protocol

- Select the target cultures and cultivate on suitable agar medium (PDA or MEA) in Petri plates.
- Sterilize and dry cotton-plugged ampoules.
- Flood the plate containing freshly growing and sporulating cultures with sterilized skim milk solution and suspend spores and hyphae.
- Transfer spore suspension into glass ampoules.
- Alternatively, mycelia instead of spores can also be freeze dried.
- Freeze spore suspension in glass ampoules at −70 °C deep freezer for 4–6 h.
- Attach frozen ampoules to a strong vacuum on the lyophilizer machine.
- Complete the freeze-drying under vacuum desiccator.
- Flame-seal the glass ampoules by sealing torch.
- Check for leakage, if any.
- Store the lyophilized ampule (s) in refrigerator or even it can be stored at ambient temperature.

Recovery of Culture

- It is general practice of culture collections to send detailed directions about how to open lyophilized culture tube.
- Different types of glass ampoules are available in market. If an ampoule is not prescored, score the neck with a file or diamond pencil.
- Wrap the scored ampoules in a sterile paper wipe moistened (but not soaking!) with ethanol and break at the scoring.
- Add sterile water or suitable liquid medium to reconstitute the culture.
- Place reconstituted culture in sterile place like laminar for about 30 min to soften and to rehydrate the dried culture.

- Mix and pipette the reconstituted mixture onto fresh agar medium.
- Incubate the culture at suitable temperature and time.
- Monitor for viability and contamination.
- Monitor for growth characteristics.

Important Tips

- Though non-sporulating cultures can also be lyophilized, spores may retain better viability than hyphae.
- Instead of skim milk solution, other suitable carrier can be used.
- Before subjecting to freezing, quality check is essential which can be performed by slide preparation and microscopy.
- It is advised to flame-seal glass ampoule while ampoules are still under vacuum by sealing torch.
- Check points are essential at every step in order to get contamination-free lyophilization of target fungal culture.

Culture Preservation on Cereal Grains

This method is generally considered as non-conventional method. There have been several reports that fungi such as *Sclerotinia*, *Magnaporthe*, *Leptosphaeria*, *Rhizoctonia* species can be stored for up to 10 years through this method of preservation. These fungi can be stored on seeds of oats, barley, wheat, rye, millet, and sorghum (Singleton et al. 1992; Gaskill 1968). Naito et al. (1993) report a low-temperature, long-term preservation method for storing *Rhizoctonia* cultures on barley grains at −20 °C with survival rates of 10 years or more. It was reported that method did not cause any changes in the isolates, and the cultures could be used directly for pathogenicity tests. Ochi and Nakagawa (2010) reported up to 3 years of viability of *Calonectria ilicicola* isolates preserved at −80 °C while 2 years at −20 °C. Different viability of fungi at different temperatures has been reported. Cryo-preserved on barley grains is also reported to be used directly as inocula, and this way the method would save a lot of time. Further, advantage of this method is that subculturing is not necessary which leads to less contamination, mutation, or mortality, commonly associated with subculturing.

Protocol

- Select fungal strain to be preserved on barley grains/seeds.
- Take about 100 g of hulled barley grains and 100 ml distilled water.
- Put the barley grains and distilled water in a 500 ml flask.

- Plug the flask with cotton and autoclave at 121 °C for 20 min.
- After cooling, inoculate flask containing sterile barley grains with a mycelial plug of selected fungus.
- Incubate the plates for at least three weeks at ambient temperature.
- Air-dry grains fully covered with fungal mycelia and spores in Petri plates at 30 °C for 24 h aseptically.
- Air-dried grains with fungus can be divided into 2–3 portions (as per requirement).
- Each set can be stored at required temperature like −20 or −80 °C in freezers.

Culture Recovery

- Take out a few air-dried and stored barley grains/seeds.
- Aseptically transfer to agar medium suitable for growth.
- Incubate at optimal temperature and time period.
- Monitor for viability and contamination.
- Monitor for the growth characteristics.

Important Tips

- Stir grains twice or thrice during incubation to avoid agglomeration of the culture.
- It is advised to completely air dry the barley grains covered with fungal mycelia and spores.
- Preserved barley grains can be used directly as inocula that would save a lot of time.
- Cultures preserved on barley grains reported to retain virulence of isolates and did not affect the mycelial growth (Ochi and Nakagawa 2010).

In addition to these methods, reports on new/revised protocols for preservation and maintenance of different groups of fungi may be found in the literature provided from time to time by researchers (Jong and Davis 1978; Singh et al. 2004; Homolka et al. 2007; Day and Stacey 2007).

Role of Culture Collections in Biotechnology/Bioprospecting

The significant presence of fungi in all major terrestrial ecosystems, freshwater, and marine make them especially valuable. Fungal diversity in inter-tropical region is largely unexplored, and their estimates are being revised upward. However, different strategies for conservation of fungi are being deliberated, as application of fungi necessitates its long-term conservation in which fungi have a vital role to play

in diverse fields such as agriculture, medicine and industrial biotechnology through successful development of mycotechnology.

Culture collections have been broadly considered as biological resource centers (BRCs) playing extremely important roles in conservation of germplasms to be maintained indefinitely. They are generally mixture of academic, public service, government, private, and commercial activities that deliver important characterized cultures as "seed" stocks for various purposes such as for the development of industrial processes, reference strains for biological assays and published scientific literature, type strains for taxonomical studies and centers for conservation of biodiversity, without depleting the collections. These culture collections are storehouses of information on cultures/strains for various purposes that can be accessed to individual collections or microorganism database (http://wdcm.nig.ac.jp/) by contact.

First collection of microorganisms was established by Professor Frantisek Král (1846–1911) in 1890 at the German University of Prague (Czech Republic). Collections are developed based on the maintenance of examples of each strain or species under controlled laboratory or field conditions. Pure cultures of the fungi are becoming increasingly important in diverse fields such as agriculture, medicine, and industrial biotechnology. Tropical fungal biodiversity deserves exploration and ex situ conservation as a prerequisite to advance researches in areas such as diseases, control, exploration for novel bioactive molecules, industrial enzymes, etc. Our knowledge of tropical fungal biodiversity as well as availability of pure cultures for R & D process needs greater emphasis to be laid for future scientific advances in fungal-based technologies.

Fungal resources of India are huge, and mycologists have been working on fungi since the turn of last century and thousands of novel and interesting forms have been described and reported from India which accommodates one of the largest biodiversity gene pools of the world. As per database of WFCC-WDCM, there are 27 culture collections currently existing in India possessing various organisms like fungi, yeast, bacteria (incl. anaerobes), cyanobacteria, actinomycetes, algae, plasmids, viruses, insects, archaea. Some of them are active and contributing to science and technology by preserving germplasm as raw materials for basic and applied research. Some of the leading culture collections of India dealing with fungal germplasms in addition to other organisms are ITCC, MTCC, NCIM, NBAIM, MCC, etc.

In addition, a "National Facility for Culture Collection of Fungi" was set up in 2008 by the Department of Science and Technology (DST), Government of India, with the primary aim to conserve germplasm of indigenous fungi in its repository, i.e., National Fungal Culture Collection of India (Acronym-NFCCI) established as an exclusive germplasm repository of fungi affiliated (as affiliate member) with World Federation for Culture Collections (WFCC) and also registered in World Data Centre of Microorganisms (WDCM-932) at MACS' Agharkar Research Institute Pune, Maharashtra. More than 4000 indigenous fungi are being maintained in NFCCI by following standard long-term preservation methods like mineral oil, glycerol, distilled water, freeze drying (lyophilization), and liquid nitrogen

(cryopreservation). The NFCCI renders various services to academia, research institution and industry, undertakes quality research and develop capacity in thrust areas of fungal taxonomy, systematics, conservation and sustainable utilization of fungal biodiversity in India. The NFCCI principally acts as service collection and perform basic functions as core activities, viz. acquisition, verification, preservation and maintenance, deposit and accession, distribution of authentic fungal strains and associated information, and documentation of fungi. Protocols provided in the text are mostly being practiced at NFCCI. Having expertise, NFCCI renders knowledge-based services like authentication of fungal cultures, mycological herbarium samples, and fungi associated with various other substrates are provided on regular basis. Authentication of fungal cultures is done based on morphological and molecular analyses. Apart from this, authenticated fungal strains are also supplied to various purposes. Annually, large numbers of academia, research institutions, and industries in India are benefited from NFCCI services.

Future Perspectives

Review of literature reveals that fungi are one among a few most fascinating organisms by rendering invaluable ecosystem services, also considered as gold-mines for innovations from cultural and scientific perspectives. But they have not been considered as an integral part of conservation biology, though fungi play crucial role in nature and in sustainable manner. Heilmann-Clausen et al. (2014) identified following five key areas in which fungi can be readily integrated into conservation as:

i. Providers of habitats and processes important for other organisms.
ii. Indicators of desired or undesired trends in ecosystem functioning.
iii. Indicators of habitats of conservation value.
iv. Providers of powerful links between human societies and the natural world because of their value as food, medicine and biotechnological tools.
v. Sources of novel tools and approaches for conservation of mega-diverse organism groups.

Therefore considering their potential and sustainable services, it is pertinent to advise conservation professionals to look into and plan conservation strategies for this unique organism which touches every facet of human life. The conservation of fungi can lead to strengthening bio-economy in long run. An International Society for Fungal Conservation (ISFC) has been established for making global awareness and strengthening fungal conservation strategies in different countries. Members from more than 40 countries are presently associated with this society.

Conclusions

Fungi are known since ancient time and embody diversified groups. They are ubiquitous and constitute a major decomposer's community in the biosphere. Out of estimated 100,000 + fungi recognized from world, nearly 30% have been recorded from tropics in the last 5–6 decades, which reflects richness of this geographic region. It is remarked that Indian subcontinent and other warmer belt of globe are the "Store House" of known and hitherto unknown fungi in nature occupying variety of substrates/habitats. Out of 27,500 described Indian fungi, 15,500 are terrestrial litter fungi, 327 are coprophilous, and about 450 are endophytic fungi. It has been assumed that global estimates of fungi are conservative, and as many as 1.43 million species remained undetected. In number terrestrial plant litter-inhabiting fungi are said to the maximum (Bhat 2010). Considering the number of fungi actually documented in India, it is important to make systematics phase-wise plan of exploration of a particular geographic location, collection and conservation of fungi which are extremely important. Selection of appropriate method is crucial for long-term preservation, and maintenance of fungi as no single method is ideal for preserving all types of fungi. Besides, red data book on fungi in India is unavailable; therefore, status of endangered/extinct fungal taxa is in state-of-flux, requiring focused efforts and clear-cut policy. It is also important to develop working model to generate knowledge based bio-economy, protection of IPR, sharing resource and information within country and build up a global partnership in conservation and sustainable use of fungal resources within framework of new regulations of CBD, OECD, Nagoya Protocol, NBA, etc. Moreover, culture collections/biological resource centers need to redefine their objectives and role for contributing toward ambitious agenda of reducing poverty and improving lives by achieving Millennium Development Goals of United Nations by utilizing these biological resources in order to produce high value bio-products and by generating employment for rural community. In addition, issues relating to the bio-security, ethical access, and use of fungal germplasm are also important for Indian bio-resource center in changing global legal framework.

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Camouflaged Mycotoxins in Some Field Crops and Forages: A Review

28

Skarma Nonzom and Geeta Sumbali

Abstract

Mycotoxins are secondary mould metabolites, which are associated with toxic effects in living forms including humans, birds and animals and are chiefly produced by some species of *Aspergillus, Penicillium* and *Fusarium*. They are produced in cereal grains and forages quite before or during or after harvesting under diverse environmental conditions. Owing to their diverse toxic effects and synergetic properties, mycotoxins are considered risky for the health of consumers ingesting foods contaminated with them. Majority of the reported mycotoxins are worldwide in distribution and impose severe health risks to all the living forms. The phytopathogenic *Fusarium* species, which are the causal agents of 'head blight' (scab) of small grain cereals and 'ear rot' of maize, occur worldwide and may accumulate several mycotoxins in the infected kernels, some of which show remarkable impact on the health of consumers. While *F. graminearum, F. culmorum*, *F. poae*, *F. avenaceum* and *Microdochium nivale* predominantly cause diseases of small grain cereals, maize is predominantly attacked by *F. graminearum*, *F. moniliforme*, *F. proliferatum* and *F. subglutinans.* Since fungal infection and subsequent mycotoxin production begins in the field prior to harvest, the focus of this review encompasses occurrence and management of various crops diseases (wheat, barley, rice, maize and sorghum) caused by toxigenic fungi and that of forage crops harbouring toxigenic endophytic fungi. The review also focuses on the influence of various factors on disease development and production of camouflaged mycotoxins in the field.

Keywords

Mycotoxins · *Fusarium* head blight · Endophytic · Toxigenic fungi
Field crops · Forages

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Introduction

Mycotoxins are secondary mould metabolites, which evoke pathological changes in man and animals. They usually remain camouflaged in food and feedstuffs, enter the food chain while maintaining their toxic properties, are quite stable and extremely difficult to eradicate. They are produced chiefly by the toxigenic strains of species belonging particularly to three fungal genera, viz. *Aspergillus*, *Penicillium* and *Fusarium*. Their presence in standing field and forage crops as well as after harvesting in storage is undesirable as they may exert number of adverse effects on the health of consumers, affecting their digestive system, nervous system, reproductive system, liver, kidneys and other organs (CAST 2003). Some mycotoxins are even immunocompromising and thus can reduce the resistance to various infectious diseases (Desjardins 2006), whereas few are known to be carcinogenic (CAST 2003). In fact, mycotoxins differ broadly in their toxicity and the toxic effects may be acute (after a single exposure) or chronic (after repeated exposures). It is quite challenging to classify them due to their myriad chemical structures and biosynthetic origins, varied biological effects and their production by a number of different fungal species. The cell biologists classify them into generic groups (such as, mutagens, teratogens, carcinogens); organic chemists have attempted to categorize them by their chemical structures (such as, lactones, coumarins); biochemists classify them according to their biosynthetic origins (such as, polyketides, amino acid-derived); physicians arrange them by the illness they cause (such as, St. Anthony's fire, aflatoxicosis, stachybotryotoxicosis); mycologists classify them by the fungi that produce them (such as *Penicillium* toxins, *Aspergillus* toxins, fusarial toxins); and clinicians often classify them by the organ they affect (such as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins).

Fungal infection and subsequent production of mycotoxins usually begins in the field during plant growth and may continue during harvesting, handling, storage and processing. Toxigenic moulds may develop under all climatic conditions on any solid or liquid support as soon as favourable nutritional and moisture levels are available. These toxigenic (mycotoxin producing) fungi can be distinguished into two groups. The first one, known as preharvest (field level) toxins, is usually produced by fungi, such as *Aspergillus flavus* and *Fusarium* species, which invade the growing plants before harvesting and produce mycotoxins like aflatoxins and various fusarial toxins (Fig. 28.1). The other group, known as postharvest (storage) toxins, is usually produced by fungi after harvesting and during crop storage, transportation and marketing, for example, ochratoxins, patulin and citrinin.

It has been estimated that 25% of the world's food production is affected by mycotoxin-producing fungi and cereal grains appear to be contaminated at higher levels (CAST 2003). International trade in various agricultural commodities such as wheat, rice, maize, barley, sorghum, groundnuts and other oilseeds amounts to around hundreds of millions of tonnes annually and a large number of these are at a high risk of contamination with diverse mycotoxins (FAO 1988). Therefore, strict regulations on these mycotoxins have been set and are implemented by most of the

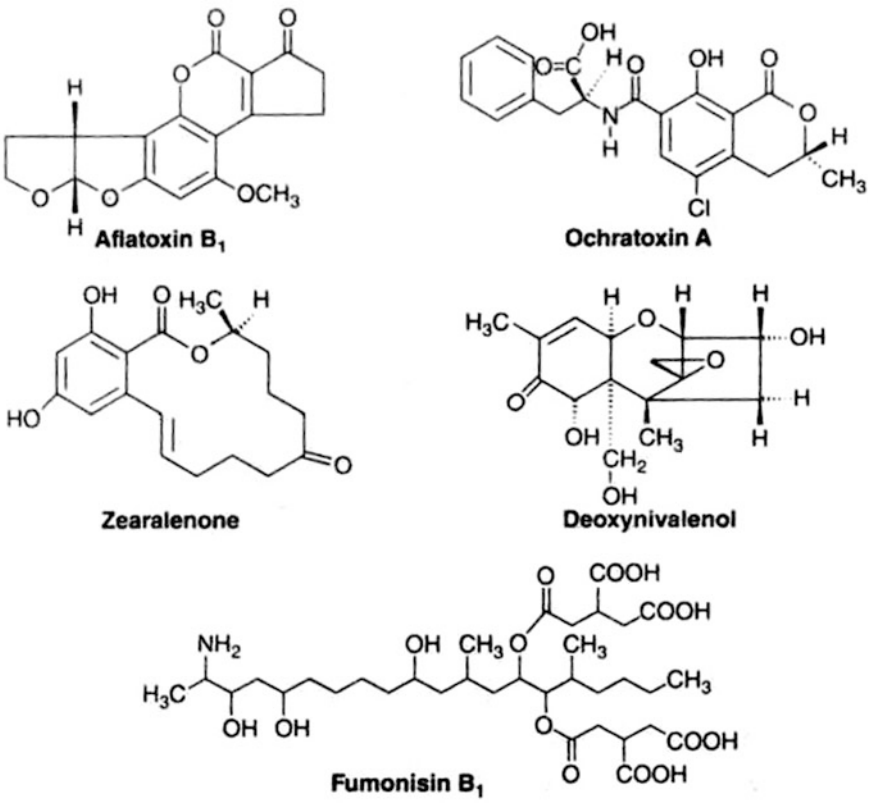

Fig. 28.1 Chemical structures of some important mycotoxins detected in field crops

importing nations, thereby influencing international trade. Further, in some of the developing countries, where agricultural commodities account for as much as 50% of the gross national exports, the economic importance of mycotoxins is considerable (Bhat and Miller 1991).

Major Groups of Field Mycotoxins

Aflatoxins: They are the best known and most intensively researched mycotoxins in the world, which are mainly produced by *Aspergillus flavus* and *A. parasiticus*. Aflatoxins are one of the most potent carcinogens known to man and have been linked to a wide array of human health problems. The major aflatoxins are B_1, B_2, G_1 and G_2, based on their fluorescence under UV light and relative chromatographic mobility during thin-layer chromatography. Of these, aflatoxin B_1 is the most potent carcinogen known and is usually the major aflatoxin produced in nature

by toxigenic strains (Squire 1981). An increased rate of mortality has been observed in farm animals fed with aflatoxin-contaminated food and thus considerably reducing grain value to be used as animal feed/export commodity. The Food and Drug Association (FDA) has established a maximum permissible level of total aflatoxins in food commodities at 20 ppb (Bhat et al. 2010). Aflatoxins are known to be responsible for not only toxicity but also carcinogenicity in human and animal populations, and diseases caused by their consumption are called aflatoxicoses. While acute aflatoxicosis results in death, chronic aflatoxicosis results in cancer, immune suppression and other slow pathological conditions. The liver is the principal target organ, with liver damage occurring in all the vertebrates, which are fed aflatoxin B_1-contaminated food/feed.

Ochratoxins: They are a group of related compounds that are produced chiefly by *Aspergillus ochraceus*, *Penicillium viridicatum* and certain other species of *Aspergillus* and *Penicillium*. The main mycotoxin in this group is ochratoxin A (OTA), which is a potent toxin that causes kidney damage and urinary tract cancers (Bhat et al. 2010). This toxin is produced in a variety of feed, food and beverages, particularly cereal and grain products. It is believed that human exposure to OTA mainly occurs from whole-grain breads (Bhat et al. 2010).

Fumonisins: These are a group of compounds produced by a number of *Fusarium* species, particularly *F. verticillioides*, *F. proliferatum* and *F. nygamai,* as well as *Alternaria alternata* f. sp. *lycopersici* on field and stored foodstuffs including corn, small grains, rice, sorghum and wheat (Marasas et al. 2001; Rheeder et al. 2002). The prime species of economic importance is *Fusarium verticillioides*, often growing as a corn endophyte in vegetative as well as reproductive tissues usually without generating disease symptoms in the host (Maria and Puia 2012). However, it may cause stalk rot, ear rot and seedling blight when weather conditions are favourable in combination with insect damage along with the presence of suitable plant and fungal genotype (Nelson et al. 1993). There are at least three naturally occurring fumonisins, viz. FB_1 (most toxic and occurs at highest concentration), followed by FB_2 and FB_3. International Arctic Research Institute, IARC (1993), studied the carcinogenicity of grains infected with *Fusarium moniliforme* and fumonisins and found them to be possible human carcinogens.

Trichothecenes: They are a group of closely related, naturally occurring *Fusarium* toxins, which include T-2 toxin, deoxynivalenol (DON), nivalenol (NIV), diacetoxyscirpenol (DAS), fusarenon-X and 3-acetyl deoxynivalenol (3-ADON). In addition to *Fusarium*, trichothecenes are also produced by a number of other fungal genera like *Myrothecium*, *Phomopsis, Stachybotrys*, *Trichoderma* and *Trichothecium* (Scott 1989). They occur singly or in combination in various cereal grains, and their thermal stability is remarkable and these often withstand the rigours of cooking. Trichothecenes come next to aflatoxins in toxicity and are implicated in some human diseases, such as Alimentary Toxic Aleukia (ATA) in Russia and Akakabi disease in Japan (Ueno et al. 1972). Toxicological characteristics of trichothecenes include inflammation of skin, digestive disorders, haemorrhagic syndrome, destruction of bone marrow and nerve disorders (Wannemacher and Wiener 1997). They are produced by many *Fusarium* species, including *F. graminearum*,

F. culmorum, *F. poae*, *F. oxysporum* and *F. sporotrichioides* (D'Mello et al. 1999). *F. sporotrichioides* and *F. poae* predominantly produce type A trichothecenes, which include T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol (DAS), whereas *F. culmorum* and *F. graminearum* produce type B trichothecenes, which include deoxynivalenol (DON), its 3-acetyl and 15-acetyl derivatives (3-ACDON and 15-ACDON, respectively) and nivalenol (NIV). These pathogens and several other species of *Fusarium* are responsible for *Fusarium* head blight (FHB) in wheat, barley and other small cereal grains (Desjardins 2006; Dill-Macky 2010) and ear rot in maize caused particularly by *F. graminearum,* which may lead to the contamination of these crops by mycotoxins particularly deoxynivalenol (Payne 1999).

Zearalenone: It is a phenolic resorcylic acid lactone with potent oestrogenic properties produced by strains of *Fusarium graminearum*, *F. tricinctum*, *F. oxysporum, F. sporotrichioides* and *F. Moniliforme,* and a period of low temperature (12–14 °C) seems essential for high yield. Zearalenone (ZEN) is encountered as a natural contaminant, particularly in maize, but occasionally in other cereals and feedstuffs also. The effects of zearalenone in animals have been observed; for example, oestrogenic syndrome in pigs and cases of reduced fertility in cattle have been reported to be associated with zearalenone (Eriksen 2006).

Historical Perspectives

Mycotoxicoses, that is, mycotoxin-associated diseases, have been recognized since long, and there are many evidences of mycotoxin poisoning. The first recognized mycotoxicosis was probably ergotism, a disease caused by the intake of grain contaminated with sclerotia of *Claviceps purpurea*. The disease was characterized by necrosis and gangrene and was known in the middle ages in Europe under the name 'Holy fire'/'St. Anthony fire'/'fire sickness' because the people affected by this mycotoxicosis used to depart to the shrine of St. Anthony to relieve the intense burning sensation experienced in their head. Another mycotoxicosis, recognized to have seriously affected human populations, is trichothecene associated 'Alimentary Toxic Aleukia' in Russia during World War II (Bhat et al. 2010). The disease was induced by eating overwintered mouldy grain and the fungi responsible belonged to the genera *Fusarium* and *Cladosporium*. Although this type of toxicoses was common during 1932–1947 in the USSR, it has not been reported since then. In 1951, an outbreak of 'bread poisoning' occurred in a small town of France due to mouldy rye that was sold illegally to avoid a grain tax, which led to the illness of around 200 people along with their pet animals who were fed with the same bread (Merhoff and Porter 1974). Another outbreak occurred in Ethiopia during 1977 and 1978 which resulted in gangrenous ergotism and affected around 140 people, of whom 34% died (King 1979).

Despite their rather high incidence, mycotoxicoses remained neglected, until 1960 when 'Turkey X disease' broke out in Great Britain. The term mycotoxin was coined in 1962 after this unusual veterinary crisis, during which approximately 100,000 turkey poults died (Blout 1961; Forgacs 1962) because of the peanut meal, which was contaminated with mycotoxins produced by *Aspergillus flavus*. After this incidence, much data and information accumulated about aflatoxins and various other mycotoxins that were discovered subsequently (Bhat et al. 2010). These included many previously reported fungal toxins, such as the ergot alkaloids, some compounds that had originally been isolated as antibiotics, such as patulin, and thereafter, a number of new secondary metabolites led to the discovery of mycotoxins, such as ochratoxins. The phase between 1960 and 1975 has been termed as the 'mycotoxin gold rush' since large number of scientists got united for the well-funded search programme for these toxigenic agents. In China, 53 outbreaks of human food poisoning occurred in 1960 and they were linked to scabby and mouldy cereals affected by *Fusarium* head blight (FHB) in the mid and lower regions of the Yangtze River valley (Pirgozliev et al. 2003). Later, during 1972–1988, approximately 884 persons were affected in 13 provinces of China when outbreaks of food poisoning reported as mouldy sugarcane poisoning (MSP) caused by an *Arthrinium* species occurred (Liu et al. 1988).

In India, one of the first outbreaks of aflatoxicosis was recorded during 1974 from Western India with 106 deaths of indigenous people whose staple food was maize (Reddy and Raghavender 2007). In 1987, an outbreak of gastrointestinal disorder in the Kashmir Valley (J&K) was associated with the consumption of toxins produced by *Fusarium* (Bhat et al. 1989). Similarly in 1995, a foodborne disease outbreak characterized by abdominal pain and diarrhoea occurred in few Indian villages of the Deccan Plateau after consumption of sorghum and maize that was damaged by unseasonal rains (Bhat et al. 1997). All the samples collected from affected households showed dominance of *Fusarium* species and mycotoxin contaminant fumonisin B1 in contrast to the samples collected from unaffected households that showed comparatively low levels of fumonisin B1. In between 1951 and 1985, surveys that were carried out reported reduction in wheat grain by 5–15%, when moderate epidemics of FHB were recorded, and up to 40%, when disease epidemics were severe (Zhuping 1994). According to Sayler (1998), wheat producers in nine US states lost 501 million bushels of grain, equivalent to $2.6 billion between 1991 and 1996. It was observed that hard red spring wheat crops were worst affected with 52% production losses, while soft red wheat and durum wheat experienced 38% and 10% production losses, respectively. Similarly, during head blight epidemics in the Northern Argentinean Pampas, yield losses between 10 and 50% were reported (Moschini et al. 2001).

Fungal Infections and Mycotoxin Contaminants in Field Crops

The genus *Fusarium* encompasses a wide range of species and patho varieties, some of which are pathogenic to a wide range of plants, especially field crops under diverse environmental conditions. These pathogenic *Fusarium* species are responsible for several diseases of small grain cereals, including *Fusarium* head blight (FHB) (also known as 'scab' or ear blight) and ear rot of maize (Parry et al. 1995). FHB was first described from England in 1884 and was considered as a major threat to wheat and barley during the early years of the twentieth century (Stack 1999, 2003). Since then, FHB has increased worldwide, and various outbreaks have taken place in Asia, Europe Canada and South America (Sutton 1982; Parry et al. 1995). FHB has been recognized by International Maize and Wheat Improvement Center (CIMMYT) as a prime factor limiting grain production in different parts of the world (Stack 1999). In USA, FHB has reached epidemic levels during several years, leading to yield losses, price discounts and reduced seed quality (Windels 2000). In the Northern Great Plains and Central USA, direct and secondary economic losses due to FHB for all crops were estimated to be $2.7 billion from 1998 to 2000 (Nganje et al. 2002). Moreover, a number of these *Fusarium* species possess the ability to produce a diverse range of toxic secondary metabolites (mycotoxins) that impose a potential health risk when contaminated grains are ingested in human and animal food products (D'Mello et al. 1999). The most important groups of fusarial mycotoxins that are based on their toxic effects on human and animal health include trichothecenes, fumonisins, zearalenone and moniliformin (D'Mello et al. 1999). According to Food and Agriculture Organization (1988), 25% of the world's crops are contaminated by mycotoxins annually, with estimated losses of about 1 billion metric tons of food and food products. These economic losses are due to yield losses that are induced by disease-causing toxigenic fungi, reduced crop value resulting from mycotoxin contamination, losses in animal productivity due to mycotoxin-related health problems and human health costs (Matny 2015). Some of the mycotoxin concentrations detected from field crops grown in various countries have been tabulated (Table 28.1) and reviewed below:

(i) **Wheat (*Triticumaestivum* L.)**: This crop is commonly affected by *Fusarium* head blight (FHB)/scab of wheat, which occurs worldwide and significantly reduces the crop yield and quality of grain (Windels 2000). It is caused by a combination of *Fusarium* species which infect spikes during flowering. So far, 19 species of *Fusarium* have been reported to be potentially associated with *Fusarium* head blight symptoms. However, it is caused chiefly by *Fusarium graminearum* (teleomorph, *Gibberella zeae*) and few other species like *F. avenaceum*, *F.culmorum* and *F. poae* and to a lesser extent by *F. acuminatum*, *F. cerealis*, *F. chlamydosporum*, *F. equiseti*, *F. langsethiae*, *F. sporotrichioides* and *F. tricinctum.* These *Fusarium* species not result in yield losses but also produce various mycotoxins in the grain when environmental conditions are favourable (Logrieco and

Table 28.1 Mycotoxins detected in some important field crops

Field crops	Mycotoxins detected	Concentration detected (mg kg^{-1})	Country	References
Wheat	DON DON DON DON 3AcDON DON + ZEN, NIV and T2	0.055–0.33 2.36 8.2 30.4 29.54 3.96–43.8	Italy Norway Austria Poland Poland Germany	Pascale et al. (2001) Elen et al. (1997) Adler et al. (1995) Visconti et al. (1986) Lepschy-von Gleissenthal et al. (1989)
	ZEN	0.330 0.120 0.120	Austria Netherlands Bulgaria	Adler et al. (1995) Vrabcheva et al. (1996) Vrabcheva et al. (1996)
	MON MON	0.88 7.2–25.2	Austria Poland	Adler et al. (1995) Lew et al. (1993)
	BEA	3.5	Finland	Kostecki et al. (1997)
	T2	0.055 0.005–0.60	Bulgaria Germany	Vrabcheva et al. (1996) Lepschy-von Gleissenthal et al. (1989)
	NIV	– 0.16–1.25 0.04–0.29	UK Krasnodarkski Krai (USSR) Germany	Turner and Jennings (1997) Leonov et al. (1990) Lepschy-von Gleissenthal et al. (1989)
Barley	DON	0.070–1.540 0.4 0.36 0.27–0.36 0.12	Italy Germany Norway Germany Poland	Pascale et al. (2000, 2001) Obst et al. (1997) Elen et al. (1997) Lepschy-von Gleissenthal et al. (1989) Chełkowski (1989)
	T2, HT2	0.302	Poland	Perkowski et al. (1997)
Maize	DON DON DON	0.78–2.99 0.5 500	Swiss Germany Austria	Eckard et al. (2011) Obst et al. (1997) Lew et al. (1991)
	ZEN ZEN	0.43 40	Swiss Austria	Eckard et al. (2011) Lew et al. (1991)
	NIV NIV	10 0.19–0.76	Austria Swiss	Lew et al. (1991) Eckard et al. (2011)
	T2 T2	0.13 992	Swiss Poland	Eckard et al. (2011) Chelkowski et al. (1987)
	HT2 HT2	0.084 642	Swiss Poland	Eckard et al. (2011) Chelkowski et al. (1987)
	Fumonisin FB1 FB1 FB2	250 5.31 26.9 6.3	Italy Italy Slovak Republic	Bottalico et al. (1995) Pietri et al. (1995) Srobarova et al. (2000)
	MON	– –	Italy Poland Austria	Logrieco et al. (1995) Lew et al. (1996), Kostechi et al. (1995) Lew et al. (1991)

(continued)

Table 28.1 (continued)

Field crops	Mycotoxins detected	Concentration detected (mg kg^{-1})	Country	References
	BEA	–	Italy	Moretti et al. (1994), Bottalico et al. (1995)
		–	Poland	Logrieco et al. (1993), Kostechi et al. (1995)
		–	Slovak Republic	Srobarova et al. (2000)

Moretti 2008). The fungus can also contaminate forage and straw, which is an additional risk for livestock (Battilani and Logrieco 2014). The toxigenic abilities of the *Fusarium* causing FHB may vary with species; for example, *F. graminearum, F.culmorum* and *F. poae* produce their own spectrum of trichothecenes including deoxynivalenol (DON) and nivalenol (NIV) along with zearalenone (ZEN). In addition, *F. poae* also produces beauvericin, whereas *F.avenaceum* produces enniatins, moniliformin and beauvericin (Xu and Berrie 2005). Different species of *Fusarium* that are dominant worldwide include *F. graminearum, F. pseudo-graminearum, F. avenaceum* and *F.culmorum* (Jacobsen 2014). In temperate regions, *F. graminearum* and *F.culmorum* are most commonly involved in FHB and trichothecene mycotoxin contamination (Jacobsen 2014). In some regions of north-western Europe, *Microdochium nivale* is also involved in FHB (Desjardins 2006; Logrieco and Moretti 2008). Similarly, *F.culmorum, F. avenaceum*, *F. sporotrichioides* and *F. langsethiae* are common in humid and cool conditions, whereas *F. poae*is considered as most important pathogen in warmer and drier environments (Rossi et al. 2001; Xue et al. 2009). This indicates that, besides genetic diversity of the pathogens, environmental factors also affect the distribution of FHB. Moreover, the various pathogenic species and strains of *Fusarium* infect different parts during different developmental stages under varied environmental conditions (Zhang et al. 2011).

FHB is known disease of flowers, the anthers of which get primarily infected by the fungal spores that gradually invade the kernels, glumes and other head parts (Fernandes et al. 2004). The spikelets which get infected become water soaked and straw coloured with subsequent loss of chlorophyll. The warm and humid weather conditions present favourable atmosphere for the production of pinkish red mycelium and conidia in the infected spikelets, and the infection later spreads to the adjacent spikelets or the entire head. The infected kernels appear shrivelled and discoloured with a pink, white or light brown scaly appearance due to the mycelial outgrowth from the pericarp (Bottalico and Perrone 2002). Some evidences suggest that wheat is susceptible even during the development of kernel (Fernando et al. 1997). The source of primary inoculum for FHB is the fungal colonies overwintering on crop residues of wheat, maize and rice (Sutton 1982; Parry et al. 1995), and this disease usually occurs in wheat–wheat, corn–wheat or rice–wheat rotations (Zhu and Fan 1989). The pathogens involved are usually spread through the

dispersal of conidia that are blown or splashed to new infection sites (Paul et al. 2004; Xu and Nicholson 2009). Wheat has been found to be susceptible to infection mostly during flowering following which a sharp decrease in susceptibility has been observed (Hart et al. 1984). The aetiological characteristic of FHB is the co-occurrence of several species of *Fusarium,* which is referred to as a 'complex'. It is quite possible to isolate as many as nine different species of *Fusarium* from a single fragment of infected tissue or up to 17 different species from freshly harvested grains collected in a limited area (Popovski and Celar 2013). Although a limited number of *Fusarium* species have been considered as pathogenic, which generally predominate in a particular host-agroclimatic system, yet numerous other strains of less pathogenic or opportunistic species of *Fusarium* also possess the capability of producing considerable amounts of mycotoxins. Hence, the toxigenic profile of a contaminated crop is determined not only by the predominant pathogenic species, but also by the opportunistic species present in the 'complex' (Burgess et al. 1997). At times, even in the absence of visible symptoms, infections may lead to the accumulation of mycotoxins like deoxynivalenol (DON), especially when prolonged wet conditions prevail during the hard dough stage (Del Ponte et al. 2007).

The toxigenic strains of *Fusarium graminearum* are classified in two chemotypes, viz. DON and NIV producers, according to the main type B trichothecenes synthesized (Popovski and Celar 2013). Further, DON-chemotype strains of *F. graminearum* are subclassified into two types, viz. 3-AcDON and 15-AcDON producers (Miller et al. 1991; Yoshizawa 1997). Field surveys reveal that the most frequently encountered mycotoxins in *Fusarium* head blight of wheat throughout the world are deoxynivalenol and its derivatives, produced by *F. graminearum* and *F. culmorum* (Bottalico and Perrone 2002). Perkowski et al. (1988) observed a high contamination of DON in triticale kernels infected by *F. graminearum* and *F. culmorum.* Similarly, in Germany, high concentration of DON was found in 87% of wheat samples studied, along with zearalenone (ZEN), nivalenol (NIV) and T2 toxin (Lepschy-von Gleissenthal et al. 1989). Later, Miller et al. (1991) showed that the production of 3-ADON and 15-ADON differed in DON producing isolates of *Fusarium graminearum* collected from different regions of the world. Concentration of DON in the kernels is well correlated to *Fusarium* head blight symptoms as the species responsible for the disease include mycotoxin-producing fungi like *F. graminearum* and *F. culmorum* (Bottalico and Perrone 2002). In the southern localities of Russia (Krasnodarski krai), widespread losses ranging from 25 to 50% and high levels of DON and its derivatives have been reported in the freshly harvested wheat grains associated with FHB (Leonov et al. 1990). On the other hand, co-occurrence of nivalenol (NIV) and fumonisins (FUS) with deoxynivalenol has frequently been found in the wheat ears associated with FHB from southern to northern European localities, where they have been attributed to the presence of NIV chemotypes of *F. graminearum* and *F. culmorum* (Bottalico et al. 1990). In Sweden and other northern countries, NIV and FUS formation has also been attributed to *F. poae* and *F. cerealis* infection (Bottalico et al. 1990; Eriksen and Alexander 1998). From Austria, Adler et al. (1995) reported high levels of DON

contamination, together with low levels of ZEN in freshly harvested kernels of durum wheat, predominantly infected by *F. graminearum* and less commonly by *F. culmorum*. Similarly, contamination of ZEN and DON has been detected from freshly harvested cereals of the Netherlands (De Nijis et al. 1996), ZEN from 69% of Bulgarian wheat samples (Vrabcheva et al. 1996), NIV and DON from grains of UK (Turner and Jennings 1997) and DON from soft wheat samples of Italy (Pascale et al. 2000, 2001).

In addition to ZEN and various trichothecenes, fumonisin B_1 has also been detected in wheat ears infected by *F. verticillioides*. It has been reported from Norway (Kosiak et al. 1997); from Bulgaria, The Czech and Slovak Republics (Parry et al. 1995; Srobarova and Pavlova 1997); from Croatia and Hungary (Toth 1997); and from South Russia and Italy (Pasquini et al. 2001; Pancaldi and Alberti 2001). In addition, fumonisins have also been detected in wheat ears infected by toxigenic strains of *F. proliferatum* from Italy, France, Portugal, Greece and Turkey (Bottalico et al. 1989; Infantino et al. 2001).

(ii) **Barley (*Hordeumvulgare* L.)**: It is an important crop that is used in brewing and malting. Incidentally, *Fusarium* head blight (FHB) or scab, caused by *F. graminearum* (teleomorph *Gibberella zeae*), a devastating disease of barley in the humid and semihumid climates, has the capacity to destroy a potentially high yield (Steffenson 2003). *F. graminearum* invades the spikes of barley after they emerge from the flag leaf sheath in the late-milk to soft dough stages of seed development (Bushnell et al. 2003). The visible symptoms include premature necrosis and a brown/grey discolouration of spike tissue. In contrast to wheat, where the fungus spreads between spikes of the ear through the rachis, the fungus does not spread in barley and the symptoms are more restrained (Boddu et al. 2006; Geddes et al. 2008). Orange sporodochia as well as some dark discolouration may be seen at the base of the infected florets, whereas the heads occasionally show the presence of perithecia. Initially, barley was not a primary host for *Fusarium,* but now it has become as vulnerable as wheat (Tekauz et al. 2000). This change might have resulted from a fundamental shift in the promoting disease or due to the cultivars that are grown (Tekauz et al. 2000). The effects of FHB, which include reduced yield and reduced grain quality collectively may reach devastating proportion during disease epidemics (Gilbert et al. 1994; McMullen et al. 1997). The disease results in a decline in grain yield due to floret sterility as well as poor grain filling and reduced kernel size. In addition, severe preharvest infection with the species of the FHB complex results in reduced seed germination and grain functionality, thereby affecting the marketability of the crop (Nielsen et al. 2014). Further, quality problems arise during brewing and malting, resulting in the occurrence of gushing and changes in flavour and colour of the finished beer (Oliveira 2012).

Barley grains affected by FHB are known to contain detectable levels of mycotoxins like nivalenol, zearalenone and deoxynivalenol (Prom et al. 1999). Among these toxins, deoxynivalenol (DON) is the main trichothecene that is detected. As *F. graminearum* is the principal pathogen involved (Clear et al. 1997; Jones and Mirocha 1999; Salas et al. 1999). Other species, including *F. poae*,

F. sporotrichioides and *F. Avenaceum,* are also found to cause infection, which normally do not produce DON, but may produce other mycotoxins (Wong et al. 1995). In market samples, only DON and 15-0-acetyl-4-deoxynivalenol have been detected (Clear et al. 1997; Abramson 1998; Jones and Mirocha 1999). However, in barley inoculated with other *Fusarium* species associated with FHB, some additional mycotoxins have also been detected (Salas et al. 1999). In barley grains, accumulation of DON largely takes place in the outer parts of the kernel, whereas in those where hulls are being shed at the time of harvesting, of more than 50% of DON concentration present initially can be lost (Clear et al. 1997).

(iii) **Maize (Zea mays L.)**: It is one of the most susceptible crops whose yield and quality are reduced by numerous fungal species and their toxins (Kommendahl and Windels 1981). In comparison with small grain cereals, which are mainly infected by *Fusarium graminearum, F. avenaceum, F. culmorum, F. poae* and *F. crookwellense* (Bottalico and Perrone 2002), maize is usually infected by a greater number of *Fusarium* species (Logrieco et al. 2002; Dorn et al. 2009; Scauflaire et al. 2011). For example, 13 different species were found in Swiss maize kernels in 2005 and 15 different species in 2006 (Dorn et al. 2009). A similar diversity was detected in the maize stalk (Dorn et al. 2009). The vast diversity of *Fusarium* species on maize plants suggests the occurrence of inter-species interactions (Reid et al. 1999). It may be colonized by strains belonging to numerous *Fusarium* species like *F.graminearum*, *F. proliferatum*, *F. verticillioides* and *F. subglutinans*, all of which can cause maize leaf blights, stalk rots, ear rots and kernel rots. A reduction in yield, quality and the feed value of the grain has been observed due to ear and kernel rots. The disease of concern is red ear rot caused by *F. graminearum* and *F. culmorum* and pink ear rot caused by *F. verticillioides*, *F. proliferatum* and *F. subglutinans*. The latter is quite common in moderately dry, warm climates, whereas the former is predominantly common in regions with frequent rainfall and low temperatures during summer and early autumn (Logrieco et al. 2002). Maize ears may get colonized by at least 15 *Fusarium* species, which are able to produce a different set of mycotoxins. In red ear rot, infection begins from the tip of the ear immediately after female flowering and proceeds towards the base, and the husks become gradually infected. On the other hand, in case of pink ear rot, infection tends to be more uniform, with no real concentration at the tip.

Some of the important mycotoxins produced by *Fusarium* species in maize include fumonisins, trichothecenes (deoxynivalenol and nivalenol) and zearalenone. In pink ear rot, elevated amounts of fumonisin B_1 have frequently been reported in freshly harvested maize ears infected with *F. verticillioides* or *F. proliferatum* (Battilani and Logrieco 2014). In comparison with the former species, the latter can grow and synthesize fumonisins under drier grain conditions (Marin et al. 1995). In addition, co-occurrence of fumonisin B_1 along with moniliformin, beauvericin and fusaproliferin is also reported (Logrieco and Moretti 2008). On the other hand, in red ear rot, elevated amounts of deoxynivalenol (DON) have been reported with more severity in cool humid areas (Battilani and Logrieco 2014). Grain that overwinters in the field can also be severely infected with red ear rot and high levels of associated mycotoxins (Patience and Ensley 2010).

Besides fusarial infection, maize can also be colonized in the field by aflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus*. The former appears as a yellow-green mould and the latter as a grey-green mould, and both are powdery in appearance. They usually appear more commonly as the climate changes from cool and wet to hot and dry and from higher latitudes to lower ones. Feeding damage resulting from ear-invading insects also contributes to the disease development and subsequent aflatoxin contamination (Woloshuk and Wise 2011). They are more common in south-eastern and south-western USA (Channaiah and Maier 2014). The greatest probability of infection is when suitable weather conditions occur during the silk and fill stage and when there is hail, drought stress, insect damage or early frost, all of which are associated with exposing the kernels to fungal infection (Patience and Ensley 2010). *Aspergillus* ear rot can produce aflatoxins, which affect grain quality and marketability, as well as livestock health if the grain is consumed. *Aspergillus flavus* produces aflatoxins B_1 and B_2, whereas *A. parasiticus* produces all the four important aflatoxins, viz. B_1, B_2, G_1 and G_2 (Diener et al. 1987). Toxicity of aflatoxin varies among animal species, but the young animals are most sensitive to this toxin (Woloshuk and Wise 2011). Furthermore, when lactating animals consume contaminated grain, aflatoxins can even enter animal's milk (Woloshuk and Wise 2011). In most of the countries of the world, aflatoxins are regulated in all the products but they are mostly regulated in maize and milk (European Commission 2006, 2010).

Besides *Aspergillus* ear rot, *Penicillium* ear rot of maize is also common and is usually caused by *Penicillium oxalicum*, although other *Penicillium* species like *P. chrysogenum*, *P. cyclopium* and *P. glaucum* are also involved in the disease complex. *Penicillium* ear rot is characterized by a distinct light blue green powdery mould that grows between kernels and on the ear surface. Infected kernels typically appear bleached and streaked. These *Penicillium* species are also known to produce mycotoxins; for example, *P. verrucosum* produces ochratoxin A (CAST 2003).

(iv) **Rice (*Oryza sativa* L.):** Rice is a versatile food crop, which is a staple food for about half of the world's population. Globally, about 700 million tons of rice are produced every year (FAO 2011). It provides about 20% of world's dietary calories in comparison with wheat (19%) and maize (5%) (Wenefrida et al. 2009). More than 90% of the world's rice is grown and consumed in Asian countries such as India, China, Japan, Indonesia, Thailand, Pakistan, Bangladesh, North and South Koreas, Myanmar, Philippines and Sri Lanka.

In the recent years, rice sheath rot has gained the status of a major disease of rice and yield loss due to this disease may vary from 9.6 to 85% (Sharma et al. 2013). It is a disease complex where a range of fungal and bacterial pathogens may be involved. Some of the important pathogens associated are *Sarocladium oryzae, Fusarium fujikuroi* complex and the bacterial pathogen *Pseudomonas fuscovaginae*. Among these, *Sarocladium oryzae* was the first pathogen to be isolated in 1922 and found to be responsible for rice sheath rot symptoms in Taiwan (Mew and Gonzales 2002). Among the various species of *Fusarium* forming the *F. fujikuroi* complex, *F. fujikuroi*, *F. verticillioides* and *F. proliferatum* have been found to cause symptoms on different plant parts and cause yield losses of 40% in Nepal

(Desjardins et al. 2000) and even up to 60% in Korea (Park et al. 2005). Symptoms of *Fusarium* sheath rot of rice include blank or partially blank panicle with reddish brown to off-white florets or kernels, which usually get covered with a white to pinkish white powder mass of microconidia and conidiophores of *F. proliferatum*. The leaf sheath of diseased plants also develops a lesion enlarging rapidly, which is initially dull to dark brown, later becoming off-white to tan with a reddish brown border that eventually occupies the entire sheath resulting in death of the leaf blade. Lower leaf sheaths may also eventually develop lesions, but rarely more than two leaf sheaths show symptoms. According to Wulff et al. (2010), some strains of *Fusarium fujikuroi* are also able to produce gibberellin A, which result in abnormal elongation of rice plants (bakanae disease).

The main species of *Fusarium fujikuroi* complex (*F. proliferatum*, *F. verticillioides* and *F. fujikuroi*) causing rice sheath rot are known to produce mycotoxins like fumonisin B and moniliformin (Wulff et al. 2010). Among these species, *F. proliferatum* is the largest producer of fumonisins and is commonly associated with rice sheath rot (Abbas et al. 1999; Quazi et al. 2013). In addition, some strains of *F. verticillioides* are also notorious for producing fumonisins (Wulff et al. 2010). Mycotoxins are generally concentrated in the outer part of the rice grain, and their concentration in the grain gets reduced to 75–80% after hulling. One of the major fumonisins, FB1 is considered as a virulence factor in *Fusarium*-induced plant diseases (Glenn et al. 2008). It has been shown that *F. proliferatum* isolates from field samples of rice with *Fusarium* sheath rot disease possess the capability to produce both fumonisins and moniliformin in culture (Bigirimana et al. 2015). The presence of fumonisins (FB_1, FB_2, FB_3) and moniliformin has also been detected in samples of commercial rice from fields exhibiting *Fusarium* sheath rot disease associated with *F. proliferatum* (Abbas et al. 1998; Gupta et al. 1991). Genome sequencing has shown the presence of a wide array of secondary metabolite gene clusters in *Fusarium fujikuroi* and *F. verticillioides*, including clusters for bikaverin, fusarubins, fusarins, fumonisins and fusaric acid (Bigirimana et al. 2015). However, beauvericin and gibberellin gene clusters were detected only in *F. fujikuroi* (Wiemann et al. 2013).

(v) **Sorghum [*Sorghum bicolor* (L.) Moenchi]**: Grain mould is an important field disease of sorghum, occurring usually when moist weather conditions prevail after flowering till maturity of grains. A number of grain mould pathogens have frequently been associated with losses in grain density (Ibrahim et al. 1985), seed mass (Somani and Indira 1999) and germination (Maiti et al. 1985). Grain mould may result in additional damages, which include food and feed processing quality, storage quality and commercial value (Hodges et al. 1999).

Moulded grains are associated with fungi belonging to more than 40 genera, although only a limited number of them infect the sorghum flowering tissues during early stages of grain development. These include *Fusarium moniliforme*, *F. pallidoroseum, Curvularia lunata* and *Phoma sorghina.* Of these, *F. moniliforme* and *C. lunata* are of worldwide significance (Williams and Rao 1981; Frederiksen et al. 1982). Initially, the infection by a grain mould pathogen occurs on the apical region of spikelet tissues and then proceeds slowly towards the base of the spikelet tissue,

either in the spikelet tissues or in the voids between these tissues. The grain itself becomes infected at the base, close to the pedicel and may interfere with grain filling and thus causes premature formation of a black layer (Frederiksen et al. 1982). Both these conditions lead to reduction in grain size. Post-maturity colonization is normally what produces the 'mouldy appearance' of grain maturing in humid conditions, and the colour of the mould usually depends on the fungi involved. Many researchers believe that head blight is distinct from grain mould (Williams and Rao 1981), although there appears to be no visible difference at the pathogen level (Frederiksen et al. 1982). According to Williams and Rao (1981), head blight is an invasion of tissues of the inflorescence by *F.moniliforme*, resulting in damage and killing of the florets up to various degrees and thus leading to complete loss of the head. The symptoms consist of discoloration and necrosis of the panicle, which may extend to the peduncle, along with reddening of pith in the affected regions. Grain mould symptoms can be induced upon inoculation with *Fusarium moniliforme*, but head blight does not necessarily occur, thereby indicating that the predisposing factors may be different for head blight and grain mould (Forbes et al. 1992).

Several fungi involved in grain mould complex possess the ability to produce mycotoxins. According to Salifu (1981), short-season sorghums, which mature during rainy seasons, tend to be more susceptible to contamination by mycotoxins compared to long-season varieties, which mature later after rainy season. A number of chemically and biosynthetically diverse mycotoxins like fumonisins, moniliformin, fusaric acid, fusaproliferin, fusarins and gibberellic acids are produced by various *Fusarium* species belonging to section *Liseola* (Leslie 1999). On the other hand, fumonisins (B1, B2 and B3) constitute a family of mycotoxins produced by *F. moniliforme* and *F. proliferatum* (Bandyopadhyay et al. 2000). Visible grain mould can indicate possible contamination by zearalenone and vomitoxin, but the absence of visible mould growth does not necessarily guarantee grains free from mycotoxin contamination (Bowman and Hagler 1991). Forbes et al. (1992) observed an increase in the concentration of mycotoxin in stored grains contaminated during preharvest stages. These moulds may also infect maize (*Zea mays*) and rice (*Oryza sativa*) apart from sorghum, and thus, the potential for the presence of fumonisins in feed and foodstuffs is high (Bandyopadhyay et al. 2000).

Factors Affecting Head Blight and Mycotoxin Production During FHB Disease

Mycotoxin concentrations in various cereal crops vary from year to year and between different regions depending upon the various factors that influence the occurrence, pattern of infestation and infection by *Fusarium* species. Some of the significant factors are environmental factors such as temperature, moisture, relative humidity; biological factors such as pathogens involved; and source of inoculum and mechanical practices like irrigation and soil tillage.

(a) **Environmental factors**: Temperature, moisture and relative humidity (RH) are the most important factors, which influence the development of *Fusarium* head blight and subsequently accumulation of mycotoxin in small grain cereals. Different species of fungi constituting the pathogenic complex responsible for head blight may differ from year to year and from one region to another, particularly as a function of climate (Parry et al. 1995; Walker et al. 1998). Climate can greatly influence these factors by either causing drought or excessive rainfall (Miller 2008). In fact, climate partly controls competition between different species. Andersen (1948) conducted detailed experiments to determine the effect of temperature and moisture on *Fusarium* head blight development in spring wheat. Later, it was detected that at flowering stage, development of *Microdochium* is favoured by lower temperatures (Pissinger 1982; Parry et al. 1995) and rain fall (Caron 2000), whereas *Fusarium* is favoured by higher temperature (Pissinger 1982) and storms (Caron 2000). Therefore, *F. Graminearum* is generally predominant in warm regions, whereas *F. culmorum* (Caron 1993; Parry et al. 1995; Jorgensen 2000), *F. avenaceum* (Parry et al. 1995) and *Microdochium nivale* (Caron 1993; Parry et al. 1995) are the predominant species of cooler regions.

It has been found that temperature and water activity (a_w) significantly control the growth and interaction between *F. moniliforme* and *F. proliferatum* and between *F. graminearum*, *F. subglutinans*, *F. proliferatum*, *Aspergillus, Penicillium, Eurotium* and *Trichoderma* species (Marin et al. 1998a). While studying the competing abilities of *Fusarium*, *Aspergillus*, *Eurotium*, *Penicillium* and *Trichoderma* species, Marin et al. (1998a) also observed that the species of *Fusarium* were dominant at higher water activity, (a_w 0.995). Earlier, Magan and Lacey (1984) also reported that among a range of field fungi, only *F.culmorum* showed competitive and dominating ability, particularly at $a_w > 0.95$. Among the *Fusarium* species, *F. graminearum* tends to have a competitive advantage over other species under cooler conditions (Marin et al. 1998b; Velluti et al. 2000). On the other hand, Marin et al. (1998b) even suggested that *F. graminearum* has a competitive advantage over *F. proliferatum* and *F. moniliforme* at 15 °C, while at 25–30 °C, these species coexisted in the same niche. Similar observations were made by Velluti et al. (2000) regardless of water activity.

There are a number of reports on the influence of climatic factors, especially humidity and temperature on *Fusarium*-associated toxins. Culler et al. (2007) showed in his field experiments that concentration of DON was lower in wheat grain from the plots that were subjected to extended irrigation (from flowering to harvest) in comparison with grains from the plots that were irrigated from flowering to the beginning of dough stage. In greenhouse experiments, Gautam and Dill-Macky (2012) demonstrated that regardless of the cultivar, DON concentrations were lower in winter wheat with spikes that were subjected to a single course of six hours wetting than those that were not wetted. Moreover, they also detected DON concentrations in runoff water from the wetted plants, confirming that it may leach out from the wheat spikes exposed to rainfall or irrigation water.

(b) **The Pathogens**: The pathogens responsible for head blight also play an important role in infection. There are more than 17 species of *Fusarium* that have been detected from naturally infected barley or wheat spikes (Shaner 2003). Each species is capable of infecting wheat and barley when spikes are inoculated, but with diverse levels of virulence (Mesterhazy 2003). Among these, *F. graminearum* is the most frequently encountered and most virulent species worldwide, but *F. culmorum* and *F. poae* have also been reported to be prevalent in some European countries (Mesterhazy 2003). For natural infection to occur, ascospores released from perithecia of *Gibberella zeae* (perfect state) usually serve as the most important primary inoculum to initiate the disease epidemics (Shaner 2003). *F. graminearum* producing macroconidia are equally infective and commonly responsible for causing disease of wheat and barley (Bai and Shaner 1994). The pathogens responsible for the disease may also vary in cultural characters, toxigenicity, virulence and vegetative compatibility groups (Dill-Macky 2003; Mesterhazy 2003).

(c) **Source of inoculum**: In case of *Fusarium* head blight (FHB) disease, the source of primary inoculum is the fungal colonies overwintering on the crop residues of wheat, maize and rice (Sutton 1982; Parry et al. 1995). Therefore, FHB usually occurs in wheat–wheat, corn–wheat or rice–wheat rotations (Zhu and Fan 1989). The pathogens usually spread via dispersal of conidia that are splashed or blown to new infection sites (Paul et al. 2004; Xu and Nicholson 2009). Wheat is one of the most susceptible crops to the infection during flowering, after which a sharp decline in susceptibility has been observed (Hart et al. 1984). In the later stages, infections may lead to accumulation of DON even in the absence of visible symptoms during the hard dough stage, especially under conditions of prolonged wetness (Del Ponte et al. 2007). *F. graminearum* can survive on a wide range of hosts, which include living plants such as wheat, corn, barley, soybean and rice as well as dead tissues of many plant species (Shaner 2003). Therefore, crop residues on the soil surface serve as important reservoir of pathogens of *Fusarium* head blight (Shaner 2003), whereas ascospores, macroconidia, hyphal fragments, chlamydospores may serve as inoculum (Bai and Shaner 1994; Dill-Macky 2003). Among these, ascospores which are released from the soil surface debris are the principal inoculums that initiate epidemics (Bai and Shaner 1994; Shaner 2003). Therefore, wheat crop grown after corn or wheat has significantly more *Fusarium* head blight in comparison with wheat grown after other crops (Shaner 2003).

(d) **Soil tillage**: It also affects head blight particularly from a qualitative point of view. For example, limited soil tillage usually enhances the frequency of head blight (McMullen et al. 1997; Dill-Macky and Jones 2000), whereas deep tillage (ploughing) decreases it (McMullen et al. 1997; Dill-Macky and Jones 2000; Krebs et al. 2000). It happens because ploughing exerts a number of direct and indirect effects not only on the structure (Guerif et al. 2001) but also on the microclimate of the soil (Guerif et al. 2001; Reicosky 2003), thereby affecting the development of fungi particularly those of the genus *Fusarium* (Guerif et al. 2001). According to Cassini (1973), about 90% of the *Fusarium* population is located in the upper 10 cm of soil and the development of this fungus depends on soil aeration.

Although this pathogen can survive for 4 years at a depth of 20–25 cm (Caron 1993), it is active and able to develop on plant debris only when present in the upper 5 cm of soil (Cassini 1973).

(e) **Irrigation practices**: They influence the microclimate of a field and may favour the development of pathogens involved in the disease syndrome. Regardless of having favourable climatic conditions for the disease in a given year, irrigation always enhances the frequency (% necrotic ears) and severity (% necrotic spikelets) of the disease over that in the non-irrigated areas (Dill-Macky and Jones 2000). When wheat is produced under irrigation, just by minimizing irrigation practices during the period from flowering to 4–5 days later, fungal infection is reduced as conidia and ascospores are dispersed by wind and rain splash (Jacobsen 2014). In Montana, where conditions are usually too dry for *Fusarium* head blight epidemics, farmers growing irrigated spring wheat try to reduce disease incidence by saturating the soil moisture prior to emergence of spike and further avoiding irrigation during spike emergence (Jacobsen 2014). In addition, drought stressed maize crops are considered more susceptible to aflatoxin and fumonisin contamination (Miller 2001; Abbas et al. 2009). Adequate practices of irrigation can minimize drought stress and subsequently reduce aflatoxin concentration in grain (Abbas et al. 2009). Similarly, *Gibberella* ear rot and related mycotoxins are often associated with high moisture levels after anthesis (Sutton 1982; Vigier et al. 1997). According to Stewart et al. (2002), prolonged wet periods promote more silk infection, resulting in higher levels of DON and ZEN contamination in maize. Therefore, in the irrigated maize crops, the amount and timing of irrigation are designed in such a way so as to minimize drought stress while optimizing yield in relation to irrigation costs. Proper irrigation practices to avoid drought stress and optimize yield benefits are recommended as the best practices for avoiding mycotoxin risk (Munkvold 2014).

Control/Management

In view of the huge impact of the incidence of mould infections and the associated mycotoxins on the health of consumers, it is extremely important to continuously prevent/manage their incidence. Preventive measures should be practiced throughout the production period, right from the field (to minimize the incidence of mycotoxins produced by moulds such as *Fusarium* and *Claviceps*) up to the production of final products (to minimize storage moulds such as *Aspergillus* and *Penicillium*). Prevention/management of fungal infections can be achieved at the field level by adopting good agricultural practices (GAP). The key objective of GAP, with respect to diseases, is to minimize the inoculum load to prevent its dispersal. Another aspect of mould prevention and management involves constant monitoring of the crop so that required preventive measures can be exercised on time, particularly during anthesis when crops like wheat and barley are susceptible to the causal agents of *Fusarium* head blight. Some of the relevant good agricultural practices are described below:

(a) **Crop Rotation**: It is an important practice that targets breaking the chain of production of infectious inoculum. For instance, by using wheat/legume rotations, management of FHB can be achieved (Mejia-Teniente et al. 2011). However, in case of FHB disease, maize crop should not be preferred for rotation as it serves as a susceptible host to the disease, and its crop residues are an excellent substrate for sporulation, which carry over the infection to wheat via stubble/crop residues (Nicholson et al. 2003). Higher risk of FHB and subsequent DON contamination has been found in wheat crop that is grown following an alternative host for the pathogen (Kabak et al. 2006). However, there is conflicting evidence that cultivation of wheat following wheat is more at risk than wheat following a non-cereal crop (Edwards 2004). Dill-Macky and Jones (2000) observed that severity of FHB disease and DON contamination was considerably different when the previous crop was maize, wheat or soya bean, with the highest levels observed in crops grown following maize and the lowest levels in crops following soya bean. It has been found that crops which serve as hosts for various *Fusarium* species (such as, grasses, maize,wheat and other cereals) if altered with those of the non-hosts (such as, potatoes, beans, onions), a considerable reduction in the risk of FHB and subsequent toxin production is achieved (Eeckhout et al. 2013). This process also facilitates the interruption of proliferating inoculum and thereby reducing its chance of survival. Crop rotation is, therefore, helpful in controlling inoculum load and minimizing the chances of infection to the host crops.

(b) **Crop Planning**: Various climatic conditions have been known to favour the development of moulds in the crop. For instance, drought stress and climatic conditions that extend the period of ripening in the field are two important environmental risk factors for *Fusarium* infection in the field (Eeckhout et al. 2013). Hence, crop planting should be carefully planned to avoid drought stress and high temperatures during seed development and maturation (Eeckhout et al. 2013). Avoidance of wet conditions prior to flowering and during harvesting should be adopted to reduce the risk of mycotoxin contamination (Eeckhout et al. 2013). Harvesting practices should be done earlier for crops which are already infected with *Fusarium* as otherwise it may lead to a higher accumulation of mycotoxin content in the infected grains (Eeckhout et al. 2013).

In addition, adoption of recommended spacing between crops/plants to prevent overcrowding can also prevent the extent of disease. Less spacing between crops/plants or a high canopy density can lead to extended periods of humid conditions during or after precipitation, which may in turn facilitate sporulation of the pathogen (Eeckhout et al. 2013).

(c) **Soil and Crop Management**: A good cultivation practice involves eradication, destruction or burial of infested crop residues. Care should be taken to ensure clean seed beds for each crop that can be achieved by right ploughing practices and by eradicating previous crop residues, which otherwise may serve as potential substrates for the possible mycotoxin-producing pathogens (Eeckhout et al. 2013). It is also very important to minimize possible plant stress that can be due to many factors, such as drought, nutrient deficiencies, cold and adverse reactions of various resources applied to the crop. For example, drought stress could be overcome by

proper irrigation practices while plant stress by providing sufficient nutrients and favourable pH that can be achieved by providing appropriate fertilizers (Eeckhout et al. 2013). Rational use of fertilizers and plant growth regulators prevents excessive plant growth and crop lodging. It has been observed that lodged grains are susceptible to increased *Fusarium* infection (Eeckhout et al. 2013).

(d) **Chemical control**: Control by means of fungicide remains an important alternative to reduce both mycotoxin accumulation and disease incidence associated with *Fusarium* species. A large number of fungicides applied as seed treatments have proved to be effective against seedling blight and reduce fungal growth considerably to allow successful establishment of the plant (Gilbert and Tekauz 1995). Foliar fungicides have also proved to be promising in controlling *Fusarium* head blight (Parry et al. 1995; McMullen et al. 1997). It has been observed that under severe conditions, even tolerant varieties require fungicide application to achieve satisfactory control (Burrows 2012). In case, the field has a history characterized by scab and sufficient cereal crop residues, then a resistant variety along with application of fungicide will decrease the risk of upcoming infection (Burrows 2012). In addition, avoiding irrigation during anthesis also reduces the incubation period (Burrows 2012). The best known fungicides used to control *Fusarium* head blight include benomyl, MBC, metconazole and prothioconazole, tebuconazole and a combination of tebuconazole and prothioconazole (Paul et al. 2007; McMullen et al. 2012). Application of fungicides well on time plays a crucial role in controlling *Fusarium* infection which should be based on weather conditions and crop development (Eeckhout et al. 2013). As *Fusarium* infection usually occurs under humid conditions during anthesis, therefore, monitoring of crop is essential along with its treatment against *Fusarium* infection during such conditions. The effectiveness of fungicide application may be enhanced by using the right type of nozzle (such as, double fan nozzles), a correct spray volume and the use of an appropriate adjuvant (Eeckhout et al. 2013).

Similarly, crops damaged by insects, rodents and birds become more susceptible to *Fusarium* infections, whereas wounds caused by insects can be reduced by using possible preventive measures, which involve use of suitable insecticides and fungicides that can control insect damage and subsequent mould infection. The use of pesticides such as alpha-cypermethrin R (Fastac), cyfluthrin R (Baythroid 2), endosulfan R, gamma-cyhalothrin R (Proaxis) and others should be in accordance with an integrated pest management programme (Eeckhout et al. 2013). In addition, it is also necessary to have a check on the weeds, particularly grasses, which may serve as collateral host for various species of *Fusarium.* In such cases, mechanical methods or herbicides such as 2,4-D or MCP, bromoxynil, thifensulfuron methyl and glyphosate can be used (Eeckhout et al. 2013).

(e) **Biological control**: In addition to chemical control, there is an increasing interest in the use of biological agents for the management of *Fusariam* head blight. Sustainable biological control of plant pathogens in an environment depends on efficient exploitation of naturally occurring microorganisms. In a balanced ecosystem, interactions between potential plant pathogens and their antagonists may prevent the initiation of infection of the host plant. Much work has been done

to develop biocontrol agents as alternatives to fungicides (Boland and Kuykendall 1998). Several biological control agents have shown promising results for reducing *Fusarium* head blight and DON accumulation in grains (Schisler et al. 2002; Palazzini et al. 2007; Xue et al. 2009). However, only few biocontrol agents have been developed commercially (Whipps and Davies 2000). But, they are often inconsistent and generally less effective than fungicides in the field.

Among the fungal biocontrol agents, various species of *Trichoderma* are known to have antagonist potential against several plant pathogens, including *Fusarium* species. For instance, *T. harzianum* has been found to be successful in colonizing wheat straw and decreasing incidence of *F. graminearum* (Fernandez 1992). These biological control agents can be effectively employed for an integrated pest management programme, although more research is required in this direction (Fernandez 1992). Bujold et al. (1999) observed that perithecia of *Gibberella zeae* show better growth on corn kernels than on wheat straw, but concluded that both wheat and corn debris serve as a suitable substrate for the evaluation of biological control agents. Although there is little information on the use of biocontrol agents against FHB, few reports have demonstrated the potential of biocontrol agents for controlling the FHB pathogens. For example, the effect of some antagonists on the development of perithecia and ascospores of *Gibberella zeae* has been observed (Bujold et al. 2001). Similarly, in vitro studies on wheat and maize residues showed that inoculating residues with a *Microsphaerosis* species significantly reduced *Gibberella zeae* ascospore production by at least 73% (Bujold et al. 2001). In the field, *Microsphaerosis* species when applied to crop residues resulted in a significant decrease in the production of perithecia, however, no visible effect was observed on the pattern of perithecia formation (Pirgozliev et al. 2003). Similarly, it has been observed that under glasshouse conditions, wheat ears when inoculated with *Phoma betae* at the time of flowering resulted in a reduction in the symptoms caused by FHB pathogen such as *F. culmorum* by around 60% (Diamond and Cooke 2003). A significant increase in the latent period of *Microdochium nivale* was observed when wheat ears were inoculated with *Pythium ultimum* or cell-free germination fluids taken from a number of pathogens involved in FHB (Pirgozliev et al. 2003).

Some bacteria like *Bacillus*, *Pseudomonas* and *Paenibacillus* have also been investigated as biocontrol agents. These microorganisms exhibit their effects by competing for nutrients and by producing antifungal antibiotics, which reduce disease severity and DON accumulation (Jacobsen 2014). Application of the bacterial strain AS 43.4 (*Bacillus* spp.) isolated from anthers of wheat has been shown to decrease disease severity of FHB under glasshouse conditions by 67–95% and mycotoxin concentration in grain by 89–97% (Khan et al. 1998). In another similar glasshouse investigation, three bacterial strains (*Bacillus* strains 43.3 and 43.4; *Cryptococcus* strain OH 182.9) out of the tested seven FHB antagonists reduced disease severity by 48–95% and decreased mycotoxin quantity in grain by 83–98% (Schisler et al. 2002). However, under field conditions, the same antagonistic strains show varied results and the studied *Bacillus* strains had no effect on either FHB severity or mycotoxin concentration in grain (Pirgozliev et al. 2003). Field studies

undertaken by McMullen et al. (2002) demonstrated significance of fungicide in controlling FHB, but *Cryptococcus* strain OH 182.9 showed no effect on disease development. However, *Clonostachys rosea* strain, a mycoparasite, has been found to reduce the production of perithecia on wheat residues as well as infection, disease severity and DON accumulation upon being sprayed on wheat spikelets (Xue et al. 2009).

(f) **Cultivar resistance**: It is an important feature of integrated control strategy against *Fusarium* head blight in all the environments. According to Hooker and Schaafsma (2005), the choice of resistant varieties is one of the most vital pre-planting strategies that affects mycotoxin risk and is the most cost-effective method available. Therefore, the most reliable and consistent strategy for controlling *Fusarium* head blight and subsequent mycotoxin production involves the use of cultivars with good resistance. Large quantitative variation for FHB resistance in wheat has been described (Buerstmayr et al. 1996). The significance of resistant wheat cultivars to FHB has been recognized since the beginning of twentieth century (Dickson and Mains 1929). Molecular mapping associated with FHB resistance, such as that carried out by Buerstmayr et al. (2002) for spring wheat, will prove helpful in marker-assisted selection and thereby will lead to a significant improvement in the development of cultivars with good resistance to *Fusarium* head blight and hence mycotoxin contamination. Similarly, a wide range of susceptibility to mycotoxin-producing fungi among commercial maize hybrids is available (Lauren et al. 2007; Parsons and Munkvold 2010).

(g) **Seed quality**: Other practices that reduce field infections and mycotoxin formation include the use of certified seeds that are treated with a fungicide treatment. The primary contribution of seed treatment is to lower seedling diseases, which can lower risks of mycotoxin contamination (Munkvold 2014). For example, if grains infected by the causal organisms of *Fusarium* head blight are sown, it may result in seedling blight (Winson et al. 2001). Similarly, drilling of *Fusarium*-infected cereal grain has been associated not only with the subsequent development of seedling blight but also with foot rot, as well as reduction in grain yield and ear number (Wong et al. 1992; Humphreys et al. 1995). However, there is evidence in wheat (Moretti 2008; Poels et al. 2008) and in maize (Causin et al. 2008) suggesting that some seed fungicides may force seed infection and contamination by DON or fumonisins by suppressing transmission of *Fusarium* species via seed. Although seed infection might play a less important role in disease transmission than survival on residues, affected seed lots should be thoroughly cleaned to eliminate shrivelled grains. This cleaning also reduces the risk of seedling blight at emergence and improves crop establishment (Duveiller et al. 2014).

Endophytic fungi and camouflaged toxins in forages

The symbiotic relationship between certain grasses and endophytic fungi ranges from mutualistic to antagonistic (Schardl et al. 2004). The grasses when infected with endophytic fungi receive important benefits such as anti-herbivory, whereas

the plant also provides the fungi with shelter, a source of food and dissemination via host propagules.

Perennial ryegrass staggers and fescue toxicosis are the common toxic forage diseases of livestock in USA, which results from the ingestion of forage having with endophyte-produced mycotoxins (Duringer et al. 2012). Among these two diseases, perennial ryegrass staggers were described as early as 1906 by Gilruth as a neurological disorder in farm animals in which outbreaks were associated with consumption of perennial ryegrass (*Lolium perenne*), which is a valuable pasture grass in temperate regions of the world, including the north-western USA, Australia and New Zealand. It was later postulated by Neill (1940) that an endophytic fungus may be involved. However, investigations by Cunningham and Hartley (1959) confirmed that the disease was associated with the ingestion of a toxin present in the grass. A similar syndrome of intoxication of sheep in Australia was reported by Everest (1974). But, it was not wholly recognized until 1981, when during a study, outbreak of ryegrass staggers occurred in a trial of ryegrass genotypes at Lincoln and the disease was found to be associated with the presence of *Lolium* endophyte (Fletcher and Harvey 1981). This finding was later confirmed at Ruakura in 1982 (Mortimer and di Menna 1983). The fungus was identified as *Acremonium* (now *Neotyphodium) loli* (Latch et al. 1984), and the main toxin involved was identified to be the alkaloid lolitrem B (Gallagher et al. 1982). Later, similar related conditions were reported, such as the ingestion of *Achnatherium inebrians* (drunken horse grass) by horses in Asia caused the syndrome known as drunken horse disease, wherein elevated levels of ergonovine and lysergic acid amide along with an endophyte similar to *Neotyphodium lolii* were found associated with the grass (Miles et al. 1996). Another grass (*Echinopogon ovatus*) associated with similar kind of disease was shown to be infected with a *Neotyphodium* endophyte similar to that of ryegrass and fescue (Miles and di Menna 1995). On the other hand, staggers syndrome has also been reported in sheep grazing on the grass *Melica decumbens* in South Africa, wherein indole-diterpenoid alkaloids other than lolitrem B have been found in the endophyte-infected grass (Miles et al. 1995a). Later, in Argentina, Miles et al. (1995b) found the grass *Poa huecu* associated with an endophyte and staggers syndrome, wherein a non-lolitrem B tremorgen and toxic glycoprotein were detected. In view of these toxic responses in grazing animals, research efforts have been concentrated at recognizing disorders associated with the presence of endophytes, the causal agents responsible, susceptibility of different groups of livestock to endophytic disorders and management practices to reduce their effects.

Endophytic fungus, *Neotyphodium lolii*, which has been found on a range of grasses, produces toxins that vary in their type and action and can be considered in two main groups, viz. the vasoactive ergot alkaloids and the indole-diterpenoid tremorgens. Structure-activity studies have shown that at least five of the indole diterpenoids produced by *N. lolii* are tremorgenic (Munday-Finch 1997). Of these, lolitrem B is the most significant and abundant in endophyte-infected ryegrass and is thought to be the main tremorgen responsible for 'ryegrass staggers' (Fig. 28.2). However, the neurotoxic effects are completely reversible (Gallagher and Hawkes 1986), and on the cessation of the feeding of endophyte-infected perennial ryegrass,

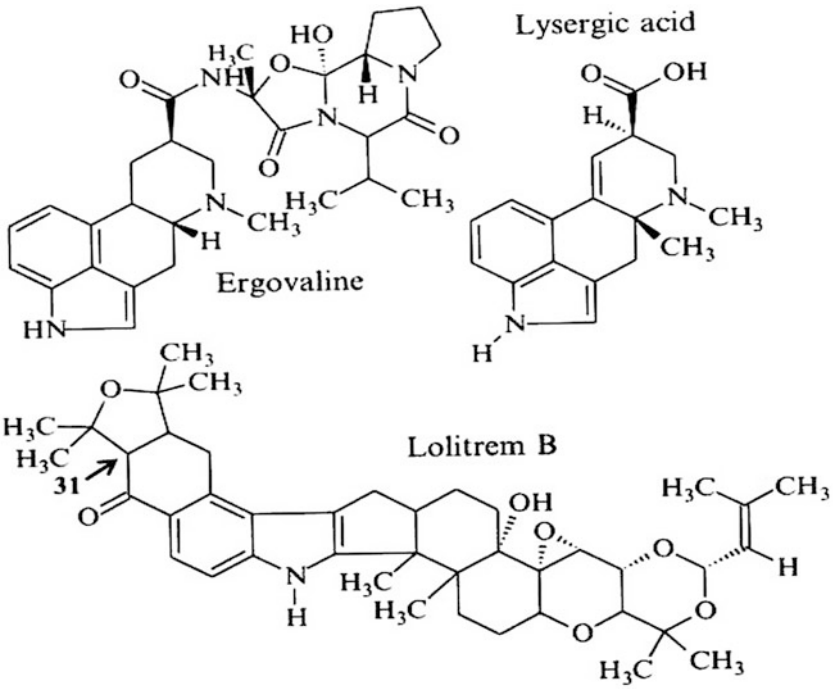

Fig. 28.2 Ergot and lolitrem alkaloids found in endophyte-infected tall fescue and perennial ryegrass

the animals showed improvement within 2–4 days, and in most cases, they show return to normal form (Smith 2002).

The spread of *Neotyphodium lolii* in ryegrass is vertical, with infection spread mainly by seed. After germination, the hyphae spread within the growing plant, with the highest concentration of hyphal elements found in the lower tillers and sheath parts (Smith 2002). Later, the embryonic seed may also become infected. Therefore, highest concentration of lolitrem B is found in the base of the plant and in the seed (Smith 2002). If seed is stored for a long time, the viability of the endophyte declines and the rate of decline are fastest in hot and humid conditions (Smith 2002). The concentration of lolitrem B may vary in response to various climatic conditions (Oldenburg 1997), plant tissues (Ball et al. 1997; Keogh et al. 1996), nitrogen content of the soil (Lane et al. 1997) and solar radiation (Fletcher et al. 2001). Keogh et al. (1996) stated that highest endophyte and toxins have been reported in the lower plant parts, in the older foliages, in the senescent potions and in the seeds of the ear.

Control of ryegrass staggers (RGS) outbreaks can be achieved by either grazing management alone or an amalgamation of grazing management with other precautions such as feeding supplements (Keogh 1983). Since the neurotoxins responsible for the development of ryegrass staggers are acquired usually from the base of pastures and are primarily associated with ryegrass leaf sheath, control over

the consumption of these portions by animals is a prerequisite for any control of RGS by grazing management. However, this type of management is required only when the early signs of RGS are first observed, thereby suggesting that pastures may prove toxic, and suitable precautions should be taken until the danger period has passed (Keogh 1983).

The second important common toxic forage disease is the tall fescue toxicosis (fescue foot and summer syndrome), which is affecting livestock in USA and results from the ingestion of forage contaminated with mycotoxins ergovaline and lysergic acid that are produced by endophytes (Fig. 28.2). Tall fescue (*Festuca arundinacea*) represents the extremely grown pasture grass in humid regions of the south-eastern and to a lesser extent in the north-western USA, with greater than 140,000 km^2 in production (Belesky and Bacon 2009). In USA, endophytic fungus, *Neotyphoidium coenophialum* of tall fescue *(Festuca arundinacea)* responsible for causing similar disorders, is extensively researched (Bacon and White 1994). In cold regions, where tall fescue hay is fed as a major portion of the diet, 'fescue foot' characteristically occurs which results from the vasoconstrictive action of ergot alkaloids on blood vessels (Klotz et al. 2008). These alkaloids cause reduced blood flow to the extremities, such as hooves, tail and ears, making them gangrenous, ultimately resulting in euthanasia of the animal. 'Summer syndrome' is common in hot, humid summer months when animals lose their ability to cool themselves due to consumption of ergot alkaloids with vasoconstrictive effects. As a result, in order to cool itself, the affected animal attempts to settle more in shade and rely more on water than grazing. Therefore, a decreased average daily gain, intolerance to high temperature, uncontrolled salivation, roughened hair coat, lower milk production and reduced conception rate are the common clinical indications of 'Summer syndrome' (Hoveland et al. 1983).

Future Perspectives

Due to increasing concerns regarding food safety, contamination of cereal grains with *Fusarium* and other fungal species is topic of concern. *Fusarium* head blight, the main disease caused by *Fusarium* species on wheat, barley and sorghum is caused by members of a diverse species complex. There are several key areas in the epidemiology of mycotoxigenic fungi that need to be investigated thoroughly before we can develop an effective strategy to minimize the risk of diseases as well as mycotoxin contamination. One of the key issues is the inter-relationship between disease incidence severity, fungal biomass and concentration of associated mycotoxins. Such knowledge will enable us not only to forecast disease development, but also the potential production of mycotoxins during the production phase in relation to various disease management methods and environmental conditions. This information together with knowledge on the production of mycotoxins during the postharvest stage can be a powerful tool to manage the health risk posed by mycotoxins in the food chain. For instance, the mycotoxin contamination profile

and related toxicological risk can vary dramatically with a range of *Fusarium* species present. Therefore, strategies to control the disease must successfully counter a range of *Fusarium* species, each with unique ecological, epidemiological and population characters. Hence, the combination of accuracy, reliability and rapidity in the diagnosis of *Fusarium* species is a major challenge for the scientists of this field. Biological control currently does not provide the level of control provided by fungicides but will be most effective when used in programmes that include resistant varieties, cultural controls and fungicides. An advantage of biological control is that it can be used post-anthesis, when fungicide applications are not allowed due to residue problems.

In addition, the ability of transgenic crops to express antifungal proteins, genes involved in plant defence responses and reduced production, accumulation or detoxification of *Fusarium* mycotoxins is significant (Makandar et al. 2006; Woriedh et al. 2011; Karlovsky 2011; Han et al. 2012). Similar transgenic approaches may be helpful in reducing fungal contamination and their mycotoxins. However, at present these transgenic technologies are not available commercially.

Conclusions

One of the serious problems faced by the world is the incidence of camouflaged mycotoxins in the food chain that are unavoidable. Therefore, an integrated strategy is required for the management of mycotoxin contamination and its associated risks so as to prevent the crop from infection that begins right from the field through harvesting, handling and storage processes. Although there are few strategies to prevent mycotoxin formation in the postharvest phase and to minimize its impact if already formed in the grain, yet an effective integrated strategy begins at preharvest level. Accordingly, a number of possible ways have been suggested for the management of disease and subsequent mycotoxin contamination at preharvest level which involves control by biological agents, physical and chemical treatments and use of resistant varieties. A very important strategy for management of mycotoxin contamination is to instruct farmers, food producers and handlers to adopt process-based guidelines, which include good agricultural practices (GAPs) at preharvest level and good manufacturing practices (GMPs) at postharvest level. As consumers are showing growing interest in food safety, therefore, the farmers and the food and feed industries must be alerted to possible mycotoxicological risks. Such strategies would reduce the risk throughout the production, handling and processing chain and can complement product standards. Also, apart from adopting good sanitary measures, general of awareness about the toxic effects of mycotoxin poisoning is also mandatory. Some effective and cheap methods of mycotoxins detoxification should be developed. International cooperation through authorized organizations should be promoted and supported, aiming the benefits for the economics and health of people of all the nations.

In the last twenty years, many studies have been carried out to investigate the biological implications of the mycotoxins contamination in human and animal foods. The teratogenicity, carcinogenicity and general toxicity of these molecules constitute a risk to animal and human health, which at present is not easy to evaluate. The development of physical, chemical and biotechnological methods to improve seed production, cultivation, harvest and storage of cereals and forages is necessary to lessen the extent of mycotoxin contamination of foods and feeds. However, it is impossible to completely eliminate moulds and their associated toxins. Wide gaps still exist on the toxicological effects of mycotoxin-contaminated feeds on various feeding animals. More research in this field is necessary as there is every possibility that the toxins will eventually enter the human food chain. Further, research needs to be focussed on the generation of data dealing with epidemiological and toxicity effects, particularly in humans. There is also a need of strict implementation of quarantine rules with respect to mycotoxin contamination at international level. In addition, more emphasis should be laid towards the development of low-cost mycotoxin detection instruments, which are portable, reliable and easy to handle at field levels. Further, development of genetically modified crops having resistance against fungal pathogens may also prove beneficial. In addition, development of novel protocols and strategies for comparing the costs and benefits of different controlling agents against fungal pathogens and mycotoxin production may also prove helpful in maintaining the economic stability of a commodity or an agricultural area.

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