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Research Paper

GREEN CAPSULE CULTURE OF MEDICINAL ORCHID *Aerides multiflora* ROXB.

Tapash Kumar Bhowmik and Md. Mahbubur Rahman

Department of Botany,
University of Chittagong,
Chittagong-4331,
Bangladesh.

Abstract

In vitro propagation of *Aerides multiflora* was attempted using 0.8% (w/v) agar solidified KC; MS; PM and MVW media with three different sources of carbohydrates *viz.* sucrose, glucose and lactose. MS basal medium proved to be best germination and PLBs formation followed by PM basal medium. The PLBs were subcultured onto MS and PM agar solidified & liquid media supplemented with various concentrations and combinations of BAP, NAA, IAA and Picloram. Best shoot growth was recorded on liquid MS medium supplemented with 1.0 mg/l Pic and 1.0 mg/l BAP (2.63 ± 0.13 cm) followed by agar solidified MS + 1.0 mg/l NAA + 1.0 mg/l BAP (2.59 ± 0.11 cm). Elongation of shoot bud was better in liquid media than agar solidified condition and MS was found better than PM medium. Seed derived seedlings were best responses as increase in length as well as the number of roots developed on agar solidified MS with 1.0 mg/l IBA (4.17 ± 0.23 cm/shoot bud and 2.93 ± 0.16 no/shoot bud) followed by MS with 0.5 mg/l IAA supplemented medium (3.73 ± 0.21 cm/shoot bud and 2.86 ± 0.17 no/shoot bud). The acclimatized seedlings were transferred to pots and watered regularly.

Key words: Acclimatization; *Aeredies multiflora*; germination; PGRs; PLBs.

INTRODUCTION

The orchidaceae is one of the highly specialised and largest families of flowering plants, comprising 25,000-35,000 species to which more and more new ones are being added every year [1]. They are well known for their strange shaped, longevity and beautiful looking flowers. They are cosmopolitan but primarily distributed in tropical areas and rarely distributed in arctic regions. Due to their ornamental and medicinal importance they demand a very high price in the international market. In Bangladesh; Chittagong,

Chittagong Hill Tracts, Cox's Bazar, greater Sylhet, Gazipur and Sundarbans mangrove forest are rich in orchid flora [2].

Beautiful fragrant foxtail orchid *Aerides multiflora* Roxb. has a great scent and comes from Assam, Bangladesh, eastern Himalayas, India, Nepal, western Himalayas, Andaman Islands, Myanmar, Thailand, Laos, Cambodia and Vietnam in semi-deciduous and deciduous dry lowland, tropical and subtropical forests at elevations of sea level to 1100 meters. Monopodial orchid *A. multiflora* blooms with colourful lip in May to June [3]. In India, *A. multiflora* is used to treat wounds [4]. In Nepal, leaf paste is also applied to cuts and wounds [5], whereas powdered leaf constitutes a tonic [6]. The tubers exhibit an anti-bacterial activity against *Klebsiella pneumonia* and *Salmonella aureus* [7-8]. Due to over exploitation and commercial demand of this orchid species required *ex situ* and *in situ* conservation [3]. Therefore conservation of this orchid is now a matter of universal concern. Tissue culture technique has been widely used for the *in vitro* mass propagation of several commercially important orchids [9-10].

Orchid seeds are produced in large numbers and they require a fungal stimulus for germination in nature. In 1922, Knudson first demonstrated the possibility of by passing fungal requirement of orchid seeds during germination *in vitro*. The technique of asymbiotic seed germination has added new dimensions to orchid propagation [11]. Nutrient composition, concentrations and different culture condition of the media have been significant role for efficient seed germination in orchid tissue culture. However, very little work has been done of this species. This paper deals with green capsule culture to develop effective *in vitro* asymbiotic germination and seedlings development protocol for the conservation of *A. multiflora* orchid.

MATERIALS AND METHODS

Green capsules of *Aerides multiflora* Roxb. were collected from Shunamgonj, Bangladesh and washed thoroughly with distilled water using savlon as a wetting agent. The capsules were then surface sterilized with 0.1% $HgCl_2$ for 10 minutes followed by 70% ethanol for 30 second then rinsed thrice with sterile distilled water. They were then split longitudinally using a sharp sterile blade under laminar air flow cabinet. Then the seeds scooped out and were inoculated onto 0.8% (w/v) agar solidified four basal media namely, KC [12]; MS [13]; [14] and MVW [15] with three

different sources of carbohydrates *viz.* sucrose, glucose and lactose. Plant Growth Regulators (PGRs) *viz.* BAP, Kn, Pic, NAA, IAA and IBA were freshly prepared. 100 ml of the media were dispensed into 250 ml culture bottles and autoclaved at 121^0C for 20 minutes at 15 lbs pressure. p^H of the medium was set at 5.8 using 0.1N NaOH or HCl prior to gelling with agar. The experiment was conducted under aseptic condition and the cultures, incubated at 25 ± 2 ^{o}C were subjected to 14h photoperiod at 4000-5000 lux intensity and 60% humidity level were maintained regularly [10]. For subsequent development of the seedlings, they were subcultured on respective media at different intervals.

Full strength MS and PM based solid & liquid eighteen types of elongation media were prepared using with different concentrations and combinations of PGRs. 0.8% (w/v) agar was also used in solid media but in liquid media no agar was added. For *in vitro* rooting of *A. multiflora*, half strength MS0 with 1.5% (w/v) sucrose and nine different types of 0.8% (w/v) agar solidified MS medium supplemented with 3% (w/v) sucrose with three auxins *viz*. IAA, IBA and NAA were used for induction of strong and stout root system.

The well developed seedlings were taken out of the culture vessels and successfully transferred to outside the culture room following successive phases of acclimatization. The seedlings were transferred to plastic pots containing a potting mixture of sterilized small brick, coal pieces, saw dust and peat moss at a ratio of 1 : 1 : 1 : 0.5 and kept in the green house (at 25-30 ^{0}C and RH 60-70%). Transplanted seedlings were watered regularly for about 2-3 months where the seedlings established and grew well in the Orchidarium.

RESULTS AND DISCUSSIONS

The results of seed culture in KC, MS, PM and MVW media supplemented with different carbohydrate source *viz*. glucose, lactose and sucrose are presented in Table-1. The germination frequency and days required for germination varied in all of the media used. 0.8% agar solidified MS medium with 3% (w/v) sucrose proved best for germination (Fig.1a) followed by PM medium with 2% (w/v) sucrose (Fig.1b). Such species medium specificity for germination of orchid seeds has been noted in *Cymbidium aloifolium* and *Spathoglottis plicata* [16], *Arundina graminifolia* [17], *Phalaenopsis amboinensis* [18] and *Dendrobium officinale* [19]. MS media is enriched

with macronutrients, micronutrients and vitamins which are enhanced for seed germination and seedling development of many orchids [20, 21]. KC medium proved better than Nitsch medium in supporting an early and higher frequency (76.66%) of Protocorm Like Bodies (PLBs) formation [11]. The seeds of *Rhynchostylis retusa* germinated well to form good seedlings in modified Knudson C medium [22]. In MS medium, PLBs are deep green in colour whereas, in PM medium are produce whitish yellow PLBs. The lowest response was found in MVW medium containing 2% (w/v) glucose. Carbon source has also great role for *in vitro* orchid seed germination. Sugar is an important component used in tissue culture research. Generally sucrose is used in the medium but in some cases other carbohydrates such as lactose, glucose, maltose, fructose, dextrose, galactose, cellulose, mannose have also been used [23-24]. Among three carbon sources, the percentage of seed germination was higher in sucrose containing medium than other lactose and glucose.

The germinated seedlings underwent elongation when subcultured on the same germination medium but the growth rate was very slow. For rapid elongation, the tiny seedlings were transferred to eighteen different kinds of solid & liquid MS and PM media with various combinations, concentrations of PGRs (BAP, IAA, NAA, and Picloram). In liquid media no agar was added. The efficiency of a medium in terms of enhancing shoot elongation was determined based on the increase in length of shoot system within 30d of culture. Different PGRs combinations and culture condition were found to be better for elongation of seed originated tiny plantlets (Table-2). The highest rate of elongation was achieved on liquid MS medium fortified with 1.0 mg/l Pic + 1.0 mg/l BAP (2.63 ± 0.13 cm; Fig. 1c) followed by agar solidified MS medium with 1.0 mg/l NAA + 1.0 mg/l BAP (2.59 ± 0.11 cm; Fig. 1d). It is established that elongation of seed originated tiny seedlings was better in liquid media than agar solidified condition. Further MS was superior to PM for elongation of shoot bud [17, 25-29].

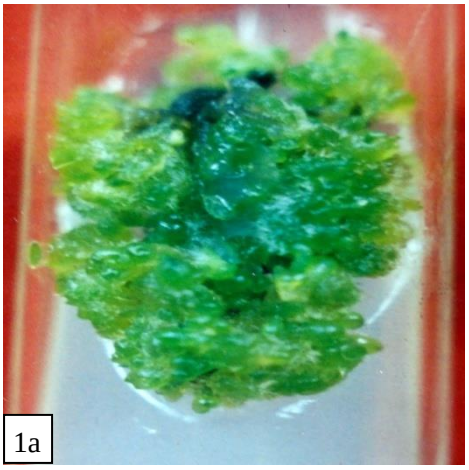
The elongated seed originated seedlings produced roots in elongation media but those were weak and few in number. Half strength MS0 and nine different types of PGR (IAA, IBA, NAA) supplemented MS media were used for induction of strong and stout root system (Table-3). The efficiency of the rooting media was evaluated based on the increase in length and number of roots developed per seedling within 30d of culture in rooting media. The increase in length as well as the number of roots developed seed

originated seedlings were more on MS medium supplemented with 3% (w/v) sucrose and 1.0 mg/l IBA Fig. 1e) followed by that on MS medium + 3% (w/v) sucrose + 0.5 mg/l IAA. Similar result was found in *Acampe praemorsa*, *Cymbidium iridioides* respectively [30-31]. IAA was effective for rooting in *Ipsea malabarica* and *Dendrobium thyrsiflorum* respectively [32-33]. The opposite result was also noted that NAA was most appropriate in inducing roots in *Cymbidium* [34]. The enrichment of medium with higher concentration of IBA or NAA along with low cytokinins content induced excellent rooting response in *Dendrobium chrysotoxum* [35]. Here also mentioned that, low concentration of auxin is more suitable than high concentration for induction of well developed root system.

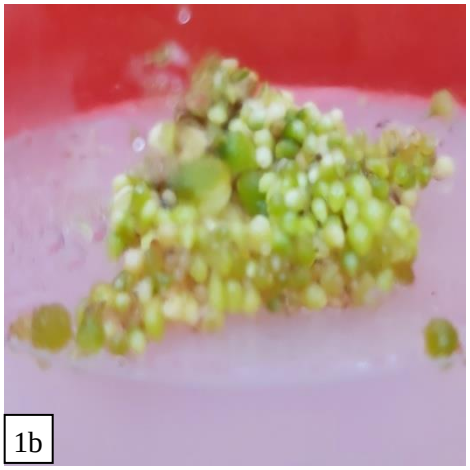
The well developed rooted plantlets were transferred from culture room to the outside environment through successive phase of acclimatization (Fig. 1f). For this purpose, the culture vessels were kept open for one day in the culture room and then kept outside of the culture room for 6h in the next day. On the third day those were kept outside of the culture room for 12h. Finally the seedlings were taken out of the culture vessels and rinsed with running tap water for removal of agar attached to the roots. Then the seedlings were transferred to plastic pots containing a potting mixture of sterilized small brick, coal pieces, saw dust and peat moss at a ratio of 1 : 1 : 1 : 0.5 and kept in the green house (at 25-30 ^{0}C and RH 60-70%). Transplanted seedlings were watered regularly for about 2-3 months where the seedlings established and grew well.

CONCLUSIONS

MS was found superior than PM, KC & MVW media and sucrose was better than glucose & lactose as a source in media for promoting germination of orchid seeds. For comparing the effectiveness in terms of enhancing seedling, liquid condition was better than solidify. Increased in root length and number of roots is higher in IBA supplemented media. However, the technique offers tremendous potential in asymbiotic seed germination, seedlings development and germplasm conservation.

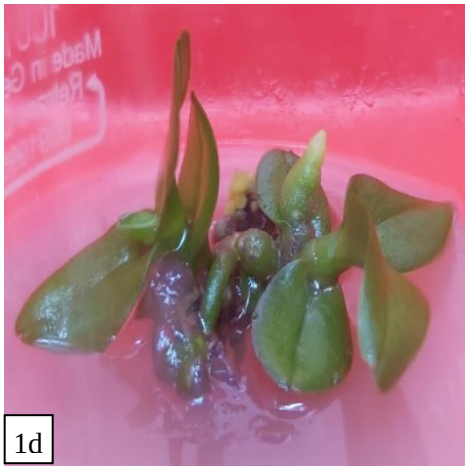


Germination of seeds on agar solidified MS medium



Germination of seeds on agar solidified PM medium

Elongated seedlings on liquid MS + 1.0 mg/l Pic. + 1.0 mg/l BAP



Elongated seedlings on agar solidified MS + 1.0 mg/l NAA + 1.0 mg/l BAP

Strong and stout root system on agar solidified MS + 1.0 mg/l IBA

In vitro developed plantlets growing in pot outside of the culture room

1. (1a-1f): *In vitro* germination, elongation, rooting and hardening of *Aerides multiflora* Roxb.

Table 1: *In vitro* germination of seeds of *Aerides multiflora* Roxb..

Nutrient medium	Carbohydrate source with concentration (w/v)	Number of culture vessels used	Number of culture vessels in which seeds germinated		Time (d) required for germination	Remarks
			No.	%		
	2% glucose	10	04	40	48 - 50	Green PLBs
KC	2% lactose	10	03	30	38 - 40	Yellowish green PLBs
	2% sucrose	10	05	50	34 - 38	Light green PLBs
	3% glucose	10	05	50	35 - 38	Yellowish green PLBs
MS	3% lactose	10	05	50	33 - 36	Green PLBs
	3% sucrose	**10**	**09**	**90**	**28 - 32**	**Deep green PLBs**
	2% glucose	10	04	40	40 - 44	Yellowish green PLBs
PM	2% lactose	10	04	40	38 - 45	Yellowish PLBs
	2% sucrose	**10**	**07**	**70**	**32 - 36**	**Whitish Yellow PLBs**
	2% glucose	10	02	20	50 - 52	Light Greenish PLBs
MVW	2% lactose	10	07	70	28 - 35	Brownish green PLBs
	2% sucrose	10	05	50	34 - 36	Yellowish green PLBs

Table - 2 : Elongation of *in vitro* germinated seedlings of *A. multiflora* on 0.8% (w/v) agar solidified and liquid media with different kinds of PGRs.

Culture medium with different combinations and concentrations of PGRs	Average initial length (cm) of *in vitro* germinated seedlings	Average length (cm) of germinated seedlings after 30d of culture on elongation medium	Increase in length (cm) of germinated seedlings within 30d of culture on elongation medium	Average initial length (cm) of *in vitro* germinated seedlings	Average length (cm) of germinated seedlings after 30d of culture on elongation medium	Increase in length (cm) of germinated seedlings within 30d of culture on elongation medium
	Solid media			Liquid media		
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.52±0.17	3.39±0.11	1.87±0.10	1.35±0.12	3.67±0.12	2.32±0.12
MS + 3% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	1.75±0.12	3.71±0.10	1.96±0.14	1.40±0.16	3.83±0.10	2.43±0.09
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	1.63±0.15	3.68±0.15	2.05±0.16	1.42±0.10	3.98±0.17	2.56±0.12
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.55±0.10	3.67±0.17	2.12±0.15	1.33±0.12	3.46±0.14	2.13±0.11
MS+3% (w/v) sucrose + 0.5 mg/l NAA+1.0 mg/l BAP	1.70±0.07	4.08±0.19	2.38±0.14	1.38±0.11	3.73±0.12	2.35±0.19
MS + 3%(w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	**1.55±0.17**	**4.11±0.12**	**2.59±0.11**	1.42±0.16	3.74±0.14	2.32±0.14
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.46±0.15	3.42±0.08	1.96±0.14	1.45±0.10	3.97±0.18	2.52±0.16
MS+3% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.52±0.13	3.65±0.14	2.13±0.12	1.38±0.14	3.96±0.15	2.58±0.11
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.60±0.17	4.08±0.13	2.48±0.15	**1.35±0.11**	**3.98±0.13**	**2.63±0.13**
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.65±0.11	3.76±0.09	2.11±0.17	1.32±0.19	3.66±0.09	2.34±0.12
PM + 2% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	1.70±0.10	3.74±0.14	2.04±0.19	1.35±0.21	3.73±0.11	2.38±0.16
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	1.58±0.19	3.73±0.16	2.15±0.08	1.45±0.16	3.90±0.13	2.45±0.09
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.74±0.16	3.97±0.21	2.23±0.11	1.50±0.12	3.72±0.19	2.22±0.17
PM + 2% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	1.68±0.14	4.03±0.19	2.35±0.14	1.48±0.10	3.90±0.12	2.42±0.14
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	1.63±0.12	4.09±0.11	2.46±0.15	1.46±0.13	3.92±0.14	2.46±0.13

PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.76±0.14	3.70±0.13	1.94±0.10	1.35±0.11	3.53±0.09	2.18±0.09
PM + 2% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.59±0.09	3.82±0.15	2.23±0.19	1.38±0.10	3.70±0.13	2.32±0.16
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.73±0.12	4.09±0.10	2.36±0.16	1.32±0.09	3.77±0.15	2.45±0.08

*All the values are mean ± SE, shoot length of each treatment contains 10 replicates.

Table - 3 : Increased length (Mean ± SE) and number of roots in seed derived seedlings of *A. multiflora* in half strength MS0 and auxin supplemented MS rooting media.

Culture medium			Average increased length and number of roots per seedling	
			Mean length (cm) ± SE	Mean no. of roots/ shoot bud ± SE
½ MS0			3.54 ± 0.19	2.72 ± 0.19
Auxin (mg/l)	**IAA**	**0.5**	**3.73 ± 0.21**	**2.86 ± 0.17**
		1.0	3.58 ± 0.22	2.52 ± 0.15
		1.5	2.54 ± 0.16	2.15 ± 0.15
	IBA	0.5	3.65 ± 0.26	2.18 ± 0.13
		1.0	**4.17 ± 0.23**	**2.93 ± 0.16**
		1.5	3.84 ± 0.22	2.57 ± 0.18
	NAA	0.5	1.83 ± 0.11	1.68 ± 0.12
		1.0	2.01 ± 0.15	1.46 ± 0.10
		1.5	1.62 ± 0.10	1.22 ± 0.08

***Root length and number of roots of each treatment contains 10 replicates.**

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