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MULTIPLE SHOOT REGENERATION IN *DENDROBIUM FIMBRIATUM* HOOK AN ORNAMENTAL ORCHID

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ABSTRACT

The prime objective of the present study was to optimize and develop efficient regeneration protocol for *in vitro* germination, micropropagation and root induction of the orchid plant *Dendrobium fimbriatum*. Three investigated culture media were Phytamax (Sigma, USA; PM), Murashige and Skoog (MS) and Modified Vacin and Went (MVW) were used. Among these media PM was found to be the most effective medium for germination of orchid seedlings. In this medium, the germination rate was 100 percent. Twelve different combinations of plant growth regulators were tested for the elongation of germinated seedlings. It was revealed that MS medium fortified with 2.0 mg/l 6-benzylaminopurine (BAP) and 0.01 mg/l indole-3-butyric acid (IBA) was the most effective for shoot elongation. The average elongation rate was 4.10 cm after 30 days of culture. Sixteen different combinations of plant growth regulators were used for induction and elongation of adventitious shoots from the nodal zone of shoot explants. MS medium supplemented with 1 mg/l BAP and 0.5 mg/l Picloram was proven to be the best for multiple shoot formation and elongation. In this medium the average number of induced shoots per explant was 4.35. Furthermore, shortest duration (16 days) for shoot induction was recorded on this medium. Half MS medium supplemented with 1.0 mg/l indole-3-acetic acid (IAA) was found suitable for effective induction and growth of adventitious roots on the micro-propagated orchid plantlets.

Key words: *Dendrobium fimbriatum,* orchid, micropropagation, callus, plant regeneration

INTRODUCTION

Dendrobium fimbriatum, a sympodial epiphytic orchid is one of the most famous orchids distributed in a few countries in South and South East Asia. It is mainly used as a decorative plant worldwide and a traditional medicine in Chinese culture. There are thousands of commercial orchid species that are now artificially grown for their beautiful flowers and glycosidal importance. An orchid plant with flowers can easily be kept within a residential area or bedroom in fresh form for many days as a symbol of beauty Rahman *et al*. (2008). Roychowdhury and Mishra (2001) reported that orchids have some medicinal properties e.g. blood clotting in wound (*Cymbidium giganteum*), antidote for poisoning and abdominal complaints (*Vanda tessellata*), healing of wounds (*Cymbidium aloifolium*), to cure hysteria (*Vanda spathulata*) and oral contraceptives (*Cymbidium madidum*).

The absence of endosperm in the orchid seed is an inhibitory factor that limits germination of its seed in nature. Orchid seeds usually germinate in symbiotic association with some species particularly mycorrhiza (a kind of symbiotic fungus) which supplies nutrient to the germinating undifferentiated orchid embryo. Moreover, orchid seeds have poor germination capability and diminish very quickly in nature for inadequate environment. In nature orchids are propagated through vegetative reproduction, but it is a very slow process. Thus *in vitro* culture techniques are now adopted for quick propagation of commercially important orchid species. Orchid seeds are artificially germinated for commercial purpose and seedlings are raised 'enmasse'. However, its number is steadily declining because of a lower rate of propagation in nature and overexploitation. Micropropagation of orchid seedlings could also be done with the use of aseptically grown seedlings. Therefore, an *in vitro* propagation technique could be a useful approach for mass scale propagation of this orchid for commercial purposes. Tissue culture techniques have been widely used for *in vitro* mass propagation of several commercially important orchid species over the past few decades Chen and Chang (2004), but the efficacy of these protocols remains far behind the optimum.

The present investigation was undertaken to establish a suitable protocol for *in vitro* germination, micropropagation and root induction of the orchid plant *Dendrobium fimbriatum*. The effects of plant growth regulators on the development of multiple shoot formation and elongation have been investigated for this species also. In the present studies we have developed a very successful and efficient micropropagation method that can be successfully employed in breeding of *Dendrobium fimbriatum* for commercial purposes.

MATERIALS AND METHODS

Preparation of plant materials: Capsules of *Dendrobium fimbriatum* were collected from the mature orchid plants grown in the Orchid House of BRAC Research Centre, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Dhaka and were used as the source of plant materials. The capsule donating orchid plants were grown in natural field conditions prior to capsule collection. The capsules were surface sterilized by submerging them in a 0.2% (w/v) mercuric chloride solution for 10 minutes and were washed 3-4 times with double distilled autoclaved water under a laminar flow hood.

In vitro seed germination: Under axenic conditions the capsules were opened with a sterile surgical blade. Seeds were then taken out with the help of sterile forceps and transferred onto tissue culture vessels containing solidified (0.8% w/v agar) medium for germination of seeds. Three different basal media were investigated for germination of seeds. These were PM (Phytamax™, Sigma Chemical Company, USA), MS (Murashige and Skoog, 1962) and MVW (Vacin and Went, 1949). All of these media were supplemented with auxin, cytokinin and/or gibberellic acid in different concentrations and combinations. Seeds were germinated for seven weeks in a growth chamber maintaining a temperature regime of 25±2°C, 98% relative humidity and a 12h day (about 2000 lux light intensity). The pH of all plant culture media was adjusted to 5.8. All media were autoclaved at 121°C for 20 minutes under a pressure of 15 lbs/sq. inch.

Shoot elongation and micropropagation: Protocorms developing from the germinated orchid seeds were isolated aseptically and transferred onto fresh culture vessels containing the same germinating medium. Further subculture of the protocorm was done at an interval of 25-30 days. Prior to each subculture the density of seedlings per vessel was reduced. For estimating rapid elongation of shoots, germinating seedlings were transferred onto different types of shoot elongation media. All shoot elongation media were based on either liquid or solidified MS (Murashige and Skoog, 1962) medium supplemented with different plant growth regulators in different combinations and concentrations (see Table 2).

For effective micropropagation of orchid shoots, the *in vitro* growing seedlings when raised up to 7-9 cm in height were cut into 2-3 segments each harboring a node. These nodal explants were transferred onto shoot induction and elongation media for development of adventitious shoots. Subculture of these explants was done at an interval of 30 days.

Newly developed adventitious shoots when reached a height of 2-3 cm were separated from each other and transferred onto rooting medium for induction of roots. For obtaining efficient root induction we have investigated MS (Murashige-Skoog, 1962) medium in combination with two plant growth regulators, IAA and IBA. A complete combination and concentration of these hormones are listed in Table 5.

Both shoot elongation and *in vitro* micropropagation of orchids were performed under controlled growth chamber conditions. The temperature was maintained at 25±2°C with a photoperiod of 16 hours/day (2000-3000 lux light intensity supplied by cool white fluorescent light). The micropropagated plantlets with well developed root system were transferred onto the soil pots and covered immediately with polythene foil to maintain complete humidity. After sufficient step-by-step acclimatization (about two weeks) in the growth chamber, the plantlets were transferred first to greenhouse (about four weeks) and then to field condition for further growth and flowering.

RESULTS

In vitro seed germination: As mentioned in the materials and methods, for estimation of the rate of germination of *Dendrobium fimbriatum* seeds we have investigated three different solidified agar media viz. PM, MS and MVW. The results of this experiment are presented in Table1. The highest percentage of seed germination (100%) was obtained on PM (Fig. 1 A), whereas the lowest on (86%) on MVW medium. It was also evident that the period of germination on PM medium was comparatively shorter (average germination time 45.5 days) than that observed on MS and MVW media. In these, the average time of germination resulted in 49 days (data not shown).

Elongation of seedlings: In order to determine the optimum rate of elongation of orchid shoots derived from the germinated seedlings we have tested MS medium supplemented with different plant hormones in 12 different combinations (Table 2). Table 2 indicates that the highest rate of shoot elongation was achieved when MS medium was fortified with 2.0 mg/l BAP and 0.01 mg/l IBA. In this combination, the average height of the shoots after 30 days of culture in liquid medium resulted in 7.56 cm, whereas it was 6.85 cm when cultured in solidified agar medium. In another combination where MS medium was supplemented with 1.0 mg/l zeatin and 0.1 mg/l IBA the rate of shoot elongation (average length 7.05 and 6.0 cm in liquid and solidified medium, respectively) was similar to that observed in MS medium fortified with 2.0 mg/l BAP and 0.01 mg/l IBA (Table 2).

Micropropagation and elongation of shoots: Micropropagation of shoots was based on induction of multiple shoot buds. Differentiation of shoot buds was limited only to the nodal zone of the explants (Figure 2C) isolated from the shoots elongated *in vitro* from the

germinated seedlings. For induction of shoot buds we have tested MS medium fortified with several plant growth regulators in 16 different combinations and concentrations. A complete list of these combinations is presented in Table 3. Shoot differentiation was first observed after 16 days of culture on micropropagative media. The highest rate of shoot induction was observed in MS medium that contained 1.0 mg/l of BAP and 0.5 mg/l of Picloram. In this combination the average number of induced shoot buds per explant resulted 4.35 (Figure 2 B). We also observed that addition of Picloram either with BAP or Zeatin to MS medium equally enhanced shoot induction from the nodal zone of cultured explants (Table 3). But in BAP/Picloram combination the average time for induction of shoot buds was shorter (17.5 days) than that observed in Zeatin/Picloram combination (20.7 days). Shoot explants that did not contain internodes failed to induce adventitious shoots (data not shown).

The induced shoots were subcultured after every 30 days by transferring them onto the same but fresh medium. The length of the shoots during subculture varied depending on combination of growth regulators in the medium. The efficiency of these media (combination and concentration of various growth hormones) on elongation rate of the induced adventitious shoots was estimated based on the difference in shoot length at the end of two consecutive subcultutres, i. e, on the 30th and 60th days of culture. These results are presented in Table 4. This table indicates that the highest shoot elongation was obtained in media that contained Picloram. The average height of the shoots after 30 days of culture on MS media containing 0.5 mg/l Picloram and 1.0 mg/l BAP or 1.0 mg/l Picloram and 1.0 mg/l BAP resulted in 3.40 cm and 3.25 cm, respectively (Table 4). In these two combinations even after 60 days of culture the rate of shoot elongation remained in similar pattern resulting 6.25 cm and 6.00 cm, respectively (Table 4).

Table 1. Effect of different media on *in vitro* germination of *Dendrobium fimbriatum* seeds.

Media	Number of culture vessels used	Number of cultural vessels in which seed germinated		Range of time for germination (days)	Mean time of germination (days)
		No.	%		
PM	30	30	100	45-46	45.5
MS	30	28	93	48-50	49
MVW	30	26	86	48-50	49

Table 2. Effect of different concentrations and combinations of plant growth regulators on the elongation of germinated seedlings.

Plant growth Regulators mg/l	0.8%(w/v) agar solidified			Without agar (liquid)		
	Average Initial length (cm) *	Average Length(cm)**	Increase in length(cm) ***	Average Initial length (cm) *	Average length(cm)**	Increase in length(cm)***
NAA + KIN						
1.0 +1.0	3.00	5.10	2.10	3.05	6.05	3.00
1.5 + 1.0	3.09	5.32	2.23	3.30	6.34	3.04
2.0+ 1.0	**3.24**	**6.34**	**3.10**	**3.22**	**6.60**	**3.38**
2.5 + 1.0	3.05	5.15	2.10	3.15	6.20	3.05
BAP + IBA						
1.0+ 0.01	2.10	3.15	1.05	3.50	5.35	1.85
1.5+ 0.01	2.67	3.90	1.23	3.66	5.52	1.86
2.0+ 0.01	**3.07**	**6.85**	**3.78**	**3.46**	**7.56**	**4.10**
2.5 + 0.01	2.95	4.10	1.15	4.00	5.90	1.90
Zeatin + IBA						
0.5 + 0.1	1.75	4.00	2.25	2.65	5.00	2.35
1.0 + 0.1	**2.88**	**6.00**	**3.12**	**3.15**	**7.05**	**3.90**
1.5 + 0.1	2.70	5.00	2.30	2.95	6.00	3.05
2.0 + 0.1	2.05	4.95	2.90	2.70	5.95	3.25

Note:
*After 60 days of germination of seedlings
**After 30 days of culture on elongation medium of seedlings
***Within 30 days of culture on elongate medium of seedlings

Table 3. Effect of different concentrations and combinations of plant growth regulators on the development of multiple shoot buds from nodal segments.

Plant growth regulators mg/l	Nodal explants	
	Time(days) required of sprouting of multiple shoot buds	Average no. of shoot Buds Sprouting in each nodal zone of shoot
BAP + IAA		**BAP + IAA**
1.0+ 1.0	26	1.0+ 1.0
2.0+ 1.0	28	2.0+ 1.0
3.0+ 1.0	**26**	**3.0+ 1.0**
4.0+ 1.0	26	4.0+ 1.0
Picloram + Zeatin		**Picloram + Zeatin**
0.50 + 1.0	20	0.50 + 1.0
1.0 + 1.0	**18**	**1.0 + 1.0**
1.5 + 1.0	20	1.5 + 1.0
2.0 + 1.0	25	2.0 + 1.0
BAP + Picloram		**BAP + Picloram**
0.50 + 0.5	16	0.50 + 0.5
1.0 + 0.5	**16**	**1.0 + 0.5**
1.5 + 0.5	18	1.5 + 0.5
2.0 + 0.5	20	2.0 + 0.5
IBA + Zeatin		**IBA + Zeatin**
1.0+ 1.0	20	1.0+ 1.0
2.0+ 1.0	**22**	**2.0+ 1.0**
3.0+ 1.0	20	3.0+ 1.0
4.0+ 1.0	20	4.0+ 1.0

Root induction and elongation: All shoots when elongated to a length of about 7-8 cm were transferred onto rooting media for induction of roots. Two kind growth regulators, IAA and IBA, were added to solidified MS medium in different concentrations for induction of roots. Results presented in Table 5 indicate that the average length of induced roots reached highest (5.2 cm) when the shoots were cultured on half strength MS medium supplemented with 1 mg/l IBA. In addition, this combination of medium was also effective for developing adventitious roots. In this medium the average number of root per shoot was 9.30 (Fig. 3).

Acclimatization and transplantation: All plantlets with well-developed root systems were removed from culture vessels and transferred to soil pots. After a two-week period of acclimatization in the growth chamber the plantlets were then adapted to greenhouse conditions. The procedure of acclimatization of the *in vitro* grown plantlets to the greenhouse environment involved a number of successive adaptive steps such as watering and maintaining high humidity. In our investigation 83.6% plantlets survived acclimatization process and grew to a normal flowering plant under field condition (Fig. 3 C).

Table 4. Effect of different concentrations and combinations of plant growth regulators on the elongation of multiple shoot buds developed from nodal segments.

Plant growth regulators mg/l	Average initial length (cm) of individual multiple shoot	Average length(cm) of individual multiple shoot after 30d of culture of elongation medium	Increase in length (cm)of individual multiple shoot within 30d of culture in elongation medium
BAP + IAA			
1.0 + 1.0	2.60	4.50	1.90
2.0 + 1.0	2.80	4.90	2.10
3.0 + 1.0	**2.25**	**4.75**	**2.50**
4.0 + 1.0	2.00	4.00	2.00
Picloram + Zeatin			
0.50 + 1.0	2.50	3.95	1.45
1.0 + 1.0	**3.25**	**6.00**	**2.75**
1.5 + 1.0	2.80	4.90	2.10
2.0 + 1.0	2.65	4.40	1.75
BAP + Picloram			
0.50 + 0.50	3.25	6.00	2.75
1.0 + 0.50	**3.40**	**6.25**	**2.85**
1.5 + 0.50	3.20	5.15	1.95
2.0 + 0.50	3.00	5.00	2.00
IBA + Zeatin			
1.0 + 1.0	2.50	4.75	2.25
2.0 + 1.0	**3.10**	**5.45**	**2.35**
3.0 + 1.0	2.50	4.25	1.75
4.0 + 1.0	2.05	4.05	2.00

Table 5. Effect of different rooting media on root induction from single shoot bud.

Rooting medium mg/l	Average length(cm) of roots after 30 days culture on rooting medium	Average no. of roots per plant
MS+ IAA		
0.50	3.00	6.00
1.0	**3.50**	**6.10**
1.5	2.95	5.90
2.0	2.50	5.00
½ MS + IBA		
0.50	4.90	8.80
1.0	**5.20**	**9.30**
1.5	4.50	9.00
2.0	4.00	8.00

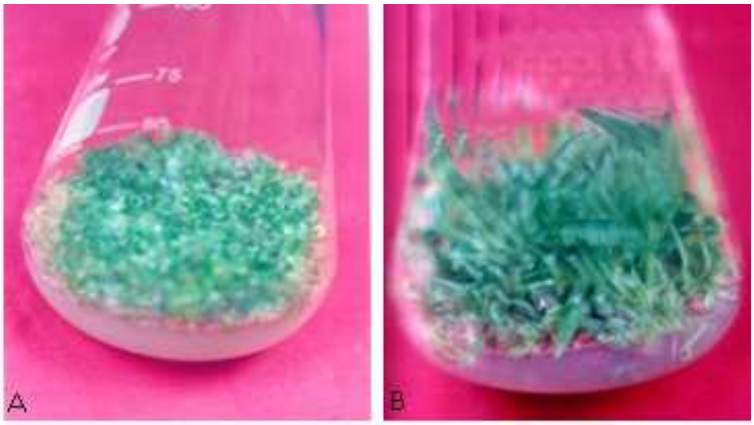

Fig. 1 In vitro seed germination
A. Germination of seeds on PM medium.
B. Seedlings undergoing elongation on agar solidified MS medium supplemented with1.0mg/l Zeatin + 0.1mg/l IBA.

Fig. 2 Shoot elongation and multiple shoot induction from shoot tip and nodal segment
A. Seedling undergoing elongation in liquid MS medium supplemented with 2.0 mg/l BAP+ 0.01 mg/l IBA
B. Multiple shoot buds developed from shoot segment with node when cultured on agar solidified MS medium supplemented with 1.0 mg/l BAP + 0.5mg/l Picloram.
C. Multiple shoot buds developed from nodal segment when cultured in liquid MS medium supplemented with 2.0 mg/l IBA and 1.0 mg/l Zeatin.

Fig. 3 Root induction and acclimatization
A. Elongated shoot buds developed root system on agar solidified MS medium with 1.0 mg/l IAA
B. Elongated shoot buds developed root on agar solidified half strength MS medium with 1.0 mg/l IBA.
C. Plantlets acclimatized in a pot outside the culture room.

DISCUSSION

This paper is a part of our approach for establishing a standard protocol for micropropagation and regeneration of the epiphytic orchid plants *Dendrobium fimbriatum*. We have developed necessary parameters that are involved in the tissue culture processes. For germination of seeds among the three investigated basal media viz. MS, PM and MVW we found PM as the most suitable one. In this medium all of the exposed seeds (100%) started to germinate (Table.1). In addition to germination rate, the time of germination was found to be the shortest also in this PM medium. Considering these two features, rate and time of germination we suggest that PM medium can be effectively used for *in vitro* germination of orchid seeds. It has been reported that PM medium was equally effective also for germination of *Epidendrum ibaguanse* species (Hossain, 2008).

One of the key steps of *in vitro* propagation is the elongation/enlargement of the donor plant materials that can be used as the source for regenerating multiple shoots. To investigate this feature we have exposed the germinated orchid seedlings to various elongation media containing different plant growth regulators in different combinations and concentrations. We found that the rate of elongation reached highest when the germinated seedlings were exposed to liquid MS containing 2.0 mg/l BAP and 0.01 mg/l IBA. In this medium the average length of germinated seedlings resulted in 7.56 cm (Table 2). However, when the seedlings were exposed to solidified (with 0.8% w/v agar) MS medium containing identical contents of the nutrients the average length of the seedlings resulted in 6.85 cm which is a bit lower than that found in liquid medium. Such type of positive effect of liquid medium for germination of seeds was reported previously by Hoque *et al*. (1994). Analyzing the results obtained in mcropragation of the induced shoots we found that the media containing Picloram and BAP developed the highest number of adventitious shoots (in average 4.35 shoots per explant). Furthermore, in this medium the time of shoot initiation was the shortest (in average 17.5 days). We believe that addition of Picloram together with BAP in the MS medium will lead to optimum micropropagation of *Dendrobium fimbriatum* species and this combination can be successfully employed by the orchid breeders. One of the major problems for orchid breeders is to generate a well-developed root system facilitating adaptation of *in vitro* grown plantlets to field conditions. For investigating this problem we tested two potential plant growth regulators IBA and IAA (Table 5). Shoots when exposed to half strength MS medium supplemented with 1 mg/l IBA developed the highest number of adventitious roots. The length of these roots was also highest in this medium (Table 5). The effect of IAA or IBA on induction of roots in other orchid species like *Vanda coerulea* was also reported by several authors (Malabadi *et al*. 2004, William *et al*. 2003, Mitra *et al*. 1976). These results are in complete agreement with those we obtained in half strength MS supplemented with 1 mg/l of IBA.

Conclusion: *Dendrobium fimbriatum* is an ornamental orchid plant commonly cultivated in many countries of Asia. The economic value and the demand of this plant make it very attracting for plant breeders. In many regions of Asia, especially in South Asia, breeding of this plant under natural field conditions is very expensive and laborious due to problems with vegetative reproduction and germination and production of fertile seeds. For overcoming these problems we have investigated a tissue culture approach. Here we propose that this method can be successfully used by the breeders for micro-propagation of orchid plants.

REFERENCES

Chen, T. Y and W. C .Chang. (2004). Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids. *Plant Cell Tissue Organ Cult.* 76: 11-15.

Hoque, M. I., A. R Roy., R .H Sarker and M. M Haque. (1994). Micropropagation of *Cymbidium bicolor* through in vitro culture. *Plant tissue culture.* 4(1): 45-51.

Hossain, M. M. (2008). Asymbiotic seed germination and *in vitro* seedling development of *Epidendrum ibaguanse* Kunth. (Orchidaceae). *African J of Biotech*. 7(20): 3614-3619.

Malabadi R. B., S. Gangadhar., Mulgund and K. Nataraja. (2004). Efficient regeneration of *Vanda coerulea*, an endangered orchid using Thidiazuron. *Plant Cell, Tissue and Organ Culture*. 76: 289-293.

Mitra, G. C., R, N Prasad and A Roychowdary. (1976). Inorganic salts and differentiation of protocorms in seed-callus of an orchid and correlated changes in its free amino acid content. *Ind. J. Exp. Biol*. 14: 350-351.

Murashige, T and F. Skoog. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.

Rahman, M.S., M. F. Hasan., M.S Hossain., A. Mandal and M. Rahman. (2008). Plant Regeneration of Orchid (*Vanda tessellata* L.) through Meristem Culture. *Int J BioRes*. 4(1): 8-16.

Roychowdhury N and H. P. Mishra. (2001). Text Book on Floriculture and Landscaping, Shyamal Ghosh Publishers, Kolkata, pp. 163.

Vacin, E and F, Went. (1949). Botanical Gazette. 110:605-613.

William S, Gangaprasad A, Seeni S, Sarojini, V (2003). Micropropagation and ecorestoration of *Vanda spathulata*, an exquisite orchid. *Plant Cell, Tissue and Organ Culture.* 72: 199-202.