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In vitro Embryo Morphogenesis and Micropropagation of *Dendrobium aggregatum* Roxb.

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Abstract

In vitro embryo morphogenesis and micropropagation of *Dendrobium aggregatum* Roxb. were described. The gradual developmental stages of embryos to seedlings were traced out. Within two weeks of culture the cells of undifferented embryos underwent repeated aniclinal and periclinal division producing a compact, green parenchymatous cell mass called spherule that emerged out by rupturing the testa. The spherules subsequently differentiated into greenish protocorms were considered as typical seed germination. Germination occurred on both (MS and Phytamax (PM) medium but MS medium proved to be more efficient. The primary protocorms underwent profuse proliferation through production of secondary (2º) protocorms when transferred to different plant growth regulators (PGRs) supplemented MS; the medium fortified with 2.0 mg/l BAP and 1.0 mg/l NAA proved to be most effective for induction of 2º protocorms and seedling development. Multiple shoot buds (MSBs) were induced in pseudobulb segments of the *in vitro* grown seedlings when cultured on different PGRs supplemented media; and the maximum number of MSBs were obtained MS + 2.0 mg/l BAP + 0.5 mg/l picloram. The MSBs underwent elongation and then they rooted when they were transferred to half strength of MS + 0.5 mg/l IAA. The well rooted plantlets were finally transferred to outside natural environment with 80% survival.

Introduction

Dendrobium aggregatum Roxb. (Syn. *D. lindleyi* Steud.) is an evergreen epiphytic orchid characterized by beautiful showy spikes of orange-yellow flowers, sporadically found in the Teknaf forest of Cox's Bazar district of Bangladesh (Ahmed 1991, Huda et al. 1999). The species has a great horticultural importance for its flowers of exquisite beauty as well as potted plant for decorating the Lobby. The natural orchid gene pool is depleting at an alarming rate due to

overexploitation for medicinal and ornamental purposes, onslaught habitat destruction by urbanization and shifting cultivation, loss of pollinators, destructive diseases, climate changes and unauthorized trade (Hossain 2009). Considering the present status of orchids, the family Orchidaceae as a whole was included in the CITES (Convention on International Trade in Endangered Species) (Shefferson et al. 2005). In the context of commercial exploitation, and to meet up future demand and conservation it is important to develop rapid, reliable and reproducible propagation techniques of this economically important orchid species. Since the discovery of *in vitro* seed germination of orchids by Knudson (1922) and the micropropagation technique by Morel (1964) the technique is routinely used for mass multiplication of economically important orchids. In fact, the discovery of tissue culture techniques added a new dimension to the production of quality plants in large quantities and propagation of exquisite and rare orchids (Stenberg and Kane 1998, Nagashima 1999, Seeni and Latha 2000, Hossain 2008, Sinha et al. 2009, Hossain et al. 2009, 2010, 2012). Although tissue culture techniques have been adapted in a number of orchid species the information for *D. aggregatum* is rare. Recently, Vijayakumar et al. (2012) reported only germination of immature seeds of *D. aggregatum*. Considering the importance, the present investigation was undertaken with a view to developing an efficient and reproducible protocol for *in vitro* germination and micropropagation of *D. aggregatum.* The protocol reported here suggests that same has a potential of providing mass supply of planting material to meet the demands of orchid growers as well as for conservation. A special attention was also paid to investigate the mode of morphogenesis of embryo during formation of protocorm and seedling development.

Materials and Methods

Plants of *Dendrobium aggregatum* Roxb. were collected from their natural habitat of Teknaf forest of Cox's Bazar district of Bangladesh and grown in the Orchidarium of the Botanical Garden of Chittagong University. Several flowers were hand pollinated on the second and third day of anthesis. The pollinated flowers were bagged with a thin transparent polythene bag for one week and then removed Several capsules were collected after three - four months of pollination and the seeds were used for *in vitro* germination. MS and PM (Phytamax; Sigma Chemical Co. USA) media were used for *in vitro* studies. pH of the media was adjusted to 5.4 for PM and 5.8 for MS prior to autoclaving at 121ºC for 20 min. at 15 psi.

The capsules were cleaned with 20% Teepol, a commercial detergent (Qualigens Fine Chemicals, Mumbai, India) and washed thoroughly under

running tap water. These were then surface sterilized by submerging the material in 0.2% (w/v) $HgCl_2$ solution for 10 min with occasional agitation followed by a dip in 70% ethanol for 1 min and rinsed two - three times with sterile distilled water and then cut longitudinally with a sterile surgical blade keeping the material on a piece of sterile aluminum slab inside a laminar air-flow cabinet. The powdery seeds were taken out and were inoculated on the surface of agar gelled nutrient medium in culture vessels (test tubes 2.5 × 15cm and conical flasks 250 cc) for germination. The culture vessels with inoculated seeds were incubated in a growth room where a cycle of 14/10 hrs light and dark regime was maintained at 60 μmol/m^2/s provided by cool white fluorescent lamps (Philips Truelight 36w/86 6500° K B7, Philips India), and 60% RH at 25 ± 2°C.

After two weeks of inoculation, some of the seeds were taken out and dispersed in one drop of water on a glass slide and observed under a light microscope. Per cent germination was calculated employing the following formula:

$$\frac{\text{No. of seeds showing swelling of the embryo} \times 100}{\text{Total no. of seeds}}$$

Once the spherules were formed, observations were recorded at one week interval to trace different stages of protocorm development. The whole operation was observed using a stereozoom microscope. Regular subculturing was done at an interval of 20 - 25 days.

For micropropagation, pseudobulb segments (0.5 - 1 cm in size) of *in vitro* grown seedlings were cultured on different PGRs supplemented media. Multiple shoot buds (MSBs) that developed from pseudobulb segments were subcultured in different PGR supplemented media for induction of rapid elongation. When the shoot buds attained a height of 3 - 4 cm they were individually grown on the rooting media for induction of strong and stout root system. Well-rooted plantlets were taken out from culture vessels, their roots were washed thoroughly under running tap water to remove traces of agar and finally transferred to plastic pots containing a potting mixture of sterilized small brick-, charcoal pieces, and peat moss at a ratio of 1 : 1 : 0.5 and kept in the greenhouse (at 25 - 30°C and RH 60 - 70%).

The experiments were designed following Complete Randomize Design (CRD). Five replicates were taken per treatment for seed culture, whereas for micropropagation and rooting 10 replicates were taken. The effects of different media on germination of seeds, induction of shoot buds and roots in the *in vitro* experiments were tested applying Duncan's multiple range test ($p > 0.5$) in one

way ANOVA. The statistical analyses were performed using the programme package Statistica ver. 7 (Statsoft, Tulsa, USA). The experiments were repeated thrice.

Results and Discussion

Germination of seeds occurred on both MS and PM media within three weeks from the date of inoculation. MS proved to be more efficient for germination of seeds (95%) with a vigorous growth of protocorms compared to PM medium (72%). Five distinct developmental stages of germinating embryos were observed during germination. **Stage 1:** No germination occurred, only viable embryos swelled up by absorbing water and nutrition (Fig. 1a), **Stage 2:** Swelling continued, cell number increased through repetitive anticlinal and periclinal cell divisions resulting in the formation of an irregularly shaped parenchymatous cell masses which came out by the rupturing of the seed coat (Fig. 1b), **Stage 3:** The parenchymatous cell mass contained dense chloroplasts, became compact with a few rhizoids at the posterior/basal part called spherule (Fig. 1c), **Stage 4:** The spherules enlarged in size and a growth appendicle appeared at the anterior portion delimiting the meristimatic zone for development of foliar organs; critically this stage is called protocorm stage and is supposed to be virtual germination of orchid seeds (Fig. 1d); **Stage 5:** The roots emerged from the basal part of the protocorms and gradually differentiated into young seedlings. Similar mode of differentiation of embryos was reported in *Cypripedium acaule* (Leroux et al. 1997), *Platanthera clavellata* (Zettler and Hofer 1998), *Habenaria macroceratitis* (Stewart and Kane 2006), *Cymbidium aloifolium* (Hossain et al. 2009), *C. giganteum* (Hossain et al. 2010) and *Dendrobium aphyllum* (Hossain et al. 2012). In fact, germination of orchid seeds followed a peculiar metamorphogenetic pathway during germination. The undifferentiated embryos gradually develop a round/ellipsoidal structure called protocorm which is an intermediate structure between the embryo and the plant (Batygina et al. 2003). When the protocorms were transferred to different PGRs supplemented MS, they multiplied profusely producing a huge number of secondary protocorms (Fig. 1e). After 2 - 3 subsequent subcultures, these secondary protocorms on the same media produced seedlings (Fig. 1f). For induction of secondary protocorms and subsequent seedling development MS fortified with 2.0 mg/l BAP and 1.0 mg/l NAA proved most effective. Induction of secondary protocorms from primary protocorms in orchids is a common phenomenon greatly influenced by the specific plant growth regulators (Hossain et al. 2010). The secondary protocorms can be regenerated directly from the outer tissues of the protocorms (Huan et al. 2004), protocorm segments or thin cell layers (Teixeira da Silva et al. 2006, 2007).

The pseudobulb segments of *in vitro* grown seedlings were cultured on different PGRs supplemented media. Thereafter, direct organogenesis occurred leading to the formation of multiple shoot buds (MSBs) on all the tested media (Table 1). The shoot buds regeneration was greatly influenced by the specific PGRs combinations. Shoot buds differentiation occurred directly from the dormant buds within four - five weeks of culture without an intervening callus phase. Differentiation continued until the end of the eighth week. Shoot buds first appeared as small, green protuberances that continued to grow and produce numerous axillary buds from the base. The highest number of MSBs (7.75 ± 0.17)

Table 1. Development of multiple shoot buds* from pseudobulb segments of *in vitro* grown seedlings of *D. aggregatum* on different PGRs supplemented MS.

PGR combinations (mg/l)	Time (days) required of sprouting of MSBs	Number of MSBs sprouted /explant (mean ± S.E.)	Length (cm) of individual shoot buds after 30 days of culture
NAA+BAP			
1.0 + 0.5	40 - 45	4.20 ± 0.18^{f}	2.30 ± 0.16e
1.0 + 1.0	30 - 35	5.42 ± 0.15^{de}	3.35 ± 0.08^{a}
1.5 + 1.0	40 - 45	6.53 ± 0.22^{ab}	3.15 ± 0.13^{bc}
2.0 + 2.0	30 - 35	5.60 ± 0.26^{cde}	3.25 ± 0.15^{ab}
BAP + picloram			
1.0 + 0.5	30 - 35	7.75 ± 0.17^{a}	2.17 ± 0.13^{f}
2.0 + 0.5	30 - 35	5.80 ± 0.18^{cd}	2.55 ± 0.15^{cde}
2.0 + 1.0	35 - 40	4.48 ± 0.18^{ef}	2.45 ± 0.10^{def}
2.0 + 1.5	25 - 35	5.70 ± 0.26^{cde}	3.25 ± 0.12^{ab}
BAP + Kn			
1.0 + 0.5	30 - 35	4.65 ± 0.25^{def}	3.10 ± 0.12^{bc}
2.0 + 0.5	25 - 30	5.05 ± 0.15^{de}	2.60 ± 0.18^{cde}
2.0 + 1.0	25 - 30	5.65 ± 0.21^{cde}	2.70 ± 0.12^{cd}
2.0 + 1.5	25 - 30	5.55 ± 0.17^{de}	2.50 ± 0.10^{de}

*Observations recorded from 10 cultured explants for each treatment.

sprouted when the explants were grown on 0.8% (w/v) solidified agar MS fortified with 1.0 mg/l BAP and 0.5 mg/l picloram (Fig. 1g). The requirement of exogenous auxins and/or cytokinins for regeneration of shoot buds and plantlet development has been reported for many orchid species (Arditti and Ernst 1993, Hossain et al. 2012). However, the combinations, concentrations, and the ratio between them are usually critically important (Hossain et al. 2010). The ratio of auxin to cytokinin for shoot bud formation varies from species to species (Teng et al. 1997). A striking synergistic effect of BAP for induction of shoot buds was reported in *Geodorum densiflorum* (Sheelavantmath et al. 2000, Bhadra and

Hossain 2003), *Rhynchostylis gigantea* (Le et al. 1999), *Vanda spathulata* (Decruse et al. 2003), *Cattleya* (Melissa et al. 1994), *Phalaenopsis* and *Doritaenopsis* (Tokuhara and Mii 1993), *Renanthera imschootiana* and *Vanda coerulea* (Seeni and Latha 2000). The shoot buds underwent elongation on the same media and the highest rate of elongation was recorded on MS + 1.0 mg/l BAP + 1.0 mg/l NAA (Table 1).

Fig.1. Seed germination and micropropagation of *Dendrobium aggregatum*: a. Seeds after one week of culture showing swelling of embryo, b. Parenchymatous cell mass coming out by rupturing testa, c. Spherules, d. A protocorm with leaf primordia, e. Profuse proliferation of protocorms producing secondary protocorms, f. Seedling development from protocorms, g. Multiple shoot bud development from pseudobulb segments, h. Induction of root system in shoot buds, and i. Establishment of *in vitro* grown seedlings in pot natural environment.

When the MSBs attained a height of 3 - 4 cm, they were individually grown on different rooting media for induction of strong and stout root system. Half strength MS + 1.5% (w/v) sucrose + 0.5 mg/l IAA proved to be more effective for induction of strong and stout root system (Table 2). Induction of roots is an inherent nature of plants which is controlled by endogenous level of hormones. Exogenous supply of root-inducing hormones like auxin enhances the process. Accordingly, the plant produces roots to uptake nutrient from nature. In nutrient deficit conditions the plant produces more roots to compensate nutrient *i.e.*,

deficiency, thereby inducing rooting. Thus the results of the present finding suggest that the combined effects of nutritional stress with IAA enhanced the development of a strong and stout root system in *D. aggregatum* (Fig. 1h). Similar results were also reported by Hoque et al. (1994), Bhadra et al. (2002, 2004), Hossain et al. (2009, 2010) in some epiphytic orchids.

The well-developed plantlets were transferred from culture room to the outside environment through successive phases of acclimation. Eighty per cent of the *in vitro* grown seedlings survived and continued to grow in pots in the greenhouse. They were finally established in Orchidarium of the Botanical Garden of Chittagong University (Fig. 1i).

Table 2. Development of root* system in individual multiple shoot buds of *D. aggregatum* when grown on different rooting media.

Rooting medium	Number of roots developed per MSB (mean ± S.E.)	Length (cm) of roots after 30 days of culture (mean ± S.E.)
½ MS	2.25 ± 0.25^{c}	2.45 ± 0.22^{b}
½MS + 0.5 mg/l IAA	3.50 ± 0.16^{a}	3.65 ± 0.18^{a}
½MS + 1.0 mg/l IAA	3.00 ± 0.16^{ab}	2.50 ± 0.10^{b}

*Observation recorded from 10 cultured shoot buds in each treatment.

The protocol developed in the present investigation for *in vitro* propagation of *D. aggregatum* offers a good opportunity to commercial orchid growers for large-scale propagation as well as for *ex situ* conservation of this orchid species. This protocol could be extended to other economically valuable, rare and endangered orchids for mass propagation and conservation.

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