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PROPAGATION OF *DENDROBIUM AGGREGATUM* THROUGH THE CULTURE OF IMMATURE SEEDS FROM GREEN CAPSULES

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ABSTRACT. An efficient protocol for propagation of *Dendrobium aggregatum* using axenic immature seeds, derived from green capsules, was developed. Immature seedfs from 120 capsules 120 days after pollination were germinated on Murashige and Skoog (MS) medium supplement with several concentration of BAP alone or in combination with NAA along with coconut water. The same media were used for induction, multiplication, elongation and rooting in vitro shoots. MS medium e containing 3% sucrose, 1.5 mg l-1 benzyl amino purine (BAP) and 15% coconut water (CW) favoured a higher percentage of germination, a higher number of protocorms, production of maximalnumber of shoots, shoot elongation, as well as root formation. During acclimatization, 95% of the plantlets survived after one month.

KEY WORDS: *Dendrobium,* protocorm like bodies, acclimatization, *in vitro*

Introduction. Orchids occupy an evolutionary high position among flowering plants and are valued for cut flower production and as potted plants, often with a very high price in the international market. The genus Dendrobium exhibits a vast diversity in vegetative and floral characteristic and is of considerable interest due to its broad geographic distribution and high value of hybrids as a floricultural commodity (Jones *et al*. 1998). Though orchids produce a large number of seeds, very few (<5%) of them germinate under natural condition because the seeds are non-endospermic, minute and require a mycorrhizal association (Rao 1977). The conventional method of vegetative propagation of orchids is a time consuming and tedious process (Sagawa & Kunisaki 1984).

Dendrobium aggregatum Roxb. (= *D. lindleyi* Steud.)[1] is a mildly fragrant sympodial orchid, grown world wide as pot plants or cut flowers (Aktat et al. 2007). It has graceful, pendulous racemes of medium sized flowers usually white in colour. Asexual propagation is extremely slow which can give rise to 2-4 plants per year.

Orchids are well known for being major trade plants in developed countries (Sagawa & Kunisaki, 1984). In vitro culture has proved particularly useful with groups of plants, which are difficult to propagate using conventional techniques (Fay 1994). When mass propagation of a new hybrid or a varity is needed within a short time, tissue culture is the only method (Goh *et al.* 1992). The orchid resources of the world in general, and Western-Ghats region of India in particular are being depleted currenbtly due to habitat loss. The collection of wild *Dendrobium* continues at levels ranging from hobbyist to large-scale illegal trade. Endemic orchids of the Western-Ghats India are facing the grim possibility of extinction under intense biotic pressures like jhum cultivation, forest fires, indiscriminate wild collection and illegal trade by the local people. Hence conservation and sustainable utilization assume greater importance to save the dwindling orchids (Kishor *et al.* 2006). Satisfying the interest of the hobbyist and demandby the traders through large-scale micropropagation is one the preferable options to prevent illegal collection from wild (Sunitibala & Rajikumar 2009).

A perusal of available literature reveals that

[1] The name *Dendrobium aggregatum* Roxb. (1832) is illegitimate, being a later homonym of *D. aggregatum* Kunth (1816), the basionym of the species presently known as *Ornithidium aggregatum* (Kunth) Rchb.f. As *D. lindleyi* is still amply cultivated and traded under the name of *D. aggregatum* Roxb., we have mantained the latter name throughout the article. [*Note by the Editor*]

micropropagation has been achieved using immature or mature seeds, shoot tip explants and from axenic node segments in *Dendrobium aphyllum* (Roxb.) C.E.C.Fisch., *D. candidum* Wall. ex Lindl. and other hybrids of *Dendrobium* (Zhang *et al.* 1993, Liu *et al.* 1988, Shiau *et al.* 2005, Xie *et al.* 2010, Nambiar *et al.* 2012). However, there is no report on clonal propagation of *D. aggregatum* using different explant sources. In this study, authors report the development of an efficient simple and reproducible one step protocol for multiple *D. aggregatum* seedlings, rooting of the microshoots and successful transplantation.

Materials and methods. Plants of *D. aggregatum* were collected from their natural habitat and kept under shade net (75%) house environment at the campus of the Government Arts College, Coimbatore. Several flowers were hand pollinated on the second day of anthesis. The pollinated flowers were bagged with butter paper for one week. Several capsules of *D. aggregatum* were harvested 120 days after pollination and brought to the laboratory for *in vitro* seed germination.

Establishment of shoot cultures by *in vitro* germination of immature seeds. The harvested capsules were soaked in aqueous solution of commercial detergent (Labolene) for 10 minutes followed by 0.5 mg/l^{-1} Bavistin (Himedia) for 20 minutes. The capsules were surface disinfected in 70% (v/v) ethyl alcohol for 30 seconds followed by 0.12% (w/v) mercuric chloride solution for 10 minutes and then rinsed 3-4 times sterile distilled water before air drying in a laminar air flow chamber for 5 minutes. Green capsules were dissected longitudinally with a sterile surgical blade. The immature seeds were scooped out of the sterilized capsules and small mass of the aggregated seeds were germinated in culture bottles (60 mm × 105 mm) each containing 30 ml of full strength Murashige and Skoog (MS) basal medium. The basal medium was comprised of full strength MS medium 30 mg l^{-1} sucrose and gelled with 8 g l^{-1} Difco bacto agar (Himedia, India). It was then supplemented with different concentration and combination of naphthalene acetic acid (NAA) and benzyl aminopurine (BAP) along with coconut water (CW) at pH 5.8 (Table 1). The cultures were incubated at 25 ± 2ºC under cool white fluorescent

Table 1.Effect of different concentration of plant growth regulators on *in vitro* development of plantlets from immature seeds of *Dendrobium aggregatum*

Concentration of BAP and NAA in MS medium + CW 150 ml/L	No. of green pods used per bottle	Capability of immature seeds forming protocorm like bodies	No. of shoots per bottle	No. of shoots with roots	Percentage of shoot forming the roots
BAP					
0.5	1	+	35	14	40.0
1.0	1	++	50	35	87.5
1.5	1	+++	75	75	100.0
2.0	1	+	41	28	68.3
2.5	1	+	32	14	36.8
3.0	1	+	20	5	25.0
BAP + NAA					
0.5 + 0.5	1	+	23	13	56.5
1.0 + 1.0	1	+	27	20	74.0
1.5 + 1.5	1	+++	37	37	100.0
2.0 + 2.0	1	++	26	15	57.8
2.5 + 2.5	1	+	20	8	40.0
3.0 + 3.0	1	+	14	3	21.4
MS Basal	1	-	-	-	-

\+ -- Very less number of protocorm like bodies formation
++ -- Less number of protocorm like bodies formation
+++ -- More number of protocorm like bodies formation

light under 14 hours photoperiods. After 15 days culture,the effects of several concentrations of BAP and NAA ranging from 0.5–3.0 mg l^{-1} individually (BAP) and combined (BAP + NAA) along with coconut water were analyzed for number of protocorms shoots and roots.

Subculture of protocorm and shoots. Shoots and protocorm were transferred to the same media and produced more protocorm like bodies, multiple shoots and roots.

Hardening. Well rooted shoots were removed from culture vessels and thoroughly washed with tap water to remove residual medium and transferred to plastic pots containing a mixture uniform, small charcoal pieces and brick pieces (1:1). They were then kept in the shade house 25% light and mist irrigated.

Experiment design and data analysis. Experiments were set up in completely randomized design. Each treatment consisted of 10 replicates. Significance of treatment effects was determined using Duncan's multiple range test (DMRT) analysis.

Results and discussion. Seeds taken from the green immature capsules were sown on the MS medium (Table 1) containing various concentrations of two plant growth regulators, namely BAP and NAA along with CW. Invariably all the embryos transferred to the MS medium with various concentrations of BAP, NAA and CW germinated within two weeks. Swelling and glistering of the embryos were first noticed within 10 days. SThe swelling of the embryo was followed by pigment synthesis. The embryos turned from yellow to yellowish green and finally becomeing green as they grew.

Due to the non-endospermic nature of the seed, germination in nature is a unique phenomenon and requires fungal infection. Germination is much more successful *in vitro*. The production of orchid seedling from seed involves sequential phases of germination, protocorm formation and seedling development. In the present investigation also same sequence of seedling development was observed when the selected orchid, *D. aggregatum* was grown on the medium. As the embryos developed into globouse protocorms, seed coats (testae) were ruptured and rhizoids and shoot initials were formed. Among the six different concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg l^{-1}) individually in combination with NAA (0.5, 1.0 1.5, 2.0, 2.5 and 3.0 mg l^{-1}) along with CW was used. MS medium contain BAP + CW (1.5 mg l + 15% in combination) was found to be the most suitable. It supported a higher rate percentage of germinations, more higher number of protocorms bodies, shoots and roots. Shoots and protocorms transferred to same media produced more protocorms, multiple shoots and roots. Incorporation of coconut water into the basal medium induces and enhances early differentiation of protocorms. Earlier Talukdar (2001) reported similar observation in *D. aphyllum*. Leetham (1974) reported that a plant growth hormone like cytokinin is present in coconut water. Protocorms which developed from the germinating seeds after 45-50 days of culture were allowed to differentiate in the same medium. The pear shaped protocorms with tiny leaf sprouting developed after 20-25 days of germination.

Auxin was the first plant growth hormone added to the seed culture. In majority of the cases auxins (mostly NAA, IAA and IBA) enhanced the germination and seedling growth (Nasiruddin *et al.* 2003). In the present study BAP and CW stimulated shoot and root growth in *D. aggregatum* as reported in *D. microbulbon* A. Rich. (Urvashi Sharma *et al.* 2007). A higher number of protocormswas produced by the medium, which contained 1.5 mg l^{-1} BAP with CW and 1.5 mg l^{-1} + 1.5 mg l^{-1} BAP and NAA with CW. Very poor results were observed ina medium which contained 3.0 + 3.0 mg 1^{-1} BAP and NAA. (Table-1). In the present investigation seedling development of *D. aggregatum* was best on MS medium supplemented with 1.5 mg 1^{-1} BAP + 15% CW. These finding are in agreement with those reported by Sharma, 2007, who observed that BAP induced better shoot and root growth in *D. microbulbon*.

A well-developed cluster was selected and subcultured a second time for root induction (Fig. 1). These subcultures were grown on the same concentrations and combinations of the same plant growth regulatorsto study their effects of the number of shoots and roots per shoot. The number of shoots and roots were counted. After 2-3 passages on the medium, the plants were taken out, washed thoroughly to remove traces of agar and transplanted to perforated plastic pots containing pieces of charcoal and bricks

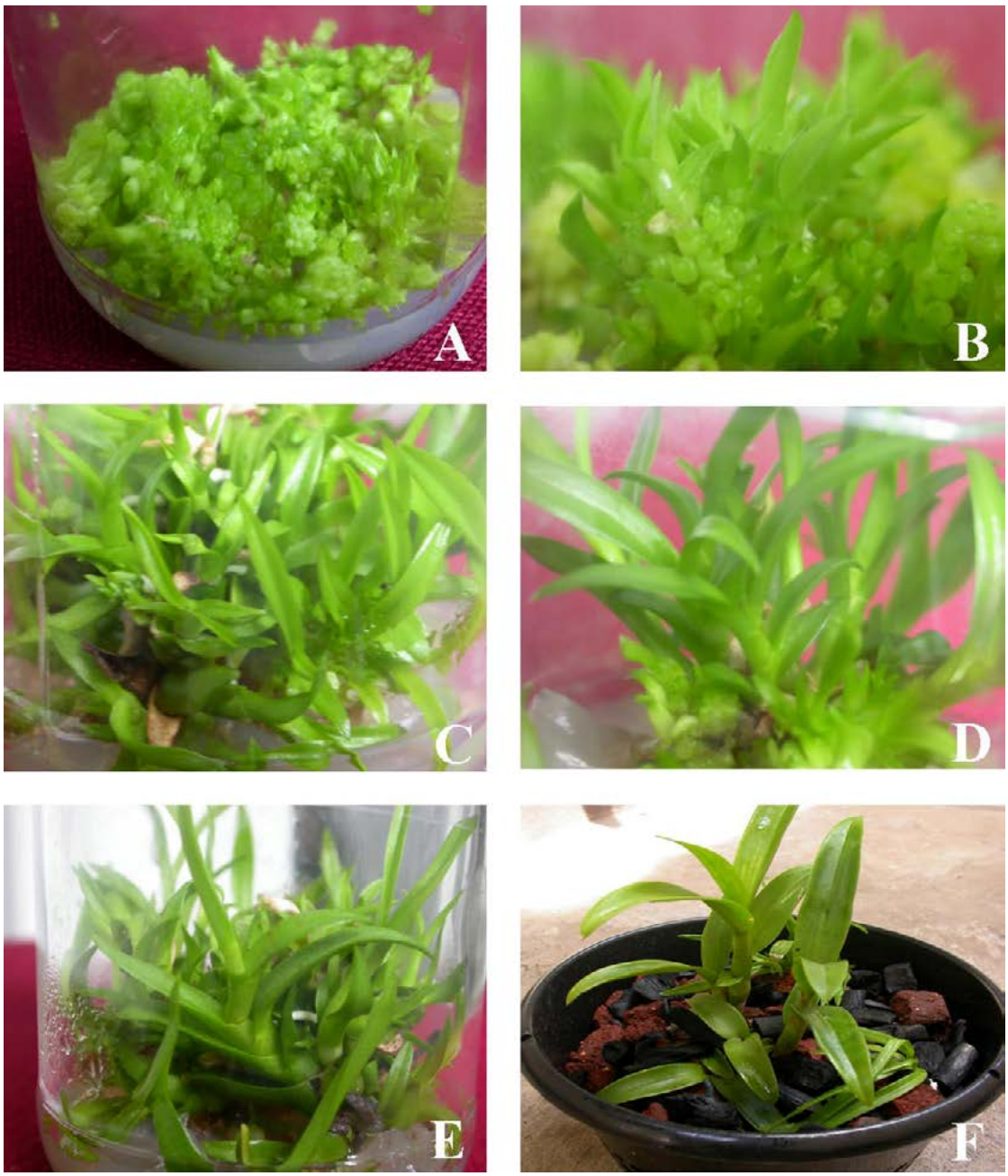

FIGURE 1. Different stages of immature seed germination and regeneration of plants of *Dendrobium aggregatum* on MS medium. A — PLBs. B — PLBs and young plants. C, D — Developed plantlets. E, F — Potted plant.

(Fig. 1). About 95% of the potted plants survived after one month in the shade house. The transplanted plants were acclimatized in the shade house for 1-2 months and transferred to the environmental condition. The present investigation revealed that concentrations of BAP and ANA along with CW in a MS medium influenced *in vitro* seed germination, production of protocorms, shoot multiplication and root initiation. The *in vitro* raised seedlings were successfully established in potting medium.

In conclusion, a simple, efficient and commercially viable protocol for germination and mass propagation of *D. aggregatum* from green capsules has been established. Using this protocol, viable, uniform and healthy plants with maximum survival rate can be produced for large scale cultivation and conservation.

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