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Asymbiotic Seed Germination and Seedling Development of a Medicinally Important Epiphytic Orchid, *Dendrobium crepidatum* Lindl. & Paxton

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Abstract

Dendrobium crepidatum Lindl. & Paxton is an important medicinal orchid with high alkaloid content having pharmacological activities. The species has been threatened due to deforestation and overexploitation therefore, a conservation strategy was needed. Asymbiotic seed germination has been used for conservation and commercial production of different orchids. The current study evaluated the effects of capsule maturity and media composition for asymbiotic seed germination and seedling development using capsules collected 118 and 148 days after pollination (DAP) of *D. crepidatum* flowers. The seed germination was highest for half strength Murashige and Skoog (MS) medium in 70 days of culture with 41±0.76% protocorm development for late capsule group (148 DAP capsules) and 36.33±0.96% for early capsule group (118 DAP capsules). Also, 148 DAP capsules showed early germination in terms of all three seed germination stages in comparison to 118 DAP capsules. The seedling development was studied across 12 different combinations of half strength MS media, where half strength MS medium supplemented with 2 ìgmL^{-1} 6-benzylaminopurine (BAP) and 1 ìgmL^{-1} 1 naphthaleneacetic acid (NAA) showed highest growth on measuring leaf number per shoot (3.25±0.47, $F_{11,36} = 7.075$ with $p < 0.05$) over 100 days of sub-culture period. Thus, relatively more mature seeds collected from 148 DAP capsules were found to be more suitable for asymbiotic seed germination with the use of half strength MS medium. The culture method thus established may facilitate conservation and large-scale cultivation of this medicinal orchid.

Keywords: Capsule, Days after pollination (DAP), Murashige and Skoog Medium, Protocorm

Introduction

Dendrobium crepidatum Lindl. & Paxton, is an epiphytic or lithophytic orchid species, flourishes in southern China, northern Indochina (Laos, Myanmar, Thailand, Vietnam) and the eastern Himalaya (Assam, Arunachal Pradesh, Sikkim, Bangladesh, Nepal) regions (Chowdhery, 2001; Raskoti, 2009; Rokaya et al., 2013). It possesses characteristic green, terete and pendulous stem which bear lanceolate leaf and white pinkish flower. *D. crepidatum* is being used in traditional Chinese pharmacopoeias (Bao et al., 2001). The alkaloids reported from the plant possess anti-inflammatory properties (Hu et al., 2016) and promote nerve growth in cell lines (Li et al., 2013). The species however, is threatened due to deforestation and overexploitation in Nepal (Raskoti, 2009). To avoid extinction and tapping of the possible pharmaceutical resources, the *D. crepidatum* species needs a conservation strategy.

An *ex situ* conservation approach involving *in vitro* techniques like seed germination, mass propagation, cryopreservation etc. have been used for conservation of endangered orchids (Decruse et al., 2003; Mohanty et al., 2012a; Bhattacharya et al., 2017). Among these, seed germination on in vitro culture system could be investigated as a conservation strategy and tool for mass production of *D. crepidatum*. Seeds are much more desirable than other tissues for in vitro culture, as plant development by seed propagation promotes inherent genetic variation ensuring survival on habitat restoration in a variety of environmental conditions (McCargo, 1998). In nature, orchid species relies on symbiotic fungus for germination (Mitchell, 1989). However, almost all species can be grown asymbiotically in presence of suitable nutrient medium conditions (Manning & van Staden, 1987). An efficient propagation technique for orchids has been developed using orchid seeds asymbiotically

(Arditti & Ernst, 1993) which has been utilized for production of different commercial and endangered orchids (Kauth et al., 2006; Stewart & Kane; 2006, Sgarbi et al.; 2009, Mohanty et al., 2012b).

The asymbiotic seed germination methods developed for many different orchid species vary in their composition and are largely species-specific (Arditti & Ernst, 1993; Zeng et al., 2013). Success of asymbiotic germination of orchid seeds depends upon maturity of seed capsule, physical germination conditions and the growth media constituents (Arditti, 1967; Zeng et al., 2013). The effect of maturity of seed capsule has been measured on the basis of number of days after pollination (DAP) as an important criterion for successful in vitro asymbiotic seed germination in *Paphiopedilum* (Lee, 2007; Long et al., 2010). Exogenous auxins are considered non-essential for seed and seedling development of orchids (Tamanaha et al., 1979) while addition of 6-benzylaminopurine (BAP) improved seed germination and protocorm proliferation (David et al., 2010; Nongdam & Tikendra, 2014). Following study was performed to evaluate the effects of capsule maturity and culture media on seed germination of *Dendrobium crepidatum* and to assess in vitro growth of its seedlings across various media combinations.

Materials and Methods

Plant materials

Unripe green capsules of *Dendrobium crepidatum* were collected from the garden of Central Department of Botany, Tribhuvan University, Kirtipur, Nepal. Flowers of *D. crepidatum* were hand-pollinated in the last week of April, 2018. Capsules were collected from the pollinated flowers in two different batches: one at the end of August 2018 categorized as early capsule group (118 DAP capsules) and the other at the end of September 2018 categorized as late capsule group (148 DAP capsules).

Surface sterilization and seed inoculation

Collected capsules were washed for 30 min. under running tap water with the addition of 3 drops of Tween 20 solution. Surface sterilization was performed using 1% Sodium hypochlorite (NaOCl) solution for 7 min. inside a laminar flow cabinet, washed five times with sterile double distilled water and finally flame sterilized for few seconds after dipping in 70% ethanol for 5 minutes. The sterilized capsules were split open longitudinally with a sterile surgical blade and seeds were inoculated on MS (Murashige & Skoog, 1962) medium and MS medium supplemented with plant growth regulators (PGRs).

Culture medium and in vitro growth conditions

MS medium was prepared utilizing their component stock solutions that included macro and micronutrients, iron salts and vitamins, prepared in concentrated solutions and stored at 4 ºC before use. During preparation of MS medium, the stock solutions were added successively with thorough mixing in a conical flask for desired volume. Sucrose (3%) was added and a near medium volume was made with double distilled water then pH 5.8 was adjusted. MS medium was supplemented with PGRs, viz., 2,4 dichlorophenoxyacetic acid (2,4-D), 1 naphthaleneacetic acid (NAA) and 6 benzylaminopurine (BAP), individually and in combinations at different concentrations of 0.5 2.0 ìgmL^{-1}. Desired volume was then made by adding double distilled water and 0.8% agar (w/v) was added. Finally, the medium was autoclaved at 121ºC for 20 min. Cultures were maintained in a culture room at 25±2ºC under equivalent light-dark cycles provided by white fluorescent tubes.

In vitro growth parameters and data recording

Initially, seed germination based on protocorm development and seed germination stages was recorded for two capsule groups using MS medium of three strengths, viz., full, half and quarter, and MS medium supplemented with 2,4-D. A single capsule from each of the capsule groups was utilized for seed germination. Using a 0.5 cm diameter spatula, seeds were suspended in double distilled water in a sterilized test tube for an hour. The seeds were then separated from their suspension using Pasteur pipettes. The seed count was performed

using a hand lens of 10X magnification in the Pasteur pipette. Finally in each MS medium, seeds were inoculated using Pasteur pipette while the double distilled water was removed as much as possible from the culture tube. Seed germination was examined by recording the protocorm development on different media compositions using the following formula:

$$\text{Protocorm development (\%)} = \frac{\text{Number of protocorms developed from inoculated seeds}}{\text{Total number of seeds inoculated}} \times 100$$

The process of seed germination was divided and recorded into three stages (seed swelling stage, green angular protocorm stage and leaf emergence stage), which were modifications of those given by Miyoshi & Mii (1995) for developmental stages of orchid embryos. These stages of seed germination were observed using a hand lens of 10X magnification and number of days for initiation of each stage was recorded across six replicates. Leaf emergence marked the initiation of seedling development.

Seedlings thus developed were transferred to the MS medium of the strength which was observed to best support the initial seed germination. Additionally, MS medium was further supplemented with NAA and BAP, individually and in combinations, and was used to establish seedling growth condition. Thus, a completely randomized experimental design with 12 different media as treatment factor over 4 replicates was performed. To assess the influence of various PGRs on in vitro seedling development, following growth parameters were measured: (1) Leaf number per shoot, (2) Leaf length, (3) Root number per shoot and (4) Root length.

Statistical analysis

In vitro seedling growth parameters viz., leaf number per shoot, leaf length, root number per shoot and root length were subjected to analysis of variance (ANOVA) with means compared by Duncan's multiple range tests (Duncan, 1955) using SPSS v25 program.

Results and Discussion

Selfing of four flowers of *Dendrobium crepidatum* resulted in 100% success of capsule formation indicating self-compatibility of the orchid species as suggested by Vasudevan & Van Staden (2010) in *D. nobile*. The major structural changes which occurred during in vitro culture of *D. crepidatum* seeds were studied with capsules collected in two groups. Initial seed swelling was observed due to absorption of water and nutrients from culture medium. The phenomenon was similar to that observed in all orchid seeds during in vitro germination prior to development of protocorm (Hossain et al., 2010). After 70 days of culture, protocorm development in initial seed germination medium was recorded. Comparatively higher percentage of protocorm development was observed

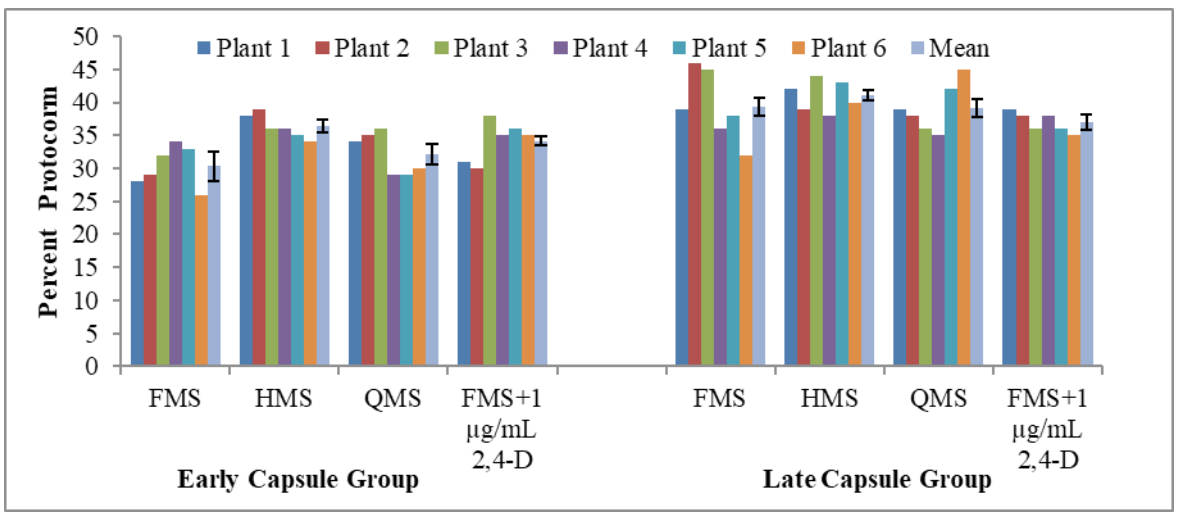

Figure 1: Effect of medium composition on protocorm development from seeds harvested from two different capsule groups of *Dendrobium crepidatum*.

in late capsule group than in early capsule group (Figure 1). This finding contradicts with the earlier observations for orchid seed germination as lower germination frequencies have been achieved by culturing mature seeds than immature seeds (Arditti et al., 1982; Rasmussen, 1995), since on maturity, integuments become impermeable to water (Kauth et al., 2008). However, to another view, sparse cuticular deposition in mature orchid seeds can make testa less hydrophobic resulting in greater germination of mature seeds (Hsu & Lee, 2012). Further, longer in vivo growth period after pollination may have resulted in greater histodifferentiation of developing embryo (Raghavan, 1997; Yeung, 2017) before in vitro culture making late capsule group more suitable for germination.

The half strength MS medium was found to be most suitable with 41±0.76% protocorm development for late capsule group and 36.33±0.96% for early capsule group. Henceforth, half strength MS medium was considered as an optimal medium for seedling development. The half strength MS medium has also been reported to show a significant seedling development in *Dendrobium transparens* (Sunitibala & Kishor, 2009) and in *D. chrysotoxum* (Kaur & Bhutani, 2011). Intervening callus formation was similar in all tested media causing decrease in protocorm development and differentiation. Cytokinin has been considered indispensable for orchid seed germination (Manning & van Staden, 1987), but for *D. crepidatum*, seeds were successfully germinated without supplementing any cytokinin which suggested presence of sufficient volume of endogenous cytokinin necessary for seed germination. Similar germination without PGR supplementation was reported in *Encyclia* aff. *Oncioides* (Znaniecka et al., 2005).

In this study, the effect of maturity of capsule on seed germination was by collecting the capsules at two different durations: early capsule group collected 118 days after pollination (DAP) and early capsule group collected 148 days after pollination. The capsules collected seeds of late capsule group showed early germination in terms of all three seed germination stages in comparison to those of early capsule group (Figure 2). The early seed germination from 148 DAP capsules may be due to appropriate age of the capsules for efficient protein mobilization during rehydration and embryonic unlignified testa allowing the permeability to nutrients (Long et al., 2010; Zeng et al., 2012). However, similar study of effect of maturity of capsule showed early seed germination from 90-120 DAP capsules in *Paphiopedilum godefroyae* (Lee, 2007) and 170-190 DAP capsules in *P. villosum var. densissimum* (Long et al., 2010). Hence, although capsule maturity affects seed germination, this effect seem to vary from orchid species to species as suggested by Deb & Pongener (2013). These germination responses, however, were slower with leaf emergence on 83.29±1.66 after inoculation for late capsule group and 89.87±1.92 days after inoculation for early capsule group, probably due to absence of PGRs in the medium as suggested by Roy et al. (2011) in *Vanda coerulea*, Decruse et al. (2013) in *Eulophia cullenii* and Nenekar et al. (2014) in *Eulophia nuda*.

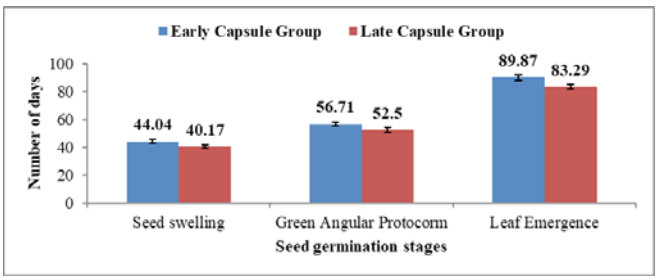

Figure 2: Changes recorded during seed germination from two different capsule groups of *Dendrobium crepidatum* over culture period.

Germinated seedlings attained maximum growth when sub-cultured on half strength MS medium supplemented with 2µg.mL^{-1} BAP and 1µg.mL^{-1} NAA (Table 1), the growth being measured on the basis of leaf number per shoot (3.25±0.47, $F_{11,36}$ = 7.075 with $p < 0.05$). This indicated a synergistic effect of auxin and cytokinin on leaf induction. Similar results of leaf development under the influence of cytokinin and auxin were observed in *Cattleya aurantiaca* (Melissa et al., 1994), *Vanda spathulata* (Decruse et al., 2003) and *Cymbidium aloifolium* (Deb & Pongener, 2011). The comparison

of leaf numbers per shoot across all tested media using Duncan's multiple range test showed similar growths between half strength MS medium supplemented with 0.5µg.mL^{-1} NAA (2.5±0.28) or 1µg.mL^{-1} NAA (2.5±0.28) and half strength MS medium supplemented with 2µgm.L^{-1} BAP and 1µg.mL^{-1} NAA medium. All tested media supported seedling development during the study (Table 1) but the growth rate measured on the basis of leaf length, root number per shoot and root length did not validate homogeneity of variances of data.

Since symbiotic orchid seed germination revealed production of cytokinin by several mycorrhizal fungi (Crafts & Miller, 1974), the effects of addition of exogenous BAP on seed germination was studied. The seed germination rate decreased with the increasing concentration of BAP (Table 1) as there was high callus induction with increasing BAP concentration similar to the observations made by Roy et al. (2007), and by Nongdam & Tikendra (2014) in *Dendrobium chrysotoxum*. Among the tested media, the half strength MS medium supplemented with 2µg.mL^{-1} BAP and 1µg.mL^{-1} NAA showed maximum seedling growth measured as the leaf number per shoot.

Conclusion

In the current stuydy, asymbiotic seed germination and seedling growth of *Dendrobium crepidatum* was evaluated in terms of protocorm, leaf and root development. The seed germination competence was found to be influenced by simple nutritional requirements without using exogenous hormones. Earlier seed germination and greater protocorm development were recorded for relatively mature capsules collected 148 days after pollination. Further experiments are necessary to support the growth response of orchid seeds with the maturity of capsule. Use of half strength MS medium supplemented with 2µg.mL^{-1} BAP and 1µg.mL^{-1} NAA caused faster seedling development when measured on the basis of leaf number per shoot. The culture conditions thus established may facilitate conservation and large-scale cultivation of this medicinal orchid.

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Table 1: Effect of different PGRs on seedling development of *Dendrobium crepidatum* after 100 days of sub culture in half strength MS medium, where values represent mean±SE while '-' represent no response

NAA (µgmL^{-1})	BAP (µgmL^{-1})	Leaf number per shoot*	Leaf length in cm	Root number per shoot	Root length in cm
-	-	1.5±0.28^{abc}	0.89±0.18	-	-
0.5	-	2.5±0.28^{de}	1.27±0.09	0.25±0.25	0.162±0.16
1	-	2.5±0.28^{de}	1.46±0.08	1.00±0.41	0.727±0.24
1.5	-	1.75±0.25^{bcd}	1.15±0.02	1.00±0.41	0.627±0.22
2	-	2.00±0.00^{cd}	1.30±0.11	1.75±0.25	1.022±0.07
-	0.5	1.00±0.40^{ab}	0.577±0.20	1.25±0.25	0.952±0.15
-	1	1.25±0.25^{abc}	0.96±0.06	1.00±0	0.865±0.07
-	1.5	0.75±0.25^{a}	0.787±0.27	1.00±0.40	0.607±0.24
-	2	1.00±0.00^{ab}	0.535±0.04	-	-
1	1	2.00±0.40^{cd}	1.317±0.22	2.25±0.47	1.36±0.13
1	2	3.25±0.47^{e}	1.327±0.12	1.75±0.25	1.112±0.08
2	1	0.75±0.25^{a}	0.600±0.21	1.75±0.47	1.382±0.13

*The values followed by the same letter are not significantly different as determined by Duncan's Multiple Range Test ($p<0.05$)

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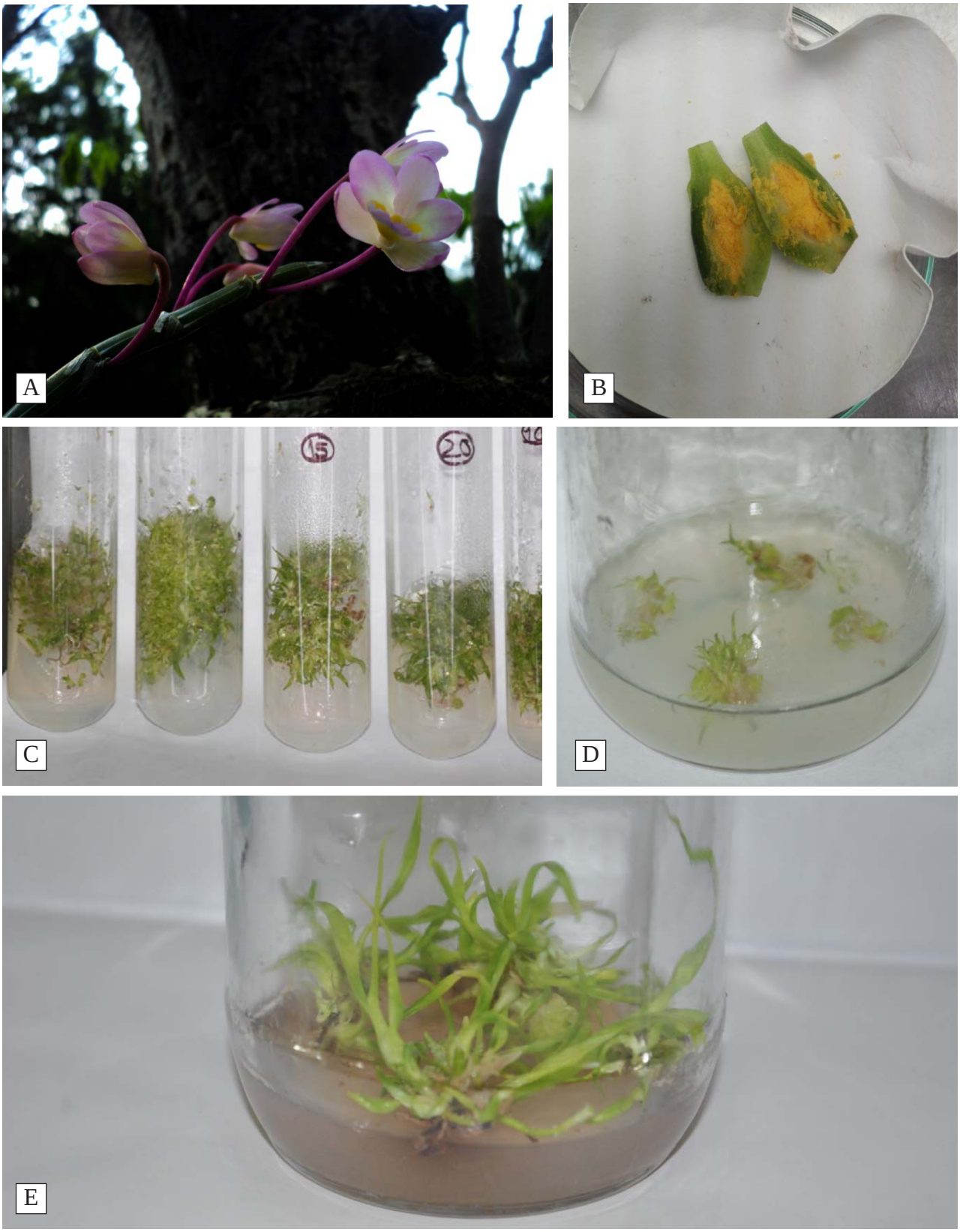

Figures: In vitro culture of *Dendrobium crepidatum* A) Flowers of *Dendrobium crepidatum* Lindl. & Paxton, B) A longitudinally dissected capsule of *D. crepidatum*, C) Seedlings developed in half strength MS medium, D) Seedlings sub cultured in half strength MS medium supplemented with 1ìgmL^{-1} NAA, E) *D. crepidatum* plantlets developed in half strength MS medium supplemented with 2ìgmL^{-1} BAP and 1ìgmL^{-1} NAA.