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IN VITRO ASYMBIOTIC SEED GERMINATION AND SEEDLING DEVELOPMENT IN *COELOGYNE FIMBRIATA* LINDL.

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Abstract

The germination potential of immature seeds of *Coelogyne fimbriata* Lindl. was evaluated on Mitra *et al.* (1976, M) medium and its combinations with and without growth additives [YE (2 gl^{-1})]; [P (2 gl^{-1})]; auxin [IAA (1 mgl^{-1}), NAA (1 mgl^{-1})]; cytokinin [KN (1 mgl^{-1})]; and AC (2 gl^{-1}). In the basal medium, 92.25±1.26% seeds showed germination response. Additional presence of IAA proved almost ineffective, however, NAA in the medium, delayed the protocorm development and subsequent morphogenetic stages leading to seedling development. Seedlings were obtained in 198.00±1.63 days. Presence of KN in the nutritional regime advanced the onset of germination and robust seedlings were obtained in 172.25±1.70 days. P and YE in the medium acted similarly during early stages of seed germination; these proved beneficial not only in considerably enhancing the germination frequency (100.00±0.00) but also in advancing the onset of germination. Seedlings with 2-3 leaves and 1-2 roots were obtained in 168.00±1.63 days. AC in the medium generally proved beneficial for enhancing the frequency and advancing the onset of germination in all the tested combination. Seedlings were transferred to clay pots containing potting mixture (brick-pieces, charcoal, moss) in ratio of 1:1:1 and nearly 75% of seedling survival was recorded.

Introduction

THE GENUS *Coelogyne* Lindl. comprises over 200 species and is distributed from the Himalayas throughout South East Asia and the tropical Pacific with the centre of distribution in Borneo. It is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2012). *Coelogyne fimbriata* Lindl. is a small to medium, cool growing plant found on limestone hills (lithophytic) or on trees (epiphytic) in evergreen forests. It inhabits tropical and sub-tropical zone at an elevation of 800-2200 m. The plant bears broad pseudobulbs, cylindric and slightly thickened towards sheathing base, arising from a stout; leaves coriaceous, elliptic-oblong, acute, tapering at the base, the short petiole; inflorescence terminal, smooth, and zigzag. It is a floriculturally important species bearing long lasting, fragrant, and buff-yellow coloured flowers with a fringed lip. The species is endangered due to habitat destruction and heavy collection pressures. Hence, present study was planned to assess the asymbiotic germination potential of seeds *in vitro* and subsequent seedling development with a view to developing mass propagation protocol for the species.

Material and Methods

Seed Sterilization

In the present study, immature seeds (38 wap) were used as explant. The green and undehisced pods of *C. fimbriata* were collected from Sikkim, India. Young capsules were first scrubbed with Teepol (0.01%), washed thoroughly under running tap water for 15-20 min, dipped in 70% ethyl alcohol for 30 sec, flamed and these were then surface sterilized for 8 min with $HgCl_2$ solution (0.1%). These were also treated with Streptomycin (0.02%) and Bavistin (0.01%) solutions for 6-7 min each and repeatedly washed 5-6 times with sterilized distilled water, so as to remove all the traces of sterilizing agents. The sterilized capsules were then split open longitudinally with a sterilized blade to scoop out the immature seeds, under aseptic conditions, in laminar air flow cabinet.

Culture Media and Incubation Conditions

The germination potential of immature seeds was tested on M medium and its combinations with and without growth additives [YE (2 gl^{-1})]; [P (2 gl^{-1})]; auxin [IAA (1 mgl^{-1}), NAA (1 mgl^{-1})]; cytokinin [KN (1 mgl^{-1})]; and AC (2 gl^{-1}). Sucrose (2%; Thermo Fisher Scientific, USA) was added as the carbohydrate source and the medium was gelled by using 0.9% agar (Thermo Fisher Scientific, USA) and pH of the media was adjusted at 5.8 by using 0.1N NaOH or HCl. The nutrient medium was also darkened by adding 0.2% w/v activated charcoal (AC) (M. Sarabhai Chemicals, Baroda). The cultures were maintained at 25±2^{0}C temperature and exposed to 12 hr illumination of 3500 lux intensity. The cultures were examined periodically (at 3 days interval) and the responses were recorded on the basis of visual observations. Sub-culturing was carried after 6 wks into fresh medium and 8 replicates were used for each treatment.

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Table 1. *In vitro* seed germination and seedling development on Mitra *et al*. (1976, M) medium, in *Coelogyne fimbriata*.

Additives	Germination response (%)	Time taken in days for						Remarks
		Onset of germination	Spherule formation	Protocorm formation	Emergence of first leaf primordium	Emergence of first root primordium	Formation of complete seedling	
M (Basal)	92.25±1.26^{abc}	19.00±0.82^{e}	33.25±0.50^{cde}	49.00± 2.45^{cde}	79.50±0.58^{d}	95.00±2.16^{de}	181.25±0.50^{e}	Healthy seedlings
AC	98.00±2.16^{def}	17.00±0.82^{cd}	31.75±0.96^{cd}	47.50±1.29^{cd}	75.00±0.82^{c}	92.25±1.25^{cd}	184.75±1.70^{ef}	Healthy seedlings
IAA	90.25±1.70^{abc}	20.00±1.63^{f}	28.25±1.25^{bc}	54.00±0.58^{f}	83.00±2.16^{de}	102.25±1.25^{efg}	186.25±0.50^{f}	Healthy seedlings
IAA + AC	96.75±0.96^{cde}	20.00±1.63^{f}	27.00±0.82^{bc}	50.50±0.96^{de}	80.75±0.50^{de}	97.25±1.70^{ef}	178.75±0.96^{e}	Healthy seedlings
NAA	94.25±1.26^{cd}	19.00±0.82^{e}	36.00±0.96^{de}	58.00±0.2.16^{fg}	91.25±2.16^{f}	112.25±2.06^{h}	198.00±1.63^{g}	Delayed seedling development
NAA+ AC	96.00±1.63^{cde}	17.00±0.82^{cd}	34.00±1.63^{cde}	56.00±0.82^{fg}	89.00±0.82^{f}	106.00±0.82f^{g}	196.00±2.16^{g}	Delayed seedling development
KN	84.25±1.26^{a}	17.00±0.82^{cd}	32.00±1.63^{cd}	50.00±1.41^{de}	79.00±2.94^{cd}	94.25±0.96^{de}	172.25±1.70^{bcd}	Better leaf and root growth
KN + AC	89.00±2.94^{ab}	15.00±0.82^{bc}	31.00±0.82^{cd}	49.00±2.16^{cde}	74.00±0.82^{c}	90.00±0.82^{cd}	170.00±1.63^{bc}	Better leaf and root growth
P	100 .00±0.00^{f}	14.00±1.63^{ab}	28.25±1.70^{bc}	42.25±0.82^{ab}	62.75±0.96^{a}	86.00±0.82^{b}	168.00±1.63^{b}	Early seedling formation
P + AC	100.00±0.00^{f}	11.75±0.96^{a}	26.00±0.82^{bc}	40.00±2.16^{ab}	60.00±1.63^{a}	83.00±2.16^{a}	160.00±1.63^{a}	Early seedling formation
YE	100.00±0.00^{f}	12.00±1.63^{a}	24.50±1.29^{b}	48.00±0.96^{cd}	66.00±0.82^{b}	90.00±1.63^{cd}	172.00±2.16^{bcd}	Protocorm multiplication; luxuriant growth of seedlings
YE+AC	100.00 ±0.00^{f}	10.25±0.50^{a}	21.25±0.50^{a}	46.25±0.82^{c}	61.75±1.25^{a}	89.50±1.29^{cd}	170.75± 0.96^{bc}	Rapid protocorm multiplication

Entries in column no. 2 to 7 are Mean ± S.E.; same alphabetical letter in the superscript denotes that the corresponding means are in the same group using Tukey test at 5%.

Acclimatization

Healthy seedlings with 2-3 well grown leaves and 1-2 roots were gradually hardened *in vitro*, by sequential elimination of growth additives, vitamins, sucrose, and minor salts from the nutrient matrix at 15 days interval. The hardened seedlings were washed thoroughly with lukewarm water to remove agar and potted in clay pots, using charcoal, moss, brick-pieces (1:1:1) as the potting media.

Statistical Analysis

Data was analyzed by SPSS software and subjected to one way analysis of variance was performed with respect to each response (average ± standard error against each additive is mentioned in Table 1). As ANOVA results showed the non significant difference of additives at 5% level of significance, various groups of additives showing identical/similar response were formed statistically. To this end, Tukey Test was performed at 5% level with respect to each response.

Result and Discussion

The immature seeds (38 wap) procured from the green undehisced capsules were found to be monoembryonate. These were inoculated on M medium and its various combinations with growth additives. The seeds showed first signs of germination (*i.e.* swelling of embryos) in all the tested combinations. The germination frequency and associated morphogenetic changes leading to seedling development were, however, differently affected by the chemical stimulus *in vitro* (Table 1). The nutritional requirements of different orchid species depend upon their inherent genetic makeup; some have very stringent needs while others can grow on a wide range of nutritional regime (Arditti, 1967). Significantly, the nutrient requirements often vary during the different developmental stages in the same taxon (Bhattacharjee and Hossain, 2015; Bhatti *et al.,* 2017; Borah *et al.,* 2015; Kaur *et al.,* 2017; Kondo *et al*., 1997; Mitra, 1986, Pathak *et al.,* 2001). However, Knudson (1921, 1922) successfully germinated the seeds of *Cattleya*, *Epidendrum,* and *Laelia* on a sugar rich nutrient medium *in vitro*, thus bypassing the fungal requirement. Since then, this technique of asymbiotic seed germination has been positively tested in a large number of orchid species and hybrids (Anuprabha and Pathak, 2012; Chen *et al*., 2015; Decruse and Gangaprasad, 2018; Dutra *et al*., 2009; Franceschi *et al*., 2019; Gangaprasad *et al*., 1999; Hossain *et al*., 2008, 2009, 2010; Jamir *et al*., 2002; Kalimuthu *et al*., 2007; Mohanty and Salam, 2017; Kondo *et al*., 1997; Lo *et al*., 2004; Markovina and Mc Gee, 2000; Pathak *et al*., 1992, 2001, 2011a, b; Parmar and Pant, 2016; Piri *et al*., 2013; Rao and Barman, 2014; Sibin and Gangaprasad, 2016; Stewart and Kane, 2006; Valletta *et al*., 2008; Vij *et al*., 1995).

In the basal medium, 92.25±1.26% seeds showed germination response. During germination, the embryos swelled and emerged out of the seed coats through lateral slits as spherules within 19.00±0.82 days. The germinating entities turned green and developed into protocorms in 49.00±2.45 days. Chlorophyll production was invariably a pre-protocorm phenomenon. According to Stoutamire (1974) and Vij *et al.* (1988), pre-protocorm development of chlorophyll is almost universal in epiphytic orchids. The protocorms differentiated first leaf and root primordia in 79.50±0.58 and 95.00±2.16 days of inoculation respectively and complete healthy seedlings were obtained in 181.25±0.50 days. Additional presence of AC in the medium proved beneficial for enhancing the frequency and advancing the onset of germination. Literature studies revealed a beneficial effect of this darkening agent in promoting germination frequency, protocorm multiplication, chlorophyll synthesis, and healthy growth of seedlings in a large number of orchid species (Arditti and Ernst, 1984; Hossain *et al*., 2009; Pathak *et al*., 2001; Piri *et al*., 2013; Vij and Pathak, 1988; Yam *et al*., 1989).

Additional presence of IAA proved almost ineffective; onset of germination and subsequent seedling development were however, further delayed (186.25±0.50 days). Literature studies also showed its inhibitory role during germination in *Dendrobium nobile* (Miyazaki and Nagamatsu, 1965), *Coelogyne viride* and *Dactylorhiza purpurella* (Hadley, 1970); seedling development was also delayed in its presence in *Cymbidium lowianum* (Sood, 1984), and *Dendrobium chrysanthum*, *Rhynchostylis retusa,* and *Vanda testacea* (Pathak, 1989).

Incorporation of AC in the medium advanced the protocorm formation (50.50±0.96 days) and seedling development (178.75±0.96 days) (Fig. 1i). NAA in the medium, however, delayed the protocorm development and subsequent morphogenetic stages leading to seedling development. Seedlings were obtained in 198.00±1.63 days. Perusal of literature revealed that it enhanced germination [*Coelogyne stricta* (Parmer and Pant, 2016), *Cymbidium aloifolium* (Deb and Pongener, 2011), *Malaxis acuminata* (Arenmongla and Deb, 2012), *Vanda dearei* (Jualang *et al*., 2014), and *Vanda testacea* (Naha *et al*., 2013)]. AC in the medium advanced the rhizogenesis (106.00±0.82 days). Presence of KN in the nutritional regime advanced the onset of germination and robust seedlings were obtained in 172.25±1.70 days (Fig. 1j). KN was reported to impair germination

frequency in *Coelogyne punctulata* (Sharma and Tandon, 1986) and retard seedling development in *Bulbophyllum leopardinum* and *Dendrobium heterocarpum* (Arora, 1990) but it was effectively used to induce seed germination in *Vanda stangeana* (Bembemcha *et al.*, 2016) protocorm multiplication in *Cymbidium eburneum* (Mahant, 1991), and *Aerides multiflora, Eria spicata*, and *Pholidata articulata* (Pathak,

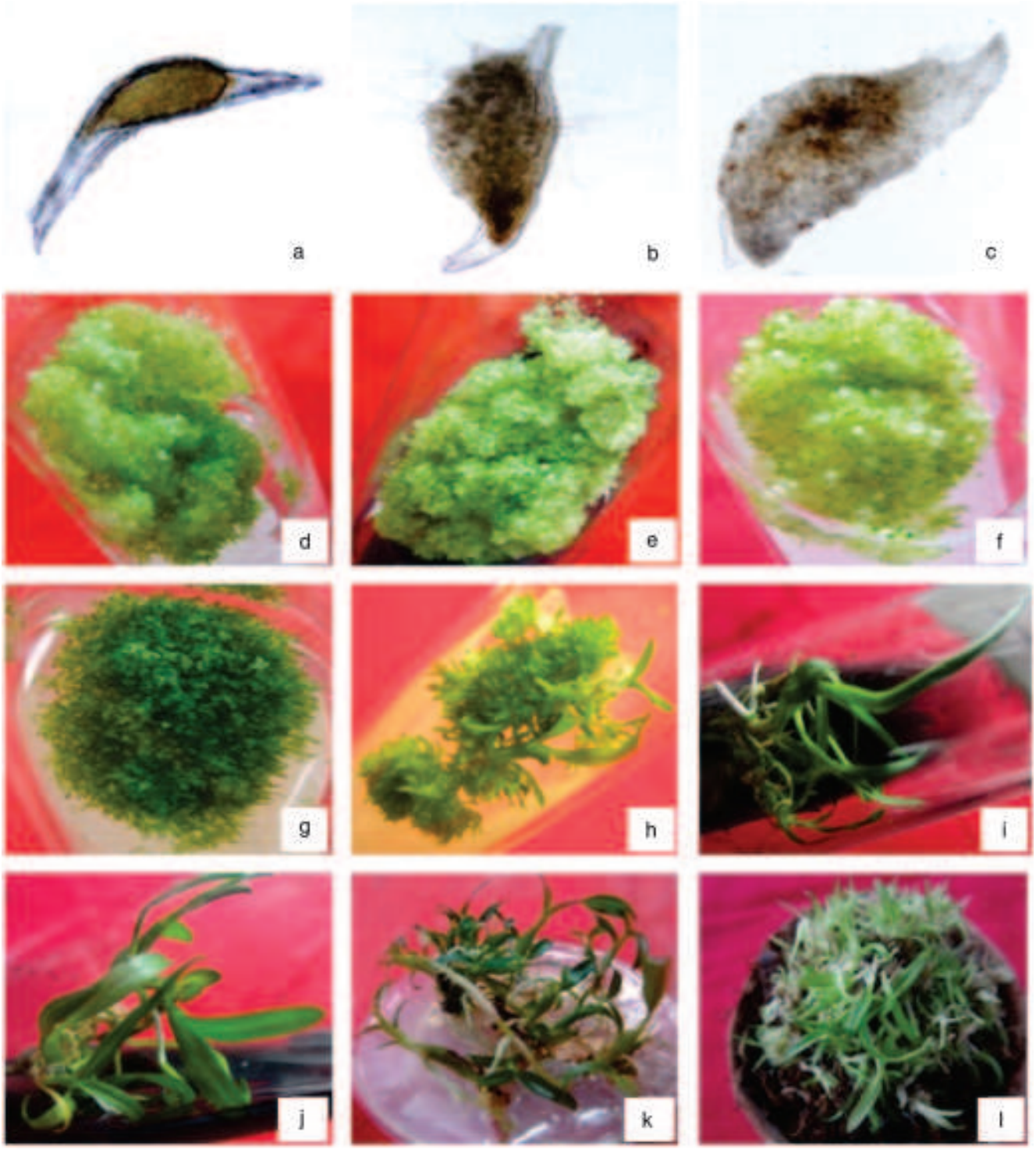

Fig 1. a-l, *In vitro* seed germination in *Coelogyne fimbriata*: a, Immature seed at the time of inoculation (×10); b, Lateral rupturing of seed coat (×10); c, Spherule formation (×10); d-g, Protocorms multiplication [M, M+IAA (1 mgl^{-1}) + AC (2 gl^{-1})], [M+KN (1 mgl^{-1})], [M+YE (2 gl^{-1})]; h, Differentiation of leaves [M+P (2 gl^{-1})]; i-j, Healthy leaves and root development [M+IAA (1 mgl^{-1})], [M+KN (1 mgl^{-1})]; k, Healthy seedlings [M+YE (2 gl^{-1})]; l, Seedlings transferred to a clay pot.

1989). It also favoured multiple shoot induction in *Satyrium nepalense* (Mahendran and Narmatha Bai, 2009) and seedling growth in *Coelogyne nervosa* (Abraham *et al*., 2012). AC enriched above combination, however, proved ineffective. P and YE in the medium acted similarly during early stages of seed germination; these proved beneficial not only in considerably enhancing the germination frequency (100.00±0.00) but also in advancing the onset of germination. Seedlings with 2-3 leaves and 1-2 roots were obtained in 168.00±1.63 days. However, YE enriched combination induced protocorm multiplication and luxuriant growth of seedling was also evident in the cultures (Fig. 1k). Literature studies revealed that YE enhanced protocorm multiplication in *Coelogyne barbata* and *Cymbidium eburneum* (Mahant, 1991); *Satyrium nepalense* and *Vanda cristata* (Pathak, 1989). YE though accelerated protocorm growth in *Rhynchostylis retusa* and *Vanda testacea* (Vij *et al*., 1981) and *Vanda dearei* (Jawan *et al.,* 2010). A perusal of literature revealed that peptone favoured germination, protocorm multiplication, differentiation thereof, and seedling growth in many species [*Cymbidium aloifolium* (Hossain *et al*., 2009), *Cymbidium macrorhizon* (Vij and Pathak, 1988), *Gastrochilus calceolaris* (Pathak *et al*., 2011), and *Phalaenopsis* hybrid (Shekarriz *et al*., 2014)]. It also favoured highest germination frequency in *Cymbidium giganteum* (Hossain *et al*., 2010), *Dendrobium aphyllum* (Hossain *et al*., 2012), *Paphiopedilum liemianum* (Utami *et al*., 2015) and promoted the growth and advanced development of protocorms in *Epidendrum ibaguense* (Hossain *et al.,* 2008). AC in the P enriched combination proved beneficial for early rhizogenesis and seedling development (160.00±1.63 days), whereas AC in YE containing cultures proved useful for rapid multiplication of protocorms. Healthy seedlings of *Coelogyne fimbriata* were gradually hardened *in vitro*, by sequential removal of growth additives, vitamins, sucrose, and minor salts from the nutrient matrix at 15 days interval. Plantlets were then potted in clay pots, filled with a potting mixture of charcoal, pine bark, brick pieces, and *Sphagnum* moss (1:1:1:1), and hardened for 8 wks in a humidity chamber. Emergence of new roots and leaves were taken as an indication for successful acclimatization of the plantlets. These were regularly irrigated and sprayed with nutrient solutions and showed 70-75% survival rate.

Conclusion

Based on results, M medium is recommended for the *in vitro* germination and protocorm development in *C. fimbriata*. However, nutrient medium containing P proved optimal for germination and subsequent seedling development, and YE enriched medium proved beneficial for culture multiplication.

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