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Research Paper

Influence of *In Vitro* Media on Asymbiotic Germination, Plantlet Development and *Ex Vitro* Establishment of *Coelogyne ovalis* Lindl. and *Coelogyne nitida* (Wall. ex Don) Lindl.

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Seed germination of two endangered orchids, *Coelogyne ovalis* and *C. nitida* was tried on four different culture media. The best germination was observed on Knudson C medium for both *C. ovalis* (87.74%) and *C. nitida* (67.82%). Protocorm volume of *C. ovalis* was better on Murashige and Skoog medium (0.021mm^3) and for *C. nitida* the protocorms were larger on Knudson C medium (0.109mm^3). Plantlets were hardened in the potting mixture containing charcoal and brick pieces (1:2) with 61% and 75% survival for *C. ovalis* and *C. nitida* respectively. This has made it possible to have large scale regeneration of the two orchids through seed germination without a requirement of symbiosis.

Key Words: Coelogyne *spp.*; Media; Asymbiotic Germination; Establishment; Threatened; Orchids

Introduction

Excessive collections, deforestation, utilization of land for agriculture, and urbanization have devastated many native orchid populations. All orchids have small seeds with undifferentiated embryos and no endosperm [1, 2], and they depend on mycorrhizal fungi for germination in nature. Increasing the ability to germinate orchid seeds is an important research topic because it is central to the propagation of orchids for cultivation and for conservation of endangered species, including the possible re-introduction of seedlings back into suitable natural habitats. Using different media and nutrients, asymbiotic seed germination in a large number of orchid species has been accomplished [3-8]. However, germination methods successful for one species are not always applicable to others and procedures for orchids of one region may not be suitable for those from another [9].

The genus *Coelogyne* comprises about 140 species [10], which produce long lasting, fragrant flowers. These are sympodial epiphytes which are distributed across India, China, Indonesia and Fiji. A few species are commonly known as 'necklace orchids', because of their long, pendant, multi-flowered inflorescence. *Coelogyne ovalis* Lindl. and *Coelogyne nitida* (Wall. ex Don) Lindl., the two species chosen for our study are distributed across Nepal, Bhutan and parts of Northeast India. The natural population of these two orchids is being threatened due to over-exploitation for trade and commerce. This communication is the first report on large scale regeneration of *Coelogyne ovalis* and *C. nitida* through asymbiotic seed germination.

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Materials and Methods

Mature capsules of *C. ovalis* and *C. nitida* were collected from plants growing in the net house of Botany Department, North Eastern Hill University, Shillong. These were washed thoroughly with tap water and sterilized by flaming three to four times after dipping in 70% alcohol. The capsules were slit open under aseptic conditions to expose the seeds. The seeds were germinated aseptically on four different nutrient media viz. Knudson C (KC) [11], Vacin and Went (VW) [12], Murashige and Skoog (MS) [13] and Mitra *et al.* (Mitra) [14], each medium supplemented with 3.0 % sucrose, 0.8 % agar and 15 % coconut milk. The pH of the medium was adjusted to 5.8 prior to autoclaving at 1.06 Kg/cm^2 and 121°C for 15 min. The culture tubes were incubated at 25≤2°C under 150μmol m^{-2} sec^{-1} light intensity and 80% humidity.

Seeds cultured on various media were observed regularly for germination. They were considered to have germinated upon emergence of the embryo from the testa. Germination percentage of seeds in different media was determined by examining the seeds microscopically after 60 days of culture. The protocorm volume was determined by using the formula given by Stoutamire [15] for an oblate spheroid (4/3p a^{2}b, where a and b are minor and major semi axes, respectively). Ten replicates were used for each treatment and the experiment was repeated twice.

Plantlets measuring around 5-6 cm with well developed roots were washed thoroughly to remove the agar-gelled medium sticking to them and were then transferred to plastic pots containing different substrata, viz. charcoal, brick pieces (1:2), charcoal, brick pieces (1:1), charcoal, brick pieces, soil (2:1:1), charcoal, brick

pieces, decaying matter (2:1:1), charcoal, brick pieces, decaying matter, soil (2:1:1:1) for hardening. The pots were kept in the glasshouse and were initially covered with polythene sheets for three weeks to maintain high humidity. The plantlets were daily fed with 1:10 diluted MS nutrient salt solution during the period. The percentage survival of the plants was calculated after 12 weeks of transfer. The data was subjected to statistical analysis.

Results

The best seed germination for both *C. ovalis* and *C. nitida* was recorded on KC medium wherein 87.74% and 67.82% germination was obtained respectively (Table 1, 2). The protocorms of *C. ovalis* were larger on MS and KC media as compared to those developed in Mitra and VW (Table 1, Fig. 1a-d). For *C. nitida,* the maximum protocorm volume was recorded in KC medium (Table 2, Fig. 2a-d). For both the species, the subsequent development of protocorms to the next stage with pointed vegetative apex was observed only in case of MS, KC and VW media but not on Mitra. The protocorms multiplied on being sub cultured and gave rise to protocorm-like bodies (plbs). The plbs when left undisturbed resulted in complete plantlets. The plantlets arising from plbs were sub cultured onto fresh KC medium for further growth and development.

Table 1. Effect of different media on seed germination and protocorm development of *C. ovalis*.

Media	% Germination[a]	Protocorm Dimensions		
		Length (mm)	Width (mm)	Volume[b] (mm^3)
Mitra	57.51 ≤ 1.89	0.33≤ 0.009	0.09 ≤ 0.004	0.012≤ 0.001
MS	32.80 ≤ 2.3	0.29≤ 0.008	0.13 ≤ 0005	0.021 ≤ 0.002
KC	87.74 ≤ 1.04	0.25≤ 0.006	0.14 ≤ 0.003	0.020≤ 0.001
VW	29.71 ≤ 2.0	0.28≤ 0.007	0.11 ≤ 0.003	0.015≤ 0.001

≤ S.E

a: For 60 days, LSD (P<0.05) = 19.06, $F_{3,16}$= 202.16*

b. For 60 days, LSD (P<0.05) = 4.66, $F_{3,36}$=26.66*

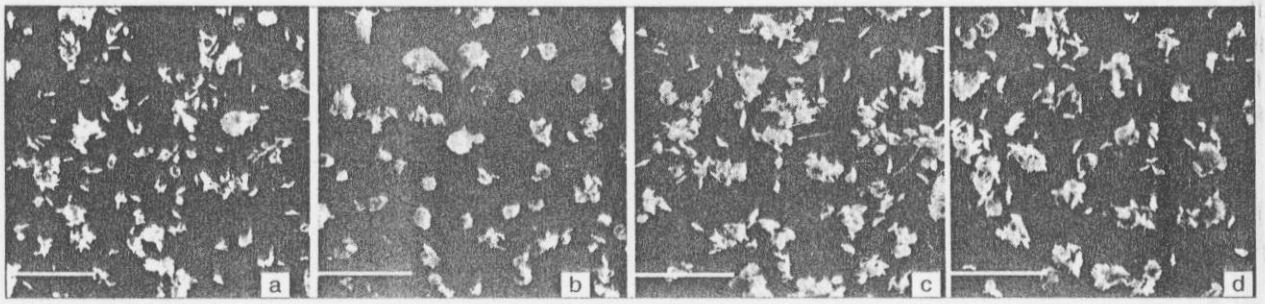

Fig. 1: Coelogyne ovalis: Asymbiotic germination in different media (a) Mitra (b) MS (c) KC (d) VW (bar = 1.0 mm)

Table 2. Effect of different media on seed germination and protocorm development of *C. nitida*.

Media	% Germination[a]	Protocorm Dimensions		
		Length (mm)	Width (mm)	Volume[b] (mm)
Mitra	43.48 ≤ 2.4	1.01 ≤ 0.02	0.13 ≤ 0.003	0.070 ≤ 0.004
MS	61.61 ≤ 2.2	1.12 ≤ 0.02	0.14 ≤ 0.003	0.085 ≤ 0.004
KC	67.82 ≤ 2.9	1.14 ≤ 0.02	0.15 ≤ 0.005	0.109 ≤ 0.008
VW	31.58 ≤ 1.3	0.97 ≤ 0.02	0.13 ≤ 0.004	0.070 ≤ 0005

≤ S.E

a. For 60 days, LSD (P<0.05) = 9.46, $F_{3,16}$= 49.86*

b. For 60 days, LSD (P<0.05) = 0.41, $F_{3,36}$=0.21ns

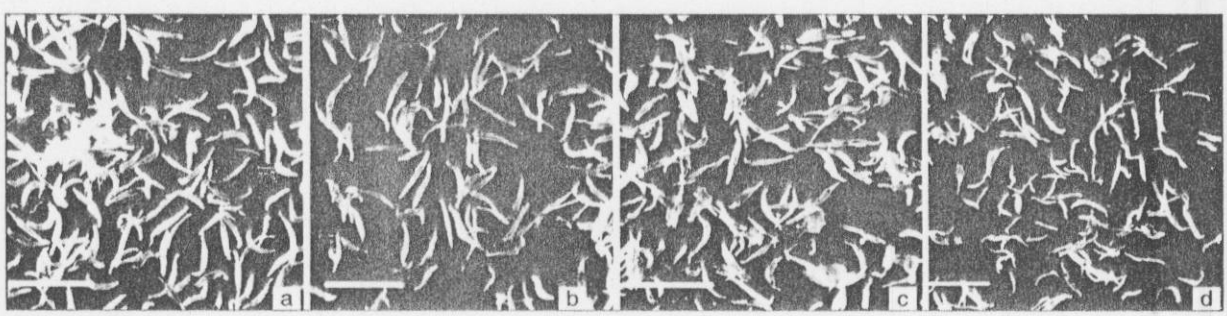

Fig. 2: Coelogyne nitida: Asymbiotic germination in different media (a) Mitra (b) MS (c) KC (d) VW (bar = 1.0 mm)

Within 4 months period, plantlets measuring around 5-6 cm in length developed which were found ideal for potting. Out of the various potting mixtures tried, the mixture containing charcoal and brick pieces was found to be the best substratum for the establishment of plantlets of both *C. ovalis* and *C. nitida*. Hardening of the plants was accomplished within 3-4 months. Around 61% of *C. ovalis* and 75% of *C. nitida* plants survived in the glasshouse after hardening. The plants have been successfully maintained for over three years in the glasshouse.

Discussion

In the present investigation, KC medium was found better for seed germination of both the Coelogyne species. This could be due to the fact that KC medium is quite rich in both macro- and micronutrients. The presence of KI, $MnCl_2$ and Na_2EDTA in larger doses in KC medium as compared to the other media tried could have facilitated better response of the seeds. The orchid seeds have been reported to show both promotory as well as inhibitory response in germination to KC medium [16-19]. This suggests that the requirements of the seeds during germination to media vary from one species to another. Addition of coconut milk (15% v/v) to the medium was found to be beneficial for germination of the seeds. These results are similar to the earlier reports on orchids where coconut water was found to improve seed germination and subsequent seedling development [20-22]. The developing protocorms were found to be round or elliptical in shape with some unicellular absorbing hairs on the basal part and an apex meristem on the tip. In both *C. ovalis* and *C. nitida*, the protocorm volume and seedling development was optimum on KC medium. The maximum protocorm volume on MS medium in case of *C. ovalis* could be due to the availability of nutrients to few developing protocorms.

Hardening of the plants is necessary to develop resistance against physical, chemical and biological factors at the time of transferring them into field conditions. A good growing medium having properties such as maximum water holding capacity, porosity and drainage is essential for proper growth and development of *in vitro* raised plants. The mixture containing charcoal and brick pieces was found to be the best substratum for the establishment of plantlets of both *C. ovalis* and *C. nitida*. These two species, being sympodial epiphytic orchids, have a specialized stem, which sits on the surface of the potting mixture and sends out feeding roots down into the mixture. They also have specially designed aerial roots in addition to the regular root system at the base of the plant. The mixture containing charcoal and brick pieces was found ideal because it not only provided structural support for the roots, but also lots of air spaces between substratum particles for roots to spread out. This study gives a complete protocol for *in vitro* mass propagation of *Coelogyne ovalis* and *Coelogyne nitida* which are two commercially important orchids of Northeast India.

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