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DEVELOPMENT OF *IN VITRO* PROPAGATION PROTOCOL FOR RAPID AND MASS PROPAGATION OF *COELOGYNE NERVOSA* A. RICH., AN ENDEMIC ORCHID OF THE SOUTHERN WESTERN GHATS USING IMMATURE SEEDS

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Abstract

Immature seeds of an endemic, rare and exquisite orchid of the Southern Western Ghats *i.e., Coelogyne nervosa* were harvested seven months after pollination and used for *in vitro* asymbiotic seed germination and subsequent seedling development. The germination of orchid seeds in nature is difficult without the symbiotic association of the mycorrhizal fungi. Therefore, the present research was focussed to study the effect of different organic additives and growth regulators on asymbiotic seed germination and their subsequent development into plantlets. Green capsule with 90% viable seeds, were cultured on liquid Mitra *et al.* (1976, M) and Knudson C (1946, KC) media containing 2% sucrose and different concentrations (0.01%-0.09%) of casein hydrolysate (CH), yeast extract (YE), peptone (P), coconut water (CW 5-25%) and different concentrations of cytokinins *i.e.*, BAP, KN, ZN and 2,i-p (0.1mgl^{-1} and 1 mgl^{-1}). Among the two different nutrient media tested for seed germination, better germination and seedling development was achieved on M medium. Of the different additives tested for seed germination, maximum seed germination was achieved on YE (0.05%) enriched M medium. Media supplemented with various cytokinins also favoured seed germination and seedling development. After 60 days, the protocorms were transferred to solid media and after the third subculture on solid medium, the healthy rooted seedlings were transferred to community pots and 60% establishment rate was recorded.

Introduction

ORCHIDACEAE IS one of the largest and most diverse families of the flowering plants, consisting of about 35,000 species listed under 800 genera (Singh *et al.*, 2007). The widely diverse climatic regions of India are reflected in the wide diversity of its orchid flora. India with 1129 species in 184 genera (Karthikeyan, 2000) is one of the major orchid habitats of the world. The Western Ghats of India is one of the richest areas of the world in terms of biodiversity. The Western Ghats of India harbours 302 species, 3 sub-species and 4 varieties in 80 genera (Kumar, 2006). Kerala has 252 species, 3 sub-species and 1 variety in 79 genera (Kumar and Manilal, 2004). Many wild orchids of Western Ghats like species of *Coelogyne* hold potential genes to contribute in breeding and thus to develop novel orchid hybrids of wider adaptability. For the past years, population of orchid species, especially those distributed in the Western Ghats region are declining due to various developmental activities. These plants can be conserved through *in situ* and *ex situ* methods. Plant tissue culture is an effective tool to conserve plant germplasm and asymbiotic germination of orchid seed and seedling development is an easy way of conservation of orchids.

The small, dust like seeds of the orchids produced in each capsule are highly fragile, nearly microscopic in size and are produced in very large numbers (Mitra, 1971); these are characterized by lack of storage tissues required for seed germination and seedling development. The orchids require a combination of multiple factors for their continued reproduction in nature; in nature, their association with a specific fungal partner, the orchid mycorrhiza, is a pre requisite for orchid seed germination. The rate of seed germination in nature is very poor (Pathak *et al.,* 2001; Vij, 2002). Even in the symbiotic germination, the seeds take a long time for germination and any disturbances in the habitat or physical environment destroys the whole population.

C. nervosa is a rare endemic orchid of Southern Western Ghats (Fig. 1) which grows as an epiphyte and is important for its beautiful flowers. An epiphyte with lemon yellow coloured *pseudobulb*, 3cm high, leaves 2 from the top of the pseudobulb, 15-18cm coriaceous. It has racemes 7-9 cm long, with 3-4 flowers with large, pretty, white *petals*. *Bracts* 2.5 cm long, creamy in bud turning brown with the opening of the flowers. *Sepals* and *petals* subsimilar, white. *Lip* white outside marked with veins of mustard colour inside, three crenulated ridges ending in red points.

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The objective of the present study was to develop a micropropagation protocol for this commercially valuable endemic orchid. The present study was undertaken with a view to establishing an efficient protocol for mass propagation and to study the effects of various organic additives and plant growth regulators (PGRs) on seed germination and growth of seedlings in *Coelogyne nervosa*, a floriculturally important endemic orchid.

Materials and Methods

Green capsules of about seven months maturity (Fig. 2) were collected from Pongalapara region (1550 m) of Agasthyamala in the Southern Western Ghats. At the time of harvest, the green capsules measured 3.6 cm in length and 2.8 cm width and 90% of the seeds were viable as evidenced from microscopic examinations (Fig. 3).

Media

Mitra *et al.* (M, 1976) liquid medium and Knudson C (1946, KC) with 2% sucrose was supplemented with different concentrations (0.01-.09%) of casein hydrolysate (CH), peptone, yeast extract (YE) and coconut water (5-25%) and various plant growth regulators like cytokinins *i.e.*, KN, BAP, ZN and 2, i-p (0.1 and 1 mgl^{-1}) were used for seed germination and protocorm growth (Fig. 4). Agar gelled (0.8%) M medium containing CH, peptone, yeast and coconut water (CW) were used for sub-culturing of the protocorms and subsequent seedling development. After the second subculture, the developing seedlings were transferred to M medium containing different concentrations of banana pulp (2.5-10%; BP) to study their effect on seedling development.

Surface Sterilization

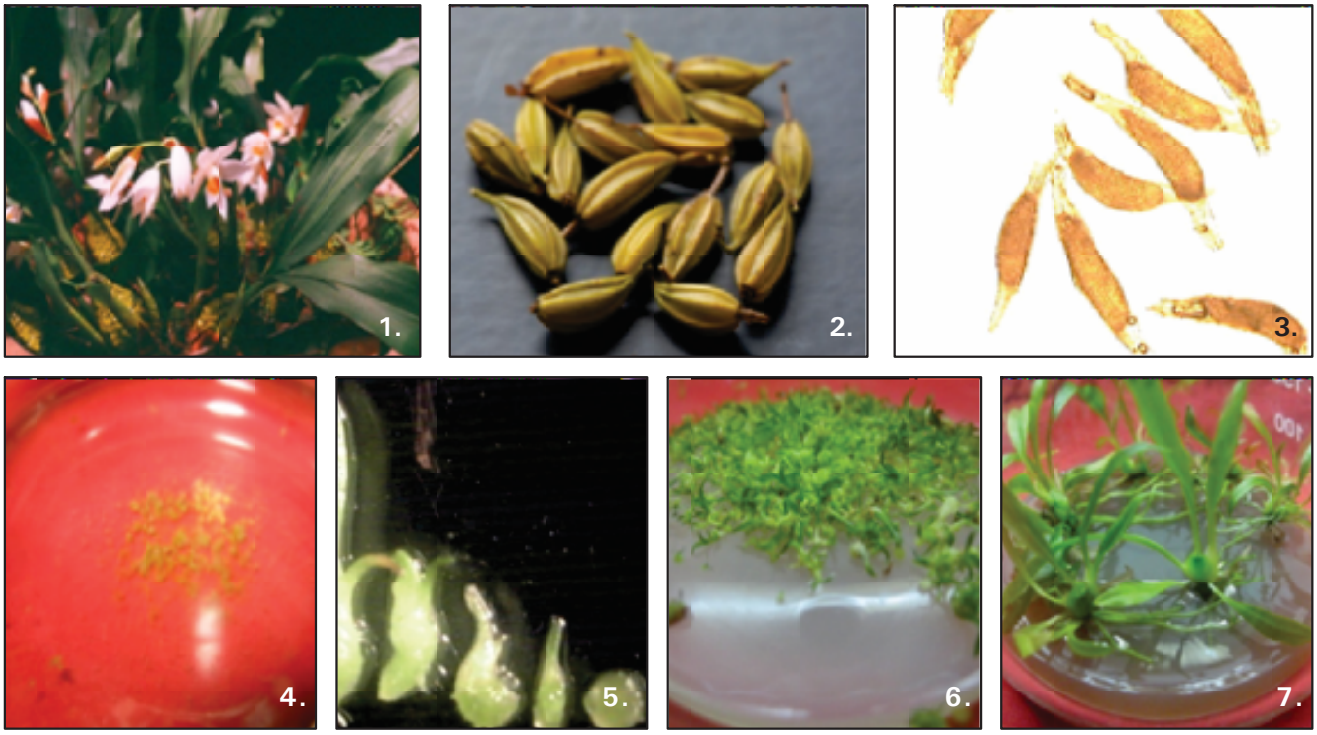
Green capsules collected from the wild growing plants were brought to the laboratory and were washed in running tap water using detergent (Labolene) and immersed in 5% sterilizing solution for 10 min. The capsules were again washed in running tap water and finally rinsed in distilled water. The washed capsules were then taken inside a laminar air flow hood where the capsules were treated with 0.1% $HgCl_2$ for 10 min followed by rinsing thrice with sterile distilled water. The capsules were then dipped in 70% ethyl alcohol and flamed for 2-3 seconds. Surface decontaminated capsules were then taken in a sterile petri plate and were split open vertically using a sterile scalpel bade. The seeds were scrapped out into 20 ml sterile DW. Aliquots of seed suspension containing approximately equal number of seeds were transferred to about 60 ml of sterilized M and KC liquid nutrient media supplemented with different organic additives and plant growth regulators in 250 ml Erlenmeyer conical flasks and kept in a gyratory shaker at 80 rpm. Seed suspension was used for testing the viability and measuring the length and width of the seeds.

All the cultures were incubated at 25 ± 2ÚC under 12 hrs photoperiod with an illumination of 1500 lux provided by Philips day light fluorescent tubes. The cultures were maintained at constant agitations at 80 rpm in a gyratory shaker (Orbitek gyratory shaker). Observations were made at weekly intervals. After 90 days of growth, the developing protocorms were collected for determining their size and fresh weight. After 90 days, the protocorms, in liquid medium were transferred to solid M medium supplemented with different organic additives. For sub-culturing, KC media was not used because of its poor performance in the initial seed germination, in liquid medium. After the second subculture, the seedlings with one or two roots and 2-3 leaves were transferred to medium containing BP (2.5-10%) to study their effect on the seedling development. After the third subculture, lasting 30 days, the seedlings were deflasked and washed in tap water and treated with 1% Indofil M45 fungicide solution. The healthy rooted seedlings were planted in clay pots filled with charcoal and tile pieces in the ratio 1:1. The potted plants were maintained in the green house conditions under diffused light; these were irrigated once in a day.

Results

During *in* vitro asymbiotic germination of *C. nervosa*, culture initiation largely depended on the nutrient media, organic additives and plant growth regulators. Of the two nutrient media used for the present study, M medium proved better than KC medium; the percentage of seed germination, however, varied with different concentrations of CH, P, YE, CW and PGRs used in the nutrient media.

The first signs of seed germination *i.e.,* swelling of embryo started after two wks of inoculation. The protocorms became creamy white or pale green in colour and in most of the protocorms, shoot primordia occurred within 50 days of inoculation (Figs. 4-5). In all the media combinations, M medium supported higher percentage of germination of seeds than KC medium (Table 1). Complex organic additives proved highly useful for the growth and development of the protocorms. M medium containing YE (0.05%) favoured maximum growth of the protocorms followed by CH, peptone and CW. An average of 12.9 mg fresh weight and 3.12 mm diameter/protocorm was obtained



Figs. 1-7. Asymbiotic seed germination of *Coelogyne nervosa*: 1, Plant in bloom; 2, Green capsules; 3, Seeds; 4, Protocorms in liquid medium; 5, Protocorm differentiation; 6, Protocorms on solidified medium; 7, Healthy seedlings (M + BP).

Table 1. Effect of different concentrations of growth additives on *in vitro* asymbiotic seed germination, in *Coelogyne nervosa*.

Nutrient Media	Additives	Germination (%)	Pigmentation	Protocorm fresh weight Mean = SD n = 2	Diameter of the protocorm (mm)
M	Nil0.	45	Pale green	3.57 ± 0.25	1.87 ± 0.87
	05%CH	75	Yellowish green	7.43 ± 0.37	2.49 ± 0.80
	0.05%YE	80	Pale green	12.90 ± 2.70	3.12 ± 038
	0.05%P	60	Pale green	7.84 ± 0.25	2.57 ± 0.25
	20%CW	63	Yellow	6.32 ± 1.25	2.41 ± 0.60
KC	Nil	40	Creamy white	3.37 ± 125	1.9 ± 0.79
	0.05%CH	52	Yellowish green	4.67 ± 1.47	2.10 ± 0.68
	0.05%YE	58	Pale green	8.78 ± 2.57	2.63 ± 0.54
	0.05%P	54	Pale green	5.43 ± 1.71	2.20 ± 0.72
	20%CW	49	Yellow	4.87 ± 0.80	2.12 ± 0.43

Observations were made after 90 days of culture

Table 2. Effect of different concentrations of CW on *in vitro* asymbiotic seed germination in *Coelogyne nervosa* on M medium.

Medium	Coconut water(mlL^{-1})	Germination(%)
M	50	50
M	100	52
M	150	56
M	200	63
M	250	60

to solid M medium containing same additives. Roots were formed simultaneously with the formation of shoots in the second subculture. After the second subculture, the developing seedlings were transferred to M medium containing different concentrations of banana pulp (2.5- 10%). Well developed healthy rooted seedlings were obtained in different concentrations of banana pulp enriched medium. After 45 days in banana pulp containing medium, the well developed seedlings were deflasked and transferred to community pots and showed an establishment rate of 60%, after six months.

Table 3. Effect of different cytokinins on the *in vitro* asymbiotic seed germination of *Coelogyne nervosa* in M liquid medium.

Plant Growth Regulators (Cytokinins)	Concentration (mgl^{-1})	Germination (%)
KN	1.0	50
KN	0.1	30
BAP	1.0	60
BAP	0.1	65
ZN	1.0	40
ZN	0.1	50
2,i-p	1.0	25
2,i-p	0.1	35

Observations were made after 60 days of culture.

Table 4. Effect of different cytokinins on *in vitro* asymbiotic seed germination of *Coelogyne nervosa* in KC liquid medium.

Plant Growth Regulators (Cytokinins)	Concentration ($mg\ l^{-1}$)	Germination (%)
KN	1.0	34
KN	0.1	30
BAP	1.0	50
BAP	0.1	46
ZN	1.0	35
ZN	0.1	32
2,i-p	1.0	25
2,i-p	0.1	35

Observations were made after 60 days of culture.

Discussion

In the present study, better seed germination and subsequent protocorm development in *Coelogyne nervosa* was recorded on M medium as compared to KC medium. M medium has been proved beneficial for seed germination of a number of orchids (Pathak *et al.*, 2001; Sibin *et al.,* 2014; Vij *et al.*, 1981). Initiation of germination, protocorm formation and subsequent growth and development of seedlings varies with the species and the medium employed (Anuprabha and Pathak, 2012; Basker and Narmatha, 2010; Pathak *et al.,* 1992, 2011; Piri *et al.,* 2013; Reddy *et al.,* 1992; Verma *et al.,* 2013; Vij and Pathak, 1988). Casein hydrolysate, yeast extract, peptone, coconut water, potato extract are among organic additives that have been commonly used in orchid tissue culture and have promoter effect on seed germination and subsequent seedling development (Lo *et al.*, 2004; Pathak *et al.*, 2001; Sylvia *et al.*, 2005). The effect of these additives varies with different species. In the present study, addition of these organic supplements at different concentrations showed significant variation in seed germination. Of the different organic additives studied, 0.05% (w/v) yeast extract considerably advanced seed germination and enhanced germination frequency (80%). YE significantly enhanced the seed germination in *Vanda dearei* (Jualang *et al.,* 2014). High nitrogen content in complex additives was reported to stimulate initial stages of seedling growth and differentiation (Paul *et al.,* 2012), in many orchids species including *V. tessellata* (Roy and Banerjee, 2002), *Dendrobium parishii* (Buyun *et al.*, 2004), and *Vanda teres* (Sinha and Roy, 2004). Yeast extract contains about 9.8% total nitrogen, comprising primarily 5.1% amino nitrogen as amino acids (Arditti and Ernst, 1993). Supplementation of organic extracts to the orchid culture medium is simple and practical method to improve culture media used for commercial production.

The seedling transferred to banana pulp (BP) containing medium in the 3rd subculture showed healthy seedlings (Fig. 7). Jualang *et al.* (2014) reported that other complex additives such as tomato juice and banana homogenate significantly enhanced protocorm growth and development especially at lower concentration (10-15%). The promoting growth effect of seedlings when transferred to banana pulp containing medium was also reported by Pierik *et al.* (1988). Banana pulp which inhibits culture initiation, has a marked growth promoting effect on a variety of plant tissues. Presently, BAP in the medium supported seed germination. Of the different cytokinins used, BAP proved better than other cytokinins like KN, ZN, 2, i-p *etc*. Optimum germination (%) was noticed in presence of BAP ($0.1\ mgl^{-1}$) in M medium. Cytokinins either alone or in combination with auxins are better used for orchid germination (Pathak *et al.,* 2001). Sonia et *al.* (2012) also reported that BAP favoured the seedling growth in *Coelogyne nervosa* in MS medium.

During the present study, *in vitro* propagation protocol was successfully developed for rapid and mass propagation of *Coelogyne nervosa,* a commerciallyvaluable and endemic orchid of the southern Western Ghats using immature seeds.

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