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MASS PROPAGATION OF *CYMBIDIUM BICOLOR* LINDL. USING *IN VITRO* ASYMBIOTIC SEED CULTURE TECHNIQUE

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Abstract

Cymbidium bicolor Lindl., a horticulturally important orchid is distributed at an altitude of 400-1000 m from Peninsular India to Srilanka. Its populations are getting rarer due to extensive collections and habitat destruction pressures. The present paper reports the propagation of this species using *in vitro* asymbiotic seed culture technique with a view to developing an efficient protocol for its mass propagation.The germination potential of its seeds at different stages of development [42, 58 wks after pollination (wap)] was tested on four different nutrient media [MS (Murashige and Skoog 1962), PDA (Potato Dextrose agar), DW+Agar (Distilled Water+Agar) and M (Mitra *et al.* (1976)]. It was observed that seeds obtained from capsules (58 wap) invariably failed to show any germination response whereas seeds procured from capsules at another stage (42 wap) positively responded to germination. Since, germination frequency was found to be highest in M medium; the germination potential of these seeds was subsequently assessed on M medium with growth additives (IAA, IBA, NAA, BAP, at1mgl^{-1} each; P, CH, AC at 2 gl^{-1} each). Healthy seedlings complete with 2-3 leaves and 1-2 roots were transferred to green house with 70% survival. Based on the present observations, different nutritional combinations are suggested for germination (M), protocorm growth and multiplication (M+AC), early differentiation stages (M+P+AC; M+AC) and seedling growth and maintenance (M+P), in *C. bicolor*.

Introduction

ORCHID SEEDS are unique in being exceedingly small. Since these contain negligible quantity of reserve food material for development of embryos (Leroux *et al.*, 1997), lack metabolic machinery, and require a symbiotic association with a suitable mycorrhizal fungus in nature (Bernard,1904), the technique of *in vitro* asymbiotic germination has added new vistas in orchid propagation. Though a large number of orchid species from diverse habits and habitats have been successfully germinated asymbiotically *in vitro* (Arditti *et al.*, 1982; Deb and Pongener, 2011; Hossain *et al.*, 2008, 2009, 2010; Pathak *et al.*, 1992, 2001, 2011; Piri *et al.*, 2013; Vij and Pathak, 1988; Vij *et al.*, 1995), data is still meagre in terms of the size of the orchid family.

Cymbidium bicolor Lindl., an epiphytic orchid species, known for its beautiful flowers is distributed at an altitude of 400-1000 m from Peninsular India to Srilanka; it blooms during March-April. Its populations are getting rarer due to extensive collections and habitat destruction pressures. The present paper reports the propagation of *Cymbidium bicolor* Lindl. using *in vitro* asymbiotic seed culture technique with a view to developing an efficient protocol for its mass propagation.

Material and Methods

Plant Material

Seeds of *Cymbidium bicolor* at different stages of development procured from capsules [42, 58 wks after pollination (wap)] were used as explants.

Surface Sterilization of Capsules

The green undehisced capsules were scrubbed with 'Teepol' as a wetting agent and cleaned with running tap water for 5-10 min. The capsules were subsequently washed with distilled water and surface sterilized with 0.1% $HgCl_2$ solution for 5-8 min followed by a treatment with streptomycin (0.03%,8 min) and Bavistin (0.01%,8 min) prior to washing thoroughly with sterile distilled water so as to remove any traces of the sterilizing agents. Further, capsules were split opened with a sterilized blade to scoop out the immature seeds.

Nutrient Media and Incubation Conditions

Four nutrient media namely [MS (Murashige and Skoog 1962), PDA (Potato Dextrose agar), DW+Agar (Distilled Water+Agar, 0.9%) and M (Mitra *et al.* (1976)] were tested to select a suitable medium for seed germination.

Since, germination frequency was found to be highest in M medium; the germination potential of these seeds was subsequently assessed on agar (0.9%) gelled M medium with and without growth additives [Indole3-acetic acid (IAA), Indole3-butyric acid (IBA), Naphthalene acetic acid (NAA), 6-Benzyl aminopurine (BAP), at1mgl^{-1} each; Peptone (P), Casein hydrolysate (CH), Activated charcoal (AC) at 2 gl^{-1} each). The pH of the media was adjusted to 5.6 with 1 N NaOH or HCl before autoclaving

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at 121°C at 1Kg cm^{-2} for 20 min.

Acclimatization

In vitro raised seedlings with 2-3 leaves and 1-2 roots were gradually hardened by sequentially removing the growth additives, vitamins, sucrose and minor salts from the nutrient matrix at 15 days interval. The seedlings were gently removed from the culture vessels with the help of a long forceps, washed thoroughly with lukewarm water, in order to remove any other agar sticking to these and were potted in clay pots (6 cm diameter), filled with a potting mixture of charcoal, pine bark, and bricks pieces (1:1:1) with topping of *Sphagnum* moss, and hardened for 6-8 wks in the green house.

Statistical Analysis

One way analysis of variance was performed with respect to each response (average ± standard error against each additive as mention 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.

Results and Discussion

Presently, seeds obtained from capsules (58 wap) invariably failed to show any germination response irrespective of any nutrient medium used whereas seeds procured from capsules at another stage (42 wap) positively responded to germination. According to Arditti *et al.* (1982), immature seeds germinate better than the mature ones and the stage at which the embryos can be cultured successfully varies with the species, genus, hybrid, nutrient medium and culture conditions. The seeds from mature pods fail to germinate due to accumulation of some inhibitory (dormancy) factors as reported in many orchids (Withner, 1959). Literature studies reveal that pretreatment of the mature seeds with cold temperature was effective in germinating saprophytic orchids (cf. Vij and Pathak, 1988). Presently, seeds obtained from capsules (58 wap) are also expected to show germination if subjected to a suitable pre-treatment; experiments are on in this direction.

Seeds in the presently investigated species showed 50%, 75% and 80% germination in DW + Agar, PDA, and MS respectively (Fig. 1), while cent per cent seed germination was achieved when seeds were inoculated on vitamin enriched M medium with relatively lower calcium and phosphorus. The amenability of some of the species to more than one media formulations reflects their wide nutritional amplitude in accord with the general ability of orchid seeds and seedlings to adapt to a variety of combinations and concentrations of inorganic salts as suggested by Arditti (1967). The nutrient regime for orchid culture is species specific and no single culture medium is universally applicable for all orchid species (cf. Arditti *et al.*, 1982; Godo *et al.*, 2010; Mahendran *et al.*, 2013; Pathak *et al.*, 2001). Since, germination frequency was found to be highest in M medium; the germination potential of these seeds was subsequently assessed on M medium with and without growth additives (IAA, IBA, NAA, BAP, at1mgl^{-1} each; P, CH, AC at 2 gl^{-1} each). The frequency and onset of, germination, time taken for morphogenetic stages leading to seedling development, however, varied with quality of growth additives (Fig. 2a-l). Literature studies reveal successful *in vitro* asymbiotic germination in a large number of orchid species from diverse habits and habitats (Arditti *et al.*,1982; Deb and Pongener, 2011; Hossain *et al.*, 2008, 2009, 2010; Pathak *et al.*, 1992, 2001, 2011; Piri *et al.*, 2013; Vij and Pathak, 1988; Vij *et al.*, 1995). Earlier, asymbiotic seed germination has been suggested as a suitable propagation method for conservation of orchids (Pathak *et al.*, 2001; Piri *et al.*, 2013; Stewart and Kane, 2006).

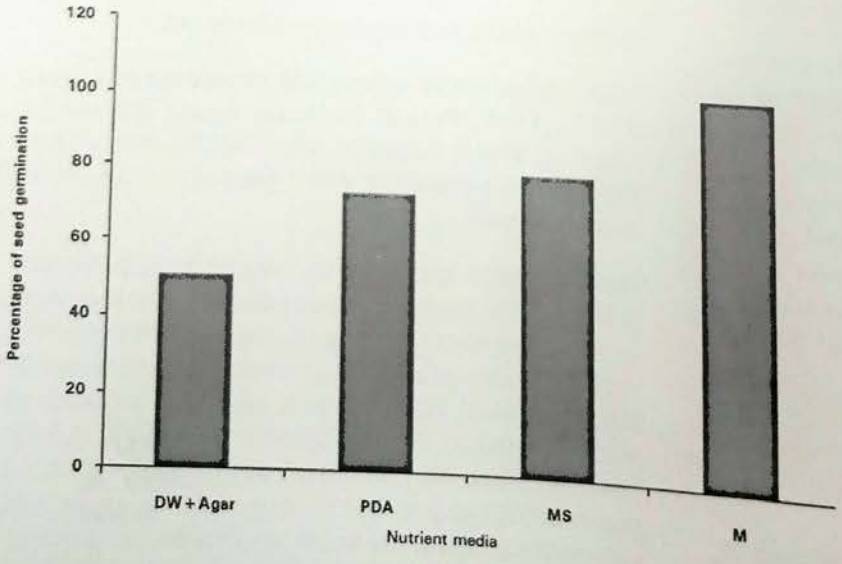

Fig1. Percentage of seed germination on different nutrient media.

In the presently investigated species, in basal M medium, 99.5 ± 1.29% seeds germinated and developed into

2013)

chlorophyllous protocorms. Healthy seedlings complete with 2-3 leaves and 1-2 roots were obtained within 190.00 ± 0.81 days. Additional presence of AC in the medium proved ineffective during early stages of germination, induced protocorm multiplication and advanced seedling development. IBA/NAA improved

Fig.2.a-l. *In vitro* asymbiotic seed culture in *Cymbidium bicolor* : a, Seed at the time of inoculation; b, Swelling of embryo and rupturing of the seed coat; c, Spherule with Rhizoids; d, Protocorm multiplication (M); e-f, Rapid protocorm multiplication and multiple shoot formation (M + AC, M + BAP + AC); g-k, Healthy seedlings (M, M + IBA + AC, M + NAA + AC, M + CH + AC); k, Seedlings being hardened *in vitro*; l, Seedlings transferred to a clay pot.

Table 1. *.In vitro* asymbiotic seed (42 wap) germination in *Cymbidium bicolor* on Mitra *et al.*(1976,M) medium.

Additives	Germination frequency (%)	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	
-	99.50±1.29[a]	9.50±1.29[a]	41.75±1.25[a]	61.25±0.95[a]	112.75±0.95[a]	152.75±0.95[a]	190.00±0.81[a]	Protocorm multiplication; Healthy seedlings
AC	99.50±1.29[a]	9.50±1.29[a]	41.00±0.81[a]	60.00±0.81[a]	110.00±0.81[a]	144.00±0.81[b]	181.25±0.95[a]	Rapid protocorm multiplication
IBA	100.00±0.81[b]	10.25±0.95[b]	44.00±0.81[b]	64.00±0.81[b]	124.25±0.95[b]	155.50±1.29[a]	187.00±0.81[a]	Protocorm formation via callusing
IBA + AC	90.00±0.81[c]	13.50±1.29[c]	56±0.81[c]	60.25±0.95[a]	115.00±0.81[a]	154.00±0.81[a]	190.75±0.95[a]	Healthy seedlings
BAP	90.00±0.81[c]	13.00±0.81[c]	80.00±0.81[d]	90.00±0.81[c]	120.00±0.81[b]	153.25±0.95[a]	187.00±0.81[a]	Healthy seedlings
BAP+ AC	100.00±0.81[b]	14.00±0.81[c]	82.00±0.81[d]	92.50±1.29[c]	119.75±0.95[b]	153.00±0.81[a]	187.75±0.95[a]	Rapid protocorm multiplication; Healthy seedlings
IAA	100.00±0.81[b]	9.50±1.29[a]	44.75±0.95[b]	63.50±1.29[b]	124.25±0.95[c]	156.75±0.95[a]	189.50±1.29[a]	Healthy seedlings
IAA+ AC	99.50±1.29[a]	10.00±0.81[b]	41.00±0.81[a]	60.00±0.81[a]	115.00±0.81[a]	146.75±0.95[b]	185.00±0.81[b]	Thicker roots
NAA	100.00±0.81[b]	10.25±0.95[b]	43.25±0.95[b]	63.50±1.29[b]	120.00±0.81[b]	155.50±1.29[a]	187.75±0.95[a]	Healthy seedlings
NAA+ AC	99.50±1.29[a]	9.25±0.95[b]	42.00±0.81[a]	63.50±1.29[b]	119.75±0.95[b]	148.75±0.95[b]	187.75±0.95[a]	Healthy seedlings
P	98.75±0.95[b]	14.00±0.81[c]	70.00±0.81[e]	79.75±0.95[d]	124.25±0.95[c]	153.25±1.25[a]	180.00±0.81[a]	Protocorm multiplication; healthy seedlings with thicker roots
P+AC	90.00±0.81[c]	13.00±0.81[c]	69.50±1.29[e]	76.00±0.81[d]	85.00±49.33[d]	157.00±0.81[a]	181.25±0.95[a]	Dark green protocorms
CH	90.00±0.81[c]	13.50±1.29[c]	91.25±0.95[f]	104.75±0.95[e]	125.00.81[c]	141.25±0.95[b]	181.25±0.95[a]	Healthy seedlings
CH+AC	80.00±0.81[c]	14.00±0.81[c]	88.00±0.81[f]	97.00±0.81[f]	112.00±0.81[a]	140.25±0.95[b]	180.00±0.81[a]	Small sized protocorms

Entries in column nos.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%.

2013)

germination frequency and induced protocorm multiplication via callusing. Healthy seedlings were obtained in AC enriched IBA/NAA supplemented medium. Similar observation were earlier made in *Cypripedium* and *Vanilla planifolia* (Hegarty, 1955); *Acampe papillosa* (Vij and Malhotra, 1988); *Eulophia dabia* (Sharma and Vij 1986), in IBA containing medium. NAA also proved beneficial during germination in hybrids of *Bletilla, Cattleya* and *Cymbidium* (Strauss and Reisinger, 1976) and *Calanthe discolor* (Miyoshii and Mii, 1995) and during seedling growth in *Cattleya, Cymbidium, Epidendrum* and *Vanda* (Arditti and Ernst, 1984). Addition of BAP in the medium though delayed early stages of seed development; healthy seedlings were obtained in the medium whereas its combination with AC in the medium induced rapid protocorm multiplication. However, BAP proved useful for seed germination earlier in *Cypripedium calceolus* (Borriss, 1969), *C. calceolus, C. candidum, Epipactis helleborine* (De Pauw and Remphhrey, 1993; Van Waes and Debergh, 1986). When BAP was replaced by IAA in the medium though seeds showed cent per cent germination, early morphogenetic stages were delayed; healthy seedlings were obtained in 189.50 ± 1.29 days. AC proved slightly beneficial during protocorm development and subsequent seedling formation; incidentally thicker roots were obtained in this combination. Perusal of literature reveals that IAA enhanced germination frequency and protocorm formation in *Laeliocattleya* (Kano,1965), *Phalaenopsis* (Ernst,1967), *Rhynchostylis retusa, Pachystoma senile* and *Vanda testacea* (Vij *et al.*,1981;1985); protocorm formation in *Rhynchostylis retusa, Saccolabium calceolare,* and *Vanda testacea* (Vij *et al.*, 1981); differentiation in *Cattleya* (Withner,1959), *Miltonia* and *Odontoglossum* (Hayes,1969). However, it was found to be inhibitory during seed germination and delayed seedling formation in *Vanda* 'Miss Joaquim' (Rao and Avdhani,1964); *Comparettia falcata* (Manrique *et al.*,2005); *Dendrobium nobile* (Miyazaki and Nagamastu,1965);and *Coeloglossum viride, Dactylorhiza purpurella, Goodyera repens,* and *Platanthera biflora* (Hadley,1970).

Incorporation of organic growth additives *i.e.*, P/CH in the medium delayed early morphogenetic stages. The former additive proved beneficial in inducing protocorm multiplication and favoured healthy seedlings with thicker root formation whereas latter additive favoured early root development and healthy seedling formation. The protocorms obtained were dark green in colour and first leaf primordia were formed as early as within 85.00 ± 49.33 days, in AC enriched P supplemented medium. However, small sized protocorms were obtained in CH + AC supplemented medium; seedlings were obtained in 180.00 ± 0.81 days. Persual of literature reveals that incorporation of P in the medium favoured germination, protocorm multiplication, and supported better seedling growth in *Cattleya, Dendrobium* and *Vanda* (Morel,1974); *Cymbidium macrorhizon* (Vij and Pathak ,1988); *Dactylorhiza maculata* (Van waes and Debergh,1986); *Goodyera biflora* (Pathak *et al.*,1992); and *Rhynchostylis* (Vij and Kher,1997). Benign effect of CH was demonstrated during germination and seedling growth in *Dactylorhiza purpurella* (Harvais, 1972); *Aerides multiflora, Rhynchostylis retusa, Saccolabium calceolare,* and *Vanda testacea* (Vij *et al.*, 1981); and *Eria spicata, Pholidota articulata,* and *Satyrium nepalense* (Pathak, 1989), combination containing CH promoted protocorm formation, their growth, and subsequent differentiation in *Eulophia dabia* (Sharma and Vij, 1986); It however, detrimentally affected the subsequent seedling growth in *Spathoglottis plicata* (Cheenaveeraiah and Patil, 1975). Presently, AC in the medium, in general proved beneficial during seed germination, protocorm multiplication and seedling development. According to Ichihashi and Kako(1973), the release of brownish exudates by germinating entities into the medium hinders the normal growth and development of young protocorms in a large number of species; AC is reported to promote germination, protocorm multiplication and healthy growth of seedlings in a large number of orchid species (Pathak *et al.*, 2001; Vij and Pathak, 1988; Werckmeister, 1970).

Healthy seedlings complete with 2-3 leaves and 1-2 roots were transferrad to green house with 70% survival. Based on the present observations, different nutritional combinations are suggested for germination (M), protocorm growth and multiplication (M + AC), early differentiation stages (M + P + AC; M + AC) and seedling growth and maintenance (M + P), in *C. bicolor*.

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