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Full Length Research Paper

Vitro seedling development and Asymbiotic seed germination of *Epidendrum ibaguense* Kunth. (Orchidaceae)

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Effects of four asymbiotic media; Murashige and Skoog (MS), Phytamax (Sigma, USA; PM), Mitra et al. (M) and Knudson 'C' (KC) were evaluated for seed germination and early protocorm development in *Epidendrum ibaguense* Kunth. Besides, effects of peptone, activated charcoal and plant growth regulators (6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D)) were also studied. Maximum seed germination was achieved on M and PM (~90%) fortified with 2 gl^{-1} peptone. Medium supplemented with 1.0 mgl^{-1} BAP improved seed germination in contrast to basal media whereas 2,4-D either singly or in combination with BAP remarkably reduced the germination rate. Activated charcoal in the medium enhanced germination of seed, induced significantly large size protocorms (1.63±18) and early rooting in protocorms. Within two weeks of culture, spherules emerged out due to cracking of the seed coat. The spherules developed into protocorms with a leaf primordium at apical portion after 3 to 4 weeks and gradually produced complete seedlings. Strong and stout root system was induced in *in vitro* seedlings on transferring in half strength PM and M media fortified with 0.5-1.0 mgl^{-1} indole acetic acid (IAA). Well-rooted seedlings were transferred to green house with 90% survival.

Key words: *In vitro*, seed germination, protocorm, *Epidendrum ibaguense*, orchid.

INTRODUCTION

Orchidaceae is the largest and most diverse family of the flowering plants, consisting of ~35,000 species under 800 genera (Singh et al., 2007). Due to poor hybridization barrier, more than 1,50,000 hybrids already have been produced and registered, many of which are multigeneric (Sheehan, 1980; Tsavkelova et al., 2001). Exquisite and perpetual flower of orchids made them doyen among ornamentals. Evident by recent increases in world floriculture trade, orchids became the second most popular cut flowers as well as potted floriculture crop with wholesale prices estimated at $126 million (USDA, 2008). Due to continuous destruction of natural habitats, unauthorized trade and ruthless collection by orchid lovers, many orchid species in nature are disappearing at an alarming rate. Furthermore, their high commercial demand has undoubtedly led to an increased emphasis on mass propagation and conservation of important orchids (Stanberg and Kane, 1998). Major obstacles for mass propagation of economically important orchids for commercial purposes as well as conservation are: (1) unavailability of efficient and reliable protocols for seed germination, (2) obligate mycorrhizal association for natural seed germination, 3) a clear understanding of early seedling growth and development and, 4) high mortality of seedlings during transplantation. Ever since the development of a method for asymbiotic germination of orchid seeds by Knudson (1922), the techniques have been used routinely for large scale propagation of a number of orchid species and their hybrids (Arditti and Earnst, 1984; Buyun et al., 2004; Shimura and Koda, 2005; Yamazaki and Miyoshi, 2006; Stewart and Kane, 2006) but a very few studies critically investigated the peculiarities of seed germination and protocorm development. Keeping this in mind, the present research programme was under taken with a view to i) develop efficient protocol for *in vitro* asymbiotic germination of seeds, ii) study the effects of plant growth regulators (PGRs), activated charcoal and peptone on seed germi-

nation and growth of seedlings, and iii) investigate the mode of morphogenesis of embryo during development of protocorm in *Epidendrum ibaguense* Kunth. a floriculturally important orchid.

MATERIALS AND METHODS

Initiation of aseptic cultures

Five to six months old green capsules of *E. ibaguense* were collected from green house grown plants. These were washed under running tap water and Teepol. The capsules were then surface sterilized by submerging in 0.1% (w/v) $HgCl_2$ solution for 10 min with occasional agitation followed by a dip in 0.04% bavistin and streptomycin sulfate solution for 20 min and washed with double sterile water for three times. Final disinfection was done by submerging the capsules in 70% ethanol for 30 s and washing thoroughly with double sterile distilled water. The sterilized capsules were then flamed and split longitudinally with a sterile surgical blade. The powdery seeds were inoculated on the surface of agar gelled nutrient medium.

Culture medium and incubation conditions for seed germination

Four different nutrient media; Murashige and Skoog (1962; MS), Phytamax (Phytamax^{TM}, Sigma, USA; PM), Mitra et al. (1976; M) and Knudson 'C' (1946; KC) supplemented with 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D), either individually or in combinations, and two additives, peptone and activated charcoal (2 gl^{-1}), were used for seed germination. The pH of the medium was adjusted at 5.8 prior to gelling with 0.8% agar. Hundred milliliters of the media were dispensed into 250 ml Erlenmeyer flasks and autoclaved at 121^{0}C for 20 min at 15 psi. Culture vessels with inoculated seeds were maintained in a culture room at 14/10 h continuous light (60 µmol $m^{2}s^{-1}$) and dark conditions at 25 $\pm$ 2^{0}C.

Percent germination and seedling development

After two weeks of inoculation, some of the seeds were scoped out and dispersed in one drop of water on a glass slide and observed under light microscope. Germination was calculated employing the following formula: Germination (%) = (No. of seeds showing swelling of the embryo X 100)/Total No. of seeds.

Once the spherules were formed, observations were recorded at an interval of one week to trace different stages of development of protocorms. These were observed using a stereozoom microscope (Nikon, SMZ1500, Japan). After protocorm development these were subcultured for 4 to 5 weeks.

Histological studies

Protocorrms were fixed in formalin : acetic acid : 50% ethanol (1:1:18; FAA) for one week and preserved in 70% ethanol until used. Longitudinal sections (12 µm) were cut with microtome (Finsee, ME, USA). Staining procedure was the same as described earlier (Prakash et al., 1999). The sections were observed under light microscope (Lobophot, Nikon, Japan).

Rooting in seedlings and transfer to green house

Half and full strength PM and M medium fortified with or without 0.5 - 1.0 mgl^{-1} indole acetic acid (IAA) were used for induction of strong and stout root system on seedlings. The seedlings at 3 to 4 cm in size with 2 to 3 leaves were individually grown on rooting media for 30 days and then transferred to green house. The potting mixture used was prepared with break pieces, charcoal pieces and peat moss at 1:1:0.25.

RESULTS AND DISCUSSION

Influence of basal media and plant growth regulators on seed germination

The seeds germinated on all the media used in study but percent seeds germinated varied on different media (Table 1). It was 70% in M basal medium followed by 60% in PM, and 50% in MS and KC. There are many reports of species-specific media for germination of orchid seeds (Arditti and Ernst, 1984). All the presently employed media differ from one another in their chemical compositions. MS is highly enriched with macro- and microelements, PM contained approximately half of MS, M contained low amount of both macro- and micro-elements but enriched with different vitamins than MS or PM, whereas KC contained comparatively low amount of both macro- and micronutrients and lacked vitamins. Maximum percent germination of seeds in M medium could be achieved due to the fact that this medium is enriched with different vitamins. Addition of various vitamins into the medium was reported to be promotive for seed germination and seedling growth of *Cymbidium elegans* and *Coelogyne punctulata* (Sharma et al., 1991). Mariat (1949) reported that vitamin B favoured germination and differentiation in *Cattleya* seedlings; thiamine, nicotinic acid and biotin were most effective in *Cattleya* hybrids. Pyridoxine was essential for chlorophyll synthesis and combination of nicotinic acid and biotin favoured better germination of *Orchis laxiflora* seeds (Mead and Bulard, 1979).

Both M and PM media supplemented with 1 mgl^{-1} BAP showed 80% seed germination but vigorous growth of protocorms was observed in M medium. Medium supplemented with 2,4-D singly or in combination with BAP showed considerably low seed germination rate. BAP is known to enhance germination frequency in *Cypripedium* spp. (Depauw, 1995), *Eulophia dabia* and *Pachystoma senile* and stimulated protocorm multiplication as well as shoot formation in *Cymbidium pendulum* (Pathak et al., 2001) and *Cattleya aurantiaca* (Pierik and Steegmans 1972). Inhibitory effects of 2,4-D on seed germination and protocorm development were recorded in many orchid species (Fornnesbech, 1972; Kusumoto, 1978; Sharma and Tandon, 1986).

Influence of activated charcoal and peptone on seed germination and protocorm development

Both M and PM media supplemented with 2 gl^{-1} peptone

Table 1. Germination of seeds of *E. ibaguense* on agar solidified different media.

Medium	PGRs (mgl^{-1})		Additives ($2gl^{-1}$)	Spherule formation (Time wks)	Protocorm formation time (wks)	Seed germination (%)
	BAP	2,4-D	AC / P			
MS	-	-	-	9- 10	12- 13	50
	-	1.0	-	9- 10	12- 13	40
	1.0	-	-	7- 8	9- 10	75
	2.0	2.0	-	7- 8	9- 10	60
	-	-	P	7- 8	8- 9	60
	-	-	AC	7- 8	8- 9	65
PM	-	-	-	6- 7	9- 10	60
	-	1.0	-	8- 9	11- 12	50
	1.0	-	-	6- 7	9- 10	80
	2.0	2.0	-	6- 7	9- 10	50
	-	-	P	7- 8	7- 8	90
	-	-	AC	7- 8	7- 8	75
Mitra	-	-	-	6- 7	9- 10	70
	-	1.0	-	7- 8	10- 11	60
	1.0	-	-	6- 7	9- 10	80
	2.0	2.0	-	6- 7	9- 10	60
	-	-	P	6- 7	6- 7	90
	-	-	AC	6- 7	6-7	80
KC	-	-	-	9- 10	12- 13	50
	-	1.0	-	9- 10	13-14	30
	1.0	-	-	8- 9	11- 12	50
	2.0	2.0	-	8- 9	11- 12	50
	-	-	P	9- 10	11- 12	65
	-	-	AC	9- 10	11- 12	60

AC = Activated charcoal; P= peptone; BAP = 6-benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid.

was found to be effective for enhancing germination and vigourous growth of protocorms (~90%, Figure 1G). Amino acids, amides and vitamin contents of peptone are suspected to be responsible for enhancing germination of seeds (Oliva and Arditti, 1984). In case of *Spiranthes cernua* and *Kingidium taenialis* seed germination, it was found to be obligatory (Mahant, 1991). The germination of seeds and growth of protocorms invariably required peptone in the medium (Pathak et al., 2001). Activated charcoal also improved seed germination (80% in M and 75% in PM) and enhanced in development of significantly large size protocorms (1.63±18, Figure 2) and induction of early rooting in the seedlings. High adsorption affinity to excessive and inhibitory compounds of activated charcoal and darkening of the media may be responsible for such behaviour. Curtis (1943) first used charcoal as darkening agent for asymbiotic germination of orchid seeds. Later on, a number of investigations favoured charcoal in asymbiotic medium (Ernst, 1974; Yam et al., 1989). The beneficial effects of charcoal in culture media are: i) adsorption of unidentified morphogeneticaly active or toxic substances (Klein and Bopp, 1971); ii) adsorption of 5-hydroxymethylfurfural which was produced by the dehydration of sucrose during autoclaving (Weatherhead et al., 1978); iii) adsorption of inhibitory phenolics and carboxylic compounds produced by the tissues (Fridborg et al., 1978); iv) adsorption of excessive hormones and vitamins in the media (Fridborg and Eriksson, 1975); and v) create dark condition that favoured rooting (Bhadra and Hossain, 2003).

Morphogenesis of embryo and development of protocorm

Within two weeks of culture the undifferentiated tissues of embryos of the seeds swelled up by imbibing water and nutrients, increasing cell number through repeated cell divisions and emerged out due to rupturing the testa. Since orchid seeds are unique due to presence of only a few celled unorganized embryo and devoid of functional endosperm, exogenous water and nutrients is obligatory for germination. Upon germination, the embryo swelled and turned to globular structure called spherules, with hairy structures at the basal part and subsequently produced protocorm (Figures 1A-C). The upper part of the

Figure 1. A. An undifferentiated embryo inside the seed coat. **B.** Spherule coming out through rupturing the seed coat (SC). **C.** Spherule with appendicle (arrow). **D.** Protocorm with first leaf premordium (LP). **E.** Protocorm of pre seedling stage with rhizoids at the base (RH). **F.** Transverse section of a protocorm showing first (FL) and second leaf (SL) premordium and development of vascular strand (VS). **G.** Seedlings growing in PM medium fortified with 2 gl^{-1} peptone. **H.** Seedling growing in pot in greenhouse (after four month of transfer).

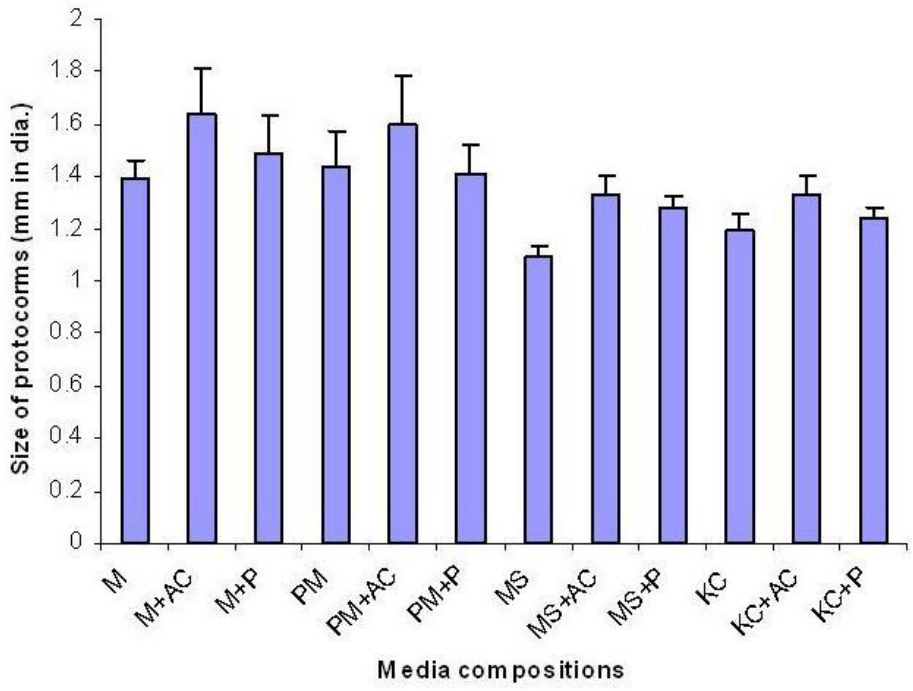

Figure 2. Protocorm size of *E. ibaguense* after 30 day of germination in different media.

protocorm (chalazal end of the embryo) formed the vegetative apex of the stem with a leaf primordium and continues to enlarge (Figure 1D). A second foliaceous primordium appeared afterward, opposite and a certain distance from the first, thus delimiting the vegetative point of the stem. In *E. ibaguense*, early protocom was green, round in shape and radially symmetrical that turned to conical shaped after 3 to 4 weeks of germination (Figure 1E).

As the shoot organs were formed, it became asymmetrical. Formation of protocorm is considered to be a peculiarity of postseminal development in orchids and

Table 2. Development of strong and stout root system in *in vitro* germinated seedlings of *E. ibaguense* after 30 days of culture on rooting media.

Culture medium	Initial No. of roots/seedling (mean ± SEM)	Initial length of Roots (cm) (mean ± SEM)	No. of roots/seedling after 30d of culture (mean ± SEM)	Length of roots (cm) (mean ± SEM)
PM	1.73±0.21	1.58±0.12	3.42 ± 0.27	2.55 ± 0.13
½PM	1.70±0.26	1.51±0.09	4.40 ± 0.22	2.76 ± 0.13
PM + 0.5 mgl^{-1} IAA	1.80±0.20	1.82±0.19	4.30 ± 0.33	3.45 ± 0.16
PM + 1.0 mgl^{-1} IAA	1.80±0.25	1.79±0.12	4.50 ± 0.27	3.12 ± 0.16
½PM + 0.5 mgl^{-1} IAA	**1.74±0.26**	**1.52±0.13**	**5.50 ± 0.31**	**4.52 ± 0.15**
½PM + 1.0 mgl^{-1} IAA	1.60±0.16	1.74±0.11	5.00 ± 0.26	4.74 ± 0.22
M	1.70±0.21	1.78±0.16	3.40 ± 0.27	2.50 ± 0.12
½M	1.65±0.22	1.75±0.16	4.22 ± 0.22	2.78 ± 0.15
M + 0.5 mgl^{-1} IAA	1.60±0.22	1.68±0.15	4.40 ± 0.22	3.32 ± 0.15
M + 1.0 mgl^{-1} IAA	1.60±0.22	1.59±0.15	4.90 ± 0.23	3.12 ± 0.16
½M + 0.5 mgl^{-1} IAA	**1.85±0.25**	**1.76±0.12**	**5.20 ± 0.30**	**4.38 ± 0.12**
½M + 1.0 mgl^{-1} IAA	1.85±0.20	1.67±0.13	4.75 ± 0.30	4.71 ± 0.18

shape of the protocorm is taxon-specific: round, oval, elongated, disk-shaped, branched, thorn-shaped, sphere-cal or spindle-shaped (Batygina et al., 2003). During protocorm formation, the basal part functioning as a "storage organ," consists of larger parenchymatous cells and are covered with hairs homologous to the hypocotyl of the embryo of flowering plants (Teryokhin, 1977). Early in germination, chlorophyll appears in the protocorm cells of epiphytic orchids (Batygina et al., 2003). The apical part of the protocorm, consisting of small number of cells formed a 'tubercle' which turns into shoot apex. In the early development of protocorm, a leaf primordium appeared at upper part that looked like a closed ridge (Figure 1C). As the primordium keeps growing, an opening formed by the edges of the ridge gradually moves to a lateral position (Figure 1F). Interpretations of the first foliar organ of orchids differ. It is referred to as either a cotyledon (Teryokhin, 1977) or as a leaf proper (Batygina and Vasilyeva, 1983). Teryokhin and Nikiticheva (1968) stated that not only the first but also the second appendicular organs formed on the protocorm are cotyledons. The term "cotyledon" is still debatable. The majority of researchers consider cotyledons to be modified leaves initiated at the embryo stage of sporophyte development in flowering plants. In view of the fact that the foliar organs in the majority of orchids appear during postseminal development, that is, at the protocorm stage, it is inexpedient to refer them as cotyledons or leaf-like organs (Batygina and Andronova, 1991). These are the shoot leaves proper, whereas the embryo itself has no cotyledons. Studies of the architecture of the surface layer and internal tissues of the protocorm demonstrated the presence of large pyramidal cells in the axial zone of the apical part of that develop shoot.

Influence of nutritional stress and IAA on induction of roots in seedlings

Both half strength PM and M media supplemented with 0.5 mgl-1 IAA proved effective for induction of strong and stout root system but quality of the seedlings were superior in PM than M in terms of dark green leaves and healthy seedlings (Table 2). Medium fortified with 1 mgl^{-1} IAA also produced good number of roots in both half strength PM and M medium but these were very thin and long. Full strength PM or M medium fortified with 0.5 or 1.0 mgl^{-1} IAA produced a few stunted roots per seedling. Considering all these features, half strength PM fortified with 0.5 mgl^{-1} IAA was the best for induction of strong and stout root system. Nutrition stress enhanced rooting significantly in *in vitro* seedling as recorded by Bhadra et al. (2002, 2004) in some orchid species. Stimulatory effects of IAA in rooting are also frequently reported in tissue culture. The present study revealed that combined effects of nutrients stress and IAA enhanced rooting in *E. ibaguense*. Well-rooted seedlings were then transferred to greenhouse where 90% seedlings survived (Figure 1H).

Conclusions

The results clearly demonstrated that M medium was most effective for high frequency germination of seeds in this orchid. The protocol offers an opportunity to commercial nurseries for large-scale propagation as well as for *ex situ* conservation of *E. ibaguense*.

REFERENCES

Arditti J, Earnst R (1984). Physiology of germinating orchid seeds. In:

Orchid Biology: Reviews and perspectives-III (ed. Arditti J), Cornell University Press, New York. pp. 177-222.

Batygina BT, Bragina EA, Vasilyeva VE (2003). The reproductive system and germination in orchids. Acta Biologica Cracoviensia Series Bot. 45(2): 21-34.

Batygina TB, Andronova EV (1991). Is there a cotyledon in the Orchidaceae? An opinion. In: Recent advances in plant biology (eds. Malik C, Abrol Y), pp. 369–374. APH Publishing Corporation, Delhi, India.

Batygina TB, Vasilyeva VE (1983). Development of the embryo and seedling of some orchids. Abstracts of the All-Union Conference "Conservation and cultivation of orchids" 1983, pp. 73-75. Kiev, USSR.

Bhadra SK, Barua AK, Bhattacarjee B, Hossain MM (2002). *In vitro* germination and micropagation of Dendrobium aphyllum (Roxb.) G.E. Fischer. Bangladesh J. Genet. Biotechnol. 3(1&2): 47-50.

Bhadra SK, Hossain MM (2003). *In vitro* germination and micropropagation of *Geodorum densiflorum* (Lam.) Schltr., an Endangered orchid species. Plant Tissue Cult. 13(2): 165-171.

Bhadra SK, Barua H, Hossain MM (2004) *In vitro* Germination and rapid micropropagation of *Bulbophyllum lilacinum* Redley. Bangladesh J. Bot. 33(2): 103-107.

Buyun L, Lavrentyva A, Kovalska L, Ivannikov R (2004). *In vitro* germination of seeds of some rare tropical orchids. Acta Universitatis Latviensis, Biology, 676: 159-162.

Curtis JT (1943). Germination and seedling development in five species of *Cypripedium*. Am. J. Bot. 30: 199-206.

Depauw MA, Remphrey WR, Palmer CE (1995). The cytokinin preference for *in vitro* germination and protocorm growth of *Cypripedium candidum*. Ann. Bot. 75: 267-275.

Ernst R (1974). The use of activated charcoal in asymbiotic seedling culture of *Paphiopedilum*. Am. Orchid Soc. Bull. 43: 35-38.

Fornnesbech M (1972). Growth hormones and propagation of *Cymbidium in vitro*. Physiol. Plant 27: 310-315.

Fridborg G, Eriksson T (1975). Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiol. Plant 34: 306-308.

Fridborg G, Pedersen M, Landstrom LE Eriksson T (1978). The effects of activated charcoal on tissue cultures: adsorption of metabolites inhibiting morphogenesis. Physiol. Plant 43: 104-106.

Klein B, Bopp M (1971). Effects of activated charcoal in agar on the culture of lower plants. Nature, 230: 474.

Knudson L (1922). Nonsymbiotic germination of orchid seeds. Bot. Gaz. 73: 1-25.

Knudson L (1946). A new nutrient medium for germination of orchid seeds. Am. Orchid Soc. Bull. 15: 214-217.

Kusumoto M (1978). Effect of combinations of growth regulating substances and organic matter on the proliferation and organogenesis of *Cymbidium* protocorms culture *in vitro*. J. Jpn. Soc. Hort. Sci. 47: 391-400.

Mariat F (1949). Action de l'acide nicotinique sur la germination et al development des embryous de *Cattleya*. Comp. Rend Acad. Sci. Paris 229: 1355-1357.

Mahant KC (1991). Green pod culture and regeneration potential in some Indian orchids: a study *in vitro*. Ph. D. Thesis, Panjab University, Chandigarh, India.

Mead JW, Bulard C (1979). Vitamins and nitrogen requirements of *Orchis laxiflora* Lamk. New Phytol. 83: 129-136.

Mitra GC, Prasad RN, Roychowdary A (1976). Inorganic salts and differentiation of protocorms in seed-callus of an orchid and correlated changes in its free amino acid content. Ind. J. Exp. Biol. 14: 350-351.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissues cultures. Physiol. Plant. 15: 473-497.

Oliva AP, Arditti J (1984). Seed germination of North American orchids. II. Native California and related species of *Aplectrum*, *Cypripedium* and *Spirenthes*. Bot. Gaz. 145(4): 495-501.

Pathak P, Mahant KC, Gupta A (2001). *In vitro* propagation as an aid to conservation and commercialization of Indian orchids: seed culture. In: Orchids: Science and Commerce (Eds. pathak P, Sehgal RN, Shekhar N, Sharma M, Sood A). pp. 319-362. Bishen Singh Mahendra Pal Singh, Dehra Dun, India.

Pierik RLM, Steegmans HHM (1972). The effect of 6-banzylaminopurine on growth and development of *Cattleya* seedlings grown from unripe seeds. Z. Pflanzanphysiol., 68: 228-234.

Prakash O, Sood A, Sharma M, Ahuja PS (1999) Grafting of micropropagated tea (*Camellia sinensis* (L.) O. Kuntze) shoots on tea seedlings- a new approach for tea propagation. Plant Cell Rep. 18: 883-888.

Sharma SK, Tandon P, Mishra RR (1991). Vitamins as related to axenic seed germination and seedling growth of *Cymbidium elegans* Lindl. And *Coelogyne punctulata* Lindl. J. Orchid Soc. Ind. 5(1,2): 25-28.

Sharma SK, Tandon P (1986). Influence of growth regulators on asymbiotic germination and early development of *Coelogyne punctulata* Lindl. In: Biology, Conservation and Culture of Orchids (ed. Vij SP) pp. 441-451. Affiliated East West Press, New Delhi, India.

Sheehan TJ (1980). Orchids. Pages 113-142 In: Introduction to Floriculture. Larson RA, ed. Academic Press, New York.

Shimura H, Koda Y (2005). Enhanced symbiotic seed germination of *Cypripedium macranthos* var. *rebunense* following inoculation after cold treatment. Physiol. Plant. 123: 281-287.

Singh MK, Sherpa AR, Hallan V, Zaidi AA (2007). A potyvirus in *Cymbidium* spp. in northern India. Austr. Plant Dis. Notes 2: 11-13.

Stanberg ML, Kane ME (1998). *In vitro* seed germination and greenhouse cultivation of *Encyclia boothiana* var. *erythronoides*, an endangered Florida orchid. Lindleyana, 13: 101-112.

Stewart SL, Kane ME (2006). Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell Tissue Org. Cult. 86: 147-158.

Teryokhin ES (1977). Parasitic flowering plants. Evolution of ontogenesis. Nauka, Leningrad.

Teryokhin ES, Nikiticheva ZI (1968). Post-seminal development of parasitic Angiospermae. I. Metamorphosis. Botani-cheskii Zhurnal SSSR 53: 39–57.

Tsavkelova EA, Cherdynseva TA, Lobakova FS, Kolomeitseva GL, Neutrosov AI (2001). Microbiota of the Orchid Rhizoplane. Microbiology, 4: 492-497.

Weatherhead MA, Bardon J, Henshaw GC (1978). Some effects of charcoal as an additive to plant tissue culture media. Z. Pflanzenphysiol., 89: 141-147.

Yam TW, Arditti J, Weatherhead MA (1989). The use of darkening agents in seed germination and tissue culture media for orchids: a review. J. Orchid Soc. India, 3(1&2): 35-39.

Yamazaki J, Miyoshi K (2006) *In vitro* Asymbiotic Germination of Immature Seed and Formation of Protocorm by *Cephalanthera falcata* (Orchidaceae). Ann. Bot. 98: 1197-1206.