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In Vitro Propagation of *Dendrobium chrysotoxum* (Lindl.)

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ABSTRACT

In vitro asymbiotic seed germination of *Dendrobium chrysotoxum* varied with fruit harvesting time and culture medium used for germinating seeds. Seeds at different developmental stages were harvested and cultured on five asymbiotic orchid seed germination media i.e. defined and undefined media, namely ½ MS, KC, M, MS and PDA. Seeds harvested 220 days after pollination showed maximum germination on all media tested. Amongst the five basal media screened, ½ MS + 3.0% sucrose was the most effective followed by M, KC, MS and undefined medium PDA. Seedlings were formed within 12.35 ± 0.12 weeks. Besides fruit harvesting time, the effect of BA, KN and α-naphthalene acetic acid (NAA) at 1 mg l^{-1} each, singly and in combination in ½ MS medium + 3% sucrose (as basal medium), on seed germination and development of seedlings was also assessed. NAA proved optimal for germination and led to early seedlings formation within 10.25 weeks. BA/KN lowered the germination frequency and delayed seedling development whereas their combination with NAA could elevate % seed germination. The efficacy of growth supplements such as banana homogenate (25, 50, 75 g l^{-1}) and peptone (1.0, 1.5, 2.0 g l^{-1}) was tested on multiplication of cultures (at the protocorm stage) in ½ MS medium. Seedlings regenerated on all medium combinations, but greatest in organic growth supplemented medium. Protocorm segments formed shoot buds which eventually differentiated into shoots with no intervening callus stage. Among the treatments, highest regeneration frequency, a maximum of 9 shoots/explant, and their accelerated development into plantlets was supported by peptone (2 g l^{-1}). Robust shoots and root formation was observed with banana homogenate (50 g l^{-1}). A higher concentration of banana homogenate (75 g l^{-1}) was detrimental to culture survival.

Keywords: growth supplements, protocorms, ornamental, medicinal orchid, seed germination
Abbreviations: **BA**, 6-benzylaminopurine; **BH**, banana homogenate; **½MS**, half-strength Murashige and Skoog medium; **KC**, Knudson C medium; **KN**, kinetin (furfuryl aminopurine); **M**, Mitra medium, **MS**, Murashige and Skoog medium; **NAA**, α-naphthalene acetic acid

INTRODUCTION

Orchid seeds are microscopic, non-endospermic and enclosed within a transparent testa cover. They are produced in large numbers but few of them germinate successfully, countless perish since they require suitable mycorrhizal associations and environmental conditions to germinate. Ever since Knudson (1922) demonstrated the possibility of germinating orchid seeds in nutrient-enriched mineral media under aseptic conditions by passing fungal requirements since then the technique has been widely employed to obtain seedlings from several terrestrial and epiphytic orchid species. Withner (1953) suggested that orchid seeds/embryos are able to germinate prior to reaching maturity. Tsuchiya (1954) also discussed the possibility of germinating orchid seeds from immature pods, since then these findings became the basis of 'Green pod' culture technique that promises for better germination percentage thus saving on time lapse between pollination and seed sowing (Sagawa 1963). The technique is widely used in rescuing hybrid embryos, propagating desired genotypes, cloning apomictic taxa, facilitating genetic transformations (Men *et al*. 2003). Use of *in vitro* protocols has been foreseen as a successful approach for conservation of rare and endangered orchids (Sarasan *et al.* 2006). The technique of asymbiotic seed germination also ensures better germination rates from immature seeds than those from mature ones due to their metabolically awakened embryos and distended testa cells as they lack dormancy or inhibitory factors (Yam and Weatherhead 1988). The appropriate stage at which the embryos can be successfully germinated varies with the genus, species, hybrids, and local conditions (Arditti *et al*. 1982a).

The addition of organic additives to the culture medium

Fig. 1 ***Dendrobium chrysotoxum.*** (**A**) Vegetative form; (**B**) during anthesis.

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to promote *in vitro* growth and proliferation of orchid is a common practice (Chai *et al*. 2002; Islam *et al*. 2003; Rahman *et al*. 2004; George *et al*. 2008). The presence of organic additives may contribute toward the development of a simple and economical plant culture media and at the same time minimize the used of exogenous PGRs, thereby possibly reducing the occurrence of undesired somaclonal variation (Lee and Phillips 1988). Complex growth supplements has the ability to influence *in vitro* regeneration, multiplication of PLBs and growth of orchid seedlings since than a variety of organic growth supplements such as apple juice, banana homogenate, beaf extract, casein hydrolysate, coconut water, corn extract, tomato juice, peptone, yeast extract etc. are tested for promoting multiplication, growth and development of *in vitro* cultures (Arditti 1967). Growth rate of the tissues can be increased by the addition of organic supplements and plant extracts (Fonnesbech 1972). The technique of protocorm slices to regenerate *in vitro* giving a large number of plants within a year through repeated slicing and culture of daughter PLBs, has emerged as a high frequency multiplication methodology for orchids (Morel 1964a, 1964b; Amaki and Higuchi 1989; Lam *et al*. 1991; Kanase and Takano 1995).

Dendrobium chrysotoxum (Lindl.) – a sympodial epiphytic species (**Fig. 1A**) flourishes in diverse habitats of south, south-east Asia including the Philippines, Borneo, Australia, New Guinea, Solomen islands and New Zealand. Endemic to north-eastern Himalaya (Assam, West Bengal, Manipur, Khasia hills) the species is distributed in evergreen semi-deciduous forests in tropical to sub-tropical climates at an altitude of 400-1000 meters (Shukla *et al*. 1998). This is one of the hardiest species of *Dendrobium* orchids as it can tolerate a very wide range of climatic conditions. Its flowers give strong fragrance of honey. They are in high demand as pot plants as their flowers can stay fresh for several days. The species is used to progenate several meritorious hybrids. Besides being great ornamental due to its appealing floral display (fiery-yellow colour, **Fig. 1B**) the species in medicinally important as well. Several isolated components from traditional medicines have been proposed as useful therapeutic agents (Gong *et al*. 2004; Lee *et al*. 2005). In the traditional chinese system of medicine, among traditional Chinese herbs, *Dendrobium*, have been used in the preparation of herbal medicines in many oriental countries for a long time. Of the *Dendrobium* medicinal plants *Dendrobium chrysotoxum* Lindl (DCL) is the most commonly used herb, the Herba Dendrobii (stems of *Dendrobium*) (Li *et al*. 2005). Medicinal property of this herb is due to the presence of polysaccharides which show potent antioxidant, immune stimulating, and anti-hyperglycemic properties (Zhao *et al*. 2007). Cytotoxic compounds are isolated from whole plant showing anti-carcinogenic activity (Chen *et al*. 2008).

Its population's decline is attributed to many factors, largely to overexploitation for its whole plant by commercial growers, poor natural regeneration and habitat destruction. Immediate conservation measures are required to mass propagate and save the species. Keeping this scenario in mind a series of experiments were conducted for 1) establishing an efficient micropropagation system, in particular, checking the influence of pod age, medium type and growth regulators on seed germination, protocorm multiplication and their successive development into seedlings 2) investigating the effect of organic and inorganic growth supplements on multiplication of PLBs and growth of plantlets.

MATERIALS AND METHODS

Plant collection and captivity under greenhouse conditions

Dendrobium chrysotoxum plants were collected from commercial grower of Darjeeling district, West Bengal, India (latitude range: 26° 31′ - 27° 13′ N; longitude range: 87° 59′ - 88° 53′ E). Plant specimens were collected during flowering stage. The healthy plants were replanted in pots (diameter 27.5 cm × 22.4 cm) containing the substrate charcoal pieces, brick pieces, bark pieces in the ratio of 1: 1: 1. Sphagnum moss covered the top surface of potting mix. The plants were maintained in green house under natural light, 70% relative humidity and 25°C/20°C day/night temperature. A voucher specimen (Herbarium number NIP-155) has been deposited in the herbarium of department of natural products, National institute of pharmaceutical education and research, Mohali, India.

Pollination

For pollination, the pollinia from completely opened flowers were picked up by fine forceps and placed on a stigma of another flower on a different plant. These hand-pollinated flowers were marked individually with tags. The capsules were allowed to grow and were plucked and sacrificed at different time intervals, to assess the effect of age of capsule (in days after pollination) on asymbiotic seed germination.

Culture media for *in vitro* seed germination

The pods harvested at different time intervals, were first scrubbed with soft brush in running tap water to remove any debris and rinsed with dish wash detergent solution. They were swabbed with ethyl alcohol under sterile (laminar) hood and surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$; Qualigens, Mumbai, India) solution containing 1-2 drops of 'Teepol' as a wetting agent for 7 min. It was followed by 2-3 washings with sterilized distilled water to remove any traces of $HgCl_2$ left on the surface of pod. Thereafter, the pods were flamed on burner and seeds were scooped out by making longitudinal slit and inoculated on different media i.e. Murashige and Skoog (1962) medium + 3.0% sucrose and ½-strength Murashige and Skoog (1962) medium + 3.0% sucrose, Knudson C (1946) medium + 2.0% sucrose, Mitra *et al*. (1976) medium + 2.0% sucrose, and potato dextrose agar medium (Hi-Media, Mumbai, India).

In a separate set of experiment, the effect of BA (1 mg l^{-1}), KN (1 mg l^{-1}) and NAA (1 mg l^{-1}) singly and in combination in ½ MS medium was also assessed on seed germination and seedling development.

Culture multiplication

Protocorms (2.0 mm long) sourced from 26-weeks old cultures, maintained on basal ½ MS medium, were used as explants. The complex growth supplements such as banana Homogenate (BH-25 g l^{-1}, 50 g l^{-1} and 75 g l^{-1} w/v), Peptone (Hi-Media) (P-1.0 g l^{-1}, 1.5 g l^{-1} and 2.0 g l^{-1} w/v) were used individually in the medium. Banana homogenate was obtained from ripened fruits purchased from market. Required quantities of pulp was weighed, homogenised in a blender and added to the medium.

All the media, except undefined medium i.e. PDA, was fortified with sucrose (Daurala Sugar Works, India) as source of nutrition and gelled with 0.8% (MS and ½ MS) and 0.9% (KC and M) agar powder (Hi-Media). The pH of the medium was adjusted to 5.7 after adding the growth regulators and organic growth supplements and dispensed in test tubes of size (25 mm × 150 mm). The media was autoclaved at 121°C at pressure of 1.06 kg/cm^2 for 15 min. Autoclaved medium was kept at 37°C to check any further contamination.

Inoculations and incubation conditions

The inoculations were done under aseptic conditions in a laminar air flow cabinet. The cultures vessels were incubated at 25 ± 2°C under a 12-h photoperiod of 3,500 Lux (fluorescent tubes-40W; Philips India Ltd, Mumbai, India). Eight replicates were used for each experiment and to check the reproducibility the experiment were repeated twice.

Percent germination

Nearly after four weeks of inoculation, some of the seeds were scoped out and dispersed in a drop of water on a glass slide and

Table 1 Grading scale used for evaluating the germination response of seeds of *Dendrobium chrysotoxum*.

Stage 1	Imbibed seed, swollen, still covered or partially covered by testa (=viable seed)
Stage 2	Enlarged seed without testa (=germination)
Stage 3	Protocorms with rhizoids
Stage 4	Protocorms with pointed shoot apex and rhizoids (appearance of shoot apex)
Stage 5	Seedlings with one or more leaf (emergence of first leaf)
Stage 6	Enlargement of first leaf and the emergence of second leaf

Table 2 Effect of pod age and basal nutrient media on *in vitro* asymbiotic seed germination and seedling formation of *Dendrobium chrysotoxum in vitro*.

Basal media	Pod age (days)	Initiation of germination (weeks)	Development (in weeks) of			
			Protocorm	1st leaf	1st root	Seedlings
½ MS	190	$5.5 \pm 0.08^{b\text{-}iklm\text{-}t}$	$8.10 \pm .08^{b\text{-}jlmo\text{-}t}$	$10.40 \pm 0.16^{b\text{-}np\text{-}t}$	$12.42 \pm .09^{b\text{-}ln\text{-}t}$	$15.0 \pm 0.40^{b\text{-}jl\text{-}t}$
	220	$3.82 \pm 0.09^{ac\text{-}p}$	$5.07 \pm .09^{ac\text{-}t}$	$7.27 \pm 0.15^{ac\text{-}t}$	$9.2 \pm 0.16^{ac\text{-}t}$	$12.35 \pm 0.12^{ac\text{-}t}$
	250	$4.0 \pm 0.00^{abd\text{-}t}$	$6.0 \pm 0.0^{abd\text{-}t}$	$8.50 \pm .08^{abd\text{-}t}$	$10.45 \pm 0.05^{abd\text{-}t}$	$14.22 \pm 0.20^{abd\text{-}ikmp\text{-}t}$
	280	$7.02 \pm 0.41^{a\text{-}lnoq\text{-}t}$	$9.02 \pm 0.12^{a\text{-}ce\text{-}hj\text{-}ln\text{-}t}$	$12.0 \pm 0.00^{a\text{-}ce\text{-}hjkm\text{-}t}$	$14.32 \pm 0.09^{a\text{-}cehjkm\text{-}t}$	$17.18 \pm 0.12^{a\text{-}ceg\text{-}kn\text{-}t}$
MS	190	$9.20 \pm 0.16^{a\text{-}df\text{-}s}$	$12.02 \pm 0.05^{a\text{-}df\text{-}s}$	$14.27 \pm 0.15^{a\text{-}dfi\text{-}t}$	$16.05 \pm 0.05^{a\text{-}df\text{-}t}$	$19.05 \pm 0.05^{a\text{-}dfi\text{-}t}$
	220	$6.28 \pm 0.09^{a\text{-}eg\text{-}jmnp\text{-}t}$	$9.0 \pm 0.0^{a\text{-}ce\text{-}gi\text{-}km\text{-}s}$	$12.42 \pm 0.09^{a\text{-}eg\text{-}t}$	$15.27 \pm 0.12^{a\text{-}cg\text{-}oq\text{-}t}$	$17.05 \pm 0.05^{a\text{-}ceg\text{-}ko\text{-}t}$
	250	$7.48 \pm 0.50^{a\text{-}fhj\text{-}oq\text{-}t}$	$10.40 \pm 0.08^{a\text{-}fh\text{-}r\text{-}t}$	$14.05 \pm 0.05^{a\text{-}fh\text{-}t}$	$16.35 \pm 0.23^{a\text{-}fh\text{-}t}$	$18.10 \pm 0.08^{a\text{-}fh\text{-}oq\text{-}t}$
	280	$10.32 \pm 0.09^{a\text{-}gi\text{-}t}$	$12.45 \pm 0.05^{a\text{-}gi\text{-}t}$	$14.50 \pm 0.08^{a\text{-}gi\text{-}t}$	$17.07 \pm 0.09^{a\text{-}gi\text{-}t}$	$19.18 \pm 0.12^{a\text{-}dfgi\text{-}t}$
M	190	$7.42 \pm 0.08^{a\text{-}fhj\text{-}t}$	$9.08 \pm 0.96^{a\text{-}ceghj\text{-}ln\text{-}t}$	$12.0 \pm 0.00^{a\text{-}ce\text{-}hjkm\text{-}o}$	$14.42 \pm 0.09^{a\text{-}ce\text{-}hjkm\text{-}t}$	$16.33 \pm 0.09^{a\text{-}hj\text{-}t}$
	220	$5.40 \pm 0.08^{b\text{-}jklmo\text{-}t}$	$7.18 \pm 0.96^{a\text{-}jk\text{-}oq\text{-}t}$	$9.15 \pm 0.05^{a\text{-}ik\text{-}np\text{-}t}$	$11.47 \pm 0.12^{a\text{-}ik\text{-}np\text{-}t}$	$14.45 \pm 0.12^{abd\text{-}ik\text{-}np\text{-}t}$
	250	$6.07 \pm 0.05^{a\text{-}eg\text{-}jlmnp\text{-}t}$	$8.05 \pm 0.05^{b\text{-}jlmo\text{-}t}$	$11.32 \pm 0.25^{a\text{-}jl\text{-}t}$	$13.02 \pm 0.05^{a\text{-}jl\text{-}t}$	$15.20 \pm 0.08^{b\text{-}jl\text{-}t}$
	280	$6.6 \pm 0.18^{a\text{-}egkmnp\text{-}t}$	$10.05 \pm 0.05^{a\text{-}km\text{-}qst}$	$12.10 \pm 0.08^{a\text{-}ce\text{-}hjkm\text{-}t}$	$14.35 \pm 0.19^{a\text{-}ce\text{-}hjkm\text{-}t}$	$17.0 \pm 0.0^{a\text{-}cegfkn\text{-}t}$
KC	190	$7.0 \pm 0.0^{ace\text{-}lnoq\text{-}t}$	$9.2 \pm 0.23^{a\text{-}ceghj\text{-}ln\text{-}t}$	$11.15 \pm 0.12^{b\text{-}ln\text{-}t}$	$12.32 \pm 0.09^{b\text{-}ln\text{-}t}$	$17.0 \pm 0.0^{a\text{-}ceg\text{-}kn\text{-}t}$
	220	$5.18 \pm 0.20^{b\text{-}ik\text{-}t}$	$8.0 \pm 0.0^{b\text{-}jlmo\text{-}t}$	$9.52 \pm 0.09^{a\text{-}mo\text{-}t}$	$11.0 \pm 0.00^{a\text{-}mo\text{-}t}$	$14.0 \pm 0.0^{abd\text{-}mo\text{-}t}$
	250	$6.38 \pm 0.12^{a\text{-}eg\text{-}jmnp\text{-}t}$	$8.45 \pm 0.05^{a\text{-}np\text{-}t}$	$10.12 \pm 0.09^{a\text{-}ik\text{-}np\text{-}t}$	$11.45 \pm 0.05^{a\text{-}ik\text{-}np\text{-}t}$	$14.52 \pm 0.05^{abd\text{-}ik\text{-}t}$
	280	$7.28 \pm 0.09^{a\text{-}cefhj\text{-}lnoq\text{-}t}$	$7.25 \pm 0.19^{a\text{-}ik\text{-}oq\text{-}t}$	$11.20 \pm 0.23^{a\text{-}eg\text{-}oq\text{-}t}$	$15.30 \pm 0.08^{a\text{-}eg\text{-}oq\text{-}t}$	$18.22 \pm 0.12^{a\text{-}fh\text{-}t}$
PDA	190	$8.40 \pm 0.14^{a\text{-}ehi\text{-}l\text{-}prt}$	$11.08 \pm 0.08^{*a\text{-}or\text{-}t}$	$0.00 \pm 0.0^{a\text{-}p}$	$0.00 \pm 0.0^{a\text{-}p}$	$0.00 \pm 0.0^{a\text{-}p}$
	220	$8.0 \pm 0.0^{a\text{-}qt}$	$10.10 \pm 0.08^{*a\text{-}km\text{-}qst}$	$0.00 \pm 0.0^{a\text{-}p}$	$0.00 \pm 0.0^{a\text{-}p}$	$0.00 \pm 0.0^{a\text{-}p}$
	250	$8.15 \pm 0.12^{a\text{-}pt}$	$10.5 \pm 0.08^{*a\text{-}fh\text{-}rt}$	$0.00 \pm 0.0^{a\text{-}p}$	$0.00 \pm 0.0^{a\text{-}p}$	$0.00 \pm 0.0^{a\text{-}p}$
	280	$9.02 \pm 0.05^{a\text{-}df\text{-}s}$	$12.08 \pm 0.09^{*a\text{-}df\text{-}s}$	$0.00 \pm 0.0^{a\text{-}p}$	$0.00 \pm 0.0^{a\text{-}p}$	$0.00 \pm 0.0^{a\text{-}p}$

Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test. * =Protocorms shifted to1/2 MS for differentiation of leaf, root primordia and seedling development.

observed under light microscope. Germination was calculated employing the following formula: Germination (%) = (Number of enlarged seeds showing swelling of the embryo × 100)/Total number of seeds. Once the spherules were formed, observations were recorded at an interval of one week to trace different stages of development of cultures. These were observed using a stereozoom microscope (Nikon, H600L, Japan).

Observations and statistical analysis

The cultures were observed regularly under binocular microscope (Olympus SZX10, Japan) and data recorded accordingly. The results were tested using one-way ANOVA test and were analyzed using Tukey's Multiple Comparison using SPSS (Version 16) software package.

Histological studies

Hand sections were cut to study the nature of meristemoids. The material was placed in potato pith and was razor cut. The thin sections floating at the surface of water were selected and placed in a drop of water on slide and observed under stereozoom microscope (Nikon, H600L, Japan).

The germination response of *Dendrobium chrysotoxum* seeds was evaluated using the grading scale (**Table 1**) as earlier implemented (Stewart and Zettler 2002).

RESULTS

Effect of developmental age of harvested pods and basal culture media on seed germination

The time of harvested pods was found to be the most crucial factor for germination of the cultured seeds. The germination of seeds under *in vitro* conditions varied significantly with respect to the time of fruit harvest and the growth medium. A significantly higher number of seeds germinated from the pod harvested at 220 DAP. The seeds procured from 220 days after pollination initiated germination after 3.38, 5.18, 5.4, 6.28 and 8.0 weeks on ½ MS, KC, M, MS and PDA medium, respectively (**Table 2**). Pods procured at 190 days after pollination took longer duration to germinate, those harvested at 160 days did not germinate and the pods of age 280 DAP showed much delayed germination. External features of pods such as diameter, length, weight was observed varying when harvested at different time intervals even colour of the seed embryo also appeared different at different stages of pod development (**Table 3**).

Table 3 Effect of fruit harvesting time on the physical growth of fruit and the seed colour of *Dendrobium chrysotoxum*

Days after pollination (DAP)	Diameter of fruit (cm)	Length of fruit (cm)	Weight of fruit (g)	Seed colour
160	2.25	3.0	2.1	Light yellow
190	2.50	3.2	2.6	Pale yellow
220	2.50	3.7	3.1	Dark yellow
250	2.75	3.7	3.2	Dark yelow

Though, the seeds germinated in all media screened i.e. defined and undefined indicating their wide nutritional amplitude and simple nutritional requirements. ½ MS was found to be the most effective for seed germination and early seedling development over other tested media followed by KC, M, MS full strength, and undefined medium i.e. PDA respectively.

Effect of growth regulators on seed germination

Besides fruit harvesting time and basal nutrient media, presence of growth adjuncts in basal medium i.e.½ MS medium + 3% sucrose, also influenced the germination frequency, onset of germination and associated morphogenetic changes leading to seedling development. The morphological development of *Dendrobium chrysotoxum* from seed to seedling was documented (**Fig. 2A-I**, **Table 4)**. Almost all the seeds were embryonate (**Fig. 2A**). Germination of

Table 4 *In vitro* seed germination of immature seeds of *Dendrobium chrysotoxum* on ½ MS medium

Growth regulators	Germination frequency (%)	Initiation of germination (weeks)	Protocorm development (weeks)	Development of (weeks)		Seedling formation (weeks)
				first leaf	first root	
Basal	75.12 ± 0.62^{def}	3.82 ± 0.09^{bd}	5.07 ± 0.09^{bcdef}	7.27 ± 0.15 ^{bcdef}	9.2 ± 0.16^{bcdef}	12.35 ± 0.12 ^{dbcdef}
BA	73.75 ± 1.5^{def}	4.42 ± 0.05 ^{adef}	7.27 ± 0.15^{adef}	11.0 ± 0.00^{adef}	14.23 ± 0.09^{ade}	18.75 ± 0.28^{adf}
KN	73.25 ± 0.9^{def}	4.0 ± 0.00 ^{d}	6.72 ± 0.60^{adf}	10.85 ± 0.54^{adef}	14.0 ± 0.00^{ade}	18.05 ± 0.38^{ad}
NAA	91.50 ± 1.2^{abcef}	2.02 ± 0.05 ^{abcef}	4.40 ± 0.14^{abcef}	6.0 ± 0.00^{abcef}	8.0 ± 0.00^{abcef}	10.25 ± 0.50^{abcef}
NAA + BA	80.15 ± 0.62^{abcd}	3.60 ± 0.11 ^{bd}	6.40 ± 0.08^{abd}	10.0 ± 0.00^{abcd}	13.20 ± 0.16^{abcdf}	17.92 ± 0.29^{ad}
NAA + KN	82.12 ± 1.0^{adef}	3.65 ± 0.10 ^{bd}	6.0 ± 0.00^{abcd}	10.12 ± 0.5 ^{abcd}	14.10 ± 0.08^{ade}	17.60 ± 0.46^{abd}

Concentration of growth regulators used = 1 mg l^{-1}.Values in a column with similar superscripts are not significantly different at $P \leq 0.05$ according to Tukey's test.

Table 5 Effect of various quantities of organic growth supplements on *in vitro* multiplication of *Dendrobium chrysotoxum* protocorm segments on ½ MS medium.

Additives	Number of shoot buds/explants	Number of shoots/explant	Complete plantlets (weeks)	Regeneration (%)
½ MS	1.0 ± 0.00^{fg}	1.0 ± 0.00^{efg}	18.20 ± 0.23^{bcdefg}	12.50 ± 1.25
½ MS + BH_{25}	1.0 ± 0.81^{fg}	1.50 ± 1.73^{efg}	19.50 ± 0.57^{acdefg}	50.00 ± 20.42
½ MS + BH_{50}	2.00 ± 0.00^{fg}	7.0 ± 0.0^{abdf}	17.40 ± 0.20^{abdefg}	75.00 ± 14.40
½ MS + BH_{75}	1.0 ± 0.00^{fg}	0.00 ± 0.00^{cdef}	0.00 ± 0.0^{abcefg}	37.50 ± 0.23
½ MS + $P_{1.0}$	1.50 ± 0.57^{fg}	2.00 ± 0.81^{cdfg}	15.88 ± 0.25^{abcdfg}	37.50 ± 0.23
½ MS + $P_{1.5}$	4.23 ± 0.12^{abcdeg}	8.25 ± 0.50^{abde}	14.08 ± 0.96^{abcdeg}	40.25 ± 12.50
½ MS + $P_{2.0}$	5.42 ± 0.62^{abcdef}	9.00 ± 0.0^{abcde}	13.31 ± 0.23^{abcdef}	80.50 ± 12.50

BH, P, concentration = g l^{-1}. Values in a column with similar superscripts are not significantly different at $P \leq 0.05$ according to Tukey's test.

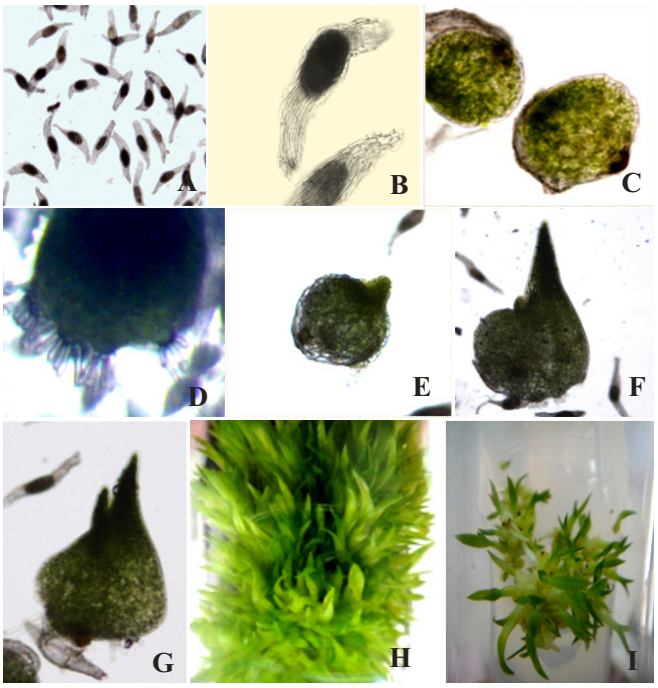

Fig. 2 Asymbiotic seed germination and seedling development of *Dendrobium chrysotoxum* on 1/2 MS medium supplemented with PGR(s). (**A**) Seeds at the time of inoculation all embryonate seeds (4X); (**B**) Swollen embryos (20X); (**C**) Spherules (20X); (**D**) Formation of protocorms with the development of rhizoids; (**E**) Emergence of primary leaf primordium (20X); (**F**) Growth of primary leaf primordium; (**G**) Emergence of secondary leaf primordium; (**H**) Development of healthy shoots in BA-enriched medium; (**I**) Seedlings in KN-supplemented medium.

seeds began within 3.82 weeks of culture in the basal medium. The swollen seeds (Stage 1, **Fig. 2B**), emerged out of the seed coat as spherules, yellowish-green in colour, which grew in size while attached to the seed coat (Stage 2, **Fig. 2C**). After 5 weeks they developed into spherical chlorophyllous protocorms with appearance of rhizoids at their basal portion (Stage 3, **Fig. 2D**). A shoot tip started developing at one end of the chlorophyllous spherule (Stage 4, **Fig. 2E**) and rhizoids at the other end. The protocorms differentiated into 1st leaf primordia (Stage 5, **Fig. 2F**) and later 2nd leaf primordia (Stage 6, **Fig. 2G**). The roots were initiated soon after at the base of leaf primordia and developed into seedlings after 12.35 weeks. NAA proved useful for early onset of germination Almost 91.50% seeds germinated, chlorophyllous protocorms obtained in 4.40 weeks which differentiated into leaf and root primordia within 6 and 8 weeks of culture respectively. Complete seedlings with 2-3 leaves and 1-2 roots were formed within 10.25 weeks.

Use of cytokinins (BA/KN) lowered germination frequency and delayed morphogenetic processes but favoured healthy shoot growth (**Figs. 2H**, **2I**). It took almost 18 weeks for seedling formation. Additional presence of NAA along with BA/KN increased the germination frequency up to 82.12% and favoured little early seedling growth as compared to individual use of BA/KN in the medium.

Effect of organic growth supplements on protocorm multiplication

In the basal medium, only 12.50% explants regenerated into a shoot bud and developed into a single plantlet per explant after 18.20 weeks. Addition of organic growth supplements favoured early initiation of regeneration response in the explants and multiplication of the regenerants (**Table 5**).

Among various concentrations of BH used, a quantity of 50 g l^{-1} of BH could initiate 7 shoots per explants which on prolonged culturing in the same medium multiplied vigorously developing into plantlets after 17.40 weeks of culture (**Figs. 3A**, **3B**). The resultant pseudobulbous shoots were healthy in appearence (**Fig. 3C**), the roots were lengthy and developed hairy out growths at their surface (**Fig. 3D**). Higher concentration of BH (75 g l^{-1}) reduced the regeneration frequency; even shoot buds failed to differentiate further into shoots and roots. They necrosed and perished soon after.

The explants in peptone (2 g l^{-1}) supplemented medium regenerated with a maximum of 87.50% frequency, which favoured vigorous multiplication of shoot buds and early plantlet development within 13.31 weeks; a significant number of shoots (9.0 shoots) per explant developed in the cultures, shoots multiplied profusely showing dense growth of plantlets (**Fig. 3**). Multiple shoots developed at the base of plantlets in the cultures (**Fig. 3G**); their histological hand sections were cut to observe structures and it appeared to be a developing shoot bud in the form of protuberance with juvenile primary leaf primordium which later developed

Fig. 3 *In vitro* culture multiplication through protocorm segments of *Dendrobium chrysotoxum* on 1/2 MS medium and its supplementation with organic growth supplements. (**A**) Shoot bud development in BH 50 g l^{-1}; (**B**) Plantlet formation in BH 50 g l^{-1} supplemented medium; (**C**) Healthy pseudobulbous shoots in BH (50 g l^{-1}) enriched medium after 17.40 weeks of culture; (**D**) Elongated roots with hairy out growths at their surface; (**E**) Development of shoot buds in P (2.0 g l^{-1}); (**F**) Profuse multiplication of shoot buds and dense growth of plantlets P (2.0 g l^{-1}); (**G**) Prolonged cultures showing development of multiple shoots; (**H**) Hand sections showing development of shoot buds in the form of small protuberance with formation of primary leaf primordium; (**I**) Growth of shoot bud showing secondary leaf primordium.

secondary leaf primordium and grew into a shoot bud (**Figs. 3H, 3I**). Growth and multiplication of pseudobulbous plantlets in the presence of growth supplemented medium was more as compared to control.

DISCUSSION

Effect of developmental age of harvested pod

The developmental age of pods was seen as the most important factor for germination of seeds. In this experiment, the pods harvested at age before 220 days or after that showed decline in germination frequency in the cultures. This could be due to the improper stage of embryos; the embryos have not reached the proper stage of maturation. Our results agree with earlier findings reported in *Cleisostoma ramaciferum* (Temjensangba and Deb 2006) and in *Vanda* where very young and mature capsules failed to germinate or resulted in a decreased germination frequency (Sharma 1998). In a study made by Vasudevan and van Staden (2010) in *Dendrobium nobile* and Lee *et al*. (2008) in *Phalaenopsis amabilis* var. *formosa*, the authors emphasized upon the correlation of internal organization of immature seeds and their germinating abilities which indicate that discontinuous cuticle layer enveloping the embryo proper, presence of gaps created by the cellular degeneration of the inner integument, and the absence of secondary wall thickenings in the outer integument all these morphological changes of seed integuments features play an important role in maximizing the germination percentages of the immature seeds. Presently such embryological study was not made in our experiment.

Effect of basal media

The nutrient regime for orchid culture is species specific and no single culture medium is universally applicable for all the orchid species as studied earlier in *Aerides rosea* on Knudson 'C', VW and MS media (Sinha *et al*.1998), *Arachnis labrosa* on M medium (Temjensangba and Deb 2005), *Cleisostoma racemiferum* on MS medium (Deb and Temjensangba 2007), *Malaxis khasiana* on MS medium (Deb and Temjensangba 2006), *Coelogyne suaveolens* on MS medium (Sungkum and Deb 2008) were reported most suitable over other nutrient media. A vast variety of culture media, differing in the quality and quantity of their major and minor salts and in the presence/absence of vitamins, have been devised on more or less empirical basis are species specific (Arditti *et al*. 1982b). In the present studies, ½ MS proved best for seed germination and seedling development as compared to other media tested. However in potato dextrose agar medium the seeds germinated with much low germination frequency but there was a delay in seedling formation as compared to the seedling development in other defined media. This could have happened due to the absence of certain chemical salts required by germinating seeds which could have been absent in PDA medium and present in defined media. On the other hand, in PDA (undefined) medium seed germination could occur due to the presence of polyamines and biosynthetic enzymes that affects growth and development of plant cells especially to nucleic acid replication and cell division in mitosis. Moreover, potato consists of useful carbohydrate, sugar, protein and vitamin for plant growth. Promotory effects of potato juice in orchid seed germination and enhanced seedling growth is already on records (Arditti and Ernst 1993) but this was not the case observed in our cultures. Nutritional requirement for optimal growth of a tissue *in vitro* may vary with the species. As such, no single medium can be suggested as being entirely satisfactory for all types of plant tissues and organs.

Effect of growth regulators

Presently, NAA favoured highest germination frequency and accelerated seedling development. Our findings have confirmed those reported earlier where benign effect of NAA is reported in several orchids (Arditti 1977). NAA was earlier reported to be promotory to seedling growth at lower concentrations in *Cymbidium* (Fonnesbech 1972), *Cattleya* and *Dendrobium* (Vajrabhaya and Vajrabhaya 1976). This auxin favoured seed germination and seedling differentiation in *Aerides odorata* (Pant and Gurung 2005). Devi *et al*. (1998) reported the promotory effect of NAA in *Vanda coerulea*. Similar results were obtained by subsequent workers (cf. Arditti and Ernst 1984). According to Hayes (1969), auxins are implicated in stimulating transport of exogenous nutrients during germinations.

In our cultures, BA did not favour better germination; this species may not require any exogenous supply of cytokinin for germination as is evident from the high frequency of germination in its absence. A literature study reveals that orchid seeds that do not require exogenous cytokinin for germination are known as cytokinin autonomous since they contain a sufficient endogenous level of cytokinin (Mercier and Kerbauy 1991; De Pauw *et al*. 1995).

Effect of organic growth supplements

The protocorm segments elicited regeneration response in the basal medium with a very low regeneration frequency. The percentage of regeneration was increased by addition of organic growth supplements in the medium. Earlier Arditti (1979) made similar conclusions indicating the beneficial effects of organic growth supplements (BH, CW, P) on growth and differentiation of protocorms and further seedling development. The positive effects of BH (50 g l^{-1}), in the present cultures, in eliciting the moderate regeneration frequency and subsequent development of healthy pseudobulbous plantlets with elongated roots could be attributed to the higher content of sucrose concentrations in BH as earlier also suggested by Aktar *et al*. (2008) in enhancing *in vitro* regeneration of *Dendrobium* orchid PLBs. Banana homogenate (10-15%) also promoted root elongation and growth of *Oncidium* plantlets (Kusumoto and Takeda 1997) and significantly increased the number of leaves in *Dendrobium nobile* cultures (Sudeep *et al*.1997). Literature survey also indicates the beneficial effects of BH in promoting highest shoot production in *Dendrobium* hybrid. In *Vanda* species BH (10%) increased the shoot length. Beneficial effects of BH (10%) on leaf size of *Spathoglottis kimballianai* is also reported (Minea *et al*. 2004).

Peptone at 2 g l^{-1}, in our cultures, proved highly beneficial in eliciting maximum regeneration response and early plantlet development. This could be attributed to the presence of very high contents of amino acids which are required for growth and development of cultures. A perusal of literature reveals that, peptone being water soluble protein hydrolysate full of amino acid content promotes growth of cultures. Literature studies also reports its beneficial effects in inducing protocorm multiplication in *Cymbidium macrorhizon* and *Cymbidium* species (Kusumoto and Furukawa 1977). Peptone is also known to have stimulated callus growth in *Phalaenopsis*, *Doritaenopsis* and *Neofinetia* (Ichihashi and Islam 1999). It has also supported better seedling growth in *Paphiopedilum*, *Phaius* and *Vanda* (Curtis 1947). In *Peristeria elata* peptone favoured early and healthy growth of seedlings (Bejoy *et al*. 2004).

CONCLUSION

From the results obtained in our work, it is concluded that seeds of *Dendrobium chrysotoxum* harvested at 220 DAP can be used for *in vitro* germination of this rare orchid using ½ MS medium and NAA. Addition of organic growth supplements such as BH and P proved beneficial for multiplication and early development of plantlets as compared to control in the cultures. Organic growth supplements contain amino acids, proteins, carbohydrates, vitamins, phenolic acids and organic compounds. Any of these component(s) could be responsible for promoting growth and development of the cultures. Further studies are required to determine which factor(s) is responsible for promotory effect of these organic additives.

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