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In vitro Seed Germination and Mass Propagation of *Cymbidium dayanum* Reichb.: An Important Ornamental Orchid of North-East India

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ABSTRACT

Cymbidium dayanum Reichb., an epiphytic orchid is one of the popular orchids in cut flower market due to its attractive white flower with distinctive red strips. Because of unrestricted exploitation of this useful ornamental plant, its population is fast receding from the natural habitat. Effective conservation methods are required to protect this beautiful orchid from extinction. The present study was carried out to develop efficient *in vitro* regeneration protocols for *C. dayanum* using seeds as explant. High seed germination response was reported in Mitra (M) and Murashige and Skoog (MS) culture media devoid of any growth regulators when 0.2% Activated Charcoal (AC) was present. Addition of 1-Naphthyl Acetic Acid (NAA) at 0.7 mg L^{-1} in Mitra medium produced maximum seed germination (94.58%) and high root proliferation. MS medium enriched with 1.2 mg L^{-1} of NAA and 0.2% AC produced highest rooting (5.32±0.35). Incorporation of 6-benzyl Amino Purine (BAP) either alone or with Indole-3- Butyric Acid (IBA) in culture media produced very poor seed germination. The few germinated seeds gave rise to small underdeveloped protocorms which subsequently died after turning brown in color on further subculturing. Seed germination improved immensely and so did the culture growth when AC was added in the medium indicating its beneficial effect in plantlet development *in vitro*. Highest shooting (5.91±0.96) was observed in Mitra medium fortified with 1.2 mg L^{-1} of BAP and 0.2% AC.

Key words: *Cymbidium dayanum*, ornamental, orchids, seed culture, protocorm, conservation

INTRODUCTION

Orchids are highly diversified groups of flowering plants which belong to family Orchidaceae. It is considered as one of the largest families of angiosperms comprising more than 25000 species, numerous hybrids and varieties (Raven *et al.*, 2005). The highly fascinating flowers of orchids with brilliant coloration, varied forms and sizes make them one of the most expensive ornamentals known today (Upadhaya and Das, 2001). They are marketed both as cut flowers and potted plants with expensive price tag making them beyond the reach of the common man. Amongst cymbidium orchids, *Cymbidium dayanum* represents a highly popular orchid in floriculture market because of its attractive beautiful white flowers with distinctive red strips (Nahar *et al.*, 2011). This species is one of the most sought after ornamentals in local cut flower market. The North-Eastern region of India which is among the 8 hottest biodiversity hotspot regions of the world has very rich biodiversity. It is considered as a treasure house for varieties of orchids harbouring more than 650 species (Medhi and Chakrabarti, 2009). The existence of cymbidium orchids in this region is more prevalent because of its varying physiological features and highly suitable climatic conditions (Nongdam, 2012). Unfortunately in recent time, the population of *C. dayanum* has declined rapidly due to unregulated heavy collection, excessive habitat destruction and deforestation. There is an increasing risk of this important orchid becoming extinct from its natural habitat if uncontrolled exploitation continues unabated. A number of useful orchids have already been included in the Red Data sheet on Indian Orchidaceae-I due to failure in restricting the unwanted human activities (Singh, 2001). *C. dayanum* had also been declared as vulnerable and threatened orchid in Red Data Book (Nayar and Sastry, 1990). Moreover, there is no proper conservation strategy to protect this vulnerable yet highly valuable orchid. Devising efficient conservation method is the need of the hour to prevent further reduction of depleting orchid population. The main obstacles in the propagation of orchids through seeds in nature are the inability of orchid seeds to germinate due to their exalbiminous nature and requirement of mycorrhizal fungal infection (Ovando *et al.*, 2005; Gutiarrez-Miceli *et al.*, 2008; Mohanraj *et al.*, 2009). However, plant tissue culture provides a new dimension to conservation and commercialization of several rare and useful orchids by propagating them rapidly at larger scale in shorter time (Vij, 2002). Orchid seeds have been successfully germinated *in vitro* without any fungal association by using plant tissue culture techniques (Alam *et al.*, 2002; Sazak and Ozdener, 2006; Long *et al.*, 2010). Though there are several reports on tissue culture of different orchids, the micropropagation of *C. dayanum* has been very less documented in the literature. Yuanhua *et al.* (2008) could obtain more than 98% of mature seed germination in *C. dayanum in vitro* after 96 days of culture on MS and Hyponex medium. Chang *et al.* (2005) were able to regenerate *C. dayanum* from germinating seeds on modified MS medium via intermediate rhizome formation. Nahar *et al.* (2011) indicated the importance of growth elicitors such as chitosan H, marine sweet and hyaluronic acid in producing plantlets *in vitro* in *C. dayanum*. The limited work on tissue culture of this species may be due to difficulty in germination of seeds *in vitro* under asymbiotic condition. This is because of the development of cuticle which lowers the seed germination potential though it helps in retention of moisture and provides physical protection to the seeds having thin seed coat (Lee *et al.*, 2006). Much work needs to be done focussing more on to developing efficient conservation methods of this rare cymbidium orchid by employing micropropagation techniques. The present study was conducted with an attempt to establish fast, reliable and useful regeneration protocols for effective conservation of this highly valuable ornamental orchid of North-East India via seed culture.

MATERIALS AND METHODS

Explant source and surface sterilization: The present study was conducted at Department of Botany, Panjab University, Chandigarh from March, 2004 to January, 2006. The five months old green capsules were collected from different areas of North- East India. The capsules were cleaned in running tap water for 10-15 min with 20% teepol followed by treatment with 0.5% of $HgCl_2$ for about 5-8 min with 1-2 drops of teepol as wetting agent. The capsules after rinsing 3-4 times in sterilized distilled water were then immersed in 70% ethanol for 15-20 min followed by flaming for 2-3 sec. The rinsing was performed to remove the mercuric chloride completely from its surface. Finally the surface sterilized capsules were split longitudinally using a sterilised knife under the laminar flow cabinet to extract the seeds to be used as explants.

Culture media preparation, inoculation and incubation: Mitra *et al.* (1976) and Murashige and Skoog (1962) media containing 2% sucrose and 0.9% agar as solidifying agent were prepared to grow *in vitro* the extracted seeds from surfaced sterilized capsules. The pH was adjusted at 5.8 after the sucrose had been properly dissolved. AC at 0.2% was also incorporated into the culture media in order to monitor its effect on the culture growth. Plant growth regulators like Indole-3- Butyric Acid (IBA), 6-Benzyl Amino Purine (BAP) and 1-Naphthyl Acetic Acid (NAA) were used singly or in combination at different concentration to ascertain the growth response of the culture. During medium preparation, appropriate concentrations of plant growth hormones were added to culture vessels prior to autoclaving. Cotton plugs were used to tightly close the culture vessels and medium was autoclaved at 1.1 kg cm^{-2} pressure and 121°C for 15-20 min. The immature seeds were inoculated under proper aseptic condition on the prepared culture media which were cooled down after autoclaving. The cultures after inoculation were incubated at 25±2°C with 60 μmol m^{-2} sec^{-1} for 10-12 h a day using fluorescent tubes.

Hardening of *in vitro* raised plants: The fully grown seedlings with well-developed leaves and roots were hardened by first culturing them for 3 weeks in a full strength medium devoid of any plant growth regulators and then in a half strength medium without vitamins and sucrose for another 2 weeks. Luke warm water was used to wash the hardened seedlings after being taken out from the culture vessels to remove the sticking agar from the roots. 0.01% of bavistin solution was used as fungicide to control any fungal infections that may be associated with the seedlings. The seedlings were then transferred to clay or plastic pots with brick, charcoal pieces and moss (1:1:1) as potting medium. The transplanted seedlings were kept in the green house condition for proper adjustment and acclimatization.

Data recording and statistical analysis: The cultures were subcultured after every 4-6 weeks and the experiment was performed twice by taking 15 replicates for each hormone treatment. The parameters evaluated for the present study were seed germination percentage, spherule, protocorm and complete seedling development in weeks. The number of shoot and root formation was also recorded to determine the appropriate culture conditions for proper shooting and rooting. The seed germination percentage was derived by using the formula given below:

$$\text{Seed germination } (\%) = \frac{\text{No. of seeds successfully germinated by swelling}}{\text{Total No. of seeds inoculated}} \times 100$$

The data obtained from all the experiments for the present study were analysed using Duncan's multiple range tests in one way ANOVA (Duncan, 1955). The statistical data analysis was conducted by employing the SPSS (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

The inoculated immature seeds started swelling in around 2 weeks of culture in basal Mitra medium devoid of any plant growth regulators. Swelling of seeds is the indication that seeds were germinating successfully. The seed coat ruptured with increase in seed size allowing the embryonal mass present inside to emerge outside. Spherical shaped parenchymatous cell bodies called spherules were formed which subsequently differentiated into elongated oval shaped chlorophyllous protocorms in 8-9 weeks (Fig. 1a). Das *et al.* (2007) and Nongdam and Chongtham (2011) observed similar development in *Cymbidium devonianum* and *C. aloifolium*, respectively. After attaining the protocorm stage, the culture took 3-4 weeks to develop into complete seedling (Fig. 1b). Chang *et al.* (2005) observed intermediate rhizome formation in *C. dayanum* two months after protocorm development which subsequently differentiated into plantlets. However in our present study we could obtain seedlings directly from protocorms without rhizome development in shorter time of 14-15 weeks (Table 1). Lu and Lee (1990) similarly, illustrated direct plantlet

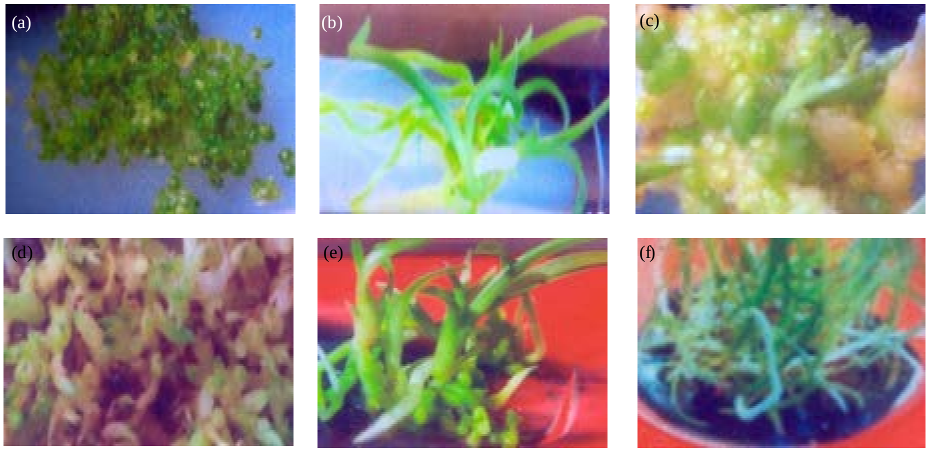

Fig. 1(a-f): *In vitro* regeneration response of *C. dayanum* in different culture conditions; (a) Spherical shaped chlorophyllous protocorms development in basal Mitra medium, (b) Differentiation of complete seedlings from protocorms in basal Mitra medium without any growth regulators, (c) Rapid protocorm proliferation in medium supplemented with 1.2 mg L^{-1} BAP, IBA and 0.2% AC, (d) Enhanced shoot multiplication in Mitra medium enriched with 0.7 mg L^{-1} BAP along with 2% AC, (e) Luxuriant leaf formation in seedlings grown on Mitra medium treated with 1.2 mg L^{-1} BAP and AC and (f) rigorous root formation in MS medium fortified with 1.2 mg L^{-1} NAA and 0.2% AC

Table 1: *In vitro* germination response of *Cymbidium dayanum* on Mitra medium with different growth regulators

PGR (mg L^{-1})			Additives	Germination	Average time (in weeks) for development of				No. of roots
BAP	IBA	NAA	(AC/2 g L^{-1})	response (%)	Spherules	Protocorm	Seedling	No. of shoots	(After 20 weeks)
-	-	-	-	80.53 ± 0.30^{f}	5-6	9-10	14-15	3.82 ± 0.51^{c}	1.82 ± 0.17^{c}
-	-	-	AC	90.17 ± 0.11^{d}	4-5	8-9	13-14	4.91 ± 0.84^{g}	2.71 ± 0.43^{a}
0.5	-	-	-	43.67 ± 0.78^{b}	11-12	19-20	F	F	F
0.7	-	-	-	44.65 ± 0.47^{b}	11-12	19-20	F	F	F
0.7	-	-	AC	84.36 ± 0.97^{a}	5-6	9-10	14-15	5.16 ± 0.71^{g}	2.27 ± 0.31^{b}
1.2	-	-	AC	86.27 ± 1.28^{a}	5-6	9-10	14-15	5.91 ± 0.96^{f}	2.41 ± 0.81^{b}
-	-	0.5	-	92.47 ± 0.42^{d}	4-5	8-9	13-14	2.41 ± 1.29^{e}	4.17 ± 1.01^{e}
-	-	0.7	-	94.58 ± 0.32^{d}	4-5	8-9	13-14	2.64 ± 1.12^{e}	4.31 ± 0.53^{e}
-	-	0.7	AC	81.25 ± 0.65^{c}	5-6	9-10	14-15	3.22 ± 1.65^{d}	4.75 ± 0.31^{d}
-	-	1.2	AC	84.34 ± 0.77^{a}	5-6	9-10	14-15	3.35 ± 0.56^{d}	4.92 ± 1.24^{d}
-	0.5	-	-	77.37 ± 0.25^{c}	6-7	10-11	15-16	3.75 ± 0.69^{f}	1.61 ± 0.63^{c}
-	0.7	-	-	79.54 ± 0.31^{c}	5-6	9-10	14-15	3.85 ± 0.51^{c}	1.75 ± 0.51^{c}
-	0.7	-	AC	81.41 ± 0.45^{c}	5-6	9-10	14-15	4.11 ± 1.24^{a}	2.25 ± 0.71^{b}
-	1.2	-	AC	86.16 ± 0.40^{a}	4-5	9-10	14-15	4.51 ± 1.01^{b}	2.63 ± 0.76^{a}
0.5	-	0.5	-	43.51 ± 0.34^{b}	11-12	20-21	F	F	F
0.7	-	0.7	-	44.25 ± 0.53^{b}	11-12	20-21	F	F	F
0.7	-	0.7	AC	84.12 ± 0.41^{a}	5-6	9-10	14-15	4.12 ± 0.54^{a}	2.55 ± 0.47^{a}
1.2	-	1.2	AC	85.34 ± 0.38^{a}	5-6	9-10	14-15	4.27 ± 0.73^{a}	2.61 ± 1.54^{a}

Results are based on the average of 15 replicates. Means followed by same letter are not significantly different at 5 % (Duncan's multiple range test). Differences between means with dissimilar superscripts are significant at 5%, BAP: 6-benzyl amino purine, IBA: Indole-3-butyric acid, NAA: 1-naphthyl acetic acid, AC: Activated charcoal, F: Failure of seedling formation, leaf and root development

Table 2: *In vitro* germination response of *Cymbidium dayanum* on MS with different growth regulator

PGR (mg L^{-1})			Additives	Germination	Average time (in weeks) for development of				No. of roots
BAP	NAA	IBA	(AC/2 g L^{-1})	response (%)	Spherules	Protocorm	Seedling	No. of shoots	(After 26 weeks)
-	-	-	-	30.10±0.21^{f}	14-15	21-22	F	F	F
-	-	-	AC	73.12±0.10^{a}	7-8	11-12	16-17	4.62±0.50^{a}	3.60±0.51^{a}
0.4	-	-	-	38.14±0.13^{b}	13-14	21-22	F	F	F
0.8	-	-	-	41.52±0.32^{b}	12-13	21-22	F	F	F
0.8	-	-	AC	82.13±0.43^{d}	5-6	9-10	14-15	4.47±0.42^{a}	2.63±0.35^{d}
1.2	-	-	AC	87.11±0.12^{f}	5-6	9-10	14-15	5.25±0.63^{d}	2.51±0.43^{d}
-	-	0.4	-	27.22±0.43^{e}	14-15	21-22	F	F	F
-	-	0.8	-	29.14±0.31^{e}	13-14	21-22	F	F	F
-	-	0.8	AC	80.15±0.52^{d}	5-6	9-10	14-15	3.26±0.62^{c}	4.51±0.23^{c}
-	-	1.2	AC	82.20±0.12^{d}	5-6	9-10	14-15	3.93±0.64^{b}	5.32±0.35^{b}
-	0.4	-	-	40.72±0.23^{b}	12-13	22-23	F	F	F
-	0.8	-	-	41.18±0.41^{b}	13-14	22-23	F	F	F
-	0.8	-	AC	45.25±0.33^{c}	12-13	21-22	F	F	F
-	1.2	-	AC	47.13±0.12^{c}	12-13	22-23	F	F	F
0.4	-	0.4	-	39.14±0.17^{b}	13-14	21-22	F	F	F
0.8	-	0.8	-	40.15±0.13^{b}	13-14	21-22	F	F	F
0.8	-	0.8	AC	73.61±0.67^{a}	7-8	11-12	16-17	4.07±0.72^{b}	3.75±0.42^{a}
1.2	-	1.2	AC	75.10±0.10^{a}	7-8	11-12	16-17	4.52±0.56^{a}	3.84±0.51^{a}

Results are based on the average of 15 replicates. Means followed by same letter are not significantly different at 5 % (Duncan's multiple range test). Differences between means with dissimilar superscripts are significant at 5%, BAP: 6-benzyl amino purine, IBA: Indole-3-butyric acid, NAA: 1-naphthyl acetic acid, AC: Activated charcoal, F: Failure of seedling formation, leaf and root development

development from protocorms after seed germination in *C. dayanum*. When seeds were grown on basal MS medium, a contrasting result was observed with only 30% of seeds showing successful germination (Table 2). The protocorms differentiating from the few germinating seeds were stunted and poor in health with brownish coloration due to loss of chlorophyll. Seedlings were not formed as the protocorms died after few subculturings. Incorporation of 0.2% AC in basal culture media significantly improved seed germination with 90 and 73% seed germination percentage recorded in Mitra and MS medium, respectively. The usefulness of AC in inducing better seed germination in many orchids was well established from different works (Miyoshi and Mii, 1995; Thomas and Michael, 2007). The promotive effect of AC might be due its ability to absorb effectively phenolic exudates released by the culture in the medium (Pan and Van Staden, 1998). In the present investigation we could also generate healthy seedlings after successful seed germination in AC treated medium. Present result further reiterated the importance of AC in producing enhanced seed germination and plantlet growth *in vitro*. Seed germination response was very poor without AC in both the culture media when BAP was present singly or with IBA at varied hormonal concentrations. The germination percentage with 0.7 mg L^{-1} of BAP alone and with IBA at the same concentration in Mitra medium was found to be 44.65±0.47 and 44.25±0.53, respectively. Similar low seed germination response was observed in MS medium with the same hormonal combination. However, Hossain *et al.* (2009) reported the favourable condition for better seed germination in *C. aloifolium* when 1.0 mg L^{-1} of BAP was incorporated in MS medium. The contrasting result as observed in our present investigation may be due to difference in the cymbidium species involved and also different hormonal compositions and concentration employed for the study. Protocorms development was extremely delayed as growth regulator combination of BAP and IBA at any level of hormone concentration was not found conducive for culture growth. Small and underdeveloped protocorms with little chlorophyll content were formed which subsequently perished as subculturing continued. Similar observation was made in *C. aloifolium* by Nongdam and Chongtham (2011). Seed germination and protocorm development were highly accelerated when 0.2% AC was added in Mitra medium supplemented either with 0.7 mg L^{-1} or 1.2 mg L^{-1} of BAP. Protocorm formation was enhanced leading to the development of big healthy and chlorophyllous protocorms (Fig. 1c). Similar response was also observed for MS medium treated with 0.8 or 1.2 mg L^{-1} of BAP and 0.2% AC. The usefulness of AC in promoting seed germination in presence of growth regulators may result from its ability to suppress potential growth inhibitors released during culture by absorbing them. It may also provide appropriate culture condition for seed germination by aerating the medium. Roy *et al.* (2007) observed similar stimulatory effect of AC on the culture growth of *Thunia marshalliana* by reducing the influence of inhibitory substances in the culture media. Also the increase in concentration level of plant growth regulators might have favoured enhance seed germination response once the presence of possible detrimental factors in culture medium was eliminated by AC. The promotive effects of plant growth regulators like cytokines and auxins on seed germination of orchids have been previously shown by Miyoshi and Mii (1995, 1998), Stewart and Kane (2006) and Godo *et al.* (2010). Shoot formation was also promoted in presence of BAP and rapid shoot multiplication was observed in the culture (Fig. 1d). Maximum number of shoots per seedling (5.91±0.96) was recorded in Mitra medium fortified with 1.2 mg L^{-1} of BAP and 0.2% AC (Table 1). The enhancing effect of BAP on shoot formation was earlier reported in *Cattleya, Cymbidium* and *Paphiopedilum* orchids by Nagaraju *et al.* (2003), Das *et al.* (2007) and Wattanawikkit *et al.* (2011), respectively. The seedlings regenerated under this growth regulator condition were healthy with luxuriantly growing chlorophyllous leaves (Fig. 1e). Supplementation of 0.4 or 0.8 mg L^{-1} of NAA in MS medium did not produce any positive response for seed germination in *C. dayanum* as very few seeds germinated successfully. This finding is substantiated with the earlier report by Rasmussen (1995) on poor seed germination of orchids in presence of auxins such as NAA, IBA and IAA. In contrasts to the above finding seed germination was very high in Mitra medium amended with NAA. In fact the highest seed germination (94.58±0.32) for *C. dayanum* in present investigation was recorded in Mitra medium fortified with 0.7 mg L^{-1} of NAA. In addition to inducing high germination response, NAA also found effective in accelerating rapid root development. Enhanced multiple rooting was seen in culture medium supplemented with 0.2% AC and NAA leading to formation of robust root system (Fig. 1f). Rooting (number of roots per plant) was maximum in MS medium enriched with 1.2 mg L^{-1} of NAA and 0.2% AC (Table 2). This result is similar with the observation previously made by Roy *et al.* (2011) in *Vanda coerulea* when they recorded maximum root number and diameter in presence of AC and NAA in the culture medium. The importance of auxins in inducing efficient rooting was also earlier illustrated in *Satyrium nepalense* and *Oncidium taka* by Mahendran and Bai (2009) and Rahman *et al.* (2005), respectively. Mitra medium was more effective in shoot production per seedling (4.92±0.84; 5.91±0.96; 4.51±1.01; 4.27±0.73) as compared to MS medium (4.62±0.50; 5.25±0.63; 3.93±0.64; 4.52±0.56) when both the culture media were supplemented with different growth regulator combinations (Fig. 2). Highest number of root

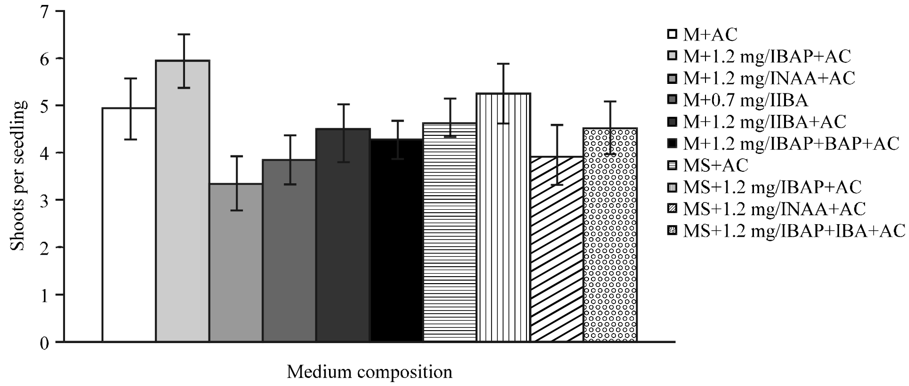

Fig. 2: *In vitro* shoot formation of *C. dayanum* on different culture medium composition

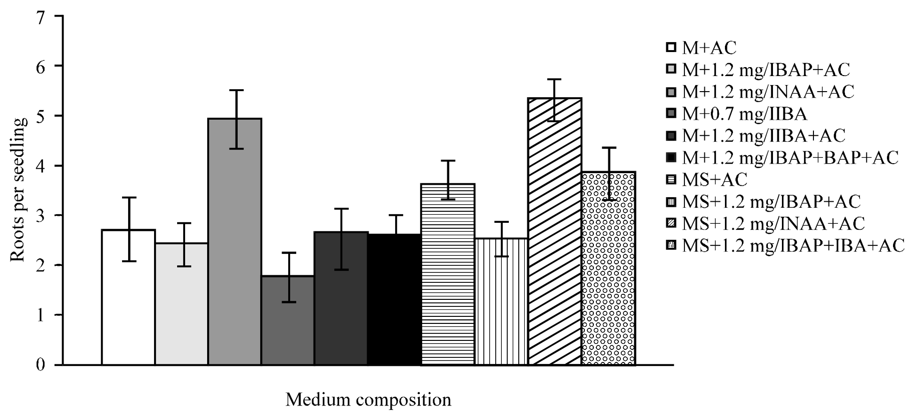

Fig. 3: *In vitro* root formation of *C. dayanum* on different culture medium composition

formation per seedling was observed for both Mitra (4.92±1.24) and MS (5.32±0.35) when the culture media were enriched with 1.2 mg L^{-1} and 0.2% AC (Fig. 3). Presence of IBA in MS medium was not conducive for plantlet growth *in vitro* as no seedling was developed after protocorm stage. However, seed germination and other cultural responses improved in Mitra medium treated with IBA and 0.2% AC. Their beneficial effect on culture growth *in vitro* had previously been demonstrated by Nongdam and Nirmala (2006) in *Cymbidium giganteum*.

CONCLUSIONS

The above study indicated variable growth response of *C. dayanum* to different culture media enriched with plant growth regulators at varied concentrations. The present study further revealed the importance of AC in seed germination of orchid *in vitro*. There was substantial improvement in seed germination in basal culture medium with AC even when no plant growth regulator was present. NAA supplemented with 0.2% of AC could be seen as the most appropriate medium for rooting for this cymbidium orchid. Protocorm formation highly delayed and protocorms poorly developed when BAP alone or with IBA was supplemented in the medium. Addition of 0.2% AC in the above hormonal combination alleviated the detrimental effects resulting in the significant improvement in seed germination and *in vitro* plantlet growth. Shooting response was also found to be very high with BAP and AC in the culture medium producing luxuriant leaf formation. The presence of AC did not help much in promoting plantlet growth in MS medium amended with IBA. Seedlings complete with 2-3 healthy green leaves and roots obtained *in vitro* were hardened and transplanted to community pots for necessary acclimatization. The *in vitro* regeneration protocols formulated from present investigation are quite useful in rapid regeneration of this rare ornamental orchid at larger scale as compared to other conventional propagation methods. The healthy *in vitro* propagated *C. dayanum* which were appropriately hardened and well acclimatized in the nursery condition could be reintroduced into its natural habitat as one of efficient conservation tactics.

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