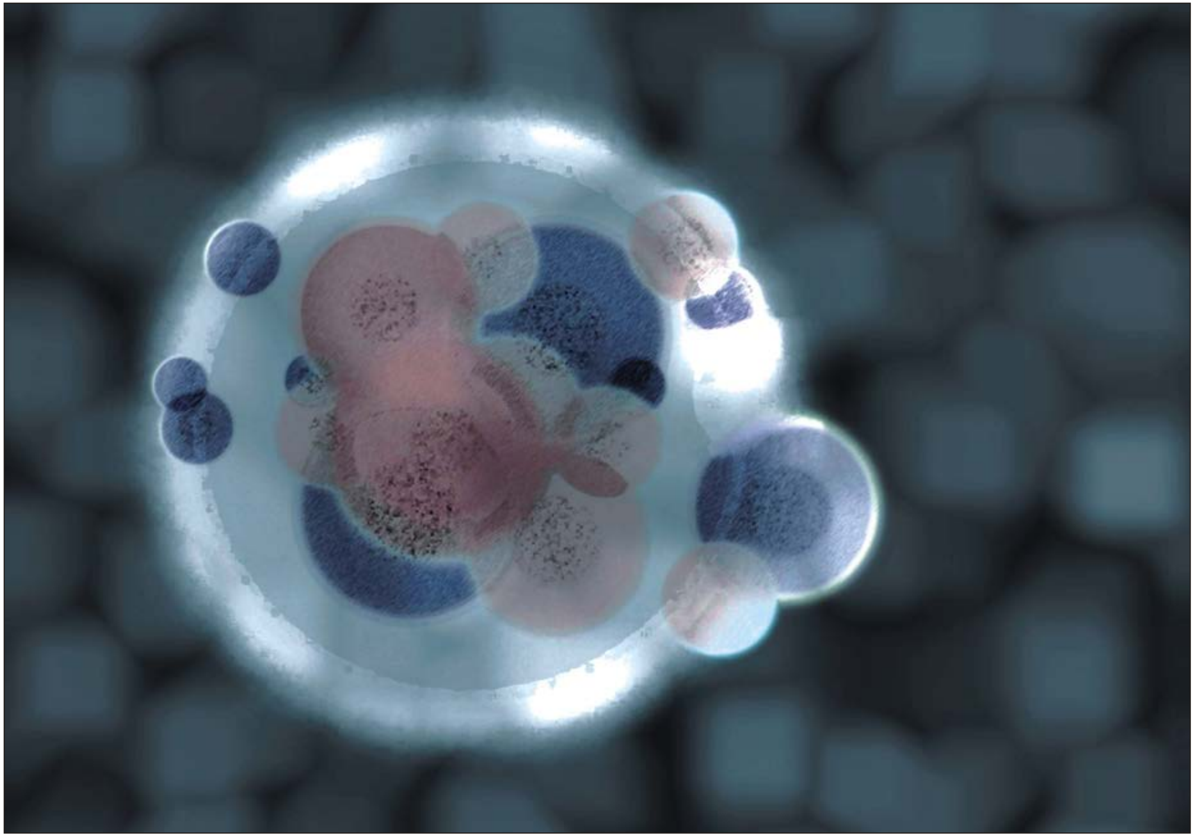

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In vitro Rapid Propagation of *Cymbidium aloifolium* (L.) Sw.: A Medicinally Important Orchid via Seed Culture

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Abstract: *Cymbidium aloifolium* (L.) Sw., an epiphytic orchid is one of the rare orchids of North-Eastern India having high medicinal and ornamental values. The present study was conducted in an attempt to preserve this important orchid by establishing an efficient *in vitro* regeneration protocol using seed culture. Two culture media viz., Mitra (M) and Murashige and Skoog (MS) supplemented with different plant growth regulators like Indole-3-butyric acid (IBA), 6-Benzyl Amino Purine (BAP) and 1-Nathyl Acetic Acid (NAA) singly or in combination were used for the present study. In 0.5 mg L^{-1} BAP supplemented media, seed germination percentage was very low and seedling formation was not observed. A similar response was observed when a combination of both IBA and BAP at 0.5 mg L^{-1} was used. Protocorm development was delayed and insignificant protocorms turned brown in color with the lost of chlorophyll and finally died even after repeated subculturing. Presence of Activated Charcoal (AC) significantly improved seed germination, protocorm formation and shoot multiplication. Shooting was best observed in MS medium supplemented with 1 mg L^{-1} BAP and AC. Auxins like NAA and IBA proved to be beneficial for rapid root initiation. However, more superior rooting was observed when M medium was enriched with 0.5 mg L^{-1} of NAA. Healthy seedlings with well developed roots were obtained which were then hardened and transplanted to community pots for further acclimatization to the nursery conditions. The regenerated plants can subsequently be used for commercial and conservation purposes.

Key words: *Cymbidium aloifolium*, orchids, seed culture, protocorm, activated charcoal, conservation

INTRODUCTION

Orchids which are considered as world's most wondrous plants represent one of the most advanced families among monocotyledons with over 25000 species, enumerable hybrids and varieties (Chugh *et al*., 2009). They have high floricultural appeals because of their extraordinarily beautiful flowers with incredible range of variation in floral shape, size, coloration and fragrance. They account for 7% of total flowering plant species which represent one of the most expensive ornamental known today and dominate the international cut flower trade (Rahman *et al*., 2005a). Besides their high ornamental values, orchids are of considerable importance in medicines as they have rich contents of alkaloids, glycerides and other useful phytochemicals (Gutierrez, 2010). Because of these properties, the plants are used for variety of folk medicines and cures by local people. Like other medicinally important orchids of this region, *Cymbidium aloifolium* also occupies a significant position in the everyday life of tribal people of North-Eastern India due to its medicinal and ornamental values. The indigenous people especially in hilly regions take immense pride in treasuring this plant because of its high utility in traditional healing and cures and floriculture trade (Medhi and Chakrabarti, 2009). The leaves are extensively used for styptic properties in the treatment of boils and fevers. The roots are tuberous and are good source of saleb and used as nutrient and demulcent. The roots are also pounded with ginger and the mixture is extracted with water and used as medicine to cure paralysis and chronic illness (Das *et al*., 2008). Some tribal people in this region used the small seeds for healing wound (Medhi and Chakrabarti, 2009). In addition to this, the whole plant can also be used as tonic and in the treatment of vertigo, weakness of eyes, burns and sores (Hossain, 2011). Apart from its medical importance, this orchid commands a good price in the floricultural market because of its perpetual, highly intricate beautiful flower with purple coloration. The orchid population once

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thriving well in its natural habitat showed alarming decline mainly due to rampant deforestation for agricultural practices and heavy indiscriminate collection by locals from wild for personal benefits (Chowdhery, 2001). An efficient conservation strategies need to be designed to not only save this valuable species from the brink of extinction but also harness its wide range of economic potential. Plant tissue culture techniques provide a new dimension to more efficient conservation and commercialization of a number of rare and useful orchid species (Vij and Aggarwal, 2003). However, the use of micropropagation techniques for propagation of orchids at commercial scale cannot be fully realised till now. This limitation is due to the non availability of efficient and reliable protocol for seed germination, very limited understanding of growth and development *in vitro* and high mortality rate during transplantation. Orchid seeds which are minute and non-endospermic rarely germinate in nature as they require appropriate mycorrhizal fungal infection for successful seed germination (Gutiarrez-Miceli *et al*., 2008; Mohanraj *et al*., 2009). The fungal association is believed to provide physiochemical stimulus required for growth initiation (Sharma and Tandon, 1990; Ovando *et al*., 2005). Orchid seeds can be germinated *in vitro* without any fungal association and many workers have successfully performed asymbiotic seed germination *in vitro* leading to plant regeneration (Tsay *et al*., 2004; Chang *et al*., 2005; Sazak and Ozdener, 2006). However, the available reports on the regeneration of *C. aloifolium* through seed culture are still very less though it has been successfully regenerated from several other explants (Das and Bhadra, 1998; Barua and Bhadra, 1999; Nayak *et al*., 2002). More efforts need to be undertaken to develop a standardized commercial scale micropropagation technique through seed culture which would help in the production of quality planting materials in larger scale. Moreover, a critical investigation is required to understand the peculiarities of seed germination, protocorm formation and seedling development *in vitro* in response to varied hormonal combinations at different concentration and also other stimulus present in the nutrient medium. The present study was undertaken with a main objective of preserving and conserving this rare multi utility orchid of this region by developing an efficient and reliable *in vitro* regeneration protocol.

MATERIALS AND METHODS

The present study was carried out at Department of Botany, Panjab University, Chandigarh from September, 2004 to June, 2006. Green unripe capsules of *C. aloifolium* were collected 24 weeks after pollination from forested areas of Manipur in North Eastern part of India. The capsules are then thoroughly washed under the running tape water with 20% teepol for 10-15 min. They were then surface sterilized for 7-8 min in 0.4% $HgCl_2$ solution with 1-2 drops of teepol as wetting agent. This was followed by repeated washing of treated capsules for 3-4 times in sterile distilled water to remove $HgCl_2$ completely from its surface. The capsules were finally dipped in 70% ethanol for 15-20 min followed by flaming for 3-4 sec. The surfaced sterilized capsules were subsequently split opened by using a sharp sterilized surgical blade and the immature seeds were scooped out to be used as explants for initiating culture. The whole procedure was performed in aseptic condition under laminar flow to prevent any form of contaminations.

The extracted seeds were inoculated on agar gelled Mitra *et al*. (1976) and Murashige and Skoog (1962) medium to determine the most appropriate medium for seed germination and development. The culture media contained 0.9% agar and 2% sucrose at pH of 5.8. The growth response was monitored with or without 0.2% of activated charcoal in the culture media. When AC was incorporated, complete dispersion was required by swirling the vessels before the medium got solidified. Plant growth regulators like Indole-3-butyric Acid (IBA), 6-Benzyl Amino Purine (BAP) and 1-Nathyl Acetic Acid (NAA) with different concentration at 0.5 and 1 mg L^{-1} were supplemented either singly or in combination to study their effect on the growth of the culture *in vitro*. The growth regulators were added prior to autoclaving and definite quantities of the medium were dispensed to test tubes and conical flasks and closed tightly with cotton plugs. The medium was autoclaved at 1.1 kg cm^2 pressure and 121°C for 15-20 min. After autoclaving, the culture vessels with sterilized medium were then placed in an appropriate position (test tubes in slanting and flask in vertical) to allow the medium to gel. The cultures were then maintained at 25±2°C which was illuminated at 60 $\mu mol/m^2/sec$ for 12 h a day using white fluorescent tubes.

Subculturing was carried out after every 4 weeks and 15 replicates were used and each treatment was repeated twice. The rate of seed germination was recorded after 3-5 weeks of culture as the seeds started swelling due to absorption of nutrients. The seed germination percentage was calculated by dividing the number of germinated seeds by total number of seeds inoculated multiplied by 100. The formation of globular structure spherules as well as the subsequent protocorm development in weeks was recorded to analyse different stages of development leading to seedling formation. The number of shoots and

roots formed in the seedlings were also recorded to determine suitable medium and growth hormone combinations for appropriate shooting and rooting.

The data recorded from the present study was subjected to Analysis of Variance (ANOVA) and significant differences were determined by Duncan's multiple range test at p = 0.05 (Duncan, 1955). The statistical data analysis was conducted using the SPSS (SPSS Inc., Chicago, USA). The well grown seedlings complete with leaves and roots were subsequently hardened by culturing them for 2 weeks in a medium (full strength) without any growth regulators followed by culturing them in the similar condition for few weeks without sucrose and vitamins in the medium. The hardened well rooted seedlings were removed and rinsed thoroughly with luke warm sterile water to remove agar and nutrients sticking to them and treat with 0.01% fungicide solution for 15-20 min. The plants are then transplanted to plastic/clay pots containing brick pieces, pine bark, charcoal pieces and moss (1:1:1) as potting mixture. The transplanted plants were finally kept in the glass house for further acclimatization to nursery condition.

RESULTS AND DISCUSSION

The immature seeds of *C. aloifolium* showed varied response depending upon the type of medium used as well as different plant growth regulators employed at varied concentrations. The two culture media used in the present study have differential combination of minerals, micro and macro elements. MS has richer contents of macro and micro-elements but lower vitamins as compared to M medium. Seed germination in the two culture basal media without any growth regulators was found to be high at around 83.52% in Mitra medium and 74.53% in MS medium (Table 1, 2).The positive influence of basal MS medium on seed germination was also observed in *Vanda coerulea* (Roy *et al*., 2011) and *Dendrobium transparens* (Alam *et al*., 2002). There are reports of species-specific media for successful seed germination of orchids *in vitro* (Bhadra *et al*., 2002). The seeds started swelling in two weeks times indicating successful germination. The swelling was due to imbibition of water and nutrients and the embryos eventually underwent several divisions to produce irregularly shaped parenchymatous cell mass called spherules which emerged out from the cracked seed coat. These globular spherules with hairy growth at the basal portion developed into protocorms which are a chlorophylous initially but subsequently turned green in color after acquiring chlorophyll in 6-7 weeks (Fig. 1a). This phenomenon was also observed by Sheelavanthmath and Murthy (2001) and Gayatri *et al*. (2006) in some terrestrial orchids. The protocorms are round, oval, elongated, branched or spindle shaped bodies which are considered as an intermediate structure between the embryos and the plants. These protocorms then underwent further morphogenetic changes resulting in the development of leaf primordia and finally to the formation of complete seedling in 14-15 weeks in Mitra and 15-16 weeks in MS medium. Nayak *et al*. (1998) reported regeneration of *C. aloifolium* by using rhizomes developed from the immature seeds when grown on the MS medium supplemented with different growth regulators like IAA, IBA and NAA. They observed the development of rhizomes from protocorms after 2-3 months which subsequently developed into the whole plant. However, in present study the chlorophyllous protocorms directly differentiated into seedlings without rhizome formation in relatively shorter time of only 14-16 weeks (Fig. 1b). Das and Bhadra (1998) had similarly reported direct seedling formation but the time required for seedling development was comparatively longer as compare to this report. When AC was incorporated, the seed germination was enhanced followed by rapid protocorm development in 6-7 weeks. This significantly reduced time required for seedling formation by another 2-3 weeks. Complete seedlings with well developed shoots and roots were regenerated in 12-13 weeks in both the Mitra and MS media. Hossain *et al*. (2009) also reported the improvement of seed germination in *C. aloifolium* when AC was incorporated in Mitra and MS media in the absence of any additives and plant growth regulators. The presence of AC might help in adsorbing the toxic substances released in the medium due to autoclaving and also by germinating seed explants. The importance of AC in asymbiotic seed germination was earlier reported by Ernst (1975) as it helped in adsorbing phenolic exudates, aerating the medium and providing near natural conditions for seed germination by absorbing light. Supplementation of 0.5 mg L^{-1} of BAP singly or in combination with 0.5 mg L^{-1} of IBA in both the culture media drastically reduced *in vitro* germination of seeds. Presence of BAP showed reduced germination percentage of 53.40±0.35 in Mitra medium and 57.67±0.78 in MS medium. However, Hossain *et al*. (2009) observed significantly high rate of seed germination in *C. aloifolium* when 1.0 mg L^{-1} of BAP was supplemented in both the culture media. This change in germination frequency may result due to difference in the concentration of plant growth regulator employed for the present study. The few germinated seeds developed into globular structure spherules which subsequently developed into small insignificant protocorms in

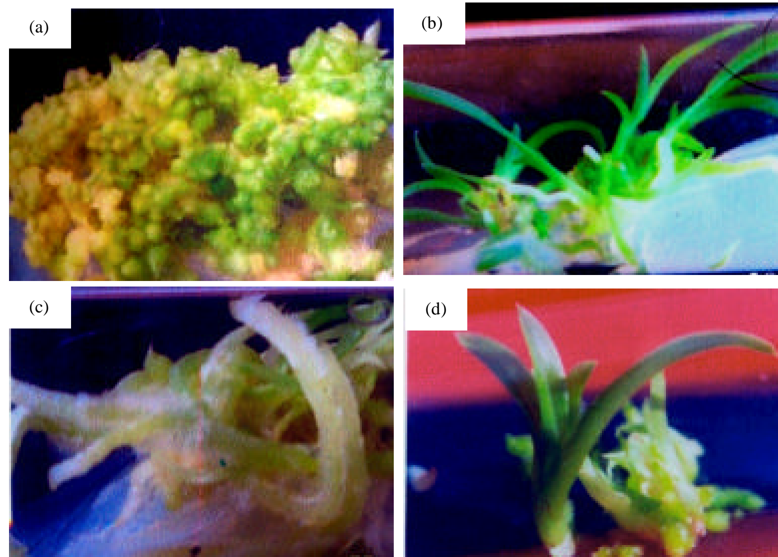

Fig. 1: *In vitro* growth response of *C. aloifolium* under different culture conditions (a) rapid protocorm proliferation in basal Mitra medium, (b) direct seedling formation from protocorms in basal MS medium, (c) robust root multiplication in Mitra medium supplemented with 0.5 mg L^{-1} of NAA and (d) enhanced leaf development in Mitra medium incorporated with 1.0 mg L^{-1} of NAA along with AC

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

		Average time taken (in weeks) for development				
Medium	Germination response (%)	Spherules	Protocorm	1st leaf primordium	2nd leaf primordium	Seedlings
M (Basal)	83.52±0.34[d]	3- 4	6-7	9-10	10-11	14-15
M*(AC)	96.47±0.34[f]	3-4	5-6	8-9	9-10	12-13
M+ (0.5 mg L^{-1}) BAP	53.40±0.35[e]	9-10	15-16	-	-	-
M*+ (1 mg L^{-1}) BAP	90.57±0.19[a]	3-4	5-6	9-10	10-11	13-14
M + (0.5 mg L^{-1}) IBA	83.90±0.76[d]	6-7	8-9	10-11	12-13	15-16
M*+ (1 mg L^{-1}) IBA	87.80±1.10[c]	4-5	6-7	9-10	11-14	13-14
M+ (0.5 mg L^{-1}) NAA	90.51±0.97[a]	3-4	5-6	7-8	8-9	10-12
M*+ (1 mg L^{-1}) NAA	90.69±0.20[a]	3-4	5-6	8-9	9-10	12-13
M+(0.5 mg L^{-1})BAP+IBA	50.20±0.45[b]	10-11	16-17	-	-	-
M*+(1mgLl^{-1}) BAP+IBA	90.41±1.48[a]	4-5	6-7	9-10	10-11	13-14

Results based on the average of 15 replicates. Means followed by same letter are not significantly different at p = 0.05. * AC was incorporated into the medium

Table 2: *In vitro* germination response of *Cymbidium aloifolium* on MS medium with different growth regulators

		Average time taken (in weeks) for development				
Medium	Germination response (%)	Spherules	Protocorm	1st leaf primordium	2nd leaf primordium	Seedlings
MS (Basal)	74.53±0.30[f]	4-5	6-7	8-9	9-10	15-16
MS*(AC)	93.17±0.11[e]	3-4	5-6	7-9	8-9	12-13
MS+ (0.5 mg L^{-1}) BAP	57.67±0.78[d]	6-7	11-12	-	-	-
MS*+ (1 mg L^{-1}) BAP	85.27±1.08[a]	4-5	6-7	8-9	9-10	13-14
MS + (0.5 mg L^{-1}) IBA	90.47±0.42[c]	4-5	7-8	9-10	10-11	13-14
MS*+ (1 mg L^{-1}) IBA	91.43±0.77[c]	3-4	5-6	7-8	8-9	12-13
MS+ (0.5 mg L^{-1}) NAA	91.37±0.25[c]	3-4	5-6	6-7	7-8	11-12
MS*+ (1 mg L^{-1}) NAA	90.46±0.40[c]	4-5	6-7	8-9	9-10	12-13
MS+(0.5 mg L^{-1})BAP+IBA	49.51±0.34[b]	8-9	12-13	-	-	-
MS*+(1 mg L^{-1}) BAP+IBA	85.34±0.38[a]	4-5	7-9	10-11	11-12	13-14

Results based on the average of 15 replicates. Means followed by same letter are not significantly different at p = 0.05. * AC was incorporated into the medium

15-16 weeks in Mitra (Table 1) and 11-12 weeks (Table 2) in MS medium. This is in contrast to earlier reports in other orchids which indicated the promotive effect of BAP in seed germination, protocorm multiplication as well as shoot multiplication (De Pauw *et al*., 1995; Chowdhury *et al*., 2003; Nhut *et al*., 2005). The presence

of both BAP and IBA also produced poor percentage of seed germination (50.20±0.45 in Mitra and 49.51±0.34 in MS medium) leading to development of small underdeveloped protocorms. These stunted protocorms did not survive long which turned brown in colour and finally died even after several subcuturing. Similar finding was reported by Hossain *et al.* (2009). Incorporation of AC with increased concentration of plant growth regulators (1 mg L^{-1}) significantly improved germination rate and protocorm formation in both the culture media. Large protocorms with high chlorophyll content were evident in short span of time. This showed that either the inhibitory effect of BAP when used singly or its antagonistic response to IBA when present together was alleviated by combined action of AC and increased concentration of growth regulator in the medium. This is also indicative of the high adsorptive nature of AC in eliminating the potential growth inhibitors released in the culture thereby producing increased germination rate and large healthy protocorm formation. Roy *et al.* (2007) earlier demonstrated the stimulatory effect of AC on culture growth of *Thunia marshalliana* by reducing the inhibitory effect of other compounds present in either MS or Phytamax media. MS medium supplemented with 1 mg L^{-1} of BAP and 2 g L^{-1} of AC was found to be most suitable for shoot multiplication as the surface of protocorms with several small green protuberances subsequently developed into multiple shoots in 7-8 weeks time. Maximum number of shoots (5.71±0.96) per seedling was recorded in this combination (Table 4). The induction of multiple shoot proliferation directly from the protocorms in presence of cytokinins was similarly reported in other orchids (Nayak *et al.*, 1997; Chang and Chang, 1998). Rooting (No. of roots per seedling) was also improved with AC (3.82±0.42 in Mitra and 2.81±0.81 in MS medium) in addition to enhanced shoot multiplication (Table 3). This finding substantiated the earlier report by Eymar *et al.* (2000) which stated that the addition of AC helped in maintaining the pH level during culture, increased nitrogen uptake and improved culture development thereby reducing the inhibitory effect of exogenous cytokinin on root growth. Seed germination was found to be significantly improved when NAA was incorporated into the culture media. The enhanced effect of NAA in seed germination was also reported in *Calanthe tricarinata* by Godo *et al.* (2010). The germinated seeds subsequently developed into healthy chlorophyllous protocorms in 5-6 weeks which was followed by rapid root initiation in 2 weeks resulting into the formation of stout root system (Fig. 1c). The promotive effect of auxin in producing enhanced

Table 3: Effect of different growth regulators on shoot and root formation of *C. aloifolium* after 16 weeks of culture on Mitra medium

Medium	Growth regulators	No. of shoots	No. of roots
M	-	3.61±0.70^{c}	2.80±0.71^{b}
M*	-	3.92±0.31^{b}	2.41±0.51^{a}
M	(0.5mgLl^{-1})BAP	0	0
M*	(1 mg L^{-1}) BAP	5.41±0.80^{e}	3.82±0.42^{d}
M	(0.5 mg L^{-1})IBA	2.61±0.26^{d}	5.41±0.69^{d}
M*	(1 mg L^{-1}) IBA	3.60±0.69^{c}	2.81±1.30^{b}
M	(0.5 mg L^{-1})NAA	3.61±0.80^{c}	5.80±1.76^{c}
M*	(1 mg L^{-1})NAA	3.92-±0.41^{b}	2.81±0.72^{b}
M	(0.5 mg L^{-1})BAP+IBA	0	0
M*	(1 mg L^{-1}) BAP+IBA	4.51±0.72^{a}	2.41±0.50^{a}

Values are Mean±SD, N = 15. Means followed by same letter are not significantly different at p = 0.05. *Activated Charcoal (AC) was incorporated into the medium

Table 4: Effect of different growth regulators on shoot and root formation of *C. aloifolium* after 16 weeks of culture on MS medium

Medium	Growth regulators	No. of shoots	No. of roots
MS	-	3.42±0.51^{a}	3.20±1.07^{b}
MS*	-	4.61±0.84^{e}	3.11±0.53^{b}
MS	(0.5 mg L^{-1})BAP	0	0
MS*	(1 mg L^{-1}) BAP	5.71±0.96^{d}	2.81±0.80^{e}
MS	(0.5 mg L^{-1})IBA	3.41±1.09^{a}	4.61±1.01^{d}
MS*	(1 mg L^{-1}) IBA	4.10±0.56^{c}	2.40±0.84^{a}
MS	(0.5 mg L^{-1})NAA	3.41±0.69^{a}	5.21±0.63^{c}
MS*	(1 mg L^{-1})NAA	4.20±1.01^{b}	3.20±0.76^{b}
MS	(0.5 mg L^{-1})BAP+IBA	0	0
MS*	(1 mg L^{-1}) BAP+IBA	3.40±0.73^{a}	2.41±1.54^{a}

Values are Mean±SD, N = 15. Means followed by same letter are not significantly different at p = 0.05. *Activated Charcoal (AC) was incorporated into the medium

rooting was also demonstrated in *Cymbidium devonium* (Das *et al.*, 2007), *Vanda coerulea* (Malabadi *et al.*, 2004), *Oncidium taka* (Rahman *et al.*, 2005b) and *Encylia maraie* (Santos Diaz and Alvarez, 2009). The presence of 0.5 mg L^{-1} IBA similarly provided a conducive environment for rigorous root formation with 5.41±0.69 and 4.61±1.01 (roots per seedling) recorded in Mitra and MS medium respectively. The effectiveness of IBA in inducing rooting in orchids had earlier been reported by many workers (Giridhar *et al.*, 2001; Nongdam and Nirmala, 2009). However, NAA was found to be more effective in producing rooting than IBA in *Cymbidium aloifolium.* Medium supplemented with 0.5 mg L^{-1} of NAA was found to be most suited for root development with maximum number of root formation recorded in Mitra medium (5.80±1.76). Surprisingly when AC was added, there was reduction in root formation even after the concentration of either NAA or IBA was increased to 1 mg L^{-1}. This might suggest the detrimental affect of AC in rooting initiation in *C. aloifolium* in the presence of auxin while promoting shoot formation. Leaf formation was enhanced and more prominent with AC in the culture medium (Fig. 1d). Such observation was also earlier reported in *Dendrobium* orchid by Nagaraju *et al.* (2004).

CONCLUSION

The present study showed that the immature seeds of *C. aloifolium* responded in accordance to the various stimuli present in the culture environment. Maximum germination response was recorded on basal Medium supplemented with AC. BAP singly or in association with IBA in absence of AC did not provide appropriate culture condition for seed germination, protocorm formation and seedling development. There was delay in the development of healthy protocorms. Instead stunted and insignificant protocorms were produced which ultimately died due to loss of chlorophyll. However, 1 mg L^{-1} BAP with AC in MS medium produced maximum shooting while rooting was significantly high when either 0.5 mg L^{-1} of NAA or IBA was used in both the culture media. The *in vitro* plants with 2-3 well developed leaves and roots were gradually hardened and acclimatized to the nursery conditions. The *in vitro* regeneration protocol established from the present investigation can be employed for the propagation of this useful orchid in larger scale which may not only help in addressing the urgent local needs for effective conservation but also meet the high pharmaceutical and ornamental requirements of this region.

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