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CONSERVATION OF THREATENED ORCHID *Aerides multiflora* Roxb. THROUGH *in vitro* ASYMBIOTUC SEED CULTURE

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ABSTRACT

The present studies were intended to expedite *in vitro* conservation of a threatened orchid *Aerides multiflora* through asymbiotic seed germination technique. The capsules were harvested at two different stages of development. The seeds were inoculated in Murashige and Skoog, 1962 (MS) medium as source of nutrition. The effect of PGRs [6-benzylaminopurine (BAP) @ 1 mg L^{-1} and auxin (α-naphthalene acetic acid; NAA @ 1 mg L^{-1}) was also assessed individually and in combination for various morphogenetic events such as seed germination percentage, morphogenetic changes and seedling development which varied with the capsule stage. The seeds obtained from undehisced light-brown capsule germinated with better percentage as compared to seeds obtained from undehisced green capsule. Cent per cent seed germination was achieved in basal MS medium alone and BAP supplemented medium. Seedling with 2-3 leaves and 1-2 roots were formed with in 20.00 ± 0.20 weeks old cultures in basal medium. The growth of germinating entities remained arrested in NAA supplemented medium and resumed growth upon shift to ½ strength medium fortified with peptone (1 g L^{-1}). BAP favoured multiplication of protocorms.

Keywords: Asymbiotic, embryo, *in vitro*, orchid seeds, testa

INTRODUCTION

The orchid plants are geologically young and belong to a monocot family Orchidaceae, which has about 25,000 species in nearly 800 genera worldwide; most of them grow in humid environment of tropical to subtropical climates, except Antarctica (Gogoi *et al*., 2012). In India, 1295 species from 179 genera are reported which are listed in CITES Appendix II (Kumar *et al*., 2024). Appendix I of CITES include *Renanthera imschootiana* (also known as "Red *Vanda*"), and approximately ten species of *Paphiopedilum* are included in it. *Liparis olivacea* has become extinct and *Dactylorhiza hatagirea* (Himalayan medicinal orchid) is enlisted as critically endangered in CITES Appendix I of convention on international trade in endangered species (Kumar *et al*., 2024).

In nature, the capsule of orchid species produces innumerable (millions in number) minute seeds (Arditti and Ghani, 2000). Only a few orchid seeds germinate and form seedling while most of them perish owing to highly undifferentiated embryos and lack of the ability to utilize their nutritional reserves. Moreover, the seeds require suitable mycorrhizal association to accomplish germination (Rasmussen, 1995). Such requirements are not met by most of the orchid seeds and most of them perish or show exceptionally low germination. This situation can be successfully overcome through rescuing the embryos, using *in vitro* asymbiotic seed germination technique. This technique is highly beneficial which ensures improved percentage of germination ensures better germination rates from

immature seeds because of their physiologically active state. Immature seeds possess metabolically awakened embryos and distended testa cells, and also lack dormancy or inhibitory factors (Kaur and Bhutani, 2014; Kendon *et al*., 2017; Koene *et al*., 2019; Nabieva, 2021). The technique is extensively utilized to save innumerable orchid embryos, to propagate desired genotype, in cloning the apomictic taxa, and various genetic transformation studies (Men *et al*., 2003). *In vitro* asymbiotic seed germination technique has been invariably tested in a variety of orchids of diverse habit and habitats (Magrini *et al*., 2011; Kaur and Bhutani, 2014; Kaur, 2021).

Orchids being highly medicinal and floriculturally significant, are collected from nature stealthily and their genetic diversity is shrinking swiftly. Consequently, whole orchidaceae family has become rare, endangered and threatened and included in appendix II orchid checklist (CITES, 2024). Thus, to conserve the gene pool of orchids a species *Aerides multiflora* (Roxb.) was selected.

A. multiflora, a fragrant, multi-flowered fox brush orchid, is a monopodial, epiphytic horticulturally important orchid species. The species bears light pink-purplish coloured fragrant flowers growing in pendulous racemose inflorescence, during spring and summer seasons (May-June). *A. multiflora* is distributed in subtropical climates of North- eastern Indian Himalayas (Assam, Nagaland, Meghalaya, Mizoram, Arunachal Pradesh) (Bose and Bhattacharjee, 1980). *A. multiflora* has a great potential for commercial exploitation in the cut-flower industry. It is quite challenging to propagate the species through vegetative means as it grows slowly in nature. Therefore, it becomes essential to save the species using biotechnological means. The study was aimed to initiate *in vitro* cultures of *Aerides multiflora* through asymbiotic seed germination technique besides checking the efficacy of Murashige and Skoog (1962) medium, growth regulators, and simultaneously analysing sequential morphogenetic milestones that seeds achieve during germination process *in vitro* in the basal medium thus devising a simple protocol.

MATERIALS AND METHODS

The apparently healthy capsules were collected from plants of *A. multiflora* growing on tree trunks of *Mangifera indica* in Cachar district, Assam, India (latitude range: 24° 50' 2.2056" N; longitude range:92° 46' 45.4152" E) in June, 2023. The capsules were procured at two different stages of development. The capsule characters such as colour, size, and development of ridges were noticed. Asymbiotic orchid seed germination medium such as Murashige and Skoog (1962) medium (Hi-media, Mumbai, India) was used to initiate the cultures. The pH of medium was adjusted to 5.7 using 1N HCl/1N NaOH before autoclaving. The effect of auxin (NAA) and cytokinin (BAP), applied @ 1 mg L^{-1}, was also checked on the asymbiotic seed germination, multiplication of protocorms and other morphological processes. The medium was /gelled with agar (Hi-media, Mumbai, India), dispensed into (25 × 150 mm) test tubes and autoclaved at 121°C at a pressure of 1.06 kg cm^{-2} for 15 min. Autoclaved medium was kept at 37°C for 2–3 days to check for any contamination. Seed viability was performed by using TTC salt (2,3,5-triphenyl-2H-tetrazolium chloride; 1%; pH 6.5) staining method (Van Waes and Debergh, 1986). The seeds were kept in dark while immersed in stain for 8 h at 30°C.

The capsules (1-2) were first gently scrubbed with an ultra-soft brush in running tap water to remove any debris from their surface. Each capsule was rinsed thoroughly with dish-wash detergent solution. The capsule was swabbed with ethyl alcohol in a laminar air flow cabinet and sequentially, disinfected with aqueous solution of 0.05% mercuric chloride (Qualigens, Mumbai, India) containing 1–2 drops of "Teepol" for 2–3 min and 0.05% streptomycin (Angel Pharma International, India) for 1-2 min. Finally, the capsule was rinsed thoroughly with sterilized distilled water. Thereafter, the capsule was incised longitudinally and the seeds were scooped out into a petridish. The cultures vessels, inoculated with seeds, were incubated under 12 h photoperiod under 40 μmol m^{-2} s^{-1} light

intensity (fluorescent tubes, Philips India Ltd, Mumbai, India) at approximately 25 ± 2°C temperature. Eight replicates were used per treatment. To check the reproducibility of the protocol, the experiments were repeated twice. The cultures were sub-cultured into their respective fresh medium as and when required.

After 4 week's inoculation, a few seeds were taken out of the culture vessel with the help of a spatula, placed in a drop of water on a glass slide and observed under a light microscope. Once the embryos emerged out of the seed coat and spherules were formed, the observations were taken at 1 week's intervals. Different development events of germinating seeds observed for morphological events were as under:

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

Photographs of different morphological events were taken using a stereozoom microscope (Nikon, H600L, Japan). Seed germination was calculated as per the formula given by Kaur and Bhutani (2014). The cultures were observed regularly under a binocular microscope (Olympus SZX10, Japan) and morphological events and data recorded. The experiment was set in complete randomized design having eight replicates per treatment. The data generated was analysed by using one-way ANOVA test (Gomez and Gomez, 1984) and the data presented by calculating mean and standard deviation. The reproducibility of the protocol was checked.

RESULTS AND DISCUSSION

In present study, *in vitro* cultures of *Aerides multiflora* were initiated through asymbiotic seed germination method in MS medium alone and in combinations with PGRs (Fig. 1). The seeds germination was 100% in basal MS medium. The germination of *A. multiflora* seeds was not obligatory to the use of plant growth regulators. Seed germination on basal MS medium (control) indicated the presence of sufficient level of endogenous hormones in seeds. Similar kind of response have earlier been reported with respect to the seed germination in MS medium in *Coelogyne flaccida* (Kaur and Bhutani, 2014), *A. multiflora* (Verma *et al*., 2015), *A. multiflora*, *Cleisocentron pallens*, *Cymbidium aloifolium*, *Dendrobium aduncum*, *D. fimbriatum*, *D. lituiflorum*, *D. moschatum*, *Phalaenopsis mannii*, *Phaius tankerville* and *Rhynchostylis retusa* (Barua *et al*., 2022). Earlier, a variety of media such as Mitra *et al*. (1976) medium, potato dextrose agar (PDA), etc. were also used for assessing the germination potential of *A. multiflora* seeds (Verma *et al*., 2015; Barua *et al*., 2022).

In present study, the seeds scooped out of their capsules were minute and dust-like. It is well known, that the size of orchid seed is quite small about 0.25 to 1.2 mm in length and 0.09 to 1.2 mm in width. A single capsule contained nearly about 4 million powdery seeds (Koene *et al*., 2019) and the present species is no exception. The seed germination of *A. multiflora* was markedly affected by the developmental stage of capsule. The seeds, from the green undehisced capsules harvested after 5 months, did not germinate in MS medium alone or in combinations with growth adjuncts. The capsule measured 2.60 ± 0.10 cm in length and 0.75 ± 0.41 cm in width having 1.00 ± 0.00 g average weight with three less prominent ridges on its external surface (Table 1; Fig. 1B-D). On the other hand, the seeds scooped out from 8 months old undehisced and light-brown capsule (2.92 ± 0.21 cm in length and 0.82 ± 0.06 cm in width) [Fig. 1E] germinated with cent per cent frequency in basal MS medium (Table 2). All the seeds were viable as their embryos turned red when stained with TTC solution (Fig. 1F). Our results are in agreement with Koene *et al*. (2019) in case of *Acianthera prolifera*, where also

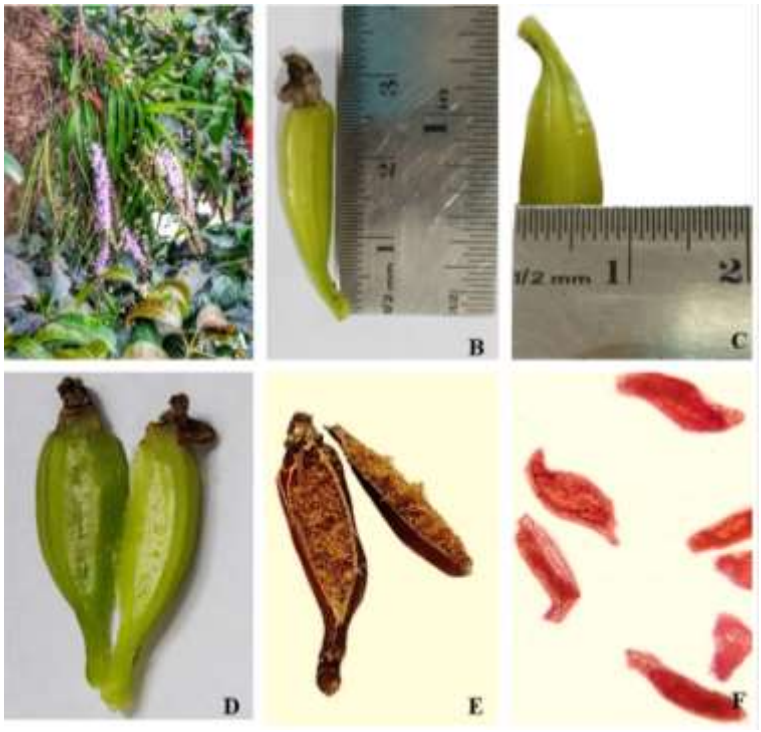

Fig. 1: *Aerides multiflora* A) Plant during flowering stage, B) Green undehisced capsules harvested after 5 months measured lengthwise, C) Pod measured width-wise, D) 5 weeks old green pod opened with longitudinal slit showing less development of seeds, E) 8 weeks old light brown colour fully filled with seeds, and F) Viable seeds with their embryo turned red when stained with TTC solution (4 x)

Table 1: Morphological features of *Aerides multiflora* capsules at different stages of development

Capsule age month)	Capsule stage	Colour	Length (cm)	Width (cm)	Weight (g)	Surface Texture	Germination (%)	Colour of capsule
5	Undehisced	Green	2.60 ± 0.10	0.75 ± 0.41	1.00 ± 0.00	3 less prominent ridges	00.00 ± 0.00	Green
8	Undehisced	Light brown	2.92 ± 0.21	0.82 ± 0.06	0.99 ± 0.05	3 well developed prominent ridges	100.00 ± 0.00	Light-brown

Data represented mean ± SD

TTC test ensured viability in seeds. *In vitro* asymbiotic seed germination success rate of orchid seeds largely depends upon the capsule's maturity stage at the time of harvesting. This is confirmed through present study on *A. multiflora* and earlier studies on *C. flaccida* (Baskin *et al*., 2006; Kaur and Bhutani, 2014; Nabieva, 2021). Similar response has been observed in *Cephalanthera falcata* wherein the seeds harvested at early stage germinated with poor frequency (Yamazaki and Miyoshi, 2006). This kind of germination response could be attributed to less developed embryo as earlier suggested by Yamazaki and Miyoshi (2006) in *C. falcata*. Literature studies point out on the metabolically awakened well-developed embryos. The results are similar to the earlier findings in *Dendrobium chrysotoxum* (Kaur and Bhutani, 2011), *Paphiopedilum Venustum* (Kaur and Bhutani, 2014), *Vanda testacea* (Kaur, 2021), *Cleisostoma ramaciferum* (Temjensangba and Deb, 2006). Earlier reports in *Phalaenopsis amabilis* var. *formosa* (Lee *et al*. (2008) and *Dendrobium nobile* (Vasudevan and van Staden, 2010) have emphasized upon the existence of correlation between internal organization of immature seeds with their germination abilities which strongly point that a discontinuous cuticle layer envelops the embryo proper, there are gaps which are formed by the degeneration of inner integument, and absence of thickenings in the secondary wall in outer integument. All these changes in seed integumentary changes play a vital role in maximizing the germination success of immature seeds. Interestingly, in our cultures, small suspensor was also found attached to the embryo. Normally, suspensor is either found absent or present in rudimentary state in orchid embryos and the present genus is not exception.

Table 2: *In vitro* asymbiotic seed germination in *Aerides multiflora* and associated morphogenetic events in MS medium and its combinations with growth adjuncts

Additive	Germination (%)	Time taken for (wks)						
		Development of			Differentiation of			Seedling development
		Spherule	Chlorophyll	Protocorm	1st leaf	2nd leaf	1st root	
Control	100 ± 0.00	7.42 ± 0.07	10.2 ± 0.06	12.56 ± 0.3	13.52 ± 0.07	14.12 ± 0.10	16.76 ± 0.25	20.00 ± 0.20
BAP	100 ± 0.00	7.2 ± 0.06	10.5 ± 0.06	11.5 ± 0.9	12.6 ± 0.08	13.44 ± 0.07	Rooting remained elusive and cultures shifted to 1/2 MS medium + NAA + P	
NAA	65 ± 0.00	9.08 ± 0.07	Cultures shifted to 1/2 MS medium + NAA + P					
BAP + NAA	70 ± 0.00	9.34 ± 0.04	Culture necrosis					

Note: MS medium: Murashige and Skoog (1962) medium; Concentration of additives, 1 mg L^{-1};
Data represents mean ± SD

Effect of PGRs on in vitro asymbiotic seed germination

PGRs such as naphthalene acetic acid (NAA) and 6-benzyladenopurine (BAP) are recognized for their efficacy in improving the seed germination and early seedling development. The efficacy of PGRs (auxins and cytokinin) on seed germination percentage, onset of seed germination and subsequent morphogenetic changes associated with seedling development was also assessed. The germination frequency varied with the kind of plant growth regulator used (Table 3). Nearly all seeds were embryonate (Fig. 2A). The seeds consisted of undifferentiated cells enclosed within a hyaline seed coat and initiated germination within 3.04 ± 0.03 weeks of culture on MS basal (hormone free) medium with the swelling of the embryo (Fig. 2B). Characteristically, the seeds were creamish-yellow in colour, transparent, and dust like. Swollen seed emerged as spherule by piercing through the hyaline

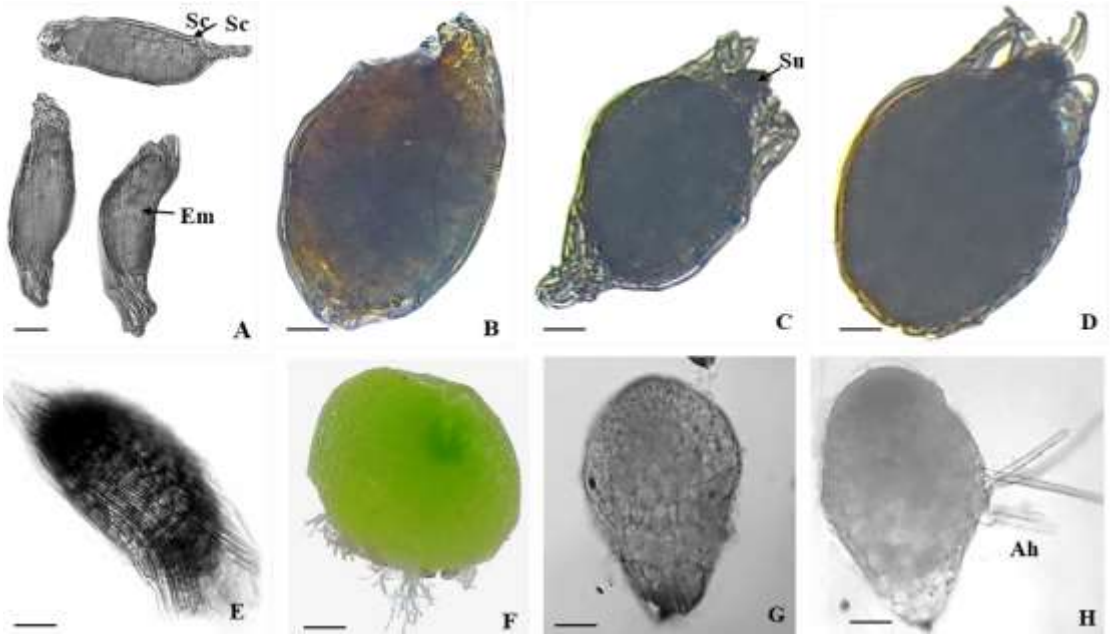

Fig. 2: *In vitro* asymbiotic seed germination and seedling development of *Aerides multiflora* in MS medium A) Embryonate seeds (Em) (4x) with seed coat (Sc), B) Swelling of embryo (4x), C) Embryo with rudimentary suspensor (Su) (4x), D & E) Swollen seed emerged as spherule by piercing through the hyaline seed coat as evident by the successive stages (20x), F) Chlorophyllous spherule with absorbing hair at the lower end of spherule (20x), G & H) spherule was dome shaped apex consisting of two layers of cells and absorbing hair (Ah) (20x)

seed coat as evident by the successive stages (Fig. 2C-E). Interestingly, the embryo had suspensor at one end of the embryo (Fig. 2C). The spherule grew in size and developed chlorophyll and absorbing hairs at the base in 10.2 ± 0.06 weeks old culture (Fig. 2F). A close observation indicated that the upper portion of the spherule was dome shaped and consisted of two layers of cells (Fig. 2G) which later elongated at apical end (Fig. 2H). The cytokinins are extremely important growth adjuncts that affect seed germination *in vitro* (Harvais, 1982). Orchid seeds contain meagre amount of reserve nutrients as lipid droplets (Arditti and Ernst, 1984), and cytokinins assist the seeds in using these complex carbohydrates without which germination could not be accomplished (De Pauw *et al*., 1995). BAP treatment favoured 100% seed germination within 3.18 weeks and developed protocorms (Fig. 3A,B). Protocorms multiplied luxuriantly in BAP treated cultures, revealing the inherent poly-embryonate potential of protocorms (Fig. 3C-D). Histological studies revealed that daughter PLB formation at the surface of the mother PLBs (Fig. 3E). The roots remained elusive in the cultures (Fig. 3F) and the cultures developed roots when shifted to ½ MS medium supplemented with NAA and peptone. Replacement of BAP with NAA in the culture medium reduced germination to only 65% after 4.26 ± 0.04 weeks (Fig. 4). The growth of spherules remained arrested unless and until shifted on ½ strength MS medium supplemented with NAA @ 1 mg L^{-1} and peptone @ 1 g L^{-1}. The cultures developed roots after 20 weeks (Table 3, Fig. 3G-H). Similar response of NAA has earlier been observed in *Vanda dearei* by Azlan *et al*. (2014) where NAA treatment delayed seed germination and induced necrosis to protocorm development. In our cultures, peptone proved most effective. The efficacy of peptone in favouring early differentiation and seedling development has been reported in *A. multiflora* (Verma *et al*., 2015), *Cymbidium pendulum* (Kaur and Bhutani, 2014), *Dendrobium* hybrid (Lekha Rani *et al*., 2005). In *Peristeria elata*, peptone favoured early and healthy growth of seedlings (Bejoy *et al*., 2004). Peptone also favoured multiplication of the cultures in *Cymbidium macrorhizon* (Kusumoto and Furukuwa, 1977) stimulated growth of the callus in *Doritaenopsis*, *Phalaenopsis*, and *Neofinetia* (Ichihashi and Islam, 1999).

Fig. 3: *In vitro* asymbiotic seed germination and seedling development of *Aerides multiflora* in MS medium A-C) Protocorm development in MS + BAP (1 mg L^{-1}), D) Multiplication of the protocorms (arrow), E) Multiplication of protocorms (arrow), F) Development of 1st and 2nd leaf primordia in MS + BAP (1 mg L^{-1}), G) Root initiation in ½ MS + NAA (1 mg L^{-1}) + peptone (1 g L^{-1}) (arrow), and H) Growth of seedlings in ½ MS+ NAA (1 mg L^{-1}) + peptone (1 g L^{-1})

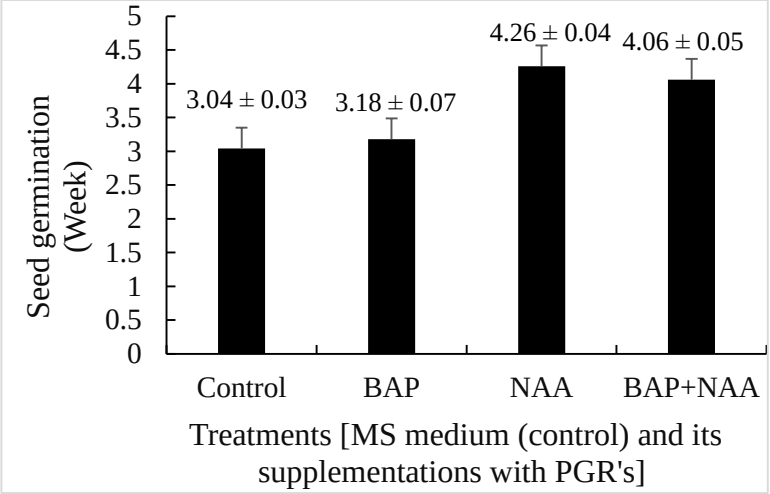

Fig. 4: Time taken in weeks by *A. multiflora* seeds for initiation of *in vitro* seed germination in MS medium and its combinations with growth adjuncts

The initiation of multiple meristematic zones composed of densely packed meristematic cells, forming new meristemoids is already on records (Ziv *et al*., 1998; Young *et al*., 2000). The cells with dense cytoplasm and conspicuous nuclei were involved in the formation of globular bodies, which is an important characteristic feature of the embryogenic cells as earlier studied in species like *Malaxis acuminata* (Kaur and Bhutani, 2010), *Dendrobium chrysotoxum* (Kaur, 2017).

Table 3: *In vitro* morphogenetic changes in germinating entities upon a shift in ½ MS medium

Additive	Time taken (wk) for					
	Development of		Differentiation of			Seedling development
	Chlorophyll	Protocorm	1st leaf	2nd leaf	1st root	
NAA + P	12.00 ± 0.06	14.01 ± 0.05	16.10 ± 0.08	18.04 ± 0.01	20.00 ± 0.00	25.00 ± 0.00

Note: ½ MS medium, half strength Murashige and Skoog medium; NAA, (1 mg L^{-1}); P, peptone (1 g L^{-1}); Data represented = mean ± SD; P = Peptone

These globules gradually grew in size, having no vascular connections with the surrounding peripheral tissues and eventually were transformed into secondary protocorms (PLBs). The PLBs proliferated to produce secondary PLBs as also reported earlier (Zhao *et al*., 2008; Kaur, 2017).

Conclusion: The present study is a one-step protocol for *in vitro* culture of *A. multiflora* orchid. MS basal medium proved to be promising for the initiation of seed germination and supported the culture growth. This simple protocol can contribute in conserving of this floriculturally important orchid species. Further studies are required to check the genetic fidelity of the regenerants and to acclimatize seedlings that are raised *in vitro*, thus, restoring them back in their natural habitat.

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