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Short Communication

Development of rapid micropropagation protocol for germplasm conservation of two orchid species - *Aerides multiflora* Roxb. and *Rhynchostylis retusa* (L.) Blume

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ABSTRACT

Aerides multiflora and *Rhynchostylis retusa* are two ornamentally and medicinally important orchids of the Himalayan region. Natural regeneration of these orchids is hampered due to declining forest cover, human interferences and problems in seed germination. The present study establishes a system for conservation and mass propagation of these two orchid species in minimal possible time. Immature seeds were used as the starting material to establish protocorms which were multiplied and used for shoot and root organogenesis. In both the species conditions were optimized for maximal plantlet conversion. In *A. multiflora*, Mitra medium supplemented with BAP (4.44µM) and NAA (5.38 µM) resulted in formation of an average 3.33 shoots and 3.67 roots per protocorm and was adjudged to be the best medium combination. Whereas, Mitra medium containing BAP (4.44 µM) alone proved to be most optimal for plantlet regeneration from protocorms of *R. retusa*. The plantlets so developed were hardened *in vitro* and conditions were standardized for *ex vitro* hardening and acclimatization. System optimization was done for potting mix, temperature and humidity conditions for plantlet growth and development under polyhouse and field conditions. Survival rate of over 80% was obtained in both the species under field conditions.

Key Words: growth regulator, growth additive, mass propagation, protocorms , hardening, acclimatization.

INTRODUCTION

Orchids, popular for their floral beauty, belong to the family Orchidaceae which is one of the largest families of monocots (including ~800 genera, 25,000-30,000 species and more than 1,20,000 registered hybrids or cultivars) (Chowdhery, 2001).

With exquisite foliage and long lasting flowers of diverse patterns; orchids are at the top of international fresh cut flower trade and are equally valued as potted plants (De *et al*., 2014). Besides their ornamental aspect, many orchids are used in traditional system of medicine as a remedy for a number of ailments (Besra *et al*., 2011).

Orchids are mainly distributed in the Indo-Malayan and Eastern Himalayan region, though they can grow vigorously under varied environmental conditions and habitats. They are majorly classified as terrestrial (growing on ground), epiphytic (growing on host trees) and saprophytic (growing on dead and decaying matter). India is home to about 1300 species of orchids (forming 9% of the flora), largely distributed in the Himalayan region and Western Ghats. Uttarakhand, a small state in the Western Himalaya, harbours around 237 orchid species (Jalal *et al*., 2008). A number of epiphytic orchids have been documented from the riverine woodlands and humid valleys in this area (500- 2500 amsl).Two such important epiphytic orchids growing on a range of host plants in Uttarakhand region are *Aerides multiflora* and *Rhynchostylis retusa.*

Aerides multiflora Roxb. (Common name – Maana), also known as Fox brush orchid, is a beautiful plant with stout, several-leafed stem with many (up to 50) flowered raceme inflorescence. Fragrant and waxy flowers are observed during the months of May- July while fruiting occurs in the months of June – August. The plant is known to possess antibacterial properties and is widely used in traditional medications *viz.* application of leaf paste on cuts and wounds (Ghanaksh & Kaushik, 2009). *Rhynchostylis retusa* (L.) Blume or Foxtail orchid (commonly called as Banda, Rasna in local language) is a monopodial, epiphyte with long, fleshy leaves, and dense many flowered raceme inflorescence (around 100-150 flowers/ inflorescence). Flowering and fruiting is observed in the months of April – June. The species is valued for its beautiful floral arrangement and extensively used in the indigenous medicine system for the treatment of menstrual disorders, asthma, rheumatism and tuberculosis (Saxena, 2020).

Both the species, *A. multiflora* and *R. Retusa*, are facing the danger of extinction, in several regions, due to the deforestation (FSI, 2017), irrational collection and inadequate germination rates in nature (Baro *et al*., 2019). However, as compared to other orchids, little attention is given towards their conservation in nature. Consequently, alternate strategies for conservation and large scale propagation of the two orchid species are the need of the hour. Micropropagation has been previously attempted to propagate both the species using different plant parts (Kumar *et al*., 2002; Vij *et al*., 2004; Thomas & Michael 2007; Parab & Krishnan, 2012; Bhattacharjee & Islam, 2015; Verma *et al*., 2015). An important feature in success of micropropagation protocol is, lab

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to land transfer capability (survival rate) of the plantlets (Kozai *et al.,* 1997; Chandra *et al*., 2010). Therefore, the present study was undertaken with the aim of developing a system that helps in conservation of these species by large scale propataion using tissue culture technology and successful *ex vitro* establishment of tissue culture raised plantlets.

MATERIALS AND METHODS

Explant collection and surface sterilization

Immature and undehisced pods of *Aerides multiflora* and *Rhynchostylis retusa* were used to initiate *in vitro* cultures. The pods of *A. multifora* (Dehradun Herbarium Reg. No. 172720) and *R. retusa* (Dehradun Herbarium Reg. No. 172719) were collected from their natural habitat in Rajpur, Dehradun, Uttarakhand (30° 21′34.02″N 78°4′2.221″E) in the month of August (Figure 2 a, b). The intact pods were treated with teepol (2-3 drops/100 ml water) followed by washing under running tap water for 10 minutes. The pods of both the species were then submerged in mercuric chloride solution (0.1% w/v) for 5 minutes with intermittent swirling. This was followed by washing with sterile water (3-4 times). Subsequently, surface sterilized pods were dipped in 70% ethanol for 10 seconds and then flamed. Flamed pods were cut open longitudinally to release the seeds. The entire procedure was carried out under laminar air flow cabinet.

Seed Viability Assessment

Tetrazolium assay (Wharton, 1955) was performed to assess the viability of *A. multiflora* and *R. retusa* seeds collected from the wild. The seeds were soaked in 1 % (w/v) solution of 2, 3, 5 triphenyl tetrazolium chloride (TTC) [HiMedia] in dark for 24 h. Thereafter, the seeds were examined under light microscope (Olympus). The seed viability was estimated by counting the number of stained embryos.

Culture Initiation

The seeds of both the species were inoculated on to basal MS medium (full and half strength) to initiate protocorm formation. For their multiplication, full strength MS medium (Murashige & Skoog, 1962) containing coconut water and yeast extract was used. After 6-8 weeks of culture, well formed protocorms were used in the second set of experiments to initiate *in vitro* plantlet formation.

Culture Establishment

To assess the efficacy of different media formulations for complete plantlet regeneration, protocorms of both the species (*A. multiflora* and *R. retusa*) were inoculated onto Mitra medium (Mitra *et al*., 1976) alone, and fortified with varying concentrations of plant growth regulators [BAP (2.22-4.44µM) and NAA (5.38 µM)], and growth additives [coconut water (10-20%), coconut milk (10-20%), and casein hydrolysate (0.1-0.2%)]. In experiments to select optimal nutrient medium combination Mitra medium devoid of any PGR was used as control. Observations on mean shoot number, mean shoot length, mean root number and mean root length were recorded after 12 weeks of culture.

Culture conditions

For both the species, Mitra medium were supplemented with 2% sucrose and gelled by using 0.65% (w/v) agar. The pH of medium was adjusted to 5.8 either with 0.1 N NaOH or HCl prior to autoclaving at 121^0 C and 105 kPa for 15 minutes. Cultures were incubated in culture room at 25 ± 2^0C and 75 % relative humidity; with a 16/8 hr (light/dark) photoperiod supplied by cool white fluorescent light [Philips, India] (light intensity of 40 µmol m^{-2} s^{-1}).

Acclimatization

Plantlets (average height ~3-5 cm with well developed shoots and roots) were used for hardening. Under *in vitro* conditions, the plantlets were hardened by shifting them to ½ X MS medium (for 2-3 weeks) followed by transfer onto ¼ X MS medium for another 2 weeks. In both the cases, MS medium used was devoid of any PGR, carbon source, and gelling agent. Thereafter, plantlets were carefully washed under tap water and transferred to polyhouse conditions (temperature range 25–30^0C and RH 60–70%). The potting mixture used for transplantation was prepared with peat moss, coconut husk, charcoal pieces (~4-6 mm), brick pieces (~4-6 mm) and mango tree bark shavings (in varying ratios) [Table 1]. All the materials used for potting-mix were pre-processed by thorough washing and air drying; followed by autoclaving at 105 kPa pressure at 121^0C for one hour. In one set of experiments, un-autoclaved potting mixture was used. Individual plantlets were placed in hyco-trays containing the potting-mix. Plant growth was monitored periodically and well developed healthy plantlets were further shifted into pots during different seasons of the year and maintained under field conditions.

Data analysis

Data was collected in the Completely Randomized Design (CRD) and was analyzed using Microsoft Excel ver. 2007 © Microsoft Technologies, USA. Each treatment was repeated thrice with ten replicates per experiment. Mean shoot/root number & length were statistically analyzed using one-way Analysis of Variance (ANOVA). Degree of variation was shown by Standard Error (SE), Critical Difference (CD) at 5%. The significance level was determined at 5% ($p <= 0.05$), 1% ($p <= 0.01$) and 0.1% ($p <= 0.001$). The significance of the data as ascertained by F-test and the CD values computed were used for comparing differences in means of various treatments.

Table 1. Different combinations of potting mix

Combination Number	Potting Mix Combinations				
	Peat Moss	Coconut Husk	Char coal	Brick Pieces	Bark Shavings
C1	1	-	2	-	1
C2	1	-	1	-	2
C3	2	-	1	-	2
C4	-	1	2	-	1
C5	-	1	1		2
C6	-	2	1	-	2
C7	2	-	-	1	2
C8	-	2	-	1	2
C9	-	-	1	1	2
C10	1	1	1	1	1

RESULTS AND DISCUSSION

Seed Viability Assessment

As the seeds of - *Aerides multiflora* and *Rhyncostylis retusa* were collected from the wild, it was important to check their viability before using them as explants (Figure 1 a, b).The tetrazolium test helped in establishing viability and germination potential of minute orchid seeds. The test results in staining of viable embryos in dark red colour while dead embryos remain unstained. In the present study the viability of seeds of *A. multiflora* and *R. retusa* was found to be 100 per cent as observed under light microscope (Figure 1 c, d). Consequently, the collected seeds were used to perform *in vitro* experiments. The immature seeds were preferred as the explants since juvenile tissues exhibit enhanced morphogenetic competence as compared to mature tissues. This is attributed to the fact that their cell wall is not yet rigid and offers more flexibility for *in vitro* manipulations (Misra & Bhatnagar, 1995).

Culture initiation

In both the species, initiation of seed germination was observed within 4 weeks of inoculation. However, development of protocorms could be observed only after 7-9 weeks of culture. Protocorms are bipolar embryonic structures having distinct shoot and root meristems. This organisation renders them efficient for complete plantlet regeneration (Ng & Saleh, 2011) hence offering a rapid method of propagation. Besides, protocorms are a favoured pathway for orchid culture as they provide genetic stability in the regenerants (Lee & Phillips, 1988).

In the present study, basal full strength MS medium was found to be most optimal for protocorm formation in both the species. For further multiplication, fortification of MS medium with coconut water (10%) and yeast extract (0.1%) proved to be most efficient (data not shown). Periodic subculturing on same media combination was done after every 3 weeks to increase the protocorm stock (Figure 2c).

Culture establishment

It has been well documented that nutrient medium compositions play a significant role on orchid organogenesis and is potential enough to modify the morphogenetic responses responses (Arditti & Ernst 1984; Vij *et al.,* 2004; Naing *et al.*, 2011; Rittirat *et al*., 2012; Sunitibala & Neelashree, 2018). With these studies as a precursor, second set of experiments were performed to assess the efficacy of nutrient media composition on protocorm proliferation and plantlet production in *A. multiflora* and *R. retusa*. Well developed protocorms established in previous experiment were used for the purpose. A study was designed where effect of nutrient medium with PGRs, and growth additives were studied for plantlet regeneration.

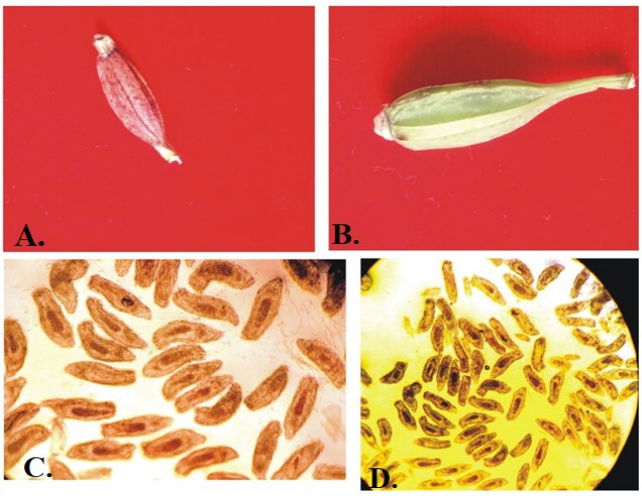

Figure 1. (a) *Aerides multiflora* pod, (b) *Rhyncostylis retusa* pod, (c) Dark stained viable embryos of *A. multiflora*, (d) Dark stained viable embryos of *R. retusa*

i) *Effect of plant growth regulators on plantlet development:* Existing literature shows variation in usage of plant growth regulators in protocorm proliferation medium. While in some species germination has been recorded in basal media devoid of any growth hormone (Mead & Bulard, 1979; Pathak *et al*., 2011; Abraham *et al*., 2012; Mohanty *et al*., 2012) in other species the role of plant growth regulators has been found to be imperative for plant growth *in vitro.* In our study it was observed that addition of cytokinin and auxin (alone or in combination) in Mitra medium gave significantly better results of plantlet development.

In *A. multiflora*, best results were obtained on Mitra medium fortified with BAP (4.44 µM) and NAA (5. 38 µM) as computed after 12 weeks of transfer (Table 2). On this combination an average of 3.33 shoots (shoot length 2.45 cm) and mean number of 3.67 roots (root length 2.47 cm) was obtained (Figure 2d). The shoot and root system was well developed with healthy shoot and long sturdy roots. On medium supplemented with cytokinin alone, shoot and root development was significantly retarded. A similar synergistic effect of BAP and NAA has been reported to be highly effective in protocorm regeneration in *Aerides species* (Vij *et al.,* 2004; Shadang *et al*., 2007; Srivastava *et al.,* 2015, Bhowmik & Rahman, 2020). These observations are supported by previous findings where cytokinin and auxin combinations have proven to stimulate proliferation and differentiation in orchid species (Seeni & Latha, 2000; Park *et al.*, 2002; Anjum *et al.*, 2006; Pathak *et al*., 2011; Mohanty *et al*., 2013; Parab & Krishnan, 2012).

Comparatively, BAP alone proved to be most efficient in inducing healthy and stout plantlets from protocorms of *R. retusa* on Mitra medium. BAP at a concentration of 4.44 µM resulted in formation of an average 4.33 shoots (shoot length 2.10 cm) and 4.33 roots (root length 2.03 cm). In *R. retusa,* addition of auxin into BAP supplemented medium did not improve plantlet development (Table 2). The results are in consonance with earlier research reports which have established the efficiency of cytokinins in germination rates in *Rhyncostylis retusa* (Sharma *et al.,* 1992; Kumar *et al*., 2002, Naing *et al.,* 2011; Bhattacharjee & Islam, 2015).

Earlier studies have opined that proliferative regenerants of orchids fail to root unless they are shifted to a rooting medium containing auxins; the need being mandatory for rooting in some orchid species like *Oncidium, Rhyncostylis* (Vij *et al*., 2002). However, in our study, proliferative protocorms could develop a healthy shoot and root system in one single step without the need of transfer to a rooting medium.

ii) Effect of growth additives on plantlet development:

In the present study, experiments were also conducted to validate the role of natural additives in protocorm

proliferation and plantlet regeneration. Growth additives being complex in nature have been frequently associated with augmenting the plant development under *in vitro* conditions (Raghavan *et al*., 1976). The growth additives used showed no promising results for both the species. Coconut water (CW) is well known for stimulating *in vitro* orchid growth due to the presence of cytokinin (Prades *et al*., 2011, Utami & Hariyanto, 2020). Addition of CW at 20% v/v to the culture medium showed results similar to the medium formulation with BAP (4.44 µM). Our results were in contrast to a previous study which recorded better results in medium supplemented with CW and other natural additive in *Rhynchostylis gigantean* (Kaewkhiew & Kaewduangta, 2010). Coconut milk (CM), on the other hand, has been previously reported to enhance germination and proliferation in orchids as it possesses growth hormone (auxin) activity (Dix *et al*., 1981). But in the present study, incorporation of CM to the medium resulted in stunted leaved and no root formation was observed. Casein hydrolysate (CH) acts as a rich source of nitrogen and hence it has been routinely used in embryo culture (Narayanswamy, 2007). Contrasting results were observed in our study, where the medium with CH showed delayed growth with subsequent drying (browning of protocorms), discouraging its use in the medium.

Acclimatization

Hardening and acclimatization of tissue-culture raised plantlets is an essential stage in micropropagation. Unless the *in vitro* raised plantlets are able to survive under natural conditions, the utility of technique is restricted. In our study, plantlets with well developed shoots and roots were hardened *in vitro*, by transferring them sequentially onto ½ X and ¼ X liquid MS medium (devoid of any PGR or carbon source) at an interval of 2-3 weeks (Figure 2e). Healthy plantlets were then screened visually and used for *ex vitro* establishment. The plantlets were carefully placed into hyco-trays filled with pre-sterilized potting mixtures and kept in polyhouse. The efficacy of different materials used as growth substrates varies from species to species in serving mechanical support to the plantlet, providing water retention, permeability for root penetration, aeration, and/ or as nutritive source in some cases (Díaz *et al*., 2010; Colombo *et al.,* 2005; Zettler *et al.,* 2007; Vasudevan & Van Staden, 2010; Gogoi *et al.,* 2012). Selecting and standardizing a suitable potting mixture is therefore an essential factor in orchid propagation. In our study a potting mixture combination of peat moss, charcoal and mango bark shavings in the ratio 2:1:2 proved to be most effective. On this combination, *A. multiflora* plantlets showed 85% survival rate; whereas *R. retusa* plantlets exhibited a higher survival rate of 92%. Peat moss is known to have a high water holding capacity (~ 30% of its dry weight). This explains its suitability as a component of potting mix often used in higher quantity (Bhattacharjee & Islam, 2015). Besides charcoal pieces provide additional mechanical support to the *in vitro* raised orchid plantlets during acclimatization (Paul & Rajeevan, 1992; Pathania *et al.,* 1998) in our study, use of mango bark shavings improved the result of acclimatization. This could be due to the fact that both the orchid species in study grow well on mango tree and might be drawing some nutrition from the bark. After about 6 weeks of maintaining the tissue culture raised plantlets in small pots; the plants were shifted to bigger pots with the same potting mix combination. The plants were covered with polybags with holes to maintain the humidity conditions and prevent excess loss of water. This also prevented excess dehydration which is an important factor in successful acclimatization (Pospíšilová *et al.,* 1999). The pots were kept in shady areas with proper air circulation and plants were checked regularly for any type of infestation or wilting.

Table 2. Effect of PGRs and additives on plantlet development of *A. multiflora* and *R. retusa*

PGR		Additives			*Aerides multiflora*				*Rhynchostylis retusa*			
					Shoot		Root		Shoot		Root	
BAP µM	NAA µM	CW %	CM %	CH %	Number	Length	Number	Length	Number	Length	Number	Length
-	-	-	-	-	1.00 ± 0.33	0.51± 0.06	1.00± 0.00	0.20 ± 0.02	1.00± 0.00	0.42±0.04	-	-
2.22	-	-	-	-	1.43± 0.33	1.48± 0.03	1.50± 0.33	0.73 ± 0.04	2.67± 0.67	0.68±0.06	2.00± 0.00	0.78± 0.02
4.44	-	-	-	-	1.66± 0.33	1.66± 0.06	2.00± 0.00	0.98 ± 0.07	4.33±0.33	2.10±0.06	3.33± 0.33	2.03± 0.03
4.44	5.38	-	-	-	3.33 ± 0.33	2.45± 0.03	3.67± 0.33	2.47 ± 0.03	3.00± 0.00	1.80±0.06	2.00± 0.00	1.72± 0.04
-	-	10	-	-	1.33± 0.33	0.68± 0.06	1.67± 0.33	0.40± 0.03	2.33± 0.33	0.85±0.03	1.80± 0.04	0.70± 0.03
-	-	20	-	-	1.33± 0.33	1.35± 0.09	2.67± 0.33	0.55± 0.05	2.33± 0.33	1.38±0.06	2.33± 0.33	0.97± 0.09
-	-	-	10	-	1.33± 0.33	0.70± 0.14	-	-	1.53± 0.33	0.52±0.04	-	-
-	-	-	20	-	1.28± 0.17	0.88± 0.06	1.33±0. 33	0.30± 0.09	2.33± 0.33	0.85±0.05	1.00± 0.00	0.55± 0.03
-	-	-	-	0.1	1.11± 0.33	0.25± 0.05	-	-	1.33± 0.33	0.50±0.17	1.33± 0.33	0.47± 0.03
-	-	-	-	0.2	1.50± 0.33	0.85± 0.14	1.33± 0.33	0.20± 0.03	2.00± 0.00	0.88±0.06	1.00± 0.00	0.59± 0.06
Significance					**	***	***	***	***	***	***	***
CD at 5%					0.23	0.06	0.27	0.06	0.60	0.60	0.47	0.77

The polybags were removed for 4-5 hours every day for first 2 weeks and completely removed thereafter. Plantlet survival in open field conditions was studied at different times of the year, April (25.9 ± 5^0C, RH ~ 46%), August (24.4 ± 5^0C, RH ~ 86%), and December (14.0 ± 5^0C, RH ~ 71%). It was observed that the plants survived in open air conditions of Dehradun during all the three month. During the months of April and August, the survival rate of plants was below 50%. Plant survival was best observed during the month of December with low temperature and optimum humidity. The plants further developed new and healthy shoots and roots (Figure 2f, g). Overall, over 80% survival of tissue- culture raised plantlets of *Aerides multiflora* and *Rhyncostylis retusa* was observed in under field conditions during December.

CONCLUSION

Tissue culture technology is an acknowledged prospective method for conservation of several difficult to cultivate plant species. This technique finds a special mention in the conservation and large scale production of many wild orchid species which are routinely harvested in an unscientific manner for commercial purposes. The present report gives a rapid *in vitro* propagation protocol for two ornamentally and medicinally important orchid species, *Aerides multiflora* and *Rhyncostylis retusa* of the Himalayan region. Standardization of nutrient medium composition for complete plantlet development in a single step is a highlight of the study as it surpasses the need of multistage culture system for root formation. This is also a complete tissue culture regeneration protocol since the plantlets have been successfully transferred from *Lab to Land* with a good survival percentage. As there are limited reports on immature seed culture of *A. multiflora* and *R. retusa*, the present system can be utilized for mass propagation and germplasm conservation of these two important species.

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Figure 2. (a) *Aerides multiflora* plant, (b) *Rhyncostylis retusa* plant, (c) Development of protocorm, (d) *In vitro* plantlet regeneration of *A. multiflora*, (d) *In vitro* hardening of tissue culture raised plantlet, (f,g) *Ex vitro* establishment of tissue culture raised plantlets of *A. multiflora* and *R. retusa* in open air conditions.

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