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In vitro propagation of *Rhynchostylis retusa* (L.) Blume through immature seed culture

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ABSTRACT

Orchids, owing to the exquisite beauty of flowers, are highly valued in the cut flower trade and as potted plants. Apart from their diversity in colour, foliage and fragrance, orchids are immensely used in folk medicines as they are endowed with phytoconstituents significant in healthcare. Given to the topographical features and climatic suitability, Uttarakhand harbours several medicinally important orchids. *Rhynchostylis retusa*, a monopodial, epiphytic orchid native to this region, is used in the treatment of rheumatic disorders, asthma, malarial fever and blood dysentery. Due to its high commercial value but slow rate of conventional propagation coupled with loss of habitat, tissue culture technology can be effectively used as an alternative to furnish the demand. In the present study different growth additives (coconut water, yeast extract, and peptone) and plant growth regulators (BA and NAA) and were screened for seed germination, proliferation and plantlet development. Optimum germination rate and PLB formation was recorded in full strength MS basal medium. Further PLB multiplication was best studied in MS medium supplemented with coconut water (10%) and highest number of leaves (4.33) and roots (4.33) per PLB were observed in MS medium with the combination of BAP and peptone. The plantlets so developed were successfully transplanted to field conditions.

Key words: *Rhynchostylis retusa, In vitro propagation, PLB, Additives, Hardening*

Introduction

Orchidaceae is the most evolved family of monocots with ~ 25,000-30,000 species (Chugh *et al*., 2009). Orchids are prized in the floriculture industry due to beautiful foliage, and long lasting flowers (Khuraijam *et al*., 2017). Orchids are also widely used in the traditional medicine system (Brinkmann, 2014, Leon and Lin, 2017). Being cosmopolitan; orchids are distributed in the Indomalayan realm and the Eastern Himalayan region. India, a biodiversity hotspot, harbours a diverse variety of orchids. Uttarakhand (500- 2500 msal), due to its well suited agro-climatic conditions and topographical features is a natural home to ~250 species of orchids (Joshi *et al*., 2009). Richness in diversity of orchid species is observed between 1500- 2000 msal and due to high humidity in this

Abbreviations

µM – micromolar, AC - Activated charcoal, BA - 6-Benzylaminopurine, CW - Coconut Water, MS - Murashige and Skoog medium, NAA - 1-Naphthaleneacetic acid, PE – Peptone, PGR – Plant Growth Regulator, PLB - Protocorm like Bodies, YE – Yeast Extract

zone most of the species are epiphytic. But anthropogenic activities coupled with natural calamities pose a major threat to the epiphytic orchids of the region. According to the reports of Government of India (MoEF) a major part (44,868 ha) of the forest cover in Uttarakhand has been commercialized for human needs due to which the forest cover has decreased. The escalating demand and slow rate of propagation of orchids necessitates the development of orchid conservation strategies and *in vitro* propagation is the best suited alternative for orchid production in this scenario.

Rhynchostylis retusa (L.) Blume. (Vern. – Banda, Rasna), Foxtail Orchid, is a monopodial, epiphytic orchid with fleshy leaves. The plants produce 3-5 unbranched racemes consisting of ~100-150 flowers/ inflorescence in the months of April – June. *R. retusa* finds mention in the Charaka Samhita and is extensively used in the indigenous medicine system for the treatment of blood dysentery, asthma, rheumatism, tuberculosis, menstrual disorders and wounds (Deorani and Sharma, 2007, Das *et al.*, 2008). It also possess leishmanicidal (Bhatnagar *et al.*, 2017), antibacterial and antifungal activities (Bhattacharjee & Islam, 2015).

With the aim of conserving *R. retusa,* commercially and medicinally important orchid species of Uttarakhand, the present work is focussed on screening the optimal nutrient medium combination for plantlet regeneration under controlled conditions.

Materials and Methods

Immature green capsules of *Rhynchostylis retusa* were collected from Dehradun, Uttarakhand. Undehisced capsules were rinsed with detergent (teepol). Further, under aseptic conditions, the intact capsules were dipped in a solution of sterilant (0.1% $HgCl_2$) with constant stirring. After 5 minutes, the capsules were thoroughly washed with sterilized water (3 times). Finally the surface sterilized capsules were dipped in dilute ethanol (70%), flamed for 20 seconds, and cut lengthwise to discharge the miniscule seeds which were uniformly inoculated onto modified MS medium (Murashige & Skoog, 1962) medium. To assess the efficacy of salt concentration and growth supplement during seed germination, full and half strength MS medium, with or without CW (10-30%) was used.

After germination, the PLBs were further multiplied and developed (1-2 leaf and root stage) onto MS medium fortified with growth additives like CW (10-20 %), YE (0.1-0.2 %) and PE (0.1-0.2%); and plant growth regulators in varying concentration (2.22-4.44µM) of BA (6- benzylaminopurine) either alone or in combination with NAA (1-naphthaleneacetic acid; 2.69-5.38µM). The seedlings were further multiplied in the medium showing best results for induction. Observations on mean shoot number/ length, and mean root number/ length were recorded at periodic intervals. In all the experiments, MS medium was supplemented with 2% sucrose and was gelled with 0.60% (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 °C and 105 kPa for 15 minutes. Cultures were aseptically incubated at 25 ± 2 °C and 75 % relative humidity with 16/8 hr (light/dark) photoperiod with a light intensity of 40µmol $m^{-2}s^{-1}$ by white fluorescent light. MS medium lacking growth supplement served as control.

Well rooted plantlets (~3-5 cm) with 3-4 fully developed leaves and 2-3 roots were selected for *in vitro* hardening and transferred to ½X basal MS medium without agar for 2 weeks. Plantlets were further hardened on ½X and ¼X basal MS medium without sucrose and agar for 2 weeks respectively. Well hardened plantlets were transferred to greenhouse (temperature range 22–30 °C and RH 60–70%). Peat moss, bark shavings, charcoal and brick pieces were mixed in varying ratio (Table 1) to formulate the potting mix. All these potting mix materials were pre-processed by thorough washing, air drying and followed by autoclaving at 105 kPa pressure and 121 °C for one hour before placing into the hyco-trays were used. The plantlets were maintained in the hyco trays for 4 weeks before shifting into bigger pots with the same potting mix. The pot-

Table 1. Different combinations of potting mix

Combination Number	Potting Mix Combinations		
	Peat Moss	Charcoal	Bark Shavings
C1	1	1	1
C2	1	1	2
C3	1	2	2
C4	2	1	1
C5	2	2	1
C6	1	2	2
C7	2	1	2
C8	2	2	2

ted plants were covered with polythene bags and maintained in a shaded place. The polythene bags were removed for 2-3 hours every day and plantlets were watered at regular intervals. Plant growth and survival was monitored after 60 days.

Data of all the experiments was collected in the Completely Randomized Design (CRD) and was analyzed using Microsoft Excel ver. 2007 © Microsoft Technologies, USA. Each treatment was triplicated with ten culture vessels per replication. The average number and length of shoots and roots 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% ($p < = 0.05$). 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.

Results and Discussion

Orchid seeds are minute in structure with undifferentiated embryo devoid of any food reserve (Arditti, 1984; Leroux *et al*., 1997). Therefore, seed germination and early stage seedling development of orchids is mycobiont mediated (Rasmussen *et al*., 2015). This limitation can be compensated, *in vitro*, by providing an exogenous supply of carbon and other minerals as furnished by the fungal partner in natural conditions. Asymbiotic germination has been well established in several orchid species (Hossain *et al*., 2009; Pathak *et al*., 2011). Many attempts have been made for propagating *R. retusa* using different plant parts (Kumar *et al*., 2002; Parab and Krishnan, 2012; Sinha and Jahan, 2012; Bhattacharjee and Islam, 2015; Sunithabala and Neelashree, 2018), and additional work is required to identify the vital growth factors.

In the present study, full strength basal MS medium was observed to be optimum for asymbiotic seed germination. The process of germination started with the swelling of embryos after 3 weeks of inoculation of the immature yellowish seed. After 5 weeks spherules were observed which were subsequently transformed to protocorm like bodies after 7 weeks. The PLBs were further proliferated to reach to a 2-3 leaf primordia and 1-2 root stage. Addition of 10 % coconut water to basal MS medium recorded the maximum proliferation. This result was in accord with the study of Piri *et al*., 2013. The beneficial effect of coconut water can be attributed to the fact that it is a vital source of calcium and magnesium; and is also known to possess gibberellic acid which aids in seed germination and development (Peixe *et al*., 2007).

The PLBs at this stage were then used for further to assess the efficacy of natural additives and PGRs (alone and in combination) for plantlet development. Natural and complex growth additives are known to enhance *in vitro* plant morphogenesis. Raghavan (1976) reported that nitrogenous and organic constituents of growth supplements promote tissue development *in vitro*. In our study, significant

Table 2. Effect of natural growth additives & PGRs on plantlet development of *R. retusa*

MS medium			Leaf		Root	
PGR (µM)		Additives	Number	Length	Number	Length
BA	NAA	(%)				
-	-	-	3.00±0.00	0.62±0.04	1.67±0.33	0.63±0.02
2.22	-	-	2.67±0.67	0.68±0.06	2.00±0.00	0.78±0.02
4.44	-	-	3.00±0.33	1.90±0.06	3.00±0.33	1.47±0.03
4.44	2.69	-	1.33±0.67	0.85±0.04	2.00±0.00	1.50±0.02
4.44	5.38	-	2.00±0.00	1.90±0.06	1.50±0.00	1.92±0.04
-	-	CW (10)	3.33±0.33	1.35±0.09	2.67±0.33	0.55±0.05
-	-	CW (20)	2.33±0.33	0.68±0.06	1.67±0.33	0.40±0.03
-	-	YE (0.1)	3.33±0.33	1.20±0.14	2.33±0.33	0.48±0.03
-	-	YE (0.2)	3.33±0.33	1.38±0.06	2.33±0.33	0.97±0.09
-	-	PE (0.1)	3.67±0.33	1.25±0.05	3.00±0.00	0.83±0.06
-	-	PE (0.2)	4.00±0.00	1.87±0.04	3.33±0.33	0.98±0.02
4.44	-	PE (0.2)	4.33±0.33	2.10±0.06	4.33±0.33	2.03±0.03
	Significance		***	***	***	***
	CD at 5%		0.60	0.47	0.77	0.40

improvement in PLB morphogenesis into plantlets was observed on supplementing the nutrient medium with additives (coconut water, yeast extract and peptone). MS medium with peptone (0.2%) resulted in formation of an average number of 3.00 leaves and 3.00 roots per PLB as observed after 12 weeks of culture (Table 2). Same concentration of peptone gave significantly better results (mean number of 4.33 leaves and 4.33 roots/ PLB) when added to MS medium having BAP (4.44µM). The plantlets so developed were stout and healthy with an average leaf and root length of 2.10±0.06 cm and 2.03±0.03 cm, respectively (Table 2). Growth promoting nature of peptone in seed germination, and PLB organogenesis has been accredited to its peptides, amino acids, amides and vitamin content (Bejoy *et al*. 2004; Pathak *et al*. 2011; Kaur and Bhutani 2014).

Well developed plantlets were hardened under *in vitro* conditions. Plantlets (~ 3 cm) were then hardened on MS basal medium (half strength) lacking any gelling agent. After 2 weeks, the plantlets were subcultured, sequentially, into ½ and ¼ strength MS basal medium lacking sucrose and agar. The cultures were monitored for 4 weeks and healthy plantlets were selected for *ex vitro* establishment. Plantlets were placed in hyco- trays containing pre sterilized potting mix. Highest plantlet survival was observed in potting mix combination with of peat moss, charcoal and mango bark shavings in the ratio 2:1:2. After 4 weeks the plantlets were shifted to clay pots with the same potting mix, covered with polybags (to avoid excessive dehydration), and kept in a cool place under shade. The plants were carefully checked for any infestation and watered regularly. The polybags were removed for 4-5 hours every day, for first few weeks, and then permanently removed. Potting mix materials are of utmost importance in acclimatization of the *in vitro* hardened plantlets by providing mechanical support, and moisture (Zettler *et al*., 2007). The success of the potting mix used in the present study can be accredited to its composition. Almost 80% survival was observed under natural conditions of Dehradun.

Fig. 1. A. Rhynchostylis retusa plant, B. PLB proliferation in MS medium supplemented with coconut water, C. Complete plantlet regeneration on MS medium with BAP and peptone, D. Ex vitro establishment of plantlet

Conclusion

The present study provides a single stage, and rapid micropropagation protocol for a medicinally important orchid species, *Rhyncostylis retusa*. Considering the rapid rate of decline in the natural habitat of the epiphytic orchid, this method can be employed for its ex vitro conservation and mass multiplication. The study concludes that the seed germination and plantlet development in orchids can be greatly enhanced by using natural growth additives.

Recommendation

Even though India is home to a large variety of orchid species (~1300), its contribution to the global orchid trade is still in its infancy. Uttarakhand has a rich orchid diversity due to the varied topography (snow capped mountains to dusty plain lands), altitudinal range (500-4500 m) and favourable climatic condition (sub tropical to alpine with high precipitation and humidity) which supports the growth of orchids. Being highly prized ornamentals (~10% global cut flower trade), and immensely revered in the traditional medicine system, the escalating market demand for orchids is leaving the species in du-

ress. The major threats to orchid survival in nature, apart from its slow rate of vegetative propagation, are the irrational collection from wild, loss of habitat due to forest clearance for developmental activities and natural disasters, and climate change. To overcome this limitation, tissue culture technology plays a pivotal role in mass propagation of the orchid species to meet the commercial demand.

Unfortunately, the orchid wealth of Uttarakhand, till date, is largely unexploited owing to lack of awareness and prioritization. Despite the richness of orchid diversity, most of the orchid species in the domestic market are exported to meet the requirements and have a market value (approx.) of Rs. 150-500 per stalk. A drawback to orchid cultivation is lack of knowledge, quality planting material, and infrastructure. In this scenario, establishment of tissue culture units, green houses and orchid farms can be a source of income generation. The floriculture industry, being labour intensive, has a potential for sustainable employment generation. The economically satisfying and ever expanding floriculture market at international level will lead to retention of rural and unemployed youth in agricultural practices.

The medicinal value of orchids is another aspect to be explored. Their use in traditional medicines as therapeutics and aphrodisiac is well stated. Due to the phytoconstituents in orchids, they are routinely harvested and used by the food processing and pharmaceutical industries. Creating linkages between the producers and the industries can effectively improve the business. Training and assistance programmes can be organised for farmers to help them cultivate and market their produce. According to the State Horticulture Mission, Uttarakhand, a huge emphasis has been on business facilitation by creating an enabling environment for industries to set up and start their operations. Thus, orchid cultivation and farming can be a developed as a lucrative business model for revenue generation and can revolutionise the Indian floriculture market if proper impetus is given by the government agencies.

References

Arditti, J. and Ernst, R. 1984. Physiology of germinating orchid seeds p. 177-222 In: J. Arditti edited. *Orchid Biology: Reviews and Perspectives Vol.III*, Cornell University Press, New York.

Bejoy, M., Kumar, S.C., Radhika, B.J. and Joemon, J. 2004. Asymbiotic seed germination and early seedling development of the dove orchid *Peristeria elata* Hook. *J. Orchid Soc. India*. 17 : 75–79.

Bhatnagar, M., Sarkar, N., Gandharv, N., Apang, O., Singh, S. and Ghosal, S. 2017. Evaluation of antimycobacterial, leishmanicidal and antibacterial activity of three medicinal orchids of Arunachal Pradesh, India. *B.M.C. Complement Altern. Med*. 17(1): 379.

Bhattacharjee, B. and Islam, S.S. 2015. The Effect of PGRs on *in vitro* development of protocorms, regeneration and mass multiplication derived from immature seeds of *Rhynchostylis retusa* (L.) Blume. *Global J. Biol. Biotech*. 4 (1) : 121-127.

Brinkmann, J.A. 2014. *Quick scan of Orchidaceae species in European commerce as components of cosmetic, food and medicinal products.* Report for Federal Food Safety and Veterinary Office, CITES Management Authority of Switzerland and Lichtenstein.

Chugh, S., Guha, S. and Rao, I.U. 2009. Micropropagation of orchids: A review on the potential of different explants. *Sci Hortic*. 122 : 507-520.

Das, P.K., Sahoo, S. and Bal, S. 2008. Ethnobotanical studies on orchids of Niyamgiri Hill Ranges, Orissa, India. *Ethnobot Leaft*. 12 : 70-78.

Deorani, S.C. and Sharma, G.D. 2007. *Medicinal Plants of Nagaland.* New Delhi, India: Bishen Singh Mahendra Pal Singh; 2007.

Hossain, M.M., Sharma, M. and Pathak, P. 2009. Cost effective protocol for *in vitro* mass propagation of *Cymbidium aloifolium* (L.) Sw.-a medicinally important orchid. *Eng. Life Sci.* 9 : 444-453.

Joshi, G., Tewari, L., Lohani, N., Upreti, K., Jalal, J. and Tewari G. 2009. Diversity of orchids in Uttarakhand and their conservation strategy with special reference to their medicinal importance. *Rep. Opin.* 1(3): 47-52.

Kaur, S. and Bhutani, K.K. 2014. *In vitro* conservation and asymbiotic propagation of *Coelogyne flaccida* (Lindl.): A threatened orchid. *Plant Biosyst*. 148(5): 935-944.

Khuraijam, J.S. and Sharma, S.C. 2017. Orchids: Potential ornamental crop in North India. *I.J.H.C.S.R*. 7: 1-8.

Kumar, A., Nandi, S.K., Bag, N. and Palni, L.M.S. 2002. Tissue culture studies in two important orchid taxa: *Rhynchostylis retusa* (L.) Bl. and *Cymbidium elegans* Lindl. p.113-124 In: S.K. Nandi, L.M.S. Palni and A. Kumar edited. *Role of plant tissue culture in biodiversity conservation and economic development*. Gyanodaya Prakashan, Nainital, India.

Leon, C. and Lin, Y.L. 2017. *Chinese Medicinal Plants, Herbal Drugs and Substitutes: an Identification Guide.* Kew Publishing, Kew.

Leroux, G., Barabé, D. and Vieth, J. 1997. Morphogenesis of the protocorm of *Cypripedium acaule* (Orchidaceae). *Plant Syst. Evol*. *205*(1-2): 53-72.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant*. 15: 473–497.

Parab, G.V. and Krishnan, S. 2012. Rapid *in vitro* mass multiplication of orchids *Aerides maculosa* Lindl. and *Rhynchostylis retusa* (L.) Bl. from immature seeds. *Indian J. Biotechnol*. 11(3): 288-294.

Pathak, P., Piri, H., Vij, S.P., Mahant, K.C. and Chauhan, S. 2011. *In vitro* propagation and mass scale multiplication of a critically endangered epiphytic orchid, *Gastrochilus calceolaris* (Buch.-Ham ex JE Sm.) D. Don. *Indian J. Exp. Biol*. 49(9): 711-716.

Peixe, A., Raposo, A., Lourenço, R., Cardoso, H. and Macedo, E. 2007. Coconut water and BAP successfully replaced zeatin in olive (*Olea europaea* L.) micropropagation. *Sci. Hort*. 113(1): 1-7.

Piri, H., Pathak. P. and Bhanwra, R.K. 2013. Asymbiotic germination of immature embryos of a medicinally important epiphytic orchid *Acampe papillosa* (Lindl.) Lindl. *Afr. J. Biotechnol*. 12(2): 162-167.

Sinha, P. and Jahan, M.A.A. 2012. Clonal Propagation of *Rhynchostylis retusa* (Lin.) Blume through in vitro culture and their establishment in the nursery. *Plant Tissue Cult. & Biotech*. 22(1): 111.

Raghavan, V. 1976. *Experimental Embryogenesis in Vascular Plants*. Academic Press, New York, London

Rasmussen, H.N., Dixon, K.W., Jersáková, J. and Tìšitelová, T. 2015. Germination and seedling establishment in orchids: a complex of requirements. *Ann Bot*. 116(3): 391-402.

Sunitibala, D.Y. and Neelashree, N. 2018. Micropropagation of the monopodial orchid, *Rhynchostylis retusa* (L.). *Int. J. of Life Sci.* 6(1): 181-186.

Zettler, L.W., Poulter, S.B., McDonald, K.I. and Stewart, S.L. 2007. Conservation-driven propagation of an epiphytic orchid (*Epidendrum nocturnum*) with a mycorrhizal fungus. *Hort Science*. 42 (1): 135-139.