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DE NOVO PLANTLET REGENERATION FROM LEAF EXPLANTS OF *RHYNCHOSTYLIS RETUSA* (L.) BLUME: A STUDY *IN VITRO*

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Abstract

The paper describes *in vitro* culture protocol of floriculturally and medicinally important orchid *Rhynchostylis retusa* using leaves as explants. Leaf explants from 16-18 wks old *in vitro* cultures were inoculated on Mitra *et al.* (1976, M) medium supplemented with growth regulators such as auxins and cytokinins (2 mgl^{-1} each), both singly and in combinations. The regeneration response frequency and time taken for the development of plantlets varied with the growth stimulus. In the basal medium, the leaf explants remained recalcitrant to regeneration, turned brown, and perished within 40 days. Additional presence of AC in the medium proved effective for initiating regeneration response via callusing and PLBs formation, at the basal end. Presence of IBA, NAA, and IAA+KN in the medium proved ineffective for initiating regeneration response. NAA and KN (2 mgl^{-1}) when used together in the medium, acted synergistically; cent per cent explants initiated regeneration response (8.75±0.25 days) via formation of callus, shoot buds and PLBs at abaxial and adaxial surface of leaf explants. Multiple PLBs were formed when the medium was augmented with KN. Development of PLBs with profuse growth of absorbing hair was observed in medium containing IBA+KN. Based on the present observations, basal medium supplemented with NAA and KN (2 mgl^{-1} each) is suggested as optimal nutrient medium for initiation, and multiple shoot formation via PLBs and healthy plantlet development.

Introduction

RHYNCHOSTYLIS RETUSA (L.) Blume is an epiphytic herbaceous orchid commonly known as *Fox-tail orchid* because of its brush-like spikes of colorful flowers. It is distributed in Bhutan, Cambodia, China, India, Indonesia, Laos, Malaysia, Myanmar, Nepal, Philippines, Singapore, Sri Lanka, Thailand, and Vietnam. In India, the plant is most common in NorthWest, NorthEast, Orissa, and Andhra Pradesh. It is used to treat rheumatic disease, blood dysentery, menstrual disorders, tuberculosis, epilepsy, gout, asthma, skin diseases, external inflammations and also as an emollient (Kumar *et al*., 2012; Pathak *et. al*., 2010). In Kurigram district of Bangladesh, people use the leaves of this plant to cure rheumatic pain (Das *et al*., 2012). It has colossal supply of biologically active compounds and sickness preventive phytochemicals properties. The natural way of propagation of this plant is usually hindered by very slow division of the pseudobulbs, so the rate of multiplication of young plants is time-consuming. Harvesting plants from the wild for medicine and horticulture purposes makes the species further endangered. Hence, development of an efficient and mass propagation method is crucial for conservation of the species. Successful attempts have been made earlier for mass multiplication of the medicinally as well floriculturally important orchids using *in vitro* asymbiotic seed germination (Anuprabha and Pathak, 2019; Bhattacharjee and Islam 2015; Bhowmik and Rahman, 2020; Gurudeva, 2019; Islam and Bhattacharjee, 2015; Kumar *et al*., 2002, 2003; Kurniasari *et al*., 2019; Madhavi and Shankar, 2019; Parab and Krishnan, 2012; Sembi *et al*., 2017; Sibin and Gangaprasad, 2016; Sunitibala and Neelashree, 2018; Thakur and Dongarwar, 2019; Thakur and Pathak, 2020; Thomas and Michael, 2007 *etc*.) and other explants such as leaf (Bhowmik and Rahman, 2020; Pathak *et al*., 2017; Sembi *et al*., 2020), root (Sood and Vij, 1986; Verma and Pathak, 2018; Vij *et al*., 1987), pseudobulb (Anuprabha and Pathak, 2020; Anuprabha *et al*., 2017; Bhowmik and Rahman, 2020), floral buds (Vasundhra *et al*., 2019), Stem nodal (Arora *et al*., 2016) *etc.*

The present study was conducted for *de novo* plantlet regeneration from leaf explants of *Rhynchostylis retusa* and subsequently for its propagation.

Material and Methods

Explant Source and Culture Medium

The mature seeds from dehisced capsules were collected from Tara Devi Hills (31˚6'N and 77˚6'E) of Shimla (Himachal Pradesh) and inoculated on the Mitra *et al.* (1976, M) medium with and without growth additives. Subsequently, whole leaves (0.5-1.0 cm) were procured from 16-18 wks (3-4 months) old *in vitro* grown *Rhynchostylis retusa* cultures and used as explants. Explants were placed on Mitra *et al.* (1976, M) medium supplemented with plant growth regulators such as Auxins (IAA, IBA, NAA) and Cytokinins (KN, BAP) at concentration of 2 mgl^{-1} each. Activated charcoal (2 gl^{-1})

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was also used, in some of the nutrient combinations. The pH of the medium was adjusted to 5.7 with 1N KOH or HCl and medium was autoclaved at 121°C at pressure of 15 psi for 20 min.

Culture and Incubation Conditions

The culturing was done under aseptic conditions in a laminar air flow cabinet. The cultures were incubated at 25±2°C under 12 hr photoperiod of 3500 lux intensity provided by white fluorescent tubes. Subculturing was done at regular intervals. For acclimatization, the complete plantlets obtained were inoculated on half strength M medium without any growth regulator for 6 wks, ¼ M medium without macro and micronutrients for 4 wks and subsequently, these were transferred on water agar medium. The plantlets were then shifted to pots containing charcoal pieces, bricks and mosses (1:1:1) as potting medium.

Observations and Statistical Analysis

The cultures were regularly observed and data recorded accordingly. In each treatment, there were four replicates and in each replicate, two explants were used. The parameters like, per cent regeneration response, meristematic loci invoked, regeneration pathway, number of regenerants obtained and time taken for completion of regeneration cycle were recorded. The results were analysed using one way ANOVA test and were analyzed using Tukey's Multiple comparison at $p \leq 0.05$ using SPSS (Version 16) software package (SPSS Inc. Chicago, US).

Results and Discussion

Regeneration competence depends upon the source and type of the explant, position of the tissue used, and the nutritional regime. Scrutiny of literature on leaf regeneration competence revealed that positive outcomes are, in general confined to the *in vitro* sourced explants than *in vivo* explants. During the present investigation, the regeneration response initiated with the formation of meristematic loci, on the explant and these meristematic loci were invoked in the basal as well as apical region of the explant. The detached leaf explants persuade various early signals including wound stimulus which alongwith the developmental status of the explant, influence the intensity of the explant's regenerative ability. The regenerative ability of young leaves is reported to be high as compared to mature leaves (Chen *et al*., 2014; Seeni and Latha, 2000; Sheelavanthmath *et al*., 2005; Vij and Aggarwal, 2003; Vij *et al*., 1986, 1994). Tissue culture methods have been used for assessing regeneration using different explants by some workers (Anuprabha *et al*., 2017; Park *et al*., 2018; Pathak *et al*., 1992, 2011, 2012, 2016; Ramesh *et al*., 2019; Regmi *et al*., 2017; Vasundhra *et al*., 2019; Verma and Pathak, 2018 *etc*.), for *in vitro* mass propagation of a number of orchids for conservation.

In the present study, initiation of morphogenetic response was both in the basal as well as apical region of the explant; it was however, mostly confined to the basal region of the leaf (Table 1, Fig. 1). According to Mathews and Rao (1985), the leaf base is the significant factor for culture initiation from foliar plants. The present study is in agreement with the earlier study (Decruse *et al*., 2003; Kuo *et al*., 2005; Martin and Madassery, 2006), where a synergistic action of auxin and cytokinin has been reported in other orchids. In the basal medium, the leaf explants failed to regenerate, turned brown, and perished within 40 days; there was a change in the color of these explants from green to brown within 7 days and brownish explants indicated the cell degeneration. The process occurs enzymatically between the enzyme polyphenoloxidase (PPO) and peroxidae (POD) with the polyphenol that forms quinon which is then polymerized to produce brown colour (Ko *et al*., 2009; Kurniasari *et al*., 2019; Ozyigit *et al*., 2007).

Additional presence of AC in the medium proved effective for initiating regeneration response in 50 per cent explants within 8.50±0.28 days via callusing and PLBs formation at the base of the leaf explant. The callus appeared on the wounded part of the explant; callus growth was characterized by the appearance of small clumps of cells. Activated charcoal generally has a strong adsorption impact, which could adsorb phenol and other harmful substances in the medium and therefore addition of activated charcoal to tissue culture establishes a dark environment appropriate for plant growth (Teixeira-da-Silva, 2006; Thomas, 2008). Kurniasari *et al.* (2019) obtained cent per cent leaf and root callus induction on pro-analyzed nutrient medium supplemented with AC and ascorbic acid, in *R. retusa*. Kaur and Bhutani (2009) also reported high regeneration frequency, early initiation, PLB proliferations, and plantlet development in either BAP (1 mgl^{-1}) alone/ AC (2 gl^{-1}) supplemented NAA (1 mgl^{-1}) containing medium in *Vanda testacea* leaves (1 cm long), sourced from 38 wks old axenic cultures. Medium containing IBA/or NAA/or IAA+KN proved ineffective for initiating regeneration response; the explants turned brown, and perished.

NAA and KN (2 mgl^{-1}) when used together in the medium, acted synergistically; cent per cent explants initiated regeneration response (8.75±0.25 days) via formation of callus, shoot buds, and PLBs at abaxial and adaxial surface of leaf explants. The augmentation of medium with the IBA+KN (2 mgl^{-1}), initiated response via callusing and PLBs at both abaxial and adaxial

Table 1. Regeneration potential of *in vitro* sourced *Rhychostylis retusa* whole leaf explants on Mitra *et al.* (1976, M) medium.

Additives*	Regeneration Response (%)	Regeneration Response Basal/Apical	Time taken in days for initiation of response	Number of meristematic loci invoked/ explant	Regeneration pathway	Number of regenerants obtained/ explant	Time taken in days for completion of regeneration cycle	Remarks
Control	-	-	-	-	-	-	-	-
AC	50.00	Basal	8.50±0.28^{b}	1.00±0.00^{b}	PLBs, Callus	1.75±0.25^{b}	112.00±0.70^{b}	Callusing and PLBs at the base
IAA	50.00	Basal	11.75±0.25^{e}	1.25±0.25^{bc}	Sb	1.00±0.00^{abc}	110.25±0.47^{b}	Shoot bud formation at the base
IBA	-	-	-	-	-	-	-	-
NAA	-	-	-	-	-	-	-	-
BAP	100.00	Basal	10.50±0.28^{d}	3.50±0.28^{de}	PLBs, Sb, Callus	2.25±0.25^{c}	120.50±0.50^{de}	Callusing, Shoot buds and PLBs at the base and at the abaxial surface
KN	50.00	Basal	14.50±0.28^{f}	1.00±0.00^{b}	PLBs	2.00±0.00^{bc}	118.50±0.64^{d}	Multiple PLBs at the base
IAA+BAP	50.00	Basal	13.50±0.28^{f}	1.50±0.28^{bc}	PLBs	1.50±0.28^{bc}	130.50±0.28^{f}	PLBs at the base
IAA+KN	-	-	-	-	-	-	-	-
IBA+BAP	100.00	Basal	10.00±0.00^{cd}	2.00±0.40^{c}	PLBs, Sb	2.00±0.40^{bc}	129.25±0.25^{f}	PLBs at the base
IBA+KN	50.00	Basal	10.50±0.28^{d}	3.50±0.28^{de}	PLBs, Callus	5.50±0.28^{d}	116.25±0.75^{c}	Callusing and PLBs at adaxial and abaxial surface; profusely hairy PLBs
NAA+BAP	100.00	Basal	9.25 ±0.25^{b}	3.00±0.00^{d}	PLBs, Sb	7.50±0.28^{e}	121.00±0.57^{e}	PLBs and Shoot buds on adaxial surface
NAA+KN	100.00	Apical and Basal	8.75±0.25^{bc}	4. 00±0.00^{e}	PLBs, Sb, Callus	8.00±0.40^{e}	110.50±0.28^{b}	Callusing, Shoot buds and PLBs on adaxial and abaxial surface

Entries in column no. 4,5,7,8 are Mean±S.E.; same alphabetical letter in the superscript denotes that the corresponding means in the same group using Tukey test at 5%. PLBs, protocorm like bodies; Sb, shoot bud(s).

*AC was used at 2 gl^{-1} and plant growth regulators were invariably used at concentration of 2 mgl^{-1}.

surface of leaf explants in 10.50±0.28 days and it took 110.50±0.28 days for completion of regeneration cycle. Development of PLBs with profuse growth of absorbing hair was observed in this medium. Incorporation of BAP (2 mgl^{-1}) in the medium not only induced morphogenetic response in cent per cent explants but also induced regeneration via callusing, shoot buds, and PLBs at the basal end of the explant as well as at abaxial surface of the leaf explants. The explants generated PLBs which correspond developmentally to one of the embryonal stages in orchid seed germination as well as callus which later differentiated into plantlets. Sunitibala and Neelashree (2018) also reported earlier, the callus induction on VW medium supplemented with NAA (0.1 mgl^{-1}) and BAP (1 mgl^{-1}) or KN (1 mgl^{-1}) in *R. retusa* leaf explants procured from 14 wks old aseptically raised plantlets. The healthy shoot bud induction was registered on medium supplemented with NAA+BAP while medium augmented with IAA induced only a few shoot buds. Multiple PLBs were noticed at the base of the explants when medium was augmented with KN (2 mgl^{-1}). Shadang *et al.* (2016) observed highest PLBs in MS+BAP (0.5 mgl^{-1}) and highest multiple shoot bud formation in MS+Coconut milk (15%)+BAP (0.5 mgl^{-1}) in *Ascocentrum ampullaceum* leaves procured from *in vitro* raised 5-6 months old cultures. Similarly, Chookoh

Fig. 1. A-L. Plantlet regeneration from *in vitro* cultured leaf explants in *Rhynchostylis retusa*: A, Plant with inflorescence; B, Leaf explants at the time of inoculation; C, Initiation response via PLB formation; D, Shoot bud formation ($M+IAA_2$); E-F, PLBs and Shoot bud formation ($M+AC_2$); G-H, Multiplication of PLBs ($M+BAP_2$; $M+KN_2$); I, Multiplication of PLBs and development of absorbing hair ($M+IBA_2+KN_2$); J-K, Plantlet development ($M+NAA_2+BAP_2$); L, Plantlets ready for transfer to the pots ($M+NAA_2+KN_2$).

et al. (2019) reported highest production of PLBs in BA (2 mgl^{-1}) and NAA (0.5 mgl^{-1}) supplemented MS medium, in *Tolumnia* Snow Fairy leaves. Jena *et al.* (2013) reported pragmatic induction of highest PLBs per explant in BAP (4 mgl^{-1}) supplemented half strength MS medium, in *Acampe papillosa* leaves, procured from *in vitro* raised seedlings. Pathak *et al.* (2017) also recommended M+KN (1.5 mgl^{-1}) as optimal nutritional combination for initiation, multiplication, and early plantlet formation for *R. gigantea* using whole leaf segments obtained from 26 wks old *in vitro* grown cultures.

Based on the present observations, basal medium supplemented with NAA and KN (2 mgl^{-1} each) is suggested as optimal nutrient medium for initiation, and multiple shoot formation via PLBs and healthy plantlet development.

Conclusion

Advancement and development of *in vitro* propagation technique has played an important role for conservation of many endangered floriculturally and medicinally important orchid species. Expanding ubiquity of orchids

for cut blossom and restorative reason has added new measurement to *in vitro* propagation procedure through which a critical number of indistinguishable clones can be raised from an explant. Consequently, methods for rapid multiplication of orchids are vital to meet the commercial demand. Likewise, the advancement of new and complex methods for phytochemical studies assumed a critical part in the quest for extra assets of natural substance for drug industry. The presently investigated species, *Rhynchostylis retusa* is medicinally as well as an economically important orchid, and lack of proper cultivation practices, obliteration of environment, unlawful and aimless assortment of the species from natural habitats represents an extraordinary danger to its endurance. Thus, present *in vitro* clonal propagation procedure using leaf explants offers a substitute strategy for viable conservation and mass propagation of *R. retusa* and the study may be extended to other related taxa as well.

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