Regeneration of Plantlets from *In Vitro* Raised Leaf Explants of an Orchid; Var. *Rhynchostylis retusa*

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Abstract

In the present investigation, young leaves were selected as the young tissue is known to regenerate better due to their less rigid cell walls. The regeneration competence was significantly influenced by the juvenility of leaves, basal media, PGRs in the medium and orientation of explants on the medium. Leaf explants showed swelling initially at their bases after 25 days of culture. In the basal medium, the explants, remained recalcitrant to regeneration and turned brown within 35 days and subsequently perished. The reactivity of the explants to BAP (benzyl adenine amino purine) almost was obligatory to the application of NAA (Naphthalene acetic acid) in the medium. The NAA in combination with IBA (Indole butyric acid) proved beneficial in activating accessory bud of regeneration. The 50% percent of explants responded to NAA+BAP (1.5mgl^{-1} each) treatment. Well-developed rooted plantlets formed either from direct shoot production or indirectly from PLBs in the induction medium. As many as 25 healthy rooted plantlets with 2-3 leaves and 1-2 roots could be obtained in 140 days of cultures.

Key words: Orchidaceae, *In vitro*, M medium, Plant Growth Regulators, *Rhynchostylis retusa*

Introduction

Orchids possessing exquisite flowers of myriad shapes, sizes, and with a variety of fragrance, brilliance in colour, and variation in form represent an order of royalty among the flowering plants. The numerical strength of these plants in terms of species has been variously estimated between 17000-35000 species in 859 genera (Dressler, 1993). Some other estimates suggest 18000 (Haywood, 1993) and 18,500 species in 778 genera (Mabberley, 1998). This handsome species ranks among the prized ornamentals due to its beautiful, densely packed, pendulous racemes with pinkish flowers having notched and spurred lip which are used in various religious rituals and adorned (as a head gear) by the ladies as a symbol of sanctity and womanhood (Hegde, 1984).

Materials and Methods

Leaves from the *in vitro* raised seedlings (170 days old) were used for the experimental purposes. About 0.6-1.2 cm long leaves of different ages were harvested and cultured on M like Mitra *et al.*,(1976) medium. All the media were fortified with sucrose (2%), and plant growth regulators (PGRs) like indole butyric acid (IBA), naphthalene acetic acid (NAA) and benzyl adenine amino purine (BAP) (0-1.5mgl^{-1}) alone or in combination. The experiment was carried out in Research Education, Agricultural and Resources Center of Balouchestan in 2016. For performing experiments, the chemically well-defined orchid culture media, M (Mitra *et al.*, 1976), were used as source of nutrition *in vitro*, with a view to identifying an optimal concentration/combination for regeneration purposes.

Results

Regeneration potential of leaf explants (either entire leaf or cut into two halves longitudinal and transverse; 0.6-1.2cm long) from *in vitro* 170 days old grown plants was tested on M medium. The regeneration competence was significantly influenced by the juvenility of leaves, basal media, PGRs in the medium and orientation of explants on the medium. The morphogenetic response to various concentrations of plant growth regulators showed variation in initiating the meristematic activity of the explants. Leaf explants showed swelling initially at their bases after 21 days of culture. In the basal medium, the explants, remained recalcitrant to regeneration and turned brown within 35 days and subsequently perished.

Moreover, the explants remained recalcitrant to regeneration even in the medium supplemented with IBA at 0.5, 1 & 1.5mgl^{-1} and NAA as well. Combination containing NAA and IBA at various concentrations and combinations also remained ineffective excepting for only one combination containing NAA(1mgl^{-1}) and IBA (0.5mgl^{-1}).

Generally, it was observed that the whole leaf yielded better result over leaf segments which produced PLBs throughout the length of the leaf, but in leaf segments PLBs formation was restricted to the cut ends only. The morphogenetic changes leading to plantlet development are illustrated in table 1. The reactivity of the explants to BAP almost was obligatory to the use NAA in the medium. Use of NAA(1mgl^{-1}) in combination with IBA(0.5mgl^{-1}) proved beneficial in activating accessory loci of regeneration.

Combination containing IBA & NAA at various concentrations and combinations also remained ineffective excepting for only one combination containing IBA(0.5mgl^{-1}) and NAA(1mgl^{-1}). The callusing of the meristemoids was highly pronounced in the additional presence of IBA+NAA(0.5+1mgl^{-1}). The callus was slightly compact, hairy chlorophyllous with many growing points each of meristemoid directly developed into shoots/Plbs. 50% percent explants responded to NAA+BAP (1.5mgl^{-1} each) treatment, and each developing a couple of meristemoids in the superficial cell layers. In the Plbs, leaf organogenesis preceded that of root. The Plbs took 65 days to differentiate into plantlets. The differentiation cycle could be significantly shortened by replacing BAP, in the NAA supplemented medium (1.5mgl^{-1} each).

It was observed that the whole leaf yielded better result over leaf segments which produced PLBs/Sb throughout the length of the leaf, but in leaf segments PLBs formation was restricted to the cut ends only. It was also recorded that younger leaves enhanced better PLBs/Sb, while older leaves almost, were recalcitrant to regenerate. Regeneration at various concentrations of BAP+IBA, almost was shoot bud mediated. Well-developed rooted plantlets formed either from direct shoot production or indirectly from PLBs in the induction medium. Up to 25 healthy rooted plantlets with 2-3 leaves and 1-2 roots could be obtained in 140 days of cultures. The nature and frequency of response was, however, markedly influenced by the quality of the growth stimulus in the medium. The results summarized in Table 1. are briefly described as follows:

Table 1. *In vitro* regeneration response of leaf explants (170 days old) of *Rhynchostylis retusa* on M medium.

Plant growth regulators Conc.(mgl^{-1})		Time taken for initiation of response (days)	Explants responded (number)	Number of meristematic formed per explants	Regeneration pathway*
Basal		35	-	-	-
IBA			-	-	-
0.5		-	-	-	-
1.0		44	-	-	-
1.50		45	-	-	-
NAA					
0.5		40	-	-	-
1.0		30	-	-	-
1.50		30	-	-	-
BAP					
0.5		33	1	3	PLB
1.0		30	2	3	PLB-Sb
1.50		30	2	4	PLB
IBA	NAA				
0.5	0.5	30	2	-	Ca
1.0	0.5	25	2	-	Ca
0.5	1.0	25	3	5	PLB-Ca
IBA	BAP				
0.5	1.0	25	1	2	Sb
1.0	1.0	25	1	4	Sb-PLB
1.50	1.0	25	5	6	Sb-PLB
NAA	BAP				
0.5	1.50	30	2	5	PLB-Sb
1.0	1.50	25	4	6	PLB-Sb
1.50	1.50	21	8	7	Sb-PLB

PLB: Protocorm like bodies, Ca: Callus, Sb: Shoot bud

The orientation of leaf on media showed a marked effect on morphogenetic response. The leaves with normal vertical orientation showed better response followed by horizontally placed leaves, while leaves cultured in reverse orientation exhibited poor PLBs formation in few explants.

Discussion

The supply of growth regulators promoted the production of shoots/Plbs from leaf explants cultured on M medium. This is probably an outcome of the habituated nature and juvenility of the explant. In the present culture, regeneration response in most of the cases is restricted to the basal region of the leaf whereas in a few cases the leaves regenerated all along the surface. The juvenility of tissues is thought to be an important factor controlling cell proliferation in several orchids (Vij and Pathak 1989; Arditti and Ernst 1993; Vij *et al*. 1997). In the present study, the response of explants to BAP in combination with auxins (NAA&IBA) and NAA&IBA differed according to the concentrations. Tokuhara and Mii (1993) reported that the combination of hormones was of key importance for the micropropagation of orchid mostly in *Phalaenopsis* Taxa.

A stimulatory effect of BAP and NAA together in the medium has been reported for certain species of orchids earlier (Kosir *et al*. 2004; Hongthongkham, J and S, Bunnag. 2014). While some authors have reported reduced induction and regeneration in medium supplemented with NAA (Arditti and Ernst 1993), others reported that an appropriate combination of NAA and BAP stimulated shoot formation (Tokuhara and Mii 1993; Tisserat and Jones 1999; Roy and Banerjee 2003). Similar results were also obtained in the current study wherein a maximum number of shoots and BFC were recorded in medium containing a combination of BAP ($1.5mgl^{-1}$) and NAA ($1.5mgl^{-1}$).

The response of the explants to PLB formation varies from species to species and from explant to explant used (Teng *et al*. 1997). The regeneration and proliferation competence of the juvenile leaves is much more than the relatively older explants. Plant regeneration from young leaf tissue could be induced either indirectly through the formation of intervening callus tissue (Hong *et al.* 2008, Huang & Chung 2010, Ng & Saleh 2011) or directly through the formation of protocorm-like bodies (Temjensangba and Chitta ranjan Deb 2005, Luo *et al.* 2008, Mayer *et al.* 2010, Naing *et al.* 2011).

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