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Research Article

REGENERATIVE COMPETENCE OF PSEUDOBULB EXPLANTS OF ENDANGERED ORCHID GENERA: A STUDY *IN VITRO*

Vishal Sharma*

Government Post Graduate College for Girls-11(Panjab University), Chandigarh

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ABSTRACT

Regeneration competence of pseudobulb segments of *Coelogyne ovalis*, procured from *in vitro* grown cultures, was assessed in Mitra medium alone and in combinations with PGRs. Juvenility of the tissues, chemical stimulus, and position of the explants (apical/basal) were the key factors in initiating the response. The explants from pseudobulbs (>3cm long) did not regenerate whereas those from younger(<3cm in length) regenerated depending upon their position in the source organ..The response frequency and time taken for the development of plantlets also varied with the growth stimulus. Maximum number of shoots was obtained from the basal segments on medium supplemented with cytokinin to auxin in ratio of 10:5 in BM+BAP(10 mg $l^{-1)}$+NAA(5 mgl^{-1}) , 40 plantlets are formed after 14 wks..The apical segments formed shoots only from apical meristem. The shoots rooted in the same nutrient medium and formed .The regenerated plantlets were acclimatized & transfered to pots filled with moss, pinebark, brick and charcoal pieces mixture with 90% survival.

INTRODUCTION

Coelogyne ovalis Lindl(Orchidaceae) is an evergreen, epiphytic and sympodial orchid with large ,white, fragrant, keeled flowers in drooping raceme .*C.ovalis* is an important herb which grows luxuriantly along Himalayan ranges from Garhwal eastwards to Arunachal Pradesh (1700-2300m).The foliar extract is favourite of the herbalist for its bone healing properties (cf.Lawler,1984), and pseudobulb extract is aphrodisiac & is used to relieve from fever, headache, burns., cough, urinary & eye infection (Pant,2013;Tsering *et al*.,2017). Besides being a victim of its own beauty and utility, it is progressively loosing its natural habitats and is getting rarer with every passage of time and figures prominently in Appendix II of the Convention on International Trade in Endangered species of Wild fauna and flora (CITES,2012,2017).

Tissue culture technique has added new dimensions to commercial exploitation of economically important plants. It is particularly useful in outbreeders like orchids which generate a great deal of heterozygosity in the progenies. Morel(1960) demonstrated the possibilities of using apical meristems for micropropagating a variety of orchids. The technique is, however, detrimental to the growth and development of mother plant, as it requires the sacrifice of the entire new growth or the only growing point .It is, thus desirable to develop an alternate and equally effective multiplication system by activating adventitious meristems in organs ,whose excision does not endanger the survival of source plant. In order to meet this objective, regenerative competence of pseudobulb explants is used for initiating *in vitro* cultures.

MATERIAL AND METHODS

C.ovalis plants were collected in nature from Arunachal Pradeshal (latitude range27º00-:27º08'N; longitude range:93º40-95º3'E and replanted in pots containing substrate such as moss, pinebark, brick and charcoal pieces(1:1:1:1) and grown under greenhouse conditions with 70% relative humidity &25/20ºc day/night temperature.

The freshly formed pseudobulbs were harvested from stock plants, were used as material for the present study. The pseudobulbs are segmented into three parts i.e distal segment (PS1), central segment(PS2) and proximal segment (PS3). These were sequentially surface sterilized with solutions of Streptomycin(0.1%,20min), Sodium hypochlorite (4%,15min) &dip in Ethanol(70%,3sec) before rinsing with sterilized distilled water. Excised pseudobulbs were segmented into 1 cm large explants and inoculated on sucrose (2%) supplemented and agar(0.9%) gelled basal medium (BM:Mitra *et al*,1976)

Corresponding author:* **Vishal Sharma
Government Post Graduate College for Girls-11(Panjab University), Chandigarh

and its various combinations with NAA(α-naphthalene acetic acid), BAP(6-Benzyl amino purine),KN(Kinetin).

The pre-inoculation medium pH was adjusted at 5.6.In parallel set of experiments 0.2% activated charcoal (AC) was used in the medium. Thirty two replicates for each treatment & the experiments were repeated a second time. All experimental manipulations were done under aseptic conditions & the cultures incubated at 25±2°c under 12 hr photoperiod of 3500 lux light intensity, were regularly observed.

Acclimatization of the Plantlet

After well-developed shoot and root formation the plantlets(3cm tall)were transfered to semisolid medium containing only half strength macro&micro salts of BM(Mitra *et al*,1976) medium; sucrose and vitamins were elimated(Fig.5) The plantlets were kept in this condition until they are 4-5cm tall, and washed with luke warm water before transfering to moss, pinebark, brick and charcoal pieces(1:1:1:1) mixture. Humidity was maintained by covering each pot with transparent polythene bag. Holes of increasing size were made in the bags to reduce the humidity level gradually. The bags were removed after 4 weeks and small plants in the pots were transferred from 90% shade to the sunlight. Survival rate was 90%.Spraying with fungicide (Bavistin 1%) twice a week was necessary to keep fungus off from the young plants.

RESULTS

The regeneration eluded the explants from well developed 1 yr old peudobulbs(>3cm long), whereas, meristematic activity could be selectively initiated in those from the freshly formed daughter pseudobulbs(<3cm long) depending upon their position in the source organ. The explants from central segments (PS2), invariably turned brown and perished soon after inoculation, whereas, those from the proximal (PS3) and distal(PS1) responded to regeneration. The response in distal segments was obligatory to combined treatment of 10mg l^{-1} treatment with either of BAP and KN. The response was, however, better expressed in AC enriched medium. Nearly 62.5% explants were embrogenetic, but the response was obligatory to a combined treatment with BAP(10 mgl^{-1}) and NAA(5 mgl^{-1}), Plbs were developed within 4 wks in each of the responding explants each of which multiplied through budding(Fig.3),prior to differentiation into plantlets ;upto 40 plantlets were obtained after 14 wks.

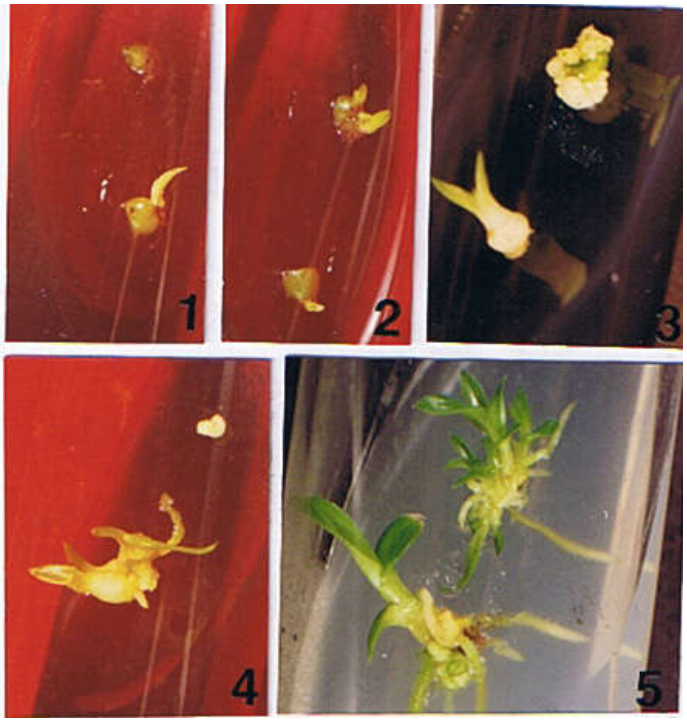

Fig 1 Direct Shoot primordial in distal segment in BM+BAP(1mg/l)+NAA(5mg/l); Fig.2. Direct Somatic embryogenesis in both distal & proximal segments in BM+BAP(10mg/l)+ NAA(5 mg/l); Fig3.Multiplication of Plbs through budding in BM+BAP(10mg/l)+NAA(5 mg/l); Fig4.Callusing at the base of plantlet in BM+BAP(10mg/l)+NAA(5mg/l) ;Fig.5.Acclimatization of the plantlet

The efficacy of multiple shoot formation differed with concentration and combinations of BAP and NAA. A low concentration of BAP(1mg l^{-1}) in NAA(5 mgl^{-1}) supplemented medium, favoured direct shoot development, whereas higher concentration of BAP(10mg l^{-1}) favoured regeneration through PLBs via callusing. The Plb proliferations were more pronounced in AC enriched regimes and plantlet were obtained in 12 wks. When BAP was replaced with KN in the medium, plb development remained elusive and proliferative shoot primordial were directly developed in proximal and distal segments.

DICUSSION

Presently pseudobulb explants were successfully used for micropropagating *C.ovalis in vitro*. The regeneration competence of the pseudobulbs seems to be markedly influenced by physiological age of the mother plant, position of donor and growth stimulus in the nutrient pool.

Table1 Regenerative response in C.ovalis pseudobulb eggplant

Growth regulators applied to BM medium	Concentrations ofGrowth Regulators (mgl^{-1})	Regeneration frequency (%)	Regeneration Pathway Plb	Shoot Primordia	No.of plantlet obtained/ explant
BA	1		-	-	-
	2		-	-	-
Kn	3		-	-	-
	5		-	-	-
BAP+NAA*	1:1	37.5	+	-	3
BAP+NAA	5:5	50	+	-	15
BAP+NAA*	10:5	62.5	+	-	40
Kn +NAA*	10:5	50	-	+	2

*medium supplemented with activated charcoal

Juvenility of the tissue emerged as the major factor since the response was more pronounced to the proximal segments due to the fact that the younger tissues with less rigid cell walls are physiologically & biochemically more active and shoe better morphogenetic potential in harmony to earlier reports (Basker and Bai, 2006; Sungkumlong and Deb,2009;Jiang *et al*.,2011; Kaur and Bhutani,2010;Kaur,2017)

The efficacy of Plant growth regulators(PGRs) in activating proliferative loci in pseudobulb explants and regulating their subsequent development into plantlets in species- specific in Orchids (Basker and Bai,2006).The regenerative pathway and differentiation varied with quality and quantity of PGRs. In present studies, a treatment with KN(10 mgl^{-1}), promoted direct development of shoot primordial (Basker and Narmatha,2006;Sungkumlong and Deb,2009;Shahinul Islam *et al*.,2015), but in BAP treated ones it favoured a switch in the regenerative pathway; the pathway was punctuated by Plb phase of development (Vij & Pathak,1989; Basker and Narmatha, 2006;Kaur,2017).The synergistic action of the combination auxin and cytokinin in inducing regeneration in explants in compliance with earlier reports (Sunitibala and Kishor,2009; Kaur and Bhutani,2010; RajKarnikar,2011; Jiang *et al.,*2011;Pant and Thapa,2012;Kaur and Bhutani,2010;Ghosh *et al*.,2014;Kaur,2017).

Addition of Activated Charcoal (AC) in the initiation media proved beneficial in maintenance of cultures (Sungkumlong and Deb, 2009)

CONCLUSION

The Pseudobulb culture is as an efficient, reliable method for clonal propagation of plantlets bereft phenotypic variations. This system open up the new avenues in conservation strategies of endangered Orchid taxa. Different permutation and combination of PGRS are used and found that nutrient regime plays an important role in the path of somatic embryogenesis.

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