J. Orchid Soc. India, 29: 75-79, 2015 ISSN 0971-5371

REVERSION OF REPRODUCTIVE PHASE TO VEGETATIVE PHASE IN THE INFLORESCENCE SEGMENTS OF *SACCOLABIUM PAPILLOSUM* LINDL. - A STUDY *IN VITRO*

Saranjeet Kaur[1] and Promila Pathak

Orchid laboratory,Department of Botany, Panjab University, Chandigarh- 160 014, India
[1]Faculty of Science, Cordia College, Sanghol-140 802, Distt., Fatehgarh Sahib, Punjab, India

Abstract

The regeneration potential of inflorescence segments of *Saccolabium papillosum* Lindl. was tested on M (Mitra *et al.*, 1976) medium and its combinations with different growth additives. The regeneration response varied with the position of explants on the donor axis. The explants, only from upper 2/3 region with undifferentiated floral buds responded to regeneration; these followed direct and callus mediated plantlet development. The percentage of regeneration in the explants was directly proportional to the level of Plant Growth regulators (PGRs) in the nutrient medium. The regeneration response was obligatory to the use of PGRs such as cytokinins [benzyladenine (BA), 6-furfurylaminopurine/kinetin (KN)], auxins [α-naphthalene acetic acid (NAA)] at 0.5 and 1.0 mg^{-l} and organic growth supplement, Peptone (P; at 1.0 gl^{-1}) in the nutrient pool. NAA favoured multiplication of Protocorm-like bodies (PLBs). Addition of activated charcoal promoted early plantlet development.

Introduction

THE GENUS *Saccolabium* includes nearly 40 species of epiphytic orchids and is distributed in the Indian subcontinent (from tropical India, Burma, throughout Indonesia to New Guinea) (Bose and Bhattacharjee, 1980). It derives its name from the bag-like shape of the labellum. Usually, the plants are dwarf, evergreen with leafy stems. The blooms are delicately colored and occasionally fragrant. Although, the flowers are small, they are produced in large numbers. Due to extermination of the forests, their natural populations are shrinking day by day. With a view to ameliorating their ever-declining wild populations, presently *S. papillosum* was selected for the purpose. *Saccolabium papillosum* Lindl. (=*Acampe papillosa* Lindl.) is a beautiful epiphytic orchid species with leaf opposed, 4-8 flowered, sub-corymbose racemes. It is distributed all along the tropical Himalaya at an altitude of 500-800 m (from Kumaon Eastwards to Arunachal Pradesh) and inhabits a variety of broad-leaved phorophytes, including *Mangifera indica* and *Shorea robusta.* While the ornamental potential of this species is yet to be explored, its therapeutic utility is well documented. Its roots are used as a substitute drug for Sarsaparila (Lawler, 1984). *S. papillosum* is faced with habitat destruction pressures that far exceed its natural regeneration. As a result, the species has become threatened in its natural habitats. The genus *Saccolabium* is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2014). Through this communication, we emphasize to conserve, propagate and multiply the species, through tissue culture techniques with a view to popularizing the species amongst amateurs, nurserymen and professionals for its cultivation, thus saving its wild populations from getting extinct.

In clonal propagation, it is immensely important to maintain genetic uniformity in *in vitro* raised progenies. In outbreeding taxa like orchids, seed raised progenies are extremely heterozygous. To maintain genetic stability of the regenerants, it is important to identify appropriate explants and their *in vitro* propagation protocols. The utility of inflorescence segments, as an effective alternative to shoot meristem for micropropagating orchids is a successful approach in this direction as this method provides opportunities to produce a large number of true to type plantlets of interest. The orchids are propagated *in vitro* by using various explants such as shoot meristem, leaves, roots, protocorms *etc.* obtained from axenic cultures but the regenerative potential of inflorescence segments as explants has been less explored as compared to the other explants. Therefore, presently an attempt was made to establish an efficient regeneration system by using inflorescence segments as explants in *S. papillosum* with a view to assessing the: i) influence of position of the explant; and ii) the effect of growth regulators individually and/or in combination on frequency and onset of regeneration and the subsequent development of regenerants into plantlets. In this paper, an efficient and reproducible single step protocol for the regeneration, multiplication and development of plantlets using inflorescence segments is reported.

Received: August 25, 2015; Accepted: September 5, 2015

Materials and Methods

Plant Collection and their Maintenance in the Greenhouse

Saccolabium papillosum Lindl. plants were obtained from a commercial grower of Dehradun, Uttarakhand, India. The healthy plants were replanted in pots (diameter 27.5 cm × 22.4 cm) containing epiphytic substrate, *i.e.*, pieces of charcoal, brick and bark in a ratio of 1:1:1. *Sphagnum* moss covered the top surface of the potting mix. The plants were maintained in a greenhouse under natural light, 70% relative humidity and 25°C/20°C day/night temperature.

Sterilization Procedure and Culture Media

The inflorescence axis was obtained from green house grown plants. It was segmented into 1.0-1.5 cm long explants. The segments were scrubbed with a soft brush under running tap water to remove any debris. Later, these segments were washed with a dish wash detergent solution. These were swabbed with ethyl alcohol under a sterile (laminar) hood and surface sterilized with 0.1% (wv^{-1}) mercuric chloride ($HgCl_2$; Qualigens, Mumbai, India) solution containing 1–2 drops of "Teepol" as a wetting agent for 4-5 min. This was followed by 2–3 rinses with sterilized distilled water. Thereafter, the ends (1.0-2.0 mm long) were severed-off and remaining explants were inoculated in M (Mitra *et al.*, 1976) medium and supplemented with growth regulators such as cytokinins [benzyladenine (BA), 6-furfurylaminopurine/kinetin (KN)] and auxins [α-naphthaleneacetic acid (NAA)] at 0.5, 1.0 mg^{-l} and organic growth supplements such as peptone (P) at 1 gl^{-l} in the medium. In a separate set of experiment, the effect of activated charcoal (AC) 2 gl^{-1} was also tested. The medium was autoclaved at 121°C at a pressure of 1.06 kg cm^{-2} for 15 min. The autoclaved medium was kept at 37°C to check any contamination in the culture medium.

Inoculation and Incubation Conditions

All inoculations were performed under aseptic conditions in a laminar airflow cabinet. The cultures were incubated at 25 ± 2°C under a 12-h photoperiod with a light intensity of 3500 lx (fluorescent tubes 40W, Philips India Ltd, Mumbai, India). Four replicates were used for each experiment and, to check the reproducibility, the experiment was repeated twice. The cultures were observed regularly under a binocular microscope (Olympus SZX10, Japan).

Statistical Analysis

One way analysis of variance was performed with respect to each response (average ± standard error against each additive as mentioned in Table 1). As ANOVA results showed the non significant difference of additives at 5% level of significance, various groups of additives showing identical/similar response were formed statistically. To this end, Tukey Test was performed at 5% level with respect to each response.

Results and Discussion

The ability of *Phalaenopsis* inflorescence segments to regenerate *in vitro* opened up exciting opportunities to use them as explants (Rotor, 1949). They have proved as an effective alternative to excised shoot meristem for micropropagating the orchids. The regeneration potential of floral buds and inflorescence segments have been tested in a few orchid species and hybrids (cf. Arditti and Ernst, 1993; Chen and Piluek, 1995; Chen *et al.*, 2002; Collins and Dixon, 1992; Goh and Wang, 1990; Ichihashi, 1992, Kher *et al.*, 1997; Lin, 1986; Martin *et al.*, 2005; Mitsukuri *et al.*, 2009; Shimasaki and Uemoto, 1991; Tokuhara and Mii, 1993; Vij *et al.*, 1986,1997).

Figs. 1-6. *In vitro* inflorescence segment culture of *Saccolabium papillosum* in M medium and its various combinations with growth adjuncts: 1, PLB mediated regeneration response in $M+NAA_{(1.0)}$; 2, Multiplication of regenerants in $M+NAA_{(1.0)}$; 3, Plantlet development in $M+NAA_{(1.0)}$; 4, Compact, chlorophyllous and organogenetic callus mediated PLB development in $M+BAP_{(0.5)}$; 5, Multiplication of PLBs in $M+BAP_{(0.5)}$;6, Healthy growth of plantlets in $M+P_{(1.0)}+AC$ enriched medium.

Presently, the undifferentiated floral-buds were amenable to transformation into vegetative ones and are paralleled with the ability of floral buds to de-differentiate and assume vegetative growth as reported earlier in epiphytic orchids such as *Dendrobium crepidatum* and *Oncidium* (Lim-Ho *et al.*, 1984). In the presently investigated species, the response in the inflorescence segments was obligatory to the use of PGRs in M medium and it varied with their position on the donor axis. Incidentally, their proliferative potential was directly proportional to the level of PGRs in the nutrient medium. However, as Rotor (1949) could obtain somatic embryos on a growth regulator free medium, it appears that the hormonal requirements, during regeneration vary with the species. The results are summarized in Table 1 and illustrated in Figs.1-6.

In our cultures, the segments from lower 1/3 region of the mother axis, with well differentiated buds, necrosed within 2 wks of culture unlike their normal development into flowers in *Ascofinetia, Neostylis* and *Vascostylis* (Intuwong and Sagawa, 1973) and *Saccolabium* (Vij *et al.*, 1986). Konar and Kitchlue (1982) hinted at the involvement of nutritional complexities during normal development of the flowers. In earlier study, a treatment with IAA, KN and CH/U was, however, obligatory for development of *S. calceolaris* buds into normal flowers (Vij *et al.*, 1986).

In our cultures, the segments from upper 2/3 region with undifferentiated floral buds were able to regenerate to the chemical stimulus in the nutrient pool. The explants followed both, direct and callus mediated PLB-plantlet development in auxin and cytokinins treated cultures. Auxin (NAA) favoured direct development of PLBs (protocorm-like bodies) without any intervening callus formation and its effect was more pronounced at 1mgl^{-1} when 74.25 ± 0.95% explants regenerated in its presence. The *neo-formations* (PLBs) multiplied profusely and up to 80 PLBs could be harvested within 15 wks (Figs. 1, 2). Plantlets were obtained in another 10 wks (Fig. 3). The importance of NAA in initiating *Oncidium* cultures from floral stalk cuttings has been demonstrated by Lim-Ho and Lee, 1987. The utility of growth additives in inducing / regulating plantlet regeneration has already been emphasized in *Dendrobium* (Vij *et al.,* 1981), *Oncidium* (Lim-Ho and Lee, 1987), *Phalaenopsis* (Lin, 1986; Tanaka and Sakanishi, 1980) and *Saccolabium* (Vij *et al.,* 1986). The benign effect of NAA with BAP/KN in inducing PLBs, callus and/or multiple shoots, is

Table 1. *In vitro* regeneration response of *Saccolabium papillosum* inflorescence segments in M medium and its combination with growth additives.

Additives	Regeneration (%)	Initiation of response (wks)	Number of regenerants	Plantlet development (wks)
-	-	-	-	-
AC	-	-	-	-
$BAP_{(0.5)}$	25.00 ± 0.81^{b}	2.50 ± 0.57^{ab}	6	29.25 ± 0.95^{def}
$BAP_{(0.5)}$ + AC	25.00 ± 0.81^{b}	2.00 ± 0.00^{a}	8	27.25 ± 0.95^{bcd}
$BAP_{(1.0)}$	50.00 ± 0.81^{c}	2.75 ± 0.50^{ab}	15	30.00 ± 0.81^{f}
$BAP_{(1.0)}$ + AC	50.00 ± 0.81^{c}	2.00 ± 0.00^{a}	20	26.00 ± 0.81^{bc}
$KN_{(0.5)}$	25.00 ± 0.81^{b}	2.75 ± 0.50^{ab}	4	31.00 ± 0.81^{f}
$KN_{(0.5)}$ + AC	25.00 ± 0.81^{b}	2.25 ± 0.50^{ab}	6	29.00 ± 0.81^{def}
$KN_{(1.0)}$	50.00 ± 0.81^{c}	2.75 ± 0.50^{ab}	10	30.00 ± 0.81^{ef}
$KN_{(1.0)}$ + AC	50.00 ± 0.81^{c}	2.00 ± 0.00^{a}	20	27.00 ± 0.81^{bcd}
$NAA_{(0.5)}$	50.00 ± 0.81^{c}	2.00 ± 0.81^{a}	20*	27.00 ± 0.81^{bcd}
$NAA_{(0.5)}$ + AC	50.00 ± 0.81^{c}	2.00 ± 0.00^{a}	18*	27.75 ± 0.95^{cde}
$NAA_{(1.0)}$	74.25 ± 0.95^{d}	2.00 ± 0.00^{a}	30*	25.00 ± 0.81^{ab}
$NAA_{(1.0)}$ + AC	75.00 ± 0.81^{d}	2.00 ± 0.00^{a}	30*	23.50 ± 1.29^{a}
$P_{(1.0)}$	10.00 ± 0.81^{a}	3.00 ± 0.00^{b}	60	34.75 ± 0.95^{g}
$P_{(1.0)}$ + AC	10.00 ± 0.81^{a}	3.00 ± 0.00^{b}	60	35.00 ± 0.81^{g}

* = Direct protocorm - like body (PLB) formation.

already on records in *Phalaenopsis* (Lin, 1986); *Diuris longifolia* (Collins and Dixon, 1992). Presently, the explants in BAP/KN (0.5 mgl^{-1}) treated cultures followed a callus mediated multiple PLB regeneration in 25.00 ± 0.81% explants; phenotypically, the callus was compact, chlorophyllous and organogenetic in nature (Fig.4). The PLBs rapidly multiplied in the respective medium (Fig.5). The response frequency was elevated to 50.00 ± 0.81% in combinations containing cytokinins at 1mgl^{-1} and their multiplicity was further accentuated if the combination was darkened with AC.

Presently, organic growth supplement (Peptone) was successfully utilized to transform undifferentiated floral buds into vegetative ones. Although, the regeneration percentage was impaired to 10.00 ± 0.81% in this combination, the regenerated callus mediated PLBs were observed to be highly proliferative, almost 60 daughter PLBs could be harvested within 18 wks. Morphogenetic processes leading to plantlet development, however, were delayed in the combination (34.75 ± 0.95 wks) but additional activated charcoal favoured healthy growth of plantlets (Fig.6). A perusal of literature reveals that peptone being water soluble protein hydrolysate loaded with very high amino acid content is able to promote growth of cultures. Similar beneficial effect of peptone was earlier observed in inducing protocorm multiplication in *Cymbidium macrorhizon* and *Cymbidium* species (Kusumoto and Furukuwa, 1977). Peptone is also known to have stimulated callus growth in *Phalaenopsis*, *Doritaenopsis*, and *Neofinetia* (Ichihashi and Islam, 1999). It also supported better seedling growth in *Paphiopedilum*, *Phaius*, and *Vanda* (Curtis, 1947). In *Peristeria elata,* peptone favoured early and healthy growth of seedlings (Bejoy *et al.*, 2004). Supplementation of organic growth additives in orchid culture medium is simple, practical, beneficial and a conventional method to improve media used for commercial production (Ichihashi and Islam, 1999). In the present studies, the used organic growth supplement contains amino acids, proteins, and organic compounds; it seems that any of these component(s) may be responsible for promoting growth and development of the present cultures. Hence, further studies are required to determine which factor(s) is responsible for promoting effect of these organic additives.

Conclusion

All these data suggest that *in vitro* regeneration potential of inflorescence segments is regulated by the developmental stage of the explant and the response is markedly influenced by the chemical stimulus in the nutrient pool. The technique can be profitably utilized as an effective alternative to shoot meristem in monopodial taxa as it is useful not only for cloning orchids but also for generating lesser number of somaclonal variations.

Acknowledgement

Financial assistance to the first author, from the Department of Biotechnology (DBT), GOI, New Delhi, during the course of study is gratefully acknowledged.

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