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IN VITRO SHOOT INDUCTION AND REGENERATION POTENTIAL OF FLORAL BUDS IN *CREPIDIUM ACUMINATUM* (D.DON) SZLACH., A MEDICINAL AYURVEDIC PLANT FROM NORTHWESTERN HIMALAYAS

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Abstract

Crepidium acuminatum (D.Don) Szlach., commonly known as Jeevak, is an endangered medicinal orchid of renowned status in Ayurveda. The dried pseudobulbs of *C. acuminatum* are one of the important ingredients of *Ashtavarga Drugs* used in the preparation of *Chyavanprash* and also to cure tuberculosis. Presently, an attempt was made to establish *in vitro* method for rapid regeneration of *C. acuminatum* using young unopened floral buds with a view to developing an efficient mass propagation protocol for its conservation. For this purpose, young unopened floral buds from immature inflorescence (8 to 10 days old) were excised, surface sterilized in bavistin (0.01 mgl^{-1}), streptomycin (0.01 mgl^{-1}), and mercuric chloride (0.1 mgl^{-1}) and subsequently cultured on M (Mitra *et al.,* 1976) medium supplemented with different growth additives (1 mgl^{-1}). Interestingly, the buds successfully regenerated into shoot buds in M medium containing IAA and IAA+KN in three wks old cultures; the best response was however, observed on medium containing IAA+KN (1 mgl^{-1} each) in which 8 to 10 pseudobulbous shoots were developed from single floral bud after 5 months of culturing. Pseudobulbous plantlets complete with roots and leaves were obtained in 21 wks old cultures. However, when BAP (1 mgl^{-1}) was added to basal medium, some buds initially showed swelling after 3 wks of culturing and subsequently, opened into as flowers. These data indicate that *C. acuminatum* can be successfully regenerated using young floral buds and this methodology may be applied to other medicinally important orchids so as to produce a large number of clones in short time.

Introduction

THE ORCHIDS account for nearly 10% of the flowering plants species and constitute one of the largest, diverse, and highly evolved families of flowering plants-Orchidaceae (Dressler, 1981). It is represented by 28,484 accepted species (Govaerts *et al.,* 2017). In India, the family is represented by 1,256 species under 155 genera (Singh *et al.*, 2019). The orchids stand distinct from other plants in having intricately fabricated and colorful flowers; microscopic seeds with highly reduced embryos and suppressed development of endosperm; and dependence on a fungal infection for germination and growth in nature (Vij, 1995). Orchids, besides their ornamental value, are also important therapeutically as they are source of curare compounds against several ailments (Arenmongla and Deb, 2012). Furthermore, herbs and herbal products are still an important part of the primary healthcare system in many parts of the world (Jawahar *et al*., 2008a; Pathak *et al.,* 2010).

Crepidium acuminatum (D.Don) Szlach. is a small sized terrestrial orchid, up to 20 cm in length, with pseudobulbous stem covered with old leafy scale at the base. Flowers are pale green to tinged purple, shortly stalked, 1-1.5 cm across with linear and minute bracts. *C. acuminatum* is highly exploited and critically endangered species (CITES, 2012, 2013) because of its extensive use in *Ayurvedic* preparations like *Ashtavarga, Jivinya Verga, Madura Verga*. It is also an ingredient of *Chyvanprash,* which is used as health tonic, blood purifier, and also as an antioxidant. It is one of the ingredients of Ashtvarga (combination of eight drugs) which is one of the core parts of the *Ayurveda* (Pathak *et al.,* 2010; Sharma *et al.,* 2011). The rate of natural propagation of this species in nature is very low (0.2-0.3%) as the seeds require a specific mycorrhizal fungus for their germination, in nature. As this species is medicinally very important, its natural populations are decreasing at an alarming rate due to ruthless collections and over-exploitation and hence this species has become rare in its natural habitats. Keeping these in mind, there is an urgent need to devise an efficient mass propagation protocol for its rapid multiplication and to save its natural population from getting extinct. Thus, *in vitro* propagation strategies are alternative methods for germination of orchid seeds which is influenced by the various factors like light and temperature conditions, developmental stages of explants, plant growth regulators (PGRs) *etc*. (Arenmongla and Deb, 2012). The rapid and efficient *in vitro* propagation of orchids can be done by using various explants including seeds (immature and mature), leaf, root, inflorescence segments, and stem nodal explants (Anuprabha *et al*., 2017; Arora *et al.,* 2016; Bhatti *et al.,* 2017; Decruse and Gangaprasad, 2018; Lekshmi and Decruse, 2018; Kaur *et al.,* 2017; Mahant, 1991; Mohanty and Salam, 2017; Pathak and Vij, 2001;

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Pathak *et al*., 2011, 2012, 2017; Sibin and Gangaprasad, 2016, Vij *et al.,* 1986, 1997). Cheruvathur *et al.* (2010) developed an efficient *in vitro* multiple shoot induction system of *C. acuminatum* using internodal explants sourced from sterile primary cultures. Kaur and Bhutani, (2009) also tested the regeneration potential of pseudobulb segments of *C. acuminatum* with different growth adjuncts. Arenmongla and Deb (2012) and Deb and Arenmongla (2013) evaluated the effects of illumination, sucrose concentration and quantity of growth regulators, developmental stages of immature embryos and successfully propagated this species by using leaf explants from *in vitro* raised plantlets. Vij *et al.* (1986) observed the *in vitro* breakdown of apical dormancy and development of vegetative shoots from inflorescence segments in *Saccolabium calceolare* Lindl. and subsequently they (1997) assessed the regeneration response of *Rhynchostylis gigantea* inflorescence segments. The goal of this investigation was to explore the possibility of regeneration from immature unopened flower buds of *C. acuminatum* as an explant source for tissue culture, to assess the maximum frequency of induction of shoots and plantlet formation and analyze the nutritional requirements. The protocol reported here is simple, easy and will help in conserving this medicinal endangered orchid.

Material and Methods

Plant Material

Pseudobulbs of *C. acuminatum* were collected from Summerhill (Distt. Shimla, Himachal Pradesh) during the months of April-May. These pseudobulbs were potted in clay pots containing sand, vermiculite, and cattle manure in 1:1:1 and kept in the Orchidarium, Department of Botany, Panjab University, Chandigarh for further experimentations. When inflorescence appeared, young unopened flower buds (2-4 mm) at various developmental stages were excised and used for *in vitro* culturing.

Surface Sterilization

The excised floral buds were washed under running tap water for 15 min to remove dust particles and other contaminants. Then these were treated with Teepol detergent for 2-3 min and washed with distilled water. Explants were surface sterilized under laminar airflow with Bavistin (Fisher Scientific) (0.01 mgl^{-1}) for 8-10 min, Streptomycin (Ambistryn®-S) (0.01 mgl^{-1}) for 5-8 min, and Mercuric Chloride (Fisher Scientific) (0.1 mgl^{-1}) for 2-3 min and subsequently rinsed 3-5 times with autoclaved distilled water.

Table 1. Regeneration potential of floral buds explants on M (Mitra *et al*., 1976) medium in *Crepidium acuminatum*.

Growth additives	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 of cycle	Remarks
-	-	-	-	-	-	Browning of explant
IAA_1	17.75±0.85[a]	1.00±0.00[a]	Shoot bud	-	-	Callusing occurred at the base of shoot bud and turned brown after 8 days and perished
BAP_1	21.75±0.25[b]	-	-	-	-	Opening of bud into flower; perished after 90 days
KN_1	25.75±0.25[c]	1.00±0.00[a]	Shoot bud	-	-	-
TDZ_1	50.75±0.25[g]	1.25±0.25[a]	Callus	-	-	-
IAA_1+TDZ_1	31.00±0.40[d]	1.00±0.00[a]	Callus	-	-	-
IAA_1+KN_1	16.75±0.47[a]	1.00±0.00[a]	Shoot bud	9.25±0.47[a]	151.25±0.62[a]	8-10 pseudobulbs were formed
IAA_1+BAP_1	33.25±0.25[e]	1.00±0.00[a]	Shoot bud	-	-	-
NAA_1	34.75±0.47[e]	1.00±0.00[a]	Shoot bud	-	-	-
AC	41.75±0.25[f]	-	-	-	-	Opening of buds into flower; perished after 40 days

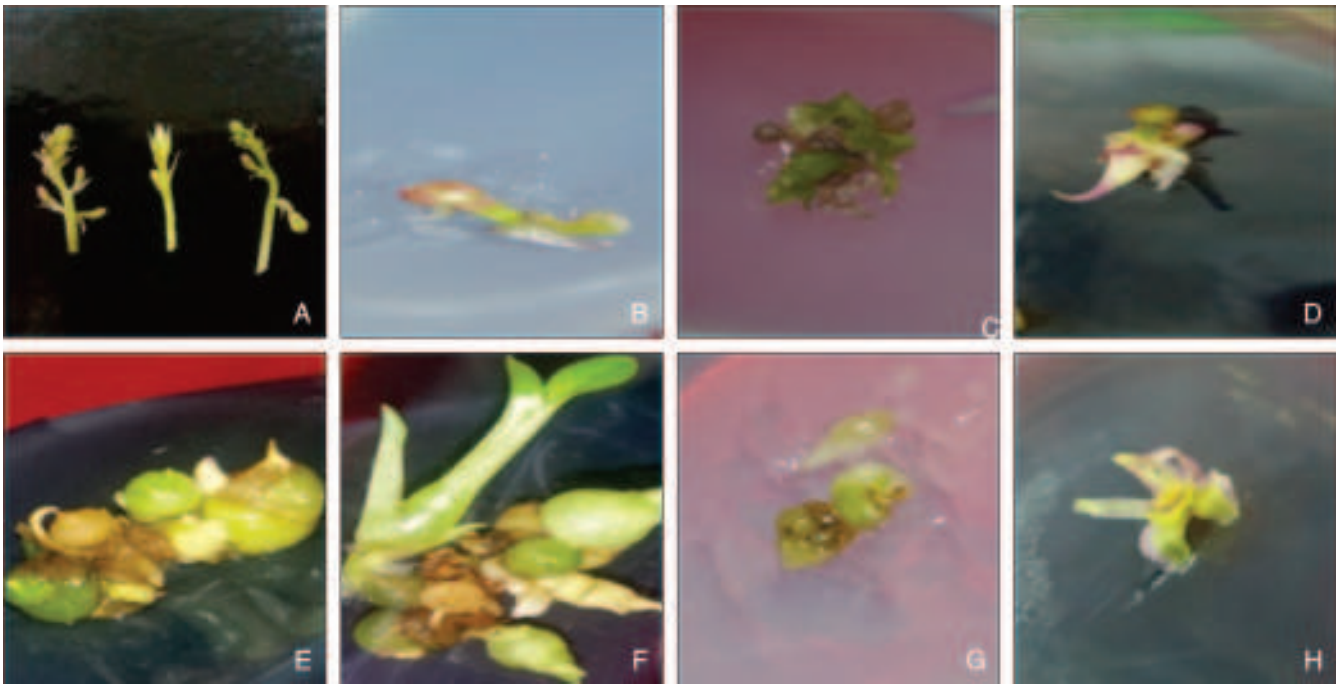

Fig 1. A-H. Induction of shoots and regeneration of plantlets of *Crepidium acuminatum* (D.Don) Szlach. from floral buds: A, Immature floral buds at the time of inoculation; B-D, Regeneration via shoot buds on the basal end of floral bud [M+IAA(1 mgl^{-1})+KN(1 mgl^{-1})]; E-F, Shoot buds developed into multiple pseudobulbous shoots and their further development into plantlets [M+IAA(1 mgl^{-1})+KN(1 mgl^{-1})]; G, Callus induction [M+TDZ (1 mgl^{-1})]; H, Opening of floral bud into complete flower (M+AC).

Culture Conditions

The explants were placed horizontally on M (Mitra *et al*., 1976) medium which was supplemented with different growth regulators like auxins: Indole-3-Acetic Acid (IAA), 1-Naphthalene acetic acid (NAA); and cytokinins: Kinetin (KN), Thidiazuron (TDZ), 6- Benzylaminopurine (BAP) (1mgl^{-1} each) singly or in combinations. Nutrient medium was solidified with 8.5 gml^{-1} agar and pH of the media was adjusted to 5.6 to 5.8 with NaOH and HCl prior to autoclaving (15-20 min) at 121^{0}C temperature and 15 psi pressure. Sucrose (2%) and Activated charcoal (AC, 2 gl^{-1}) was also added. The cultures were incubated at 25±2^{0}C temperature with 3500 lux light intensity.

Acclimatization of Regenerants

Healthy plantlets with 2-3 well grown leaves and 1-2 roots were gradually hardened *in vitro*, by sequential elimination of growth additives, vitamins, sucrose, and minor salts from nutrient matrix at 15 days interval. The hardened seedlings were washed thoroughly with lukewarm water to remove agar and potted in clay pots containing sand, vermiculite, and cattle manure in 1:1:1.

Experimental Design

Experiments were performed in a randomized design and repeated twice. Each treatment had four replicates and each replicate comprised 2 explants. Morphogenetic response from floral bud explants was evaluated after regular interval of 3 days. Average number of days for initiation response, number of meristematic loci, plantlets obtained, and completion of cycle were also recorded. These data were statistically analyzed using one way ANOVA and means were compared using Tukey's honest significance test by Statistical Package for the Social Science (SPSS) 16.0 Version.

Results and Discussion

In basal medium, floral bud explants failed to initiate any regeneration response. These, however, showed morphogenetic response in the presence of medium supplemented with growth regulators (Table 1). AC (2 $gl^{-1)}$ and BAP (1 mgl^{-1}) when used alone, proved ineffective for inducing regeneration and the explants followed their own natural pathway of opening of flowers after swelling; these, however, perished after 40 days and 90 days respectively (Fig. 1 H). When IAA (1 mgl^{-1}) was added in the basal medium, the explants regenerated via shoot bud formation at the base in 17.75±0.85 days, the shoot buds, however, failed to show further development. Addition of KN in combination with IAA (1 mgl^{-1} each) enhanced the regeneration response to cent per cent and completion of life cycle took place in 151.25±0.62 days (Table 1); 8 to 10 pseudobulbous shoots were formed which

subsequently developed into complete plantlets (Fig. 1 E-F). In medium containing NAA (1 mgl^{-1}), initial response of explants was delayed (34.75±0.47 days); but incorporation of KN (1 mgl^{-1}) into the basal medium, proved effective for early shoot bud initiation (25.75±0.25 days) as compared to NAA (1 mgl^{-1}). Earlier, deleterious effects of higher level of NAA were reported in *Rhynchostylis retusa* by Vij *et al*., (1981). TDZ (1 mgl^{-1}) induced regeneration via callus formation, whereas addition of IAA (1 mgl^{-1}) in combination proved beneficial for early regeneration response via shoot bud formation. Shadang *et al.* (2009) studied young inflorescence (3-5 cm) procured from *in vivo* grown plants of *Hygrochilus parishii* on VW and modified KC medium. Callus prior to regenerating PLBs was observed. Goh and Wong (1990) used inflorescence explants from *in vivo* grown plantlets of *Aranda* Deborah on liquid KC medium and observed formation of PLBs.

Sawa and Hara (1973) made attempt to culture *Cymbidium goeringii* inflorescence tissue and first floret explants were successful in showing swelling growth followed by callus formation; both cytokinins and Auxins, however, had limited effect on induction of rhizome development and subsequent plantlet regeneration. Presently, the use of PGRs was found to be obligatory for inducing regeneration response in the *in vitro* sourced explants. Vij *et al.* (1991) studied floral stalks procured from *in vitro* grown plantlets of *Dendrobium crepidatum* and *D. pierardii.* It was observed that growth additives proved obligatory for vegetative transformation of floral buds. The explants acquired meristematic activity along the cut ends with each meristemoid subsequently developing into shoot buds. Kaur and Vij (1995) examined the inflorescence segments (0.4-0.6 cm) procured from *in vivo* grown plantlets of *Rhynchostylis retusa* on VW medium, leafy shoots were directly developed in NAA and BAP (1 mgl^{-1} each). The role of growth additives in invoking meristematic activity and promoting proliferation in the explants during regeneration is well documented in orchid literature (Abdul Karim and Hairani, 1990; Nayak *et al.,* 1997; Temjensangba and Deb, 2005; Vij *et al.,* 1986, 1994, 1997). Martin *et al.* (2005) used floral stalks (10-15 mm) procured from *in vivo* grown plants of *Dendrobium* hybrids Sonia 17 and 28 on half and full strength MS medium with different PGRs like NAA, BAP, KN, and CW (15%) evoked bud break. KN showed better growth of the initiated buds. Medium with KN (6.97 µm) showed conversion of more than 90% PLBs to shoots. In present study, the meristematic activity in the explants was restricted mostly to the basal end. Similar observations were made earlier in *Paphiopedilum* Armani white, where explants only from the base tissue group of the large floral buds produced shoots or plantlets (Liao *et al.,* 2011). Wangner *et al.* (2007) worked on *Doritaenopsis* Purple Gem 'Chin Hua' immature apical flower buds (5.0 mm), immature lateral buds (5.0 mm), flower stem nodes (10-20 mm) and flower pedicel sections (5-10 cm) procured from *in vivo* grown plants were inoculated on MS medium. Immature apical flower buds were the only explants that showed induction and multiplication of shoots *in vitro*. Earlier, inflorescence explants were also successfully for regeneration purposes in *Rhynchostylis gigantea* (Vij *et al.,* 1997) and *Saccolabium calceolare* (Vij *et al.,* 1986). During the present investigation, M medium supplemented with IAA and KN (1 mgl^{-1}) proved optimal for inducing regeneration in the floral bud explants, multiple shoot production, and subsequent complete plantlet development in *C. acuminatum*.

Conclusion

Present results indicate that floral buds can be effectively utilized for orchid micropropagation. The efficacy of M medium in supporting high germination response is further confirmed, as it supported growth in selected combinations of plant growth regulators. PGR's can be effectively utilized for invoking regeneration response in floral bud explants but their requirements are very specific and these vary during initiation, multiplication, and differentiation of cultures. The technique of floral bud culture has significantly shortened the time for the production of the plantlets. Data indicates that utility of such explants may be explored in many other related orchid taxa for *in vitro* propagation purposes.

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