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Effect of Copper Sulphate ($CuSO_4.5H_2O$) and Pre-cold Treatment on Labellum Explants in Endangered Orchid Taxa

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ABSTRACT

The regenerative competence in Labellum (petal) explants in *Coelogyne ovalis* and *Vanda cristata* depends significantly on physiological stage of donor tissue, pre-cold treatment, genotype and medium components. Juvenility of tissue emerged as the major factor controlling the activation of proliferative loci in labellum explants. The efficacy of PGRs in regeneration is species specific; Kn was effective in *C. ovalis* cultures and BAP in those of *V. cristata*. The maximum callus induction and growth in labellum explants was observed on medium supplemented with cytokinin to auxin in ratio of 10:1. The regenerated plantlets were acclimatized and transferred to pots filled with moss, pinebark, brick and charcoal pieces mixture with 90% survival.

INTRODUCTION

Application of tissue culture techniques has added new dimensions to the propagation and commercialization of floriculturally significant plants. It is particularly useful in outbreeders like orchids which generate a great deal of heterozygosity in the progenies. Resident meristem (apical, axillary) are frequently used as explant tissues for micropropagating orchids. Several workers have reported induction of callus, Plbs (Protocorm-like bodies) and whole plants from shoot meristem (Roy et al. 2007, Pant & Thapa 2012, Haque & Ghosh 2013), foliar culture (Park et al. 2002, Deb & Pongener 2013), root culture (Park et al. 2003, Deb & Pongener 2012), young inflorescence stalk (Chen & Chang, 2000, Martin et al. 2005, Sinha et al. 2007) intact floral bud (Lim Ho et al. 1984, Liao et al. 2011), but attempts to assess a similar competence of labellum (petal), have remained almost negligible (cf. Arditti & Ernst 2008). In this paper, we report the possibility of using labellum for initiating *in vitro* cultures of *Coelogyne ovalis* and *Vanda cristata*. *C. ovalis* commonly known as Jivanti is used in ayurvedic formulations for its aphrodisiac properties (Lawler 1984, Pant 2013), whereas, *V. cristata* has been used as breeding material for raising floriculturally significant hybrids. Besides being victim of its own beauty and utility, both are progressively losing its natural habitat and heading towards extinction in absence of suitable remedial measures.

MATERIALS AND METHODS

C. ovalis and *V. cristata* plants were collected in nature from Garhwal Himalayas eastwards to Arunachal Pradesh (1000-1800m) and grown under greenhouse conditions at Punjab University, Chandigarh. The twigs with young inflorescence harvested from stock plants were used as material for the present study. The unopened floral buds were harvested and stored overnight at 4°C. Subsequently floral buds were squentially surface sterilized with solutions of streptomycin (0.1%, 20 min), sodium hypochlorite (4%, 15 min) and dip in ethanol (70%, 3 sec) before rinsing with sterilized distilled water. To obtain labellum explants, the sepal and outermost petals were removed from the bud; and the remaining petals were then placed with the abaxial side on sucrose (2%) supplemented and agar (0.9%) gelled (Sharma 2012) (SM) medium and its various combinations with NAA (α-naphthalene acetic acid), BAP (6-benzyl amino purine) and KN (Kinetin).

Culture media: Effects of medium composition on callus induction were studied in the present work. On the basis of the preliminary experimental results, a refined experiment was designed, where Murashige & Skoog (1962) medium and Mitra et al. (1976) medium, were compared with modified (Mitra et al. 1976 and Sharma 2012) medium. It is clear that modified (Mitra et al. 1976 and Sharma 2012) medium was more conducive to the induction of callus than Murashige & Skoog (1962) medium and Mitra et al. (1976) medium when supplemented with the BAP, Kn and NAA. The composition of medium is one of the important factors determining not only the success of labellum culture, but also the mode of development. Since explants failed to regenerate despite repeated subcultures on different media,

Table 1: Effect of pre-cold treatment on callus induction of labellum explants.

Pre-cold Treatment time	Callus formation rate(%) *C. ovalis*	*V. cristata*	Remarks
0 hrs	-	-	Labellum failed to respond and become necrotic.
8 hrs	-	-	Labellum failed to respond and become necrotic.
24 hrs	62.5%	50%	Compact, warty, acholophyllous and organogenetic callus is formed.
48 hrs	-	-	Labellum become necrotic within one week.
72 hrs	-	-	Labellum failed to respond and become necrotic.

Table 2: Number of plantlets per callus in response to PGRS in labellum (petal) explants.

(i) *C. ovalis*			
Growth regulators applied to SM medium	Concentrations of growth regulators (mg L^{-1})	Calli producing shoots (%)	No. of plantlet obtained/explant
BA	1	-	-
	2	-	-
Kn	3	-	-
	5	-	-
Kn+NAA	1:1	-	-
Kn+NAA	5:5	50	40
Kn+NAA	10:5	62.5	60
(ii) *V. cristata*			
Growth regulators applied to SM medium	Concentrations of growth regulators (mg L^{-1})	Calli producing shoots (%)	No. of plantlet obtained/explant
BA	1	-	-
	2	-	-
Kn	3	-	-
	5	-	-
BAP+NAA	1:1	-	-
BAP+NAA	5:5	50 ±12.5	30
BAP+NAA	10:5	75	70

the effect of copper sulphate ($CuSO_4.5H_2O$) on the labellum regenerative competence, was tested by varying the $CuSO_4.5H_2O$ level (0.08, 0.20, 0.80, 1.2, 1.8 and 2.2 mg/L) in the media. The concentration (2.2 mg/L) in the medium favours regeneration in labellum explants. 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 were used and the experiments were repeated four times. All experimental manipulations were done under aseptic conditions and the cultures incubated at 25±2°C under 12 hr photoperiod of 3500 lux light intensity, were regularly observed.

Effect of pre-cold treatment on the callus formation from labellum: The pre-treatment of floral buds with low temperature is essential step for enhancing the ratio of responding labellum explants in the present studies (Table 1). Thus, maintaining floral buds for periods ranging from 24-48 hrs at 4°C prior to labellum culture stimulates the regeneration in both species. Analysis of variance of the callus induction rate indicated that callus formation was significantly affected by pre-cold treatment and callus formation rate of both species increased after labellum pre-treated under 4°C condition for 24 hrs. On the contrary, when cold treatment (4°C) was 0 hrs, 8 hrs, 48 hrs and 78 hrs either no labellum formed callus or became necrotic within one week.

For assessing the cytological status of the regenerants, the root tips were pre-treated in saturated aqueous solution of 8-hydroxy quinoline (3 hrs), fixed in Carnoy's liquid (24 hrs) and stored in 70% alcohol, until use. These were sequentially hydrolysed for 8-10 min in HCL at 60°C, washed thoroughly with water and stained with feulgen nuclear reaction before squashing in 2% acetocarmine solution.

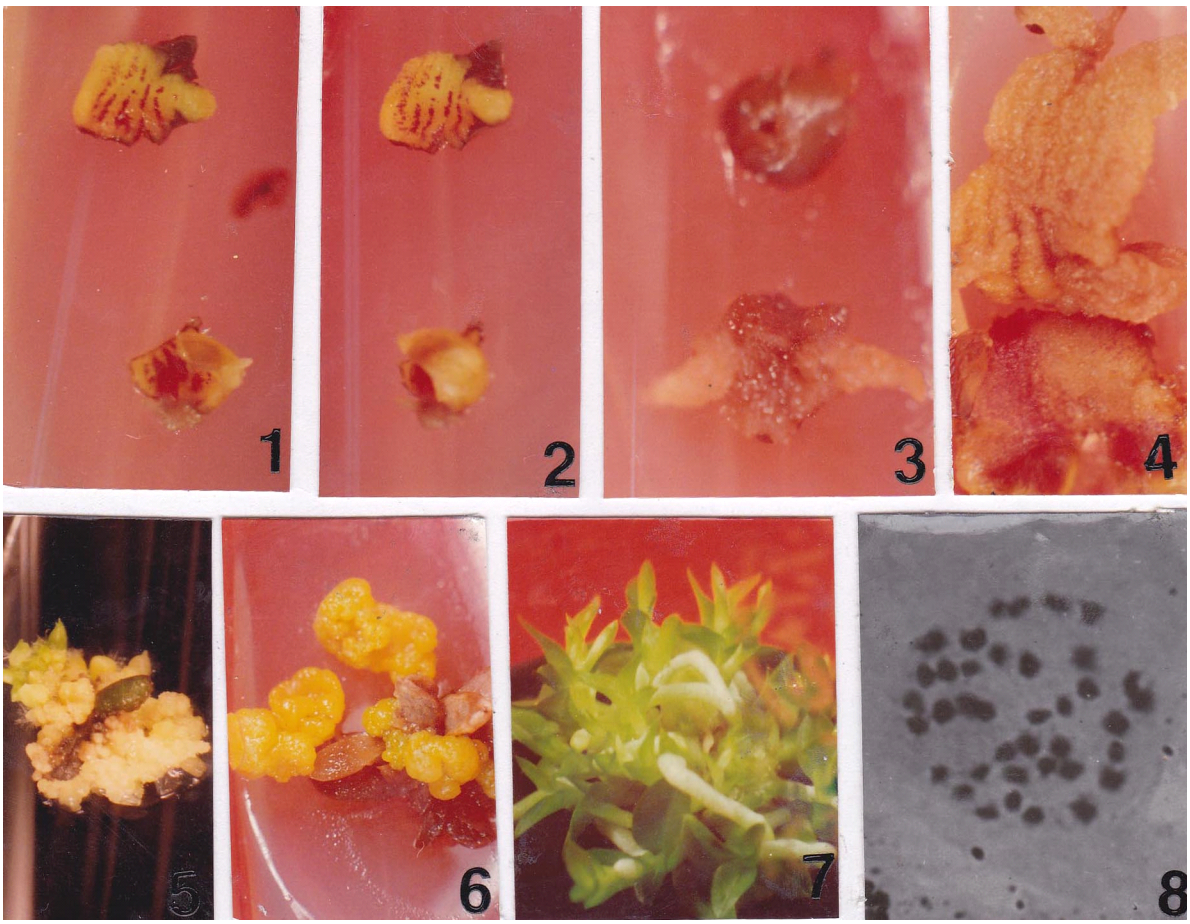

Fig. 1: 1- Labellum culture (*V. cristata*) in SM+BAP (5 mgL^{-1}) + NAA (5 mgL^{-1}); 2- Swelling in the lower labellum explant; 3- Two lateral protuberances in SM+BAP (10 mgL^{-1}) + NAA (5 mgL^{-1}); 4- Labellum culture (*C. ovalis*) in SM+Kn (10 mgL^{-1}) + NAA (5 mgL^{-1}); 5, 6- Differentiation in callus in SM+NAA (0.5 mgL^{-1}) + Sucrose (0.1%); 7- Complete plantlet; 8- Cytological study of *V. cristata* (2n=38)

ACCLIMATIZATION OF THE PLANTLET

After well-developed shoot and root formation, the plantlets (3 cm tall) were transferred to semisolid medium containing only half strength macro and micro salts of Sharma et al. (2012) medium; sucrose and vitamins were eliminated. The plantlets were kept in this condition until they were 4-5 cm tall (Fig. 1.7), and washed with lukewarm water before transferring 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 regenerative competence of the labellum explants seems to markedly influenced by maturity level of the tissues, pre-cold treatment and growth stimulus in the nutrient pool. The labellum of *V. cristata* and *C. ovalis* from open flowers (2 days of anthesis) remained recalcitrant despite variations in chemical regime, whereas those from buds (14 days prior to anthesis) responded and the cell proliferations were obligatory to a combined treatment of cytokinin (BAP/Kn) and NAA in modified medium (Mitra et al. 1976, Sharma 2012).

In *C. ovalis,* 62.5% explants responded to a treatment with Kn (10 mg L^{-1}) and NAA (5 mg L^{-1}) by callusing all along the surface (Fig. 1.1). However, in *V. cristata* maximum regeneration (75%) occurs in medium containing 10 mg L^{-1} BAP and 5 mg L^{-1} NAA. A lower dose of BAP (5 mg

L^{-1}) in the latter combination impaired the regeneration to 50±12.5% (Table 2). In both the cultivars, two types of callus are recognized according to colour, texture and time of callus initiation. The callus is hard, fast growing and callus initiation was observed on whole petal explants and somewhat warty in appearance in *C. ovalis*, whereas in *V. cristata*, the explants representing proximal regions of labellum regenerated, each responding developed two lateral protuberances which developed into callus. The callus was achlophyllous, compact and irregular in shape (Figs. 1.2-1.4). Differentiation eluded the neo-formation unless removed from cytokinin influence and treated with 0.5 mg/L NAA under a low osmoticum (0.1%) in a nutrient pool (Figs. 1.5-1.6). Plantlet complete with 2-3 leaves and 1-2 roots were harvested after 15 wks of subculture in both the cases. Since regenerants were true to their mother plant in chromosome number (2n=38), the utility of labellum explants in micropropagating *C. cristata* and *V. cristata* is acclaimed (Fig. 1.8).

DISCUSSION

Juvenility of the tissue emerged as the major factor controlling the activation of proliferative loci in labellum explants due to the fact that younger tissues with less rigid cell walls are physiologically and biochemically more active showing better morphogenetic potential as has already been demonstrated in several plant groups including orchids (Misra & Bhatnagar 1995, Kumar & Kanwar 2006, Niknejad et al. 2011).

The labellum from open flowers failed to respond due to enhanced production of ethylene in the flower from anthesis onwards (Zuping & Wei 1994, Tous et al. 2000, Sharma 2012), as ethylene is inhibitory to regeneration (Chae & Park 2012, Dong et al. 2012, Park et al. 2012, Silva 2013). In present studies, the efficacy of PGRs in regeneration is species specific due to the variation in genotype (Kumar & Kanwar 2006). The efficacy of BAP, Kn and NAA in the present studies is in accordance with similar reports in *Vanda cristata* (Vij & Sharma 1996), *Cymbidium* hybrid, Chrysanthemum, Tuberose, Carnation, *Oncidium* (Kim & Kako 1984, Nahid et al. 2007, Karami 2008, Kadam et al. 2010, Thakur & Dongarwar 2013). However, in the present study, higher organogenetic responses are observed in explants when BAP, Kn was used in dose double than that of NAA in compliance to earlier reports (Nahid et al. 2007, Mohanty et al. 2012, Ramar & Ayyadurai 2015).

The beneficial and promotary effects of elevated concentrations of copper on *in vitro* cultures have been emphasized in several dicots and monocot plants (Purnhauser & Gyulai 1993, Sharma & Vij 1997, Joshi & Kothari 2007, Kaul et al. 2010, Kowalska et al. 2012, Silva 2013, AL-Mayahi 2014, Makowska et al. 2017, Sarrapoulou & Maloupa 2017). Since Cu^{+2} is a component and/or factor of many important enzymes involved in electron transport, and protein and carbohydrate biosynthesis. In this connection, it is worthwhile to mention that zygograms of certain enzymes are considered as useful markers of morphogenesis in tissue raised cultures.

Application of pre-cold treatment has become an essential measure to increase the efficiency of floral parts in regeneration in accordance with earlier reports (Vij & Sharma 1996, Datta 2005, Herath et al. 2009, Arjunappa et al. 2016, El Goumi et al. 2017). The ability of callus to organogenerate under low sucrose environment conform to the earlier studies (Meir et al. 1985, Ichihashi et al. 1986, Javed & Ikram 2008, Kamle & Baek 2017). The plants raised were cytologically stable as reported earlier by Haque & Ghosh (2013).

CONCLUSION

Juvenility of the tissue, pre-cold treatment and increased concentrations of copper had a positive effect on the regeneration frequency. The growth adjuncts are obligatory to regeneration and the efficacy, but vary with their quality and quantity in the nutrient pool. A negative correlation between osmotic stress of the medium and the organogenetic changes true to type and cytologically stable plants are raised in labellum explants. The study contributes to the conservation of endangered orchid species, and this *in vitro* mass propagation protocol provides material for future pharmacological and biotechnological studies.

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