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In vitro Axillary Shoot Regeneration and Direct Protocorm-like Body Induction from Axenic Shoot Tips of *Doritis pulcherrima* Lindl.

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Abstract

An efficient protocol for *in vitro* propagation of an important ornamental terrestrial orchid, *Doritis pulcherima* Lindl. through axillary shoot and direct protocorm-like body (PLB) formation from shoot tip explants derived from six-month-old axenic seedlings has been described. Shoot tips were cultured on modified nutrient medium of Knudson's C supplemented with 0.1% peptone and combination of various concentrations of NAA and BAP. The effect of NAA and BAP on axillary shoot formation, protocorm-like body induction and root regeneration from the explants was significant. The highest frequency of axillary shoot formation was recorded in the medium containing 2 mg/l BAP and the PLB production was higher in the medium containing 2 mg/l NAA. A higher concentration of BAP showed inhibitory effects on the axillary shoot formation and PLB induction. Efficient root regeneration was observed in low concentration of NAA. However, the profuse root formation was common in the PGR free medium. Rooted plantlets were hardened successfully through the stepwise acclimation protocol and platelets were finally established in the potting mixture containing small pieces of dead tree bark of mango, charcoal pieces and broken bricks in 1 : 2 : 1 ratio.

Introduction

Doritis pulcherrima Lindl. is a sympodial, terrrestrial orchid belonging to the family Orchidaceae, subfamily Epidendroideae, and tribe Vandeae. The genus *Doritis* has a wide distribution, ranging from the Himalayas of Northern India, through Thailand and Burma, to Malaysia and Indonesia (Treuscher 1977). It

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bears beautiful flowers and is a valuable material for the orchid hybridization. *D. pulcherrima*, synonymous with *Phalaenopsis pulcherrima* (Lindl.) J. J. Smith (Sweet 1980, Christenson 1995), has been used extensively in breeding with *Phalaenopsis* to produce floriculturally important hybrid *Doritaenopsis* superior cultivars. Although hybridization has long been practiced with this plant species for breeding superior cultivars, available information about the micropropagation of *Doritis* is relatively meager.

Orchid requires a combination of multiplicity of factor for the continued reproduction in nature. The propagation of orchids through sexual means is a very slow process as their seeds lack endosperm and require the fungal association for germination in nature. The fungus is believed to augment the carbohydrate, auxin and vitamin transport in the orchid (Arditti *et al.* 1982). Orchids are highly heterozygous and their vegetative propagation through division of clumps of rhizomes, bulbs or by the rooting of off shoots requires a long time. Moreover, it is extremely difficult to generate the desired number of plants. It is, therefore, critical to take initiative for ~~the~~ mass propagation of orchids and subsequently their successful establishment in nature. Thus, tissue culture technique is considered as a potent alternative method for mass scale propagation of these orchids.

Shoot tip culture in sympodial orchids is an efficient system for the production of large numbers of plantlets in a short period of time (Chug et al. 2009). Propagation of orchids through shoot tip culture has been successful in *Anacamptis*, *Anoectochilus*, *Arudina*, *Cymbidium*, *Dendrobium*, *Paphiopedilum*, *Phaius*, *Phalaenopsis*, *Vanda* and *Vanilla* (Morel 1970, Huang 1988, Nagaraju and Parthasarathy 1995, Devi et al. 1997, Seeni and Latha (2000), Roy and Banerjee (2003), Subramanium and Taha (2003), Ket et al. (2004), Kalimuthu et al. (2006), Roy et al. (2007). A few genera such as *Vanda* (Seeni and Latha 2000), *Cymbidium* (Subramaniam and Taha 2003), and *Dendrobium* (Roy et al. 2007) are reported to be the best orchids in inducing PLBs by shoot tip culture. The aim of the present study is to develop a suitable protocol for micropropagation of *D. pulcherrima* from shoot tips and to elucidate the impact of exogenous cytokinin and auxin on the rate of shoot elongation, axillary branching, PLB formation and root induction.

Materials and Methods

The material used ~~for~~ in the present investigation was small axenic shoot tips of 2 - 4 mm length from six-month-old seedlings that have been raised *in vitro* through asymbiotic seed culture of *D. pulcherrima*.

All inorganic macro- and micro-salts, iron-EDTA, sucrose, and potassium iodide were procured from Sisco Research Laboratory (SRL), Mumbai, India. The solidifying agent agar and peptone were purchased from Merck Ltd., Mumbai, India. BAP and NAA were procured from Sigma Chemicals Co., St Louis, USA.

In the present investigation, the culture medium used was modified nutrient solution of Knudson's C (1946) supplemented with or without various concentrations and combinations of BAP and NAA to assess the effect of PGR on PLB formation, shoot multiplication and rooting of *D. pulcherimma* Lindl. The medium was supplemented with 2% sucrose and 0.1% peptone. The original iron source was replaced with iron-EDTA of MS and the media were solidified with 0.8% w/v agar. The pH of the media was adjusted to 5.2 prior to autoclaving at 121°C for 20 min.

For culture initiation, 2 - 4 mm long shoot tips were excised aseptically and transferred to Knudson's C (KC) basal media and also on to KC media supplemented with various concentrations and combinations of BAP (1.0 - 4.0 mg/l) and NAA (1.0 and 2.0 mg/l). The experiment was planned with a total 12 treatments having five replicates for each with ten explants for each replica.

After inoculation, the cultures were incubated at 25 ± 2°C with 14 hrs photoperiod of 3000 lux light intensity. The observations were recorded at regular intervals of one week up to the eighth weeks.

After three - four weeks of culture initiation, shoot tips exhibited either axillary shoot formation or adventitious PLB formation without callusing. In histological studies hand sections through suitable explants were made, mounted in 10% glycerin solution and observed under a binocular microscope Olympus CH-30. Rootless axillary shoots developed in all the tested media except in KC with NAA 2.0 mg/l and BAP 4 mg/l. Later rooting was achieved in the treatments devoid of BAP. In general, PLB production was observed in all the treatments.

For the induction of root**s,** regenerated single shoots were cultured both on KC + peptone and KC media supplemented with low concentrations of NAA (0.5 - 1 mg/l). The cultures were maintained for induction and the ramification of roots.

The regenerated rooted plantlets were further subjected to *ex vitro* hardening. The plantlets were taken out from the culture vials and were washed thoroughly under running tap water to remove the traces of adhering agar. Those were then treated with 0.1% (w/v) bavistin (fungicide) solution and again washed with water. The rooted plantlets were transferred to the potting mixture containing small pieces of dead tree bark (mango), charcoal pieces and broken

bricks in 1 : 2 : 1 ratio. The plants were totally covered with plastic bags initially to maintain humidity. Plants became acclimated to a reduced relative humidity by gradually removing the plastic cover and then, were completely uncovered and finally hardened to greenhouse conditions.

Somatic chromosomes were studied from actively growing root tips. Excised root tips were split longitudinally and the root caps and velamen tissues were carefully removed with a sharp scalpel. Root tips were pretreated with a mixture of saturated solution of para-dichloro benzene (PDB) and 2 m M 8-hydroxy-quinoline (1 : 1) for 4 h at 18ºC, fixed in modified Carnoy's fluid (ethanol: chloroform: glacial acetic = 2 : 1 : 1) at 4ºC for 24 h, hydrolyzed in 1 (N) HCl for 10 min at 60ºC, stained in 2% aceto-orcein solution for 2 h and finally squashed in 45 % acetic acid.

All observed data were analyzed by one-way ANOVA test, followed by the Duncan's multiple range comparison at 0.05 significance level using SPSS (version 16) software package. Statistical analyses were performed on the arcsine transformed values of the data presented in Tables 1 and 2.

Results and Discussion

In the present investigation, two plant growth regulators were used either individually or in various combinations to fortify KC basal media for culturing shoot tips of *D. pulcherrima.* Depending upon the chemical composition of the media, cultured shoot tips followed two different pathways of growth and morphogenesis. In the first route, multiple shoots regenerated on explant surface through direct axillary shoot bud proliferation; and in the second pathway plantlets originated via PLB formation. In most of the cases, responses were directly observed from the meristematic dome of the cultured shoot tips. Formation of axillary shoots (Fig. 1A) and direct PLBs (Fig. 1B) from cultured shoot tips were initiated after 15 - 30 days which proliferated further for another three - four weeks.

Initially all explants swelled with no intervening callus formation. Necrosis of the explants was commonly observed in all the treatments in varying amounts. The frequency of occurrence of necrosis in the explants was high in the medium supplemented with NAA (Table 2). Addition of increasing concentrations of BAP either individually or in combination with NAA in the medium lowered the rate of necrosis. Tissue blackening and necrosis of the explants have been frequently encountered in this study; necrosis might have caused by the oxidation of phenolic compounds by polyphenol oxidase present in the explants. It has been reported earlier that the addition of organic acids and frequent subculturing of the propagules exhibited satisfactory result (Morel 1974,

Table 1. Effects of NAA and BAP in varying concentrations on *in vitro* response of shoot tips after 60 days of culture.

Culture media	Plant growth regulators (mg/l)		Axillary shoot forming explants (%)	No. of shoots/ responding explants	PLB forming explants (%)	No. of PLB per responding explants
	NAA	BAP				
N0B0	-	-	12.82 ± 9.2^{abc}	0.92 ± 0.5^{ab}	16.67 ± 2.4^{bc}	1.23 ± 0.1^{abc}
N0B1	-	1	39.68 ± 8.8^{c}	1 ± 0^{b}	7.69 ± 4.4^{abc}	0.67 ± 0.3^{ab}
N0B2	-	2	45.48 ± 13.6^{c}	1.47 ± 0.3^{b}	7.51 ± 4.4^{ab}	0.67 ± 0.3^{ab}
N0B4	-	4	11.43 ± 5.9^{abc}	0.67 ± 0.3^{ab}	2.38 ± 2.3^{a}	0.33 ± 0.3^{a}
N1B0	1	-	19.44 ± 10^{abc}	0.67 ± 0.3^{ab}	19.23 ± 8.4^{bc}	7.33 ± 3.2^{d}
N1B1	1	1	20.97 ± 8.3^{abc}	1.22 ± 0.2^{b}	20.79 ± 7.5^{bc}	3.833 ± 1.6^{cd}
N1B2	1	2	28.04 ± 3.2^{bc}	1.17 ± 0.2^{b}	10.07 ± 2.7^{abc}	1.17 ± 0.2^{abc}
N1B4	1	4	5.56 ± 5.5^{ab}	0.33 ± 0.3^{ab}	4.94 ± 2.5^{ab}	0.67 ± 0.3^{ab}
N2B0	2	-	17.78 ± 9.6^{abc}	0.67 ± 0.3^{ab}	24.44 ± 8^{c}	1.83 ± 0.3^{bc}
N2B1	2	1	15 ± 7.6^{abc}	0.67 ± 0.3^{ab}	7.17 ± 0.3^{abc}	1 ± 0^{ab}
N2B2	2	2	19.17 ± 10.8^{abc}	0.89 ± 0.5^{ab}	15.45 ± 0.7^{bc}	1.67 ± 0.3^{abc}
N2B4	2	4	0 ± 0^{a}	0 ± 0^{a}	4.94 ± 2.5^{ab}	0.67 ± 0.3^{ab}

Data shown are the mean of five replicates. In each column, mean values followed by the same letter are not significantly different at the 0.05 % level (DMRT).

Table 2. Effects of plant growth regulators on *in vitro* rooting of plantlets and necrosis of explants.

Culture media	Plant growth regulators (mg/l)		Root forming plantlets (%)	No. of roots per explant	Explant necrosis (%)
	NAA	BAP			
N0B0	-	-	28.57 ± 4.1^{c}	1.83 ± 0.4^{b}	24.44 ± 4.4^{bc}
N0B1	-	1	0 ± 0^{a}	0 ± 0^{a}	12.82 ± 2.6^{abc}
N0B2	-	2	0 ± 0^{a}	0 ± 0^{a}	7.7 ± 4.4^{a}
N0B4	-	4	0 ± 0^{a}	0 ± 0^{a}	14.29 ± 4.1^{abc}
N1B0	1	-	17.8 ± 6.7^{b}	2.41 ± 0.4^{c}	32.3 ± 12.6^{c}
N1B1	1	1	0 ± 0^{a}	0 ± 0^{a}	20.32 ± 3.6^{bc}
N1B2	1	2	0 ± 0^{a}	0 ± 0^{a}	19.96 ± 2.3^{bc}
N1B4	1	4	0 ± 0^{a}	0 ± 0^{a}	21.16 ± 3.3^{bc}
N2B0	2	-	24.44 ± 2.2^{c}	1.67 ± 0.2^{b}	28.57 ± 4.1^{bc}
N2B1	2	1	0 ± 0^{a}	0 ± 0^{a}	21.32 ± 3.9^{bc}
N2B2	2	2	0 ± 0^{a}	0 ± 0^{a}	24.36 ± 6.2^{bc}
N2B4	2	4	0 ± 0^{a}	0 ± 0^{a}	12.27 ± 2.6^{ab}

Data shown are the mean of five replicates. In each column, mean values followed by the same letter are not significantly different at 0.05% level (DMRT).

Ernst 1994). Specifically in orchids, oxidation of phenolics was reported to account for browning of explants followed by necrosis (Morel 1974, Vendrame and Maguire 2007).

In general, the type, concentration and combination of cytokinins play a key role in *in vitro* propagation of many orchid species (Arditti and Ernst 1993). In the present study, it was clearly observed that reduced concentrations of BAP (1 or 2 mg/l) resulted in the maximum axillary shoot formation (Fig. 1D). Furthermore, this response of BAP varied significantly ($p < 0.05$) from the remaining treatments. However, the basal medium + 0.1% peptone as control failed to produce any significant effect on the axillary shoot formation. Among the different combinations tested in this study, NAA (1 mg/l) and BAP (2 mg/l)

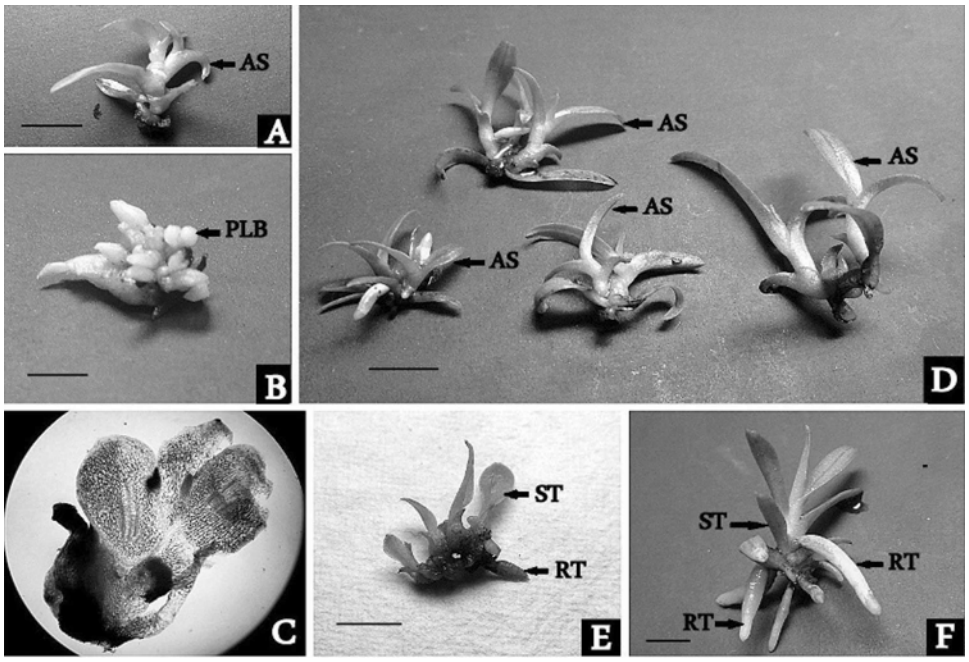

Fig. 1. *In vitro* multiplication and plant regeneration from shoot tips of *Doritis pulcherrima*: A. Initiation of axillary shoot (AS) after 25 - 30 days. B. Profuse multiplication through PLB formation after 20 - 25 days. C. Histological details of PLB formation from shoot tip explants. D. Development of healthy axillary shoots (AS) after 40 - 45 days. E. Differentiation of shoots (St) and roots (Rt) from PLB. F. *In vitro* rooting of the regenerants with well-developed roots (Rt). Bar = 1cm.

were found effective in shoot multiplication. High concentration of BAP and low concentration of NAA favored the induction of multiple shoots in *Dendrobium fimbriatum* (Rajkarnikar and Niraula 1994), in *Cymbidium karnan* (Chung et al. 1998), in *Dendrobium* orchid (Talukdar et al. 2003), in *Aerides odorata*

(Pant and Gurung 2005) and in *Dendrobium transparens* (Sunitibala and Kishor 2009).

On the contrary, increased levels of BAP exhibited suppression of shoot proliferation as well as enhancement of shoot elongation in *D. pulcherrima*. Such findings are in agreement with those reported on the endangered terrestrial orchid *Anoectochilus elatus* (Sherif et al. 2012). The present study also indicated that exogenous auxin along with cytokinin suppressed the apical dominance, which led to the proliferation of axillary shoots. The combined NAA + BAP treatments increased the formation of shoots compared to BAP alone. Thus, the regeneration potential of explants was markedly influenced by their physiological status and chemical stimulus provided by the medium. The quality, quantity and the nature of growth regulators have a significant effect on the regeneration efficiency of shoot tip explants.

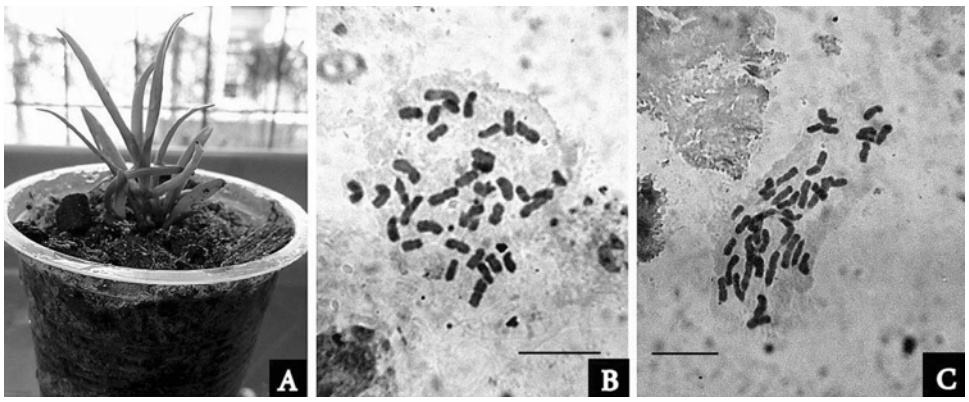

Fig. 2. Plantlets acclimation and its mitotic chromosome complement: A. Establishment of micropropagated plant in hardening mixture; B&C. The normal diploid chromosome number of 2n = 38 at mitotic metaphase in a somatic cell. Bar = 10 μm.

In this study, regeneration of plants via PLB formation was another pathway initiated from the shoot apices. For differentiation of PLBs, some orchid species do not require exogenous PGRs for PLB formation (Chen and Chang 2000, Roy and Banerjee 2003, Huan et al. 2004), while others are PGR-dependent (Lin et al. 2000, Lee and Lee 2003, Lu 2004, Wu et al. 2004). In the present study, even though PLB formation was quite efficient in the PGR free condition, the rate was further enhanced with the addition of BAP and NAA (Table 1). Histological observations of the responding explants showed the developing PLBs with vascular differentiation (Fig. 1C). Highest number of PLBs has been achieved in the medium containing NAA (2 mg/l). However, 1 mg/l NAA either individually or in combination with 1 mg/l BAP showed moderate effect on the frequency of

PLB formation (Table 1). This type of NAA efficacy of regeneration contradicts the previous reports in *Bulbophyllum careyanum* (Vij et al. 2000) and *Dendrobium moschatum* (Kanjilal et al. 1999). Number of PLBs was severely reduced in the medium containing 4 mg/l BAP. It is interesting to note that the frequency of PLB production in the presence of NAA alone was markedly reduced when increased level of BAP was added in this medium. This finding is in agreement with the results of Kalimuthu et al. (2007). In contrast, NAA has more or less no influence on the BAP activity regarding PLB production. PLB undergoes shoot and root differentiation for regeneration of the plantlets (Fig. 1E).

For induction of roots, the *in vitro* generated shootlets of *D. pulcherrima* were harvested and sub-cultured on KC + peptone basal medium as well as on the medium containing low levels of NAA. Regeneration of maximum number of roots was observed in PGR free medium suggesting that the KC + peptone medium alone is sufficient for root induction in this species. The medium containing NAA (1 - 2 mg/l), showed moderate root regeneration. On the contrary, the medium containing BAP either individually or in combination with NAA failed to generate any roots. This observation indicates that BAP has inhibitory effect on root induction. In the rooting medium, no morphological abnormalities, such as irregular leaf shape and color or abnormal root development were observed (Fig. 1F).

Further, somatic chromosome analysis from randomly selected root tips of *in vitro* regenerated plants and donor plants was carried out to assess the chromosomal stability during the *in vitro* multiplication of this species. Root tip cells of both *in vitro* regenerated and donor plants showed 38 chromosomes in somatic cells (Fig. 2B, C). Thus the present study reaffirms the chromosomal stability of the species during *in vitro* propagation.

The *in vitro* generated rooted plantlets of *D. pulcherrima* (Fig. 2A) were successfully hardened on a potting mixture containing small pieces of dead tree bark (mango), charcoal pieces and broken bricks in 1 : 2 : 1 ratio. About 65% of the plantlets survived and initiated new growth. This piece of work suggests that broken charcoal and bricks are favorable material for the establishment of terrestrial orchids such as *D. pulcherrima*.

In summary, the present study describes two efficient morphogenetic pathways of *in vitro* propagation involving PLB formation and multiple axillary shoot regeneration in *D. pulcherrima*, using shoot tips as explants. The direct PLB formation without any intervening callus could be initiated best with 2 mg/l NAA. Hence the application of NAA at 2 mg/l is recommended for optimum PLB production. Second, axillary shoot regeneration could also be induced from shoot tips, for which 1 or 2 mg/l BAP could be used. The results reported here

confirm the fact that the exogenous cytokinin is the most crucial growth regulator for the suppression of apical dominance (Kane 1996, Roy and Banerjee 2002), a process which leads to axillary shoot formation. The advantage of the second pathway is that shoot multiplication occurs through stimulation of axillary growth, which is the most preferred pathway for obtaining genetically stable plants (Kane 1996, Roy and Banerjee 2002). Furthermore, the genetic stability of the regenerated plants at chromosomal level has a direct relationship with the protocol system used. Thus the present protocol proves to be efficient and reproducible and can be used for large scale multiplication to fulfill the commercial demand of this valuable orchid species in orchid industry.

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