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Comparative Efficiency Analysis of Different Explants and Contribution of Polyamines on *in vitro* Propagation of *Dendrobium* Hybrid Sonia

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Abstract

An efficient method of plant regeneration and subsequent growth and development of *Dendrobium* hybrid Sonia was developed using intact seedling, shoot tip and thin cell layer. Maximum number of PLBs was obtained in MS liquid medium supplemented with 0.5 mg/l NAA and 1mg/l BAP through shoot tip and thin cell layer culture (TCL). Highest number of adventitious shoot formation was recorded in 0.5 mg/l of NAA and 2 mg/l of BAP through shoot tip culture. In shoot tip and TCL cultures, the necrosis was checked in presence of NAA (0.5 mg/l) and BAP (1 mg/l). Maximum callus frequency was recorded in NAA and BAP (0.5 mg/l) through thin cell layer culture. Direct PLB formation was better at 1.0 µM concentration in all the three polyamines tested. Three polyamines tested were effective in direct PLB formation as well as adventitious shoots.

Introduction

Orchids belong to the largest and most diverse family Orchidaceae, comprising of approximately 700 - 800 genera and near about 25,000 - 30,000 species (Begum et al. 2000, Xiang and Chan 2003) and considered as the doyens among ornamental plants. Attractive and enthralling flowers with vibrant colors, capability of blooming up to perfection and their ability to long distance transport enabled them to achieve uppermost position in international cut flower market. However, *Dendrobium* hybrids occupied the leading position for their high number of flowers per inflorescence, year round production with long lasting flower (Martin et al. 2006). Although, most of the orchid

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species produce millions of seeds in a single capsule, in natural condition less than 1% of them, get opportunity to germinate due to lack of sufficient nutrient supplements. To overcome the difficulties in propagation, *in vitro* technique, chiefly micropropagation, provides a solution for producing large number of propagules within a limited time. Micro-propagation of orchids is the most frequently used convenient technique for their exploitation as a major trade in developed countries (Goh and Tan 1982, Sagawa and Kunisaki 1982). Presently the available method of mass clonal propagation using tissue culture techniques is very common (Goh and Tan 1982). Although orchids can be rapidly propagated through *in vitro* technique using shoot tips (Saiprasad et al. 2002), leaf (Chen et al. 1999, Chen and Chang 2000) and stem nodes (Pathania et al. 1998) yet difficulties arise regarding their protocorm regeneration and plantlet formation. That's why all the procedures mentioned above are indeed inadequate for meeting up the commercial need for vegetative propagation. Even though some of these methods gave rise to a large number of protocorms, but their subsequent development is rather slow. Therefore, the present study deals with a bi-directional technique; on one hand it focused on to the *in vitro* propagation of the *Dendrobium* hybrid Sonia using intact seedling, shoot tips and thin cell layer (TCL) tissues as explants along with the different combinations of two synthetic phytohormones BAP and NAA. On the other hand, it deals with the optimization of the concentration and combination of plant growth regulators for better plantlet regeneration and their subsequent growth and development.

So far as the polyamines (an organic compound) are concerned, it has two or more primary amino groups $-NH_2$, found in all living organisms and considered as an essential element for growth and development in both prokaryotes and eukaryotes (Tabor and Tabor 1984, Tiburcio et al. 1993) and may well be functional as a growth regulator in plants. Unlike phytohormones, very limited investigations were carried out on the effect of polyamines on shoot multiplication in commercial orchids (Saiprasad et al. 2004) and subsequently a large knowledge gap is formed in this area. Here we used different concentrations of three polyamines; putrescine, spermine and spermidine in the culture medium along with BAP as control-phytohormone and observed their effects on shoot tip culture of *Dendrobium* hybrid Sonia.

Materials and Methods

Seeds of *Dendrobium* hybrid Sonia were collected from six-month-old capsules. For surface sterilization, the mature capsules were rinsed with 90% (v/v) ethanol for 1 min, followed by treatment with 0.1% (w/v) mercuric chloride solution for 20 minutes and finally washed thrice with sterile distilled water. After opening the capsules aseptically, the seeds were spread on the surface of modified Knudson C peptone (KCP) basal medium (Knudson et al.1946) for germination and seedling development. Shoot tips (6 - 8 nm) were excised from 6-month-old aseptically raised seedlings and cultured in half-strength agar solidified MS basal medium supplemented with 8 µM BAP and their

responses were critically recorded in presence of polyamines. Five replicates were made for each treatment each with eight explants cultured on 100 ml modified KCP medium. The cultures were incubated at 25 ± 2°C with a photoperiod of 12 - 14 hrs at 3000 lux light intensity by using white fluorescent light. Half strength MS basal medium was used for culturing the explants. The medium amendments include replacement of the original micronutrients with half-strength microsalts (iron-EDTA in full strength) of MS, inclusion of 0.1% (w/v) peptone and 0.9% (w/v) agar. The MS was supplemented with BAP and various concentrations (0.5 and 1.0 µM) of three polyamines i.e., putrescine, spermine, spermidine were made. The pH was adjusted to 5.6 - 5.8 before autoclaving at 121^0C for 20 min under 15 lb/inch pressure.

Intact seedlings, shoot tips, and thin cell layers were excised from 6-months-old axenic seedlings and grown in half-strength MS basal medium supplemented with different concentrations and combinations of BAP (0.5, 1 and 2 mg/l) and NAA (0.5 mg/l) to determine the efficiency of different nutrient media, growth regulators and medium supplements for the favorable propagation of the orchid *Dendrobium* hybrid Sonia. The cultures were incubated at 26 ± 1^0C under approximately 200 lux light for 16/8 hrs light/dark cycle. Data were collected after 90 days of inoculation of explants for recording direct PLB formation, callus induction, adventitious shoot formation and necrosis frequency per sample. For *ex vitro* establishment, 10 well-rooted plantlets were rinsed thoroughly with tap water to eradicate the remaining nutrient agar from the surface of the roots and transplanted to clay pots containing a mixture of dried coconut husk, small pieces of brick and charcoal (1 : 1 : 1 v/v). Instantaneously after transplanting, the plantlets were sprayed with 0.1% (w/v) thiram solution (tetramethyl thiuram disulphide; Sree Ramcides Chemicals Pvt. Ltd., Chennai, Tamil Nadu, India), a broad-spectrum fungicide. The transplants were set aside in a well-ventilated position in the experimental garden under subdued light for further growth and development.

All the data of different morphogenetic responses were subjected to ANOVA and subsequent mean separation was performed by DMRT. The experimental units were assigned to RCBD with single replicate per block, according to Little and Hills (1978).

Results and Discussion

Effect of polyamines on shoot tip culture of *Dendrobium* hybrid Sonia through direct PLB formation, adventitious shoot formation, callus formation, callus mediated PLB formation and necrosis phenomenon's were described. In case of control set, there was no direct PLB formation. Whereas, in both 0.5 and 1.0 µM concentrations of spermidine (Spd) the frequency of direct PLBs were 60% i.e., out of five replicates three exhibited direct PLB formation. But the mean number of direct PLB differed, Spd (1.0 µM) had higher mean number of direct PLB formation with 6 per explant than 0.5 µM concentration of Spd which generated 5.33 PLBs per explant. Direct PLB formation was not recorded at a concentration of 1.0 µM Spm, but at 0.5 µM concentration, low

frequency of direct PLB (20%) was observed (Table 1). However, in case of putrescine (Put), the results were strikingly different. At 1.0 µM Put high frequency (80%) direct PLB formation was observed as compared to 0.5 µM concentration.

Development of adventitious shoots from shoot tip explant is summarized in Table 1. The highest frequency of adventitious shoot formation (60%) was recorded at 0.5 µM Spd concentration. However, adventitious shoot formation was entirely absent at 0.5 µM concentration of Spm. A 1.0 µM of Spm showed 60% shoot formation and the mean number of adventitious shoots was 1.33 per explants. Putrescine exhibited adventitious shoot formation only at 1.0 µM concentration with 60% frequency.

Callus formation was recorded only in the medium containing spermine and putrescine. In case of Spm, both 0.5 and 1.0 µM formed callus and the frequencies were 20 and 40%, respectively. However, in Put callus was formed only at 0.5 µM with 20% frequency (Table 1).

In both Spm and Put at 0.5 µM, the mean number of callus mediated PLB was 6 per explant which was greater than the mean number of direct PLBs in these treatments. The mean numbers of callus mediated PLB in Spm at 1.0 µM was 7.5 per explant in which direct PLB formation was absent. Callus mediated organogenesis (Leaf) was also observed in Spm at 1.0 µM. Callus derived PLB development was critically reviewed through histological studies by freehand section cutting (Fig. 1) (Table 1). Interestingly necrosis frequency, in general, was very low including that of the control (Table 1, Fig. 1).

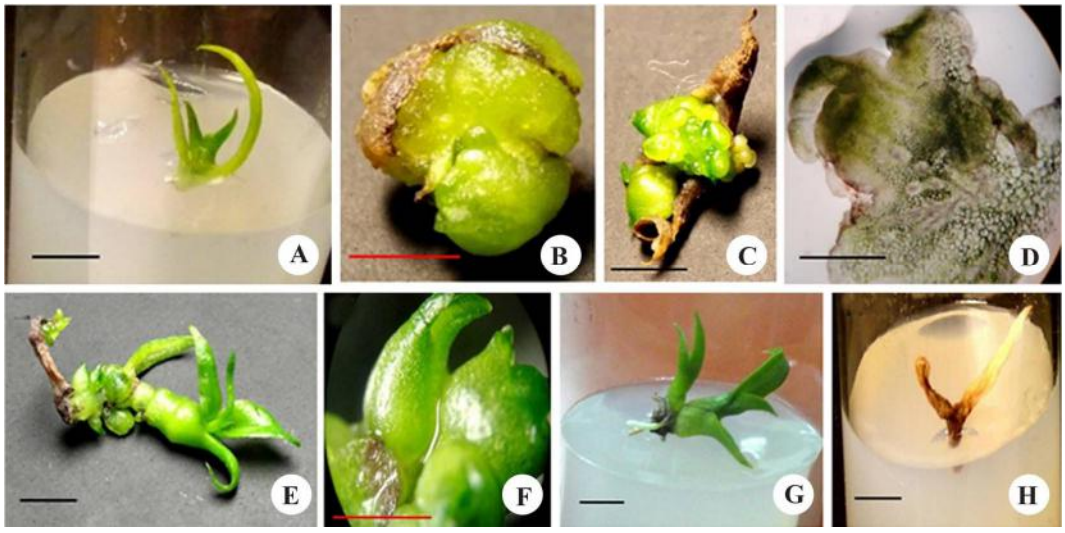

Fig. 1. Different developmental stages of the studied plant during polyamine treatment. A. Explant in aseptic condition, B. Compact callus, C. Callus undergoing differentiation, D. Histological view of callus derived PLB, E. Shoot tip derived direct PLB (Scale = 5 mm), F. Direct PLB formation from explant surface. G. Axillary shoot formation, H. Explant necrosis (Scale: D. 200 µm), rest = 5 mm).

Table 1. Effects of polyamines - spermidine (Spd), spermine (Spm) and putrescine (Put) on shoot tip culture of *Dendrobium* hybrid.

Treatment (in μM)	Frequency of direct PLB formation (%)	Mean no. of direct PLB	Adventative shoot frequency (%)	Mean no. of adventative shoots	Frequency of callus formation (%)	Mean no. of callus mediated PLB	Explant necrosis frequency (%)
Control	0	0.00	40	1.50 ± 0.14	-	-	-
Spd- 0.5	60	5.33 ± 0.37*	60	1.33 ± 0.13	-	-	-
Spd- 1.0	60	6 ± 0.07*	40	1.50 ± 0.12	-	-	20
Spm- 0.5	20	2 ± 0.07*	-	-	20	6 ± 0.15*	20
Spm- 1.0	-	-	60	1.33 ± 0.06	40	7.50 ± 0.11*	20
Put- 0.5	20	1 ± 0.10*	-	-	20	6 ± 0.25*	20
Put- 1.0	80	4.50 ± 0.17*	60	1 ± 0.05*	-	-	-

Data shown are the mean of five replicates ± standard error. *indicates that the character is significantly different from each other (ANOVA, $F_{crit} > F_{value}$, $p < 0.05$).

Table 2. Effects of NAA and BAP in varying concentrations on *in vitro* response of intact seedling culture after 90 days.

Treatment	Plant growth regulators mg/l		Callus frequency (%)	Mean no. of callus mediated PLB	Frequency of direct PLB formation (%)	Mean no. of direct PLB	Frequency of adeventious shoot formation (%)	Mean no. of shoots	Explants necrosis frequency
	NAA	BAP							
Control	-	-	0	0	40	1±0.11	0	0	0
N1B1	0.5	0.5	0	0	50	5.5 ± 0.30*	25	1 ± 0.05*	0
N1B2	0.5	1	0	0	20	2 ± 0.15*	0	0	0
N1B3	0.5	2	20	5 ± 0.14*	20	16 ± 0.2*	0	0	0

Data shown are the mean of five replicates ± standard error. *indicates that the character is significantly different from each other (ANOVA, $F_{crit} > F_{value}$, $p < 0.05$).

Results regarding the effect of polyamines from the present study indicated that more or less in all three polyamines tested (Spd, Spm and Put), at 0.5 and 1.0 µM except Spm at 1.0 µM, direct PLB formation was triggered with a highest frequency of 80%. Whereas, in case of Put, 1.0 µM was found very effective for direct PLB formation (6 per explant). While the control with only 8µM BAP showed no direct PLB formation, incorporation of polyamines in culture media in certain concentrations enhanced direct PLB formation in shoot tip culture. An earlier study in cv. Rungnappa Red, showed an exponential increase in shoot production with the use of increasing concentration of from 0.25 to 1.0 mM. (Kumari and George 2011). Moreover, similar enhancement of shoot production in polyamine supplemented media was also reported in *Achras sapota* (Purohit et al. 2007) which was probably due to the stimulatory effect on cell division and/or to the inhibitory effect on ethylene production (Bais et al. 2000). Corroborating those observations the present study also shows formation of adventitious shoots in Spd, Spm and Put supplemented media at certain concentrations (0.5 and 1.0 µM) along with the control. Bertoldi et. al. (2004) observed that the medium containing Put produced the greatest percentage of nonresponding anthers (22) and of anthers with callus (51.4). However, the present investigation shows callus and further callus mediated PLB formation only in media containing Spm at both 0.5 and 1.0 µM and also in Put (0.5 µM). Although the callus forming frequency was low (20 - 40%), the mean number of callus mediated PLB formation was quite high (6 - 7.5 per explant).

Callus, direct PLB and adventitious shoots formation were considered as major parameters for observing the effect of *in vitro* propagation using different explants and synthetic phytohormones. In intact seedling culture, frequency of 20% callus formation was found in case of N_1B_3, whereas callus formation was absent in case of N_1B_1 and N_1B_2 (Table 2) and also in the shoot tip cultures (Table 3). However, explants using TCL tissue showed highest frequency of callus formation in case of N_1B_1 (40%). However, 50 % direct PLB formation frequency was found in case of intact seedling culture in N_1B_1 hormonal combination and the lowest was detected in N_1B_2, N_1B_3 (Table 2). Moreover, in case of shoot tip culture, frequency of 80% direct PLB formation was recorded with a mean number of 7 per explants in N_1B_2 whereas the N_1B_3 showed the least value (Table 3). TCL system also showed similar results (80% direct PLB formation) with a mean number of 6.25 per explants in case of N_1B_2 and lowest direct PLB formation was detected in control treatment (Table 4).

For adventitious shoots formation, intact seedling culture exhibited 25% adventitious shoot formation in case of N_1B_1 while it was absent in case of N_1B_1, N_1B_3 and control treatments (Table 2). Whereas, shoot tip culture showed 60% adventitious shoot formation in N_1B_3 and least adventitious shoot formation in N_1B_1 (Table 3). On the other hand, in case of TCL culture highest adventitious shoot formation was found in case of N_1B_1 (40%) and the number was less recorded in N_1B_2, N_1B_3 and control treatments (Table 4). Necrosis was completely absent in intact seedling cultures system. In shoot tip and thin cell layer explants, the necrosis was completely inhibited in N_1B_2.

Table 3. Effects of NAA and BAP in varying concentrations on *in vitro* response of shoot tips culture after 90 days.

Treatment	Plant growth regulators mg/l		Callus frequency	Mean no. of callus mediated PLB	Frequency of direct PLB formation (%)	Mean no. of direct PLB	Frequency of adventious shoot formation (%)	Mean No of shoots	Explants necrosis frequency (%)
	NAA	BAP							
Control	-	-	0	0	20	1 ± 0.05	20	1 ± 0.05	20
N_1B_1	0.5	0.5	0	0	40	6 ± 0.23*	0	0 ± 0*	20
N_1B_2	0.5	1	0	0	80	7 ± 0.14*	40	2 ± 0.16*	0
N_1B_3	0.5	2	0	0	0	0	60	1.6 ± 0.13*	20

Data shown are the mean of five replicate s± standard error. *indicates that the character is significantly different from each other (ANOVA, $F_{crit} > F_{value}$, $p < 0.05$).

Table 4. Effects of NAA and BAP in varying concentrations on *in vitro* response of thin cell layer tissue culture after 90 days.

Treatment	Plant growth regulators mg/l		Callus frequency (%)	Mean no of callus mediated PLB	Frequency of direct PLB formation (%)	Mean No of direct PLB	Frequency of adventious shoot formation (%)	Mean No of shoots	Explants necrosis frequency (%)
	NAA	BAP							
Control	-	-	0	0 ± 0	20	1 ± 0.05*	20	1 ± 0.32	20
N_1B_1	0.5	0.5	40	12.5 ± 0.2*	40	5.5 ± 0.32*	40	1 ± 0.55	20
N_1B_2	0.5	1	20	11 ± 0.29*	80	6.25 ± 0.07*	20	1 ± 0.45	0
N_1B_3	0.5	2	20	13 ± 0.07*	60	2.66 ± 0.12*	20	1 ± 0	20

Data shown are the mean of five replicates ± standard error. *indicates that the character is significantly different from each other (ANOVA, $F_{crit} > F_{value}$, $p < 0.05$).

So far as the *in vitro* propagation using different explants in various combinations of phytohormones is concerned, all treatment combinations of 0.5 mg/l NAA and 0.5 mg/l BAP were found suitable for callus induction along with callus mediated PLB formation through thin cell layer system. This response differs from earlier observation made on *Dendrobium sonia*-28 where percentage of formation of callus, cultured in half-strength MS semi-solid medium with 0.05 mg/l NAA and 0.1 mg/l 2,4-D was 34.29% (Mei et al. 2012) where PLBs were used as explants. Furthermore, present data completely differ with the study of Mei et al. (2012) on *Dendrobium* where the formation of callus was significantly reduced when 0.1 mg/l NAA and 0.1 mg/l 2,4-D were used (Mei et al. 2012).

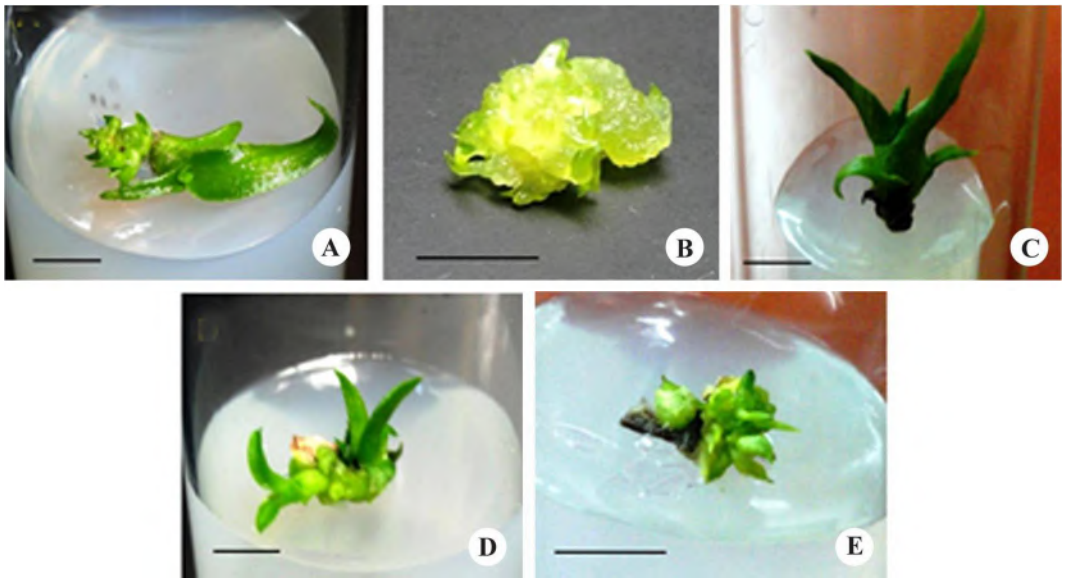

Fig. 2. Different developmental stages during *in vitro* propagation of the plant using various explant and synthetic phytohormone. A. Intact seedling derived direct PLB, B. Callus undergoing differentiation, C. Normal shoot elongation, D. Adventitious shoot formation from shoot tip explants, E. Callus derived PLB from thin cell layer tissue (Scale: 5 mm).

Although most of the callus induction media incorporate cytokinin together with auxin for better response, a cytokinin had little inducing effect on its own and is required for preventing necrosis of callus and improved growth (Lin et al. 2000, Roy and Banerjee 2003, Lu et al. 2004). In case of differentiation of PLBs, two types of orchid calli are generally observed. Some species do not require exogenous PGRs for PLB formation (Chen and Chang 2000, Roy and Banerjee 2003, Takamura et al. 2004), while others are PGRs dependent (Lin et al. 2000, Lee and Lee 2003, Lu 2004, Wu et al. 2004). In the present study 0.5 mg/l NAA and 0.5 mg/l BAP were found suitable for callus mediated PLB formation through thin cell layer system and 0.5 mg/l NAA and 1 mg/l BAP were the most suitable for direct PLB formation in shoot tip culture. This response also differs

from earlier observation in other orchid explants (Park et al. 2002, 2003) where low level TDZ over other cytokinins was involved in direct PLB formation. Usually, necrosis is a major problem encountered during callus culture of orchids (Chang and Chang 1998, Lin et al. 2000, Lu 2004, Wu et al. 2004). However, in the present study necrosis was completely absent in intact seedling culture system although in case of shoot tip culture and thin cell layer culture necrosis was only inhibited in N_1B_2.

Although a lot of research has been conducted on the effect of polyamines in tissue culture, the effect of polyamines on shoot multiplication in commercial orchids is seldom appreciated. Present data indicated that all three polyamines (Spd, Spm and Put) were effective in direct PLB formation and adventitious shoot formation. However, they were less effective in callus formation**.** Apart from this, the present study also revealed that thin cell layer culture method, in the long run, will become very effective system for generating various responses particularly in callus formation and direct PLB formation in orchid.

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