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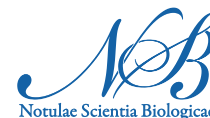

Original Article

In Vitro Organogenesis of a Slipper Orchid, *Paphiopedilum* 'Alma Gavaert'

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Abstract

The aim of the present study was to improve the regeneration efficiency of callus lines in a slipper orchid, *Paphiopedilum* 'Alma Gavaert'. Three kinds of vegetative tissues, root, stem and leaf segments, were used as explants to induce callogenesis; out of these, only root explants formed callus and was subcultured in the presence of 5 mg/L dicamba and 5 mg/L 2,4-D combined with 1 or 2 mg/L TDZ. The resulting four callus lines, assigned as 5Di1T, 5Di2T, 5D1T and 5D2T, respectively, were used to test the effect of NAA to BA ratios on re-differentiation, wherein the highest number of shoots (approximately 2 shoots/0.1 g callus clump) were obtained in callus line 5D2T at ratios of 0.001 and 0.002. A largely improvement of shoot regeneration efficiency was obtained by continuous selection of callus lines which derived from different explant positions. Eventually, six callus lines, including 5D2T-T6-G5 to 5D2T-T6-G10, were able to produce approximately 10 times of shoots per callus clump when compared with the parental callus line 5D2T.

Keywords: callus; heterogeneity; organogenesis; shoot formation; slipper orchid

Introduction

Paphiopedilum (slipper orchids), which have an impressively beautiful flower with pouch-shaped specialized lip or labellum, is one of the most popular orchid genera (Sheehan and Sheehan, 1994). Although wild populations of this genus are threatened, a number of *Paphiopedilum* species are commercialized as pot-plant products with high value (Zeng *et al.*, 2015).

For inducing callus and subsequent re-differentiation, seed-derived protocorms of a *Paphiopedilum* hybrid (*P. callosum* 'Oakhi' × *P. lawrenceanum* 'Tradition') were used to induce callus, but only a few plantlets could be obtained via protocorm-like-body (PLB) formation (Lin *et al.*, 2000). Compton (2005) also used seed-derived protocorms to induce callus formation, and the resulting PLBs could further be converted into plantlets. A progress for enhancing of regeneration capacity is using seeds from green capsules of *Paphiopedilum* 'Alma Gavaert' to directly induce callus (Hong *et al.*, 2008); although only 4.7 shoots were obtained from the parent callus clump in the cited report, subsequent multiplication of the shoots resulted in 25 shoots and eventually 75 plantlets.

In *Paphiopedilum rothschildianum*, secondary PLB formation was successfully induced from stem-derived callus (Ng *et al.*, 2011). More recently, seed-derived protocorms of *P. hangianum* were used as explants to induce PLB or callus formation, and eventually regenerated plantlets obtained showed no obvious phenotypic variation (Zeng *et al.*, 2013).

For inducing shoots, Huang *et al.* (2001) used shoots of *in vitro* seedlings to induce multiple shoot formation and they found thidiazuron inhibits shoot multiplication and also subsequent root formation in *Paphiopedilum*. Instead, Chen *et al.* (2002, 2004) used stem nodal and leaf explants of *P. philippinense* hybrids to induce shoot formation or shoot multiplication and successfully obtained a few regenerated plantlets. A more efficient protocol was established by Ng *et al.* (2010), whereas stem nodal and single shoot explants of *P. rothschildianum* were used as explants to induce multiple shoot formation; they obtained 2.8 - 2.9 shoots per explant. An *in vitro* cutting method was developed by Udomdee *et al.* (2012) and was applied to produce multiple shoots from *in vitro* seedlings of *P.* 'Hsinying Rubyweb'.

The vegetative propagation of this genus has always been restricted and time consuming especially via tissue culture protocols for plant regeneration (Ng *et al.*, 2010; Zeng *et al.*, 2015). Based on knowledge, although there are ample number of protocols on *Paphiopedilum* regeneration, commercial propagation by growers has still been entirely

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via seed germination (Long *et al*., 2010; Zeng *et al*., 2013; Chen *et al*., 2015; Zhang *et al*., 2015). Thus, for commercial species or cultivars, there is a highly demand to develop more efficient and reliable tissue culture protocols for its breeding, conservation as well as commercial propagation.

The aim of the present study is to largely enhance the regeneration efficiency of callus culture of *Paphiopedilum* 'Alma Gavaert' which is one of the most popular commercial cultivars.

Materials and Methods

Culture conditions

In vitro plantlets obtained from seed-derived calli were used as donor plantlets. The culture conditions were followed as in Hong *et al*. (2008) protocol. The plantlets were cultured on a plant growth regulator (PGR)-free 1/2 MS (Murashige and Skoog, 1962) medium with a subculture period of 2 months. The basal medium (BM) was a half strength MS medium containing 2.215 g/L powder (Product No. M519, Murashige and Skoog basal medium with vitamins, PhytoTechnology Lab., Lenexa KS, USA) and supplemented with 1 g/L peptone, 30 g/L sucrose and 3.5 g/L Gelrite. The pH of all media was adjusted to 5.2 and then sterilized using an autoclave at 121 °C and 15 psi for 20 min.

Induction of re-differentiation from vegetative tissues and subsequent callus proliferation (in darkness)

Root tip segments (approximately 1 cm in length), stem segments (approximately 0.5 cm in length) and leaf segments (approximately 1 cm in length) were excised from 6-month-old donor plants and were used as explants for inducing callus. The culture containers were 120 mm × 25 mm test tubes, and each contained 10 ml of the culture medium.

Two auxins, dicamba (3,6-dichloro-2-methoxybenzoic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid) combined with one cytokinin, thidiazuron, TDZ and 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea, were used to test their effects on callus induction. All the cultures were kept in darkness and a temperature of 22 ± 1 °C. The subculture period was of approximately four weeks. To calculate the percentage of callus formation, each treatment contained four dishes (four replicates) and each dish contained four explants. Data were scored after 90 days of culture.

To calculate proliferation rate of callus, each treatment contained four test tubes (four replicates) and each test tube contained one callus clump. The proliferation rate of callus was calculated as the ratio of the final fresh mass to the initial fresh mass of callus clumps after 60 days of culture.

Effects of NAA to BA ratios on re-differentiation from callus lines (in light)

The resulting callus lines (5Di1T, 5Di2T, 5D1T and 5D2T) obtained from the above experiment (Table 1), were used in the experiment. The callus lines were referred as DixTy or DxTy (Dix means dicamba at x mg/L, Dx means 2,4-D at x mg/L, Ty means TDZ at y mg/L, respectively). To test NAA to BA ratios on re-differentiation from callus lines, NAA (1-naphthaleneacetic acid) at 0, 0.01, 0.1, 1, 5 and 10 mg/L were combined with BA (benzyl adenine) at 0, 0.01, 0.1, 1, 5 and 10 mg/L for 18 combinations, i.e. NAA to BA ratios (Table 2). PGRs were added to the BM according to the experimental design and the pH was 5.2. To calculate the number of shoots, each treatment contained five test tubes (five replicates) and each test tube contained one callus clump (approximately 0.1 g). The culture containers were 120 mm × 25 mm test tubes. All the calli were cultured in a growth chamber, under a 16 h/8 h (light/dark) photoperiod, at 28-36 μmol m^{-2} s^{-1} (daylight fluorescent tubes FL-30D/29, 40 W, China Electric Co., Taipei, Taiwan). The temperature was set at 22 ± 1 °C. The number of shoots per callus clump was counted under a stereoscope (SZH, Olympus, Tokyo, Japan) and the data were scored after 120 days of culture.

Enhancement of re-differentiation capacity by selection of callus lines (in light)

The callus line 5D2T from root explants was further separated according to the plantlets origin from tips, middle parts and lower parts of root explants, assigned as 5D2T-T, 5D2T-M and 5D2T-L, respectively. To test the re-differentiation capacity, the callus lines were cultured at two kinds of NAA to BA ratios, 0.001 (NAA/BA = 0.01/10) and 0.002 (NAA/BA = 0.01/5). To test the effects of different explants, ten callus lines from ten different root tips were assigned as 5D2T-T1 to 5D2T-T10. The callus lines were cultured at 0.001 NAA to BA ratio (NAA/BA = 0.01/10). To select high re-differentiation capacity, the callus clump of line 5D2T-T6 was divided into two parts and two callus lines, 5D2T-T6-G1 and 5D2T-T6-G2, resulted. The callus clump of line 5D2T-T6-G2 was divided into two parts and two callus lines, 5D2T-T6-G2 and 5D2T-T6-G3, resulted. After 9 times of divisions, ten callus lines, from 5D2T-T6-G1 to 5D2T-T6-G10 were obtained. Each callus line was sub-cultured and proliferated more for evaluation. To induce re-differentiation from callus lines, the culture conditions, light regime, culture period and data evaluation were the same as the above experiment. To calculate the number of shoots, each treatment contained five test tubes (five replicates) and each test tube contained one callus clump (approximately 0.1 g).

Rooting of callus-derived shoots (in light)

Callus-derived shoots were used to test the effect of NAA on rooting. Five concentrations of NAA were tested, 0, 0.1, 0.5, 1 and 5 mg/L. The culture containers were 250 ml flasks. Except for PGRs, the culture conditions were the same as the above experiment. The number of roots per shoot was counted under a stereoscope and the data were scored after 120 days of culture. Each treatment contained five shoots (each with 3 - 4 leaves) for five replicates.

Scanning electron microscopy

The samples were fixed in a solution that contained 2.5% glutaraldehyde and were then dehydrated using a graded series of ethanol (Dawns, 1971). They were then further dehydrated using a critical point dryer (HCP-2, Hitachi Ltd., Japan). Samples were coated with gold in an ion coater (IB-2, Giko Engineering Co., Japan) and examined under a scanning electron microscope (DSM-950, Carl Zeiss, Germany).

Statistic and data analysis

All the experiments were designed with a Completely Randomized Design. Replication and sample size for each experiment were described as in above paragraphs. Analysis of variance (ANOVA) was used for data evaluation. The data expressed as percentages were transformed using arc sine prior to ANOVA and then converted back to the original scale (Compton 1994).

All treatment means were compared by following Duncan's Multiple Range Test (DMRT, Duncan 1955) and significant differences between means were presented at the level of $p \leq 0.05$.

Table 1. Effects of dicamba, 2,4-D and TDZ on callus formation and proliferation from root explants of *Paphiopedilum* 'Alma Gavaert'

PGRs (mg/l)			Percentage of callus formation (%)[A]	Proliferation rate of callus[B]	Survival callus lines
Dicamba	2,4-D	TDZ			
0	0	0	0 b	-	
1	0	1	0 b	-	
2	0	1	6.3 ± 12.5 b	necrotic	
5	0	1	68.8 ± 12.5 a	1.60 ± 0.05 a	5Di1T
10	0	1	6.3 ± 12.5 b	necrotic	
0	1	1	0 b	-	
0	2	1	0 b	-	
0	5	1	62.5 ± 14.4 a	1.65 ± 0.08 a	5D1T
0	10	1	6.3 ± 12.5 b	necrotic	
1	0	2	0 b	-	
2	0	2	6.3 ± 12.5 b	necrotic	
5	0	2	62.5 ± 14.4 a	1.48 ± 0.06 a	5Di2T
10	0	2	6.3 ± 12.5 b	necrotic	
0	1	2	0 b	-	
0	2	2	6.3 ± 12.5 b	necrotic	
0	5	2	68.8 ± 12.5 a	1.78 ± 0.09 a	5D2T
0	10	2	6.3 ± 12.5 b	necrotic	

[A] Data were scored after 90 days of culture. Four replicates (dishes) each with four root explants were performed for each treatment.
[B] Data were scored after 60 days of culture. Four replicates (tubes) each with one callus clump were performed for each treatment.

Table 2. Effects of NAA and BA on the number of shoots per callus from different callus lines of *Paphiopedilum* 'Alma Gavaert'

NAA/BA ratio	PGRs (mg/l)		Callus lines			
	NAA	BA	5Di1T	5Di2T	5D1T	5D2T
PGR-free control	0	0	0.2 ± 0.5 b	0.2 ± 0.5 c	0.2 ± 0.5 bc	0.6 ± 0.6 b
1,000	10	0.01	0 b	0 c	0 c	0 b
100	10	0.1	0 b	0 c	0 c	0 b
10	10	1	0 b	0 c	0 c	0 b
500	5	0.01	0 b	0 c	0 c	0 b
50	5	0.1	0 b	0 c	0 c	0 b
5	5	1	0 b	0 c	0.2 ± 0.5 bc	0.2 ± 0.5 b
100	1	0.01	0 b	0 c	0 c	0 b
10	1	0.1	0 b	0 c	0 c	0 b
1	1	1	0 b	0 c	0.2 ± 0.5 bc	0.2 ± 0.5 b
0.001	0.01	10	1.2 ± 0.5 a	1.0 ± 0.0 a	0.8 ± 0.5 a	2.2 ± 0.5 a
0.01	0.1	10	0.6 ± 0.6 ab	0.6 ± 0.6 b	0.6 ± 0.6 ab	1.0 ± 0.0 b
0.1	1	10	0.2 ± 0.5 b	0.2 ± 0.5 c	0.4 ± 0.6 abc	0.8 ± 0.5 b
0.002	0.01	5	0.4 ± 0.6 b	0.6 ± 0.6 b	0.6 ± 0.6 ab	2.0 ± 0.7 a
0.02	0.1	5	0.2 ± 0.5 b	0.4 ± 0.6 bc	0.4 ± 0.6 abc	0.8 ± 0.5 b
0.2	1	5	0 b	0 c	0.4 ± 0.6 abc	0.6 ± 0.6 b
0.01	0.01	1	0 b	0 c	0 c	0 b
0.1	0.1	1	0 b	0 c	0 c	0 b

Data were scored after 120 days of culture. Five replicates (tubes) each with one callus clump (about 0.1 g) were performed for each treatment.
Data in the same column followed by the same letters are not significantly different by Duncan' multiple range test at $P < 0.05$.

Results and Discussion

Callus induction and proliferation

It has been reported that stem nodal and leaf explants have no ability to form callus in the presence of low dosages of TDZ or combined with 2,4-D in *P. philippinense* hybrids (Chen *et al*., 2002, 2004). Similar results were found in the present study, whereas stem and leaf explants had no ability to form callus in the combinations of TDZ and 2,4-D. By contrast, it was found that only root explants could form callus (Fig. 1a). Eleven combinations of plant growth regulator (PGR) were found to be effective for callus induction from root explants (Table 1).

The calli were yellowish and compact when induced from tips (T), middle parts (M) and lower parts (L) of root explants (Fig. 1a). The highest percentages of callus formation (from 62.5 to 68.8%), as well as subsequent successful proliferation (with proliferation rate from 1.48 to 1.78) were obtained in four treatments, including 5 mg/L dicamba combined with 1 mg/L TDZ (the resulting callus line was named as 5Di1T), 5 mg/L dicamba combined with 2 mg/L TDZ (callus line 5Di2T), 5 mg/L 2,4-D combined with 1 mg/L TDZ (callus line 5D1T) and 5 mg/L 2,4-D combined with 2 mg/L TDZ (callus line 5D2T) (Table 1).

These callus lines were maintained for further selections and induction of re-differentiation. In general, a vigorous callus mass of *P.* 'Alma Gavaert' showed bright yellowish and compact texture (Fig. 1b). In plant tissue culture, dicamba was found to be effective on induction of callus formation, somatic embryogenesis, as well as an enhancement of regeneration capacity (Immonen, 1996; Płażek *et al*., 1999; Osuna and Barrow, 2004; Steinmacher *et al*., 2007; Wang *et al*., 2010; Chen, 2012). In *P.* 'Alma Gavaert', 5 mg/L dicamba combined with 1 or 2 mg/L TDZ could induce callus formation from root explants (Table 1) and the resulting callus could be maintained on the same medium with a range of proliferation rates, varying from 1.48 to 1.60 (Table 1). Otherwise, during subculture, no morphological abnormalities were found.

In plant tissue culture, TDZ was proved to have multidimensional effects and could induce both auxin- and cytokinin-like responses (Murthy *et al*., 1998; Guo *et al*., 2011; Wang *et al*., 2016). TDZ itself or combined with 2,4-D usually gave an efficient response and played a multiple role on induction of organogenesis, somatic embryogenesis, as well as embryogenic callus in orchids (Chen, 2012). Furthermore, TDZ combined with 2,4-D played a crucial role in callogenesis in *Cymbidium*, *Oncidium*, *Paphiopadilum* and *Phalaenopsis* orchids (Chang and Chang, 1998; Chen and Chang, 2000; Chen *et al*., 2000; Lin *et al*., 2000). By contrast, it has been reported that TDZ inhibits shoot proliferation and rooting in *Paphiopedilum* (Huang *et al*., 2001).

In *P.* 'Alma Gavaert', although TDZ combined with 2,4-D could induced callus formation from root explants, the capacity of subsequent re-differentiation, i.e. shoot formation, was found to be relatively low when compared with the cases of *Cymbidium*, *Oncidium* or *Phalaenopsis* orchids (Chen and Chang, 2000; Chen *et al*., 2000; Wu *et al*., 2004; Chen, 2012).

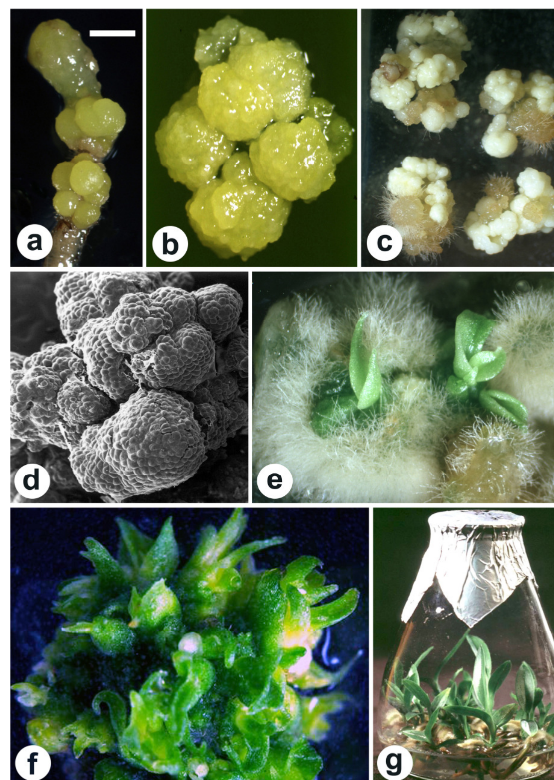

Fig. 1. Plant regeneration via callus cultures from root explants of *Paphiopedilum* 'Alma Gavaert' (The scale bar in upper left refers to all panels); **(a)** callus formation from the tip (T), middle (M) and basal (B) regions of a root explant (*Scale bar* 2 mm); **(b)** several masses of proliferated calli (*Scale bar* 2.5 mm); **(c)** re-differentiation of calli showed white masses (WM) and absorbing hairs (AH) (*Scale bar* 4 mm); **(d)** a scanning electron microscopic photo of a white mass (*Scale bar* 200 µm); **(e)** shoot buds formed from the white mass (*Scale bar* 4.5 mm); **(f)** shoot buds formed form the callus line 5D2T-T6 (*Scale bar* 5 mm); **(g)** regenerated plantlets (*Scale bar* 1.8 cm)

Effects of NAA to BA ratios on shoot formation from callus lines

Four callus lines obtained from the above experiment, namely 5Di1T, 5Di2T, 5D1T and 5D2T, were used to test the effects of NAA to BA ratios on shoot formation (Table 2). In the PGR-free control, all the callus lines could form shoots, but the numbers of shoots were quite low (Table 2). In callus lines 5Di1T and 2Di2T, five kinds of NAA to BA ratios, including 0.001, 0.002, 0.01, 0.02 and 0.1, could induce shoot formation, whereas an average of 0.2 - 1.2 and 0.2 - 1.0 shoots were obtained from a callus clump (0.1 g), respectively (Table 2). In long-term callus cultures induced by dicamba combined with TDZ, a morphological abnormality on somatic embryogenesis in betel nut and *Oncidium* 'Gower Ramsey' have been reported (Wang *et al.*, 2010; Chen, 2012). In *P.* 'Alma Gavaert', since the callus culture was maintained less than two years, there were no morphological abnormalities of shoots. In callus lines 5D1T and 2D2T, eight kinds of NAA to BA ratios, including 0.001, 0.002, 0.01, 0.02, 0.1, 0.2, 1 and 5, could induce shoot formation, and an average of 0.2 - 0.8 and 0.2 - 2.2 shoots were obtained from a callus clump (0.1 g), respectively (Table 2). Overall, the callus line 5D2T produced higher shoot numbers per callus clump at 0.001 and 0.002 of NAA to BA ratios, and the shoot numbers were 2.2 and 2.0, respectively (Table2). The importance of auxin to cytokinin ratio on regulation of organogenesis and development *in vivo* and *in vitro*, has long been proved (Skoog and Miller, 1957; Smigocki and Owens, 1989; Thorpe, 1994). It has been reported that the NAA to BA ratios played a crucial role on organ formation from calli and the effects followed the regular pattern (Tsai *et al.*, 2016). Interestingly, in *P.* 'Alma Gavaert', although relatively lower ratios promoted shoot formation, relatively higher ratios did not promote root formation from callus (Table 2).

When transferred onto re-differentiation media, i.e. media supplemented with NAA and BA, white masses gradually formed from the parent callus (Fig. 1c). Based on scanning electron microscopic observation, shoot buds were originated from these white masses (Fig. 1d). During subculture on the re-differentiation medium, these shoot buds developed and numerous absorbing hairs formed around (Fig. 1e). In previous reports, the re-differentiation from callus cultures all via PLB formation for the intervening of plant regeneration was noted (Lin *et al.*, 2000; Compton, 2005; Hong *et al.*, 2008; Ng *et al.*, 2011; Zeng *et al.*, 2013). Interestingly, the present study is the first to obtain shoot regeneration, but not PLB formation from callus culture of *Paphiopedilum*. It was found that root-derived callus has quite different in regard to morphological capacity when compared with the seed-derived or protocorm-derived calli.

Effect of explant heterogeneity on capacity of shoot formation

The callus line 5D2T was further separated into three callus lines, 5D2T-T, 5D2T-M and 5D2T-L, from tips, middle parts and lower parts of explants, respectively. At 0.001 of NAA to BA ratio, 5D2T-T produced significantly highest number of shoots per callus clump when compared with callus lines 5D2T-M and 5D2T-B, with an average of 4.2 shoots (Table 3).

Enhancement of shoot formation by selection of callus lines

Ten callus lines from different root tips, from 5D2T-T1 to 5D2T-T10, showed no significant difference in proliferation rate (Table 4). However, their different capacities to form shoots was found (Table 4). At 0.001 of NAA to BA ratio, 5D2T-T6 produced significantly the highest number of shoots (7.8 shoots/callus clump) when compared with other callus lines (Table 4). Ten callus lines obtained by continuous division of 5D2T-T6, from 5D2T-T6-G1 to 5D2T-T6-G10, showed no significant differences in proliferation rate (Table 5). However, these callus lines had different capacity to form shoots (Table 5). Six callus lines, 5D2T-T6-G5, 5D2T-T6-G6, 5D2T-T6-G7, 5D2T-T6-G8, 5D2T-T6-G9 and 5D2T-T6-G10, resulted in significantly higher number of shoots (20.4 – 22.4 shoots/callus clump) when compared with other callus lines (Table 5). In tissue culture, due to the heterogeneity of explants, selection of callus lines may provide an opportunity to obtain a higher capacity of regeneration or to develop a more efficient micro-propagation pathway (Rout *et al.*, 1998). In the present study, by continuous division and selection of callus lines, the capacity of shoot regeneration was remarkably promoted (Fig. 1f; Table 5).

Rooting of regenerated shoots and plantlet growth

In the PGR-free control, regenerated shoots could form an average of 1.8 roots per shoot after 120 days of culture (Table 6). Dealing with rooting of callus-derived shoots in *P.* 'Alma Gavaert', 5 mg/L NAA gave the highest root numbers per shoot (2.7 root/shoot) after 60 days of culture (Hong *et al.*, 2008). A similar result was found in the present study, in the presence of 5 mg/L NAA, a significantly higher number of shoots (3.6 roots/shoot) was obtained after 120 days of culture (Table 6). Plantlets were successfully obtained via rooting of regenerated shoots and normal growth and morphology were found (Fig. 1g).

Table 3. Effects of callus lines on number of shoots per callus in *Paphiopedilum* 'Alma Gavaert'

Callus lines	NAA/BA ratio	
	0.01/10 = 0.001	0.01/5 = 0.002
5D2T-T	4.2 ± 0.5 a[A], a[B]	2.6 ± 0.6 a, b
5D2T-M	1.6 ± 0.6 b, a	1.2 ± 0.5 b, a
5D2T-L	0.6 ± 0.6 c, a	0.4 ± 0.6 c, a

[A] Data in the same column or [B] in the same line followed by the same letters are not significantly different by Duncan' multiple range test at $P < 0.05$.
Data were scored after 120 days of culture. Five replicates (tubes) each with one callus clump (about 0.1 g) were performed for each treatment.

Table 4. Effects of callus lines on proliferation rate and number of shoots per callus in different callus lines of *Paphiopedilum* 'Alma Gavaert'

Callus lines	Proliferation rate	Number of shoots per callus
5D2T-T1	3.30 ± 0.04 NS[a]	4.2 ± 0.5 b
5D2T-T2	3.42 ± 0.10 NS	4.4 ± 0.9 b
5D2T-T3	3.48 ± 0.05 NS	3.8 ± 0.8 b
5D2T-T4	3.50 ± 0.05 NS	3.6 ± 0.6 b
5D2T-T5	3.56 ± 0.05 NS	4.6 ± 0.6 b
5D2T-T6	3.52 ± 0.08 NS	7.8 ± 0.5 a
5D2T-T7	3.52 ± 0.06 NS	4.4 ± 0.6 b
5D2T-T8	3.56 ± 0.09 NS	4.4 ± 0.9 b
5D2T-T9	3.60 ± 0.10 NS	4.2 ± 0.5 b
5D2T-T10	3.45 ± 0.17 NS	4.6 ± 0.6 b

Means ± Standard Deviation (SD) in the same column followed by the same letters are not significantly different (Duncan' multiple range test at P ≤ 0.05). [a] NS = non-significantly different. Data were scored after 120 days of culture. Five replicates (tubes) each with one callus clump (about 0.1 g) were performed for each treatment.

Table 5. Effect of clonal selection on proliferation rate and number of shoots per callus in the callus line 5D2T-T6 of *Paphiopedilum* 'Alma Gavaert'

Generation	Callus lines	Proliferation rate	Number of shoots per callus
1	5D2T-T6-G1	3.56 ± 0.10 NS[a]	8.4 ± 0.9 d
2	5D2T-T6-G2	3.52 ± 0.21 NS	12.6 ± 0.6 c
3	5D2T-T6-G3	3.58 ± 0.19 NS	14.2 ± 1.9 bc
4	5D2T-T6-G4	3.60 ± 0.11 NS	15.4 ± 1.5 b
5	5D2T-T6-G5	3.42 ± 0.08 NS	21.0 ± 1.6 a
6	5D2T-T6-G6	3.56 ± 0.10 NS	21.2 ± 1.9 a
7	5D2T-T6-G7	3.50 ± 0.59 NS	22.0 ± 1.2 a
8	5D2T-T6-G8	3.52 ± 0.06 NS	22.4 ± 2.1 a
9	5D2T-T6-G9	3.42 ± 0.10 NS	22.2 ± 1.8 a
10	5D2T-T6-G10	3.50 ± 0.06 NS	20.4 ± 3.2 a

Means ± Standard Deviation (SD) in the same column followed by the same letters are not significantly different (Duncan' multiple range test at P ≤ 0.05). [a] NS = non-significantly different. Data were scored after 120 days of culture. Five replicates (tubes) each with one callus clump (about 0.1 g) were performed for each treatment.

Table 6. Effect of NAA on root formation from callus-derived shoots of *Paphiopedilum* 'Alma Gavaert'

NAA (mg/l)	Number of roots per shoot
0	1.8 ± 0.4 b
0.1	2.0 ± 0.7 b
0.5	2.4 ± 1.1 b
1	2.6 ± 0.5 b
5	3.6 ± 0.5 a

Means ± Standard Deviation (SD) in the same column followed by the same letters are not significantly different (Duncan' multiple range test at P ≤ 0.05). Data were scored after 120 days of culture. Five replicates (tubes) each with one shoot bud were performed for each treatment.

Conclusions

The selected callus lines 5D2T-T6-G5, 5D2T-T6-G6, 5D2T-T6-G7, 5D2T-T6-G8, 5D2T-T6-G9 and 5D2T-T6-G10 gave remarkably higher capacity of shoot formation when compared with the parent callus line 5D2T. The number of shoots was promoted up to approximately 10 times (22.4/2.2) by this method. In addition, an optimized ratio of NAA to BA was found at 0.001 (NAA/BA = 0.01/10) for inducing shoot formation from callus cultures. The protocol developed in the present study was proposed to apply in other species of *Paphiopedilum* or other genera of orchids which are difficult to regenerate *in vitro*.

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