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SHORT COMMUNICATION

Plant regeneration via protocorm-like body formation and shoot multiplication from seed-derived callus of a maudiae type slipper orchid

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Abstract Tiny seeds from 5-month-old green capsules of a maudiae type slipper orchid, *Paphiopedilum* Alma Gavaert, were induced to form totipotent callus on 1/2 strength MS medium supplemented with 22.60 μM 2,4-D and 4.54 μM TDZ in darkness. The callus was proliferated more and maintained without any morphogenesis on the same medium with a 2-month interval of subculture for more than 2 years. When transferred to 1/2 MS medium supplemented with 26.85 μM NAA, an average of 4.7 protocorm-like bodies (PLBs)/shoot buds formed from each explant after 120 days of culture. After another 72 and 240 days of culture on the same medium, 25 shoot buds and eventually 75 plantlets were obtained through shoot multiplication from the original culture. Kinetin at 4.65 μM was suitable for shoot multiplication and could induce an average of 3.0 shoots from a single young shoot after 60 days of culture. The regenerated plantlets grew normally when transplanted to containers with sphagnum moss in a shaded greenhouse.

Keywords Auxin · Cytokinin · Plant regeneration · Protocorm-like body · *Paphiopedilum*

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	N^6-[2-Isopentenyl]-adenine
BA	N^6-Benzyladenine
BAP	N-benzyl-9-[2-tetrahydropyranyl]adenine
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kinetin	6-Furfurylaminopurine
MS	Murashige and Skoog (1962) medium
NAA	Naphthaleneacetic acid
PLB	Protocorm-like body
TDZ	Thidiazuron, 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea
Zeatin	6-[4-Hydroxy-3-methylbut-2-enylamino]purine

Introduction

Paphiopedilum are popularly known as slipper orchids because of the resemblance of the pouch-shaped lip to a lady's slipper (Sheehan and Sheehan 1994) and a number of species are marketed as pot plants with high value. There are only a few reports describing aseptic culture methods for regenerating *Paphiopedilum* orchids (Stewart and Button 1975; Huang et al. 2001; Lin et al. 2000; Chen et al. 2002, 2004), mostly because the explants are difficult to be maintained in culture. Due to the limited success in tissue culture protocols, commercial *Paphiopedilum* propagation by growers has still been entirely through asymbiotic germination.

Stewart and Button (1975) used shoot apex of *Paphiopedilum* to induce callus, and occasionally, a few plantlets were obtained during subculture. However, the callus was difficult to maintain and eventually failed to survive. Lin

et al. (2000) induced callus from seed-derived protocorms of a *Paphiopedilum* hybrid and a few plantlets were formed via protocorm-like-body (PLB) formation from the callus. Therefore, poor callus proliferation and low regeneration capacity of callus cultures were the major impediments limiting the utility of in vitro culture for large-scale *Paphiopedilum* propagation. In this communication, callus was induced directly from seeds of a *Paphiopedilum* orchid and a method for plantlet formation via multiplication of callus-derived shoots was established.

Materials and methods

Callus induction and maintenance

Five-month-old green capsules by self-pollination were collected from 5-year-old potted plants of *Paphiopedilum* Alma Gavaert grown under greenhouse condition at Academia Sinica, Taipei, Taiwan (Fig. 1a, b). The surfaces of capsules were rubbed with 70% ethanol for 3–5 s, followed by agitation for 20 min in a solution of 1% sodium hypochlorite and 0.05% Tween 20. The capsules were then thoroughly rinsed with sterile distilled water for three times. For callus induction, seed from the capsules (Fig. 1c) were sown on 1/2 modified MS (Murashige and Skoog 1962) medium containing half-strength macro- and micro-elements of MS salts and supplemented with: *myo*-inositol (100 mg/l), niacin (0.5 mg/l), pyridoxine HCl (0.5 mg/l), thiamine HCl (0.1 mg/l), glycine (2.0 mg/l), peptone (1 g/l), NaH_2PO_4 (170 mg/l), sucrose (20 g/l), Gelrite (2.2 g/l), 2,4-D (22.60 μM), and TDZ (4.54 μM). The pH of media was adjusted to 5.2 with 1 N KOH or HCl prior to autoclaving (at 121°C and 15 psi) for 15 min. The seeds were placed on the surfaces of culture media and were incubated in 120 × 25 mm test tubes in darkness and at 25 ± 1°C.

PLB induction and plantlet formation

Auxins (2,4-D at 0.45, 2.25, 4.52 and 22.60 μM; IAA at 0.57, 2.86, 5.71 and 28.55 μM; IBA at 0.49, 2.46, 4.92 and 24.60 μM; NAA at 0.54, 2.69, 5.37 and 26.85 μM), cytokinins (2iP at 0.49, 2.46, 4.92 and 24.60 μM; BA at 0.44, 2.22, 4.44 and 22.20 μM; BAP at 0.32, 1.62, 3.23 and 16.15 μM; kinetin at 0.47, 2.33, 4.65 and 23.25 μM; zeatin at 0.46, 2.28, 4.56 and 22.80 μM), 2,4-D (2.26, 4.52, 13.56, 22.60 and 67.80 μM) combined with TDZ (0.45, 1.36, 4.54 and 13.62 μM), NAA (2.69, 5.37, 16.11, 26.85 and 80.55 μM) combined with TDZ (0.45, 1.36, 4.54 and 13.62 μM) were used to test their effects on proliferation and morphogenesis of subculture seed-derived callus. Growth regulators were added to the media (1/2 modified MS medium as described above but devoid of 2,4-D and TDZ) according to the experimental design. The pH of the media was 5.2. Pieces of callus (about 5 mg in fresh weight) were inoculated on the surfaces of culture media and were incubated in 120 × 25 mm test tubes under a 16:8 h photoperiod at 28–36 μmol m^{-2} s^{-1} (daylight fluorescent tubes FL-30D/29, 40 W, China Electric Co., Taipei, Taiwan) at 25 ± 1°C. Twenty replicates were performed for each treatment. The percentage of callus browning, PLB formation and the number of PLBs and/or shoots per explant formed from each responding callus were counted under a stereoscope (SZH, Olympus, Tokyo, Japan) and the data were scored after 90 or 100 days of culture. Data expressed as percentage were transformed using arc sine prior to ANOVA and converted back to the original scale (Compton 1994). Treatment means were compared using Duncan's multiple range test (Duncan 1955).

Shoot multiplication and plantlet formation

Regenerated shoots on 26.85 μM NAA containing medium were used to test the effects of IAA (2.86, 5.71, 28.55 μM), NAA (2.69, 5.37, 26.85), kinetin (2.33, 4.65, 23.25 μM) and zeatin (2.28, 4.56, 22.80 μM) on shoot multiplication, root formation and shoot vitrification. Three replicates were used for each treatment and data were scored after 2 months of culture. Basal medium, culture conditions and statistic analysis were the same as described above.

Histological observation

Tissues for histological observations were fixed in FAA (95% ethyl alcohol:glacial acetic acid:formaldehyde:water, 10:1:2:7), dehydrated in a tertiary-butyl-alcohol series, embedded in paraffin wax, sectioned at 10-μm thickness and stained with 0.5% safranin-O and 0.1% fast green (Jensen 1962).

Results and discussion

Callus induction and maintenance

Initially, seeds of *Paphiopedilum* Alma Gavaert became swelling on 1/2 modified MS medium supplemented with 22.60 μM 2,4-D and 4.54 μM TDZ within 1 month of culture in darkness. After another 2 months of culture on the same medium, the swelling seeds became median compact callus tissue with bright yellowish color (Fig. 1d). Lin et al. (2000) reported that protocorm-derived callus of a *Paphiopedilum* hybrid grew and proliferated well at 4.52–45.20 μM 2,4-D + 0.45–4.54 μM TDZ, and the most suitable was at 22.60 μM 2,4-D and 4.54 μM TDZ. This

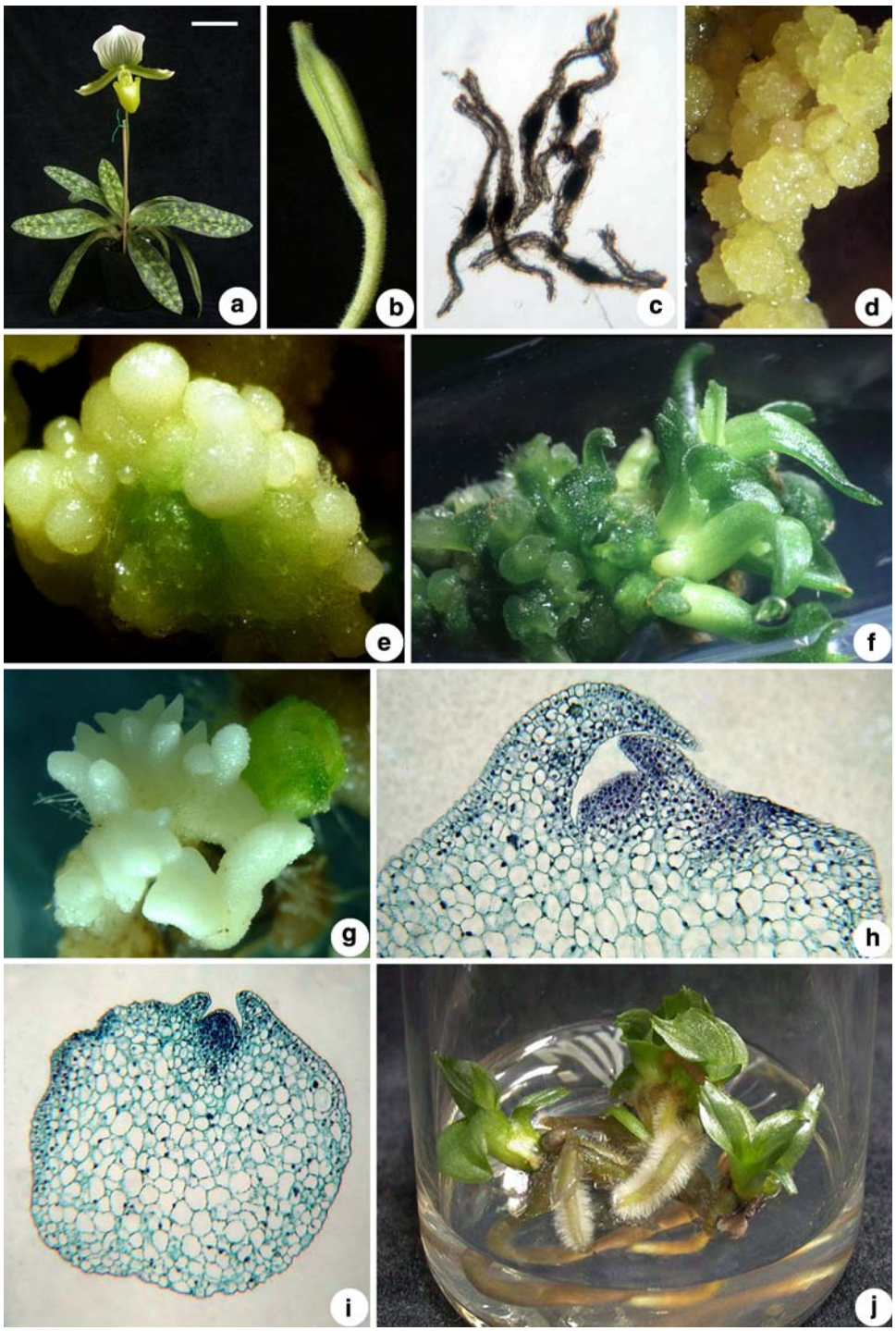

Fig. 1 Plant regeneration via callus cultures from seeds of *Paphiopedilum* Alma Gavaert. (the *bar* in upper left refers to all panels). **a** A blooming potted plants ready for self-pollination (*bar* 6.5 cm); **b** a 5-month-old green capsule (*bar* 850 μm); **c** seeds for callus induction (*bar* 30 μm); **d** yellowish seed-derived callus (*bar* 65 μm); **e** a cluster of white granular structures formed from callus (*bar* 60 μm); **f** PLBs and shoot buds formed from callus (*bar* 350 μm); **g** multiple shoot buds (*bar* 150 μm); **h** longitudinal section of a shoot bud (*bar* 50 μm); **i** longitudinal section of a PLB (*bar* 80 μm); **j** callus-derived plantlets (*bar* 1 cm)

combination is also performed well when dealt with proliferation and maintenance of seed-derived callus of *Paphiopedilum* Alma Gavaert. Devoid of 2,4-D, callus failed to proliferate and gradually turned pale or dark brown. However, occasional PLB formation was found at 4.54 μM TDZ, and only one plantlet developed. Higher dosage of 2,4-D at 67.80 μM was not suitable for callus subculture and partially due to the high browning rate.

PLB induction and plantlet formation

Auxins (2,4-D, IAA, IBA and NAA) at different concentrations were used to test their effects on PLB formation from callus of *Paphiopedilum* Alma Gavaert. Except for browning, when cultured on these media in light, several types of structures were found, such as pale to bright yellowish callus with mediate to highly compact textures,

greenish compact granular masses with more or less absorbing hairs, clusters of white granules (Fig. 1e), white compact irregular masses with or without hairs, soft watery tissues and abnormal root-like structures. The white granules subsequently turned green and developed into protocorm-like bodies and then formed shoot (Fig. 1f). The histological observation reveals that the protocorm-like body consists of the shoot apical meristem (SAM) and two primodia of sheath leaves at the interior region and larger cells act as the storage area at the posterior region (Fig. 1i). PLB formation from callus was obtained at low frequencies in five treatments and the best response was found at 26.85 μM NAA induced 4.7 PLBs/shoots per explant (5 mg of callus piece) after 120 days of culture. In the previous report by Lin et al. (2000), seven shoot buds formed from 100 mg of protocorm-derived callus of a *Paphiopadilum* hybrid after 150 days of culture. Therefore, the regeneration capacity of seed-derived callus of *P.* Alma Gavaert was obviously higher when compared with protocorm-derived callus induced by Lin et al. During subculture, some white clusters of shoot buds multiplicated from the basal or lateral region of the parent PLBs or shoots (Fig. 1g). After 8 months of culture on the same medium, 75 plantlets were obtained eventually from original 4.7 shoot buds induced at 26.85 μM NAA. These shoots also consisted SAM and leaf primordia (Fig. 1h) but connected with each other at their basal parts. Continuous subculture of shoot buds highly promoted the amount of plantlets when compared with the previous report that apparently providing unsatisfied results (Lin et al. 2000).

Except for 4.65 μM kinetin, all the cytokinins (2iP, BA, BAP, kinetin and zeatin) tested gave no response on PLB formation from callus. High frequencies (65–100%) of callus browning were obtained in all cytokinin treatments.

Combinations of NAA and TDZ at low and relative high dosages showed different results on PLB induction. Higher dosages of NAA at 26.85, 80.55 μM combined with 4.54 and 13.62 μM TDZ gave no response on PLB induction. However, alone used NAA at 26.85 μM induced four PLBs/shoots per callus after 100 days of culture. In addition, further subculture on the same medium for another 92 days resulting 25 shoot buds. Combinations at low dosages of NAA (2.69, 5.37, 16.11 μM) and TDZ (0.45, 1.35 and 4.54 μM) all induced PLB formation from callus culture (Table 1). In addition, the frequencies of PLB formation were from 5 to 30% and each callus formed 1–3 PLBs (Table 1). Generally, the regeneration capacity of seed-derived callus of *P.* Alma Gavaert was similar to protocorm-derived callus of a *Paphiopadilum* hybrid induced by Lin et al. (2000). However, the protocol of callus induction using seeds as explants was more efficient when compared with the method that using protocorms as explants (Lin et al. 2000).

Shoot multiplication and plantlet formation

TDZ is effective in induction of in vitro morphogenesis in shoot regeneration and multiplication (Ernst 1994; Chen and Piluek 1995; Nayak et al. 1997; Chen and Chang 2000a) and direct somatic embryogenesis (Chen et al. 1999; Chen and Chang 2000a) of several orchids. Moreover, TDZ combined with 2,4-D are required for callus induction in *Cymbidium* (Chang and Chang 1998), *Oncidium* (Chen and Chang 2000a, b), *Phalaenopsis* (Chen et al. 2000) and *Paphiopedilum* (Lin et al. 2000). However,

Table 1 Effects of NAA and TDZ on PLB formation and plant regeneration from callus of *Paphiopedilum* Alma Gavaert

NAA (μM)	TDZ (μM)	% browning	% PLB formation	No. PLBs/shoots per explant	% rooting plantlets	No. roots per plantlet
0	0	100 a	0 b	0	0 a	0
0	0.45	95 a	0 b	0	0 a	0
0	1.36	75 ab	0 b	0	0 a	0
0	4.54	60 bc	0 b	0	0 a	0
2.69	0	40 cd	0 b	0	5 a	1
2.69	0.45	20 cde	30 a	2.8	0 a	0
2.69	1.36	15 de	15 ab	3	5 a	1
2.69	4.54	15 de	5 b	1	0 a	0
5.37	0	15 de	0 b	0	5 a	4
5.37	0.45	10 de	5 b	1	0 a	0
5.37	1.36	5 de	20 ab	1.3	0 a	0
5.37	4.54	0 e	5 b	1	0 a	0
16.11	0	100 a	0 b	0	0 a	0
16.11	0.45	40 cd	5 b	1	0 a	0
16.11	1.36	25 de	5 b	1	0 a	0
16.11	4.54	0 e	10 b	1.5	0 a	0

Data were scored after 120 days of culture. Data expressed as percentage were transformed using arc sine prior to ANOVA and converted back to the original scale for demonstration in table (Compton 1994). Means of 20 replicates with the same letters are not significantly different at $P < 0.05$ (Duncan 1955)

Table 2 Effects of IAA, NAA, kinetin and zeatin on multiplication of callus-derived shoots of *Paphiopedilum* Alma Gavaert

Treatments	Shoot multiplication rate	No. root per shoot	% vitrification
Control	1.0 a	2.0 a	0 b
IAA (μM)			
2.86	1.3 a	1.3 ab	0 b
5.71	2.0 a	1.3 ab	30 ab
28.55	1.7 a	0.7 bc	70 ab
NAA (μM)			
2.69	1.3 a	2.3 a	0 b
5.37	2.0 a	2.0 a	0 b
26.85	2.3 a	2.7 a	0 b
Kinetin (μM)			
2.33	2.0 a	1.3 ab	30 ab
4.65	3.0 a	2.0 a	0 b
23.25	3.0 a	0.3 bc	30 ab
Zeatin (μM)			
2.28	2.0 a	0.3 bc	100 a
4.56	1.3 a	0.3 bc	30 ab
22.80	1.3 a	0 c	70 ab

Data were scored after 60 days of culture. The shoot multiplication rate was counted as final shoot numbers dividing by initial shoot numbers. Data expressed as percentage were transformed using arc sine prior to ANOVA and converted back to the original scale for demonstration in table (Compton 1994). Means of three replicates with the same letters are not significantly different at $P < 0.05$ (Duncan 1955)

Huang et al. (2001) reported that TDZ inhibits shoot proliferation and rooting in *Paphiopedilum*. In addition, Chen et al. (2002) reported that the best treatment of 4.54 μM TDZ induced only 20% of leaf explants to form shoots. Therefore, other cytokinins (kinetin and zeatin) and two auxins (IAA and NAA) were tested on shoot multiplication in this study (Table 2). The best response of shoot multiplication rate was found at 4.65 and 23.25 μM kinetin and could induce an average 3.0 shoots from one single shoot after 60 days of culture (Table 2). However, there was 30% of shoot vitrification at 23.25 μM kinetin (Table 2). During shoot multiplication and development, roots developed from the basal parts of shoots that resulting plantlet formation (Fig. 1j). NAA at 26.85 μM gave the highest root numbers per shoot when compared with other treatments (Table 2).

Conclusion

According to the present results, the suitable procedures for plantlet formation from callus culture of *P.* Alma Gavaert are: (1) callus induction from seeds of green capsules and further maintenance were on 1/2 MS medium supplemented with 22.60 μM 2,4-D and 4.54 μM TDZ in darkness; (2) PLB induction was at 2.69 μM NAA combined with 0.45 μM TDZ; (3) Shoot multiplication was at 4.65 μM kinetin; (4) plantlet development was at 26.85 μM NAA.

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