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Plant Regeneration through Somatic Embryogenesis from Callus-derived PLBs of Tropical Slipper Orchid (*Paphiopedilum niveum* (Rchb.f.) Pfitz.)

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ABSTRACT

We have developed a protocol for the indirect production of protocorm-like bodies (PLBs) from germinating seeds and subsequently regenerated plants of a slipper orchid (*Paphiopedilum niveum* (Rchb.f) Pfitz.). To solve the serious problem of necrosis that occurs during normal culture and the subsequent stages of development, the incorporation of activated charcoal (AC) and polyvinylpolypyrrolidone (PVPP) into the culture media during the stage of callus induction was also examined. Embryogenic callus was induced from the germinating seeds and showed no browning or only a few necrotic tissues on modified Vacin and Went (VW) solid medium supplemented with 0.1 mgl^{-1} 1-phenyl-3-(1,2,3-thiadianol-5-yl) urea (thidiazuron) (TDZ), 1 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2% AC. These calli developed further along the route for production of PLBs on modified VW medium containing a combination of plant growth regulators (0.1 mgl^{-1} 1-napthaleneacetic acid (NAA) and 0.5 mgl^{-1} TDZ) and 1% sucrose. The regenerated PLBs eventually formed the highest number of shoots and roots on modified Murashige and Skoog (MS) solid medium supplemented with 20 and 50 gl^{-1} banana homogenate, respectively. About 90 regenerated shoots were obtained from about 10 mg of initial PLBs. Samples of the obtained plantlets grew well after being transplanted into mini-pots placed in a shaded greenhouse. A histological study showed that PLBs originated from the surface of the embryogenic callus. Some PLBs could produce secondary PLB resulting in greater PLB proliferation. The PLB-derived plantlets had shoot and root poles indicating that plant regeneration could be considered as a pathway for somatic embryogenesis.

Keywords: callus induction, activated charcoal, PLB formation, tissue browning, plant regeneration
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **AC**, activated charcoal; **DW**, distilled water; **MS**, Murashige and Skoog; **NAA**, 1-napthaleneacetic acid; **PGR**, plant growth regulator; **PLB**, protocorm-like body; **PVPP**, polyvinylpolypyrrolidone; **TDZ**, 1-phenyl-3-(1,2,3- thiadianol-5-yl) urea (thidiazuron); **TTC**, 2,3,5 triphenyl tetrazolium chloride; **TZ**, tetrazolium salts; **VW**, Vacin and Went medium

INTRODUCTION

Paphiopedilum niveum (Rchb.f.) Pfitz. (Orchidaceae), commonly known as 'Slipper orchid', is an endangered species in the wild due to overexploitation. For conservation and commercial purposes, there is an increasing demand for production of potted plants that have high value (Chen *et al*. 2004; Hong *et al*. 2008). Of most importance is the urgent need to develop an efficient method for mass scale propagation of this genus in order to ensure its conservation. Tissue culture techniques have been widely used for *in vitro* mass propagation of several commercially available orchids. There have been reports of several explants being used such as root tips (Park *et al.* 2003), shoot tips (Roy and Banerjee 2003; Jheng *et al.* 2006; Teixeira da Silva *et al.* 2006a), shoot tip-derived suspension (Tokuhara and Mii 2003), apical buds (Meesawat and Kanchanapoom 2002; Roy *et al.* 2007), floral stalks (Chen *et al*. 2002), stem nodes (Chen *et al*. 2002; Jarnathanam and Seshadri 2008), leaves (Lee and Lee 2003; Chen *et al.* 2004; Chen and Chang 2006; Jarnathanam and Seshadri 2008) protocorm segments (Chen *et al*. 2000; Lin *et al*. 2000; Lu 2004; Zhao 2008), protocorm-like bodies (PLBs) (Ishii *et al.* 1998; Huan *et al*. 2004; Teixeira da Silva *et al.* 2006a, 2006b), PLB thin cell layers (TLCs) (Teixeira da Silva and Tanaka 2006; Teixeira da Silva *et al.* 2006b), callus (Chen and Chang 2000), mature seeds (Hong *et al.* 2008), to obtain PLBs (through direct or indirect somatic embryogenesis) and/or shoots, then subsequently plantlets. Cloning is a suitable method for rapid mass propagation. An improved method is of great importance for both commercial exploitation and for conservation because it will provide an alternative method for collection from the wild. It is also a prerequisite to pursue genetic transformation studies (Tokuhara and Mii 2003; Quiroz-Figueroa *et al*. 2006; Ruan *et al*. 2009). An efficient protocol for regenerating *P. niveum* (Rchb.f.) Pfitz through callus-derived PLBs is limited because of the problems associated with obtaining callus. One of the critical problems during callus culture, used to develop a system for cloning, is the tissue browning that results from phenolic accumulation and causes a loss of growth capacity and tissue death. So, this important problem needs to be solved. A histological study was carried out to obtain callus, free from phenolics, for use as an effective explant for plant regeneration via callus-derived PLBs. Since there is no previous reliable method for vegetative propagation and no report of plant regeneration from PLBs of *P. niveum* (Rchb.f.) Pfitz species, the present study aims to establish if the developmental stages and structure of the callus formed in the presence of activated charcoal (AC) could be used effectively for plant regeneration and to identify an efficient protocol for subsequent plant regeneration.

MATERIALS AND METHODS

Plant material and seed preculture

A number of flowers of *Paphiopedilum niveum* (Rchb.f) Pfitz were self-pollinated by hand and the six-month-old capsules were col-

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Original Research Paper

lected. They were surface sterilized with 20% (v/v) clorox (5.25% (w/w) active chlorine) containing 1-2 drops of Tween 20 for 20 min and rinsed 2-3 times with sterile distilled water (DW). After the pods were cut longitudinally and aseptically into halves, the seeds were scooped out with forceps into a 125 ml Erlenmeyer flask containing 20 ml of sterile DW. The seeds were then incubated in darkness on a shaker at 25 ± 2°C for 2 weeks. These imbibed seeds were used as donor explants for the callus induction experiment. To evaluate the fertility of seeds in each capsule, the viable seeds were examined by tetrazolium (TZ) analysis or the TTC test (Vujanovic *et al.* 2000). Seeds were placed in 1 ml of 1% 2,3,5 triphenyl tetrazolium chloride (TTC; Merck, Germany) at room temperature in darkness. After 24 h of incubation, the seeds were washed with DW three times and then observed under a stereo microscope (Zeiss, Stemi DV 4). Only the dark red-colored embryos were scored as viable. Two replicates were performed and five times (each replicate) were sampled. Seed viability (SV) was reported as a percentage which was calculated from the equation:

% SV = [Red embryos/(Numbers of red embryos + colorless embryos)] × 100

Callus induction

From preliminary results (unpublished data), modified Vacin and Went (VW) medium containing 0.1 mgl^{-1} 1-phenyl-3-(1,2,3- thiadianol-5-yl) urea (thidiazuron or TDZ; Sigma, USA), 1 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma) and 0.2% Phytagel (Sigma, USA) was chosen as the standard medium for callus induction experiments. To decrease the amount of necrotic tissues, which is common in callus cultures, the antioxidant polyvinylpolypyrrolidone (PVPP; MW 40,000; Amresco, USA) (at 0, 0.2 and 0.5%) and 0.2% AC (Riedel-de Haën AG, Germany) were added as optional additives according to the experimental objectives. The pH of the media was adjusted to 5.3 with 1 N NaOH or HCl prior to autoclaving at 121°C for 20 min. The precultured-seeds were sprinkled onto the above media and incubated in darkness at 25 ± 2°C for 3 months, followed by a 16-h photoperiod at irradiance of 23 $\mu molm^{-2}s^{-1}$ provided by Philips white fluorescent lights. The subculture intervals were 14 days and data were recorded after 3 months of culture. The morphological changes and visual characteristics during callus induction were observed and recorded (Digital camera, Panasonic DMC-FZ 18). After 3 months of cultures, the percentage of callus production [% Callus production = (Number of seeds producing callus/Number of viable seeds inoculated) × 100], were recorded and the data were analyzed statistically. The cultures of 3-month-old calli were also collected for histological observations.

PLB formation

One piece of callus (8 mg in fresh weight) was placed on the surface of culture medium in a bottle containing 10 ml of modified VW medium supplemented with plant growth regulator (PGRs), 0.1 mgl^{-1} 1-napthaleneacetic acid (NAA; Fluka) and 0.5 mgl^{-1} TDZ. This best medium formula was obtained from preliminary experiments (data not shown). To examine the effect of sucrose with and without PGRs on the formation of PLBs, three levels (10, 20 and 30 gl^{-1}) of sucrose were separately added to the media with and without PGRs. The cultures were maintained under a 16-h photoperiod at illumination conditions similar to those used in the callus induction experiment, as previously described. Subculturing was conducted monthly. The increase in fresh weight of callus-derived PLBs (Final fresh weight – Initial fresh weight inoculated) and the percentage of PLB formation (% PLB formation) were evaluated after 4 months of culture. This percentage was calculated from the equation:

% PLB formation = (Number of callus pieces forming PLBs/Number of callus pieces inoculated) × 100.

Plant regeneration

To study the effects of adding potato (*Solanum tuberosum*) and banana homogenates (*Musa* AAA group, cv 'Kluai Hom Thong') during the shoot and root induction stages, PLBs were transferred onto PGR-free medium for 1 month to nullify any carry-over effects of the inductive PGRs. Then clusters of about 2 mm-long PLBs (20 mg fresh weight/bottle) were inoculated onto modified MS medium supplemented with potato homogenate (20 and 50 gl^{-1}) or banana homogenate (20 and 50 gl^{-1}). The fresh potatoes and ripe bananas were peeled, cut into small pieces (1 cm^3 sections). The small pieces of potatoes were boiled for 10 min with 100 ml of DW and blended. The banana homogenate was prepared by blending pieces of banana with 100 ml DW. These homogenates were added to the medium before the pH was adjusted (Lee and Lee 2003). Ten bottles were used for each treatment. The incubation conditions were the same as previously described. The numbers of shoots and roots that formed from each responding PLB were recorded after 4 months of culture and was evaluated as the mean number of shoots or roots per 10 mg initial PLBs which was calculated from the equation: Shoots (or roots) = [Number of shoots (or roots) observed]/2. The mean numbers of shoots and roots per 10 mg initial PLBs were determined although the initial fresh weight of PLBs was about 20 mg/clump, inoculated in each bottle. Because the minimal amount of 20 mg/clump of initial PLBs was required for further development (personal obs.). Thereafter, some plantlets were transplanted into mini-pots and placed in a shaded greenhouse at an intensity of 30 $\mu molm^{-2}s^{-1}$ photosynthetic photon flux (PPF) under natural conditions (approximately 28 ± 2°C).

Statistical analysis

Experiments were assigned in a completely randomized design (CRD). Ten replicates were performed for each treatment in all experiments, namely, callus induction, PLB formation and plant regeneration. For callus induction, observations were made every month for 3 months. For formation of PLBs and regeneration of plants, increases in fresh weight of PLBs as well as the number of shoots and roots were recorded after 4 months of culture, respectively. All data were separately analyzed statistically by a one-way ANOVA for each set of experiments and treatment means were compared by Duncan's multiple range test (DMRT) and the LSD test at a significance level of $P = 0.05$.

Histological and histochemical observations

Tissues samples were fixed in FAA II (formaldehyde: glacial acetic acid: 70% ethyl alcohol; 1: 1: 18 v/v/v) for 48 h, dehydrated in a tertiary-butyl-alcohol series, embedded in paraplast plus, sectioned at 6 μm (for callus and PLBs) and 8 μm (for plantlets) thickness using a rotary microtome (AO, 820 SPENCER). The sections were stained with Delafield's hematoxylin and safranin (Johansen 1940; Ruzin 1999) to investigate the developmental patterns of the callus-derived plantlets. To investigate the accumulation of phenolics, carbohydrates and lipid, the sections of treated calli were stained with 1% toluidine blue O (TBO) (Ruzin 1999), Periodic acid Schiff (PAS) (Feder and O'Brien 1968; Ruzin 1999) and Sudan IV (Ruzin 1999), respectively. These sections were observed with a light microscope (Olympus, BX 51) and an in-built digital camera (Olympus, DP 71).

RESULTS AND DISCUSSION

Determination of seed viability

Seed viability of 6-month-old *Paphiopedilum niveum* capsules was about 23-30%.

Callus induction

Seeds were cultured on modified VW solid medium supplemented with 0.1 mgl^{-1} TDZ and 1 mgl^{-1} 2,4-D. Seed-derived calli were obtained within 3 months after culture on all media, namely, modified VW medium (control), modified VW medium with 0.2% AC and modified VW medium containing 0.5% PVPP (**Fig. 1**). There were no significant differences among the number of calli obtained from the

Table 1 Effect of polyvinylpolypyrrolidone (PVPP) and activated charcoal (AC) on callus induction of *Paphiopedilum niveum* (Rchb.f.) Pfitz. Data after 3 months of culture.

Treatments [a]	**Percentage of seeds producing calli** [b] **(mean ± S.E.)**	**Visual observations of browning** *
Modified VW (control)	60.00 ± 18.71 ab	++
Modified VW + 0.2% AC	88.89 ± 7.35 a	-
Modified VW + 0.2% PVPP	0	unavailable
Modified VW + 0.5% PVPP	38.89 ± 13.89 b	+

[a] All treatments were cultured in dark conditions for a month followed by a 16/8 light/dark photoperiod at an intensity of 23 µmol m^{-2} s^{-1}.
[b] The different letters indicate significant differences at $P < 0.05$ with Duncan's multiple range test
++, dark brown; +, brown; -, light brown or no browning; unavailable, no callus growth

control and other treatments (**Table 1**). Thus, AC and PVPP had no effect on the stage of callus induction. The results clearly show that *in vitro* explants required exogenous PGR to regulate cell division, a process essential for callus formation (Roy and Banerjee 2003). The highest amount of browning was obtained from the medium without any additional antioxidant (control) (**Fig. 1A**). In contrast, the lowest amount of tissue browning was obtained in medium containing 0.2% AC. Moreover, the callus from this medium was vigorous and was yellow (**Fig. 1B**). The callus cultured on medium supplemented with 0.5% PVPP was light brown in color (**Fig. 1C**). The presence of either AC or PVPP decreased necrosis during culture, although the necrosis of explants was common and varied in frequency depending on the PGR concentration (Roy and Banerjee 2003). The addition of 0.2% AC was required for improved callus production. From visual observations, there was no callus growth on modified medium containing 0.2% PVPP and this proved to be inhibitory. It was possible that callusing from this treatment was extremely slow. So, 0.2% AC was an important component to add to, reduce or avoid the accumulation of phenolic compounds. AC has a very large specific area ranging from 600 to 2000 m^2/g available for adsorption. It has an adsorption preference for moderately polar (phenolic compounds) rather than apolar or highly polar organics (Pan and van Staden 1998). Hoque and Arima (2002) reported that phenolic compounds excreted by explants during some stages of callus induction resulted in the loss of growth capacity and tissue death. Our histological and histochemical investigations confirmed that the cultures on medium supplemented with 0.2% AC had a greatly increased potential to induce callus from seeds compared with other treatments. The calli formed clusters of meristematic tissues comprising small isodiametric cells with large nuclei, numerous small vacuoles and no intercellular spaces (**Fig.**

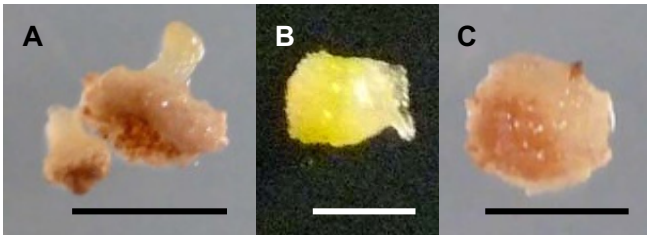

Fig. 1 Photographs showing 3-month-old seed-derived callus of *Paphiopedilum niveum* (Rchb.f.) Pfitz. (**A**) Necrosis callus on modified VW (control). (**B**) Compact, bright yellowish callus on modified VW medium supplemented with 0.2% AC and showing no tissue browning. (**C**) Pale brownish callus on modified VW containing 0.5% PVPP. Bars (in µm): **A** = 3000; **B** = 1000; **C** = 1500.

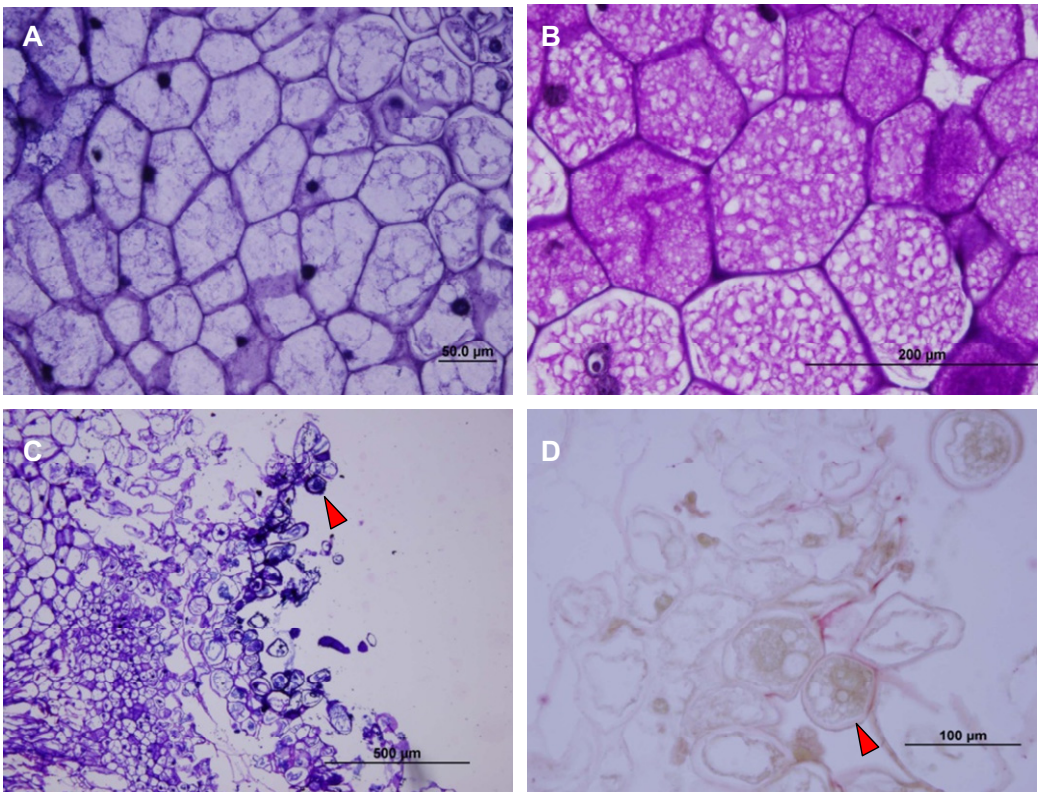

Fig. 2 Histological features of calli cultured on (A-B) modified VW supplemented with 0.2% AC and (C-D) without AC (as control). (**A**) Calli showing the characteristics of meristematic tissue and (**B**) carbohydrate accumulation (PAS test). (**C**) Calli showing the dark blue color (arrow head) of phenolic accumulation (TBO test) and (**D**) the brownish orange (arrow head) of lipid compounds (Sudan IV test). Bars (in µm): **A** = 50; **B** = 200; **C** = 500; **D** = 100.

Table 2 Effect of sucrose and plant growth regulators (PGR) on the formation of callus-derived PLBs of *P. niveum* (Rchb.f.) Pfitz. after 4 months of culture.

Presence of PGR	Sucrose (%)	Increased fresh weight of PLBs [a] (mg)	PLBs formation[b] (%)	Color of explant
+	0	0.00 ± 0.00 b	0	Brown
+	1	142.86 ± 84.52 a	57.16	
+	2	48.14 ± 31.74 ab	28.58	
+	3	28.00 ± 28.00 b	14.29	
-	0	0.00 ± 0.00 b	0	Brown
-	1	0.00 ± 0.00 b	0	Brown
-	2	0.00 ± 0.00 b	0	Brown
-	3	0.00 ± 0.00 b	0	Brown

Comparison of the mean values was analyzed using Duncan's multiple range test. Values with different letters indicate significant differences at $P < 0.05$.
[a] Initial fresh weight of each callus piece was 8 mg. Increased in fresh weight of PLBs (IFW) was calculated from the equation: IFW = Final fresh weight – Initial fresh weight
[b] Percentage of PLB formation = (Number of callus piece providing PLBs/Number of callus piece inoculated) * 100

2A) (Esau 1964). The calli cultured on medium supplemented with 0.2% AC showed that cells accumulated carbohydrate (PAS test) (**Fig. 2B**) with no accumulation of phenolic compounds (TBO test). This indicated the presence of more active protoplasts (Esau 1964) than the control. The calli cultured on medium without any antioxidant (control) exhibited tissue browning, accumulation of phenolic compounds (**Fig. 2C**) (TBO test), reserve lipid (Sudan IV staining) (**Fig. 2D**) and no carbohydrate accumulation.

PLB formation

PGRs and sucrose at different concentrations were tested for their effects on PLB proliferation and PLB or somatic embryo (Ishii *et al.* 1998) formation. Germination of PLBs occurred in all cultures treated with a combination of PGRs and at all concentrations of sucrose. There were significant differences between the increased fresh weight of PLBs and the different percentages of sucrose in all media containing PGR. The highest increase of fresh weight (142.86 ± 84.52 mg) and the highest percentage (57.16) of formed PLBs was derived from the callus cultured on medium supplemented with PGRs (0.1 mgl^{-1} NAA and 0.5 mgl^{-1} TDZ) and 1% sucrose. In the absence of PGRs either with or without sucrose as well as the presence of PGRs in the absence of sucrose, the calli were brown, failed to proliferate and eventually formed no PLBs (**Table 2**). There was no increase in the fresh weight of PLBs and no newly formed PLBs appeared on the medium to which PGRs had been added and that lacked sucrose and all PGR-free medium with or without sucrose. It was therefore essential to transfer the callus of *P. niveum* to a PGR medium supplemented with sucrose for PLB formation. This callus required PGRs and sucrose for PLB differentiation. An interactive effect of PGRs and sucrose was clearly reported as an important role in the induction of somatic embryogenesis. PGRs play a role in the induction of either unorganized callus growth or polarized growth leading to somatic embryogenesis in *Psidium guajava* L. cv. 'Banarasi local' (Rai *et al*. 2007). It was reported that, in most cases, PGRs particularly auxin, is required for the induction of somatic embryogenesis but inhibitory for somatic embryo development. Embryo development and maturation were observed in the absence of PGRs. Similarly, in this present study PLB (somatic embryo) development and maturation of *P. niveum* were reported in PGR-free medium. The sugars probably play multiple roles during somatic embryogenesis. They serve mainly as carbon and energy sources, osmotica, stress protectants and signal molecules (see review by Lipavská and Konrádová 2004). They also revealed that PGRs have a primary directing effect while exogenous sugar is a medium component playing a dominant role during conifer somatic embryogenesis. Iraqi *et al*. (2005) showed that sucrose not only acts as a carbon source for embryogenetic tissues but also functions as factors modulating the genes coding for enzymes involved in carbohydrate metabolism during somatic embryogenesis in black spruce. Li *et al*. (2005) reported that the entire sucrose-pretreated PLBs could induce new PLBs and pretreating PLBs with 0.5 M sucrose increased single-cell embryogenesis 3-to 4-fold, which might be suitable for genetic transformation of *Oncidium*. However, the present study was not similar to PLB formation in *Phalaenopsis* Richard Shaffer 'Santa Cruz'. It was reported that sucrose influenced the formation of callus-derived PLB. Thus, the *Phalaenopsis* calli easily formed PLBs after being transferred to a medium without sucrose (Ishii 1998).

The histological study revealed that a cluster of cells appeared from the surface of the callus mass (**Fig. 3A**). These cell clusters contained small cells, dense cytoplasm and large nuclei that were characterized as meristematic. These cells continued to grow (**Fig. 3B**) and emerged as yellow globular PLBs. Then, these PLBs eventually formed shoot and root buds. PLBs originated from the surface layer of callus masses. This is similar to *Phalaenopsis*, in which pro-embryo-like structures formed on the surface of the totipotent callus mass before formation of PLB clusters (Chen *et al.* 2000). In addition, *P. niveum* PLBs could further produce secondary PLBs that resulted in an increase of the fresh weight of PLBs (**Fig. 3C**). This is a characteristic feature of many orchids such as in *Dendrobium fimbriatum* (Roy and Banerjee 2003).

Plant regeneration via PLBs

PLBs (20 mg initial fresh weight/PLB cluster) were transferred to PGR-free modified MS media for 1 month; they were then cultured on the same media with various additives (potato and banana homogenates). Regeneration of plantlets was induced from PLBs on modified MS medium with different concentrations of potato and banana homogenate (0, 20 and 50 gl^{-1}) (**Table 3**). The mean number of regenerated shoots was evaluated after 4 months of culture. Most shoots (**Fig. 4C**) were present when PLBs were cultured on medium with 20 gl^{-1} banana homogenate (90 ± 45). Huang *et al*. (2001) reported that medium containing 20 gl^{-1} banana powder could enhance shoot proliferation of *P. phi-*

Table 3 Effect of potato and banana homogenates on regeneration of *P. niveum* (Rchb.f.) Pfitz. plants.

Organic supplement (gl^{-1})		Mean shoot number per 10 mg initial PLBs ± S.E.[a]	Mean root number per 10 mg initial PLBs ± S.E.[a]
Potato homogenate	Banana homogenate		
0	0	16.7 ± 4.20 b	0.80 ± 0.80 ab
20	0	39.7 ± 18.1 ab	0.10 ± 0.10 b
50	0	58.1 ± 37.6 ab	0.10 ± 0.10 b
0	20	89.6 ± 45.5 a	1.00 ± 0.75 ab
0	50	31.8 ± 17.6 ab	3.00 ± 1.35 a

Comparisons of the mean values were analyzed using Duncan's multiple range test. Values with different letters indicate significant differences at $P < 0.05$.
[a] Initial of PLB clump was 20 mg. The mean numbers of shoots and root per 10 mg initial PLBs were shown. Means shoots (or roots) numbers per 10 mg initial PLBs = [Number of shoots (or roots) observed]/2.

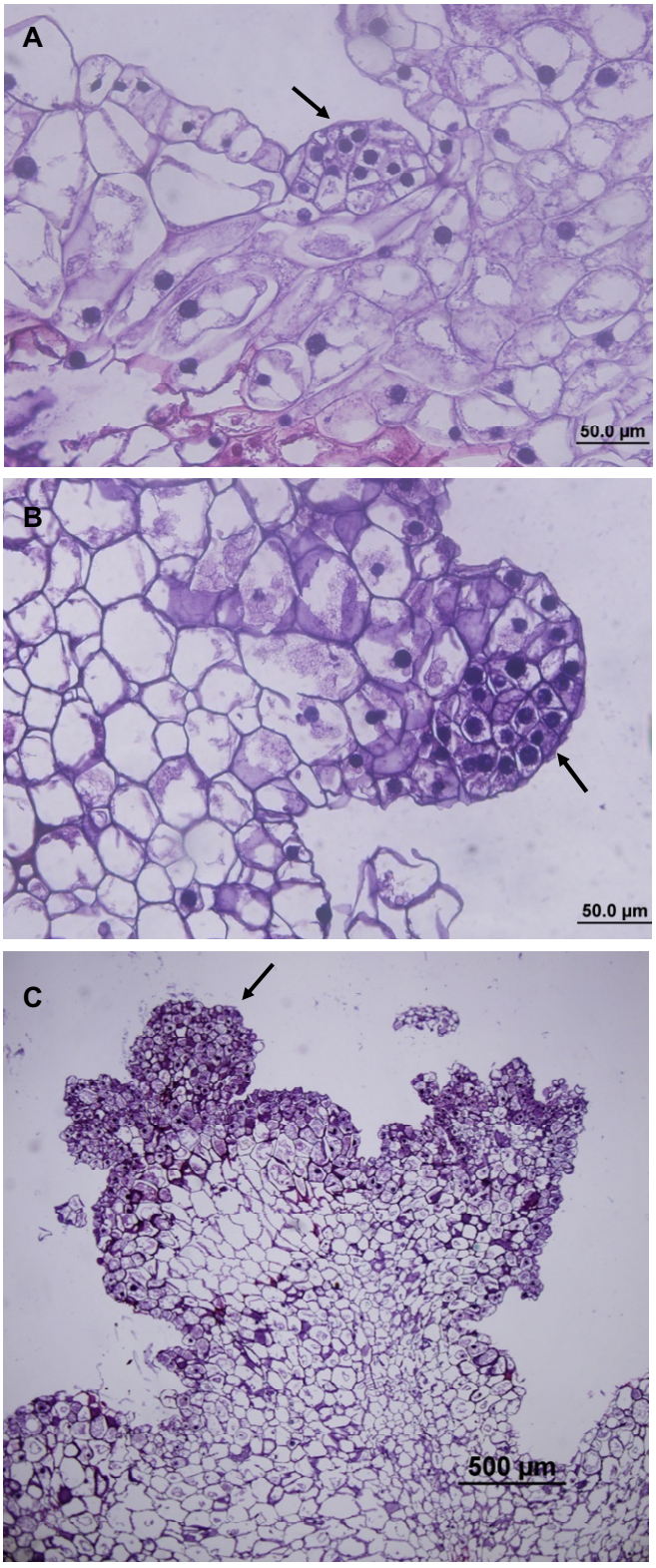

Fig. 3 Micrographs of somatic embryo development of *Paphiopedilum niveum* (Rchb.f.) Pfitz. obtained from callus cultured on PGR-modified VW medium supplemented with 10 gl^{-1} sucrose. (**A**) A 1-month-old pro-embryonic stage (12 cells stage) originates at the periphery of the callus mass (arrow) and (**B**) eventually forms the early stage of a globular-shape (arrow). (**C**) Secondary PLBs (arrow) formed on the surface of the somatic embryo. Bars (in µm): **A, B** = 50; **C** = 500.

lippinense × *P.* Susan Booth. Homogenized banana is often reported to promote growth in orchid culture but the reason for the stimulatory effect of this addition is unknown. Some reports have mentioned that the medium supplemented with banana autoclaved at high concentration, high pressure and acidic conditions might liberate growth-promoting natural additives such as biotin, vitamins B1, B2 and C, amino acids (lysine, cysteine, methionine, arginine), minerals (K, P, Ca and Fe; Tawaro 2005 cited in Barnell 1940) and PGRs (GA_7 and GA_x; Vyas *et al*. 2009), IAA and zeatin (Vyas *et al*. 2009). On the other hand, a significant increase of the rooting response was obtained from shoots cultured on 50 gl^{-1} of blended banana (6.0 ± 2.7). Tawaro (2005) (cited from Arditti and Ernst 1993) reported that Fe in banana is present in a utilizable form that stimulates the growth and root formation of orchids.

Histological observations also showed that the shoots produced consisted of shoot and root apical meristems (SAM and RAM) and this could indicate the initiation of plant regeneration. These shoots were connected to each other at their base (**Fig. 4E**). From visual observation, plantlets on this medium also produced healthy shoots and roots (**Fig. 4F**). This result appeared to be similar to those shown for protocorms of *Dendrobium lituiflorum* Lindl. that developed into rooted and strengthened plantlets on Knudson C medium supplemented with 25% (v/v) banana extract (Vyas *et al*. 2009). Arditti and Pridgeon (1997) also suggested that the addition of banana homogenate to a medium enhanced the growth of plantlets. In contrast, medium supplemented with potato homogenate promoted PLB survival and rooting in a tropical slipper orchid, *Paphiopedilum* hybrid (Lin *et al.* 2000) and a temperate slipper orchid, *Cypripedium formosanum* (Lee and Lee 2003). However, the effect of organic additives on various orchid species has indicated that different orchids require different organic compounds. Thus, incorporation of organic additives into the medium is not always effective and their effects are not consistent. The effectiveness of inducing changes depends on the basal medium and the species of orchid. After transplanting into mini-pots in the shade greenhouse, plantlets grew well (**Fig. 5**).

This is the first report of a *P. niveum* (Rchb.f) Pfitz orchid in which the production of calli occurred from germinating seeds in the presence of a low concentration of PGR and AC. Although the frequency of calli formation was still low, the calli exhibited regenerative potential to form PLBs and subsequently regeneration of plants. Plantlets obtained from callus-derived PLBs usually involve somatic embryogenesis (Zhao *et al*. 2008; Yu *et al*. 2009).

In conclusion, calli of *P. niveum* (Rchb.f) Pfitz orchid were induced from germinating seeds on modified VW medium containing 0.1 mgl^{-1} TDZ, 1 mgl^{-1} 2,4-D and 0.2% AC. The subcultured calli were transferred to modified VW medium supplemented with 0.1 mgl^{-1} NAA, 0.5 mgl^{-1} TDZ and 1% sucrose to produce PLBs, including secondary PLBs. The embryogenic calli of this orchid species required both sucrose and PGRs for PLB differentiation through the embryogenenic pathway. The PLBs developed into plantlets (with shoot and root poles) on modified PGR-free MS medium supplemented with 20-50 gl^{-1} banana homogenate as an organic additive. Histological evidence of somatic embryogenesis demonstrated that plant regeneration via formation of PLBs from seed-derived callus was an effective system for large-scale propagation of *P. niveum*. It was estimated that by using the protocol described here approximately 90 regenerated shoots could be formed from 10 mg fresh weight of a PLB cluster after being transferred to modified MS medium containing 20 gl^{-1} of blended banana.

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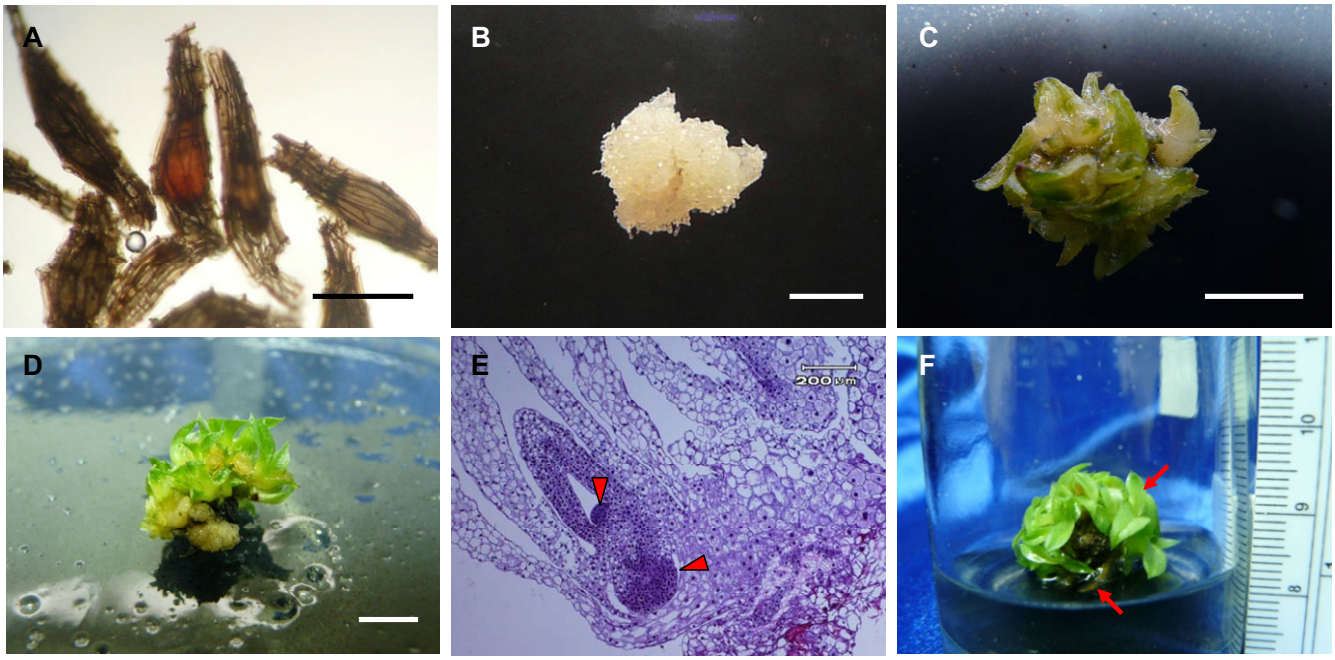

Fig. 4 Plant regeneration from seed-derived calli of *Paphiopedilum niveum* (Rchb.f.) Pfitz. (**A**) Viable seeds for callus induction. (**B**) Mass of yellow callus with small protuberances. (**C**) Cluster of regenerated shoots on modified MS medium supplemented with 20 gl^{-1} banana homogenate. (**D**) Cluster of PLB-differentiated shoots and roots cultured on modified MS medium supplemented with 50 gl^{-1} banana homogenate. (**E**) Longitudinal section of a shoot cluster at the stage shown in **D** shoot (arrow) and root (arrow head) apical meristems. These shoots connect with each other at their base. (**F**) PLB-derived plantlets with healthy shoots and roots (arrow) after 4 months of culture on modified MS medium supplemented with 50 gl^{-1} banana homogenate. Bars (in µm): **A** = 250; **B** = 1000; **C** = 5000; **D** = 6000; **E** = 200.

Fig. 5 A sample of a complete plantlet of *Paphiopedilum niveum* (Rchb.f.) Pfitz. on vermiculite after being transferred to the greenhouse for a month.

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