Propagation of Ornamental Plants
Vol. 00, № 0, 2000: 00-00

FACTORS AFFECTING THE DIRECT SOMATIC EMBRYOGENESIS FROM LEAF EXPLANTS OF *PHALAENOPSIS APHRODITE* RCHB. F. SUBSP. *FORMOSANA* CHRISTENSON

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Abstract

The effects of NaH_2PO_4, sucrose, activated charcoal, polyvinylpyrrolidone (PVP), and strength of MS medium, were studied to optimize direct somatic embryogenesis from leaf explants of *Phalaenopsis aphrodite* subsp. *formosana*. The results showed that full- and quarter-strength macroelements of MS medium were not suitable for direct embryo induction from leaf explants. Thus, a half-strength macro- and full-strength microelements of MS nutrients plus full-strength of MS vitamins, 170 mg l^{-1} NaH_2PO_4, 1 g l^{-1} peptone, 3 mg l^{-1} TDZ, and 20 g l^{-1} sucrose is proposed as a suitably modified medium. In addition, PVP at 0.25 g l^{-1} significantly promoted direct embryogenesis on the cut ends of the explants, but activated charcoal at 0.5 - 1 g l^{-1} was inhibitory.

Key words: activated charcoal, embryogenic competence

INTRODUCTION

Phalaenopsis orchids are popular in international flower markets and have high commercial value as cut flower and potted plant production. Conventional *in vitro* culture protocols had been developed for propagation of this genus mainly via protocorm-like body formation, shoot multiplication and callus culture (Tanaka et al. 1975, Arditti and Ernst 1993, Tokuhara and Mii 1993, Ernst 1994, Chen and Piluek 1995, Duan et al. 1996, Ishii et al. 1998, Islam and Ichihashi 1999, Chen et al. 2000, Park et al. 2000, 2002, Tokuhara and Mii 2001). Recently, more efficient regeneration systems through direct somatic embryogenesis had been developed using leaf cultures (Kuo et al. 2005, Chen and Chang 2006, Gow et al. 2008, 2009). However, further systematic investigations on medium composition and physiological status are needed to optimize the protocol for practical use in regenerating transgenic plants or mass propagation of this orchid. The aim of this report is to study the effects of NaH_2PO_4, sucrose, and strength of MS medium, activated charcoal and polyvinylpyrrolidone on direct somatic embryogenesis using leaf culture system of *Phalaenopsis aphrodite*.

MATERIALS AND METHODS

Plant materials

In vitro grown seedlings of *Phalaenopsis aphrodite* Rchb.f. subsp. *formosana* Christenson (formerly also referred to as *Phalaenopsis amabilis*) were purchased from Taiwan Sugar Corporation (TSC), Chiayi, Taiwan. The plants were maintained on a plant growth regulator (PGR)-free half-strength MS (Murashige and Skoog 1962) medium in 250 ml flasks with a 2-month-interval subculture period and 2 subcultures. All of the cultures were incubated under a 16/8-h (light/dark) photoperiod at photosynthetic photon flux density of 32 μmol m^{-2} s^{-1} (daylight fluorescent tubes FL-30D/29, 40 W, China Electric Co., Taipei, Taiwan) and temperature of 26 ± 1°C. The seedlings with 3-5 leaves and 2-4 roots were used as donor plants.

Somatic embryo induction in darkness

The basal medium for somatic embryo induction was a modified MS medium containing half-strength macro- and full-strength microelements and supplemented with (mg l^{-1}) myo-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1,000), NaH_2PO_4 (170), sucrose (20,000), thidiazuron (3.0), and Gelrite (2,500). The pH of the variants of medium was adjusted to 5.2 with 1M KOH or HCl prior to autoclaving at 121°C for 15 min. Leaf tip segments (about 1 cm in length) taken from the donor plants were used to induce direct somatic embryogenesis on different variants of the medium.

Received: November 7, 2013 *Accepted: January 10, 2014*

The leaf explants were placed adaxial-side-up on the culture medium and were incubated in 90 × 15 mm^2 Petri dishes under darkness for 2 months in an incubator at temperature of 26 ± 1°C. Modification of medium composition including sucrose (0, 10, 20, 30, and 40 g l^{-1}), NaH_2PO_4 (0, 42.5, 85, and 170 mg l^{-1}), MS medium strength (full-strength macro- and microelements, half-strength macro- and full-strength microelements as half-strength, and quarter-strength macro- and full-strength microelements as quarter-strength), activated charcoal (0, 0.5, 1.0, and 2 g l^{-1}), and polyvinylpyrrolidone (PVP; 0, 0.1, 0.25, and 0.5 g l^{-1}) were used to test their effects on direct somatic embryo formation.

Somatic embryo development in light

Leaf-derived embryos were transferred onto a PGRs-free half-strength MS medium in 250 ml flasks under a light condition with 16/8-h (light/dark) photoperiod at photosynthetic photon flux density of 32 µmol m^{-2} s^{-1} and temperature of 26 ± 1°C for 45 days.

Histological analysis

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).

Scanning electron microscopy (EM) observations

Samples for scanning EM were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 4 h at 4°C, and then dehydrated in ethanol (Dawns 1971), dried using a critical point dryer (HCP-2, Hitachi), and coated with gold in an ion coater (IB-2, Giko Engineering Co.). A scanning EM (DSM-950, Carl Zeiss) was used for examination and photography of the samples.

Data analysis

The percentage of explants forming somatic embryos was recorded as those formed from entire explants or different parts of the explants (LT: leaf tips; Ad: adaxial sides; Ab: abaxial sides; CE: cut ends). The number of embryos formed from each responding explant was counted under a stereomicroscope (SZH, Olympus, Tokyo, Japan) at the protocorm stage. Data were scored after 60 days of culture. Five replicates (dishes) each with four leaf explants were provided for each treatment. The data expressed as percentages were transformed using arcsine prior to ANOVA and than converted back to the original scale (Compton 1994). All means were compared by following Duncan's Multiple Range Test (Duncan 1955). Significant differences between means were presented at the level of $p \leq 0.05$.

RESULTS AND DISCUSSION

The morphogenetic pathway of direct embryogenesis

When leaf explants of *P. aphrodite* were cultured on TDZ-containing half-strength MS medium supplemented with 20 g l^{-1} sucrose, pale yellow-green globular embryos were obtained after 45 days of culture in darkness (Table 1, Fig. 1A). These embryos subsequently turned green, enlarged and developed scale leaves 45 days after transfer onto PGRs-free half-strength MS medium in light condition (Fig. 1B). The histological study revealed that the epidermal cells had undergone a process of dedifferentiation and gained mitotic ability to form meristematic cells (Fig. 2A). Subsequently, the

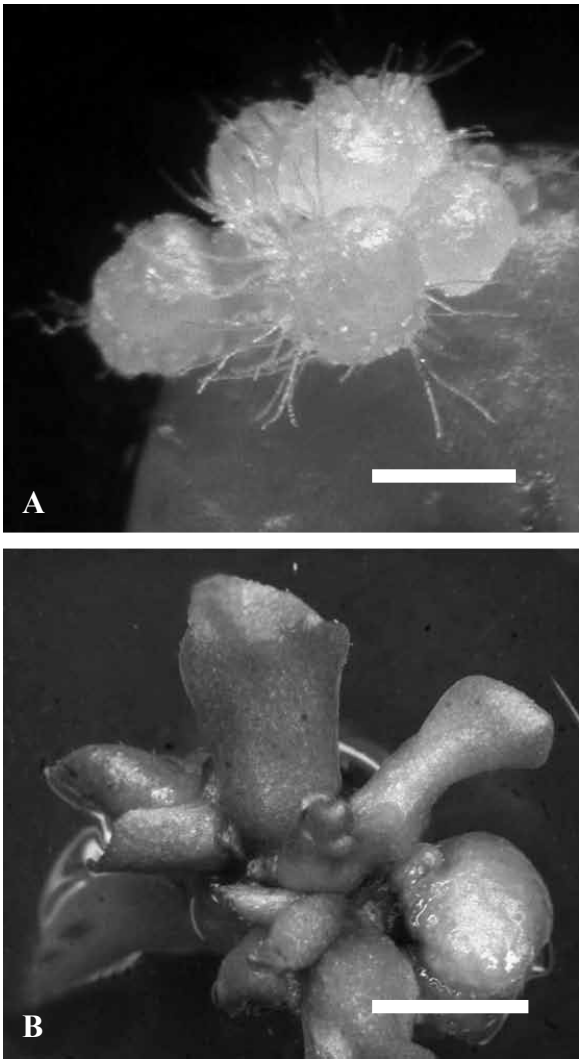

Fig. 1. Direct somatic embryogenesis from leaf explants of *P. aphrodite.* A) Somatic embryos with absorbing hairs formed on a leaf explant after 45 days of culture on half-strength MS medium with 3 mg l^{-1} TDZ in darkness (*Scale bar* = 1.5 mm), B) Green, enlarged embryos with developing leaves after 45 days of culture after transfer the somatic embryos shown in (A) to PGR-free half-strength MS medium in light (Scale bar = 4 mm).

Table 1. Effect of sucrose on direct somatic embryogenesis from leaf explants of *P. aphrodite*.

Sucrose (g l^{-1})	Explants with embryogenesis (%)	Explants with browning (%)	Part of explant with embryogenesis (%)				No. of embryos per responding explant
			CE	Ad	Ab	LT	
0	0 ± 0.0 d	65 ± 3.5 a	0 ± 0.0 c	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 c
10	40 ± 9.1 ab	35 ± 7.4 ab	25 ± 3.5 b	30 ± 3.5 a	0 ± 0.0 b	5 ± 3.5 b	7.5 ± 0.4 b
20	65 ± 7.9 a	15 ± 3.5 b	55 ± 7.6 a	25 ± 3.5 a	15 ± 3.5 a	40 ± 3.5 a	7.8 ± 0.6 b
30	10 ± 3.5 cd	50 ± 7.9 a	0 ± 0.0 c	10 ± 0.0 ab	0 ± 0.0 b	0 ± 0.0 b	10.5 ± 1.1 a
40	5 ± 3.5 bc	65 ± 3.5 a	10 ± 3.5 c	10 ± 3.5 ab	0 ± 0.0 b	0 ± 0.0 b	11.0 ± 1.4 a

Means within a column followed by the same letter are not significantly different according Duncan's multiple range test at $p \leq 0.05$ (Duncan 1955).

meristematic cells gave rise to form somatic embryos without the intervening of callus tissues (Fig. 2B). These leaf-derived embryos developed and consist of scale leaves and the vascular tissue on the parent explants (Fig. 2C). Scanning EM observation revealed that the subepidermal cells were also able to divide into meristematic cells, thus forming protuberances through the epidermis (Fig. 3A). Single-state embryos formed on surfaces of explants with scattering dedifferentiated leaf cells (Fig. 3B). When a mass of leaf cells were induced to dedifferentiate, it became easier to form multiple-state of embryos (Fig. 3C). In addition, asynchronous formation of embryos was frequently found on the explants (Fig. 3C). The foliar embryos had the ability to form secondary embryos from their anterior end when they were still on the parent explants (Fig. 3D).

Effect of sucrose

In *Oncidium* orchid tissue culture, concentration of sucrose significantly affected somatic embryogenesis from leaf explants (Chen and Chang 2002, Su et al. 2006). Without sucrose, leaf explants of *P. aphrodite* failed to form embryos with necrosis after 2 months of culture on TDZ-containing medium (Table 1). Sucrose at 20 g l^{-1} gave the most suitable results with highest percentage of explants with embryogenesis from the entire explant and lowest percentage of explants with browning (Table 1). Except for the adaxial side, sucrose at 20 g l^{-1} gave significantly higher percentage of explants with embryogenesis when compared with other concentrations on the leaf parts (Table 1). Higher concentrations of sucrose resulted in lower embryogenic

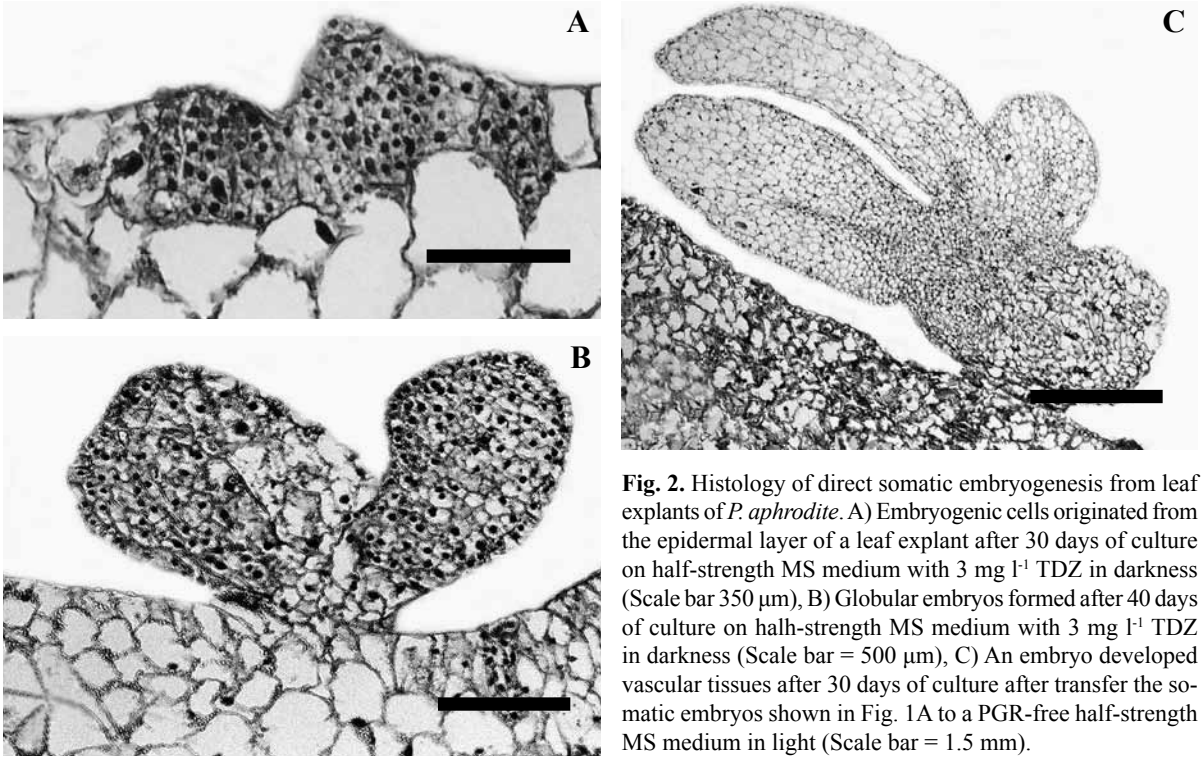

Fig. 2. Histology of direct somatic embryogenesis from leaf explants of *P. aphrodite*. A) Embryogenic cells originated from the epidermal layer of a leaf explant after 30 days of culture on half-strength MS medium with 3 mg l^{-1} TDZ in darkness (Scale bar 350 μm), B) Globular embryos formed after 40 days of culture on halh-strength MS medium with 3 mg l^{-1} TDZ in darkness (Scale bar = 500 μm), C) An embryo developed vascular tissues after 30 days of culture after transfer the somatic embryos shown in Fig. 1A to a PGR-free half-strength MS medium in light (Scale bar = 1.5 mm).

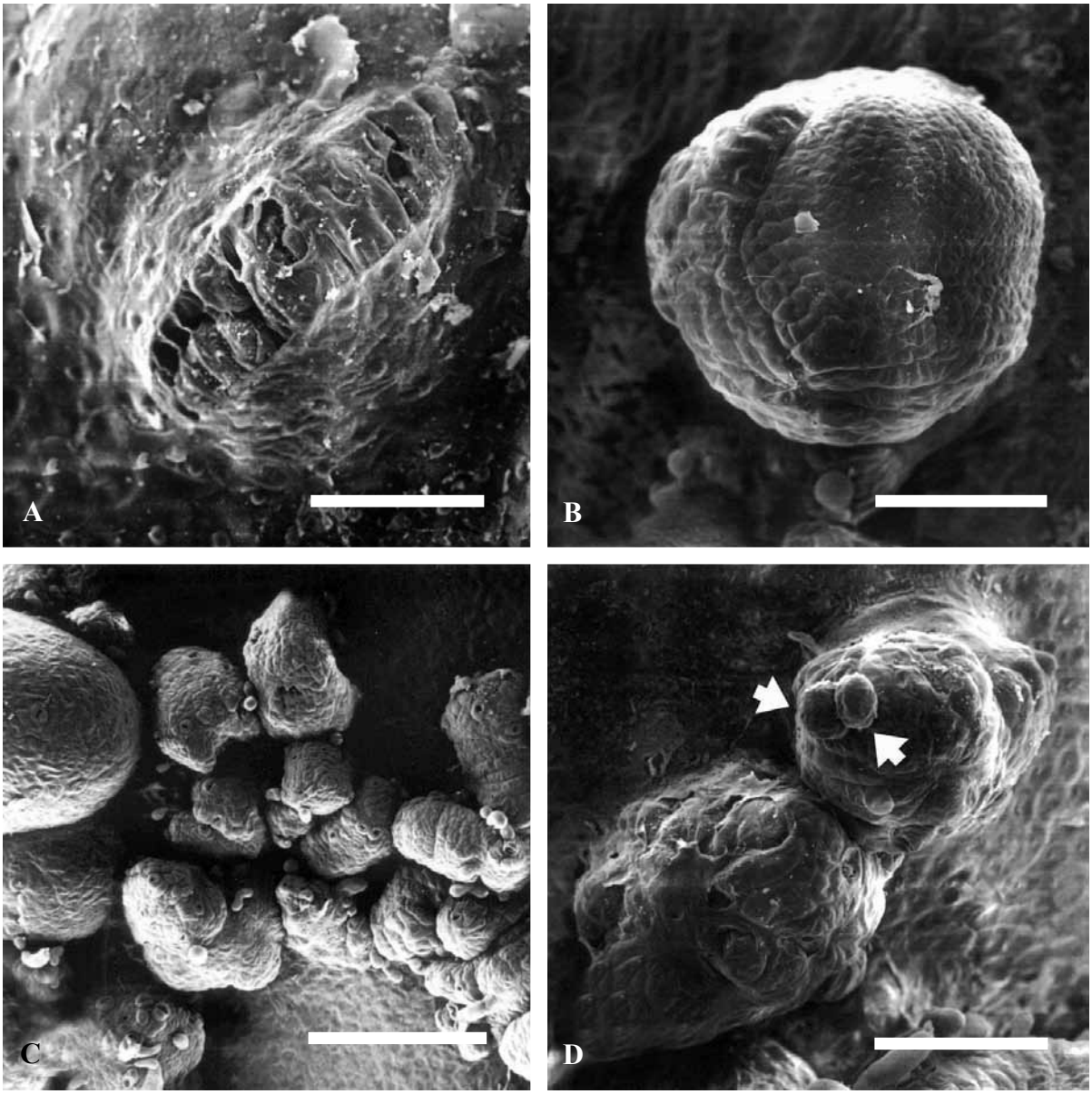

Fig. 3. Scanning electron microscopic observation on direct somatic embryogenesis from leaf explants of *P. aphrodite*. A) An early event of direct embryogenesis from subepidermal cells (Scale bar = 100 μm), B) A globular embryo (Scale bar = 200 μm), C) Embryos with scale leaves formed on a leaf explant (Scale bar = 350 μm), D) Secondary embryos (arrow) formed on a primary embryo (Scale bar = 200 μm.

Table 2. Effect of NaH_2PO_4 on direct somatic embryogenesis from leaf explants of *P. aphrodite.*

NaH_2PO_4 (mg l^{-1})	Explants with embryogenesis (%)	Explants with browning (%)	Part of explant with embryogenesis (%)				No. of embryos per responding explant
			CE	Ad	Ab	LT	
0	10 ± 3.5 b	50 ± 14.0 a	0 ± 0.0 b	10 ± 3.5 a	5 ± 0.0 ab	5 ± 3.5 b	13.5 ± 0.6 a
42.5	25 ± 3.5 b	30 ± 7.0 a	10 ± 3.5 b	25 ± 14.0 a	0 ± 0.0 b	10 ± 3.5 b	13.5 ± 0.5 a
85	20 ± 3.5 b	30 ± 3.5 a	10 ± 3.5 b	10 ± 7.0 a	0 ± 0.0 b	10 ± 3.5 b	4.3 ± 0.4 c
170	65 ± 7.9 a	15 ± 7.0 a	55 ± 7.6 a	25 ± 14.0 a	15 ± 7.0 a	40 ± 3.5 a	7.8 ± 0.6 b

Means within a column followed by the same letter are not significantly different according Duncan's multiple range test at $p \leq 0.05$ (Duncan 1955).

responses, higher browning rates but higher number of embryos per responding explant (Table 1).

Effect of NaH_2PO_4

Phosphate plays an important role in plant growth and development, and the process of somatic embryogenesis may be greatly influenced by phosphate (Pedroso and Pais 1995). NaH_2PO_4 was usually supplemented in media as an additive phosphate source for *in vitro* culture in *Dendrobium*, *Epidendrum*, *Oncidium*, and *Paphiopedium* (Chen et al. 1999, 2002, Chen et al. 2000, Chen et al. 2004, Chung et al. 2005, 2007). In *Oncidium*, NaH_2PO_4 was found to be effective in induction of direct embryogenesis from leaf cultures and the optimal concentrations was between 85 to 170 mg l^{-1} (Chen and Chang 2002). NaH_2PO_4 at 170 mg l^{-1} resulted in the highest efficiency of direct embryogenesis with 65% of explants forming an average of 7.8 embryos per responding explants. By contrast, other concentrations of NaH_2PO_4 had no significant effects on direct embryogenesis. Except for adaxial and abaxial sides, NaH_2PO_4 at 170 mg l^{-1} gave significantly higher percentage of explants with embryogenesis when compared with other concentrations on the leaf locations (Table 2).

Effect of medium strength

High concentration of nutrients in culture medium seems did not favor *in vitro* culture of *Phalaenopsis* (Arditti and Ernst 1993). In the present study, the result showed that half-strength MS medium was the most suitable one for induction of direct embryo formation from leaf explants of *P. aphrodite* (Table 3). Both full-strength and quarter-strength MS gave lower embryogenic responses and higher browning rates (Table 3). Indeed, half-strength MS medium was used as basal medium for *in vitro* culture of *Dendrobium*, *Epidendrum*, and *Oncidium* (Chen et al. 1999, 2002, Chen et al. 2000, Chung et al. 2005, 2007). Except for the adaxial side, half-strength MS medium gave significantly higher percentage of explants with embryogenesis when compared with other strength on the leaf locations (Table 3).

Effect of activated charcoal

Activated charcoal was usually used in conventional *in vitro* culture medium of *Phalaenopsis* to reduce the toxic effect of phenolic compounds secreted by explants (Arditti and Ernst 1993). However, in the present study, the application of activated charcoal gave a negative effect on direct embryo induction from leaf explants of *P. aphrodite* (Table 4). Activated charcoal doses of 0.5, 1.0, and 2.0 g l^{-1} were all totally inhibitory and likely could be related to the obtained explant browning rates between 55 to 80% (Table 4). Suggestion is that the activated charcoal may absorb TDZ or reduce its activity to induce embryogenesis.

Effect of polyvinylpyrrolidone

Polyvinylpyrrolidone (PVP) is soluble in water and binds to polar molecules exceptionally well, owing to

Table 3. Effect of MS medium strength on direct somatic embryogenesis from leaf explants of *P. aphrodite*.

Strength of MS medium	Explants with embryogenesis (%)	Explants with browning	Part of explant with embryogenesis (%)				No. of embryos per responding explant
			CE	Ad	Ab	LT	
Full	30 ± 3.5 b	40 ± 14.0 a	15 ± 3.5 b	25 ± 10.5 a	0 ± 0.0 b	0 ± 0.0 b	5.2 ± 0.5 c
Half-strength	65 ± 7.9 a	15 ± 7.0 a	55 ± 7.6 a	5 ± 0.0 ab	15 ± 3.5 a	40 ± 3.5 a	7.8 ± 0.6 b
Quarter-strength	20 ± 3.5 b	40 ± 20.0 a	20 ± 3.5 b	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 b	10.0 ± 0.4 a

Means within a column followed by the same letter are not significantly different according Duncan's multiple range test at $p \leq 0.05$ (Duncan 1955).

Table 4. Effect of activated charcoal on direct somatic embryogenesis from leaf explants of *P. aphrodite*.

Activated-charcoal (g l^{-1})	Explants with embryogenesis (%)	Explants with browning (%)	Part of explant with embryogenesis (%)				No. of embryos per responding explant
			CE	Ad	Ab	LT	
0	65 ± 7.9 a	15 ± 3.5 b	55 ± 7.6 a	25 ± 3.5 a	15 ± 3.5 a	40 ± 3.5 a	7.8 ± 0.6 a
0.5	0 ± 0.0 b	55 ± 7.6 ab	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 b
1	0 ± 0.0 b	70 ± 17.5 a	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 b
2	0 ± 0.0 b	85 ± 14.0 a	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 b

Means within a column followed by the same letter are not significantly different according Duncan's multiple range test at $p \leq 0.05$ (Duncan 1955).

Table 5. Effect of PVP on direct somatic embryogenesis from leaf explants of *P. aphrodite.*

PVP (g l^{-1})	Explants with embryogenesis (%)	Part of explant with embryogenesis (%)				No. of embryos per responding explant
		CE	Ad	Ab	LT	
0.00	45 ± 10.5 a	25 ± 3.5 b	20 ± 7.9 a	0 ± 0.0 a	25 ± 14.0 a	7.8 ± 0.3 b
0.10	50 ± 3.5 a	40 ± 7.0 ab	30 ± 7.0 a	5 ± 0.0 a	25 ± 10.5 a	10.7 ± 2.6 a
0.25	60 ± 14.0 a	60 ± 7.9 a	35 ± 3.5 a	10 ± 7.0 a	45 ± 21.0 a	15.5 ± 3.4 a
0.50	50 ± 3.5 a	50 ± 7.0 a	25 ± 14.0 a	5 ± 3.5 a	25 ± 3.5 a	10.6 ± 2.8 a

Means within a column followed by the same letter are not significantly different according Duncan's multiple range test at $p \leq 0.05$ (Duncan 1955).

its polarity. In plant tissue culture media, PVP adsorb not only toxic exudates (phenolics) but also growth regulators and nutrients (Bhat and Chandel 1991). In the present study, the use of PVP significantly enhanced direct embryo induction from cut ends of explants (Table 5). In *Dioscorea alata* L., the exudate from the cut end of the explant was responsible for browning of the culture medium (Bhat and Chandel 1991). Therefore, the suggestion is that PVP may absorb the toxic exudate(s) from cut ends and this way promoted the somatic embryogenesis. According to experimental results, a suitable concentration of PVP would be 0.25 g l^{-1} (Table 5).

According to the present results, a modified MS medium with half-strength macro-, full-strength microelements, and vitamins, 170 mg l^{-1} NaH_2PO_4, 0.25 g l^{-1} PVP and 20 g l^{-1} sucrose could be proposed as a suitable medium for direct somatic embryogeneisis in *Phalaenopsis aphrodite* subsp. *formosana.*

REFERENCES

Arditti J., Ernst R. (1993). Micropropagation of Orchids. New York, John Wiley, Volume 2: 467-520.

Bhat S. R., Chandel K. P. S. (1991). A novel technique to overcome browning in tissue culture. Plant Cell Reports, 10: 358-361.

Chen J. T., Chang C., Chang W. C. (1999). Direct somatic embryogenesis on leaf explants of *Oncidium* 'Gower Ramsey' and subsequent plant regeneration. Plant Cell Reports, 19: 143-149.

Chen J. T., Chang W. C. (2002). Effects of tissue culture conditions and explant characteristics on direct somatic embryogenesis in *Oncidium* 'Gower Ramsey'. Plant Cell, Tissue and Organ Culture, 69: 41-44.

Chen J. T., Chang W. C. (2006). Direct somatic embryogenesis and plant regeneration from leaf explants of *Phalaenopsis amabilis*. Biologia Plantarum, 50: 169-173.

Chen L. R., Chen J. T., Chang W. C. (2002). Efficient production of protocorm-like bodies and plant regeneration from flower stalk explants of the sympodial orchid *Epidendrum radicans*. In Vitro Cellular & Developmental Biology-Plant, 38: 441-445.

Chen T. Y., Chen J. T., Chang W. C. (2004). Plant regeneration through shoot bud formation from leaf explants of *Paphiopedilum* orchids. Plant Cell, Tissue and Organ Culture, 76: 11-15.

Chen Y., Piluek C. (1995). Effects of thidiazuron and N^6-benzylaminopurine on shoot regeneration of *Phalaenopsis*. Plant Growth Regulation, 16: 99-101.

Chen Y. C., Chang C., Chang W. C. (2000). A reliable protocol for plant regeneration from callus culture of *Phalaenopsis*. In Vitro Cellular & Developmental Biology-Plant, 36: 420-423.

Chung H. H., Chen J. T., Chang W. C. (2005). Cytokinins induce direct somatic embryogenesis of *Dendrobium* Chiengmai Pink and subsequent plant regeneration. In Vitro Cellular & Developmental Biology-Plant, 41: 765-769.

Chung H. H., Chen J. T., Chang W. C. (2007). Plant regeneration through direct somatic embryogenesis from leaf explants of *Dendrobium*. Biologia Plantarum, 51: 346-350.

Compton M. E. (1994). Statistical methods suitable for the analysis of plant tissue culture data. Plant Cell, Tissue and Organ Culture, 37: 217-242.

Dawns C. J. (1971). Biological Techniques in Electron Microscopy. Barnes and Noble, New York, 193 pp.

Duan J. X., Chen H., Yazawa S. (1996). *In vitro* propagation of *Phalaenopsis* via culture of cytokinins-induced nodes. Journal of Plant Growth Regulation, 15: 133-137.

Duncan D. B. (1955). Multiple range and multiple F test. Biometrics, 11: 1-42.

Ernst R. (1994). Effects of thidiazuron on *in vitro* propagation of *Phalaenopsis* and *Doritaenopsis* (Orchidaceae). Plant Cell, Tissue and Organ Culture, 39: 273-275.

Gow W. P., Chen J. T., Chang W. C. (2008). Influence of growth regulators on direct embryo formation from leaf explants of *Phalaenopsis* orchids. Acta Physiologiae Plantarum, 30: 507-512.

Gow W. P., Chen J. T., Chang W. C. (2009). Effects of genotype, light regime, explant position and orientation on direct embryo formation from leaf explants of *Phalaenopsis* orchids. Acta Physiologiae

Plantarum, 31: 363-369.
Ishii Y., Takamura T., Goi M., Tanaka M. (1998). Callus induction and somatic embryogenesis of *Phalaenopsis*. Plant Cell Reports, 17: 446-450.
Islam M. O., Ichihashi S. (1999). Effects of sucrose, maltose and sorbitol on callus growth of *Phalaenopsis*, *Doritaenopsis* and *Neofinetia*. Journal of the Japanese Society for Horticultural Science, 68: 1124-1131.
Jensen W. A. (1962). Botanical Histochemistry. Freeman, San Francisco, 408 pp.
Kuo H. L., Chen J. T., Chang W. C. (2005). Efficient plant regeneration through direct somatic embryogenesis from leaf explants of *Phalaenopsis* 'Little Steve'. In Vitro Cellular & Developmental Biology-Plant, 41: 453-456.
Murashige T., Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15: 495-497.
Park S. Y., Murthy H. N., Paek K. Y. (2000). Mass multiplication of protocorm-like bodies using bioreactor system and subsequent plant regeneration in *Phalaenopsis*. Plant Cell, Tissue and Organ Culture, 63: 67-72.
Park S. Y., Murthy H. N., Paek K. Y. (2002). Rapid propagation of *Phalaenopsis* from floral stalk-derived leaves. In Vitro Cellular & Developmental Biology-Plant, 38: 168-172.
Pedroso M. C., Pais M. S. (1995). Factors controlling somatic embryogenesis. Plant Cell, Tissue and Organ Culture, 43: 147-154.
Su Y. J., Chen J. T., Chang W. C. (2006). Efficient and repetitive production of leaf-derived embryos of *Oncidium*. Biologia Plantarum, 50: 107-110.
Tanaka M., Hasegawa A., Goi M. (1975). Studies on the clonal propagation of monopodial orchids by tissue culture. I. Formation of protocorm-like bodies from leaf tissues in *Phalaenopsis* and *Vanda*. Journal of the Japanese Society for Horticultural Science, 44: 47-58.
Tokuhara K., Mii M. (1993). Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. Plant Cell Reports, 13: 7-11.
Tokuhara K., Mii M. (2001). Induction of embryogenic callus and cell suspension culture from shoot tips excised from flower stalk buds of *Phalaenopsis* (Orchidaceae). In Vitro Cellular & Developmental Biology-Plant, 37: 457-461.