Factors Affecting Thidiazuron-Induced Direct Somatic Embryogenesis of *Phalaenopsis aphrodite*

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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 thidiazuron (TDZ)-induced 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 macroelement 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 are 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.

Keywords

Activated charcoal · Direct somatic embryogenesis · Embryogenic competence · Polyvinylpyrrolidone

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16.1 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, 2001; 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). 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 present 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 the leaf culture system of *Phalaenopsis aphrodite*.

16.2 Materials and Methods

16.2.1 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 for two times of subculture. All of the cultures were incubated under a 16/8-h (light/dark) photoperiod at photosynthetic photon flux density of 32 $\mu molm^{-2}\ s^{-1}$ (daylight fluorescent tubes FL-30D/29, 40 W, China Electric Co., Taipei, Taiwan) and temperature of 26 ± 1 °C. The seedlings with three to five leaves and two to four roots were used as donor plants.

16.2.2 Somatic Embryo Induction (in Darkness)

The basal medium for somatic embryo induction was a modified MS medium containing half-strength macroelements and full-strength microelements and supplemented with [mg l^{-1}]: myoinositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1000), NaH_2PO_4 (170), sucrose (20,000), thidiazuron (3.0), and Gelrite (2500). The pH of variants of the 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. 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 macroelements and full-strength microelements as half-strength, and quarter-strength macroelements 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}) was used to test their effects on direct somatic embryo formation.

16.2.3 Somatic Embryo Development (in Light Condition)

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 μmolm^{-2} s^{-1} and temperature of 26 ± 1 °C for 45 days.

16.2.4 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).

16.2.5 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.

16.2.6 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 arc sine prior to ANOVA and

then 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 $p \leq 0.05$.

16.3 Results and Discussion

16.3.1 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 16.1; Fig. 16.1a). These embryos subsequently turned green, enlarged, and developed scale leaves 45 days after transfer onto PGR-free half-strength MS medium in light condition (Fig. 16.1b). The histological study revealed that the epidermal cells had undergone a process of dedifferentiation and gained mitotic ability to form meristematic cells (Fig. 16.2a). Subsequently, the meristematic cells gave rise to form somatic embryos without the intervening of callus tissues (Fig. 16.2b). These leaf-derived embryos developed and consist of scale leaves and the vascular tissue on the parent explants (Fig. 16.2c). Scanning EM observation revealed that the subepidermal cells were also able to divide into meristematic cells, thus forming protuberances through the epidermis (Fig. 16.3a). Single-state embryos formed on surfaces of explants with scattering dedifferentiated leaf cells (Fig. 16.3b). When a mass of leaf cells were induced to dedifferentiate, it became easier to form multiple-state of embryos (Fig. 16.3c). In addition, asynchronous formation of embryos was frequently found on the explants (Fig. 16.3c). The foliar embryos had the ability to form secondary embryos from their anterior end when they were still on the parent explants (Fig. 16.3d).

Table 16.1 Effect of sucrose on direct somatic embryogenesis from leaf explants of *P. aphrodite*

Sucrose (g l^{-1})	% of explant with embryogenesis	% of explant with browning	% of each part of explant with embryogenesis				No. of embryos per responding explant
			CE	Ad	Ab	LT	
0	0 d	65 a	0 c	0 b	0 b	0 b	0
10	40 ab	35 ab	25 b	30 a	0 b	5 b	7.5
20	65 a	15 b	55 a	25 a	15 a	40 a	7.8
30	10 cd	50 a	0 c	10 ab	0 b	0 b	10.5
40	5 bc	65 a	10 c	10 ab	0 b	0 b	11.0

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)

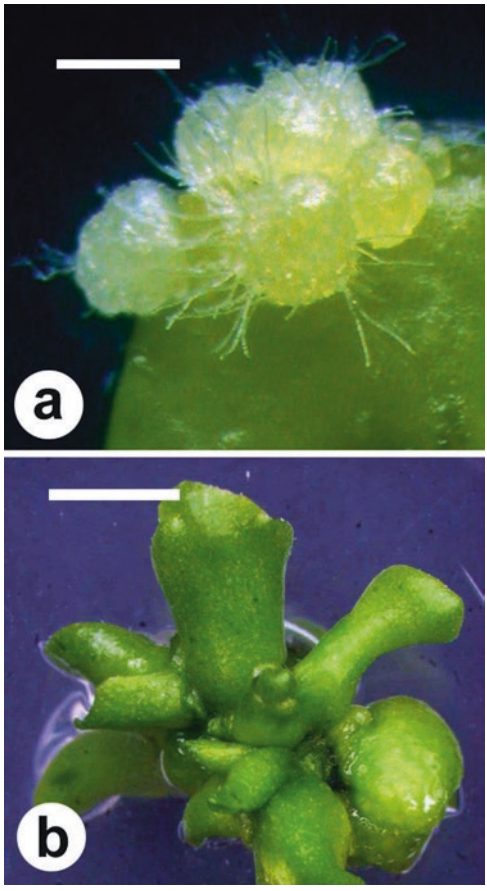

Fig. 16.1 Direct somatic embryogenesis from leaf explants of *P. aphrodite*. (**a**) Somatic embryos with absorbing hairs formed on a leaf explant after 45 days of culture on half-strength MS medium with 3 mg l^{-1} TDZ in darkness (scale bar = 1.5 mm). (**b**) Green, enlarged embryos with developing 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)

16.3.2 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 16.1). 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 16.1). 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 16.1). Higher concentrations of sucrose resulted in lower embryogenic responses, higher browning rates, but higher number of embryos per responding explant (Table 16.1).

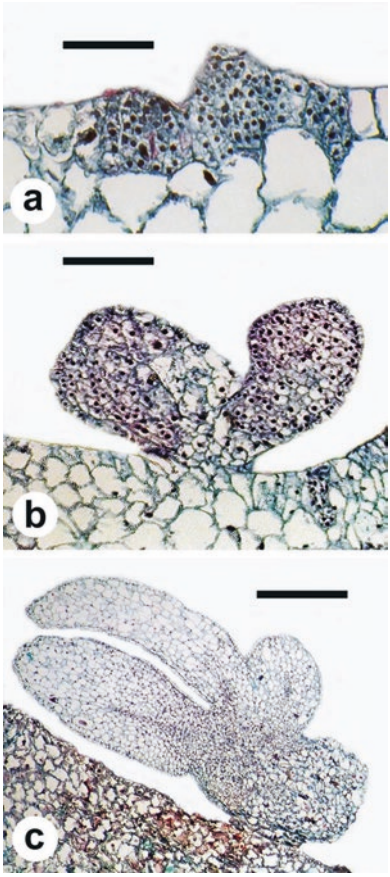

Fig. 16.2 Histology of direct somatic embryogenesis from leaf explants of *P. aphrodite*. (**a**) Embryogenic cells originated from the epidermal layer of a leaf explant after 30 days of culture on half-strength MS medium with 3 mg l^{-1} TDZ in darkness (scale bar 350 μm). (**b**) Globular embryos formed after 40 days of culture on half-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. 16.1a to a PGR-free half-strength MS medium in light (scale bar = 1.5 mm)

16.3.3 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 *Paphiopedilum* (Chen et al. 1999, 2000, 2002, 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 and 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 16.2).

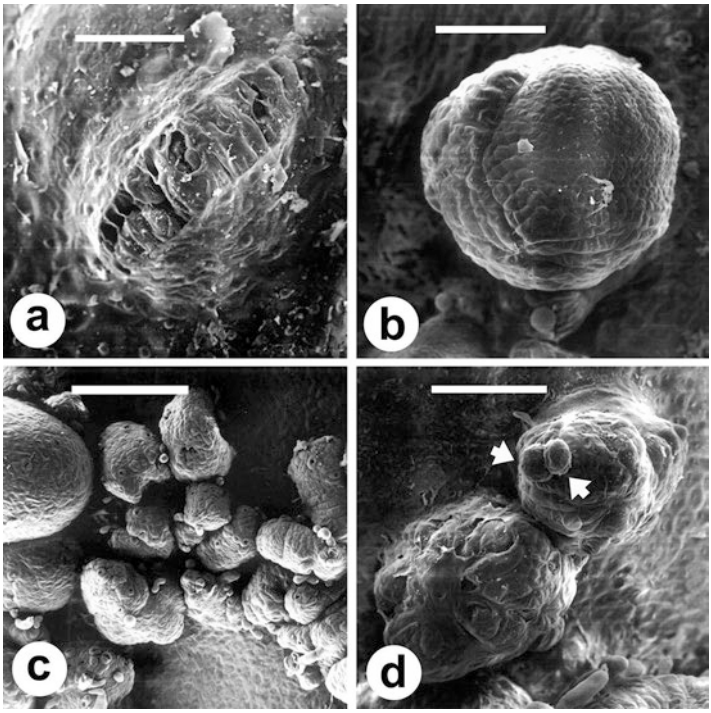

Fig. 16.3 Scanning electron microscopic observation on direct somatic embryogenesis from leaf explants of *P. aphrodite*. (**a**) An early event of direct embryogenesis from subepidermal cells (scale bar = 100 μm). (**b**) A globular embryo (scale bar = 200 μm). (**c**) Embryos with scale leaves formed on a leaf explant (scale bar = 350 μm). (**d**) Secondary embryos (arrow) formed on a primary embryo (scale bar = 200 μm)

Table 16.2 Effect of NaH_2PO_4 on direct somatic embryogenesis from leaf explants of *P. aphrodite*

NaH_2PO_4 (mg l^{-1})	% of explant with embryogenesis	% of explant with browning	% of each part of explant with embryogenesis				No. of embryos per responding explant
			CE	Ad	Ab	LT	
0	10 b	50 a	0 b	10 a	5 ab	5 b	13.5
42.5	25 b	30 a	10 b	25 a	0 b	10 b	13.5
85	20 b	30 a	10 b	10 a	0 b	10 b	4.3
170	65 a	15 a	55 a	25 a	15 a	40 a	7.8

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

Strength of MS medium	% of explant with embryogenesis	% of explant with browning	% of each part of explant with embryogenesis				No. of embryos per responding explant
			CE	Ad	Ab	LT	
Full	30 b	40 a	15 b	25 a	0 b	0 b	5.2
1/2	65 a	15 a	55 a	5 ab	15 a	40 a	7.8
1/4	20 b	40 a	20 b	0 b	0 b	0 b	10.0

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)

16.3.4 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 16.3). Both full-strength and quarter-strength MS gave lower embryogenic responses and higher browning rates (Table 16.3). Indeed, half-strength MS medium was used as basal medium for in vitro culture of *Dendrobium*, *Epidendrum*, and *Oncidium* (Chen et al. 1999, 2000, 2002; 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 16.3).

16.3.5 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 16.4). 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% and 80% (Table 16.4). Suggestion is that the activated charcoal may absorb TDZ or reduce its activity to induce embryogenesis.

16.3.6 Effect of Polyvinylpyrrolidone

Polyvinylpyrrolidone (PVP) is soluble in water and binds to polar molecules exceptionally well, owing to its polarity. In plant tissue culture media, PVP adsorb not only toxic exudates (phenolics) but also growth regulators and nutrients (Bhat and

Table 16.4 Effect of activated charcoal on direct somatic embryogenesis from leaf explants of *P. aphrodite*

Activated charcoal (g l^{-1})	% of explant with embryogenesis	% of explant with browning	% of each part of explant with embryogenesis				No. of embryos per responding explant
			CE	Ad	Ab	LT	
0	65 a	15 b	55 a	25 a	15 a	40 a	7.8
0.5	0 b	55 ab	0 b	0 b	0 b	0 b	0
1	0 b	70 a	0 b	0 b	0 b	0 b	0
2	0 b	85 a	0 b	0 b	0 b	0 b	0

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

PVP (g l^{-1})	% of explant with embryogenesis	% of each part of explant with embryogenesis				No. of embryos per responding explant
		CE	Ad	Ab	LT	
0	45 a	25 b	20 a	0 a	25 a	7.8
0.1	50 a	40 ab	30 a	5 a	25 a	10.7
0.25	60 a	60 a	35 a	10 a	45 a	15.5
0.5	50 a	50 a	25 a	5 a	25 a	10.6

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)

Chandel 1991). In the present study, the use of PVP significantly enhanced direct embryo induction from cut ends of explants (Table 16.5). 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 16.5).

According to the present results, a modified MS medium with 1/2-strength macroelements, 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 embryogenesis in *Phalaenopsis aphrodite* subsp. *formosana.*

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