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INDUCTION OF REPETITIVE EMBRYOGENESIS FROM SEED-DERIVED PROTOCORMS OF *PHALAENOPSIS AMABILIS* VAR. *FORMOSA* SHIMADZU

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Summary

An *in vitro* culture procedure was established for repetitive embryogenesis and plant regeneration from seed-derived protocorms of *Phalaenopsis amabilis* var. *formosa* Shimadzu (*Orchidaceae*). Seed-derived protocorms were cultured on modified half-strength Murashige and Skoog (1962) basal medium (½MS) devoid of plant growth regulators. After 45 d, 28.1% of protocorms formed embryos from their posterior regions. 1-Phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ; 0.45, 4.54, and 13.62 μM) promoted direct embryo formation. The best response was at 13.62 μM TDZ, and 100% of the protocorms formed a mean number of 13.5 embryos after 45 d of culture. By contrast, naphthaleneacetic acid (NAA) at 0.54 and 5.37 μM inhibited direct embryo formation. On basal medium devoid of plant growth regulators, 18.8% of primary proliferating embryos could form more embryos. TDZ (0.45, 4.54, and 13.62 μM) also promoted this process. Proliferating embryos/protocorms were transferred to basal medium devoid of plant growth regulators for plantlet formation. Plantlets were successfully obtained from the embryos after 4–6 wk. Following subculture every 6 wk for three passages, the plantlets were transferred to sphagnum moss in a container for acclimatization in the greenhouse. The survival rate was 100%.

Key words: direct embryo formation; moth orchid; protocorm.

Introduction

Phalaenopsis, 'moth orchids', have long arching sprays and are among the most beautiful flowers in the world. This genus has economic value for pot plant and cut flower production. They are distributed throughout Southeast Asia with a few species extending from Taiwan, Sikkhim to Australia and the Pacific (Teob, 1989). Many protocols for *in vitro* culture of *Phalaenopsis* explants have been developed (Arditti and Ernst, 1993), and their morphogenetic pathways are usually through shoot multiplication (Ernst, 1994; Chen and Piluek, 1995; Duan et al., 1996), protocorm-like body (PLB) proliferation (Tanaka et al., 1975; Tokuhara and Mii, 1993; Islam and Ichihashi, 1999; Chen et al., 2000; Young et al., 2000), and somatic embryogenesis (Ishii et al., 1998). Here we describe a method for inducing repetitive embryogenesis from seed-derived protocorms of *Phalaenopsis amabilis* var. *formosa* Shimadzu, an important native orchid in Taiwan.

Materials and Methods

Plant materials and culture conditions. Green capsules were collected from potted plants of *Phalaenopsis amabilis* var. *formosa* Shimadzu for 3 mo. after self-pollination. The capsules were immersed in 70% ethanol for 30 s, followed by agitation for 15 min in a solution of 2% sodium hypochlorite and 0.05% Tween 20. Seeds from these capsules were sown on half-strength modified MS (Murashige and Skoog, 1962) basal medium (½MS) containing half-strength macro- and micro-elements of MS salts supplemented with (mg l^{-1}): *myo*-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1000), NaH_2PO_4 (170), sucrose (20 000), and Gelrite™ (2200). After 80 d of culture, these seeds germinated into zygotic protocorms. Plant growth regulators were added to other versions of the media prior to autoclaving. The pH of the media was adjusted to 5.2 with 1 *N* KOH or HCl prior to autoclaving for 15 min at 121°C. Zygotic protocorms were placed on the surfaces of the culture medium and were incubated in 60 × 15 mm Petri dishes (Greiner Labortechnik, France) under a 16:8 h photoperiod at 28–36 $\mu mol\, m^{-2}\, s^{-1}$ (daylight fluorescent tubes FL-30D/29, 40 W, China Electric Co.,Taipei) at 26 ± 1°C.

Induction of direct embryo formation from zygotic protocorms. Eighty-day-old seed-derived protocorms (about 400–500 μm in diameter) were used to test the effects of naphthaleneacetic acid (NAA; 0, 0.54, 5.37 μM) plus 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ; 0, 0.45, 4.54, 13.62 μM) on direct embryogenesis. Eight replicates each with four zygotic protocorms were used for each treatment. The percentage of seed-derived protocorms forming embryos was recorded. The number of embryos formed from each responding seed-derived protocorm was counted under a stereomicroscope (SZH, Olympus) and the data were recorded as the mean number of embryos per seed-derived protocorm after 45 d of culture. Treatment means were compared using Duncan's multiple range test (Duncan, 1955).

Induction of repetitive embryogenesis. Primary embryo-derived protocorms (about 400–500 μm in diameter) were used to test the effects of TDZ (0, 0.45, 4.54, 13.62 μM) on repetitive embryogenesis. The experimental design and the statistical method were the same as the first experiment.

Histological and scanning EM observation on direct embryogenesis from seed-derived protocorms. 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). Samples for scanning electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.0) for 4 h at 4°C, dehydrated in ethanol (Dawns, 1971), critical-point dried (HCP-2,

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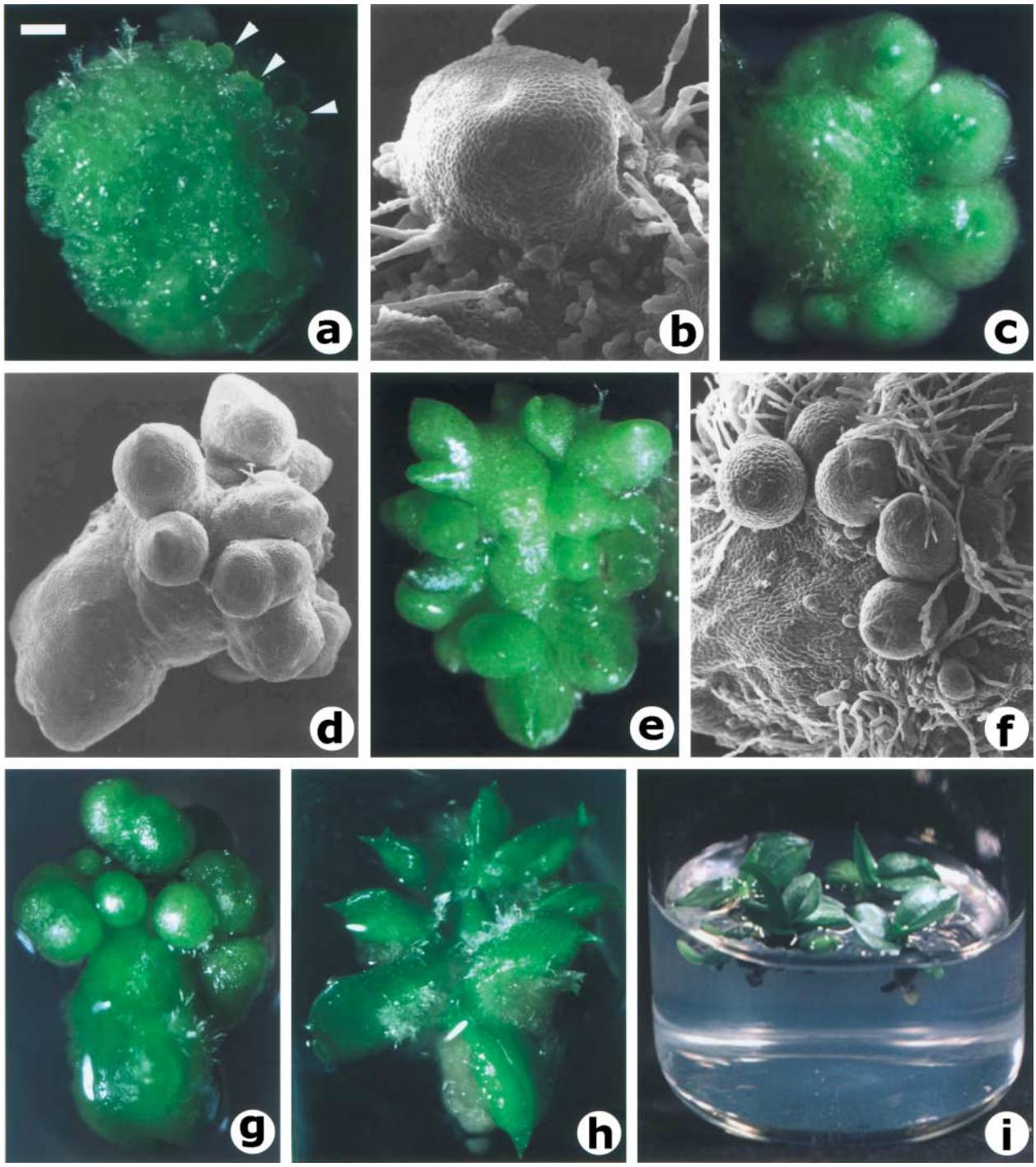

FIG. 1. Repetitive embryogenesis from seed-derived protocorms and plantlet formation of *Phalaenopsis amabilis* var. *formosa* Shimadzu (*bar* in *upper left* refers to all panels). *a*, Embryos (*arrows*) formed from a seed-derived protocorm after 20 d of culture on hormone-free ½MS medium (*bar* = 250 μm). *b*, Scanning EM photograph of an embryo/young protocorm that was formed from the seed-derived protocorm (*bar* = 120 μm). *c*, Embryos formed from a seed-derived protocorm after 45 d of culture on hormone-free ½MS medium (*bar* = 450 μm). *d*, Scanning EM photograph of embryos formed from the posterior region of a seed-derived protocorm after 45 d of culture on hormone-free ½MS medium (*bar* = 500 μm). *e*, Embryos formed from a seed-derived protocorm after 45 d of culture on ½MS medium supplemented with 13.62 μM TDZ (*bar* = 400 μm). *f*, Scanning EM photograph of secondary embryogenesis when cultured on ½MS medium supplemented with 13.62 μM TDZ for 30 d (*bar* = 180 μm). *g*, Secondary embryogenesis from protocorm-derived embryos when cultured on ½MS medium supplemented with 13.62 μM TDZ for 45 d (*bar* = 400 μm). *h*, Proliferating embryos germinated on basal medium devoid of growth regulators (*bar* = 1 mm) after transferring from TDZ-containing medium. *i*, Plantlets derived from proliferating embryos (*bar* = 8.5 mm).

Hitachi), and coated with gold in an ion coater (IB-2, Giko Engineering Co.). A scanning electron microscope (DSM-950, Carl Zeiss) was used for examination and photography of the specimen.

Results and Discussion

Direct embryogenesis from seed-derived protocorms. When 80-d-old seed-derived protocorms were cultured on hormone-free modified ½MS medium, posterior regions of some protocorms directly formed embryos within 20 d (Fig. 1*a*). After another 2–4 wk of culture, these embryos further developed into protocorms with sheath leaves and absorbing hairs (Fig. 1*b–d*). Seed-derived protocorms (28.1%) formed a mean number of 4.1 embryos per dish (each dish contains four seed-derived protocorms) (Table 1). TDZ (0, 0.45, 4.54, 13.62 μM) promoted direct embryo formation. TDZ at 13.62 μM resulted in the best response for the mean number of embryos per dish (59.5), and one single seed-derived protocorm formed an average of 14.8 embryos (Table 1; Fig. 1*e*). In contrast, NAA (0.54 and 5.37 μM) used alone slightly decreased both the percentage of embryogenesis and the embryo numbers, but this effect was not statistically significant ($P = 0.05$). In the presence of 4.54 and 13.62 μM TDZ, NAA significantly decreased embryo numbers. In *Oncidium* (*Orchidaceae*), TDZ promoted direct somatic embryogenesis from leaf explants, but NAA was found to be inhibitory (Chen et al., 1999; Chen and Chang, 2001). As demonstrated here, TDZ-containing medium was also effective in inducing direct embryogenesis in *P. amabilis* var. *formosa* Shimadzu, and NAA inhibited direct embryo formation.

Histology of direct embryogenesis from seed-derived protocorms. Regenerated embryos (each embryo contains many dark-stained embryonic cells) were generally formed from the epidermal layers of posterior regions of seed-derived protocorms, and surrounded by absorbing hairs (AH, Fig. 2*a*). As differentiation progressed, the embryos enlarged and epidermal cell layers were developed (Fig. 2*b*). Subsequently, the anterior regions developed small meristematic cells (<20–30 μm in diameter), but the posterior and central regions contained larger cells (>80–100 μm) (Fig. 2*b*). The embryos enlarged and subsequently developed into young protocorms (Fig. 2*c*).

TABLE 1

EFFECTS OF NAA AND TDZ ON DIRECT EMBRYO FORMATION FROM SEED-DERIVED PROTOCORMS OF *PHALAENOPSIS AMABILIS* VAR. *FORMOSA* SHIMADZU

NAA (μM)	TDZ (μM)	Protocorms with direct embryogenesis (%)	No. of embryos per dish
0	0	28.1 b	4.1 f
0	0.45	100 a	22.9 cde
0	4.54	100 a	54.1 a
0	13.62	100 a	59.5 a
0.54	0	25 b	3.9 f
0.54	0.45	100 a	20.0 de
0.54	4.54	100 a	26.1 cd
0.54	13.62	100 a	43.5 b
5.37	0	12.5 b	1.1 f
5.37	0.45	87.5 a	16.9 e
5.37	4.54	100 a	29.3 c
5.37	13.62	100 a	40.1 b

The frequency of embryo-forming protocorms and the mean number of embryos per dish were scored after 45 d in culture.

Means of eight replicates (dishes, each dish contained four seed-derived protocorms) with the same *letters* within *columns* are not significantly different at $P < 0.05$ (Duncan, 1955).

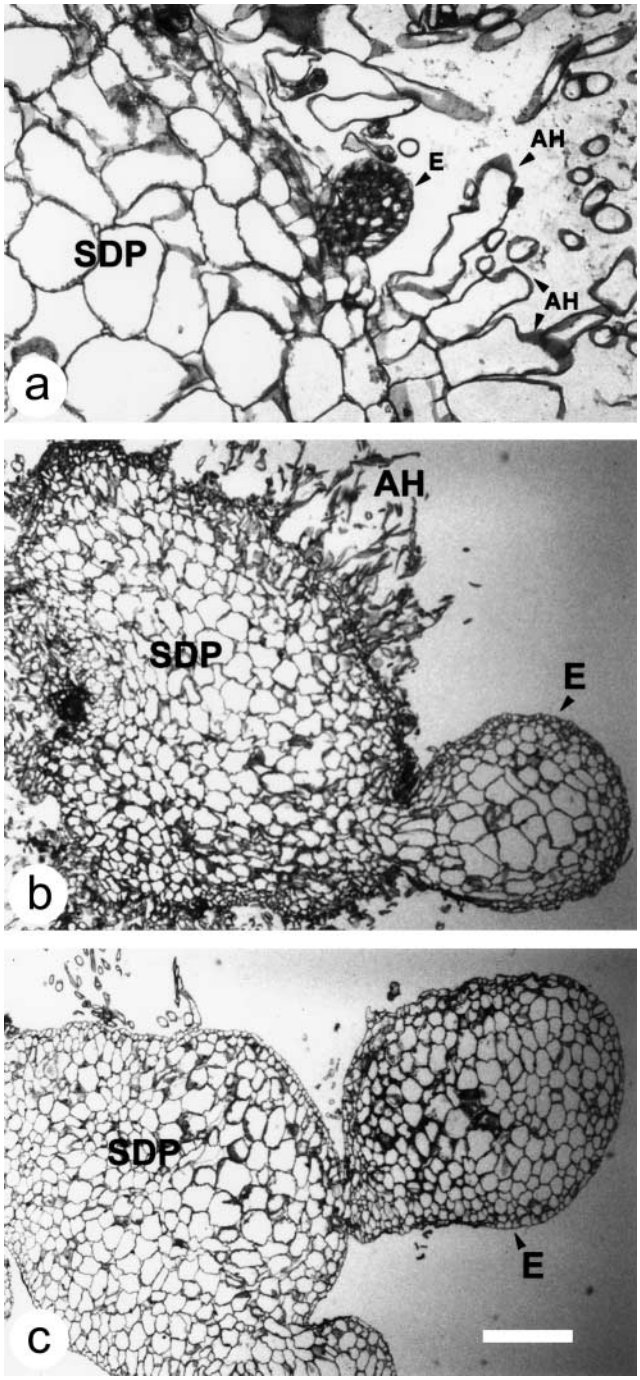

Fig. 2. Histology of direct embryogenesis from seed-derived protocorms of *Phalaenopsis amabilis* var. *formosa* Shimadzu (*bar* in *c* refers to all panels). *a*, An embryo (E), containing small and densely stained embryonic cells, formed on the posterior surface of the seed-derived protocorm (SDP). This embryo was surrounded by absorbing hairs (AH) of the SDP (*bar* = 200 μm). *b*, A developing embryo (E) containing small embryonic cells in the anterior region and larger cells in the central and posterior regions (*bar* = 500 μm). *c*, An embryo developed into a young protocorm (*bar* = 500 μm).

TABLE 2

EFFECT OF TDZ ON REPETITIVE EMBRYOGENESIS OF *PHALAENOPSIS AMABILIS* VAR. *FORMOSA* SHIMADZU

TDZ (μM)	Embryos with secondary embryogenesis (%)	No. of secondary embryos per dish
0	18.8 b	3.3 d
0.45	96.9 a	19.6 c
4.54	100 a	28.0 b
13.62	100 a	44.1 a

The frequency of embryogenesis and the mean number of embryos per dish were scored after 45 d in culture.

Means of eight replicates (dishes, each dish contained four primary proliferating embryos) with the same *letters* within *columns* are not significantly different at $P < 0.05$ (Duncan, 1955).

Repetitive embryogenesis. After 50–60 d of culture on basal medium with or without TDZ, secondary embryos formed from the surface of primary embryos (Fig. 1*f*). On basal medium devoid of growth regulators, 18.8% of primary embryos formed a mean number of 3.3 secondary embryos per dish. TDZ (0, 0.45, 4.54, 13.62 μM) promoted secondary embryogenesis, and 13.62 μM resulted in the best response for the mean number of embryos per dish (44.1) (Table 2; Fig. 1*g*).

Embryo germination and plantlet formation. Proliferating embryos were transferred onto basal medium devoid of growth regulators and kept under a 16:8 h photoperiod. Under this condition, embryos continued developing into protocorms and further formed shoots (Fig. 1*h*). Plantlets were obtained after 4–6 wk of culture (Fig. 1*i*). After subculturing every 6 wk for three passages, 108 plantlets with five or six leaves and three or four roots were transferred to sphagnum moss in a container for acclimatization in the greenhouse. The survival rate was 100%.

Conclusion

A tissue culture protocol for inducing repetitive embryogenesis from seed-derived protocorms of *Phalaenopsis amabilis* var. *formosa* Shimadzu was established. This protocol is simple, easy to carry out, and could provide a large number of embryos in a short period of time. Therefore, it is a suitable system for further studying the morphological and physiological events during embryogenesis of this orchid.

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