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Zygotic and Somatic Embryogenesis of *Phalaenopsis*

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ABSTRACT

Tissue culture protocols for zygotic and somatic embryo induction of a popular economical important orchid, *Phalaenopsis*, are reviewed in this report. In *P. amabilis*, repetitive embryogenesis was achieved using seed-derived protocorm as explants. In *P. amabilis*, *P.* 'Little Steve' and *P.* 'nebula', direct somatic embryogenesis and subsequent secondary embryogenesis were induced from leaf explants. Factors including explant type, explant orientation, culture period, subculture period, light requirement, basal medium, medium composition and growth regulators for embryo induction are comprehensively described in this review.

Keywords: growth regulator, medium composition, orchid, protocorm, somatic embryogenesis

CONTENTS

INTRODUCTION

Phalaenopsis (Orchidaceae), commonly known as moth orchids, are distributed throughout Southeast Asia with a few species extending from Taiwan, Sikkhim to Australia and the Pacific (Teob 1989). Most members of this genus are epiphytic shade plants, and a few are lithophytes. *Phalaenopsis* shows a monopodial growth habit and an erect growing rhizome produces from the top one or two alternate, thick and fleshy, elleptical leaves a year. They have long arching sprays and are among the most beautiful flowers in the world. Potted *Phalaenopsis* production has high economical value in flower markets. In previous reports, *in vitro* culture protocols were developed for members of this genus (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; Young *et al*. 2000; Tokuhara and Mii 2001; Park *et al*. 2002). However, only two among them reported somatic embryogenesis (Ishii *et al*. 1998; Tokuhara and Mii 2001). In this review, we describe protocols and morphogenetic events for somatic embryo induction, secondary embryogenesis and plantlet formation in this genus that were recently developed by our research group.

ZYGOTIC EMBRYOGENESIS (Chen and Chang 2004)

The seeds of *Phalaenopsis* are extremely tiny and the rudimentary embryo contains no endosperm as an energy source for seed germination and further growth (Nishimura 1981). Under the *in vitro* culture condition with external sources of nutrition, the seed germinates into a globular-shaped embryo (Nishimura 1981). The embryo further develops into a protocorm that consists of a shoot apical meristem and sheath leaves on the inside, and larger storage cells which contain numerous starch grains on the outside (Nishimura 1981). A number of epidermal cells differentiate into absorbing hairs around the posterior region (Nishimura 1981). Basically, it was recognized that protocorms are almost equal to the heart to heart-torpedo stage zygotic embryos of dicotyledonous plants (Nishimura 1981; Chen and Chang 2004).

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Invited Mini-Review

Requirements for induction of zygotic embryogenesis *in vitro*

Green capsules of *Phalaenopsis* potted plants were collected for about 3 months after 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): *myo*-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1000), NaH_2PO_4 (170), sucrose (20000), and Gelrite (2200). 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. The cultures are placed under a 16:8 h photoperiod at 28-36 μmol m^{-2} s^{-1} at 26 ± 1°C.

After 2-3 months of culture on a modified ½MS medium devoid of growth regulator, seeds germinated into globular embryos and then protocorms with a high germination rate. During embryo development on the same medium, about 30% of seed-derived protocorms spontaneous formed regenerated embryos from the epidermal cells with a mean number of one regenerated embryo per seed-derived protocorm. In the presence of thidiazuron (TDZ), the capacity of embryo formation was enhanced. TDZ at 13.62 μM resulted in the best response with 100% of seed-derived protocorms could form a mean number of about 15 regenerated embryos. By contrast, NAA (0.54 and 5.37 mM) slightly decreased both the percentage of embryogenesis and the regenerated embryo numbers, but the effects were not statistically significant. However, in the presence of 4.54 and 13.62 mM TDZ, NAA significantly decreased regenerated embryo numbers per seed-derived protocorm.

Repetitive embryogenesis

About 19% of primary embryos formed a mean number of 0.8 secondary embryos from their posterior epidermal cells when cultured on hormone-free medium. Basically, the younger seed-derived embryos/protocorms have higher capacity to form secondary embryos. TDZ at 0.45-13.62 μM promoted secondary embryogenesis, and 13.62 μM resulted in the best response for the mean number of secondary embryos. Using this method, we could continuously obtain embryos without waiting for the long duration of flowering, pollination and capsule maturation. It is a suitable system for studying morphogenesis and physiology of embryo development in *Phalaenopsis*.

Morphogenetic events of zygotic embryogenesis *in vitro*

Initially, embryogenic cells showed on the posterior surfaces of seed-derived protocorms (SDP) (3-5 mm in diameter) and then gave rise to form proembryos (E) (**Fig. 1**). Based on the histological data, cell division originated from the epidermal cell layers of parent protocorms. These proembryos further developed, became larger (1.5-2 mm in diameter) and subsequently became globular (**Fig. 2**). The regenerated embryo contains large amount of dark stained embryonic cells. Generally, each of the embryogenic cells had a larger nucleus and a smaller cell size than parent protocorm cells. As differentiation progressed, the embryos enlarged and their epidermal cell layers were developed. 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). The regenerated embryos enlarged and subsequently developed into regenerated protocorms (RP) on the parent seed-derived protocorm (SDP) (**Fig. 3**), each consisting of a shoot apical meristem at the summit of the interior region surrounded by two sheath leaves.

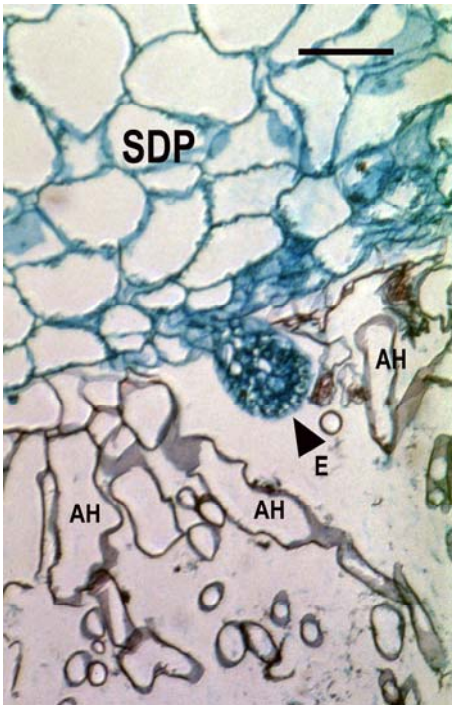

Fig. 1 Embryo formation in *Phalaenopsis*. An embryo (E), containing small and densely stained embryonic cells, formed on the posterior surface of the seed-derived protocorm (SDP). This embryo was surrounded by absorbing hairs (AH) of the SDP (bar = 200 μm).

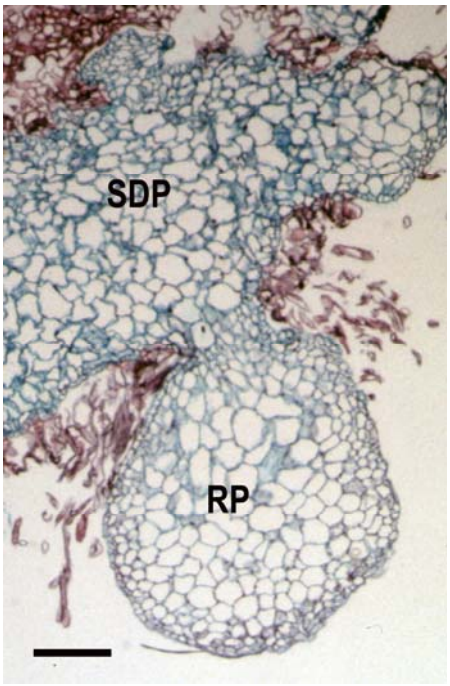

Fig. 2 Embryo to protocorm development. A regenerated embryo develops into protocorm (RP) containing small embryonic cells in the anterior region and larger cells in the central and posterior regions (bar = 500 μm).

Plantlet conversion

Regenerated embryos were transferred onto basal medium devoid of plant growth regulators and kept under a 16:8 h photoperiod. Under such condition, embryos continued developing into protocorms and further formed shoots. Plantlets were obtained after 4-6 wk of culture on the same condition. After subculture every 6 wk for several passages, normal and healthy plantlets with leaves and roots could be transferred to sphagnum moss in a container for acclimatization in the greenhouse. A high survival rate was achieved.

SOMATIC EMBRYOGENESIS IN *PHALAENOPSIS*

Somatic embryogenesis has often been considered to be a suitable system for plant propagation and for obtaining transgenic plants. It also provides a model system for studying plant morphology and physiology during early development of higher plants (Zimmerman 1993). In recent years, somatic embryogenesis protocols have been success-

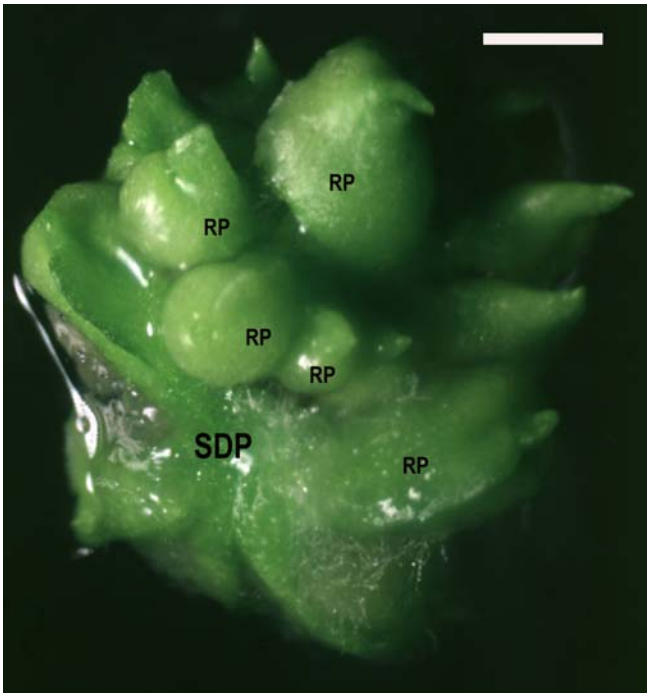

Fig. 3 Protocorm regeneration. Regenerated protocorms (RPs) formed on a seed-derived protocorm (SDP) (bar = 800 μm).

ful with several orchids, including *Cymbidium* (Chang and Chang 1998; Nayak *et al*. 2006), *Dendrobium* (Chung *et al*. 2005; Chung *et al*. 2007), *Oncidium* (Chen *et al*. 1999), and *Phalaenopsis* (Ishii *et al*. 1998; Tokuhara and Mii 2001; Kuo *et al*. 2005; Chen and Chang 2006). We summarized protocols for induction of three cultivars of *Phalaenopsis* developed by our group (Gow 2005; Kuo *et al*. 2005; Chen and Chang 2006).

Direct somatic embryo induction

Phalaenopsis amabilis (Chen and Chang 2006)

Phalaenopsis amabilis var. formosa is an important native orchid in Taiwan. Leaf explants of *P. amabilis* could form clusters of somatic embryos directly from epidermal cells without an intervening callus within 20-30 d when cultured on ½MS medium supplemented with 0.3-1 mg/l of TDZ. In the presence of TDZ, somatic embryos directly formed from the surface of leaf explants after 20 d of culture. After another 10 d of culture on the same TDZ-containing media, the embryos enlarged and more embryos were formed. The embryos turned green and developed into protocorms for about 2 weeks of culture. TDZ was effective in direct embryo induction, but NAA at 0.1-1 mg/l was inhibitory.

Phalaenopsis 'Little Steve' (Kuo et al. 2005)

Leaf segments of another important cultivar *P.* 'Little Steve' were used as explants testing the effects of 2,4-D (0.1-1 mg/l) combined with kinetin (0.3-3 mg/l), BA (0.3-3 mg/l) and TDZ (0.3-3 mg/l) on the induction of direct somatic embryogenesis. After one month of culture in darkness, clusters of somatic embryos formed from leaf surfaces and wounded regions of explants on ½MS medium supplemented with BA and TDZ. However, kinetin showed no response on direct embryo induction. In addition, 2,4-D highly retarded the frequency of embryogenesis that was induced by TDZ. Generally, adaxial surfaces near wounded regions had the highest embryogenic competency compared to other regions of explants. Besides, the histological study revealed that somatic embryos mostly arose from epidermal cell layers of the explants.

Effects of culture period and explant length (Gow 2005)

Two species of *Phalaenopsis* orchids, *P. amabilis* and *P.* Nebula, were used to investigate the effects of induction period (30, 45, 60 days), subculture period (30, 45, 60 days), explant length (1, 1.5, 2 cm) on direct somatic embryogenesis from different locations (the leaf tip, the adaxial side, the abaxial side, the cut end) of leaf explants. In general, the cut end had the highest competence to form embryos than the other locations of leaf explants. The suitable culture conditions for direct embryo formation are 60 days for induction period, 45 days for subculture period and 1 cm for length, respectively.

Effects of genotype, light regime, explant position and orientation (Gow 2005)

The influence of light regime, explant position and orientation on direct embryo formation from leaf explants of two *Phalaenopsis*, *P. amabilis* and *P.* Nebula, were investigated to optimize the protocol for regenerating of this orchid. When explants were cultured in light, direct embryogenesis was highly retarded in both species. Embryos showed whitish to pale green in color and larger size than those cultured in darkness. Furthermore, light regime induced explant browning, embryo necrotic and eventually low plantlet conversion rate. Sixty days of culture in darkness is the most suitable duration for direct embryo induction. Explant orientation also highly affected the amount of direct embryogenesis, and explants placed adaxial-side-up on culture medium had higher embryogenic response than abaxial-side-up orientation. In both species, the cut end had highest embryogenic competence than other leaf locations. Moreover, the leaf basal segment also had higher embryogenic competence than the leaf tip segment.

Effects of growth regulators (Gow 2005)

Leaf explants of two *Phalaenopsis*, *P. amabilis* and *P.* Nebula, were used to test the effects of auxins (2,4-D, IAA, IBA, NAA at 0.3-3 mg/l), cytokinins (2iP, BA, kinetin, TDZ, zeatin at 0.3-3 mg/l), GA_3 at 1-5 mg/l, ancymidol at 1-5 mg/l, polyamines (putrescine, spermine, spermidine at 1-5 mg/l), ACC at 5-50 μM, $AgNO_3$ at 5-50 μM and $CoCl_2$ at 5-50 μM on the amount of direct embryo formation on different leaf locations (the cut end, the adaxial side, the abaxial side and the leaf tip). The results showed that there was a genotypic effect on direct embryo formation induced by cytokinins that 3 mg/l BA and 0.5 mg/l 2iP was the most effective in *P. amabilis* and *P.* Nebula, respectively. Besides, explant position highly affected embryogenic competence of leaf cells in both species that the cut end showed highest embryogenic response, the adaxial side was the second, and then the abaxial side and the leaf tip. Altogether, cytokinins tested were all effective in both species, and ACC, ACC and $AgNO_3$ showed some embryogenic response in *P.* Nebula. However, auxins, GA_3, ancymidol and polyamines were inhibitory in both species.

Effects of other medium compositions (Gow 2005)

Medium compositions (NaH_2PO_4, sucrose and MS medium strength) and additives (active charcoal and polyvinylpyrrolidone [PVP]) were tested to optimize the culture medium for direct embryo induction from leaf explants of *P. amabilis*. The results showed that full- and ¼-strength macro-elements of MS medium were not suitable for direct embryo induction from leaf explants. Thus, a modified medium composition is proposed as ½-strength macro- and full-strength micro-elements of MS salts plus MS vitamins, 170 mg/l NaH_2PO_4 and 20 g/l sucrose. In addition, PVP at 0.25 g/l significantly promoted direct embryogenesis on the cut end of the explant, but active charcoal at 0.5-1 g/l were all inhibitory.

Secondary somatic embryogenesis (Chen and Chang 2006)

Excised leaf-derived embryogenic masses were used to test the effect of TDZ on secondary embryogenesis of *P. amabilis*. In light, the masses turned brown and became necrotic after 45 d of culture on the hormone-free medium.

In the presence of TDZ (0.01, 0.1, 1 and 3 mg/l), somatic embryos were induced from the leaf-derived masses after one month of culture. The embryos developed into protocorms and some of them formed secondary embryos on the same TDZ-containing media. The numbers of embryos could be repetitive increased by subdivision of the embryo clusters. TDZ at 3 mg/l resulted in a best response on secondary embryo induction.

Plantlet development (Gow 2005; Kuo *et al*. 2005; Chen and Chang 2006)

Whole leaf cultures with embryos/protocorms were transferred onto hormone-free medium and kept under a 16-h photoperiod. Under this condition, protocorms continued developing and further formed shoot. Plantlets were obtained after about 6-8 weeks of culture. After subculture every 6 weeks for three passages, the plantlets developed several leaves and roots. These plants were potted in sphagnum moss for acclimatization in greenhouse with normal growth.

CONCLUSION

A tissue culture protocol for inducing repetitive embryogenesis from seed-derived protocorms of *P. amabilis* was established on a basal medium containing ½-strength macro- and full-strength micro-elements of MS salts plus MS vitamins, 170 mg/l NaH_2PO_4, 20 g/l sucrose and 1-3 mg/l TDZ. Proliferating of repetitive embryos was obtained on the same TDZ-containing medium after subculture of primary embryos. It is a suitable system for further studying the morphological and physiological events of zygotic embryogenesis of this orchid.

Induction of direct embryo formation of three *Phalaenopsis* species (*P. amabilis*, *P.* 'Little Steve' and *P.* Nebula) were achieved by using leaf segments as explants. Generally, cytokinins tested were effective in direct embryo induction in all of the three species, and ACC, ACC and $AgNO_3$ showed some embryogenic response in *P.* Nebula. However, auxins, GA_3, ancymidol and polyamines were inhibitory. Besides, there were also genotypic and explant position effects on direct embryo induction in *Phalaenopsis*. Suitable factors for direct embryo induction from leaf explants are proposed as: 1) basal medium: ½-strength macro- and full-strength micro-elements of MS salts plus MS vitamins, 170 mg/l NaH_2PO_4, 0.25 g/l PVP and 20 g/l sucrose; 2) explant type: the basal leaf segments; 3) explant orientation: adaxial-side-up on the culture medium; 4) induction period: 60 days in darkness; 5) subculture period: 45 days; 6) leaf explant length: < 1 cm.

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