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ORIGINAL PAPER

Influence of growth regulators on direct embryo formation from leaf explants of *Phalaenopsis* orchids

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Abstract Leaf explants of two *Phalaenopsis*, *P. amabilis* and *P.* Nebula, were used to test the effects of auxins (2,4-D, IAA, IBA, NAA), cytokinins (2iP, BA, kinetin, TDZ, zeatin), GA_3, ancymidol, polyamines (putrescine, spermine, spermidine), ACC, $AgNO_3$ and $CoCl_2$ 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 13.32 μM BA and 4.92 μM 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 at 20 μM had 35% of embryogenic response in *P. amabilis*. However, auxins, GA_3, ancymidol and polyamines were inhibitory in both species.

Keywords Auxin · Culture condition · Cytokinin · Embryogenic competence · Explant position · Genotype · Moth orchid

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	N^6-2-Isopentenyl adenine
ACC	1-Aminocyclopropane-1-carboxylic acid
BA	N^6-Benzyladenine
GA_3	Gibberellin A_3
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
kinetin	N^6 Furfuryladenine
MS	Murashige and Skoog (1962) medium
NAA	1-Naphthaleneacetic acid
TDZ	1-Phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (thidiazuron)
Zeatin	6-[4-Hydroxy-3-methylbut-2-enylamino]purine

Introduction

Phalaenopsis orchids have highly economical value for cut flower and pot plant production in international flower markets. The conventional in vitro culture protocols had been developed in this genus and usually for the propagating purpose via protocorm-like body formation, shoot multiplication and callus culture (Tanaka et al. 1975; Arditti and Ernst 1993; Tokuhara and Masahiro 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). Recently, more efficient regeneration systems through direct somatic embryogenesis had been developed using leaf cultures (Kuo et al. 2005; Chen and Chang 2006). However, further systematic investigations on culture condition, medium composition and physiological status are needed to optimize the protocol for practical use in

Table 1 Effects of cytokinins on direct somatic embryogenesis from different locations of leaf explants of *Phalaenopsis amabilis*

Cytokinins (μM)	Percentage of emrbyogenesis on the entire explant (%)	Percentage of embryogenesis on different explant locations (%)				No. of embryos per responding explant
		CE	Ad	Ab	LT	
Control	0 h	0 f	0 d	0 b	0 c	0
2iP						
2.46	40 cdef	20 cde	30 abc	5 ab	0 c	3.8
4.92	45 bcde	35 abc	35 ab	10 ab	10 bc	7.2
14.76	50 bcde	50 ab	30 abc	15 a	15 abc	7.4
BA						
2.22	35 defg	25 bcd	10 cd	0 b	10 bc	2.3
4.44	50 bcde	35 abcd	30 abc	5 ab	15 abc	4.4
13.32	80 a	55 a	45 a	10 ab	30 ab	6.6
Kinetin						
2.32	15 fgh	15 cdef	0 d	0 b	0 c	0
4.65	5 h	5 ef	0 d	0 b	0 c	1
13.94	20 gh	15 def	0 d	0 b	10 bc	1.8
TDZ						
2.27	30 efg	25 cde	10 cd	0 b	10 bc	3.2
4.54	65 abc	55 a	5 d	0 b	10 bc	3
13.62	65 abc	55 a	25 bc	15 a	40 a	7.8
Zeatin						
2.28	45 bcde	30 abcd	30 ab	5 ab	10 bc	3.7
4.56	35 cdef	35 abc	10 cd	5 ab	5 c	4.9
13.68	60 abcd	55 a	40 ab	15 a	15 abc	7

LT Leaf tips; *Ad* adaxial sides; *Ab* abaxial sides; *CE* cut ends

Data were scored after 60 days of culture. The amounts of direct embryo formation were counted at entire explants or at different explant locations. Data expressed as percentage were transformed using arc sine prior to ANOVA and converted back to the original scale for demonstration in table (Compton 1994). Five replicates (dishes) each with four leaf explants were performed for each treatment. Mean with the same letters within columns are not significantly different at $P < 0.05$ (Duncan 1955)

regenerating transgenic plants or mass propagation in this orchid. In this report, the leaf culture system of *Phalaenopsis* was used to study the effects of growth regulators on direct somatic embryogenesis.

Materials and methods

Two *Phalaenopsis* orchids were used in this study, *Phalaenopsis amabilis* Shimadzu var. *formosa* with large white flowers (the most beautiful native orchid species in Taiwan) and *P.* Nebula (one of the most popular commercial cultivar). In-vitro-grown plants of *P. amabilis* were purchased from Taiwan Sugar Corporation (TSC), Taiwan. While the seedlings of *P.* Nebula were collected by in vitro seed germination followed the protocol described by Chen and Chang (2006). The basal medium is 1/2-strength modified Murashige and Skoog (1962; 1/2 MS) medium containing half-strength macro- and micro-elements supplemented with myo-inositol (100 mg/l), niacin (0.5 mg/l), pyridoxine HCl (0.5 mg/l), thiamine HCl (0.1 mg/l), glycine (2 mg/l), peptone (1 g/l), NaH_2PO_4 (170 mg/l), sucrose (20 g/l) and Gelrite (2.2 g/l). Plant growth regulators were added according to the experimental design and the pH of the media was adjusted to 5.2 with 1 M KOH or HCl prior to autoclaving for 15 min at 121°C. The plants were maintained with a 2-month-interval subculture period on a hormone-free 1/2 MS medium in 250 ml flasks under a 16-h photoperiod at irradiance of 28–36 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 three to five leaves and two to four roots were used as donor plants.

Leaf tip segments (about 1 cm in length) taken from the donor plants were used to induce direct somatic embryogenesis on a 1/2 MS basal medium supplemented with growth regulators as the experimental design. The leaf explants of two genotypes (*P. amabilis*, *P.* Nebula) were placed adaxial-side-up on the culture medium and were incubated in 90 × 15 mm^2 Petri dishes under darkness for

Table 2 Effects of cytokinins on direct somatic embryogenesis from different locations of leaf explants of *Phalaenopsis* Nebula

Cytokinins (μM)	Percentage of emrbyogenesis on the entire explant (%)	Percentage of embryogenesis on different explant locations (%)				No. of embryos per responding explant
		CE	Ad	Ab	LT	
Control	0 e	0 e	0 c	0 c	0 b	0
2iP						
2.46	30 bcd	25 bcd	10 abc	0 c	0 b	3.7
4.92	60 ab	50 ab	15 ab	25 a	20 a	8.8
14.76	30 bcd	30 bcd	10 abc	5 bc	0 b	4.2
BA						
2.22	40 abc	40 ab	10 abc	10 c	10 ab	13.1
4.44	30 bcd	25 bcd	5 bc	5 bc	5 b	10.2
13.32	15 cde	15 cde	0 c	0 c	0 b	1.7
Kinetin						
2.32	15 cde	15 cde	0 c	0 c	0 b	7.0
4.65	15 cde	15 cde	5 bc	5 bc	0 b	16.0
13.94	10 de	5 de	5 bc	0 c	0 b	2.0
TDZ						
2.27	15 cde	5 de	0 c	5 bc	0 b	10.3
4.54	35 abcd	25 bcd	5 bc	20 ab	5 b	6.7
13.62	65 a	45 ab	25 a	5 bc	10 ab	6.1
Zeatin						
2.28	30 bcd	20 cde	15 abc	0 bc	5 b	8.8
4.56	45 abc	35 abc	10 abc	5 bc	5 b	8.7
13.68	60 ab	60 a	5 bc	15 ab	0 b	4.7

LT Leaf tips, *Ad* adaxial sides, *Ab* abaxial sides, *CE* cut ends

Data were scored after 60 days of culture. The amounts of direct embryo formation were counted at entire explants or at different explant locations. Data expressed as percentage were transformed using arc sine prior to ANOVA and converted back to the original scale for demonstration in table (Compton 1994). Five replicates (dishes) each with four leaf explants were performed for each treatment. Mean with the same letters within columns are not significantly different at $P < 0.05$ (Duncan 1955)

2 months. Growth regulators including auxins (2.26, 4.52 and 13.57 μM 2,4-D; 2.85, 5.71 and 17.13 μM IAA; 2.46, 4.92 and 14.76 μM IBA; 2.69, 5.37 and 16.11 μM NAA), cytokinins (types and concentrations were shown in Tables 1, 2), GA_3 (2.89, 7.22 and 14.44 μM), ancymidol (3.90, 9.75 and 14.44 μM), polyamines (11.34, 28.36 and 56.72 μM putrescine; 4.94, 12.36 and 24.71 μM spermine; 6.88, 17.21 and 34.42 μM spermidine), ACC, $AgNO_3$ and $CoCl_2$ (concentrations were shown in Table 3) were used to test their effects on the amount of direct embryo formation from different locations (LT: leaf tips; Ad: adaxial sides; Ab: abaxial sides; CE: cut ends) on leaf explants. Five replicates (dishes) each with four leaf explants were performed for each treatment.

The percentage of explants forming somatic embryos was recorded as formed from entire explants or different locations of the explants. The number of embryos formed from each responding explant was counted under a stereomicroscope (SZH, Olympus, Tokyo, Japan) at the protocorm stage. Data expressed as percentage were transformed using arc sine prior to ANOVA and converted back to the original scale (Compton 1994). Treatment means were compared by following Duncan's Multiple Range Test (Duncan 1955).

Results and discussion

Effects of auxins

Leaf explants of *P. amabilis* formed small transparent granules from cut ends in all of the auxin treatments, but these granular structures failed to further develop. Severe browning was found on all explants especially on 2,4-D-containing medium. In fact, almost all the explants became necrotic after 2 months of culture. In the previous report by Kuo et al. (2005), it was found that leaf explants became necrotic and no embryos were obtained when cultured on medium supplemented with 0.45–4.52 μM 2,4-D. Besides, exogenous auxins also played an inhibitory role in direct

Table 3 Effects of ACC, $AgNO_3$ and $CoCl_2$ on direct somatic embryogenesis from different locations of leaf explants of *Phalaenopsis amabilis*

Treatments	Percentage of emrbyogenesis on the entire explant (%)	Percentage of browning (%)	Percentage of embryogenesis on different explant locations (%)				No. of embryos per responding explant
			CE	Ad	Ab	LT	
Control	0 b	15 bc	0 b	0 b	0 b	0 b	0
ACC (μM)							
5	15 ab	5 c	15 ab	5 b	5 b	0 b	7.0
10	15 ab	15 bc	15 ab	10 b	0 b	0 b	10.0
20	35 a	25 abc	20 a	25 a	0 b	0 b	10.6
50	10 b	65 a	5 ab	10 b	0 b	5 b	4.5
$AgNO_3$ (μM)							
5	0 b	60 a	0 b	0 b	0 b	0 b	0
10	5 b	30 abc	5 ab	0 b	0 b	0 b	6.0
20	0 b	20 bc	0 b	0 b	0 b	0 b	0
50	0 b	45 ab	0 b	0 b	0 b	0 b	0
$CoCl_2$ (μM)							
5	5 b	60 a	5 ab	0 b	0 b	0 b	7.0
10	0 b	40 abc	0 b	0 b	0 b	0 b	0
20	10 b	45 ab	5 ab	5 b	0 b	0 b	10.5
50	10 b	60 a	5 ab	5 b	0 b	0 b	3.5

LT Leaf tips, *Ad* adaxial sides, *Ab* abaxial sides, *CE* cut ends

Data were scored after 60 days of culture. The amounts of direct embryo formation were counted at entire explants or at different explant locations. Data expressed as percentage were transformed using arc sine prior to ANOVA and converted back to the original scale for demonstration in table (Compton 1994). Five replicates (dishes) each with four leaf explants were performed for each treatment. Mean with the same letters within columns are not significantly different at $P < 0.05$ (Duncan 1955)

embryo formation of *Oncidium* and *Dendrobium* (Chen and Chang 2001; Chung et al. 2005, 2007). In *P. amabilis*, Chen and Chang (2006) had reported that NAA retards direct embryo formation from leaf explants and reduced the amount of embryos induced by TDZ. Another work by Kuo et al. (2005) in *Phalaenopsis* "Little Steve", 2,4-D showed highly inhibitory in direct embryo induction from leaf explants even in the presence of TDZ. The present result showed that four kinds of auxins (including 2,4-D, IAA, IBA and NAA) all highly retarded direct embryogenesis from leaf explants of *P. amabilis*.

Effects of cytokinins

Working with *P. amabilis*, TDZ promoted repetitive embryogenesis from zygotic protocorms (Chen and Chang 2004), and induced a higher frequency of embryogenesis from leaf explants (Chen and Chang 2006). In *P.* "Little Steve", TDZ induced a higher frequency of embryogenesis from leaf explants than BA and kinetin (Kuo et al. 2005). In this present study, Table 1 shows effects of different cytokinins on direct embryo formation from leaf explants of *P. amabilis*. Except for the control treatment, all of cytokinin tested had embryogenic response after 2 months of culture. The highest percentage of embryogenesis (80%) was found at 13.32 μM BA, while the highest number of embryos per explant was 7.8 at 13.62 μM TDZ (Table 1). Altogether, BA at 13.32 μM had the highest enhancement on the amount of direct embryogenesis (80% of embryogenesis × 6.6 embryos per explant) than other cytokinins (Table 1). Leaf position could affect direct embryogenesis, and different locations showed distinct ability to form embryos (Table 1). The cut ends had highest embryogenic competence and adaxial sides were the second (Table 1). By contrast, abaxial sides and leaf tip locations only formed a small numbers of embryos (Table 1).

In *P.* Nebula, the highest percentage of embryogenesis and number of embryos per explant were at 13.62 μM TDZ and 4.65 μM kinetin, respectively (Table 2). Altogether, 4.92 μM 2iP had the highest response on the amount of embryogenesis (60% of embryogenesis × 8.8 embryos per responding explant) than other cytokinins (Table 2). Obviously, there was a genotypic effect on the type and concentration of cytokinin using in direct embryo induction (Tables 1, 2). A position effect was also effective in *P.* Nebula, the order of locations' embryogenic competence was almost the same with *P. amabilis* as the cut end, the adaxial side was the second, and then the abaxial side and the leaf tip (Table 2). Both in two species, the cut end had

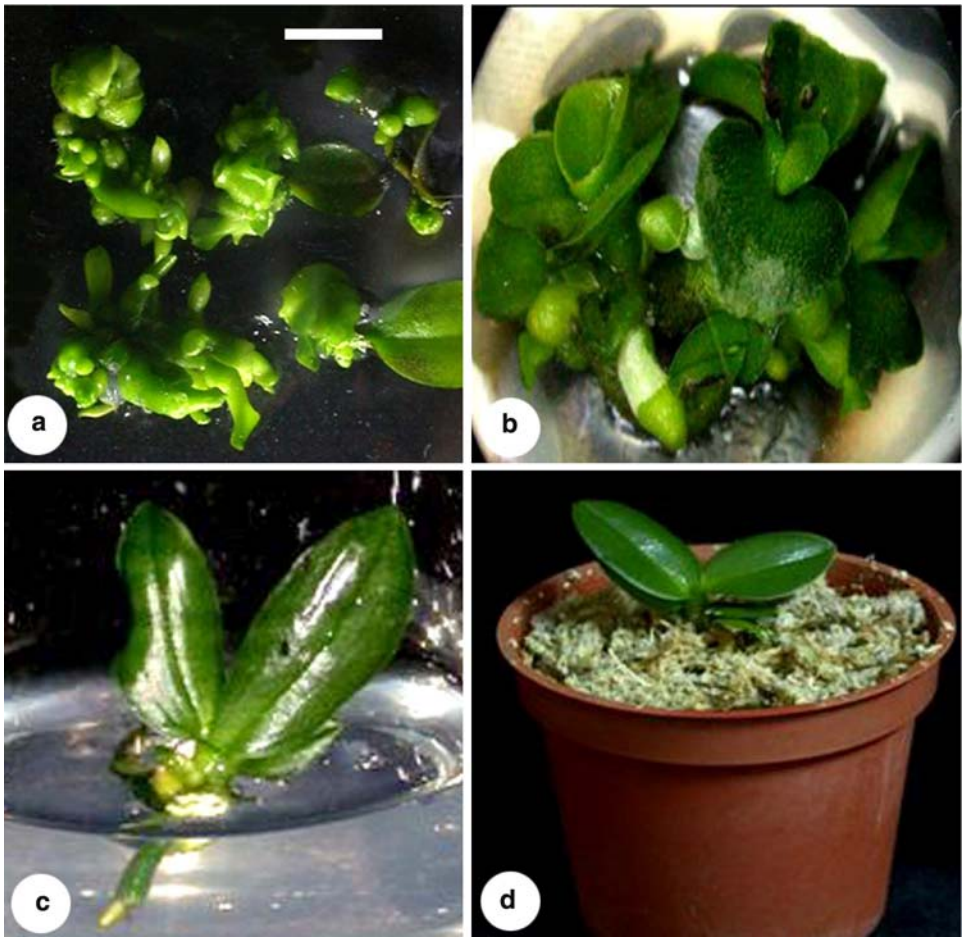

Fig. 1 Plant regeneration via direct somatic embryogenesis from leaf explants of *Phalaenopsis amabilis* on 13.32 μM BA containing medium. **a** embryos formed on leaf explants after 2 months of culture in darkness (*Scale bar* 8 mm); **b** embryos further enlarged and formed roots after subculture on hormone-free 1/2 MS medium for 2 months of culture (*Scale bar* 5 mm); **c** an embryo-derived plantlet (*Scale bar* 10 mm); **d** a 6-month-old regenerated plantlet acclimatized in greenhouse (*Scale bar* 25 mm)

the best embryogenic response than other leaf locations (Tables 1, 2). However, in *Oncidum* leaf cultures, the leaf tip had a highly embryogenic competence than the cut end and other locations (Chen et al. 1999; Chen and Chang 2001). Further investigations on more species are needed to verify the difference in position effects between *Phalaenopsis* and *Oncidium* leaf cultures.

Effect of GA_3 and ancymidol

Leaf cultures of *P. amabilis* showed no embryogenic response in all of the GA_3 and ancymidol treatments. Although occasionally a small number of transparent granules was found on cut ends of explants. All of these protuberances failed to develop and became necrotic with the parental explants eventually after 2 months of culture.

Effects of polyamines

Except for one explant formed a single embryo at 11.34 μM putrescine, all of the polyamine treatments resulted in no embryogenic response from leaf cultures of *P. amabils*.

Effects of ACC, $AgNO_3$ and $CoCl_2$

In *P. amabilis*, ACC at 20 μM significantly induced 35% of leaf explants to form 10.6 embryos per explant (Table 3). However, there were no significant effects induced by other treatments of ACC, $AgNO_3$ and $CoCl_2$ (Table 3). In addition, all of the ACC, $AgNO_3$ and $CoCl_2$ treatments had no embryogenic response on leaf cultures of another species, *P.* Nebula. Further intensive investigations using more genotypes should be performed to clarify the action of ethylene on direct embryo formation in *Phalaenopsis*.

Plantlet conversion

After 2 months of culture in darkness, embryos (about 2–4 mm in diameter) formed on surfaces of leaf explants (Fig. 1a). These regenerated embryos showed no obvious morphological difference between growth regulator

treatments. Embryos further enlarged and formed roots after 4 months of culture (Fig. 1b). Plantlets derived from embryos were successfully established on hormone-free 1/2 MS medium and grew normally (Fig. 1c). After 6 months of culture, several dozens of plantlets were transplanted to sphagnum moss and acclimatized in greenhouse with 100% survival rate (Fig. 1d).

In summary, promotion of direct embryo formation could be achieved by adjusting the type and concentration of growth regulators in culture medium. Generally, cytokinins tested were all effective in direct embryo induction of both species, and ACC at 20 μM showed 35% of embryogenic response in *P. amabilis*. However, auxins, GA_3, ancymidol and polyamines were all inhibitory in both species. Besides, genotype and explant position also affect direct embryo induction in *Phalaenopsis*.

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