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Factors Affecting to Somatic Embryogenesis and Plant Regeneration from Callus and *In Vitro* Ontogeny of *Doritaenopsis* Taisuco Ladylip

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The efficient plant regeneration protocols were developed for *Doritaenopsis* Taisuco Ladylip through callus culture and somatic embryogenesis. The embryogenic calli were cultured on half strength Murashige and Skoog (MS) medium supplemented with 0.5 mg·L⁻¹ thidiazuron, 2.5 mg·L⁻¹ 2,4-dichlorophenoxyacetic acid, 30 g·L⁻¹ sucrose and 3 g·L⁻¹ Gelrite for proliferation. Basal salts, carbohydrate of medium obviously affected the somatic embryogenesis of callus. The highest frequency of somatic embryogenesis was obtained when embryogenic calli were cultured on half strength MS medium containing 20 g·L⁻¹ maltose or 10 g·L⁻¹ trehalose. The protocorm-like bodies well developed into plantlets after changing the basal salts to Hyponex, and carbohydrate to sucrose. The histological processing of somatic embryogenesis from callus was observed with light microscopy, and somatic embryo was clearly origin from single cell.

Key words: ammonium nitrate, carbohydrate, *Phalaenopsis*, protocorm-like body, somatic embryo

INTRODUCTION

Doritaenopsis genus is an intergeneric hybrid between *Doritis* and *Phalaenopsis*. In commercial practice, plants of *Phalaenopsis*, *Doritis* and *Doritaenopsis* are collectively named as phalaenopsis orchids. At present, the Royal Horticultural Society (RHS) committee has decided to sink *Doritis* into *Phalaenopsis*; therefore *Doritis* and *Doritaenopsis* are now considered to be *Phalaenopsis* by RHS (Royal Horticultural Society, 2013).

Phalaenopsis orchid has been a favorite consumer product due to its attractive color, graceful and long-lasting flowers. Nowadays, pot phalaenopsis has become one of the most important orchids in the trade. For phalaenopsis orchid, an efficient plant regeneration system is beneficial for the micropropagation of selected elites and genetic transformation studies. Various micropropagation systems have been reported for micropropagating orchids; the generation of protocorm-like bodies (PLBs) is one of the important techniques (Arditti and Ernst, 1993). PLBs were first noted in the shoot-tip culture of *Cymbidium* orchid by Morel (1960). Since the general characteristics of growth and structure are similar to those of protocorms, the regenerated structures are termed PLBs.

PLBs of phalaenopsis can be induced directly from various explants, such as shoot tips (Tokuhara and Mii, 1993), flower stalk buds (Ichihashi, 1992), root tips

(Tanaka *et al.*, 1976), and leaf segments (Park *et al.*, 2002). The indirect regeneration of PLBs from embryogenic callus culture has also been documented using solid (Ishii *et al.*, 1998; Tokuhara and Mii, 2001) and liquid suspension cultures (Tokuhara and Mii, 2003). In the literature, it is often suggested that PLBs are somatic embryos simply based on morphology (Huan *et al.*, 2004; Ishii *et al.*, 1998) or that somatic embryogenesis is an early step in PLB formation (Zhao *et al.*, 2008). Somatic embryogenesis is the development of somatic cells into differentiated plants through embryo stages under appropriate conditions (Zimmerman, 1993). Are PLBs somatic embryos? Do they take on characteristics similar to zygotic embryos prior to developing into PLBs? To answer these questions and to determine whether PLBs are indeed somatic embryos, we carried out a detailed ontogenetic study of PLBs from a friable embryogenic callus of *Phalaenopsis* using histological and histochemical methods (Lee *et al.*, 2013). This study demonstrates that during the early stages of PLB formation, the cells show cytological characteristics and cell wall markers similar to zygotic embryo development, justifying the statement that PLBs are indeed somatic embryos of orchids.

At present, the various micropropagation systems have been developed for the research and the application of *Doritaenopsis* (Amaki and Higuchi, 1989; Chowdbury *et al.*, 2003; Ernst, 1994; Islam *et al.*, 2003; Islam and Ichihashi, 1999; Park, *et al.*, 2002; Park *et al.*, 2003; Tokuhara and Mii, 1993; Zhou, 1995). Although phalaenopsis seedling main production methods are by *in vitro* sowing, lateral bud of flower stalk culture and the protocorm-like body multiplication system, yet related researches are still continuous developing for mass production and genetic engineering. It is even more important, plant regeneration through somatic embryogenesis in genetic transformation has the certain superiority. The published reports about *Doritaenopsis* plant

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regeneration from callus were already considerable plentiful (Chowdbury *et al.*, 2003; Islam and Ichihashi, 1999; Islam *et al.*, 2003). Somatic embryogenesis offers vast potential as the method not only for clonal propagation but also genetic transformation. Therefore, in the view of long-term researches of molecular breeding in phalaenopsis, it is necessary to establish the cell culture systems of high regeneration efficiency and demand for genetic transformation.

MATERIALS AND METHODS

Plant material and callus proliferation

The initial callus was generated from *in vitro* grown protocorms of the breeding line *Doritaenopsis* Taisuco Ladylip. The callus was cultured and maintained on 1/2-strength macroelements and full-strength microelements of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 100 mg·L⁻¹ glutamine, 1 mg·L⁻¹ biotin, 100 mg·L⁻¹ myo-inositol, 30 g·L⁻¹ sucrose, 3 g·L⁻¹ Gelrite, 2.5 mg·L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.5 mg·L⁻¹ thidiazuron (TDZ). The pH of the medium was adjusted to 5.7 with 1 N KOH prior to autoclaving at 121°C for 20 min. The callus was incubated in darkness at 25±2°C.

The same medium as above and another six media with different supplements of plant growth regulators, including 2 or 3 mg·L⁻¹ 2,4-D combined with 0, 0.1 or 1.0 mg·L⁻¹ TDZ, were prepared by the same protocol as above and adopted to test the effect of plant growth regulator treatments on callus proliferation. Each treatment contained 0.2 g fresh weight callus per 9-cm diameter plastic Petri dish containing 20 mL medium with four replicate dishes. Data were scored after six weeks of culture in darkness at 25±2°C.

Somatic embryogenesis and PLB formation

For somatic embryogenesis and PLB formation from callus, 1/2 strength MS basal media (1/2 MS) containing 100 mg·L⁻¹ glutamine, 1 mg·L⁻¹ biotin, 100 mg·L⁻¹ myo-inositol, 3 g·L⁻¹ Gelrite supplemented with three types of carbohydrates (sucrose [Bio Basic INC.], maltose [Sigma] and trehalose [Sigma]) at concentration of 5, 10 and 20 g·L⁻¹, and two combinations of plant growth regulators (2 or 3 mg·L⁻¹ 2,4-D combined with 1 mg·L⁻¹ TDZ) separately were prepared by the same protocol as above and adopted to evaluate the effects of different types and concentrations of carbohydrate source, and growth regulator combinations.

This three-factorial experiment was carried out in terms of a completely randomized design with 18 different treatments and four replicates of each treatment. Calli gained on media containing 2 mg·L⁻¹ 2,4-D+1 mg·L⁻¹ TDZ and 3 mg·L⁻¹ 2,4-D+1 mg·L⁻¹ TDZ from prior experiment of callus proliferation were respectively used for treatments with 2 and 3 mg·L⁻¹ 2,4-D combined with 1 mg·L⁻¹ TDZ in this experiment. For each replicate, the 0.3 g fresh weight callus initially divided into 10 masses was inoculated on a 9-cm Petri dish containing 20 ml medium. Data were scored after three months of culture

at 25±2°C with 16 h light/8 h dark and photon flux of 22–35 μmol·m⁻²·s⁻¹ (daylight fluorescent tube FL-20D/18; 20 W). The events investigated included the fresh weight and number of PLBs formed from callus. The osmotic pressure of media without solidifying agent were also measured by osmometer (5500 Vapor pressure osmometer, Wescor Inc.), and in unit of mOsm·kg⁻¹.

Plantlet growth and development

To investigate the effects of sucrose and ammonium nitrate concentrations on growth of plantlet regenerated from callus, tests were done as a two-factorial experiment in a completely randomized design with 9 different treatments and four replicates of each treatment.

PLBs regenerated from prior experiment of somatic embryogenesis and PLB formation were separately cultured on basal media containing 3 g·L⁻¹ Hyponex#1, 0.1 g·L⁻¹ citric acid, 1 g·L⁻¹ tryptone-peptone, 1 g·L⁻¹ activated charcoal, 8 g·L⁻¹ agar supplemented with sucrose at concentration of 10, 20 and 30 g·L⁻¹, and ammonium nitrate at concentration of 0, 412.5 (1/4 MS) and 825.0 mg·L⁻¹ (1/2 MS). The pH of the media was adjusted to 5.6 prior to autoclaving. Ten PLBs (on average 0.15 g fresh mass) were placed on a 9 cm Petri dish containing 20 mL solid medium. Each Petri dish was regarded as a replicate. After three months of culture at 25±2°C with 16 h light/8 h dark and photon flux of 22–35 μmol·m⁻²·s⁻¹, explants were evaluated in terms of plantlet growth indexes including weight, number and length of leaf and root.

Morphological and histological investigations

Cytological observations were begun at the onset of somatic embryogenesis. The materials were observed and taken pictures in the stereomicroscope (Olympus SZX-RFA) and were squashed and examined using a light microscope (Zeiss Axioskop2). For histological observations, samples were fixed in FAA solution (70% ethanol, 5% glacial acetic acid, and 5% formaldehyde) for 24 hours in room temperature, then were dehydrated with a graded series of ethanol and butanol solutions and embedded in paraffin referred to Berlyn and Miksche (1976). Tissue was sectioned at 8–10 mm thickness, stained with Delafield's hematoxylin solution (Merck, Germany) for 10 min, and examined under a light microscope (Zeiss Axioskop2).

RESULTS

Effect of 2,4-D and TDZ on callus proliferation

This experiment was performed to evaluate the effects of the type and concentration of plant growth regulators on callus proliferation in *Doritaenopsis* Taisuco Ladylip. In this study the effects of 2,4-D and TDZ at various concentrations were tested for their potential on callus fresh weight gain and morphogenetic change. The combination of 2 mg·L⁻¹ 2,4-D and 1 mg·L⁻¹ TDZ or 2.5 mg·L⁻¹ 2,4-D and 0.5 mg·L⁻¹ TDZ were most effective on callus fresh weight. The reduction of TDZ concentration decreased callus fresh weight. When 2 or 3 mg·L⁻¹

2,4-D was combined without TDZ, necrotic browning callus was formed (Table 1). Under the TDZ supple-

ment, the calli looked more fresh yellow in 3 mg·L⁻¹ 2,4-D than in 2 mg·L⁻¹ 2,4-D (Fig. 1A).

Table 1. Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) and thidiazuron (TDZ) on callus proliferation in *Doritaenopsis* Taisuco Ladylip

Plant growth regulators (mg·L ⁻¹)		Callus		
2,4-D	TDZ	Fresh weight (g)	Increased weight (%)	Morphology
2.0	0	0.3 d	48 d	Browning death
2.0	0.1	1.0 bc	416 bc	
2.0	1.0	1.4 a	616 a	
2.5	0.5	1.3 a	558 a	
3.0	0	0.3 d	51 d	Browning death
3.0	0.1	0.9 c	358 c	
3.0	1.0	1.1 b	466 b	

Basal medium was 1/2 MS, 100 mg·L⁻¹ glutamine, 1 mg·L⁻¹ biotin, 100 mg·L⁻¹ myo-inositol, 30 g·L⁻¹ sucrose, 3 g·L⁻¹ Gelrite, pH5.7.

For each treatment, four replicates of 0.2 g callus in each dish were cultured.

Data were scored after six weeks of culture in the dark.

Means separated within columns by Duncan's multiple range test at $P \leq 0.05$.

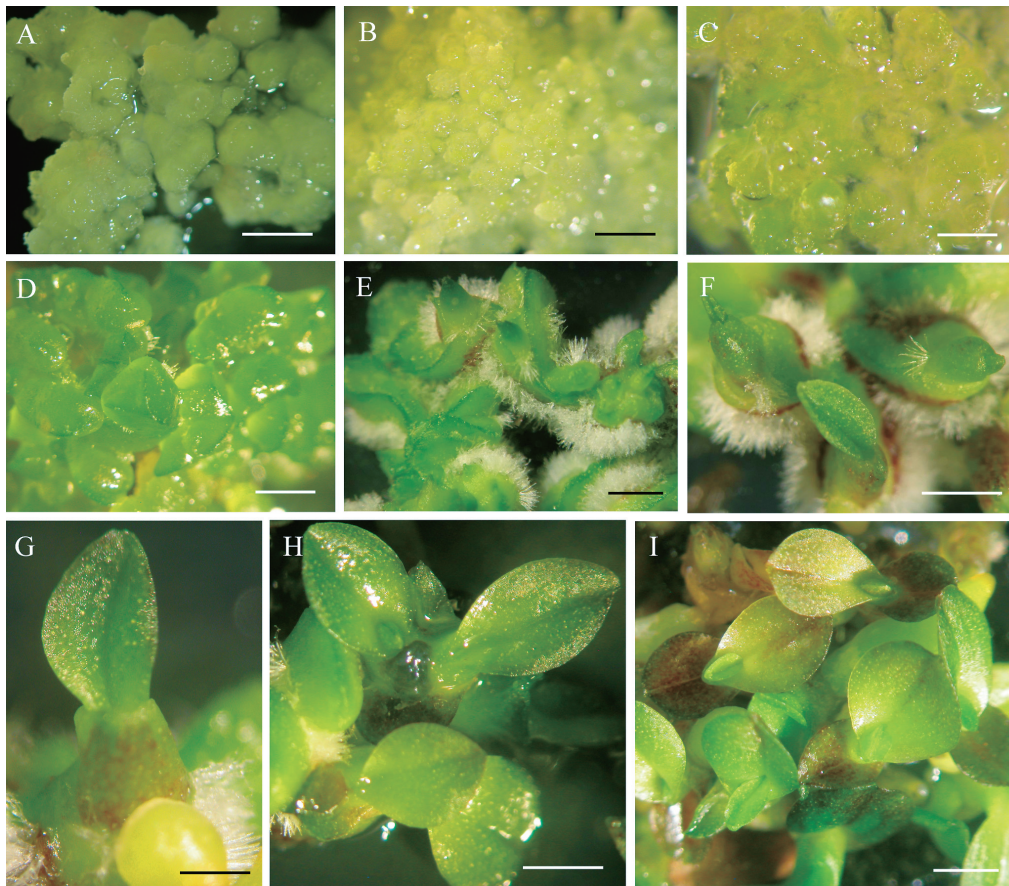


Fig. 1. Plant regeneration through somatic embryogenesis of callus in *Doritaenopsis* Taisuco Ladylip. Continuous subculturing of the initial callus derived from *in vitro* grown protocorms resulted in the formation of the friable yellowish-white embryogenic callus (A). After transferred to the protocorm-like body (PLB) induction medium, the callus gradually turned greenish (B) and then gave rise to some of initial PLBs within two months of culture (C). After three months culture, somatic embryos developed to PLBs with morphological and functional polarity (D). Rhizoids formed on surface of mature PLBs with leaves after five months of culture (E, F), and roots protruded after six months of culture (G, H). PLBs developed into intact plantlets within seven months of culture (I). Bar=2 mm (A, D, E, F, H, I) ; 1 mm (B, C, G).

Effects of different types and concentrations of carbohydrate source, and growth regulator combinations on somatic embryogenesis and PLB formation from callus

This three-factorial experiment was carried out to investigate the effects of maltose, trehalose and sucrose at various concentrations, and two growth regulator combinations on somatic embryogenesis and PLB formation from callus in *Doritaenopsis* Taisuco Ladylip. Significant differences in fresh weight gain, PLB formation and plantlet development were all observed among the types and concentrations of carbohydrate source tested (Table 2); however the interactions between the types and concentrations of carbohydrate source were also significant. Calli gained on media containing 2 mg·L⁻¹ 2,4-D+1 mg·L⁻¹ TDZ (D2T1) and 3 mg·L⁻¹ 2,4-D+1 mg·L⁻¹ TDZ (D3T1) from prior experiment of callus proliferation and respectively used for treatments with 2 and 3 mg·L⁻¹ 2,4-D combined with 1 mg·L⁻¹ TDZ had no significant effect on fresh weight gain, PLB formation and plantlet development (Table 2). In addition, the two-factor interaction between type of carbohydrate source and growth regulator combination and the interaction between concentration of carbohydrate source and growth regulator combination, and the three-factor interaction between type and

concentration of carbohydrate source and growth regulator combination were all not significant (Table 2).

The rise of concentration in all three carbohydrates tended to increase explant fresh weight, although the treatments employed trehalose at concentration of 20 g·L⁻¹ were the most ones (Table 3). The treatments used sucrose at concentration of 20 g·L⁻¹ were no useful on PLB formation (Table 3), the explants still remained in a callus stage. When sucrose was supplemented at 5 and 10 g·L⁻¹, the formation of the PLBs were observed (Table 3); however the PLBs were almost smaller than 2 mm in diameter in treatments of sucrose at 10 g·L⁻¹. The treatments employed maltose at concentration of 10 and 20 g·L⁻¹ and trehalose at 10 g·L⁻¹ were most effective on PLB formation in quantity (Table 3), about 300 PLBs were produced from each 0.3 g callus after three months of culture.

Comparing the development of PLB with three different carbohydrates, based on the proportion of PLBs with leaf, it was observed that maltose was the most effective carbohydrate source with average of 12.4%, obviously higher than 8.7% of the trehalose and 2.8% of the sucrose (Table 2). To simultaneously take account of the root development, maltose at 20 g·L⁻¹ was obvious the best choice for PLB formation and development (Table 3).

Table 2. Analysis of variance for the effects of three different disaccharides and concentrations, and two growth regulator combinations on fresh weight and the protocorm-like body (PLB) formation from the callus in *Doritaenopsis* Taisuco Ladylip

Treatments	Fresh weight (g)	No. of PLB(>2 mm) (A)	No. of PLB with leaf(>2 mm) (B)	No. of PLB with leaf(>2 mm) (C)	((B+C)/A) * 100	No. of PLB with root
maltose	3.7 b	256.1 a	30.0 a	3.4 a	12.4 a	2.9 a
trehalose	4.8 a	158.6 b	15.5 b	1.8 b	8.7 b	1.3 a
sucrose	3.9 b	20.0 c	1.0 c	0.5c	2.8 c	0.0 c
Carbo	***	***	***	***	***	***
5 g·L ⁻¹	2.3 c	109.3 b	5.9 b	0.9b	6.9 a	0.4 b
10 g·L ⁻¹	4.7 b	204.8 a	22.6 a	2.7a	8.5 a	2.0 a
20 g·L ⁻¹	5.4 a	120.7 b	18.0 a	2.1 ab	8.5 a	1.9 a
Con	***	***	***	**	ns	*
Carbo * Con	***	***	***	***	***	***
D2T1	4.1 a	144.2 a	16.1 a	2.1 a	8.4 a	1.3 a
D3T1	4.1 a	145.7 a	14.9 a	1.7 a	7.5 a	1.5 a
DT	ns	ns	ns	ns	ns	ns
Carbo * DT	ns	ns	ns	ns	ns	ns
Con* DT	ns	ns	ns	ns	ns	ns
Carbo * Con* DT	ns	ns	ns	ns	ns	ns

Calli gained on media containing 2 mg·L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D)+1 mg·L⁻¹ thidiazuron (TDZ) (D2T1) and 3 mg·L⁻¹ 2,4-D + 1 mg·L⁻¹ TDZ (D3T1) from prior experiment of callus proliferation were respectively used for treatments with 2 and 3 mg·L⁻¹ 2,4-D combined with 1 mg·L⁻¹ TDZ in this experiment.

Basal medium supplemented with 1/2 MS, 100 mg·L⁻¹ glutamine, 1 mg·L⁻¹ biotin, 100 mg·L⁻¹ myo-inositol, 3 g·L⁻¹ Gelrite, pH 5.7. For each treatment, four replicates of 0.3 g callus in each dish were cultured. Data were scored after three months of culture at 25±2°C with 16 h light/8 h dark and photon flux of 22–35 μmol·m⁻²·s⁻¹.

Means separated within columns by Duncan's multiple range test at $P \leq 0.05$.

NS, *, **, ***: Not significant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table 3. Effects of three different disaccharides and concentrations, and two growth regulator combinations on fresh weight and the protocorm-like body (PLB) formation from the callus in *Doritaenopsis* Taisuco Ladylip

Callus from the culture medium	Carbo-hydrate	Conc. (g·L ⁻¹)	mOsm·kg ⁻¹	F.W. (g)	No. of PLB (>0.2 mm)	No. of PLB with leaf (<2 mm)	No. of PLB with leaf (>0.2 mm)	((B+C)/A)* 100	(C/A)* 100	No. of PLB with root
					(A)	(B)	(C)			
D2T1	maltose	5	68	2.1e	119.3cd	0.5d	7.5d	8.1de	7.6de	0.3d
		10	83	4.2d	347.0a	2.8bcd	30.3c	9.5cde	8.7bcde	2.8bcd
		20	110	4.6cd	289.3b	8.8a	52.5a	21.2a	17.8a	4.8ab
	trehalose	5	66	1.9e	141.8c	0.3d	5.3d	4.0ef	3.7ef	0.8cd
		10	79	5.5b	276.3b	6.0ab	40.8abc	17.1ab	14.9abc	3.0bcd
		20	109	6.8a	71.3de	0.0d	4.5d	5.8ef	5.8ef	0.5cd
	sucrose	5	69	2.6e	47.5ef	1.0d	4.3d	10.4bcde	8.4cde	0.0d
		10	82	4.3d	5.3f	0.0d	0.0d	0.0f	0.0f	0.0d
		20	113	5.2bc	0.0f	0.0d	0.0d	0.0f	0.0f	0.0d
D3T1	maltose	5	68	2.3e	145.3c	1.8cd	12.5d	9.5cde	8.3de	0.3d
		10	83	4.2d	312.5ab	2.8bcd	28.5c	10.0bcde	9.1bcde	3.5abc
		20	110	4.6cd	323.5ab	3.8bcd	49.0ab	16.2abc	15.1ab	6.0a
	trehalose	5	66	2.0e	141.0c	0.0d	4.3d	2.9ef	2.9ef	1.0cd
		10	79	5.5b	281.5b	4.8bc	36.0bc	14.4abcd	12.8abcd	2.8bcd
		20	109	7.0a	40.0ef	0.0d	2.0d	8.0de	8.0de	0.0d
	sucrose	5	69	2.5e	61.0e	2.0cd	1.8d	6.2ef	2.5ef	0.3d
		10	82	4.4cd	6.5f	0.0d	0.0d	0.0f	0.0f	0.0d
		20	113	4.5cd	0.0f	0.0d	0.0d	0.0f	0.0f	0.0d

Calli gained on media containing 2 mg·L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D)+1 mg·L⁻¹ thidiazuron (TDZ) (D2T1) and 3 mg·L⁻¹ 2,4-D+1 mg·L⁻¹ TDZ (D3T1) from prior experiment of callus proliferation were respectively used for treatments with 2 and 3 mg·L⁻¹ 2,4-D combined with 1 mg·L⁻¹ TDZ in this experiment.

Basal medium supplemented with 1/2 MS, 100 mg·L⁻¹ glutamine, 1 mg·L⁻¹ biotin, 100 mg·L⁻¹ myo-inositol, 3 g·L⁻¹ Gelrite, pH 5.7.

For each treatment, four replicates of 0.3 g callus in each dish were cultured. Data were scored after three months of culture at 25±2°C with 16 h light/8 h dark and photon flux of 22–35 μmol·m⁻²·s⁻¹.

Means separated within columns by Duncan's multiple range test at $P \leq 0.05$.

Effects of sucrose and ammonium nitrate concentrations on growth of plantlet regenerated from callus

This two-factorial experiment was carried out to evaluate the effects of sucrose and ammonium nitrate concentrations on growth of plantlet regenerated from callus in *Doritaenopsis* Taisuco Ladylip. No significant differences in total number of leaves, total leaf length and weight measured per treatment were observed among the sucrose concentrations as well as among the ammonium nitrate concentrations tested (Table 4). However, significant differences in total number of roots, total root length and weight measured per treatment were all observed among the sucrose and ammonium nitrate concentrations (Table 4); in addition, the interactions between the sucrose and ammonium nitrate concentrations were not significant. Among the sucrose concentrations, treatments of 30 g·L⁻¹ gave the highest total number of roots and 10 g·L⁻¹ the poorest, and treatments of 20 and 30 g·L⁻¹ offered both the highest total root length and weight (Table 4). Among the ammonium nitrate concentrations, treatments without additional supplementation and treatments of 412.5 mg·L⁻¹ (half strength

employed in MS) gave the highest total number of roots, and treatments without additional supplementation offered both the highest total root length and weight and 825.0 mg·L⁻¹ (quarter strength employed in MS medium) the least (Table 4).

In terms of the ratios of root to leaf in fresh weight, in length and in number, there were all significant differences among the sucrose and ammonium nitrate concentrations (Table 5); however the interactions between the sucrose and ammonium nitrate concentrations were not significant. Among the sucrose concentrations, treatments of 30 g·L⁻¹ gave the highest ratios of root to leaf in fresh weight, in length and in number and 10 g·L⁻¹ the least (Table 5). Among the ammonium nitrate concentrations, treatments without additional supplementation and treatments of 412.5 mg·L⁻¹ gave the highest ratios of root to leaf in fresh weight, and treatments without additional supplementation offered both the highest ratios of root to leaf in length and in number and 825.0 mg·L⁻¹ the least (Table 5).

In considering the growth and balance of root and shoot (Table 4 and 5), basal media containing 3 g·L⁻¹ Hyponex#1 supplemented with sucrose at concentration

Table 4. Analysis of variance for the effects of sucrose and ammonium nitrate concentrations on growth of plantlet regenerated from callus in *Doritaenopsis* Taisuco Ladylip

Treatments			Total no. of leaves	Total leaf length (cm)	Total leaf weight (g)	Total no. of roots	Total root length (cm)	Total root weight (g)
Sucrose (A)	(g·L ⁻¹)	10	32.0a	24.7b	1.2a	22.0c	17.8b	0.5b
		20	30.1a	27.5a	1.4a	32.3b	36.4a	1.1a
		30	31.0a	25.0ab	1.2a	36.3a	37.8a	1.2a
			ns	ns	ns	***	***	***
NH ₄ NO ₃ (B)		0	29.6a	26.0ab	1.3a	33.8a	1.2a	37.4a
		1/4MS	31.8a	27.2a	1.2a	30.9a	1.0b	30.8b
		1/2MS	31.7a	24.1b	1.2a	25.9b	0.7c	23.8c
			ns	ns	ns	***	***	***
A*B			ns	ns	ns	ns	ns	ns

Basal medium supplemented with 3 g·L⁻¹ Hyponex#1, 0.1 g·L⁻¹ citric acid, 1 g·L⁻¹ tryptone–peptone, 1 g·L⁻¹ activated charcoal, 8 g·L⁻¹ agar, pH 5.6.

Concentration of ammonium nitrate (NH₄NO₃): 1/4 MS (412.5 mg·L⁻¹), 1/2 MS (825.0 mg·L⁻¹).

For each treatment, four replicates of ten protocorm-like bodies (on average 0.15 g fresh mass) were cultured. Data were scored after three months of culture at 25±2°C with 16 h light/8 h dark and photon flux of 22–35 μmol·m⁻²·s⁻¹.

Values followed by the same letter are not significantly different at $P \leq 0.05$. NS, ***: Not significant or significant at $P \leq 0.001$, respectively (two-way ANOVA, Duncan's multiple range test).

Table 5. Analysis of variance for the effects of sucrose and ammonium nitrate concentrations on root/leaf ratio of plantlet regenerated from callus in *Doritaenopsis* Taisuco Ladylip

Treatments			Root/leaf ratio		
			Fresh weight	Length	Number
Sucrose (A)	(g·L ⁻¹)	10	0.43c	0.72c	0.69c
		20	0.84b	1.31b	1.08b
		30	1.07a	1.49a	1.18a
		***	***	***	
NH ₄ NO ₃ (B)		0	0.97a	1.44a	1.16a
		1/4MS	0.81a	1.12b	0.98b
		1/2MS	0.56b	0.96c	0.81c
		**	***	***	
A*B		ns	ns	ns	

Basal medium supplemented with 3 g·L⁻¹ Hyponex#1, 0.1 g·L⁻¹ citric acid, 1 g·L⁻¹ tryptone–peptone, 1 g·L⁻¹ activated charcoal, 8 g·L⁻¹ agar, pH 5.6.

Concentration of ammonium nitrate (NH₄NO₃): 1/4 MS (412.5 mg·L⁻¹), 1/2 MS (825.0 mg·L⁻¹).

For each treatment, four replicates of ten protocorm-like bodies (on average 0.15 g fresh mass) were cultured. Data were scored after three months of culture at 25±2°C with 16 h light/8 h dark and photon flux of 22–35 μmol·m⁻²·s⁻¹.

Values followed by the same letter are not significantly different at $P \leq 0.05$. NS, **, ***: Not significant or significant at $P \leq 0.01$, or 0.001, respectively (two-way ANOVA, Duncan's multiple range test).

of 20 and 30 g·L⁻¹ and without additional supplementation of ammonium nitrate were both suitable for plantlet growth in *Doritaenopsis* Taisuco Ladylip.

Morphological and histological observations of somatic embryogenesis and PLB development

Continuous subculturing of the initial callus derived from *in vitro* grown protocorms of the breeding line *Doritaenopsis* Taisuco Ladylip resulted in the formation

of the friable yellowish–white embryogenic callus (Fig. 1A). After transferred to the PLB induction medium and cultured at 25±2°C with 16 h light/8 h dark and photon flux of 22–35 μmol·m⁻²·s⁻¹, the callus gradually turned greenish (Fig. 1B) and then gave rise to some of initial PLBs within two months of culture (Fig. 1C). After three months culture, somatic embryos developed to PLBs with morphological and functional polarity (Fig. 1D). A number of mature PLBs with leaves protruded from the

tops and rhizoids from the bottoms were clearly visible after five months of culture (Fig. 1E, F). Until sixth month, the true leaves continuously grew and radicles penetrated from the bottoms of PLBs (Fig. 1G, H). PLBs developed into intact plantlets with some leaves and roots within seven months of culture (Fig. 1I).

To determine whether single cells from the friable callus are capable of giving rise directly to somatic embryo, we first focused on the structural changes to the loosely packed callus cells. A brief vortex treatment in liquid medium separated the friable cell masses into small clumps and individual cells. The materials at various regeneration periods were smeared to observe process of somatic embryogenesis by a phase contrast microscope. Isolated single cell (Fig. 2A, left) and two-celled somatic proembryo (Fig. 2A, right), with a smaller terminal cell and a larger basal cell from friable callus, are both observed at the initial stage of differentiation. Derivatives of the terminal cell continue to divide several times and proembryo continues to expand by anticlinal and periclinal divisions (Fig. 2B–D). As development progresses,

the proembryo with suspensor cell (Fig. 2E) and the globular proembryo (Fig. 2F–H) are formed orderly. As it develops further, the somatic embryo becomes elongated and a distinct protoderm is formed (Fig. 2I) and then reaches more mature stage (Fig. 2J) with obvious visible shoot pole.

During the PLB development, secondary PLB can often be observed on the surface of the PLB. Histological studies were also conducted to determine the origin of secondary PLB from PLB. Cross sections of the PLB developed on the Hyponex medium revealed that mitotic divisions occurred in epidermal cells of PLB and resulted in the formation of embryonic origin (Fig. 3A). Continual cell divisions within embryonic origin resulted in the formation of a small protuberance by 8 cells (Fig. 3B) and 16 cells (Fig. 3C). Continual cell divisions within the small protuberance result in the formation of a PLB (Fig. 3D). As it develops further, the PLB reached more mature stage with shoot apex (Fig. 3E) and with distinct leaf primordia (Fig. 3F).

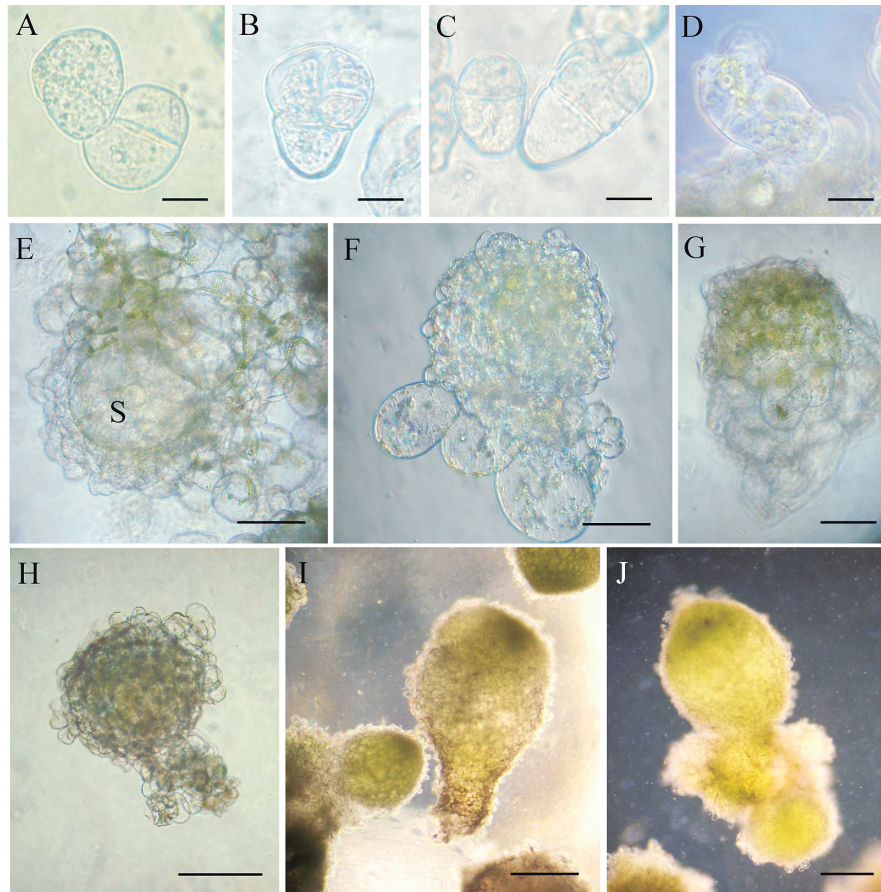


Fig. 2. Somatic embryogenesis from friable embryogenic callus of *Doritaenopsis* Taisuco Ladylip. (A) Isolated single cell (left) and two-celled somatic proembryo (right) with a smaller terminal cell and a larger basal cell from friable callus. Derivatives of the terminal cell continue to divide and proembryo continues to expand by anticlinal and periclinal divisions (B, C, D). As development progresses, the proembryo with suspensor cell (E) and the globular proembryo (F, G, H) are formed orderly. As it develops further, the somatic embryo becomes elongated and a distinct protoderm is formed (I) and then reaches more mature stage with obvious visible shoot pole (J). Bar=20 μ m (A, B, C, D); 50 μ m (E, F, G); 0.5 mm (H, I, J).

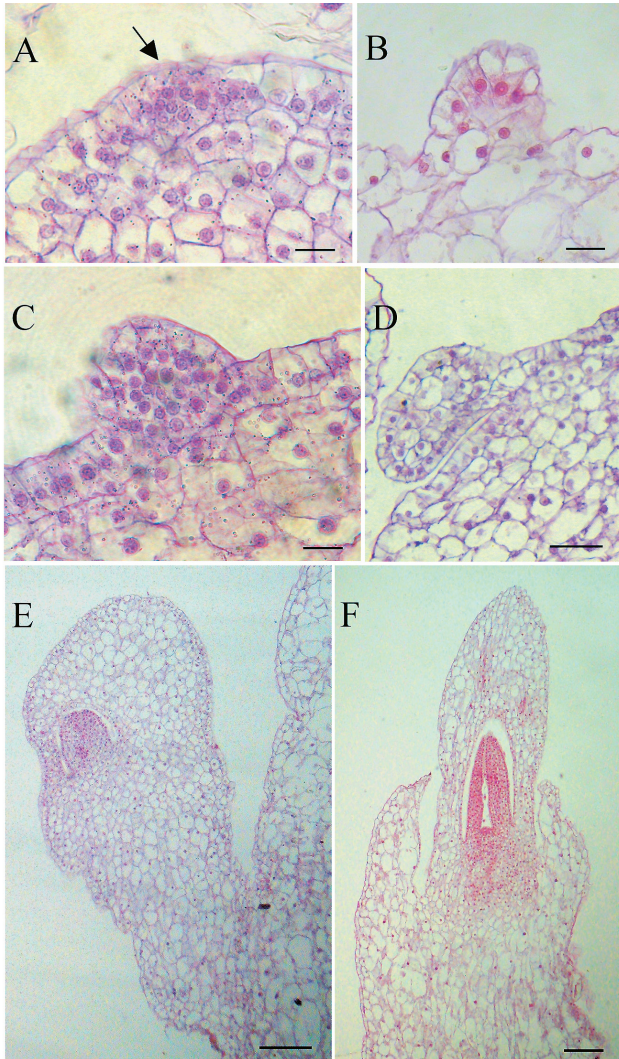


Fig. 3. Light micrographs of the formation of secondary protocorm-like body (PLB) from PLB in *Doritaenopsis* Taisuco Ladylip. (A) Mitotic divisions occurring in epidermal cells of PLB and formation of embryonic origin (arrow-head). Continual cell divisions within embryonic origin result in the formation of a small protuberance by 8 cells (B) and 16 cells (C). (D) Continual cell divisions within the small protuberance result in the formation of a PLB. (E) PLB with shoot apex (E) and with distinct leaf primordia (F). Bar= 50 μm (A, B, C); 100 μm (D); 0.5 mm (E, F).

DISCUSSION

BA (benzyl adenine), NAA (naphthalene acetic acid) (Tokuhara and Mii, 2001) or 2,4-D (Ishii *et al.*, 1998) are applied frequently to induce and proliferate callus in *Phalaenopsis* and in *Doritaenopsis* (Chowdhury *et al.*, 2003). The experimental results were published that combination of 2,4-D and TDZ was suitable for callus induction and proliferation in *Phalaenopsis* (Chen *et al.*, 2000). In this experiment, combination of 2,4-D and TDZ also was used to proliferate callus, however 2,4-D in 2 mg·L⁻¹ was benefit of fresh weight gain than in 3 mg·L⁻¹.

A lot of research papers pointed out replacing sucrose of maltose had the better effects in embryogenic callus production (Wang and Wei, 2004), somatic embryogene-

sis (Reidiboyam-Talleux *et al.*, 1999; Strickland *et al.*, 1987), embryo maturation, and plant regeneration (Druart, 1990; Hristoforoglu *et al.*, 1995; Junaid *et al.*, 2006). In *Doritaenopsis*, Islam *et al.* (1999) indicated the callus-derived plant production depends on the components of culture media; for instance, sucrose is suitable for callus proliferation, while maltose and sorbitol are suitable for PLB proliferation. They explicitly deducted that sucrose was easily absorbed and utilized to be advantageous to callus proliferation; but maltose and sorbitol were not easily absorbed and utilized being advantageous to PLB multiplication. Strickland *et al.* (1987) showed that at equal osmolarity maltose serves to improve yield and development of somatic embryos compared to sucrose and concluded that the maltose effect was primarily nutritional and not osmotically mediated. The effect of maltose on the induction of embryogenesis was specific and can possibly be attributed to its slow hydrolysis by plant cells, exerting starvation conditions early in culture and stable osmolarity of the medium later on (Indrianto *et al.*, 1999). Nørgaard *et al.* (1997) suggested that the slow metabolism of maltose would lead to sufficient oxygen being present in the cells to allow survival and subsequent somatic embryo development. Drop of carbohydrate availability within the cell may create a signal (Koch, 1996) that will reorientate development program (Blanc *et al.*, 1999).

Trehalose (α -D-glucopyranosyl- [1,1]- α -D-glucopyranoside) is made of two glucose molecules linked in an alpha-1,1 configuration and thus with no reducing ends Trehalose is a non-reducing disaccharide sugar that is widely distributed in nature (Elbein, 1974), and largely known as a stress protectant and a reserve (Goddijn and van Dun, 1999). Unlike sucrose, trehalose does not constitute a major source of carbon or energy in plants, with its concentration in most cases being at least an order of magnitude lower than that of sucrose (Goddijn and van Dun, 1999). The new conceptual framework to address the mechanisms by which trehalose metabolism and sugar signalling modulate growth and development in plants, trehalose metabolism could play a more direct role in regulating cell division as an integrator of nutritional status and growth (Gómez *et al.*, 2006). In *Arabidopsis*, addition of even fairly low amounts of external trehalose to the growth medium results in a significant inhibition of seedling root elongation (Wingler *et al.*, 2000). Liu *et al.* (2006) concluded that trehalose amended NP and KC media brought about better results than sucrose containing media for PLB proliferation of *Phalaenopsis* and *Doritaenopsis*.

In the experiments of somatic embryogenesis in *Doritaenopsis* Taisuco Ladylip, the strong orientation towards somatic embryogenesis induced with the maltose or trehalose treatments and the opposite effects, i.e., the low orientation towards somatic embryogenesis and callus proliferation was greater, obtained with the sucrose treatment. The somatic embryogenesis induction medium contained maltose or trehalose, rather than sucrose, in equimolarity, this result could be explained by Blanc *et al.* (2002) indicated the slow growth of the

maltose treatment was not due to a carbon nutrition deficit, but rather to a specific physiological state due to the cells entering the embryonic pathway.

Sucrose of 20 g·L⁻¹ and the 412.5 mg·L⁻¹ ammonium nitrate (1/4MS) combined with 3 g·L⁻¹ Hyponex#1 was suitable culture medium for *Doritaenopsis* Taisuco Ladylip plantlet development. Hinnen *et al.* (1989) indicated that increased NH₄⁺ and NO₃⁻ concentrations promoted shoot growth, but decreased root growth of *Phalaenopsis* hybrid seedlings. Hyponex medium was found to be suitable for conversion of PLBs into plantlets and 83% of PLBs transformed into plantlets on this medium (Young, 2000).

In general, the protocorm is considered as an intermediate structure between the embryo and the plant in Orchidaceae, i.e. protocorm like body is as an intermediate structure between the somatic embryo and the plantlet. Swamy (1949) distinguished embryogeny of the Orchidaceae into two groups according to the presence or absence of a suspensor. The first group is composed in part of Cyripedioideae, the embryo consists mostly of two terminal cells and a middle cell and, to a lesser degree, a suspensor initial which does not contribute much material to the make-up of the embryo. Almost all Orchidaceae species including *Phalaenopsis* in which the suspensor initial remains distinct and does not participate in the construction of the "mature" embryo belong to the second group (Leroux *et al.*, 1997). Quiroz-Figueroa *et al.* (2006) pointed out somatic embryos originate by two pathways, unicellular or multicellular, and both direct and indirect somatic embryogenesis embryos can derive from single or various cells. During indirect somatic embryogenesis of *Doritaenopsis* Taisuco Ladylip, we could observe initial embryogenic single cell of callus proceeding unequal division to form the top and basal end of two-celled stage, succeeded to periclinally and anticlinally divide of the top cell and formed 3–7 cells of embryonal mass. This system where embryos originate from a single cell is a very desirable system for the plant regeneration of genetically transformed cells, and avoiding chimeras. However, the secondary somatic embryos produced during its development in *Doritaenopsis* Taisuco Ladylip, and the secondary somatic embryos derived from mitotic divisions occurring in PLB epidermal layer through unicellular or multicellular pathway. An embryogenesis secondary system that source of material is somatic embryos can be useful to clone the selected individual after transformation.

In conclusion, we describe the efficient system of *Doritaenopsis* Taisuco Ladylip that utilizes callus culture and somatic embryogenesis for plant regeneration; a thousand PLBs produce averagely from 1 g calli after 3 months culture. Maltose and trehalose are being substituted for sucrose, is superior for somatic embryogenesis, and PLBs normally transfer to plantlets on the medium supplemented with Hyponex salts. This system of plant regeneration would be the basis of molecular breeding in *Doritaenopsis*.

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