

Somatic embryogenesis from stem thin cell layers of *Dendrobium aqueum*

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Abstract

An efficient *in vitro* regeneration protocol through somatic embryogenesis was established from stem transverse thin cell layers (tTCLs) of *Dendrobium aqueum* Lindley, an imperiled orchid. This study outlines the induction and successive maturation stages of *D. aqueum* somatic embryos (SEs). The tTCLs (~ 0.5 mm thick) cultured on half-strength Murashige and Skoog (MS) medium containing cytokinins and auxins, either individually or in combination, produced embryogenic callus (EC). Treatment with 0.5 mg dm⁻³ zeatin induced EC in 41.42 % of tTCLs. As many as 42.66 globular SEs per tTCL were formed in the presence of 1.5 mg dm⁻³ N⁶-(2-isopentyl) adenine (2iP) but only on 10.33 % of explants. The combined treatment of 2iP (1.5 mg dm⁻³) and 0.5 mg dm⁻³ 6-benzyladenine resulted in 34 globular SEs on 14.7 % of tTCLs whereas the combination of 2iP and 1.0 mg dm⁻³ indole-3-butyric acid (IBA) induced 7.4 globular SEs on 52.33 % of tTCLs. Supplementation of activated charcoal, amino acids, and antioxidants alleviated browning at all the concentrations tested, but the EC response declined. The addition of 0.5 mg dm⁻³ polyvinylpyrrolidone to 1.5 mg dm⁻³ 2iP and 1.0 mg dm⁻³ IBA produced 24 SEs on 19.89 % of tTCLs suggesting that the EC and SEs can be effectively induced by individual cytokinins whereas the synergistic treatments with other compounds can only enhance the induction of EC. Histological observations of EC showed the formation of globular SEs from sub-epidermal regions. Successive developmental stages of globular SEs and the intermediate stage of protocorm like bodies until the formation of plantlets were observed. The plantlets obtained through SEs showed no morphological variations, and inter simple sequence repeat profiles also confirmed the genetic fidelity of *in vitro*-derived progeny with high monomorphism (97.78 %). In conclusion, the use of stem tTCLs is an effective method to produce SEs through indirect somatic embryogenesis in *D. aqueum*.

Additional key words: auxins, cytokinins, epiphyte, embryogenic callus, *in vitro* culture, ISSR, protocorm like bodies.

Introduction

The *Orchidaceae*, one of the largest, most highly evolved, and diverse families of flowering plants, contains about 35 000 species belonging to 850 genera (Hossain *et al.* 2013). *Dendrobium* is a large genus of the *Orchidaceae* with about 1 600 species worldwide, and 116 can be found in India (Misra 2007). *Dendrobium aqueum* Lindley is an endemic orchid distributed in the Kolli hills (Namakkal) and Shervarayans (Salem) of Eastern Ghats, Tamil Nadu, India (Matthew 1983). Natural *D. aqueum* populations are declining due to

anthropogenic activities such as the collection of orchids and habitat destruction. This species was categorized as near threatened in 2000 by the International Union for Conservation of Nature and Natural Resources (Kumar *et al.* 2001), and subsequent field studies at the Kolli hills have recorded a continual decline of this natural population (Sudhakar Reddy *et al.* 2005). Studies on the phenology and *in vitro* propagation through asymbiotic seed germination and protocorms have been already achieved in *D. aqueum* (Parthibhan *et al.* 2012,

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Abbreviations: 2iP - N⁶-(2-isopentyl) adenine; AC - activated charcoal; BA - 6-benzyladenine; DSE - direct somatic embryogenesis; EC - embryogenic callus; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; ISSR - inter simple sequence repeat; KN - kinetin; MS - Murashige and Skoog; NAA - 1-naphthalene acetic acid; PGR - plant growth regulator; PLB - protocorm-like body; PVP - polyvinylpyrrolidone; SE - somatic embryo; tTCL - transverse thin cell layer; TDZ - thiadiazuron; ZEA - zeatin.

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2015a,b).

Conventional propagation of *Dendrobium* by seed germination requires a suitable mycorrhizal fungus due to the absence of a mature endosperm in the seed (Teixeira da Silva *et al.* 2015c), although seeds can be germinated *in vitro* under asymbiotic conditions (Teixeira da Silva *et al.* 2015b). In *Dendrobium*, a wide range of explants including apical buds, flower buds, rhizomes, seeds, leaves, and root tips can produce protocorm-like bodies (PLBs), callus, or directly shoots, which ultimately result in the formation of plantlets (Teixeira da Silva *et al.* 2015a). Thin cell layers (TCLs) which consist of a small amount of tissue comprising very few cell layers can be prepared from almost any plant organ. These, when cut longitudinally, are referred to as ITCLs, and when cut transversally, are referred to as tTCLs (Teixeira da Silva 2013, Teixeira da Silva and Dobránszki 2013). The regeneration of PLBs and shoot buds from TCLs has been possible from shoots, shoot tips, nodes, or PLBs in *Dendrobium* species (Table 1 Suppl.). The PLBs in orchids are *de facto* somatic embryos (Teixeira da Silva 2014, Teixeira da Silva and Winarto 2016), a phenomenon that was previously confirmed by histology in this and in another orchid genus, *Phalaenopsis* (Lee *et al.* 2013). Somatic embryogenesis or PLB induction in orchids is often contingent upon the type and ratio of auxin and cytokinin during *in vitro* propagation (Hossain *et al.* 2013).

Dendrobium species possess various medicinal properties (Teixeira da Silva and Ng 2017) and *D. aqueum* is known for its free-radical scavenging and antiglycation potential, and phytochemicals such as

flavonoids, cyanogenic glycosides, and tannin (Maridass *et al.* 2008, Mukherjee *et al.* 2012).

The TCLs system has a few applications in *Dendrobium in vitro* culture (Table 1 Suppl.). When cultured on a Murashige and Skoog (1962; MS) basal medium without plant growth regulators (PGRs), *D. draconis* tTCLs remain green for up to 1 - 2 weeks and then gradually become brown and die (Rangsayatorn 2009). Browning or blackening explants and calluses occurs as a result of phenolic secretions from the wounded cells, during sectioning tissue from shoot tips, leaves, or PLBs (Kaewubon *et al.* 2015). The incidence of browning in the medium and explants on TCL cultures were frequently reported in several orchids such as *D. fimbriatum*, *D. chrysotoxum*, *D. candidum*, *D. draconis*, *D. nobile*, *Cymbidium* spp., and *Phalaenopsis cornu-cervi* (Roy and Banerjee 2003, Malabadi *et al.* 2005, Teixeira da Silva *et al.* 2006, Roy *et al.* 2007, Zhao *et al.* 2007, Rangsayatorn 2009, Vyas *et al.* 2010, Rittirat *et al.* 2012). In these studies, shoot production and embryogenic response of the explants decrease. However, the addition of coconut water at 5 % (v/v) or optimization of PGR concentrations decreases necrosis and enhances PLB production (Roy and Banerjee 2003, Roy *et al.* 2007, Vyas *et al.* 2010).

As a part of a wider *ex situ* conservation strategy for *D. aqueum*, the present research was carried out to develop a protocol to induce somatic embryos (SEs) from tTCLs. Furthermore, the genetic fidelity of SE-derived plantlets was assessed using inter simple sequence repeat (ISSR) markers.

Materials and methods

Explants and culture conditions: Shoots of uniform height (~ 5 cm) with a minimum of four nodes obtained from *in vitro* grown seedlings were used as the explant source (Parthibhan *et al.* 2012). The shoots (~ 4.5 cm) were prepared by removing the shoot tip, leaves, protocorm, and roots. Stems were then cut transversely into slices of 0.5 mm thick (*i.e.*, tTCLs) using a surgical blade (*Glass Van*, Faridabad, India) and used as explants for embryogenic callus (EC) induction.

The tTCLs were cultured on a half-strength (macronutrients) MS medium supplemented with 2 % (m/v) sucrose and different concentrations (0.1 - 3.0 mg dm⁻³) of cytokinins, *viz.*, 6-benzyladenine (BA), N⁶-(2-isopentyl) adenine (2iP), kinetin (KN), thidiazuron (TDZ), and zeatin (ZEA) to induce an embryogenic callus (EC). To evaluate the synergistic effect of cytokinins and auxins as a way to improve EC induction and proliferation, the cytokinins that responded best on EC induction, BA, 2iP, and KN, were combined with three auxins, *viz.*, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 1-naphthalene acetic acid (NAA), in a wide range of concentrations (0.01 - 2.0 mg dm⁻³). In order to enhance EC production by preventing or minimizing the browning of explants and EC, activated

charcoal (AC; 1 %, m/v), an amino acid arginine (5 - 100 mg dm⁻³), and two antioxidants glutathione (5 - 100 mg dm⁻³) and polyvinylpyrrolidone (PVP; 0.1 - 1.0 mg dm⁻³) were tested in the MS medium supplemented with 1.5 mg dm⁻³ 2iP and 1.0 mg dm⁻³ IBA. All the culture media were solidified with 0.7 % (m/v) agar. The pH was adjusted to 5.7 ± 0.2 with 0.1 M NaOH or 0.1 M HCl before autoclaving at 121 °C and 1.06 × 10⁵ N m⁻² for 15 min. The cultures were incubated at a temperature of 23 ± 2 °C, a 16-h photoperiod, and a photosynthetic photon flux density of 35 μmol m⁻² s⁻¹ provided by cool white fluorescent lamps (*TL-D Super 80*, *Philips*, Gurgaon, India). All chemicals and reagents were purchased from *HiMedia* (Mumbai, India).

Histology: Some of the EC with SEs were fixed in an FAA (formalin + acetic acid + 70 %, v/v, ethanol, 1:1:18, v/v/v) solution (Zhao *et al.* 2007). The EC was neither dehydrated in an ethanol series nor embedded in wax since the EC with developing SEs tended to shrink during the dehydration process. Fine sections, which were made manually using thin commercial blades (*SuperMax-SS*, Thane, India) were stained with 1 % (m/v) safranin and then observed and photographed under a triangular

compound microscope (*GTR-22A*, *GETNER*, Haryana, India).

Genomic DNA extraction, primers, PCR amplification, and ISSR markers: Genomic DNA was extracted from leaves of an *in vitro* mother plant and six plantlets (randomly selected) raised from somatic embryogenesis using the cetyltrimethyl ammonium bromide method as described by Wang *et al.* (2009) with a modification. Five ISSR primers, UBC-812 [(AC)8C], UBC-826 [(GA)8YC], UBC-866 [(AC)8AT], I-2 [(AC)8AG], and I-4 [(AC)8TG], which were already reported to show a high polymorphism in *Dendrobium* species (Wang *et al.* 2009), were randomly selected for the study. PCR amplification was performed in a thermal cycler (*Eppendorf*, Hamburg, Germany) using 10 mm³ of reaction mixture containing 7 mm³ of PCR master mix, 1 mm³ of genomic DNA, 1 mm³ of sterile double distilled water and 1 unit of Taq DNA polymerase, and PCR conditions were adopted from Wang *et al.* (2009).

PCR products were electrophoresed on 1.2 % (m/v) agarose gels which contained 3 mm³ of ethidium bromide stain. The electrophoretic patterns of the PCR products were recorded digitally using a *Gel-Doc-2000* image analysis system (*Bio-Rad*, Hercules, PA, USA). The amplified fragments were scored for the presence (1) and absence (0) of homologous bands, and a matrix of ISSR data was assembled. The experiment was repeated again, and only the reproducible fragments were scored for polymorphism analysis.

Results

Stem tTCLs cultured on a half-strength MS basal medium (control) remained green for two to three weeks and then gradually turned brown, becoming completely necrotic. In contrast tTCLs cultured on the same medium supplemented with cytokinins remained green and induced EC as well as direct SEs after five weeks. A high percentage of explants (41.42 %) induced green compact EC in response to 0.5 mg dm⁻³ ZEA (Table 1). When the concentration of ZEA increased, EC induction decreased, and yellowish-green callus was produced. Subsequently, on the media with 0.5 and 0.1 mg dm⁻³ BA, 16.83 and 16.27 % of tTCLs induced a green EC with compact globular SEs (Fig. 1A).

On the other hand, 2iP at all concentrations (0.1 - 3.0 mg dm⁻³) produced pale green to green and yellow friable ECs with loosely packed SEs at low frequencies. The highest percentage of explants (10.75 %) produced a yellow EC in response to 1.5 mg dm⁻³ 2iP (Fig. 1E,F). KN and TDZ at 1.5 mg dm⁻³ induced yellowish-green and green callus, respectively in 8.40 and 8.50 % of tTCLs. A few explants (2.74 %) induced a green compact callus on the TDZ-containing medium. Morphologically, the growth of developing globular SEs was better in response to BA than to other cytokinins.

In ZEA-induced callus, the green callus turned yellow

Ex vitro establishment of plantlets: The SE-derived plantlets with 2 - 3 roots from all cultures (25 weeks old) were hardened and acclimatized as in Parthibhan *et al.* (2015a). In brief, the plantlets with 4 - 5 fully expanded leaves and roots were cultured on a half-strength MS basal medium without sucrose for 5 weeks, then transferred into small paper pots (4 cm wide × 6 cm high) containing a 1:1 (m/m) mixture of brick pieces and charcoal. Moss was laid over the potting mixture and covered with polyethylene bags to maintain high relative humidity. The substrate was regularly moistened with a half-strength MS liquid medium and kept in a culture room at 25 ± 1 °C, a 16-h photoperiod, and a photosynthetic photon flux density of 35 μmol m⁻² s⁻¹ for 4 - 6 weeks. Finally, the well-hardened plantlets were transferred to a greenhouse.

Experimental design and data analysis: Experiments were completely randomized. Each treatment had 12 explants per Petri dish and with three replicates. The quality and frequency of EC and SE were recorded after eight weeks of culture. The number of globular SEs was counted under a dissecting microscope (*DM-2*, *Weswox Optik*, Haryana, India) after 15 weeks of culture. The percentage data were arcsine transformed before statistical analysis. All data were analyzed by ANOVA using Statistical Package for the Social Sciences (*SPSS v. 18*) software (*IBM SPSS*, Chicago, IL, USA). Means were differentiated at the α = 0.05 level of significance using Duncan's multiple range test (DMRT).

and became necrotic after eight weeks of culture (Table 1). On the other hand, green and yellowish-green callus that formed in the presence of 0.5 mg dm⁻³ BA, 1.5 mg dm⁻³ 2iP, and 1.5 mg dm⁻³ KN continued to proliferate well and developed globular SEs (except for BA) after 8 - 12 weeks on the same medium. Therefore, to enhance the frequency of ECs, 0.5 mg dm⁻³ BA was combined with 1.5 mg dm⁻³ KN and 1.5 mg dm⁻³ 2iP. In addition, 0.5 mg dm⁻³ BA and 1.5 mg dm⁻³ 2iP were combined with different auxins to improve EC and SE production.

The combination of two cytokinins, BA (0.5 mg dm⁻³) and KN (1.5 mg dm⁻³) improved EC induction frequency to 20 % via the formation of a yellowish-green callus. In contrast, 0.5 mg dm⁻³ BA with 1.5 mg dm⁻³ 2iP reduced the frequency of EC to 14.67 %, and pale green to green callus formed (Table 2). From the two cytokinin combinations, callus that formed on medium with BA+2iP was healthier (no browning) than on medium with BA+KN. Callus that formed in response to the BA+KN combination was yellowish-green, and further proliferation was severely affected by tissue browning.

Auxins (IAA, IBA, and NAA) at 0.01 - 1.0 mg dm⁻³ effectively increased the frequency of EC induction when combined with the cytokinins BA and 2iP (Table 2). The

synergistic effect of 2iP (1.5 mg dm^{-3}) with these auxins was more effective than that of BA on both EC induction and callus proliferation. The highest (53.33 %) induction of green and yellowish-green calluses was recorded on 2iP (1.5 mg dm^{-3}) with 1.0 mg dm^{-3} IBA (Fig. 1B), followed by 2iP with 1.0 mg dm^{-3} IAA (41.67 %). Among the BA (0.5 mg dm^{-3}) and auxin combinations, the best response (33.33 %) was on the medium with 0.1 mg dm^{-3} NAA and 1.0 mg dm^{-3} IAA, respectively (Table 2). Poor EC induction between 8.33 and 16.00 % was noted when the highest auxin concentration

(2.0 mg dm^{-3}) was used.

Treatments employing AC, arginine, PVP, and glutathione prevented early browning in tTCLs, but EC induction did not improve. The tTCL explants cultured on the medium supplemented with AC failed to respond further. The highest number of tTCLs formed green ECs (18.33 %) on the medium supplemented with 50 mg dm^{-3} arginine (Fig. 2) and 0.5 mg dm^{-3} PVP whereas only 11.67 % of green ECs was formed in response to 100 mg dm^{-3} glutathione (Figs. 3, 4).

The EC induced from any treatment developed further

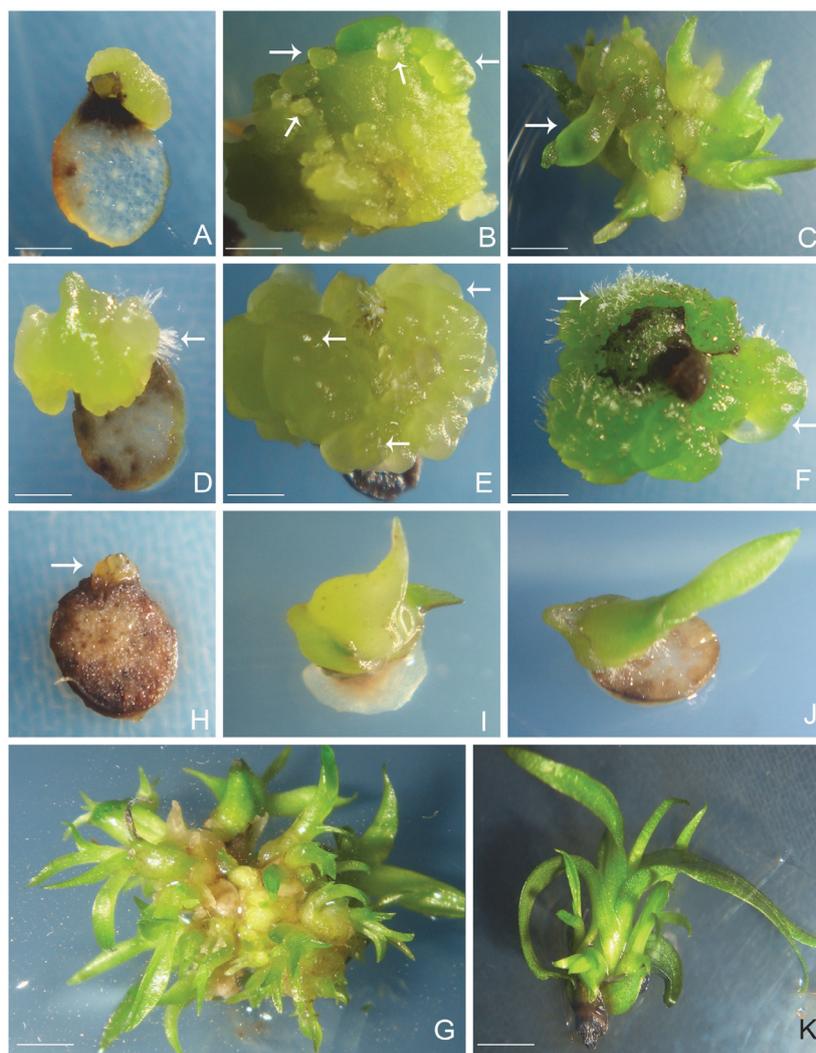


Fig. 1. Indirect somatic embryogenesis (A-G) and direct somatic embryogenesis (H-K) on half-strength MS from stem thin cell layers (TCLs) of *Dendrobium aqueum*: A - a dedifferentiated green compact embryogenic callus (EC) induced from the sub-epidermal region of a stem TCL in the presence of 0.5 mg dm^{-3} BA; B - redifferentiated globular and heart-shaped somatic embryos (SEs) developed from the compact surface of proliferated embryonic callus (EC) in response to 1.5 mg dm^{-3} 2iP and 1.0 mg dm^{-3} IBA; C - a redifferentiated, elongated, and torpedo-like SE with a leaf primordium (the arrow) and late-stage protocorm-like bodies (PLBs) with a leafy shoot initial emerging in response to 1.5 mg dm^{-3} 2iP with 0.01 mg dm^{-3} NAA; D - dedifferentiated green, compact EC with sunken globular embryos (with rhizoids) in response to 0.1 mg dm^{-3} BA; E and F - aerial and inverted views of proliferated EC with multiple, compact, sunken globular SEs and PLBs with a leafy shoot initial and rhizoids in response to 1.5 mg dm^{-3} 2iP; G - SE-derived shoots with more than two leaves; H-J - direct SE (PLB) induction (arrow) and development in response to 0.1 mg dm^{-3} of KN, BA, and ZEA each, respectively; K - multiple shoot formation obtained from a direct SE in response to 1.5 mg dm^{-3} 2iP, 0.01 mg dm^{-3} NAA, and 0.3 mg dm^{-3} polyvinylpyrrolidone. Scale bars: 2 mm (A to K).

Table 1. Embryogenic callus induction from stem transverse thin cell layers of *Dendrobium aqueum* on half-strength Murashige and Skoog medium supplemented with different cytokinins. The experiment was repeated 3 times with 12 explants per treatment. Means \pm SEs, $n = 12$. Values within each column with different letters are significantly different ($P \leq 0.05$; DMRT). 2iP - N⁶-(2-isopentyl) adenine; BA - 6-benzyladenine; EC - embryogenic callus; G - green; KN - kinetin; LG - light/pale green; SE - somatic embryo; TDZ - thidiazuron; W - white; Y - yellowish; YG - yellowish green; ZEA - zeatin; - means no response.

Cytokinins	Concentration [mg dm ⁻³]	Indirect somatic embryogenesis			Direct somatic embryogenesis		
		callus response [%]	colour of EC	SE number [explant ⁻¹]	Response [%]	SE number [explant ⁻¹]	shoot length [cm]
BA	0.1	16.27 ^d	G	2.33 \pm 0.57 ^{ghi}	8.33	1.00	0.70
	0.5	16.83 ^c	G	16.00 \pm 1.00 ^b	-	-	-
	1.0	8.36 ^f	YG	4.33 \pm 0.57 ^{ef}	-	-	-
	1.5	0.00 ^l	-	0.00 \pm 0.00 ^k	-	-	-
	2.0	0.00 ^l	-	0.00 \pm 0.00 ^k	-	-	-
	3.0	0.00 ^l	-	0.00 \pm 0.00 ^k	-	-	-
2iP	0.1	6.66 ^g	G	3.00 \pm 1.00 ^{fgh}	8.33	1.00	0.60
	0.5	8.38 ^f	G	3.33 \pm 0.57 ^{fg}	-	-	-
	1.0	8.82 ^f	PG	15.00 \pm 1.00 ^{bc}	-	-	-
	1.5	10.75 ^e	Y	42.67 \pm 0.58 ^a	-	-	-
	2.0	6.52 ^g	Y	15.00 \pm 1.00 ^{bc}	-	-	-
	3.0	5.43 ^h	Y	13.67 \pm 1.53 ^c	-	-	-
TDZ	0.1	0.00 ^l	-	0.00 \pm 0.00 ^k	-	-	-
	0.5	0.00 ^l	-	0.00 \pm 0.00 ^k	-	-	-
	1.0	4.48 ⁱ	G	1.67 \pm 0.58 ^{hij}	-	-	-
	1.5	8.50 ^f	G	5.67 \pm 0.58 ^{de}	-	-	-
	2.0	2.75 ^k	G	0.67 \pm 0.58 ^{jk}	-	-	-
	3.0	0.00 ^l	-	0.00 \pm 0.00 ^k	-	-	-
KN	0.1	3.70 ^j	PG	0.67 \pm 1.15 ^{jk}	8.33	1.00	0.55
	0.5	5.33 ^h	G	6.67 \pm 1.53 ^d	-	-	-
	1.0	6.85 ^g	YG	3.00 \pm 2.00 ^{fgh}	-	-	-
	1.5	8.40 ^f	YG	1.00 \pm 1.00 ^{ijk}	-	-	-
	2.0	4.53 ^l	G	0.00 \pm 0.00 ^k	-	-	-
	3.0	2.28 ^k	G	0.00 \pm 0.00 ^k	-	-	-
ZEA	0.1	8.53 ^f	G	0.67 \pm 0.58 ^{jk}	8.33	1.00	0.35
	0.5	41.42 ^a	G	3.67 \pm 1.15 ^{fg}	-	-	-
	1.0	26.02 ^b	YG	1.00 \pm 1.00 ^{ijk}	-	-	-
	1.5	16.83 ^c	YG	0.67 \pm 0.58 ^{jk}	-	-	-
	2.0	8.50 ^f	YG	0.00 \pm 0.00 ^k	-	-	-
	3.0	4.55 ^l	YG	0.00 \pm 0.00 ^k	-	-	-

and continued to grow on the same medium. Except for tissue browning, all cultures, irrespective of the callus induction medium employed, showed callus proliferation, and SE formation and multiplication. Developing EC displayed four colors, green, pale green, yellowish-green, and yellow. Green, pale green, and some yellowish-green callus formed SEs whereas yellow and yellowish-green callus turned brown and became necrotic.

Microscopic and histological observations of EC sections revealed different maturation stages of the SEs. Stem tTCLs consisted of an epidermis and a few layers of small polyhedral cells (Fig. 5A), which were packed at sub-epidermal regions, followed by large, scattered, and vacuolated cells. Initially, a green protuberance originated from the sub-epidermal region by periclinal and anticlinal cell divisions (Fig. 5B,C). Subsequent proliferation of these cells formed an EC, consisting of

cells with a dense cytoplasm, prominent nucleus and vacuoles (Fig. 5D,E). However, successive growth of ECs and formation of SEs (Fig. 5E-G) depended on the type and combination of PGRs employed.

The structure of the ECs was generally compact and slightly friable at the surface (Fig. 1A,D). Globular SE initials were observed as either protuberances or closely packed clusters inside and/or on the surface of the callus mass, creating a friable surface (Fig. 5E). Clusters of SEs initially consisted of only globular SEs (Fig. 1 Suppl., Fig. 5E). However, subsequent lowering concentration of nutrients in the medium, and interruption of browning and necrosis in EC, instigated differences in maturation of the SEs. The globular SEs continued to enlarge and formed an asymmetric heart-shape SEs (Fig. 1 Suppl.). At maturity, the early heart-shape SEs formed either typical heart-shaped SEs (asymmetric) (Fig. 1 Suppl.,

Fig. 5E) or elliptical SEs that subsequently formed typical elongated torpedo-shaped (asymmetric) SEs or enlarged PLBs (Fig. 1 Suppl.). Bipolar SEs were characterized by the formation of a pointed shoot apex or cotyledonary leaf initiation and rhizoidal hairs, which appeared on opposite poles of the PLBs and torpedo-shaped SEs (Fig. 1 Suppl.). Very few globular or heart-shaped SEs that formed on yellowish-green or yellow calluses failed to develop further due to severe browning. Mature torpedo SEs and PLBs with a cotyledonary leaf (Fig. 5G) grew into rooted, leafy shoots (Fig. 1 Suppl.). The successive stages of SE maturation from globular to torpedo stages were hardly visible and difficult to measure since the SEs were compactly packed. In such cases, early globular SEs or shoots that developed later were evaluated.

The rate of SE formation and the quality of SEs

varied with the concentration, type, and combination of PGRs. The highest number of SEs per tTCL (42.66) was recorded on the medium with 1.5 mg dm^{-3} 2iP alone followed by 1.5 mg dm^{-3} 2iP + 0.5 mg dm^{-3} BA (34 SE per explant) whereas the PVP (0.5 mg dm^{-3})-supplemented medium formed only 24 SEs per tTCL (Fig. 3).

The cytokinins 2iP (1.0, 2.0, and 3.0 mg dm^{-3}) and BA (0.5 mg dm^{-3}) individually induced about 14 - 16 SEs per tTCL. When these concentrations were raised, or when other cytokinins were used, poor development of SE was observed forming only 1 - 13 SEs per tTCL. When BA (0.5 mg dm^{-3}) was combined with any auxin, it had no distinct effect on the formation of SEs or PLBs except when used with 1.0 mg dm^{-3} IAA at a later stage. On the other hand, 2iP when combined with auxins effectively induced EC and SEs. The highest values of

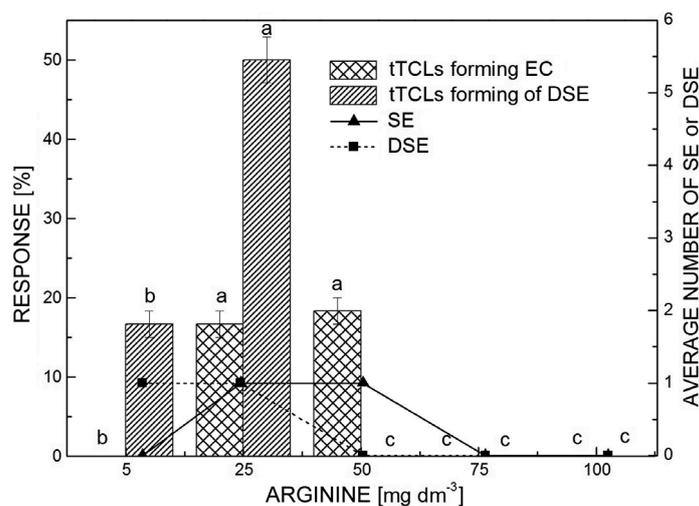


Fig. 2. Embryogenic callus (EC) induction and the number of somatic embryos (SEs) from stem transverse thin cell layers (tTCLs) of *Dendrobium aqueum* in response to arginine. Means \pm SDs, $n = 12$, different letters indicate significantly different means ($P \leq 0.05$; DMRT). DSE - direct somatic embryogenesis.

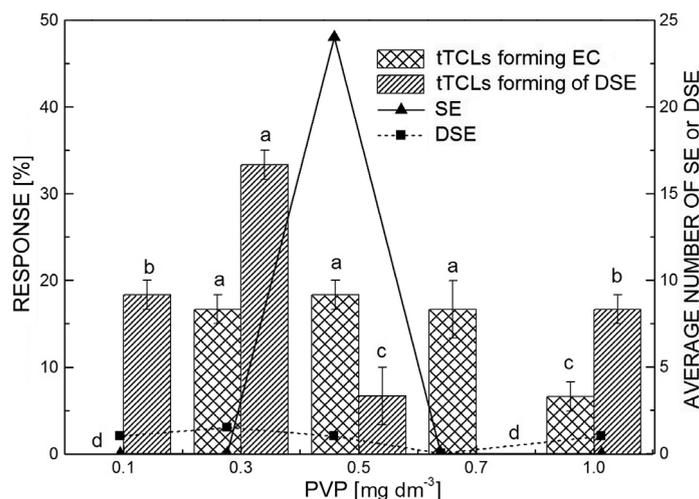


Fig. 3. Embryogenic callus (EC) induction and the number of somatic embryos from stem transverse thin cell layers (TCLs) of *Dendrobium aqueum* in response to polyvinylpyrrolidone (PVP). Means \pm SDs, $n = 12$, different letters indicate significantly different means ($P \leq 0.05$; DMRT). DSE - direct somatic embryogenesis.

Table 2. Effects of combined cytokinins and auxins on embryogenic callus induction from stem transverse thin cell layers (tTCLs) of *Dendrobium aqueum*. Means \pm SEs, $n = 12$ (experiment was repeated 3 times with 12 explants per treatment). Values within each column with different letters are significantly different ($P \leq 0.05$; DMRT). IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; NAA - 1-naphthalene acetic acid; PGR - plant growth regulator; - = no response.

PGRs	Concentrations [mg dm ⁻³]	Indirect somatic embryogenesis callus response [%]	SE number [explant ⁻¹]	Direct somatic embryogenesis response [%]	SE number [explant ⁻¹]	shoot length [cm]
BA + 2iP	0.5 + 1.5	14.67 ⁱ	33.67 \pm 1.53 ^a	25.00 ^a	1.00	0.35
BA + KN	0.5 + 1.5	20.00 ^{hi}	1.67 \pm 0.58 ^l	0.00 ^e	-	-
BA + IAA	0.5 + 0.01	21.67 ^{gh}	4.67 \pm 0.58 ^{hij}	11.67 ^{ab}	1.00	0.30
BA + IAA	0.5 + 0.1	26.66 ^{defg}	5.00 \pm 0.00 ^{ghi}	6.67 ^{bc}	-	-
BA + IAA	0.5 + 1.0	33.30 ^c	6.33 \pm 0.58 ^{def}	3.33 ^{cd}	-	-
BA + IAA	0.5 + 2.0	16.00 ^{ij}	5.33 \pm 0.58 ^{ghij}	0.00 ^d	-	-
BA + IBA	0.5 + 0.01	11.00 ^{jk}	5.33 \pm 0.58 ^{ghij}	3.30 ^{cd}	-	-
BA + IBA	0.5 + 0.1	30.00 ^{cde}	5.67 \pm 0.58 ^{fghi}	0.00 ^d	-	-
BA + IBA	0.5 + 1.0	20.00 ^{hi}	5.00 \pm 0.00 ^{ghi}	0.00 ^d	-	-
BA + IBA	0.5 + 2.0	8.33 ^k	4.33 \pm 0.58 ^{ijk}	0.00 ^d	-	-
BA + NAA	0.5 + 0.01	11.67 ^{jk}	5.67 \pm 0.58 ^{fghi}	10.00 ^{ab}	1.00	0.35
BA + NAA	0.5 + 0.1	33.33 ^c	6.33 \pm 0.58 ^{def}	3.35 ^{cd}	-	-
BA + NAA	0.5 + 1.0	25.00 ^{efgh}	6.67 \pm 0.58 ^{de}	0.00 ^d	-	-
BA + NAA	0.5 + 2.0	8.33 ^k	3.67 \pm 0.58 ^k	0.00 ^d	1.00	0.30
2iP + IAA	1.5 + 0.01	11.67 ^{jk}	5.25 \pm 0.25 ^{ghij}	3.30 ^{cd}	-	-
2iP + IAA	1.5 + 0.1	28.33 ^{cdef}	5.83 \pm 0.29 ^{fgh}	10.00 ^{ab}	1.00	0.40
2iP + IAA	1.5 + 1.0	41.67 ^b	11.33 \pm 0.58 ^b	0.00 ^d	-	-
2iP + IAA	1.5 + 2.0	8.33 ^k	6.67 \pm 1.04 ^{de}	0.00 ^d	-	-
2iP + IBA	1.5 + 0.01	21.67 ^{ghi}	5.92 \pm 0.14 ^{fgh}	0.00 ^d	-	-
2iP + IBA	1.5 + 0.1	23.33 ^{fgh}	5.96 \pm 0.14 ^{fgh}	0.00 ^d	-	-
2iP + IBA	1.5 + 1.0	53.33 ^a	7.25 \pm 0.25 ^d	3.35 ^{cd}	-	-
2iP + IBA	1.5 + 2.0	11.67 ^{jk}	5.58 \pm 0.14 ^{fghi}	11.67 ^{ab}	1.00	0.45
2iP + NAA	1.5 + 0.01	31.66 ^{cd}	9.00 \pm 0.50 ^c	16.67 ^a	1.00	0.75
2iP + NAA	1.5 + 0.1	21.67 ^{ghi}	6.33 \pm 0.29 ^{def}	11.60 ^{ab}	-	-
2iP + NAA	1.5 + 1.0	11.60 ^{jk}	5.75 \pm 0.25 ^{fgh}	3.30 ^{cd}	-	-
2iP + NAA	1.5 + 2.0	8.66 ^k	3.75 \pm 0.25 ^{jk}	0.00 ^d	-	-

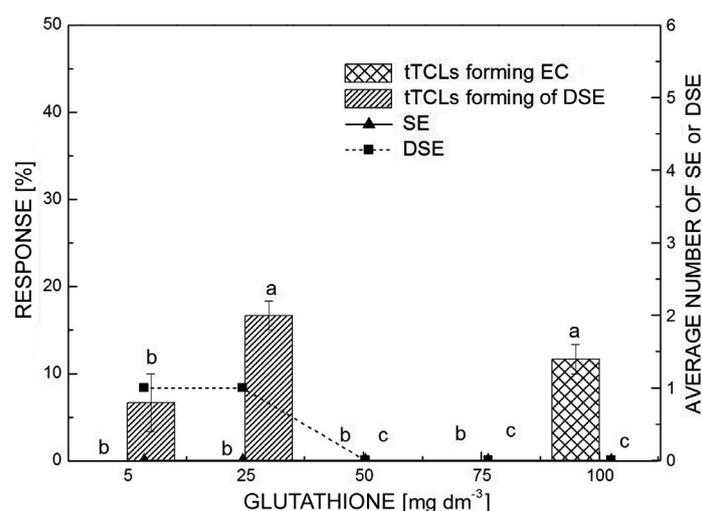


Fig. 4. Embryogenic callus (EC) induction response and somatic embryo (SE) development from stem transverse thin cell layers (tTCLs) of *Dendrobium aqueum* in response to glutathione. Means \pm SDs, $n = 12$, different letters indicate significantly different means ($P \leq 0.05$; DMRT). DSE - direct somatic embryogenesis.

11 and 7.2 globular SEs per tTCL were recorded when 2iP was combined with 1.0 mg dm⁻³ IAA and 1.0 mg dm⁻³ IBA, respectively (Fig. 1B). Unlike its combination with BA, NAA at 0.01 mg dm⁻³ induced 9.0 SEs per tTCL when combined with 1.5 mg dm⁻³ 2iP (Table 2).

When arginine was supplemented at 5 - 50 mg dm⁻³, it effectively prevented explant browning and supported both green EC induction and globular SE formation (Fig. 2). However, subsequent somatic embryogenesis was poor, forming only 1.00 PLBs per tTCL at 50 mg dm⁻³. Antioxidants glutathione and PVP equally inhibited explant browning more than arginine, but glutathione did not induce EC while 0.5 mg dm⁻³ PVP induced 24.33 SEs per tTCL.

In addition to EC induction, some of the tTCLs with an initial green protuberance induced globular SEs directly on the medium with selected PGRs and additives. The SEs formed either by direct conversion or by emergence from the initial green protuberance of tTCLs. These globular SEs developed PLBs with rhizoids at the basal end and a shoot tip at the opposite pole and did not develop an intermediate callus. All the cytokinins, except only TDZ at 0.1 mg dm⁻³, induced a single SE in 8.33 % of explants (Table 1, Fig. 1H-J) whereas the BA+2iP combination yielded a higher frequency (25.00 %) of embryo induction with no increment in the number of SEs (Table 2). Synergistic treatments, BA and 2iP with different auxins, showed only a slight improvement on direct SE induction than the individual treatments, but not higher than BA+2iP. In terms of cytokinin+auxin combinations, a maximum response of 16.67 % of explants induced direct SEs on the medium with 1.5 mg dm⁻³ 2iP and 0.01 mg dm⁻³ NAA (Table 2). All auxins at a high concentration of 2.0 mg dm⁻³ (except for IBA) when combined with 2iP or BA failed to form SEs directly. The use of additives at low concentrations increased direct SE formation in 2iP+IBA combination. Most tTCLs (50 %) formed one SE directly in response to 25 mg dm⁻³ arginine (Fig. 2) whereas on the medium with 0.3 mg dm⁻³ PVP, 1.5 SEs per TCL was formed on a maximum of 33.33 % explants (Figs. 1K and 3). Glutathione-supplemented medium resulted in a stunted shoot growth.

Finally, more than 90 % of the hardened plantlets survived after three to four weeks in the greenhouse. No variations were observed in morphology and growth characteristics among the potted plantlets. Genetic fidelity analysis attempted with 5 ISSR primers produced scorable bands from 325 to 1900 bp (Table 2 Suppl., Fig. 2 Suppl.). All the five primers, collectively produced 45 scorable bands, of which 44 (97.78 %) bands were monomorphic. This revealed the genetic fidelity of the plants raised through somatic embryos. Primer I2 produced only 1 polymorphic band (2.22 %) out of 11 bands.

Discussion

In orchids, TCLs have been prepared from shoots, stem nodes, or PLBs (Table 1 Suppl.). TCLs have been widely

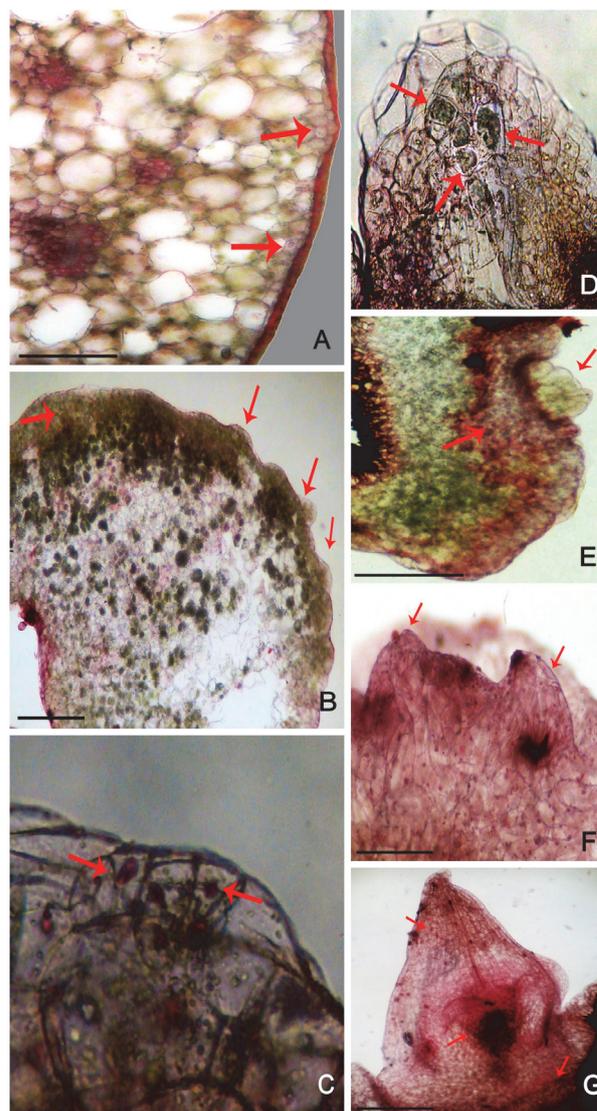


Fig. 5. Histology of stem transverse thin cell layers (tTCLs), callus and protocorm-like bodies (PLBs) of *Dendrobium aqueum*. A - Hand-made section of a stem tTCL showing small polyhedral cells (SPC) in sub-epidermal regions (the arrows); B - a green compact embryogenic callus (EC) showing dense meristematic regions, with multiple protuberances of somatic embryo (SE) initials (the arrows); C and D - embryogenic cells with dense cytoplasm, a prominent nucleus, and vacuoles showing both anticlinal (AC) and periclinal (PC) divisions on EC (the arrows); E and F - formation of globular SE on EC surface and protuberance from the meristematic regions of ECs; G - an SE derived protocorm-like body (PLB) with a leafy shoot initial (the upper arrow) and a dense meristematic protocorm (the middle arrow), not connected with the EC (the lower arrow). Scale bars: 100 μ m (A and B), 200 μ m (E and F), and 650 μ m (G).

applied in tissue culture, somatic embryogenesis, and genetic transformation of many higher plants including

orchids (Teixeira da Silva 2003, 2013). A previous study on the *in vitro* propagation of *D. aqueum* induces multiple shoots from protocorms on a half-strength MS medium supplemented with cytokinins, auxins, and additives (Parthibhan *et al.* 2015a). Unlike that study, where the regeneration of shoots was completely organogenic showing clear vascular traces connecting the shoot to the explant, in the present study, regeneration from stem tTCLs occurred mostly through the formation of EC leading to SE development, without any vascular traces (Fig. 1A,D, 5C). Previous studies on *Dendrobium* TCLs produced either direct PLBs or shoot buds, and the outcome and frequency were related to the species and the type of explant used (Table 1 Suppl.). The SE development started with the formation and appearance of globular or slightly elliptic structures from a compact or friable EC. These structures were very similar to the swollen embryos of germinating seeds (*i.e.*, protocorms) in *D. aqueum* (Parthibhan *et al.* 2012). On development, these globular SEs formed PLBs; additionally, there were successive stages of somatic embryogenesis where heart- and torpedo-shaped structures formed. It is common for monocotyledonous species to have no general stages of embryogenesis, unlike dicotyledonous species. There are different developmental patterns in monocotyledonous somatic embryogenesis that make it difficult to generalize different stages (Hirimburegama 1995). Nevertheless, somatic embryogenesis in monocots may be specific to a particular species, genus, or family (Poddudanya-Arnoldi 1967, Natesh and Rao 1984). Developing globular SEs in monocots comprises an incipient shoot meristem and a single cotyledonary primordium side by side, which makes them appear asymmetric, unlike in dicots (Jürgens *et al.* 1994). Similar asymmetric shapes have been reported in several monocotyledonous species, equivalent to heart (*Halophila*), torpedo, and cotyledonary stages (*Phoenix dactylifera*), torpedo, heart, and globular embryos (*Triticum aestivum*), and torpedo shape (*Agave tequilana*) have been reported in several monocotyledonous species (Jürgens *et al.* 1994, Gutiérrez-Mora *et al.* 2004, Eshraghi *et al.* 2005, Portillo *et al.* 2007, Shahinul Islam 2010, Gutiérrez-Mora *et al.* 2012).

Explant browning followed by necrosis on a PGR-free basal medium, in our case on a half-strength MS, occurs mainly due to lack of plant growth-promoting substances as was reported in *D. nobile*, *D. candidum*, and *D. draconis* (Malabadi *et al.* 2005, Zhao *et al.* 2007, Rangsayatorn 2009). In contrast, around 2 - 3 % of TCLs of *Cymbidium aloifolium* and *D. nobile* TCLs cultured on a MS basal medium containing full-strength macronutrients induced 1 - 2 PLBs per TCL (Nayak *et al.* 2002).

The addition of individual cytokinins to TCL culture has been reported to encourage the frequency of EC and multiple SE formation in many orchids (Hossain *et al.* 2013). Of the five cytokinins tested in this study, EC induction was more efficient on ZEA than BA and 2iP, although SE formation was significantly lower on ZEA than on 2iP and BA. Similarly, TCLs obtained from

D. nobile PLBs also fail to induce and multiply PLBs on a medium with ZEA (Nayak *et al.* 2002). In contrast, ZEA and ZEA riboside formed many SEs or PLBs from different explants (a root-derived callus and leaf), including TCLs of PLBs in other orchids, namely *Cymbidium aloifolium* and *Oncidium* spp. (Chen and Chang 2001, Nayak *et al.* 2002, Wu *et al.* 2004). These studies indicate that ZEA can efficiently induce EC, but the frequency of response and embryogenic outcome may depend on the explant type and species. Moreover, TCLs from PLBs and callus used in these studies, often comprising an undifferentiated mass of meristematic cells, resulted in a higher frequency of embryogenic response than the TCLs derived from leaf explants. Similarly, in our study, tTCLs obtained from the stems of *D. aqueum*, composed of highly differentiated cells with few meristematic cells, displayed a lower frequency of EC. The presence of other tissues or even other nutrients and PGRs present at the site of regeneration may be responsible for a lower frequency of EC induction from tTCLs (Lakshmanan *et al.* 1995).

The outcome of SE formation was highly contingent on the cytokinin used. Previous reports that employed TCLs for *D. gratiosissimum*, *D. draconis*, and *D. nobile* showed the highest PLB production only with individual cytokinins, *e.g.*, 2.0 mg dm⁻³ KN or 2.5 and 5.0 mg dm⁻³ BA (Nayak *et al.* 2002, Rangsayatorn 2009, Jaiphet and Rangsayatorn 2010). Individual cytokinins (2iP, BA, KN, ZEA, and TDZ) have also been reported to induce direct SEs from leaf explants of *Dendrobium* cv. Chiengmai Pink (Chung *et al.* 2005) whereas tTCLs obtained from the nodes of *D. candidum* produced direct shoot buds on a BA-containing medium more than on KN or NAA (Zhao *et al.* 2007). Similar responses of PLB or SE induction by individual cytokinins were recorded in other orchid species, such as *Phalaenopsis cornucervi* and *Oncidium* spp., using different explants (Chen and Chang 2001, Wu *et al.* 2004, Rittirat 2012). In this study, SEs that formed in response to 0.5 mg dm⁻³ ZEA turned necrotic whereas the EC induced on 1.5 mg dm⁻³ 2iP or 0.5 mg dm⁻³ BA produced a high number of SEs (42.67 and 16.00 SEs per tTCL, respectively), which then developed to successive stages of SEs (Table 1). When these optimal levels were exceeded, intense yellow callus was formed with a reduced number or no PLBs, similar to a report on *Aranda* nodal TCLs (Lakshmanan *et al.* 1995). These results indicate that the outcome of EC proliferation and SE production in *Dendrobium* are dependent on the PGRs used as well as on the explant type. This is further evidenced by an experiment from PLB-derived TCLs, in which zeatin riboside induces more PLBs in *C. aloifolium* whereas BA forms more PLBs in *D. nobile* (Nayak *et al.* 2002).

Of all the combined treatments tested, BA with 2iP increased SE formation considerably (33.67 SEs per tTCL) rather than inducing a callus. Likewise, 2iP in combination with auxins also formed slightly more SEs and a higher EC frequency than any BA+auxin combination (Table 2). To date, the synergistic ability of

2iP on somatic embryogenesis has not yet been reported for any *Dendrobium* genotype. On the other hand, BA (0.5 mg dm^{-3}) combined with KN (1.5 mg dm^{-3}) or with auxins improved the EC response more than individual treatments, but SE formation was highly reduced (Table 2). The BA+NAA combination has constantly been reported to improve EC induction or PLB production in *Dendrobium* including when TCLs have been used (Kanjilal *et al.* 1999, Nhat and Dung 2006, Rangsayatorn 2009, Teixeira da Silva *et al.* 2015a, Table 1 Suppl.). However, the combinations BA+KN and BA+IAA used in this study were less effective than the 2iP+IAA combination. KN, when used alone, appeared to inhibit the activity of BA during both callus induction and SE formation. Unlike KN, all other auxins used with BA enhanced the frequency of EC but inhibited SE development due to necrosis (Table 2). A similar negative influence of KN on PLB production has already been reported in *Oncidium* cv. Gower Ramsey, *D. nobile*, and *D. draconis* (Chen and Chang 2001, Nayak *et al.* 2002, Rangsayatorn 2009).

Amino acids are infrequently used in orchid tissue culture except for some seed germination studies (Hossain *et al.* 2013). Somatic embryogenesis using amino acids has been reported in other plants, including *Phoenix dactylifera* (Sghaier *et al.* 2009) and *Saccharum spontaneum* (Nieves *et al.* 2008). Amino acids, especially arginine, promote accumulation of soluble protein in embryogenic calluses during SE differentiation and maturation (Lee *et al.* 2007, Nieves *et al.* 2008, Sghaier *et al.* 2009). Moreover, amino acids are the constituents of seed storage proteins and play an important role in germination and seedling development (Muntz 1998). For these reasons, it is likely that the early green protuberances and EC initials subsequently developed into plantlets after PLBs formed in *D. aqueum* in response to arginine. The rapid shoot bud development from PLBs and EC achieved in this study in response to 25 to 50 mg dm^{-3} arginine was also observed in *Cymbidium pumilum* and *C. goeringii* (Ueda and Torikata 1968, 1972, Nieves *et al.* 2008).

Browning explant tissues and a regenerative callus ultimately leading to necrosis are major problems in orchid somatic embryogenesis (Wu *et al.* 2004, Roy *et al.* 2007, Majumder *et al.* 2010). In orchids, when explants are obtained from mature plants, they either release exudates into the medium or accumulate phenolic substances within their tissues during culture, and these become toxic to cells when oxidized (Chugh *et al.* 2009, Kaewubon *et al.* 2015). In the present study, the stems used to obtain tTCLs were about 5 cm in height and 2 - 3 mm in diameter, green and hard. There was no exudation during the preparation of tTCLs whereas after 3 - 4 weeks in culture, the edges of some of the explants turned brown and explants became necrotic. Apart from the negative impact on explants, callus browning affected callus proliferation and SE formation. The PGR concentrations and combinations used induced explant browning at various levels, 0 - 100 %, 9 - 66 %, and 0 - 86 % in

D. fimbriatum, *D. farmeri*, and *Dendrobium* cv. Indonesia Raya, respectively (Roy and Banerjee 2003, Majumder *et al.* 2010, Rachmawati *et al.* 2015). In our study, some of the callus that turned completely brown, as well as the explant from which it formed, initiated green EC and continued to proliferate, and could be subcultured to form shoots. This might be due to the regenerative competence of PGRs on stem TCLs of *D. aqueum* similarly as reported earlier in *D. crepidatum* and *D. pierardianum* (Vij *et al.* 1991), and *Coelogyne stricta* (Basker and Narmatha Bai 2006), where regenerated shoot buds grow normally with the help of PGRs even after the explants become brown.

Active charcoal is frequently employed to control explant browning (tissue oxidation) and to promote *in vitro* micropropagation, somatic embryogenesis, synthetic seed production, protoplast culture, anther and microspore culture, and rooting in plants, including orchids (Sharma *et al.* 2007, Thomas 2008, Rittirat *et al.* 2012, Teixeira da Silva *et al.* 2015a). In this study, AC controlled browning effectively, but completely failed to support callus induction. Similarly, glutathione did not support EC induction. In general, when glutathione is supplemented to culture medium, rooting is favoured, EC fresh mass and SE yield increase, and it can effectively protect *in vitro* cultured tissues against oxidative stress (Tyburski and Tretyn 2010). In the present study, 5 to 75 mg dm^{-3} arginine did not induce callus, and callus that formed at a higher concentration (100 mg dm^{-3}) became necrotic (Fig. 4). On the other hand, lower concentrations of glutathione (5 - 25 mg dm^{-3}) directly induced SEs, which turned immediately into shoots *via* PLBs (Fig. 4). The ratio of reduced to oxidized glutathione can influence cell extension, growth, and division (Standardi and Micheli 1988). This might explain the direct formation of SEs rather than *via* callus in *D. aqueum*. Unlike AC and glutathione, PVP significantly inhibited explant browning and supported EC induction following SE formation as well as direct SE formation (Fig. 3). The addition of PVP to the culture medium helps to oxidize polyphenols, which are released by explants into the medium and promotes organogenesis (Rout *et al.* 2006). However, in this study, the rate of conversion of initial green protuberances into direct SEs was observed at low concentrations of PVP (0.1 - 0.3 mg dm^{-3}), proliferation of EC at 0.3 - 0.5 mg dm^{-3} PVP, and effective formation of SEs at 0.5 mg dm^{-3} PVP (Fig. 3).

Overall, the plantlets obtained from SEs showed no morphological variation among the treatments (Fig. 1G). Generally, the use of synthetic PGRs at sub- and supra-optimal levels supposedly induce somaclonal variation in orchids (Arditti and Ernst 1993). However, the ISSR marker profiles of the SE-derived *D. aqueum* plants showed a high rate of monomorphism (97.78 %) and confirmed the genetic fidelity of plantlets derived from various PGR treatments.

In conclusion, this is the first report on somatic embryogenesis of *D. aqueum* from stem tTCLs. We also report the successive maturation stages of *D. aqueum*

SEs. The individual and combined effects of cytokinins, auxins, arginine, glutathione, and/or PVP on somatic embryogenesis until the shoot development in *D. aqueum*

could be applied to the regeneration of other important orchids.

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