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Organogenic Potential of *Dendrobium* Floral Tissues for Stable Transformation Applications

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This study aimed to examine a promising procedure for *in vitro* direct organogenesis from floral tissues of *Dendrobium* Sonia 'Earsakul' as a new potential regeneration system for transformation in orchids. Petal and sepal explants from floral buds (2.0–2.3 cm) survived on ½ MS solid medium supplemented with or without 1-naphthaleneacetic acid (NAA) or/and benzylaminopurine (BA), but only petal explants generated protruding meristemoid tissues when they were cultured on ½ MS medium supplemented with both NAA and BA. A significant increase in the number of protruding meristemoid tissues was observed in the liquid medium. The ½ MS liquid medium supplemented with 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BA was successfully used to induce meristemoids of petal tissues transiently transformed by *A. tumefaciens* strain EHA105 carrying pCAMBIA–1301 via agroinfiltration and the subsequent use of 20 mg L^{-1} meropenem was effective in eliminating *Agrobacterium* from the infected explants. The transformed status of the meristemoid tissues was confirmed by GUS expression analysis, indicating that the T-DNA of the vector used had been successfully incorporated into the genome. For the first time, the researchers have demonstrated the potential methodology for direct organogenesis from agroinfiltration-transformed petal tissues of *D.* Sonia 'Earsakul'.

Key words: *Dendrobium*, floral tissue, meristemoid, organogenesis, tissue culture, transformation

INTRODUCTION

Dendrobium is the second largest genus in the orchid family (Orchidaceae) and contains the greatest diversity of horticulturally interesting specimens, accounting for about 85% of the total orchid cut flower trade worldwide (Puchoaa 2004, Teixeira da Silva *et al*. 2015). *Dendrobium* hybrids have dominated international trade due to their floriferous nature, with interesting flower colors, sizes and shapes, year-round availability, and long vase life. *D.* Sonia 'Earsakul' is a common favorite hybrid orchid in Thailand, and it has become popular in many countries due to its spectacular form and color. The high demand for this clone and other varieties of *D*. Sonia lines have prompted researchers to develop innovative tissue culture techniques dedicated to this hybrid line (Kumari *et al*. 2013, Puchoaa 2004) – aiming for varietal improvement, high regeneration yield, clonal stability, as well as for transgenic applications (Julkifle *et al*. 2012).

Dendrobium micropropagation has been well documented in various forms using different explants such as nodal segments, shoot tips, leaves, pseudobulbs, axillary buds, and floral stalks (Teixeira da Silva *et al*. 2015). Depending on the purpose, the choice of explant mainly relies on the ability of the selected tissue to regenerate into a complete

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plant either indirectly (callus phase) or directly (*i.e*., somatic embryogenesis, direct organogenesis) (Chugh *et al*. 2009). Although callus culture systems can be commonly observed in orchid tissue culture, researchers tend to use direct regeneration to avoid the incidence of somaclonal variation. Regeneration by direct organogenesis skips the intervening callus phase, which eliminates undesirable somaclonal variation associated with the prolonged callus culture period – making it a favorable method for transformation experiments. Direct organogenesis has been undertaken in orchids using shoot tips, leaves, rhizome and root segments, and inflorescence stalks (Chugh *et al*. 2009). Nevertheless, with the growing need for varietal improvement in orchids, it is interesting to determine the regeneration ability of other possible explant materials such as petal and sepal tissues. The use of floral tissues (petals or sepals) can be a good alternative for micropropagation of *Dendrobium* hybrids as the technique would not require the sacrifice of the mother plant and its growing shoots. Previous studies for other plant genera such as *Dianthus* (Frey & Janick 1991), *Sinningia* (Pang *et al*. 2012), and *Fragaria* (Debnath 2005) have demonstrated the high regeneration potential of petal and sepal tissues, and have shown that these tissues can be exploited for genetic transformation experiments (Frey & Janick 1991, van Altvorst *et al*. 1996). The use of inflorescence parts and floral buds of orchids have been reported as effective explants for micropropagation (Chugh *et al*. 2009); however, to date, there is no available information on the *in vitro* regeneration ability of petal and sepal tissues from orchids. Recently, successful utilization of *D.* Sonia 'Earsakul' petal and sepal tissues for transient transformation via agroinfiltration has been established generating an interesting array of opportunities for developing flower color modification technology in orchids (Pinthong *et al*. 2014, Ratanasut *et al*. 2015). Regeneration from transiently transformed petal or sepal explants will be a promising tool for stable transformation of orchids. Therefore, this work aimed to demonstrate the applicability of petal and sepal tissues to *in vitro* culture regeneration, and to apply this technique to regenerate transiently transformed floral tissues to produce stable transformants of *D.* Sonia 'Earsakul'.

MATERIALS AND METHODS

Floral Tissue Culture Conditions

Dendrobium Sonia 'Earsakul' plants from a commercial farm served as the source of explants. For surface sterilization, stage 2 floral buds measuring 2.0–2.3 cm (Ratanasut *et al*. 2015) were washed under running tap water for 15 min followed by soaking in diluted fungicide solution (0.1% captan, 0.1% carbendazim, and 0.1% metalaxyl; w/v) for 15 min. Floral buds were then treated with 15% (v/v) commercial bleach (6% sodium hypochlorite) containing two drops of Tween 20 for 15 min and subsequently rinsed three times with sterile distilled water. Sepals and petals were separated and cut into pieces of about 1.0 cm x 0.5 cm prior to culturing in half-strength (Murashige & Skoog 1962) (½ MS) medium, supplemented with 1-naphthaleneacetic acid (NAA; 0.1, 0.5, 1.0 mg L^{-1}) or benzylaminopurine (BA; 0.1, 0.5, 1.0 mg L^{-1}) alone or in combination, 15% (v/v) coconut water and 15 g L^{-1} sucrose (pH 5.7) and solidified with 7 g L^{-1} agar. Meanwhile, for comparing the efficiency of medium types (semi-solid vs. liquid), hormone concentrations were limited to levels 0.5 and 1.0 mg L^{-1} of both NAA and BA and their combinations, respectively, since no significant results were observed at 0.1 mg L^{-1} levels for both phytohormones during the previous experiment using semi-solid media. Explants were placed abaxially on the surface of the solid medium or immersed in the liquid medium, and then incubated at 22±2 °C with a 16 h photoperiod (20 µmol m^{-2} s^{-1}). The liquid cultures were shaken at 80 rpm.

Agroinfiltration of Petal Tissues

Agrobacterium tumefaciens strain EHA105 harboring the pCAMBIA–1301 was used for infiltrating petal tissues of *D.* Sonia 'Earsakul' following the method of Pinthong *et al*. (2014). The pCAMBIA–1301 carries the T-DNA region containing the *β-glucuronidase* (*gusA*) with an intron to ensure that expression of glucuronidase activity is derived from the plant cells and not from residual *A. tumefaciens* cells. After infiltration, co-cultivation was carried out for two days at room temperature (25 °C), after which the infiltrated flowers were harvested for aseptic culture.

In Vitro Culture of Infiltrated Petal Tissues

Infiltrated flowers were soaked in diluted fungicide solution for 15 min followed by 15% (v/v) commercial bleach (6% sodium hypochlorite) for 5 min and then rinsed three times in sterile distilled water containing 5 mg L^{-1} meropenem. The surface-sterilized petal tissues were cultured in ½ MS liquid medium supplemented with 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BA. To evaluate the efficiency of antibiotics for eliminating *Agrobacterium,* the standard 250 mg L^{-1} cefotaxime – as well as the different levels of meropenem (5, 10, and 20 mg L^{-1}) – were tested. The antibiotics were filter-sterilized before being added to the liquid medium. The cultures were incubated according to the conditions described above and sub-cultured to antibiotic-free medium after four weeks.

Determination of Putative Transformants

The histochemical GUS assay was performed according to the protocol of Jefferson & Kavanagh (1987). The four-week old petal tissues infiltrated with pCAMBIA–1301 were immersed in 10 mL of GUS staining solution. The

GUS stained samples were incubated overnight in a vacuum at 37 °C. Afterwards, the staining solution was replaced with 70% ethanol to decolorize the plant tissues. Tissue samples were observed under a stereomicroscope.

Histological Analysis of Meristemoid Tissues

Before inoculation and during regeneration (after 4 and 8 weeks of incubation), tissues were collected and fixed in 105 mL of modified FAA solution (Johansen 1940) [50 mL of 95% (v/v) ethanol, 45 mL of distilled water, 5 mL of 100% formalin, and 5 mL of glacial acetic acid] for 18 h. Samples were then subjected to dehydration, paraffin embedding, and staining following the protocol of Kermanee (2009). The stained tissues were observed under a compound light microscope.

Experimental Design and Statistical Analysis

A completely randomized design was used in all experiments. Each treatment was replicated at least five times and each replicate contained two explants. The first experiment on the growth response of both petal and sepal explants to different hormone combinations was performed once, while tests on the effect of media type in meristematic response of petal explants were conducted three times (3 trials) using the selected hormone combinations (with higher incidence of growth response) from the previous experiment. Data on the observable growth induction rate (%) was computed as the percentage of responding explants per treatment, whereas the means (n) from total number of visible organogenic tissues (OT) were counted per explant and averaged per treatment. The approximate size of the growing meristemoid tissues were segregated into two ranges (<0.5 mm and >0.5–2.0 mm) and were counted as means per explant and averaged per treatment. Explants that remained green or purple after 4 and 12 weeks of incubation, regardless of growth induction response, were counted for survival rate (%) per treatment. For test with antibiotics, bacterial contamination and survival rate were recorded after 4 weeks of incubation (with antibiotics), while bacterial regrowth incidence and survival rate were observed on week 12 (8 weeks on antibiotic-free medium). Data were subjected to Analysis of Variance (ANOVA) and Duncan Multiple Range Test (DMRT) using the SPSS 17.0 software. Significance was set at $p \leq 0.05$.

RESULTS

Growth Response of Petal and Sepal Explants to Different Hormone Combinations

Morphogenic response of the petal and sepal explants of *D.* Sonia 'Earsakul' to various concentrations of NAA and BA and the combinations is shown in Table 1. Sepal explants cultured in any of the media supplemented

Table 1. Influence of NAA and BA combinations on *in vitro* meristematic tissue (MT) induction of *D.* Sonia 'Earsakul' petal and sepal tissues.

Hormone Combinations* (mg L^{-1})		Petal**			Sepal**		
NAA	BA	Growth Induction (%)	Mean MT per Explant (n)	Survival (%)	Growth Induction (%)	Mean MT per Explant (n)	Survival (%)
0	0	0^{e}	0^{c}	91.7 ± 8.3^{a}	-	-	58.3 ± 20.1^{ab}
	0.1	0^{e}	0^{c}	50.0 ± 0.0^{abc}	-	-	75.0 ± 17.1^{a}
	0.5	0^{e}	0^{c}	50.0 ± 12.9^{abc}	-	-	58.3 ± 15.4^{ab}
	1	0^{e}	0^{c}	91.7 ± 8.3^{a}	-	-	75.0 ± 17.1^{a}
0.10	0	0^{e}	0^{c}	16.7 ± 16.7^{c}	-	-	33.3 ± 16.7^{ab}
	0.1	0^{e}	0^{c}	50.0 ± 12.9^{abc}	-	-	50.0 ± 18.3^{ab}
	0.5	0^{e}	0^{c}	58.3 ± 20.1^{abc}	-	-	33.3 ± 16.7^{ab}
	1	0^{e}	0^{c}	50.0 ± 12.9^{abc}	-	-	25.0 ± 17.1^{ab}
0.50	0	0^{e}	0^{c}	33.3 ± 10.5^{bc}	-	-	58.3 ± 20.1^{ab}
	0.1	0^{e}	0^{c}	50.0 ± 18.3^{abc}	-	-	16.7 ± 10.5^{b}
	0.5	50.0 ± 18.3^{bc}	1.1 ± 0.4^{b}	75.0 ± 7.1^{ab}	-	-	75.0 ± 11.2^{a}
	1	75.0 ± 11.2^{a}	2.5 ± 0.5^{a}	75.0 ± 11.2^{ab}	-	-	50.0 ± 18.3^{ab}
1.00	0	0^{e}	0^{c}	33.3 ± 16.7^{bc}	-	-	58.3 ± 15.4^{ab}
	0.1	33.3 ± 16.7^{bc}	0.7 ± 0.4^{bc}	50.0 ± 18.3^{abc}	-	-	50.0 ± 18.3^{ab}
	0.5	58.3 ± 8.3^{ab}	2.1 ± 0.4^{a}	91.7 ± 8.3^{a}	-	-	8.3 ± 8.3^{b}
	1	25.0 ± 11.2^{de}	0.6 ± 0.3^{bc}	91.7 ± 8.3^{a}	-	-	33.3 ± 21.1^{ab}

*Plant growth regulators were added to the medium.
**Data were collected at week 4 after incubation. The values represent the means ± standard error (SE) of 6 replicates. Means followed by the same letters within columns are not significantly different at p=0.05 (DMRT).

with different hormone combinations did not develop shoots or calli even after 4 weeks of incubation. On the other hand, only meristemoid tissues originating from the abaxial epidermis in contact with the medium of petal cultures (Figure 1a, 1b) starting from week 2 were observed in some NAA and BA combinations, but not in NAA or BA alone. Cultures in the medium supplemented with 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BA exhibited the highest growth response ratio (75%) with an average of 2.5 observable meristemoid tissues per explant, while the lowest response was observed in 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} BA (16.67%) with an average of 0.6 meristemoid tissues per explant. This morphogenic response suggests that the ability of the petal tissues of *D.* Sonia 'Earsakul' to acquire totipotent competence can be triggered within the range of 0.5–1.0 mg L^{-1} NAA and 0.1–1.0 mg L^{-1} BA.

Effects of Plant Growth Regulators and Medium Types in Meristematic Response of Petal Explants

All explants started to swell after two weeks and the first signs of growth were observed in both solid and liquid media supplemented with phytohormones. Petal tissues cultured in liquid medium were 2–3 times larger than petal tissues placed on solid medium after four weeks. Meanwhile, all sepal explants did not exhibit any sign of growth to any of the media type and hormone combinations tested (data not shown). At week 3, growth initiation was also detected in some petal cultures in the hormone-free liquid medium (5%); however, the cultures did not progress further even after eight weeks. Less meristemoid tissues were generated from petal tissues cultured on solid medium with hormones (Figure 1a, 1b) as compared to the liquid counterpart even after 8 weeks (Figure 1c, 1d). After four weeks of incubation, the highest induction rate was observed in liquid medium supplemented with 1.0 mg L^{-1} NAA and 1.0 mg L^{-1}BA

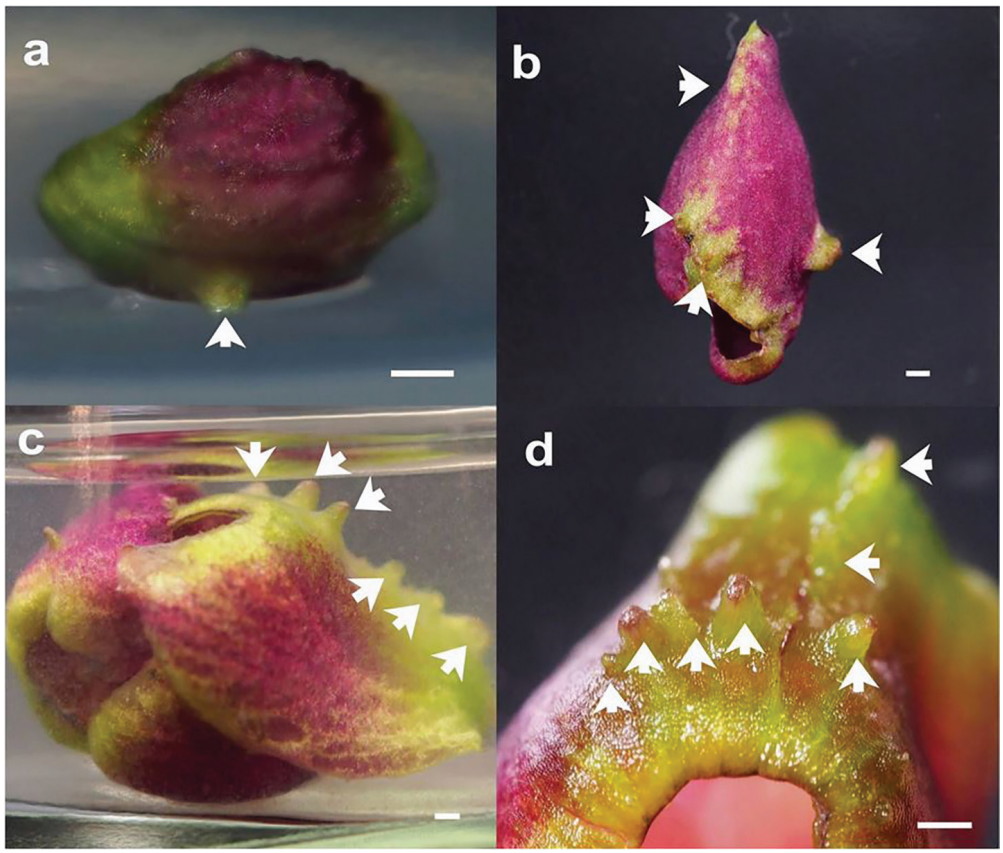

Figure 1. *In vitro* meristematic growth induction of *D.* Sonia 'Earsakul' petal tissues. (a) the 4-week old petal culture planted abaxially in the solid ½ MS medium supplemented with 1.0 mg L^{-1} NAA and 0.1 mg L^{-1} BA showing the growing tissue originating from the abaxial epidermis (arrow) in contact with the medium; (b) the meristematic tissues emerging (arrows) from the 8-week old petal tissue (removed from the vessel) cultured on the solid medium supplemented with 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BA; and (c, d) the meristematic tissues (arrows) from the 8-week old petal tissue cultured in the liquid medium supplemented with 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BA. Scale bar = 1 mm.

(88.6%), but no significant difference was observed when compared with other liquid media supplemented with other hormone combinations. However, the highest number of larger (0.5–2.0 mm) meristemoid tissues were observed in the liquid medium supplemented with 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BA, while the liquid medium with 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} BA had the highest frequency of small meristemoid tissues (<0.5 mm). Overall, the results showed that petal explants cultured in liquid medium produced a higher percentage response than the solid medium counterpart (Table 2).

Histological Analysis of Meristemoids Induced in Petal Tissues

Histological observations of petal tissue samples were presented in Figure 2. Cross-section of the petal tissue before inoculation (Figure 2a) revealed higher number of active cells characterized by the stained epidermal cells' nuclei on the abaxial surface than the adaxial side. On the other hand, cross-section of sepals before and during culture (Figure 2b and 2d, respectively) did not exhibit any stained nuclei, indicating the possible absence of active meristematic cells. Meanwhile, in all the petal tissues, no callus was observed but meristematic protuberances on the abaxial epidermis in both solid and liquid cultures starting from week 2. The visible regenerated tissues on all responding treatments have the same characteristics of determined organ formation as they continue to grow in week 4 (Figure 2c), varying only in the numbers of meristemoid tissues formed per explant and sizes in response to hormone concentrations. Protruding tissues with densely stained meristematic centers (meristemoids) continue to develop via cell division and new possible organogenic centers were also formed (Figure 2e, 2f).

Effect of Different Antibiotics on Efficiency of *Agrobacterium* Elimination and on Growth and Development of Infiltrated Petals *In Vitro*

The results regarding the effects of meropenem and cefotaxime on the efficiency of *Agrobacterium* elimination and on growth and development of infiltrated petals *in vitro* are shown in Table 3. As expected, infected petal explants inoculated in antibiotic-free medium were all severely damaged by bacterial contamination starting from week 1 of incubation. At week 4, on the other hand, no contamination was observed in media supplemented with antibiotics except at 10 mg L^{-1} meropenem (10%). Although no significant differences were observed in the mean number of meristemoid tissues among the treatments, larger sizes of protruding tissues (~1–2 mm) were observed in media with meropenem – particularly at 20 mg L^{-1} – while those grown in cefotaxime had smaller regenerants (<0.5mm), which ceased to grow at week 4. Even after all explants were transferred to antibiotic-free medium at week 4, a notable decline in the survival rate was still observed in cultures derived from cefotaxime treated medium (10%), while cultures in 20 mg L^{-1} meropenem remained high (90%) until week 12.

Determination of Putative Meristematic Transformants

To determine the feasibility of orchid petal tissue culture as a potential transformation system, histochemical GUS assay was performed to random samples from four-week old petal cultures infiltrated with *A. tumefaciens* EHA105 harbouring pCAMBIA–1301. As illustrated in Figure

Table 2. Effects of NAA and BA combinations and medium types on meristematic initiation in *D.* Sonia 'Earsakul' petal tissues after 4 weeks of incubation.

Hormone Combinations (mg L^{-1})		Media Type	Growth Induction (%)	Mean Meristematic Tissues per Explant	Average Size Frequency Meristematic Tissues per Explant (n)		Survival (%)
NAA	BA				< 0.5 mm	> 0.5–2.0 mm	
0	0	Solid	0^{c}	0^{d}	0^{c}	0^{c}	75.7 ± 16.0^{abc}
		Liquid	5.0 ± 5.0^{bc}	0.1 ± 0.1^{d}	0.1 ± 0.1^{c}	0^{c}	96.7 ± 3.3^{a}
0.5	0.5	Solid	22.2 ± 4.7^{bc}	0.5 ± 0.3^{cd}	0.4 ± 0.3^{c}	0.1 ± 0.1^{c}	47.9 ± 16.3^{c}
		Liquid	73.9 ± 12.4^{a}	2.8 ± 0.3^{abc}	2.2 ± 0.1^{abc}	0.6 ± 0.3^{ab}	83.3 ± 12.0^{ab}
	1	Solid	36.1 ± 20.0^{b}	1.1 ± 0.7^{bcd}	0.4 ± 0.2^{c}	0.7 ± 0.5^{ab}	65.3 ± 5.0^{abc}
		Liquid	83.9 ± 9.6^{a}	4.3 ± 1.2^{a}	3.6 ± 1.0^{a}	0.6 ± 0.2^{ab}	98.3 ± 1.7^{a}
1	0.5	Solid	37.5 ± 12.0^{b}	1.1 ± 0.5^{bcd}	0.9 ± 0.3^{bc}	0.2 ± 0.2^{c}	70.8 ± 10.5^{abc}
		Liquid	75.0 ± 8.7^{a}	4.0 ± 0.7^{a}	3.0 ± 0.6^{ab}	1.0 ± 0.1^{a}	95.0 ± 5.0^{a}
	1	Solid	20.8 ± 4.2^{bc}	0.4 ± 0.2^{cd}	0.3 ± 0.1^{bc}	0.1 ± 0.1^{c}	60.4 ± 17.5^{bc}
		Liquid	88.6 ± 3.4^{a}	4.8 ± 1.8^{a}	4.1 ± 1.7^{a}	0.7 ± 0.1^{ab}	93.9 ± 3.1^{a}

*The values represent the average ± standard error (SE) of three independent experiments with at least 5 replicates per experiment. Means followed by the same letters within columns are not significantly different at p=0.05 (DMRT).

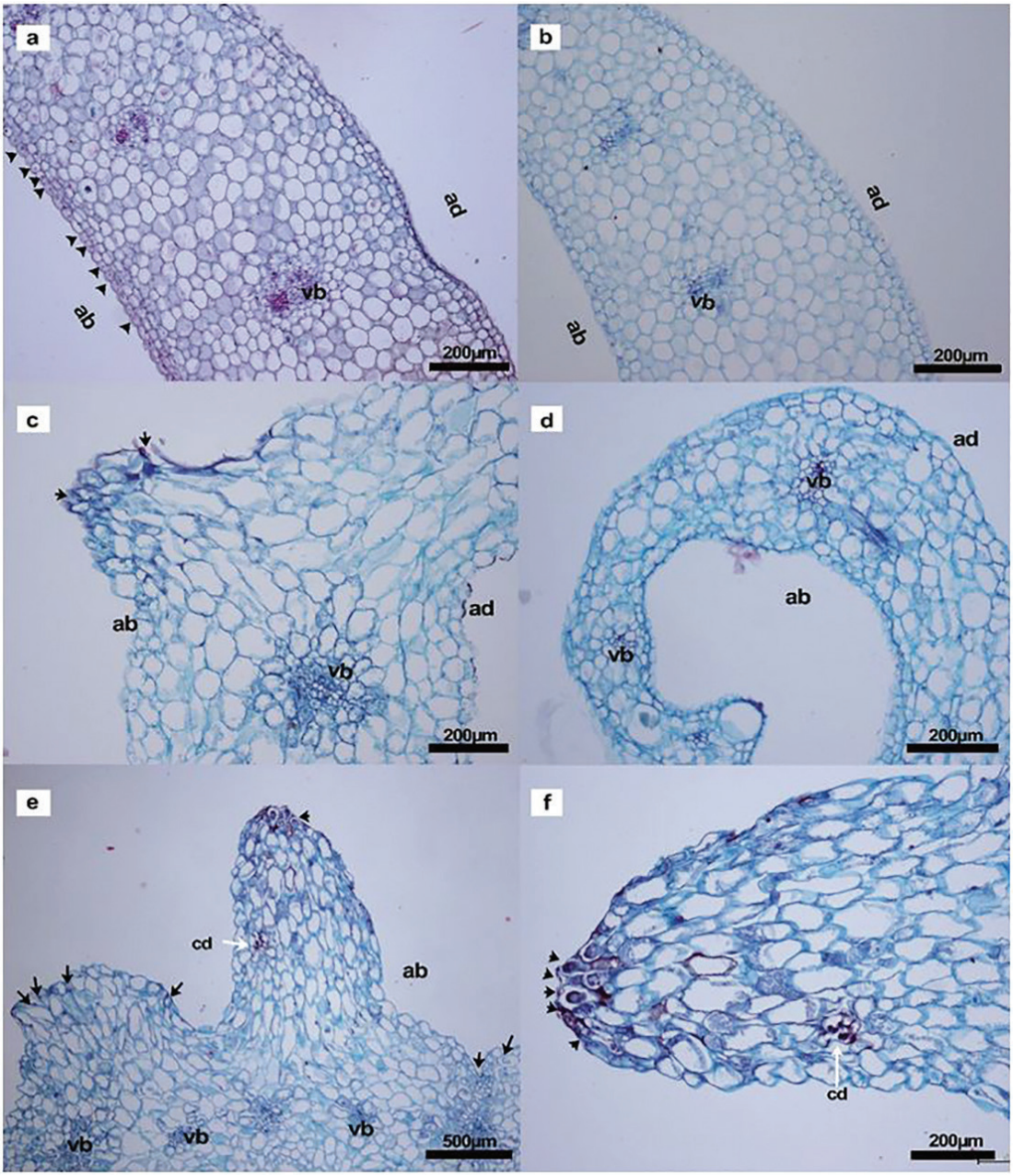

Figure 2. Histology of meristemoids in petal tissues of *D.* Sonia 'Earsakul'. (a) Cross-section of petal explant before inoculation showing the stained nucleus of the active cells (arrow heads) of the abaxial epidermis; (b) cross-section of sepal explant before inoculation; (c) longitudinal section of a protruding meristemoid tissue (black arrows) on the abaxial epidermis in contact with the semi-solid medium in a 4-week-old petal culture; (d) cross-section of 4-week-old sepal culture inoculated in semi-solid medium; (e) longitudinal section of elongated organogenic tissue with darkly stained meristematic region (arrow head) of an 8-week-old petal culture grown in liquid medium and newly protruding and developing meristematic centers (black arrows); (f) detail of the elongated meristematic tissue (right side of c) with densely stained meristemoids (arrow heads) and cell division (white arrow). ab – abaxial epidermis; ad – adaxial epidermis; vb – vascular bundle; cd – cell division.

3, the GUS expression detected in the infiltrated petals characterized by the blue stain confirmed the transgenic nature of the developing meristemoids, which are possible organogenic tissues.

DISCUSSION

Choice of explant and optimization of plant phytohormone ratio are important in establishing a plant tissue culture protocol towards callus or organ formation. To the

Table 3. Effects of antibiotic concentration on *in vitro* culture of agroinfiltrated petal tissues of *D.* Sonia 'Earsakul.'

Treatment	Mean Meristematic Tissue per Explant after 4 Weeks (n)*	Bacterial Contamination Rate (%) after 4 Weeks*	Survival Rate (%) after 4 Weeks*	Survival Rate (%) after 12 Weeks*	Bacterial Regrowth Incidence (%) after 12 Weeks***
5 mg L^{-1} meropenem	1.4±0.5	0[a]	90±10.0[a]	50±16.7[b]	40±16.3
10 mg L^{-1} meropenem	1.0±0.5	10±10[a]	50±16.7[b]	40±16.3[b]	10±10.0
20 mg L^{-1} meropenem	1.9±0.5	0[a]	90±10.0[a]	90±10.0[a]	20±13.3
250 mg L^{-1} cefotaxime	1.3±0.4	0[a]	100±0.0[a]	10±10.0[b]	10±10.0
Antibiotic-free medium	ND**	100±0[b]	0[c]	ND**	ND**

*The values represent means ± SE of 10 replicates. Means followed by the same letters within columns are not significantly different at p=0.05 (DMRT).
**ND – no data
***The explants were sub-cultured in antibiotics-free medium at weeks 4–12.

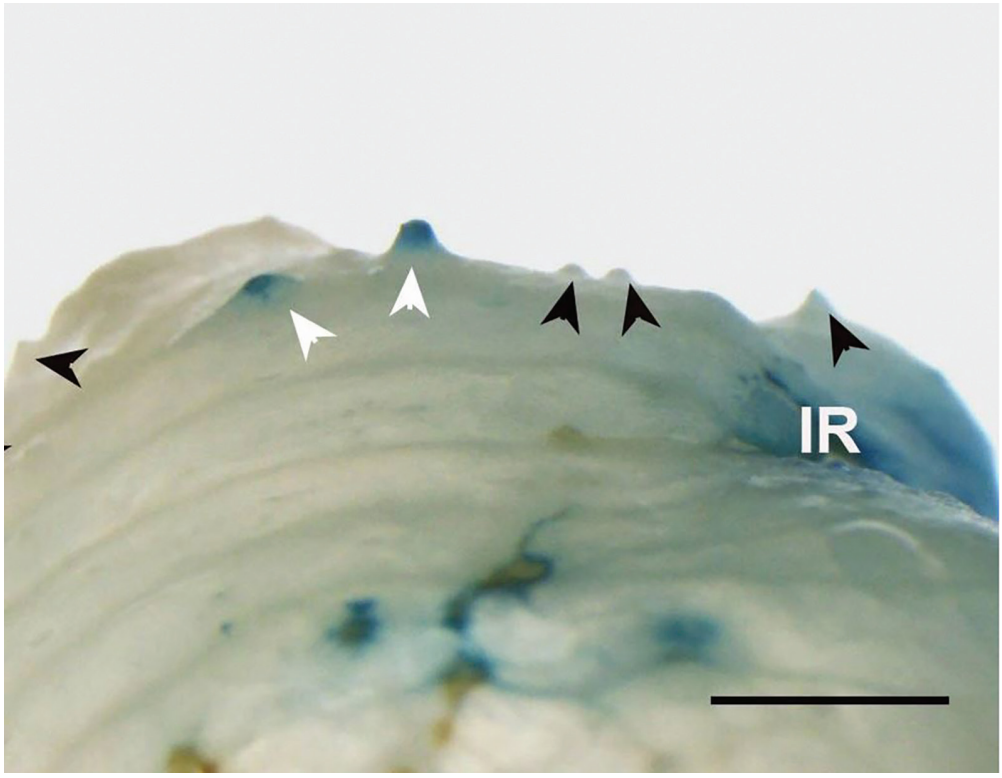

Figure 3. Histochemical GUS assay of a 4-week-old meristematic culture derived from a petal explant of *D*. Sonia 'Earsakul' infiltrated with *A. tumefaciens* strain EHA105 harboring pCAMBIA-1301. Transformed organogenic tissues were stained blue (white arrow heads) and non-transformed organogenic tissues were clear (black arrow heads). IR – infiltration region. Scale bar = 1 mm.

researchers' knowledge, the present study is the first to describe the *in vitro* regeneration potential of *D.* Sonia 'Earsakul' petal and sepal explants. Sepal explants did not show any signs of meristematic response to any hormone and/or media type combinations. This phenomenon may be considered recalcitrance to phytohormones (Delporte *et al*. 2012), and nutrient medium used in the present study may not be sufficient to induce totipotency in sepal tissues of *D.* Sonia 'Earsakul'. Meanwhile, only the petal explants of *D.* Sonia 'Earsakul' exhibited the ability for meristemoid formation regardless of the levels of phytohormones present in the media; this also showed no clear indication of single effects of an auxin and a cytokinin, but suggest their synergistic effect on the meristematic response. Synergistic effects of NAA and BA leading to higher growth response has been previously observed in *Dendrobium* shoot tips (Roy & Banerjee 2003), nodal segments (Kumari *et al*. 2013), and leaf

cultures (Puchoaa 2004) – as well as in *Phalaenopsis* (Park *et al*. 2002), *Cymbidium* (Nayak *et al*. 2002), and other orchid genera (Chugh *et al*. 2009). In non-orchidaceous plants, the presence of cytokinin was sufficient to induce multiple shoots in petal tissues of *Streptocarpus* (Chaudhury *et al*. 2010) and *Chrysanthemum* (Song *et al*. 2011). However, adding both auxin and cytokinin in various ratios was necessary for the regeneration of petal explants of *Dianthus* (Frey & Janick 1991), *Sinningia* (Pang *et al*. 2012), and *Sedum* (Wojciechowicz 2009) – generating either calli, shoots, or somatic embryos.

The benefits of using a liquid culture system instead of the conventional semi-solid media have been reported for several plants (Mehrotra *et al*. 2007), including orchids (Nayak *et al*. 2002, Puchoaa 2004). Growing cultures in a liquid medium allows for a closer contact between the medium and the tissue, which enhances nutrient uptake and increases aeration – resulting in enhanced growth and development (Mehrotra *et al*. 2007). The observable expansion in the size of the explants in liquid media possibly indicates efficient nutrient absorption resulting in activation of meristematic cells of the petal epidermis. Nayak *et al*. (2002) reported an increased growth response and PLB formation frequency in *C. aloifolium* and *D. nobile* thin cell layers (TCL) using a suspension culture maintained in an 80 rpm rotary shaker. Three different culture media – Knudson C, Vacin and Went (VW), and MS; in both solid and liquid states – were previously tested with *D.* Sonia leaf explants for PLB production, and it was found that the highest PLB frequency was obtained using full strength liquid MS medium supplemented with 1.0 mg L^{-1} NAA and 0.1 mg L^{-1} BA (Puchoaa 2004). Thus, the culture response is not only modulated by the specific medium but also by specific species, as well as nutritional and hormonal requirements. Results showed that half strength liquid MS medium supplemented with both auxin and cytokinin was more favorable than semi-solid culture medium for maximizing the totipotency of *D.* Sonia 'Earsakul' petal tissues for potential organogenesis.

The histological analysis confirmed the meristematic nature of the developing organ tissues from petal explants of *D.* Sonia 'Earsakul,' which resembles the meristematic cells observed in the early stage of somatic embryogenesis in *Phalaenopsis amabilis* leaf epidermis (Chugh *et al*. 2009), developing PLBs of *Dendrobium* Sonia 28 (Julkifle *et al*. 2012), as well as in organogenic tissue formation in *Sedum* petal tissues (Wojciechowicz 2009). This stage of development can proceed to independent organ formation or whole plant regeneration if given the proper nutritional and hormonal requirements as well as appropriate culture conditions, and can therefore be an interesting material for genetic transformation studies in orchids.

The success of *Agrobacterium*-mediated transformation can be determined through the recovery of developing transgenic plant or tissues and elimination of bacterial residues. The researchers therefore cultured the infiltrated petals in liquid medium supplemented with either cefotaxime or meropenem for eliminating *Agrobacterium*. By using the conventional cefotaxime, elimination of *Agrobacterium* was achieved but it appeared to be toxic to petal tissues, even after being withdrawn from culture on week 4. The effective concentration of cefotaxime for eliminating *Agrobacterium* (250 mg L^{-1}) has been reported to cause severe necrosis in transformation studies in rice, tomato, and tobacco (Farzaneh *et al*. 2013, Priya *et al*. 2012). On the other hand, the same effectivity of eliminating bacterial residues was observed in cultures grown in medium supplemented with meropenem but had little inhibitory effect on the *in vitro* development of *D.* Sonia 'Earsakul' petal tissues. The results support the efficiency of meropenem over cefotaxime for eliminating bacterial residues in *Agrobacterium*-mediated transformation, which has been reported for several plants (Farzaneh *et al*. 2013, Konagaya *et al*. 2013, Ogawa & Mii 2007) and has also been applied to orchids because of its effectiveness at low concentrations and less inhibitory effects on plant development (Cao *et al*. 2006, Zhang *et al*. 2010).

Bacterial regrowth was visibly observed at week 12, some two months after withdrawing both meropenem and cefotaxime from the medium. This indicates that bacterial growth was efficiently suppressed and persisting *Agrobacterium* required a long recovery time. Cao *et al*. (2006) observed bacterial regrowth in *D. phalaenopsis* PLBs as early as 10 days after removing 50 mg L^{-1} meropenem from the medium. In other reports, occasional *Agrobacterium* regrowth was detected in putatively transformed shoots of tomato, tobacco, and rice grown in meropenem-supplemented medium at week 16 (Ogawa & Mii 2007). In the present analysis, the researchers have deduced that in the transient transformation procedure, the *in vivo* co-cultivation for two days – which allowed the bacterial solution to dry up after agroinfiltration – might have contributed to the efficiency of the antibiotics in suppressing the growth of the *Agrobacterium in vitro*. Conventional co-cultivation usually requires the growing of the plant tissues in a bacterial solution *in vitro* for some period of time, followed by transfer to a medium with antibiotics to eliminate *Agrobacterium*. This facilitates bacterium-plant tissue contact resulting in efficient transformation; however, eliminating bacterial regrowth becomes problematic when the use of antibiotics will also deter the growth of the sensitive transgenic tissues (Cao *et al*. 2006). Adjusting the co-cultivation conditions into an environment that will reduce bacterial population is therefore an important consideration for increasing the survival of transformed tissues. It has been reported that a certain degree of dryness in the environment during co-cultivation with filter paper wicks

hampered the growth of *Agrobacterium* in rice and Japanese cedar without necessarily compromising transformation efficiency (Konagaya *et al*. 2013). Meanwhile, the surface sterilization procedures applied in infiltrated petals prior to aseptic culture should have also removed bacterial solution residues. With these conditions, bacterial populations on the surface of the petal tissues were reduced prior to antibiotic exposure facilitating efficient bacterial elimination, thereby allowing the efficient regeneration of agroinfiltrated petal tissues. Regeneration of agroinfiltrated leaves of *Nicotiana benthamiana* (Nekrasov *et al*. 2013) and *Vitis vinifera* (Zottini *et al*. 2008) demonstrated the potential of converting transiently transformed tissues into stable transformation lines. Thus, by combining the advantages of a transient transformation system previously developed (Pinthong *et al*. 2014, Ratanasut *et al*. 2015) and the new tissue culture procedure using petal tissues reported here, the researchers have established an efficient protocol for eliminating *Agrobacterium* and a promising method of turning transient transformants to stable transformation for orchids.

CONCLUSION AND RECOMMENDATION

This study has successfully demonstrated a potential organogenic pathway from petal tissues of *D.* Sonia 'Earsakul' and *in vitro* culture of transiently transformed petal tissues as a novel transformation procedure. *In vivo* co-cultivation of agroinfiltrated petals significantly contributes to the reduction of bacterial overgrowth prior to aseptic culture, resulting in the efficient suppression of *Agrobacterium* using meropenem. Furthermore, as revealed by GUS expression analysis, the resulting organogenic tissues were putative to contain stable transformants. Given enough time and suitable growing environment, these transformed tissues can proceed to the formation of a complete organ or plants. Although the researchers have successfully developed a strategy for combining transient transformation technique and petal tissue culture for possible generation of stably organogenic transformed tissues from transient transformants in *Dendrobium* and other orchids, further studies are still being conducted to optimize the growing conditions of transiently transformed petal *in vitro.*

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