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Direct Somatic Embryogenesis and Regeneration of an Indonesian orchid *Phalaenopsis amabilis* (L.) Blume under a Variety of Plant Growth Regulators, Light Regime, and Organic Substances

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Abstract

Phalaenopsis amabilis is an Indonesian native orchid often used as parent to produce various orchid hybrids. However, this natural orchid is increasingly difficult to find growing naturally in the forest due to over-harvesting and destruction of its natural habitat. The objectives of this study were to investigate the effect of plant growth regulators (PGRs), light regime, and organic substances on the induction and regeneration of somatic embryos (SEs) of *P. amabilis* orchid. Root, stem, leaf, and protocorm explants were cultured on New Phalaenopsis (NP) medium supplemented with thidiazuron (TDZ) (0.0, 1.0, 2.0, 3.0 mg L^{-1}) in combination with α-napthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and indole acetic acid (IAA) with concentrations of 1.0, 2.0, and 3.0 mg L^{-1}. Light and dark conditions were tested for their effectiveness to induce the formation of SEs, and the resulting SEs were cultured on NP medium supplemented with various organic substances (banana, bean sprout, tomato, and potato extracts) with concentrations of 50, 100, 150, 200, and 250 g L^{-1}. Results showed that the highest number of SEs (36.45 ±0.26 embryos) was found in stem explants cultured in NP medium supplemented with 3.0 mg L^{-1} TDZ and 1 mg L^{-1} NAA. When explants were cultured in dark conditions, the number of SEs significantly increased with the highest number of SEs achieved in stem explants culture in 3.0 mg L^{-1} TDZ and 1.0 mg L^{-1} NAA. NP medium supplemented with 150 g L^{-1} tomato extract was the most effective medium for growth of SEs-derived plants. Seedlings of this treatment produced an average of 4.20 ±0.17 leaves and 3.20 ±0.11 roots after 12 weeks of culture. In conclusion, SEs can be produced effectively from stem explants with a combination of 3.0 mg L^{-1} TDZ and 1.0 mg L^{-1} NAA, one month early in dark conditions, and regenerated on NP medium with addition of tomato extract.

Keywords Organic substances, plant growth regulators, *Phalaenopsis amabilis*, somatic embryogenesis

1. Introduction

Orchidaceae is one of the largest and diverse families of flowering plants. About 5000 of 20,000 species of orchid live naturally and distributed throughout Indonesia (Schuiteman, 2010). *P. amabilis* is one of the most important orchids in Indonesia. This orchid has preeminent flowering characteristics with beautiful flower shape and graceful inflorescence; it also has been used extensively in the breeding program as parent plant to create new superior hybrids (Semiarti *et al*., 2010). However, the availability of this orchid is hindered due to illegal trade and deforestation; hence, other means of propagation would be required such as tissue culture technique.

Somatic embryogenesis is a powerful system for plant mass propagation through tissue culture technique and has been extensively used for orchid conservation (Bhattacharyya *et al*., 2016; Moradi *et al*., 2017). In plant tissue culture systems, the addition of plant growth regulators (PGRs) into the culture medium is the most preferred way to induce somatic embryogenesis (Borpuzari and Borthakur, 2016; Méndez-Hernández *et al*., 2019). Usually, the combination of auxin and cytokinin is the most utilized PGRs during the initiation of somatic embryos (SEs) in orchid plants (Shen *et al*., 2018; Soonthornkalump *et al*., 2019; Zanello and Cardoso, 2019). Previously, we successfully developed an efficient protocol to induce SEs formation using thidiazuron (TDZ) (Mose *et al*., 2017).

Structurally, TDZ is different from natural purine-based cytokinins which have a typical 5-carbon side chains (Tarkowski *et al*., 2009). However, TDZ's action in development is much closed to cytokinin metabolism and associated with isopentenyl adenine that lead to rapid cell division and initiation of organogenesis (Guo *et al*., 2011). TDZ is widely used in plant somatic embryogenesis, either alone or conjugated with other PGRs (Hong *et al*., 2010; Guo *et al*., 2011). Jainol and Gansau (2017) reported that combination of TDZ and α-napthalene acetic acid (NAA) successfully induced high number of SEs from leaf tip explants of *Dimorphorchis lowii* orchid. Moreover, Moradi

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et al. (2017) reported that combination of TDZ and 2,4-D induced SEs formation from single node, crown, apical bud, and protocorm explants of *Epipactis veratrifolia* orchid.

It has been found that incubation of explants with NAA or 2,4-D during somatic embryogenesis induction produces an increase of endogenous auxin in some species (Pasternak *et al.*, 2002; Vondráková *et al.*, 2011). Ayil-Gutiérrez *et al.* (2013) reported that the addition of NAA in the culture medium during somatic embryogenesis induction increased the free IAA and IAA amide conjugates levels in *Coffea canephora* explants, where part of the increase of the auxin content is due to de novo synthesis. Ceccarellil *et al.* (2000) reported that 2,4-D induced tryptophan-dependent synthesis of IAA in *Daucus carota* suspension culture during the induction of SEs. Furthermore, considerable efforts have been made to identify light conditions that are needed to optimize the protocol for somatic embryogenesis induction in plants (Baharan *et al.*, 2015). Light is one of the crucial factors affecting plant tissue culture (Hew and Yong, 2004). The intensity and condition of light has been reported to affect somatic embryogenesis induction in *Cattleya* and *Oncidium* orchids (Cueva-Agila *et al.*, 2016; Sampaio *et al.*, 2010). In *Phalaenopsis* orchid, low intensity of light is known to accelerate *in vitro* shoot formation (Tanaka *et al.*, 1988).

Various kinds of organic substances have also been used in large-scale for orchid tissue culture including banana pulp, potato extract, coconut water, corn extract, and beef extract (Nambiar *et al.*, 2012). Yong *et al.* (2009) reported that certain organic substances contain growth factors such as cytokinin and auxin which were found to have potential for promoting growth of tissue cultured plants. *Trans*-zeatin riboside (ZR) and *trans*-zeatin (Z) which are cytokinins contained in banana pulp (Ge *et al.*, 2008), auxin and gibberellin in tomato extract (Shuiying *et al.*, 2016) and bean sprout extract (Sanjaya *et al.*, 2019), and cytokinin in potato extract (Anstis and Northcote, 1975; Lomin *et al.*, 2018) are very beneficial for balancing nutrient availability in the culture medium.

Organic substances promote growth of orchid seeds, increase the size of protocorm-like bodies (PLBs), and help regeneration of plantlets (Abbaszadeh *et al.*, 2018; Chew *et al.*, 2018; Dulić *et al.*, 2018). It was reported in Abbaszadeh *et al.* (2018) that organic substances contained not only PGRs, but also vitamins, inorganic ions, amino acids, and sugars. Hence, the present investigation was to know the effect of PGRs and light regime on the induction of SEs, and the effect of organic substances on the regeneration of SEs in *P. amabilis* orchid.

2. Materials and Methods

2.1. Plant materials, growth conditions, and culture medium

Green siliques were collected from *P. amabilis* potted plants following 120 days of self-pollination. The siliques were dipped in 70% ethanol, passed over a Bunsen gas burner fire and waited until the fire went out. This process was repeated three times. After being sterilized and cut open the seeds were taken out and sown on New Phalaenopsis (NP) solid medium (Islam *et al.*, 1998; Semiarti *et al.*, 2010). Cultures were maintained at a temperature of 25 ±1°C with 14 µmol m^{-2} s^{-1} intensity of continuous light.

2.2. PGRs treatment to induce SEs formation

To evaluate the effect of PGRs on SEs formation, we performed a test with 144 combinations of four kinds of explants (protocorm, leaf, stem, and root), three different kinds of auxins (NAA, 2,4-D, and IAA), and 12 combinations of TDZ concentrations (0.0, 1.0, 2.0, 3.0 mg L^{-1}) and NAA or 2,4-D or IAA concentrations (1.0, 2.0, 3.0 mg L^{-1}).

Roots, stems, and leaves of 6-month-old *in vitro* plantlets and 4-week-old protocorms were used as explants (Figure 4a, f, k, and p). Explants were cut transversely (± 0.5 cm) and planted on NP solid medium supplemented with the combination of PGRs. Cultures were maintained at a temperature of 25 ±1°C in dark conditions for the first 14 days of culture and then transferred to 16 h light conditions. Subcultures were conducted every two weeks and observed every day using dissecting microscope (Eschenbach, Germany). Photographs were taken once a week for eight weeks using digital camera (Canon Power Shot A2400, Japan).

2.3. Light treatment for SEs formation

In order to determine the role of light and dark conditions in the formation of SEs in *P. amabilis* orchid, we selected the best combination of TDZ and NAA, or 2,4-D, or IAA in all types of explants and repeated the assays culturing the explants in the dark or in 16 h light photoperiod for the first month of culture. After one month, cultures in dark conditions were transferred to 16 h light conditions. Cultures were maintained with a temperature of 25 ±1°C. Observations were conducted every day using dissecting microscope and photographed once a week for eight weeks using digital camera.

2.4. Histological analysis of SEs development

Histological sections of SEs were prepared using paraffin method according to Ruzin (1999). The sections were examined under light microscope (Olympus, Japan) and photographed using Optilab Microscope Camera (Miconos, Indonesia).

2.5. Additional organic substances in the culture media for plant regeneration

Four different organic substances (banana, bean sprout, tomato, and potato extracts) with five different concentrations (50, 100, 150, 200, 250 g L^{-1}) were evaluated for SEs regeneration. Banana (*Musa paradisiaca* var. *sapientum*), bean sprout from mung bean (*Vigna radiata*), tomato (*Lycopersicon esculentum*) cultivar 'Arthaloka', and potato (*Solanum tuberosum*) cultivar 'Granola' was obtained from local markets. Banana, tomato, and potato were separately prepared by cutting them into 1 cm^3 cubes, while bean sprout was cut into two parts. Each material was homogenized, and homogenate was filtered through a steel mesh with a 150 µm pore size. Each organic substance was added into the media prior to autoclaving. Mature SEs produced from the best combination of PGRs in all types of explants (experiment 2.2) that have formed leaf primordia were planted on NP solid medium supplemented with organic substances. NP basal medium without addition of organic substances was

used as control. Cultures were maintained at a temperature of 25 ±1°C with 14 µmol m^{-2} s^{-1} intensity of continuous light. Observations were conducted using dissecting microscope and photographed once a week for 12 weeks using a digital camera.

2.6. Experimental design and data analysis

In the experiments, each explant and plant were referred to as one replicate. Twenty replicates for SEs induction and regeneration were used in each experiment. For SEs induction, explants were cultured in Petri dishes (∅100 mm x 15 mm). Each Petri dish contained five explants. For regeneration of SEs, 100 mL culture flasks were used. Each flask contained 3 – 4 plantlets. Data were subjected to analysis of variance (ANOVA) and comparisons between the mean values of treatment made by the Duncan's Multiple Range Test calculated at the confidence level of $p \leq 0.05$. The statistical package SPSS (Version-22) was used for the analysis.

3. Results

3.1. Effect of PGRs on direct SEs formation

The results showed that among the treatments, the highest number of SEs (36.45 ±0.26) was observed in stem explants cultured on NP medium supplemented with 3 mg L^{-1} TDZ and 1.0 mg L^{-1} NAA (Figure 1). Furthermore, we found that the combination of TDZ and 2,4-D highly inhibited SEs formation in all types of explants. SEs failed to form in root explants cultured at all concentrations of 2,4-D alone with no TDZ (Figure 2). In addition, root and leaf explants cultured on NP medium supplemented with 1.0 mg L^{-1} TDZ and 3.0 mg L^{-1} 2,4-D, and root explants cultured on NP medium supplemented with 1.0 mg L^{-1} TDZ and 2.0 mg L^{-1} 2,4-D did not form any embryos. In combination of TDZ and IAA, the highest number of SEs was found in stem explants cultured on NP medium supplemented with 3.0 mg L^{-1} TDZ and 1.0 mg L^{-1} IAA, resulted in 25.20 ±1.96 embryos per explant (Figure 3).

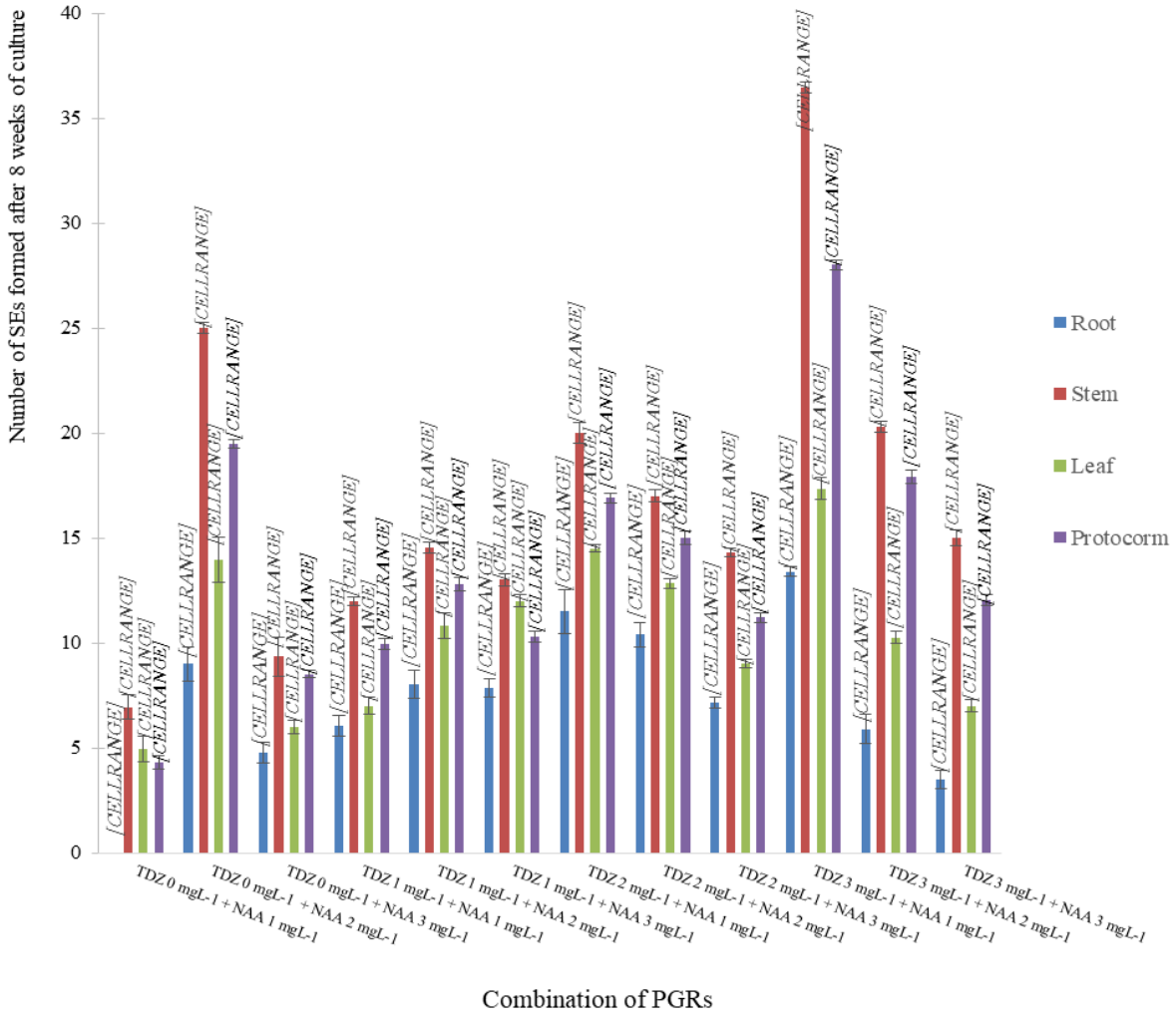

Figure 1. Effects of combination of TDZ and NAA on the formation of SEs from various types of explants in *Phalaenopsis amabilis*. Data in the bars followed by the same letters are not significantly different by Duncan's multiple range test at $p \leq 0.05$.

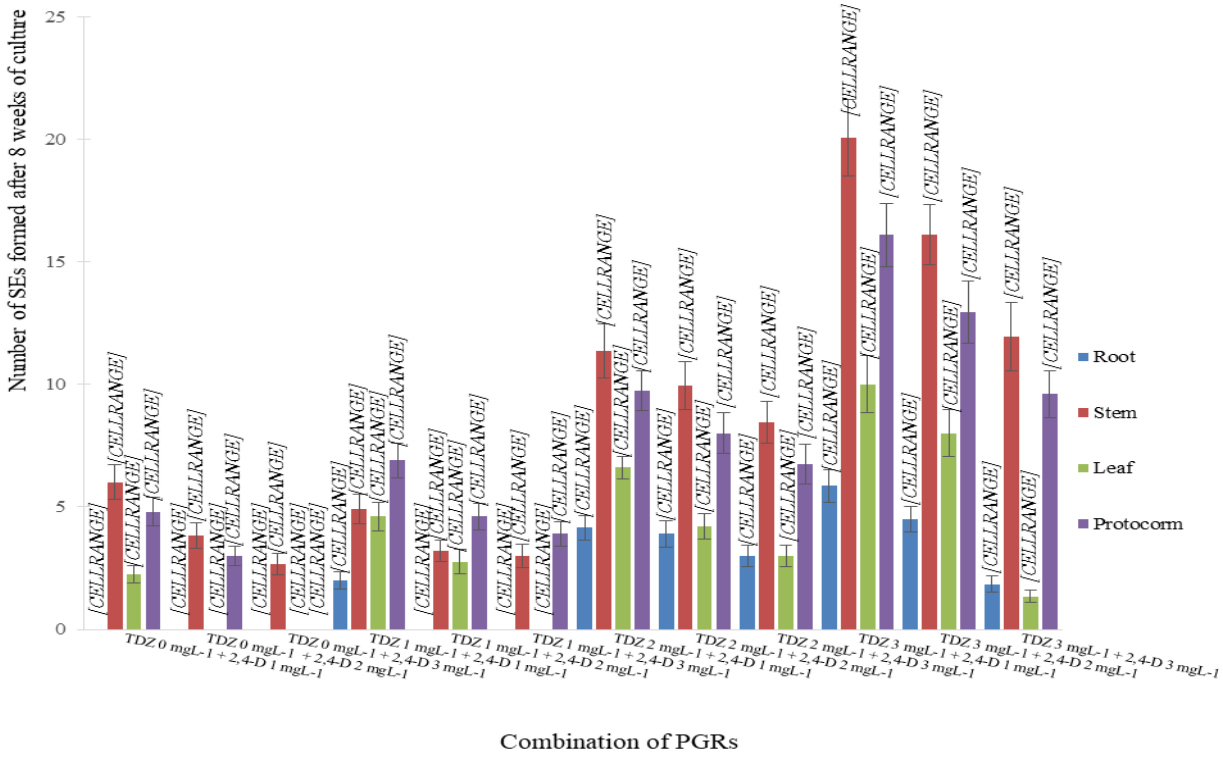

Figure 2. Effects of combination of TDZ and 2,4-D on the formation of SEs from various types of explants in *Phalaenopsis amabilis*. Data in the bars followed by the same letters are not significantly different by Duncan's multiple range test at $p \leq 0.05$.

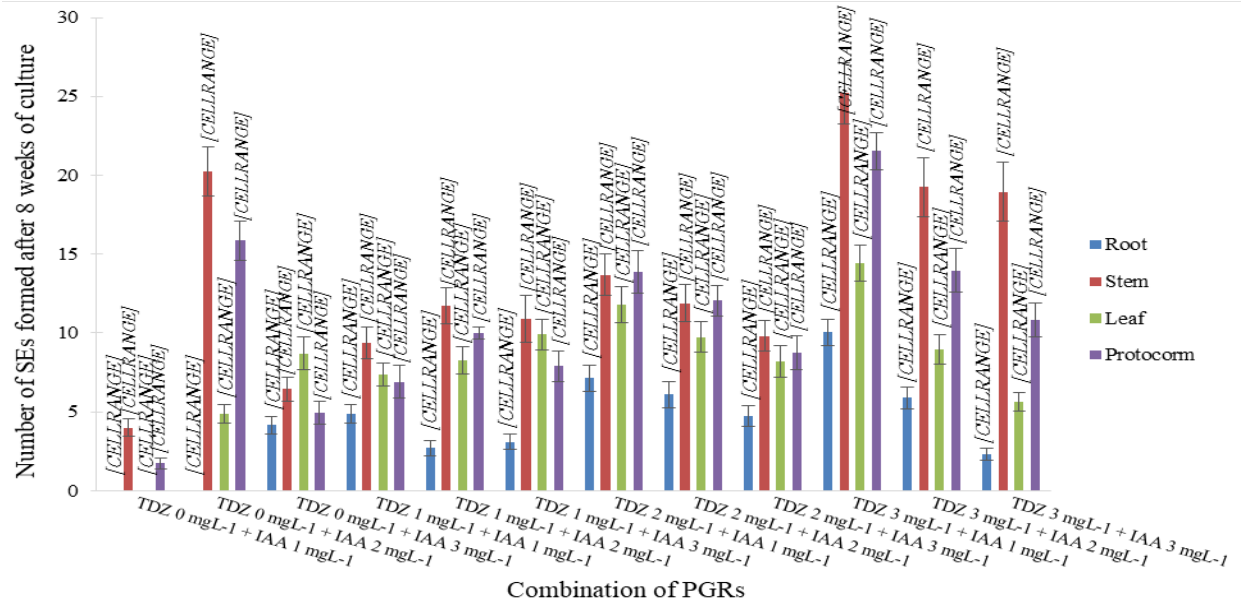

Figure 3. Effects of combination of TDZ and IAA on the formation of SEs from various types of explants in Phalaenopsis amabilis. Data in the bars followed by the same letters are not significantly different by Duncan's multiple range test at $p \leq 0.05$.

Histological analysis showed that pro-embryos consisted of small, thick-walled cells, and clearly distinguishable from the surrounding cells by the dense cytoplasm and conspicuous nucleus (Figure 5a). Pro-embryos enlarged and subsequently formed globular embryos with suspensor at the basal region (Figure 5b-c). Globular embryos developed into scutellar embryos with notch at the apical region of embryo (Figure 5d). Shoot apical meristem (SAM) eventually formed from the notch, surrounded by two leaf primordia, coleoptile, and procambium in the middle part of embryo (Figure 5e), marked the development of a mature embryo.

The present results showed that direct somatic embryogenesis was achieved in all treatments. After first subculture, pro-embryos emerged from the wounding site of protocorm and stem explants (Figure 4h and r), and from the tissue near the cut side of root and leaf explants (Figure 4c and m). These embryos further developed, progressively enlarged, became globular and eventually formed a distinctive feature on the apical region (Figure 4d, i, n, s) before forming leaf primordia (Figure 4e, j, o, and t).

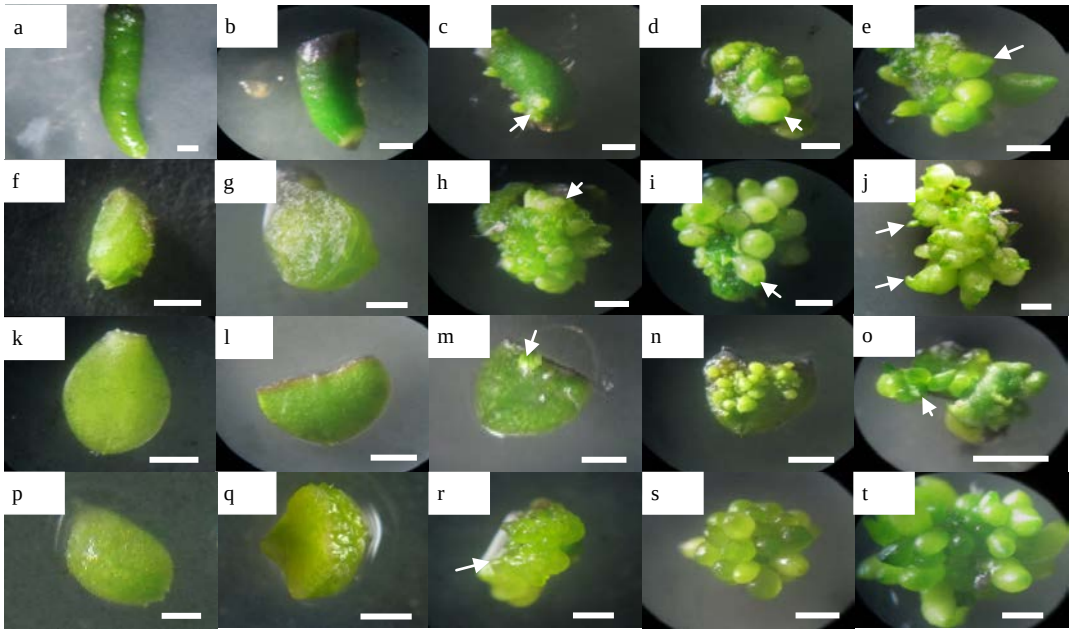

Figure 4. Developmental stages of SEs of *Phalaenopsis amabilis* from various explants. (a-e) Root (f-j) stem (k-o) leaf and (p-t) protocorm explants, (b, g, l, q) Section of explants were cultured in the treatment medium, Pro-embryos appeared from the wounding area of stem (h) and protocorm (r) explants, and near the cut side of root (c) and leaf (m) explants, and subsequently formed globular structure (arrows), (d, i, n, s) Embryos formed a distinctive feature on the apical region (arrows), (e, j, o, and t) Embryos with leaf primordia (arrows). Scale bars (a, f, k, p – t): 100 µM, Scale bars (b – e, g – j, l – o): 200 µM.

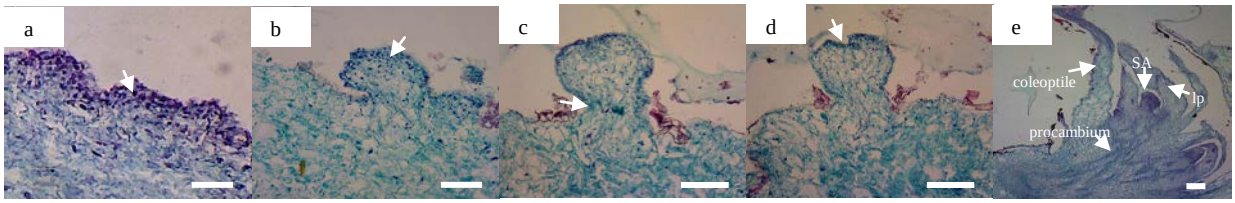

Figure 5. Anatomy of SEs development in *Phalaenopsis amabilis*. (a) Pro-embryos consisted of cells with large and densely-stained nuclei (arrow); (b) Pro-embryo developed into globular embryo (arrow) and formed suspensor (arrow) at the basal region (c); (d) Scutellar embryo with notch at the apical region (arrow); (e) Mature embryo consisted of SAM, leaf primordia (lp), coleoptile, and procambium. Scale bars: 200 µm.

3.2. Effect of light regime during SEs formation

When explants were cultured in dark conditions for the first month of culture, higher number of SEs were obtained (Figure 6). The highest number of SEs was found in stem explants cultured on NP medium supplemented with 3 mg L-1 TDZ and 1 mg L-1 NAA, which gives values of 40.12 ±0.31 embryos per explant. Meanwhile, light conditions highly retarded SEs formation in all explants. Explants formed whitish to yellow-green SEs in darkness (Figure 7a), and turned green after one week transferred to light conditions (Figure 7b). On the other hand, explants easily become browned and eventually necrotic in light conditions, and the SEs formation rate was reduced (Figure 7c-d). In both treatments, stem explants had the best embryogenic response than other types of explants.

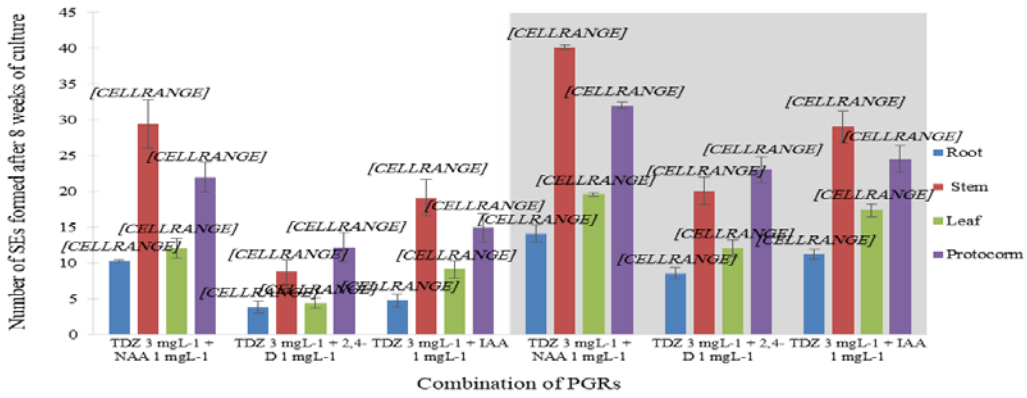

Figure 6. Effects of light regime on the formation of SEs from various types of explants in *Phalaenopsis amabilis*. Data in the bars followed by the same letters are not significantly different by Duncan's multiple range test at $p \leq 0.05$.

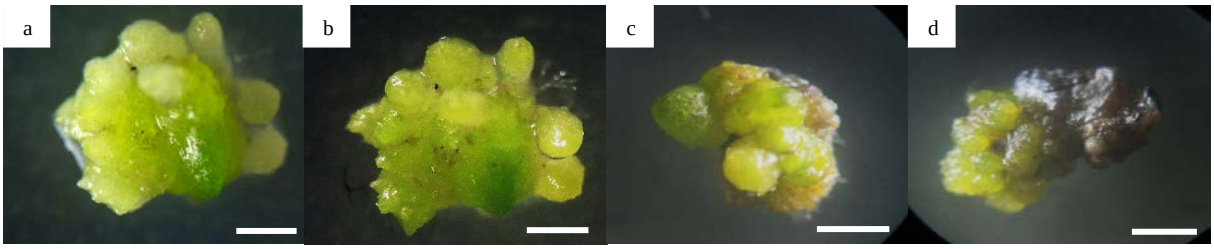

Figure 7. Growth of SEs after cultured in dark and light conditions for one month. SEs from stem explant cultured in dark conditions appeared whitish to yellow-green in color (a) and turned green after one week transferred to light conditions (b); Explant cultured in ligh conditions turned into brown and the number of embryos decreased (c-d). Scale bars: 500 µm.

3.3. Effect of additional various organic substances on regeneration of SEs

SEs of *P. amabilis* responded variably when cultured on different organic substances. The seedling development process began with the formation of leaf primordia at the apical region of SEs (Figure 8a). First leaf initiated after 2 weeks of subculture (Figure 8b), followed by a formation of second leaf and first root one month later (Figure 8c). The formation of second root started after 8 weeks, and it was followed by the elongation of roots (Figure 8d). After 12 weeks of culture, a seedling could produce up to two leaves and two roots (Figure 8e).

Figure 8. Growth of SEs into plantlets on NP basal medium. (a) Eight-week-old mature SEs; (b) Leaf primordia fully grown into leaves after 2 weeks of subculture; (c) After 4 weeks, the second leaf and root has formed; (d) Plantlet after 8 weeks and (e) 12 weeks of culture. Scale bars: 0.5 cm.

The effects of organic substances on the regeneration of SEs into seedlings are shown in Table 1 and Figure 9. The results revealed that the regeneration of SEs had significantly enhanced by the addition of organic substances. Among the different organic substances, tomato extract was found to be the most effective. It was observed that SEs treated in NP medium containing 150 g L^{-1} tomato extract successfully produced 4.20 ±0.17 leaves after 12 weeks of culture with an average length of leaves at 25.21 ±0.31 mm, and the average width of leaves at 8.06 ±0.05 mm per responsive explant. Moreover, the addition of 150 mg L^{-1} of tomato extract also promoted the highest number of roots (3.20 ±0.11 roots) with an average length of roots at 25.03 ±0.19 mm.

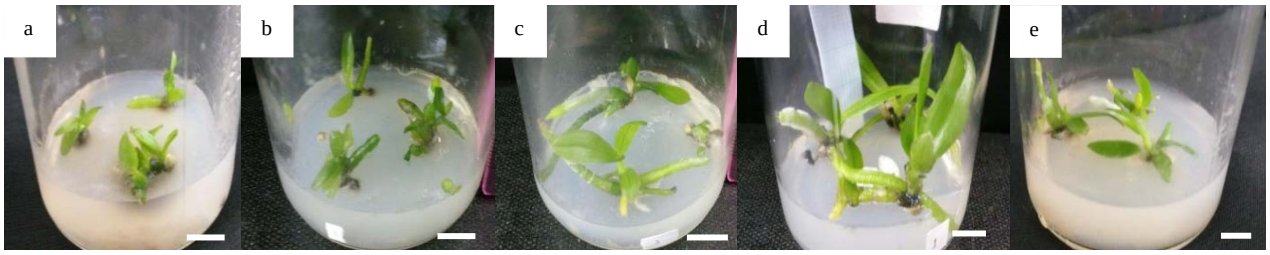

Figure 9. SE-derived plantlets after 12 weeks of culture on NP media supplemented with different organic substances. (a) Control; (b) Banana extract; (c) Bean sprout extract; (d) Tomato extract; (e) Potato extract. Scale bars: 1 cm.

Table 1. Effect of additional organic substances on regeneration of SEs after 12 weeks of culture

Organic substances (g L^{-1})	Number of leaves	Leaf length (mm ±SE)	Leaf width (mm ±SE)	Number of roots	Root length (mm ±SE)
Control	2.00 ±0.14^{a}	5.73 ±0.09^{a}	3.71 ±0.07^{a}	1.70 ±0.12^{a}	8.33 ±0.49^{a}
Banana					
50	2.15 ±0.16^{abc}	6.31 ±0.22^{b}	4.08 ±0.08^{bc}	1.90 ±0.12^{ab}	10.61 ±0.92^{b}
100	2.40 ±0.22^{abcd}	10.88 ±0.16^{e}	4.88 ±0.07^{de}	2.00 ±0.12^{abcd}	13.28 ±0.50^{d}
150	2.70 ±0.23^{cdef}	13.38 ±0.12^{g}	5.91 ±0.14^{hi}	2.40 ±0.11^{defghi}	16.29 ±0.78^{hi}
200	3.40 ±0.19^{gh}	11.26 ±0.18^{ef}	5.69 ±0.09^{gh}	2.35 ±0.13^{cdefgh}	14.56 ±0.50^{ef}
250	3.10 ±0.24^{fgh}	9.06 ±0.23^{d}	5.28 ±0.07^{f}	2.20 ±0.13^{bcdef}	11.03 ±0.11^{bc}
Bean sprout					
50	2.10 ±0.13^{ab}	11.00 ±0.11^{e}	3.92 ±0.03^{b}	2.10 ±0.14^{abcde}	14.34 ±0.85^{e}
100	2.30 ±0.17^{abcd}	14.57 ±0.11^{h}	4.21 ±0.05^{c}	2.25 ±0.14^{bcdefg}	17.51 ±0.62^{j}
150	3.15 ±0.19^{fgh}	19.04 ±0.18^{k}	4.86 ±0.06^{de}	2.50 ±0.13^{efghi}	20.39 ±0.13^{m}
200	3.55 ±0.17^{h}	17.35 ±0.17^{j}	5.33 ±0.05^{f}	2.70 ±0.10^{hi}	16.62 ±0.26^{i}
250	3.35 ±0.24^{gh}	15.81 ±0.10^{i}	5.00 ±0.05^{e}	2.30 ±0.14^{bcdefgh}	15.24 ±0.12^{fg}
Tomato					
50	3.00 ±0.17^{efgh}	17.80 ±0.25^{j}	6.68 ±0.03^{j}	2.10 ±0.12^{abcde}	19.69 ±0.18^{l}
100	3.55 ±0.22^{h}	21.00 ±0.32^{l}	7.44 ±0.06^{l}	2.50 ±0.11^{efghi}	22.00 ±1.02^{o}
150	4.20 ±0.17^{i}	25.21 ±0.31^{n}	8.06 ±0.05^{n}	3.20 ±0.11^{j}	25.03 ±0.19^{p}
200	3.60 ±0.11^{h}	23.09 ±0.26^{m}	7.64 ±0.02^{m}	2.80 ±0.13^{i}	21.26 ±0.13^{n}
250	3.45 ±0.18^{h}	18.57 ±0.15^{k}	7.06 ±0.07^{k}	2.70 ±0.12^{hi}	18.06 ±0.99^{jk}
Potato					
50	2.60 ±0.16^{bcdef}	7.36 ±0.14^{c}	4.69 ±0.05^{d}	1.95 ±0.15^{abc}	12.70 ±0.60^{d}
100	2.80 ±0.17^{defg}	11.61 ±0.15^{f}	5.04 ±0.04^{e}	2.40 ±0.13^{defghi}	16.58 ±0.40^{i}
150	3.20 ±0.15^{fgh}	15.31 ±0.13^{i}	5.66 ±0.04^{g}	2.65 ±0.13^{ghi}	18.68 ±0.44^{k}
200	3.00 ±0.19^{efgh}	17.32 ±0.18^{j}	6.01 ±0.05^{i}	2.55 ±0.11^{fghi}	15.66 ±0.65^{gh}
250	2.45 ±0.15^{abcde}	14.69 ±0.18^{h}	5.80 ±0.04^{gh}	2.35 ±0.15^{cdefgh}	11.60 ±0.58^{c}

Note: Data in the same column followed by the same letters are not significantly different by Duncan's multiple range test at $p \leq 0.05$.

4. Discussion

4.1. Effect of PGRs on SEs formation

PGRs are key in impacting somatic embryogenesis (Mujib *et al.*, 2016). In most investigated cases, SEs induction initially requires auxin and cytokinin added together in the culture medium. In orchids, various classes of auxins have been widely employed and reported in a number of species like *Cymbidium* Twilight Moon 'Day Light' (Teixeira da Silva and Tanaka, 2006), *Malaxis densifolia* (Mahendran and Bai, 2016), *E. veratrifolia* (Moradi *et al*., 2017), *P. aphrodite* (Gow *et al*., 2018), and *Paphiopedilum niveum* (Soonthornkalump *et al*., 2019). In this study, TDZ in combinations with various auxins (NAA, 2,4-D, IAA) were incorporated in NP medium to evaluate their effects on SEs formation in various types of explants of *P. amabilis* orchid. Among the combination of PGRs, 3.0 mg L^{-1} TDZ and 1.0 mg L^{-1} NAA produced the highest number of SEs from stem explants. It is worth noting that Khoddamzadeh *et al.* (2011) reported a high level of direct SEs induction (72%) when *P. bellina* leaf tissues were cultured on the same medium. Nevertheless, Zanello and Cardoso (2019) reported low SEs formation when using lower concentration of TDZ and high concentration of NAA (0.125 mg L^{-1} TDZ and 1.0 mg L^{-1} NAA) on leaf tissues of *Phalaenopsis* hybrid 'RP3' and 'Ph908'.

In other reports, combination of 2,4-D with TDZ at appropriate concentrations was effective for SEs induction in *Oncidium* 'Gower Ramsey' (Chen and Chang, 2000), *Cattleya maxima* (Cueva Agila *et al*., 2013), and *E. veratrifolia* (Moradi *et al*., 2017). On the contrary, we found that the use of 2,4-D and TDZ highly retarded the formation of SEs in *P. amabilis* orchid. In accordance with our results, Shen *et al.* (2018) also reported the inhibitory effect of 2,4-D and TDZ on SEs formation in *Tolumnia* Louise Elmore 'Elsa' orchid. No globular embryos were found at this combination that resulted in high percentage of browning (100%). Yam and Arditti (2017) stated that the effects of the same auxin may differ in respect to one species and may not be the same with another orchid. In line with this, Naranjo *et al*. (2016) reported that different genetic makeup plays a role in tissue culture responsiveness. Moreover, we also anticipate the difference between the basal medium in each research. Oyamada and Takano (1985) found that the response of

SEs to certain PGRs depended on the particular chemical level in the basal solution.

Apart from the kind of PGRs, explant types also affect the formation of SEs in orchid (Chugh *et al*., 2009). Leaves are the most common target tissue used for SEs induction in orchid (Jainol and Gansau, 2017). Different protocols have been developed for a number of orchid species through somatic embryogenesis using leaf explants (De Conti *et al*. 2018; Shen *et al*., 2018, Zanello and Cardoso, 2019). However, orchid leaves are known to be recalcitrant to regeneration and possessed high oxidation rate (Temjensangba and Deb, 2005; Kaur and Bhutani, 2009). Recently, SEs were regenerated from seed-derived protocorm and stem explants (Mose *et al*., 2017; Soonthornkalump *et al*., 2019). Here, we found that stem explants successfully produced high number of SEs in all PGRs combinations. Consistent with our results, Kanjilal *et al*. (1999) also reported that SEs were effectively induced from stem disc culture of *Dendrobium moschatum* orchid.

4.2. Effect of light regime during SEs formation

Light is known to affect induction and maturation of somatic embryogenesis (Meneses *et al*., 2005; Chung *et al*., 2007). In some orchid plants, somatic embryogenesis induction is more effective in the absence of light (Gow *et al*., 2009; Sampaio *et al*., 2010). Cueva-Agila *et al*. (2016) reported that light inhibits the formation of SEs in leaf explants of *Cattleya maxima* orchid. In the present research, it was perceived that explants cultured in light conditions for the first 30 days of culture produced lower number of SEs compared to explants cultured in dark conditions. In addition, Gow *et al*. (2009) reported that leaf explants of *P. amabilis* Shimadzu var. *formosa* and *P. nebula* showed high intensity of browning after 60 days of culture in light conditions. Explant browning is greatly affected by light (Patel *et al*., 2018). Light induced the production and accumulation of phenolic compounds (von Aderkas *et al*., 2015). North *et al*. (2010) reported that oxidation of phenolic compounds produced quinones that are toxic and causing tissue necrosis and death of explant. In studies on leaf explants of date palm plants (*Phoenix dactylifera*), light stimulated secretion of phenolic compound and inhibited callus formation (Baharan *et al*., 2015).

4.3. Effect of additional organic substances on regeneration of SEs

Regeneration response of SEs into plantlets are generally slow in many orchid genera including *Phalaenopsis* (Yam and Arditti, 2017). Hence, the media and its formulation are important to maximize orchid's vigour in the tissue culture conditions (Gnasekaran *et al*., 2010). Chew *et al*. (2018) reported that addition of organic substances in the culture medium increased the percentage of SEs regeneration response. Besides containing hormones, proteins and minerals, organic substances have also become natural carbon source that is needed for orchid development (Nambiar *et al*., 2012). Carbon sources of culture medium supplement the low CO_2 concentration and light energy deficiency to the plants under *in vitro* conditions (Sarmah *et al*., 2017). Organic substances such as banana and tomato contain carbon source in the form of simple or complex sugars (Gnasekaran *et al*., 2010). Sugars enter into the metabolic pathways and breaking down of sugar produces the required energy for *in vitro* orchid germination, growth of cells, buds, and even plantlets growth (Zahara *et al*., 2017).

In this study, medium supplemented with 150 g L^{-1} of tomato extract gave the highest plantlet growth rate. Our result is also in agreement with a study on the growth enhancement effect of tomato extract in *Dendrobium* hybrids (Hapsoro *et al*., 2018), *Vanda helvola* (David *et al*., 2015), and *Vanda* Kasem's Delight (Gnasekaran *et al*., 2012). Furthermore, Gnasekaran *et al*. (2010) reported that tomato extract serves to promote cell division through cytokinin and increased the percentage of *Phalaenopsis violacea* PLBs proliferation rate.

According to Semiarti *et al*. (2010), tomato (*L. esculentum* 'Arthaloka') extract contained 3.70% total sugars, 1.78% total proteins, 1.05% crude fibre, 1.84% total carotene, 0.042% vitamin C, 0.024% antioxidants (DPPH: 1,1-diphenyl-2-picrylhydrazyl), and other components. Gnasekaran *et al*. (2012) reported that the presence of antioxidants in the culture medium successfully prevented browning of PLBs of *Vanda* Kasem's Delight orchid. Moreover, Cunningham *et al*. (1996) reported that carotenoids with cyclic end-groups were essential com6ponents of photosynthetic membranes and play a role in preventing photo-oxidation. Apart from carotenoid and DPPH, tomato also contains lycopene. Lycopene served as strong antioxidant which inhibits free radical formation, assists wound cells repairing process, and inhibits DNA oxidation (Halliwel, 1996). The presence of these antioxidants successfully prevented browning of culture and maximized the growth of SEs into plantlets.

5. Conclusion

Direct somatic embryogenesis in *P. amabilis* orchid can be effectively induced from various explants by using a combination of auxin and cytokinin, with the best combination being 3.0 mg L^{-1} TDZ and 1.0 mg L^{-1} NAA. Stem of *in vitro* seedlings was the best explant to be used to induce direct somatic embryogenesis in *P. amabilis*. Dark condition was needed for SEs formation, and addition of tomato extract has effectively increased the regeneration rate of SEs. These findings provided efficient protocols for commercial propagation and conservation of *P. amabilis* orchids.

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