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Original Research Article

The Influence of Thidiazuron on Direct Somatic Embryo Formation from Various Types of Explant in *Phalaenopsis amabilis* (L.) Blume Orchid

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ABSTRACT

Phalaenopsis amabilis is an important national flower of Indonesia as a parent for orchid breeding, so that needs a good strategy to produce high number of plants. The objective of this research is to analyze the use of thidiazuron (TDZ) for producing high number of plantlets, through directly induction of somatic embryos (SEs) from various explants. The method was used 20 each of protocorms, leaves, stems and roots as explants. The explants were dissected transversely, then put on various culture media: New Phalaenopsis (NP) and NP + (1, 2, 3) mgL^{-1} TDZ. Cultures were maintained at 25°C with continous white light. The formation of SEs was observed every week for 8 weeks. The results showed that SEs formation increased inline with the addition of TDZ concentration to the NP medium, for both velocity and amount of SEs formation. In NP0, SEs were formed at (26.07 ± 0.73) days after inoculation of protocorm, whereas on NP + (1, 2, and 3 mgL^{-1}) TDZ, SEs were formed at (17.85 ± 0.67) days, (15 ± 0.64) days, and (11 ± 0.64) days, respectively. All types of explants formed SEs on NP + TDZ (1–3 mgL^{-1}), whereas only 14 of 20 protocorms produced SEs (70%), and 8 of 20 stems formed SEs (40%) in NP0. In roots, SEs was formed on NP + 2 mgL^{-1} TDZ and NP + 3 mgL^{-1} TDZ. For stems, the highest amount of SEs (28.25 ± 1.07) was reached on NP + 3 mgL^{-1} TDZ, followed by protocorm (23.30 ± 1.13) SEs and roots (8.25 ± 0.68) SEs. In contrast, in NP0, the amount of SEs was very low (1.25 ± 0.46) from stem and (1.50 ± 0.65) from protocorms, there was no evidence of SEs formation in the leaves and roots.

1. Introduction

Somatic embryogenesis is defined as a process in which a bipolar structure, resembling a zygotic embryo, develops from a non-zygotic cell without vascular connection with the original tissue (Arnold et al. 2002). In that process, somatic cell differentiate into a plant without the involvement of fertilization or gamete fusion (Smertenko and Bozhkov, 2014). Somatic embryogenesis has emerged as a powerfull tool for studying plant development because somatic embryos (SEs) resemble zygotic embryos and undergo almost the same developmental stages (Elhiti et al. 2013). Therefore, somatic embryogenesis has been considered to be a suitable system for plant mass propagation and for regeneration of transgenic plants (Bhattacharyya et al. 2016).

In recent years, somatic embryogenesis protocols have been successfully developed in several orchids, including *Dendrobium* (Bhattacharyya et al. 2016; Kaewubon and Meesawat, 2016), *Cymbidium* (da Silva and Winarto, 2016), *Oncidium* (Mayer et al. 2010), *Cattleya* (Cueva-Agila et al. 2016), *Vanda* (Hardjo et al. 2016) and *Phalaenopsis* (Winarto et al. 2016). However, the selection of suitable types and sources of explant are critical factors for obtaining a successful culture in somatic embryogenesis system (Feng and Chen, 2014).

Currently, some techniques of propagation have also been developed for a number of orchid species through somatic embryogenesis from various types of explant including leaves (Jainol and Gansau, 2017), roots (Meilasari and Iriawati, 2016), shoot tips (Van et al. 2012), stem nodal (Hong et al. 2010), seed-derived protocorms (Mahendran and Bai, 2016), and protocorm-like bodies (PLBs) (Li and Xu, 2009). Furthermore, the proccess of somatic embryogenesis could be induced either directly from epidermal and sub-epidermal cells of explants (Moradi et al. 2017) or indirectly via intervening callus (Niknejad et al. 2011). However, plant regeneration from callus is often associated with genetic and cytological variation making the strategy less desirable for large-scale clonal multiplication (Anjaneyulu and Giri, 2011). Direct

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somatic embryogenesis is beneficial with its reduced time for plant propagation as well as with minimized culture-induced genetic changes (Jayanthi et al. 2011).

Generally, somatic embryogenesis is considered to occur in response to modifications in the level of available growth regulators, especially auxins and cytokinins in tissue culture media (Moradi et al. 2017). Thidiazuron (TDZ) is a potent regulator for *in vitro* propagation system in a wide variety of plants (Guo et al. 2011). For some years, TDZ has been generally used to culture orchid tissue, which could induce organogenesis and high-frequency of direct somatic embryogenesis, either alone or in combination with other growth regulators (Mahendran and Bai, 2016; Wu et al. 2012). Moreover, many studies reported that the use of TDZ alone could induced direct SE in *Phalaenopsis* orchid (Feng and Chen, 2014). TDZ displayed primaryly cytokinin-like activity and can be used as a subtitute for both auxin and cytokinin (Kou et al. 2016). Besides, Bhataccharyya et al. (2016) reported that TDZ showed better efficacy over other purine-type cytokinins (BA or Kinetin) even at low concentrations.

Phalaenopsis amabilis is a native Indonesian orchid and one of the most important parental species of *Phalaenopsis* hybrids (Semiarti et al. 2007). Semiarti et al. (2013) reported that the number of propagated plants of this orchid using seeds was still limited because of highly dependent on the existance and the quality of siliques that resulted from pollination. Moreover, the continuity of mass propagation of this orchid to produce large number of uniform seedlings in a relatively short time to meet the market demands is also still limited. Therefore, to solve that problem, this study was taken to evaluate the influence of TDZ on direct SEs formation from various types of organs of *P. amabilis* as explants.

2. Materials and Methods

2.1. Plant materials and culture conditions

A silique of *P. amabilis* (Java ecotype) plant (Figures 1A and 1B) following 120 days of pollination was collected from Titi Orchids Nursery, Harjobinangun-Pakem, Sleman, Yogyakarta. The silique was wiped with 70% alcohol then passed over a fire and waited until the fire went out. This work is done for three times. After sterilized, the seeds were taken from the silique and sown on a solid New Phalaenopsis (NP) medium (Islam et al. 1998; Semiarti et al. 2010). Thereafter, the cultures were incubated in 100 mL flask at temperature of 25 ± 1°C with 1000 lux of continuous light. Four-week-old protocorms (developing orchid embryos) (Figure 1C) and 6-month-old orchid seedlings (Figure 1D) were used as the source of explants.

Roots and leaves of the 6-month-old seedlings and 4-week-old protocorms that were cut transversely, and stems were used as explants. The explants were put on NP solid medium supplemented with TDZ (0, 1, 2, 3 mgL^{-1}), and cultures were maintained at temperature of 25 ± 1 °C with 1000 lux intensity of continuous light. Subcultures were conducted every two weeks. Detailed observation on morphology of SEs formation was conducted every day and photographs were taken once a week for eight weeks using dissecting microscope (Eschenbach, Germany).

2.2. Histological analysis on the differentiation of SEs

Histological analysis on the differentiation of SEs was observed for each developmental stage of SEs in the surface of explants by anatomic preparation using paraffin method according to Ruzin (1999). The anatomic samples in the glass slides were observed using light microscope (Olympus, Japan).

2.3. Data analysis

A total samples of 320 explants grouped randomly into 16 treatment groups. Each group consisted of 20 explants cultured in 4 Petri dishes (diameter 100 mm × height 15 mm), each Petri dish contained 5 explants. All treatment means were compared by following Duncan's Multiple Range Test. Significant differences between means were presented at the level of $P \leq 0.05$.

3. Results

3.1. Induction of SEs

Based on percentage data of SEs formation after 8 weeks of culture, it was known that SEs could be induced in all treatments, except on leaf and root explants that cultured on hormone-free medium and root explants cultured on NP medium supplemented with 1 mgL^{-1} TDZ (Table). Within four weeks some explants turned to yellow and became necrotic. However, only 70% of protocorms and 40% of stem explants were successfully formed SEs in TDZ-free medium.

In the presence of TDZ in culture medium, 100% of SEs were successfully formed from protocorm and stem explants. The initial embryos emerged as protruding nodular masses from the surface of

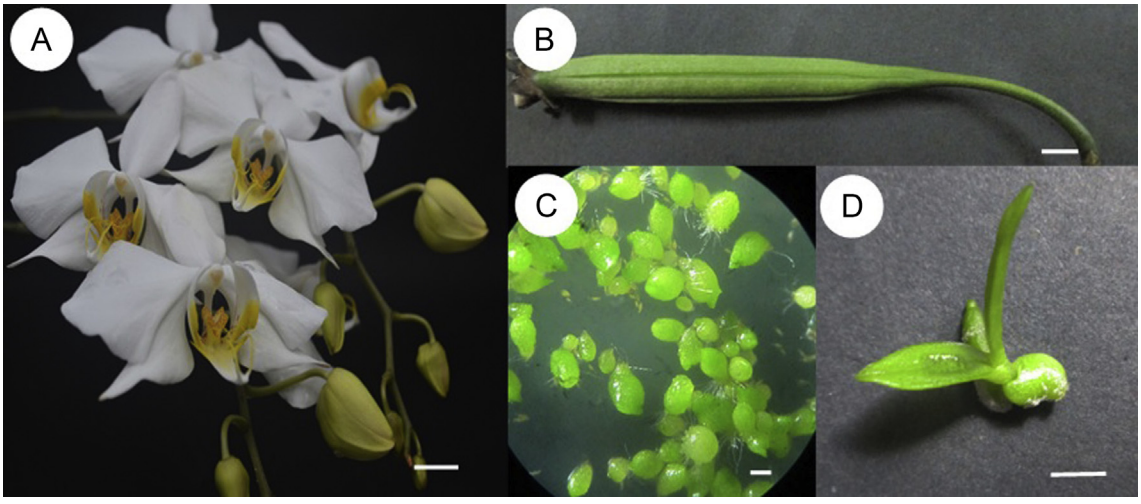

Figure 1. Phenotype of *P. amabilis*. (A) A bunch of flowers for the material of self-pollination to get silique. (B) 4-month-old silique. (C) 4-week-old protocorms. (D) 6-month-old seedling (bars a, b, d = 1 cm; bar c = 1 mm).

Table. Effects of various concentrations of TDZ on the formation of SEs from various types of explant of *P. amabilis*

Types of explant	Concentrations of TDZ (mgL^{-1})	Percentage of SEs formation (%) after 8 weeks of culture (n = 20)	SEs induction time (days)	Number of SEs formed after 8 weeks of culture
Protocorm	0	70	26.07 ± 0.73^{f}	1.50 ± 0.65^{b}
	1	100	17.85 ± 0.67^{d}	9.85 ± 0.93^{g}
	2	100	15.00 ± 0.64^{c}	16.05 ± 0.89^{i}
	3	100	11.00 ± 0.64^{b}	23.30 ± 1.13^{k}
Leaf	0	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	1	75	35.00 ± 0.65^{j}	3.20 ± 0.68^{c}
	2	90	31.78 ± 0.73^{i}	4.83 ± 0.38^{e}
	3	100	27.05 ± 0.68^{g}	7.75 ± 0.44^{f}
Root	0	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	1	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	2	65	36.36 ± 1.15^{k}	4.21 ± 1.05^{d}
	3	80	32.25 ± 0.93^{i}	$8.25 \pm 0,68^{f}$
Stem	0	40	31.00 ± 0.76^{h}	1.25 ± 0.46^{b}
	1	100	22.15 ± 0.98^{e}	13.90 ± 0.91^{h}
	2	100	18.30 ± 0.86^{d}	21.05 ± 0.89^{j}
	3	100	14.95 ± 0.75^{c}	28.25 ± 1.07^{l}

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

the wounding part of explants. This structure appeared to be produced directly without intervening of callus phase (Figure 2A). After two weeks of subcultured, the embryos become progressively enlarges, and more embryos were formed. In addition, numerous white absorbing hair formed in the basal region (Figure 2B). It indicated that the embryos continued to form a striking structure on the apical region (Figure 2C) before producing a shoot (Figure 2D).

Under periodical observation it was known that inline with the high concentration of TDZ, both the induction time and the number of SEs produced were also increased (Table). The results showed that NP medium supplemented with 3 mgL^{-1} TDZ was the most suitable medium for embryos formation, which resulted in the fastest SEs formation on protocorm explants (11.00 ± 0.64 days) and the highest SEs number on stem explants (28.25 ± 1.07). In contrast, protocorms and stems produced the lowest number of SEs when cultured on NP basal medium, that is 1.25 ± 0.46 SEs from stems and 1.50 ± 0.65 from protocorms.

3.2. Histological analysis on the differentiation of SEs

The results of histological analysis showed that SEs originated from the epidermal and sub-epidermal cells of explants as proembryonic masses that consisted of small, thick-walled cells with large and densely stained nuclei (Figures 3A and 3B). There were no vascular connections between the proembryo and mother tissue. At four weeks after initiation of culture, some proembryos enlarged and formed a globular-shape embryo with a protoderm (Figure 3C) that surrounds the embryo. The structure consisted of a layer of cells, arranged regularly and thightly. The globular embryo further elongated and formed a suspensor in the basal region (Figure 3D).

By the time of culture, globular embryo developed into a scutellar embryo which consisted of two protuberances at the apical part and a notch between the protuberances (Figure 3E). In subsequent development, a shoot meristem was formed from the notch. The shoot meristem was enclosed by two leaf primordia and coleoptile (Figure 3F). In this stage, we also noticed the formation of root meristem at the basal part of embryo. This structure became prominent by its dense cytoplasm and distinct nuclei. Next, the embryo elongated followed by the formation of procambium in the middle part of embryo (Figure 3G). Eventually, leaf primordia elongated indicating a mature embryo development (Figure 3H).

4. Discussion

4.1. Induction of SEs

Plant regeneration via SEs from various vegetative parts of orchid are essential and has been done in many species (Chugh et al. 2009). Meilasari and Iriawati (2016) reported that SEs were successfully induced from roots and leaves of *Phalaenopsis* 'Join Angle X Sogo Musadian' cultured in half-strength Murashige and Skoog (MS) medium supplemented with various combinations of plant growth regulators (PGRs). While, Hong et al. (2010) reported that about 55,5% of stem nodal segments of *Zygopetalum mackayi* orchid were formed SEs when planted on MS medium enriched with 4,54 μM TDZ. Here, SEs were successfully induced from stem, root, leaf, and protocorm explants of *P. amabilis*. Among those types of explant, stem and protocorm showed the best response by production of the highest number of SEs. Similar results were reported by Moradi et al. (2017) when they assesed the potential of different types of explant of *Epipactis veratrifolia* orchid. They found

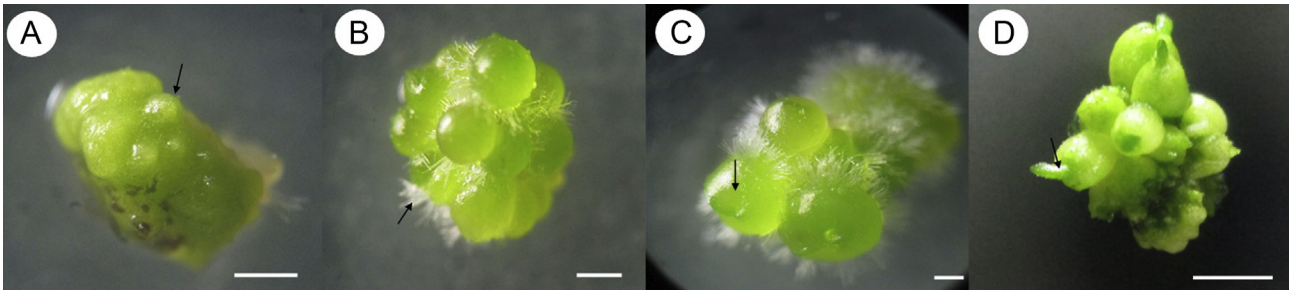

Figure 2. Developmental stages of SEs of *P. amabilis*. (A) SEs (arrow) were formed from the surface area of the wounding part. (B) SEs enlarged and formed absorbing hair (arrow) at the basal region. (C) SEs formed a striking structure on the apical region. (D) SEs with a shoot (arrow) (bars = 50 mm).

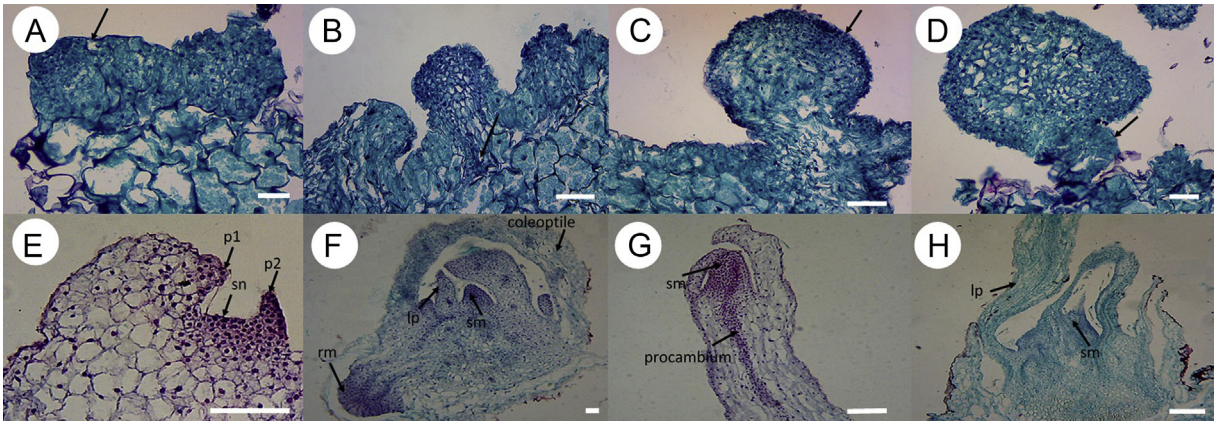

Figure 3. Anatomy of direct SE development from stem of *P. amabilis*. (A) The structure resemble to proembryo (arrow) originated from epidermal and (B) sub-epidermal cells layers. (C) Globular embryo, surrounded by protoderm (arrow). (D) Globular embryo elongated with a suspensor (arrow) on the basal region. (E) The embryo formed two protuberances (p1, p2) and a scutellar notch (sn) between two protuberances. (F). SE consisted of shoot meristem (sm), leaf primordia (lp), enclosed by the coleoptile, and root meristem (rm) at the basal part of embryo. (G). The formation of procambium. (H) Leaf primordia elongated (bars = 100 μm).

that protocorm had the highest embryo induction frequency (100% embryogenesis) among leaf segment, apical bud, single node, and crown. SEs were also formed from protocorm and stem explants when cultured on hormone-free medium. This result suggests that the endogenous hormone inside the explants might still have a role in embryo formation. Consistent with our result, Kaewubon and Meesawat (2016) also reported that bisected protocorm of Pigeon orchid (*Dendrobium crumenatum*) cultured on PGRs-free medium could formed SEs.

However, leaf and root explants cultured on TDZ-free medium did not formed any embryos. Similar results were also reported by Khoddamzadeh et al. (2011) in *Phalaenopsis bellina,* in which leaf explants planted on basal medium failed to form embryos and the explants tend to be necrotic. As we used various concentrations of TDZ (0, 1, 2, 3 mgL^{-1}), we found that, inline with the higher concentration of TDZ, the number of embryos increased. Similar results were obtained by Balilashaki et al. (2015) using leaf tip segments of *P. amabilis* cv. 'Surabaya' planted on MS medium supplemented with TDZ (0.5, 1, 2, and 3 mgL^{-1}). Moradi et al. (2017) also reported that addition of 3 mgL^{-1} TDZ produced the highest number of SEs, both in protocorms (2,5 embryos) and leaves (0,33 embryos) of *E. veratrifolia*.

TDZ is a diphenylurea derivative with influential cytokinin activity and have been suggested as one of the most effective PGRs for SEs induction in comparison to other cytokinins (Gantait and Sinniah, 2012; Sujjaritthurakarn and Kanchanapoom, 2011). TDZ affected plant physiology, such as cellular, nutrient, transport and alters the endogenous levels of PGRs (Ouyang et al. 2016). Wu et al. (2012) demonstrated that TDZ was more effective to induce direct SEs from leaf explants of *Renanthera* Tom Thumb'Qilin' orchid, which was significantly higher than BAP, kinetin, and NAA.

TDZ was generally effective in inducing SEs at concentration ranging between 0.01 to 5 mgL^{-1} (Gantait and Sinniah, 2012). Whereas, the use of TDZ with concentration of 1 mgL^{-1} failed to formed SEs in root explants. Lang and Hang (2006) also examined various concentrations of TDZ affecting the production of SEs from root explants of *Vanda coerulea* orchid found that, concentration of TDZ below 3 mgL^{-1} did not produced any embryos.

4.2. Histological analysis on the differentiation of SEs

Most tissue culture studies in the Orchidaceae family have reported that SEs were derived from the epidermal cells of mother tissue (Chen and Hong, 2012; Cueva-Agila et al. 2016). However, we found that the SEs originated from the epidermal and the sub-epidermal cells of explant. This result also reported in *C. bicolor* (Mehendran and Bai, 2012) and *D. crumenatum* (Kaewubon and Meesawat, 2016). Although the process of SEs formation has occured earlier at the cellular level, but visually SEs were seen as a proembryo mass that rapidly differentiated to formed a globular-shape embryo. This embryo undergo differentiation by formed a terminal notch. The notch marks the future location in the shoot apex and represents the earliest morphological sign of coleoptile development (Alcantara et al. 2014). At this stage, cell divisions were most frequent in the terminal cells that eventually forms shoot and root meristem. Matsumoto et al. (1996) mentioned that the formation of these features designated the bipolarity of SE and fulfill the anatomical requirements of a true SE.

In conclusion, NP medium supplemented with 3 mgL^{-1} TDZ was the best medium to induce direct SEs from all various explants of *P. amabilis*. While protocorm and stem appeared to be the most responsive explants due to the fastest and the highest number of SEs produced. The easy procedure for SEs production using TDZ-containing medium will support and give high benefit for orchid conservation as well as orchid industry.

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Conflict of Interest Statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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