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Clonal Fidelity of Micro-propagated *Phalaenopsis* Plantlets Based on Assessment Using Eighteen *Ph-Pto* SNAP Marker Loci

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ABSTRACT

Phalaenopsis amabilis is an Indonesia native orchid species having large, white flowers with yellow labellum coloration. This studies aimed to develop *Phal. amabilis* micropropagation methods and evaluate the regenerated plantlet fidelity. Media supplemented with Thidiazuron (TDZ) and Polyvinylpyrrolidone (PVP) and medium pH adjustment effects to induce protocorm-like bodies (PLBs) from leaf explants and proliferate secondary PLBs were investigated. Clonal fidelity among regenerated plantlets was evaluated using eighteen SNAP marker loci. The results showed that the ½ MS medium supplemented with 3 mg L^{-1} TDZ and 0.5 g L^{-1} PVP was the best for PLB induction while the ½ MS medium supplemented with 0.5 mg L^{-1} TDZ was the best for PLB proliferation. For PLB induction, the media pH was adjusted into pH=7 for efficient PLB regeneration. Based on the assessment using 18 SNAP marker loci, four variant alleles in three loci (11.8%) out of a total 34 plantlets were detected. The mutation frequency at the evaluated SNAP marker loci was 2.5 x 10^3 (0.25%). Changes in SNP alleles may not always result in phenotype changes and allele variant occurrences may not affect phenotype fidelity of micro-propagated *Phal. amabilis* plantlets. Therefore, further studies about the phenotype fidelity among plantlets are necessary.

INTRODUCTION

Phalaenopsis amabilis, commonly known in Indonesia as 'Anggrek Bulan' is one of the *Orchidaceae* species typically having large (7-12 cm), pure white flowers with yellow coloration and some red stripes on its labellum (Handoyo, 2010). Based on their perianth morphology, there are six types of *Phal. amabilis*, such as Taiwan, Sumatera, Irian Jaya, Java, Borneo, and Grandiflora types. The Ph*al. amabilis* from Sumatera has smaller perianth than those from Java and Borneo while *Phal. grandiflora* from Borneo is characterized as having the largest flower and more than 5 cm petals (Ikedo, n.d.).

As in many orchid species, illegal harvest, forest destruction, and climatic changes may contribute to the decline of their natural populations (Crain & Tremblay, 2014; Crain & Tremblay, 2017; Fay, 2018). Therefore, it is necessary to develop concerted efforts for ex situ conservation of endangered orchid species before it becomes extinct. The development of ex-situ conservation requires the use of tissue culture as the alternative technology for clonal propagation (Merritt, Hay, Swarts, Sommerville, & Dixon, 2014).

Various tissues and organs have been used as explants to initiate propagation of *Phalaenopsis*, such as leaf (Balilashaki, Naderi, Kalantari, & Soorni, 2014), inflorescence nodes, flower stalk buds, seed derived protocorm-like bodies (Balilashaki, Naderi, Kalantari, & Soorni, 2014; Soetopo & Purnamaningsih, 2012) or seedlings (Feng & Chen, 2014). Protocorm-like bodies (PLBs) are somatic embryos of orchids (Lee, Hsu, & Yeung, 2013), which can be used as explants for secondary PLB proliferation (Chugh, Guha, & Rao, 2009; Mahendran & Bai, 2016; Mose, Indrianto,

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Purwantoro, & Semiarti, 2017; Niknejad, Kadir, & Kadzimin, 2011).

Browning of the explants is a challenge in establishing in vitro culture of *Phalaenopsis* species using vegetative tissues. Explant browning is the common problem occurring in the early stages of plant tissue culture (Ru, Lai, Xu, & Li, 2013). The tissue browning problem is because of the plant phenolic compound enzymatic oxidation, which in turn inhibits absorption of nutrients and causes explant death (Ahmad et al., 2013). One strategy to alleviate such problem is adding antioxidants onto the establishment medium (Ahmad et al., 2013) and PVP is one of the antioxidants commonly used for in vitro culture of orchids.

Plant growth hormones are critical for orchid micropropagation and an addition of cytokinin is necessary for shoot organogenesis (Hill & Schaller, 2013). Gow, Chen, & Chang (2008) obtained the highest number of embryogenesis responses and the largest number of generated SE from leaf explants on medium supplemented with 13.6 µM (3 mg L^{-1}) TDZ. However, an addition of plant growth hormones as part of the in vitro propagation methods might also result in somaclonal variation. Therefore, the preferred orchid tissue culture protocols should support the increase in PLBs multiplication, total yield, and plantlet conversions without inducing somaclonal variations (Pornpienpakdee et al., 2010). Samarfard et al. (2014) found that a medium supplemented with a combination of 0.1 mg TDZ and 10 mg L^{-1} chitosan gave the lowest frequency of somaclonal variation on *Phalaenopsis gigantea* micropropagation.

Although in vitro seed germination has been successful for generatively propagating *Phalaenopsis*, clonal propagation using vegetative tissues (i.e. leaves and inflorescence nodes) is less successful because of the high frequency of somaclonal variations (Chen, Yu, Chen, & Huang, 2008). The presence of somaclonal variation can be done by evaluating the morphological, physiological or biochemical characters of the regenerated plantlets, or by using molecular markers (Bairu, Aremu, & Van Staden, 2011). A number of molecular markers have been used to identify somaclonal variation among *Phalaenopsis*, such as random amplified polymorphic DNA (RAPD, Khoddamzadeh et al., 2010) and inter-simple sequence repeat (ISSR, Samarfard, Kadir, Kadzimin, Ravanfar, & Saud, 2013).

However, the use of co-dominant marker, such as a single nucleotide amplified polymorphism (SNAP) marker (Pesik, Efendi, Novarianto, Dinarti, & Sudarsono, 2017; Sukma, Elina, Giyanto, & Sudarsono, 2017; Sutanto, Sukma, Hermanto, & Sudarsono, 2014) and SSR markers (Fattmah & Sukma, 2011) may be more robust than those of dominant markers (i.e. RAPD, ISSR or AFLP). The SNAP markers may be developed based on the presence of nucleotide sequence variabilities of the target genes (Pesik, Efendi, Novarianto, Dinarti, & Sudarsono, 2017; Sutanto, Sukma, Hermanto, & Sudarsono, 2014). Under tropical conditions, most of the *Phalaenopsis* accessions are sensitive to soft rot disease because of *Dickeya dadantii* infection. Therefore, developing soft rot resistance *Phalaenopsis* varieties is beneficial for *Phalaenopsis* grower in the tropics.

The *Pto* was reported as the gene confering resistance to a bacterial pathogen in tomato (Sun, Zhao, Wang, Pei, & Yang, 2011). Therefore, PTO protein may also be associated with *Phalaenopsis* responses against soft rot disease. The *Pto* genes from various *Phalaenopsis* species have been isolated and characterized (Elina, Sukma, Giyanto, & Sudarsono, 2017) and 18 SNAP marker loci have been identified based on partial *Pto* gene sequences (Sukma, Elina, Giyanto, & Sudarsono, 2017). Effectiveness of these *Pto* based SNAP markers to evaluate soft rot disease responses across a number of *Phalaenopsis* species and their hybrids has been tested (Sukma, Elina, Giyanto, & Sudarsono, 2017). Therefore, the identified 18 SNAP marker loci may be used to evaluate changes in the *Pto* gene sequences during *Phalaenopsis* clonal propagation and monitor the presence of *Phalaenopsis* somaclonal variants with changing responses to pathogens.

The aim of the studies were to develop a method for *Phalaenopsis amabilis* micropropagation and evaluated the clonal fidelity of the regenerated plantlets using *Pto* based SNAP markers. The effects of media supplemented with Thidiazuron (TDZ) and Polyvinylpyrrolidone (PVP) and the medium pH adjustment to induce protocorm-like bodies (PLBs) from young leaf explants and to proliferate secondary PLBs were investigated. Clonal fidelity across the regenerated plantlets was evaluated using eighteen *Pto* based SNAP marker loci.

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MATERIALS AND METHODS

All in vitro activities in this research were conducted at the Plant Tissue Culture Lab. (PTC Lab.), Department of Agronomy and Horticulture, Bogor Agricultural University, Bogor, Indonesia. Meanwhile, all molecular analysis was done at Plant Molecular Biology Lab. (PMB Lab.). Both activities were done during the period of December 2015 to January 2017.

Exp. 1. Effects of TDZ and PVP on PLB Induction from Leaf Explants

In this in vitro experiments, leaf explants were harvested from four months old *Phalaenopsis amabilis* plantlets regenerated from in vitro germinated seeds. The seed germination and plantlet regeneration as explants sources were on Knudson C medium (Knudson, 1946). Leaf sections from in vitro propagated *P. amabilis* plantlets (ca. 1 cm^2) were cut transversely using a surgical blade (Fig. 1A) before culturing them onto PLB induction medium. In this experiment, segments of the leaf explants were induced to generate protocorm-like bodies (PLBs) on half ionic strength of MS (½ MS, Murashige & Skoog, 1962) basal medium. The basal medium for PLB induction also contained the following organics and vitamins, such as 100 mg L-1 Myoinositol, 0.5 mg L^{-1} of Niacin, 0.5 mg L^{-1} Pyridoxine HCl, 0.1 mg L-1 Thiamine HCl, 2.0 mg L^{-1} of Glycine, and 1 g L^{-1} Peptone. Sucrose at 30 g L-1 and agar at 7 g L^{-1} were also added to the medium. To induce PLBs, four concentrations of TDZ (0.1, 0.5, 1, or 3 mg L^{-1}) with or without addition of 0.5 mg L^{-1} PVP were evaluated. In this experiment, medium pH was adjusted to 5.2 using either 1 M KOH or HCl before sterilization. The experimental unit consisted of three explants, and each treatment was replicated three times.

The cultures were kept in the dark for one month and transferred under 16 hours photoperiod for one month. The percentages of surviving explants and explant-forming PLBs, and the numbers of regenerated PLBs per explant were recorded at eight weeks after culture. When appropriate, the data were subjected to statistical analysis using Statistical Tool for Agricultural Research (STAR) software version 2.0.1 developed by IRRI (Gulles et al., 2014).

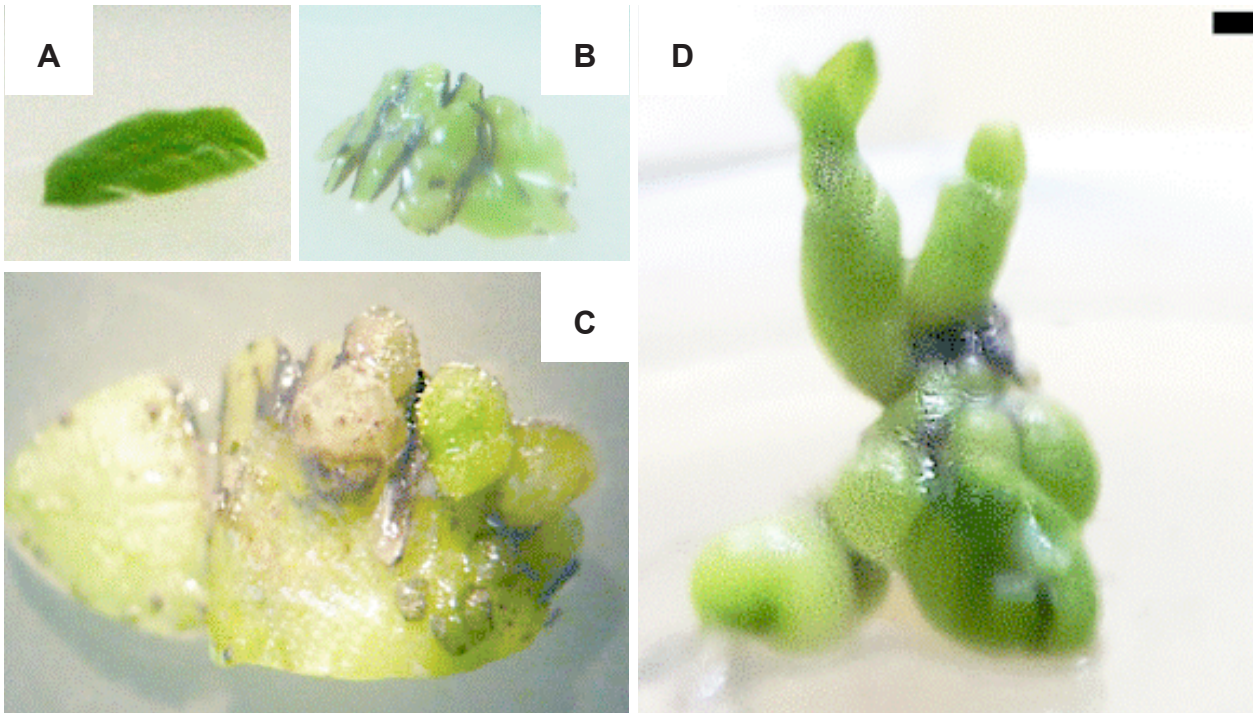

Fig. 1. Growth and development of leaf explants and induction of protocorm-like bodies (PLBs) from injured tissues. The explants were grown on ½ MS medium supplemented with 1 mg L^{-1} Thidiazuron (TDZ) and 0.5 g L^{-1} PVP. (A) The leaf explant at 0 weeks after planting (WAP); (B) swelling of the leaf explant at 6 WAP; (C) formation of PLBs at 8 WAP; and (D) plantlet regeneration from PLBs at 12 WAP. The bars in image A to D was 10 mm

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Exp. 2. Effects of pH Medium on PLB Induction from Leaf Explants

PLB induction from *Phal. amabilis* leaf explant was also evaluated on PLB induction medium having pH adjusted to 4, 5, 6, or 7. The PLB induction medium composition and the explant used in this evaluation were the same as in the previous investigation. Thidiazuron (1 mg L^{-1}), and PVP (0.5 g L^{-1}) were added to the PLB induction medium. The cultures were kept in the dark for two months and transferred under 16 hours photoperiod for one month. The percentages of surviving explants and explant-forming PLBs, and the numbers of regenerated PLBs per explant were recorded at two months after culture. When appropriate, the data were subjected to statistical analysis as previously described.

Exp. 3. Effects of TDZ and PVP on PLB Proliferation

Seeds of *Phal. amabilis* were grown on the Knudson C (Knudson, 1946) semi-solid medium for three months. The regenerated protocorms were used as explants. In this experiment, the basal medium for PLB proliferation was the same as those of PLB induction medium in Exp. 1. To proliferate PLBs from protocorms, four concentrations of TDZ (0.1, 0.5, 1, or 3 mg L^{-1}) with or without addition of 0.5 mg L^{-1} PVP to the basal medium were used and the responses of explants were evaluated. In this experiment, the medium pH was adjusted to 5.2 before sterilization.

The protocorms were cultured with the basal part exposed to the medium. The cultures were kept in the dark for one month and transferred under 16 hours photoperiod for another month. The percentages of the surviving explants, the explant-forming PLBs, and the numbers of regenerated PLBs per explant were observed at eight weeks after culture. When appropriate, the data were subjected to statistical analysis as previously described.

Exp. 4. Clonal Fidelity Evaluation Using SNAP Markers

The clonal fidelity of plantlets developed from regenerated PLBs on medium containing 1 mg L^{-1} TDZ and 0.5 g L^{-1} PVP were assessed by evaluating their SNAP marker profiles. DNA isolation used CTAB method (Doyle & Doyle, 1987), which had been adopted for orchid species (Fatimah & Sukma, 2011; Sukma, Elina, Giyanto, & Sudarsono, 2017; Sudarsono, Haristianita, Handini, & Sukma, 2017) and various tropical crops (Ajijah, Hartati, Rubiyo, Sukma, & Sudarsono, 2016; Larekeng, Maskromo, Purwito, Mattjik, & Sudarsono, 2015; Maskromo, Larekeng, Novarianto, & Sudarsono, 2016; Oktavia, Kuswanhadi, Dinarty, Widodo, & Sudarsono, 2017). The DNA quality and its estimated quantity were determined by comparing to DNA standard using agarose gel electrophoresis.

Elina, Sukma, Giyanto, & Sudarsono (2017) have identified eighteen SNAP marker loci based on nucleotide sequence variabilities of *Pto* gene from various *Phalaenopsis* species. Those primer sets were used to evaluate the clonal fidelity of the clonally propagated *Phal. amabilis* plantlets. All mother plants and regenerated plantlets were subjected to SNAP genotyping using the appropriate primers, and their SNAP allele profiles evaluated.

The SNAP markers were amplified by PCR in a volume of 12.5 µL, consisting of 2 µL genomic DNA sample as a template, 6.25 µL of KAPA2G Fast ReadyMix (Kapa Biosystems Inc., USA), 3.85 µL double-distilled water (ddH_2O), and 0.2 µL each of forward and reverse primers. The DNA amplification was performed in a BioRad T100™ thermocycler, using the following steps: a pre-denaturation step of 3 minutes at 95 ºC; 35 cycles of denaturation for 15 seconds at 95 ºC, annealing for 15 seconds at 47–60 °C (according to the appropriate Ta for each primer pairs), and extension at 72 ºC for 1 second; and a final extension at 72 ºC for 10 minutes. PCR products were electrophoresed at 80 V for half an hour using 1 % (w/v) agarose in 1x Sodium Borate (SB) Buffer, stained with 20 µL L^{-1} GelRed™ and visualized on a UV transilluminator. The gels were photographed using digital camera and stored for scoring. Genotyping of the individual mother plants and the regenerated plantlet were done by scoring the presence or absence of the reference and the alternate alleles, respectively. Differences in the allele profile among the mother plants and the regenerated plantlets indicated they were plantlet variants. Frequencies of the plantlet variants were calculated by dividing the number of plantlet variants over the entire regenerated plantlets. Moreover, the frequency of allele variants was estimated by dividing the number of observed allele variants over the total alleles (2 x number of loci x number of plantlets).

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RESULTS AND DISCUSSION

Exp. 1. Effects of TDZ and PVP on PLB Induction from Leaf Explants

The addition of PVP into medium positively affected the percentage of surviving explants, explants with PLBs, and average numbers of regenerated PLBs per explant (Table 1). The addition of 0.5 g L^{-1} PVP into the culture medium containing TDZ resulted an increase of explant survival and the average number of regenerated PLBs per responding explant (Table 1). On the other hand, the absence of PVP from the induction medium tended to reduce all observed parameters because the increased in the explant browning frequencies. Browning occurs because the oxidation of phenolic compounds released by the injured explant tissues, the oxidized compounds difused into the medium and inhibits explant growth and development (Chen, Chen, Wang, Xu, & Li, 2012). Therefore, the beneficial effects of PVP supplementation may be because of reducing browning and improving explant response in the PLB induction. The previous report indicated a low concentration (0.1-0.5 g L^{-1}) of PVP reduced browning in sugar cane (Shimelis, Bantte, & Feyissa, 2015).

On the other hand, the addition of TDZ into induction medium only positively affected the percentage of surviving explant, the explant forming PLBs, and the numbers of regenerated PLBs per responding explants (Table 1). The effects of increasing TDZ concentration on the percentage of surviving explants and explants forming PLBs were not statistically significant (Table 1). The highest average number of PLBs per responding explant after eight weeks was obtained in the induction medium supplemented with 3 mg L-1 TDZ. Mose, Indrianto, Purwantoro, & Semiarti (2017) have previously reported that media supplemented with 1-3 mg x L^{-1} TDZ are the best media for PLB induction from *Phalaenopsis amabilis* explants.

Gow, Chen, & Chang (2010) reported that the induction of PLBs from leaf explants required at least two months culture period in dark. In this study, PLB structures were first observed at 25 days after cultures, which was much faster than that previously reported. The formation of PLBs was initially started surrounding the injured tissue of the leaf explants (Fig. 1A and 1B). In the higher levels of TDZ, PLBs regenerated in the wounded green leaf tissues and the yellow ones (Fig. 1C). After two months of incubation in the dark, the responding explants (Fig. 1C) were transferred onto the same medium composition. Subsequently, the cultures were incubated in incubation room with 16 hours photoperiod for two months. Four weeks after transferring onto the fresh medium and culturing them under the light, PLBs continued proliferating and forming shoots (Fig. 1D). Once they formed the shoot, the PLBs were then sub-cultured periodically onto HP regeneration medium every four months and incubated under the light until the plantlets were ready for acclimatization.

Table 1. Effects of Thidiazuron (TDZ) and Polyvinylpyrrolidone (PVP) concentrations on percentages of surviving explants, explant with primary protocorm-like bodies (PLBs) and the average number of PLBs per leaf explant at eight weeks after primary cultures

Treatments	TDZ (mg L^{-1})				Average over TDZ
	0.1	0.5	1.0	3.0	
PVP (g L^{-1})	% of surviving explants				
0	33.33	44.4	66.7	77.8	55.6 b*
0.5	55.57	88.9	88.9	100.0	83.3 a
Average over PVP	44.45	66.7	77.8	88.9	CV: 8.8 %
PVP (g L^{-1})	% of explants with PLBs				
0	22.20 Aa	22.2 b	55.6	55.6	38.9
0.5	11.10 Ba	89.0 a	66.7	77.8	61.2
Average over PVP	16.65	55.6	61.1	66.7	CV: 7.1 %
PVP (g L^{-1})	Average numbers of PLBs per explants				
0	0	0.5	2.0	3.9	1.6 b
0.5	0	2.7	3.2	5.0	2.7 a
Average over PVP	0.00 C**	1.6 B	2.6 B	4.5 A	CV: 12.7 %

Remarks: CV = coefficient of variation. For each variable, * = the average over TDZ values followed by different small letters; or ** = the average over PVP values followed by different capital letters – were significantly different at $p < 0.05$ according to Tukey's HSD Test

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As one of the synthetics cytokinin, TDZ was an effective plant growth regulator for inducing somatic embryos from *Phalaenopsis* leaves (Antensari, Mariani, & Wicaksono, 2014; Khoddamzadeh et al., 2011). In this study, most of the explants grown in PLB induction medium supplemented with 0.1 mg L^{-1} TDZ became yellow in color, and some died. Some explants grown in PLB induction medium supplemented with 0.1 mg L^{-1} TDZ stayed green, but there was no PLB formation.

Effects of pH Medium on PLB Induction from Leaf Explants

Table 2 presents significant effects of medium pH on percentages of surviving explants, explant with PLBs, and the average numbers of PLBs per explant. The lower medium pH, the larger the adverse effects on the observed parameters are. The agar solidified poorly under the lower medium pH and caused the explants to submerge into the medium, which in turn causes poor PLB development from the explants. Medium pH > 5 was equally good for percentages of both the surviving explants and the explant with PLBs (Table 2). On the other hand, medium pH = 7 was the best for the average numbers of PLBs per responding explant (Table 2).

Medium pH may affect in vitro explants development. Santarem, Pelissier, & Finer (1997) showed pH = 7 was the best for inducing somatic embryos from soybean explants. In another study, Hofmann, Nelson, & Korban (2004) demonstrated that soybean somatic embryo development was the best when medium pH was adjusted 5.7. The explant type and plant cultivars might probably contribute to the response differences. The medium pH is also affected in-vitro explant growth and development (George, Hall, & De Klerk, 2008). Without pH regulation, the ionization of acidic and basic compound groups might affect embryo somatic development (Santarem, Pelissier, & Finer, 1997). On the other side, Shi, Yang, Yan, & Du (2017) stated that medium pH has minimal effects on apple tissue culture. However, the lower medium pH, the more difficult for them to solidify and resulted in handling problems. Moreover, Chen, Bates, & Carlson (2015) stated that subcultures practice every 21-day may best maintain medium pH level and desirable explant growth.

Table 2. Effects of pH medium on percentages of surviving explants, explant with protocorm-like bodies (PLBs) and the average number of PLBs per explant at eight weeks after primary cultures

The pH of medium	% surviving explants	% explant with PLBs	Average numbers of PLBs per explant
7	100.0 a*	100.0 a*	5.5 a*
6	77.8 a	77.8 a	3.3 b
5	88.9 a	66.7 a	2.0 b
4	33.3 b	0.0 b	0.0 c
F Test	**	**	**
CV (%)	18.1	5.9	8.3

Remarks: CV = Coefficient of variation and PLB = Protocorm-like body. * = For each variable, the mean values followed by different letters were significantly different ($p < 0.05$) according to Tukey's HSD Test. ** = Indicated F-tests of the analysis of variance, were highly significant ($p < 0.01$).

Effects of TDZ and PVP on PLB Proliferation

Most of the secondary PLBs developed from the injured tissues (Fig. 2) after removal of the primary PLB apical parts to prevent apical dominance. Huang, Tsai, Cheng, Chen, & Chen (2014) have reported the formation of many secondary PLB structures in the epidermal layer around the excised PLBs.

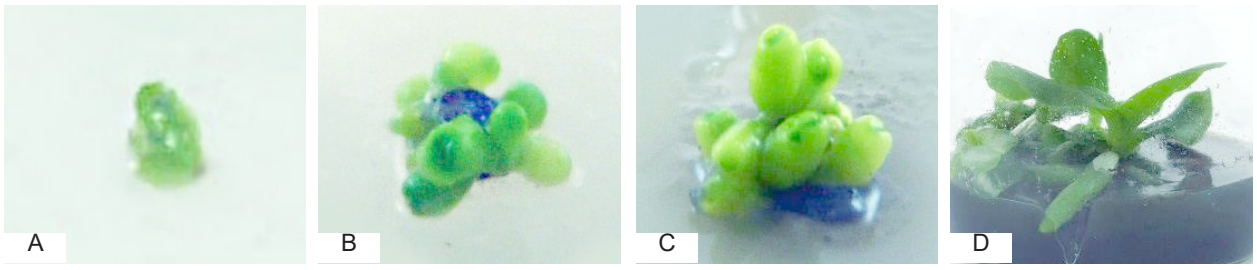

Fig. 2. Development of protocorm-like bodies (PLBs) on medium containing 0.5 g L^{-1} PVP + 1 mg L^{-1} TDZ at (A) 0 week after planting (WAP); (B) 6 WAP; (C) 8 WAP; and (D) 34 WAP. The size bar on the image A-C was 1 mm while that in the image D was 10 mm. The red arrow indicated the position of the wounded leaf explant where the PLBs regenerated

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Table 3. Effects of Thidiazuron (TDZ) and Polyvinylpyrrolidone (PVP) concentrations on percentages of surviving explants, explants with secondary protocorm-like bodies (PLBs) and the average number of secondary PLBs per explant in the PLB proliferation stage at eight weeks after primary cultures

Treatments	TDZ (mg L^{-1})				Average over TDZ
	0.1	0.5	1.0	3.0	
PVP (g L-1)	% of surviving explants				
0	96.3	96.3	88.9	88.9	92.6
0.5	96.3	92.6	100.0	100.0	97.2
Average over PVP	96.3	94.5	94.5	94.5	CV (%): 6.3 %
PVP (g L-1)	% of explants with PLBs				
0	92.6	92.6	85.2	81.5	88.0 b*
0.5	96.3	96.3	96.3	100.0	97.2 a
Average over PVP	94.5	94.5	90.8	90.8	CV (%): 8.8 %
PVP (g L-1)	Average numbers of PLBs per explants				
0	4.3	6.0	5.7	6.0	5.5
0.5	4.7	5.1	4.4	5.8	5.0
Average over PVP	4.5	5.5	5.0	5.9	CV (%): 18.8

Remarks: CV = coefficient of variation. * = For each variable, the average over TDZ values followed by different letters were significantly different ($p < 0.05$) according to Tukey's HSD Test.

The addition of TDZ to the PLB proliferation medium did not show any significant effect on all parameters during the PLB proliferation (Table 3). The result in this study was in line with that of Chen, Chen, & Chang (2002) which showed supplementation of TDZ (0.1 - 1.0 mg L^{-1}) to the PLB proliferation medium did not significantly affect the proliferation rate and the PLBs numbers on Epidendrum. In different circumstances, Samarfard et al. (2014) showed that the addition of 0.1 mg L-1 TDZ significantly increased proliferation of Phal. gigantea PLBs than that of 0.5 mg L^{-1} TDZ in the PLB proliferation stage. The response differences may be because of the different *Phalaenopsis* species used. Moreover, somatic embryo development is a complex processes controlled by not only plant growth hormones but also by various factors (Fehér, 2015). Since PLBs are orchid somatic embryos, the same complex processes may also control PLBs development.

The significant effect of PVP supplementation to the proliferation medium was only for the percentage of explant forming secondary PLBs (Table 3). The proliferation medium supplemented with 0.5 g L^{-1} PVP results in 97.2 % explants forming secondary PLBs while that without PVP yielded only 88.0 % (Table 3). The absence of PVP in the media was associated with browning and resulted in low percentages of explant forming PLBs. Browning occurred in almost all of the proliferation media without PVP supplementation. Browning can lead to inhibition of nutrient uptake and may eventually result in death (Ahmad et al., 2013).

The PLBs regenerated into plantlets with shoot and roots if they were sub-cultured onto HP media and maintained for 16 weeks. After 34 weeks in HP media, the regenerated plantlets with 2-3 roots and 3-4 leaves were ready for acclimatization. In this experiment, plantlets regenerated in a low concentration of TDZ were visually healthier and normal than those regenerated in a high concentration of TDZ (Fig. 3).

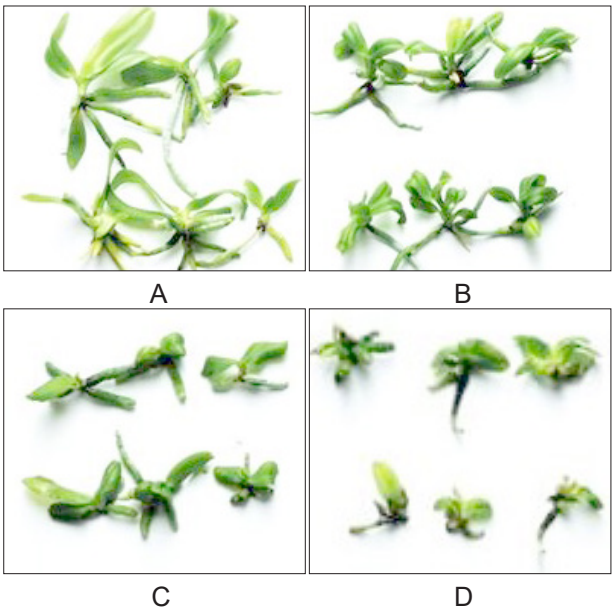

Fig. 3. Regenerated plantlets from protocorm-like bodies (PLBs) on regeneration medium containing: (A) 0.1 mg L^{-1}, (B) 0.5 mg L^{-1}, (C) 1 mg L^{-1}, and (D) 3 mg L^{-1} of Thidiazuron (TDZ) at 48 weeks after initiation of plantlet regeneration. The bar represent 10 mm in length

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Clonal Fidelity Based on Ph-Pto SNAP Marker Analysis

In this study, a total of 34 plantlets regenerated from five independent PLBs were evaluated. These clones were evaluated using 18 loci of SNAP markers. All the SNAP primer sets used to assess the plantlets were able to amplify and yield the appropriate PCR products. Five mother plants used to generate plantlets exhibited the same allele profiles for the 18 SNAP marker loci. The genotypes of the mother plants for the evaluated 14 SNAP marker loci were in the heterozygous, while for the other four loci were in homozygous configurations (locus Pto-37, Pto-64, Pto-223, and Pto-292). Moreover, most of the regenerated plantlets (30 out of 34 plantlets) also showed the same banding patterns to the mother plants for 18 SNAP marker loci.

In this experiment, there were at least four potential plantlet variants exhibiting changes in allelic pattern in three of the evaluated SNAP marker loci (locus Pto-52, Pto-72, and Pto-355). Fig. 4 presents the changes in banding pattern for the evaluated SNAP markers. The calculated percentages of plantlet fidelity were indicated by percentages of similar SNP allele profiles to the original mother plant of *Phalaenopsis amabilis*. Table 4 presents the plantlet and the locus identity exhibiting variant SNP alleles. As much as 88.2 % of regenerated plantlets showed genetic fidelity since their SNAP marker profiles were the same as their mother plants (Table 4). While, 11.8 % of the regenerated plantlets showed changes in allele profiles in three SNAP markers than the mother plants, indicating they might be the variant plantlets. Furthermore, base substitution mutation frequency can be estimated using the occurrences of such SNAP allele variants. The estimated substitution mutation frequencies among the 18 loci of SNAP markers in the regenerated plantlets were 2.5 x 103 (0.25 %). The estimation was calculated using data presented in Table 4 (3 allele changes/[18 loci x 2 x 34 plantlets]).

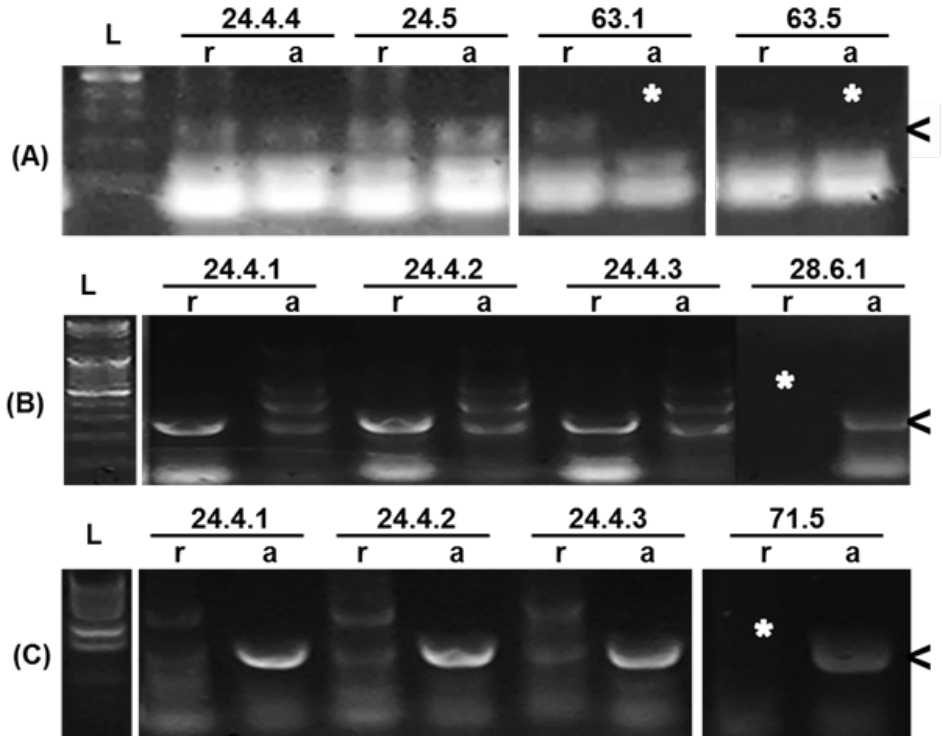

Fig. 4. The changes in allele banding patterns of the three SNAP marker loci among regenerated *Phalaenopsis amabilis* plantlets. Changes in allele banding patterns at the (A) Pto-52, (B) Pto-72, and (C) Pto-355 SNAP loci found among regenerated plantlets. The r – represented reference allele and the a – alternative allele. The < indicated the positions of the expected amplified products. The * indicated the plantlet showed allele changes in the respective loci. The L indicated 100 bp. DNA size ladders

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Table 4. Percentages of plantlet fidelity as indicated by similarities and differences to the original mother plant of *Phalaenopsis amabilis* for the eighteen loci of SNAP markers generated using the *Pto* gene

Mother plants ID	Σ plantlets evaluated	% of plantlets fidelity	The plantlets and the locus identity exhibited the variant SNAP allele
24	10	100.0	-
28	9	88.9	28.6.1 (Locus Pto-72)
30	5	100.0	-
63	5	60.0	63.1 (Locus Pto-52);63.5 (Locus Pto-52)
71	5	80.0	71.5 (Locus Pto-355)
Total	34	88.2	4 plantlets

In previous studies, a successful detection of somaclonal variation in *Phalaenopsis* using molecular markers has been reported. Khoddamzadeh et al. (2010) have used RAPD markers to detect somaclonal variation among PLBs of *Phalaenopsis bellina*. The results indicated there were no differences in RAPD profiles between the mother plant and the regenerated three months old culture of PLBs. However, they detected 17 % in RAPD marker profile variations among the proliferated, six months old culture of PLBs. In various elucidation using ISSR markers, Samarfard et al. (2014) reported the presence of 20 % in ISSR marker profile variations in *Phal. gigantea* among the mother plant and the 20 weeks old culture of secondary PLB. In this research, the presence of 11.8 % plantlet variants was reported based on SNAP marker analysis. Although gene-specific and co-dominant SNAP marker based on nucleotide sequence variations of *Pto* gene were used, this finding was in line with the previous study using genome-wide dominant markers of either RAPD or ISSR (Khoddamzadeh et al., 2010; Samarfard et al., 2014).

Other studies have also confirmed the presence of induced SNP changes during plant tissue culture. In *Arabidopsis thaliana*, the spontaneous mutation rate is about 7 x 10^{-9} base substitutions per site per generation, as estimated by whole-genome sequencing with individuals after 30 generations (Ossowski et al., 2010). As estimated by whole-genome sequencing after approximately 20 replications in the rice cell culture, the rice mutation rate was 1.74 x 10^{-6} base substitutions per site (Miyao et al., 2012). Although the SNP changes based on SNAP marker profiles occurring among plantlets regenerated from PLBs of *Phal. Amabilis* have been estimated, further research to accurately elucidate the base substitution mutation frequency in the Phalaenopsis genome is needed.

A simple method for *Phalaenopsis amabilis* clonal propagation by inducing PLBs from leaf explants on medium supplemented with Thidiazuron (TDZ) and PVP has been developed. The best medium for PLB induction from leaf explants was the ½ MS medium supplemented with 3 mg L^{-1} TDZ and 0.5 g L^{-1} PVP. The media pH for PLB induction were adjusted into pH = 7 for efficient PLB regeneration from injured leaf explants. For PLB proliferation, the best medium was the ½ MS medium supplemented with 0.5 mg L^{-1} TDZ. Subsequently, Phal. amabilis clonal propagation can be done by culturing young leaf explants of the selected mother plants using those define methods. Clonal fidelity is important in clonal propagation of *Phalaenopsis*. This study using SNAP markers, developed based on nucleotide sequences variation of the *Pto* gene, indicated the presence of at least 11.8 % possible variants. These variants may have changed their responses to disease infection. Previous study have indicated that the variation in *Pto* based-SNAP marker profile might be associated with differences in the soft rot disease response (Sukma, Elina, Giyanto, & Sudarsono, 2017). Since phenotypes of the regenerated plantlets have not been evaluated, association among the changes in SNAP allele profiles to the disease infection responses cannot be established yet. To validate the presence of somaclonal variations, regenerated plantlets have to be established under ex vitro conditions and evaluated for the presence of morphological, flower, responses to diseases infection, and other character variations. Such data will be collected in different studies.

CONCLUSION

The best medium composition for PLB induction from leaf explants and protocorm of Phalaenopsis amabilis was the ½ MS medium supplemented with 3 mg L^{-1} TDZ and 0.5 mg L^{-1} PVP.

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The medium pH should be adjusted to 7 for PLBs induction from leaf explants. There were 11.8 % possible variants out of 34 evaluated plantlets based on the assessment results using 18 loci of *Ph-Pto* SNAP markers. The estimation used the numbers of detected mutant alleles, the mutation frequencies at 18 *Ph-Pto* SNAP marker loci were 2.5 x 103 or 0.25 %. Since changes in SNP allele may not always result in phenotype changes, the frequency of allele variant occurrences do not always affect the phenotype fidelity of Phal. amabilis plantlets. However, further phenotype fidelity studies among plantlets regenerated using the outlined procedures in the future are suggested.

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