BIODIVERSITAS
Volume 22, Number 3, March 2021
Pages: 1077-1090

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d220301

Morphological, molecular and resistance responses to soft-rot disease variability among plantlets of *Phalaenopsis amabilis* regenerated from irradiated protocorms

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Manuscript received: 20 November 2020. Revision accepted: 28 January 2021.

Abstract. *Putri HA, Purwito A, Sudarsono, Sukma D. 2021. Morphological, molecular and resistance responses to soft-rot disease variability among plantlets of* Phalaenopsis amabilis *regenerated from irradiated protocorms. Biodiversitas 22: 1077-1090. Phalaenopsis amabilis* (L.) Blume is a prominent donor for the white petal and sepal trait in *Phalaenopsis* breeding. However, it has an undesirable character, such as susceptible to soft-rot disease. Therefore, developing soft-rot resistance mutants through gamma irradiation could be explored. This study aimed to evaluate the variability of plantlets regenerated from irradiated and non-irradiated protocorms using morphology, stomatal size and molecular markers and to test responses of the plantlets against soft-rot disease. The plantlets were regenerated from irradiated (5, 10, 15 or 20 Gy) and non-irradiated protocorms. The results showed that *P. amabilis* plantlet variants were successfully identified based on their leaf morphology and stomatal size variations. A few plantlets have low stomatal densities, large stomatal size, and high chloroplast numbers, which indicated they were polyploids. Leaf disc assay for soft-rot disease response grouped most of the plantlets into very susceptible or susceptible. Moreover, four soft-rot resistant plantlets regenerated from irradiated and non-irradiated protocorms were successfully identified. The resistant plantlets were identified after three consecutive periods of inoculations with pathogens causing soft-rot disease. The evaluation also confirmed nucleotide variation in the *Pto* gene isolated from different levels of plantlet variant resistance responses.

Keywords: *Dickeya dadantii*, irradiation, *Phalaenopsis amabilis*, *Pto* gene, SNAP markers, soft-rot disease

INTRODUCTION

Phalaenopsis amabilis ssp. *amabilis*, a subspecies in the *P. amabilis* complex, is distributed widely in the southern part of the Philippines (Palawan) and the western part of Indonesia (Borneo, Sumatera, and Java) (Tsai et al. 2015). It is one of the native orchids species in Indonesia (Handoyo and Prasetya 2006). The other known species in the complex of *Phalaenopsis* include *P. amabilis* ssp. *moluccana*, *P. amabilis* ssp. *rosenstromii*, *P. aphrodite* ssp. *aphrodite*, *P. aphrodite* ssp. *formosana* and *P. sanderiana* (Christenson 2001). *Phalaenopsis amabilis* has flower characteristics that are favorable in the horticultural industry. Therefore, solving various problems associated with *P. amabilis* cultivation in Indonesia will help further develop this important *Phalaenopsis* species.

Flowers of *P. amabilis* have a white color and a large size of both petals and sepals. *Phalaenopsis amabilis* is used as the main source of white petal and sepal characters for developing advanced white hybrid varieties (Tang and Chen 2007). *P. amabilis* flowers well under natural conditions in either the low elevation or the high elevation regions and this orchid species grow in broad geographic ranges (Tsai et al. 2015). In general, the flower inflorescences of *Phalaenopsis* stay fresh for 2-4 months (Bose et al. 1999). The long inflorescence vase life is a good character for this important floriculture commodity (Guo et al. 2012; Liu et al. 2013). Unfortunately, *P. amabilis* species is susceptible to soft rot disease (Sukma et al. 2017; Raynalta et al. 2018) due to *Dickeya dadantii* infection (Sudarsono et al. 2018), which is undesirable under tropical environments. Therefore, the development of soft rot resistance *P. amabilis* variety is important to support resistance breeding of this orchid species.

Although soft rot disease exists in orchid nurseries worldwide (Keith et al. 2005), the disease is even more devastating under tropical conditions (McMillan et al. 2007; Joko et al. 2011). Leaf of *Phalaenopsis* infected with soft rot disease shows small-water-soaked spots as initial symptoms. The symptoms spread rapidly in the initially infected leaf and result in soft rot having a foul odor. Optimum conditions for bacterial growth result in the spread of symptoms throughout entire plants within 2-3 days (Joko et al. 2011) and the plants could die. If available, soft-rot resistance *P. amabilis* may be used as a donor for developing soft-rot resistance lines through breeding programs (Raynalta et al. 2018). Unfortunately, *P. amabilis* accession having such resistance mechanisms has never been identified. Most of the *P. amabilis* accessions from West Java, Indonesia, are very susceptible to soft-rot disease (Sukma et al. 2017; Sanjaya et al. 2020). Development of new *P. amabilis* genetic variants having

soft-rot resistance characters through such an alternative approach as somaclonal variation and induced mutation may be conducted.

The induced mutation has been used to widen orchid genetic diversity (Thinh et al. 2011) while somaclonal variation arises among tissue culture regenerated plantlets (Kaeppler et al. 2000; Bairu et al. 2011; Wang and Wang 2012; Currais et al. 2013; Ajijah et al. 2016). Although it is undesirable in clonal propagation, somaclonal variation may be useful to orchid breeders since they provide novel or beneficial variants (Khrisna et al. 2016). Reported phenotypic variants among *Phalaenopsis* plantlets include changes in leaf and shoot characters, ploidy levels and peloric or semi-peloric characters (Chen et al. 2008). Such somaclonal variations occurred in *P. gigantea* (Samarfard et al. 2014), *P.' Wedding Promenade'* (Lee et al. 2017), and *P. amabilis* (Raynalta et al. 2018). Meanwhile, somaclonal variation has contributed to the generation of new variants in several ornamental species, such as Saintpaulia (Matsuda et al. 2014); Chrysanthemum (Miler and Zalewska 2014); gerbera (Bhatia et al. 2009; 2011) and *Hedicyum coronarium* (Parida et al. 2013).

Moreover, disease resistance mutants have been regenerated through induced mutation in other plants such as abaca, banana, coffee and peanut (Yusnita et al. 2005; Purwati et al. 2007; Ibrahim et al. 2018; Indrayanti et al. 2018; Li et al. 2019). Furthermore, irradiation may be employed to augment variation among regenerated plantlets (Jain 2005; 2012) and yielded abiotic stress-tolerant (Widoretno et al. 2003; Penna et al. 2012; Widoretno et al. 2012; Oladosu et al. 2015; Rahayu and Sudarsono 2015; Suprasanna et al. 2016) and biotic stress resistant (Yusnita et al. 2005; Purwati et al. 2007; Rai et al. 2011; Sutanto et al. 2014; Indrayanti et al. 2018) mutant lines. Therefore, somaclonal variation and induced mutation by gamma irradiation might also be used as an alternative approach to generating soft-rot disease resistance *P. amabilis* mutants.

Changes in morphology and ploidy levels may indicate either somaclonal or induced mutations (Chen et al. 2008; Lee et al. 2017). However, such morphological variations may also be due to epigenetics or environmental factors (Landey et al. 2015; Anil et al. 2018). Moreover, somaclonal variation was detected using various techniques, including morphological, cytological, and molecular analyses (Bairu et al. 2011; Samarfard et al. 2014; Landey et al. 2015; Ghimire et al. 2016; Sebastiani and Ficcadenti 2016; Lee et al. 2017). Genetically controlled somaclonal or induced mutations may be evaluated using various molecular markers, such as RAPD, ISSR, AFLP and SNAP markers or by sequencing of certain target genes (Chen et al. 2004, 2008; Samarfard et al. 2014; Raynalta et al. 2018).

Limited reports are available discussing the existence of certain nucleotide sequence variability of the target genes and their possible association with the mutant phenotypes. *Pseudomonas syringae* pv. tomato (*Pto*) resistance gene is the gene associated with hypersensitive responses against bacterial pathogens (Xiao et al. 2001; Choi et al. 2011). Raynalta et al. (2018) and Elina et al. (2017) have shown *Pto* nucleotide sequence variability among *Phalaenopsis* species. Those researchers have also shown the limited association between *Pto* nucleotide sequence variability and the level of resistance against soft-rot disease. Therefore, further studies to evaluate the association among PTO nucleotide sequence variability and the associated responses of the putative *P. amabilis* plantlet variants against soft-rot disease will be beneficial.

This study aims to evaluate in vitro plantlet morphological and stomatal characters of *P. amabilis* regenerated from irradiated protocorms, examine the presence of *Pto* nucleotide sequence variability among the regenerated plantlets based on SNAP marker analysis and determine the responses of the plantlets against soft-rot disease to identify the existence of soft-rot resistance variants among the regenerated plantlets. The evaluation results indicated the presence of variability among in vitro plantlets of *P. amabilis* regenerated from irradiated protocorms for several leaf characters, stomatal sizes and densities, and chloroplast number in guard cells-indicating the presence of different ploidy levels. Changes in PTO gene nucleotide sequences were also observed among the evaluated *P. amabilis* plantlets. Moreover, higher resistance responses of a few regenerated plantlets than the original *P. amabilis* mother plants were also observed. In this paper, the possible occurrences of induced soft-rot disease resistance mutants of *P. amabilis* plantlets regenerated from irradiated protocorms and *Pto* nucleotide sequences due to irradiation are discussed.

MATERIALS AND METHODS

Plant materials

A flower of *P. amabilis* was self-fertilized and the developed pod was harvested five months after pollination. After pod sterilization, the seeds were sown on Knudson C medium for six months and the developed protocorms were irradiated using gamma-ray at either 5, 10, 15, or 20 Gy. The irradiated protocorms were germinated in half-strength MS medium (Murashige and Skoog 1962) supplemented with coconut water (15%) and activated charcoal (2 g/L) for 16 weeks. Subsequently, the developed protocorms were transferred into the Hyponex medium containing activated charcoal (2 g/L) and continually sub-cultured for every 12 weeks to the same fresh medium until they developed into plantlets. In this study, we randomly sampled 25 in vitro regenerated plantlets having at least 3-4 leaves (Figure 1) for each irradiation level and used them for analysis.

In vitro plantlet morphological characterization

Leaf morphological characters (thickness, shape, tip shape, surface texture, and symmetry) were characterized according to Orchid Ornamental Plant Characterization Guide (IOCRI 2004). Leaf thickness was measured on the tip and the middle part of the biggest and fully developed leaf using calipers. Both leaf and leaf tip shapes were classified into seven types, leaf edge shape into three types, leaf surface texture into one, and leaf symmetry into two

(Table 1). The frequency of leaf morphological characters among the evaluated plantlets was counted and the percentage was calculated.

In vitro plantlet stomatal characterization

Fully developed leaf (ca. length=5 cm, width=1 cm) samples were taken from in vitro grown plantlets. One leaf sample was taken from each plantlet for stomatal characterization. The adaxial side of the epidermal layer was peeled off from leaf samples and used for stomatal characterization. The characterization was conducted under an Olympus CX23LEDRFS1 microscope with 40× magnification. Stomatal density (SD), stomatal length (SL), stomatal width (SW) and chloroplast number (CN) in each guard cell were recorded. Stomatal counting was done in triplicate with Image J software (Schneider et al. 2012), and stomatal density (SD) was calculated per mm^2. Stomatal length and width were measured in triplicate for each leaf sample with Image Raster 2.1 software (Miconos, Indonesia). The chloroplast numbers were also counted in triplicate for each leaf sample, both manually with Image J software (Schneider et al. 2012) and automatically using CellProfiler software (Carpenter et al. 2006). The stomatal character and chloroplast number data were analyzed for boxplot using Statistical Tool for Agricultural Research (STAR, IRRI 2013) and cluster gram heatmap using R package d3heatmap software (Cheng et al. 2016).

Plantlet responses against soft-rot disease

The bacterial isolate causing the soft-rot disease was isolated from infected *Phalaenopsis* sp. leaf and showed soft-rot symptoms (Figure 1). To minimized contamination of other microbes, the leaf surface of the infected sample was wiped using alcohol (70%). Subsequently, part of the infected leaf was inserted in a 15 ml sterile plastic tube containing 9 ml of sterile ddH_2O and hand shook to release the leaf sap. Leaf sap suspension (1 mL) was taken from the plastic tube and diluted 10× using sterile ddH_2O. Three times serial dilutions (10×, 100× and 1000×) were subsequently done and the suspensions (100 µL) were plated into nutrient agar (NA) medium. The growing bacterial colonies were subjected to Koch Postulate and the single colony bacterial isolate causing soft-rot symptoms in *P. amabilis* leaf disc assay was used for resistance evaluation. The selected bacterial isolate was inoculated into liquid Lactose broth (LB, 15 mL) medium, shaken for 21 h at 100 rpm and harvested by centrifugation at 8000 rpm for 6 min. The bacterial pellets were washed twice using liquid LB medium and resuspended in sterile ddH_2O to a final volume of OD_{600}=0.2. For subsequent resistance evaluation of the *P. amabilis* plantlets, the fresh bacterial suspension was diluted 10×.

Detached leaf disc assay for evaluating resistance response was done following Sudarsono et al. *(*2018). Leaf disc (2-4 cm^2) was taken from in vitro plantlets, the middle of the leaf discs was injured using a needle and 10 µL of bacterial suspension was deposited in the injured tissue. The inoculated leaf discs were incubated in a plastic box under 100% humidity. Three leaf discs were tested for each plantlet. Occurrences of soft-rot symptoms were recorded at 72 hours after inoculation and the soft-rot diameters were noted. Disease intensity was calculated based on criteria as described previously (Sudarsono et al. 2018).

Plantlet molecular characterization using SNAP marker

Total genomic DNA was isolated from the leaf of 95 plantlet samples and the original mother plant of *P. amabilis*. DNA isolation was done using the standard CTAB method (Doyle and Doyle 1990) with minor modifications that have routinely been used for various orchid species and tropical perennial crops (Handini 2014; Sutanto et al. 2014; Elina et al. 2017, Sukma et al. 2017; Sudarsono et al. 2017; Pesik et al. 2017; Raynalta et al. 2018). Eleven loci of single nucleotide amplified polymorphism (SNAP) markers were used for molecular characterization of the in vitro plantlets regenerated from non-irradiated and irradiated protocorms. The SNAP marker loci were developed previously based on the *Pto* gene nucleotide variability (Elina et al. 2017).

Table 1. Leaf morphological diversity among *Phalaenopsis amabilis* plantlets regenerated from irradiated and non-irradiated protocorms

Leaf characters	Plantlets population origin	
	Control (non-irradiated protocorm) (%)	Irradiated protocorm (%)
Leaf shape		
Subulate	5	3
Linear	0	4
Oblong	0	1
Lanceolate	95	67
Oblanceolate	0	8
Ovate	0	15
Obovate	0	1
Leaf apex shape		
Acute	50	58
Acuminate	5	4
Apiculate	18	11
Mucronate	0	11
Obtuse	18	8
Truncate	0	1
Retuse	9	5
Leaf edge shape		
Entire	77	77
Undulate	18	22
Erose	5	1
Leaf surface texture		
Glabrous	100	100
Leaf symmetry		
Symmetry	50	56
Asymmetry	50	44

Note: The evaluated number of *Phal. amabilis* plantlets were 22 for those regenerated from non-irradiated protocorms and a total of 73 for those regenerated from irradiated protocorms

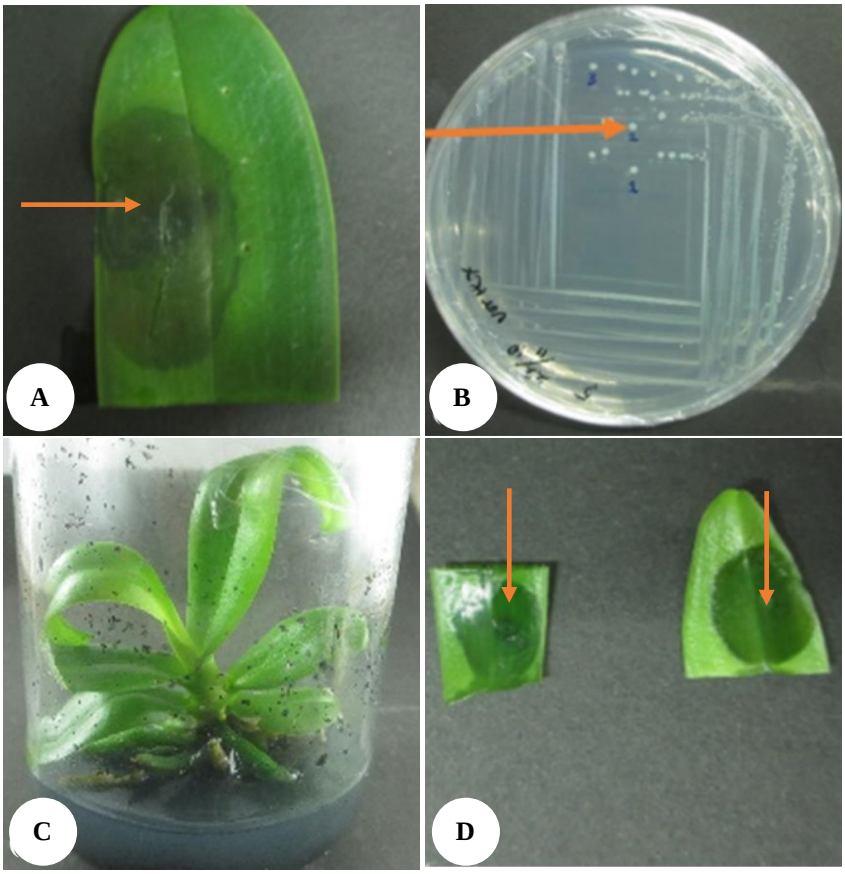


Figure 1. Inoculum sources and regenerated plantlets of *Phalaenopsis amabilis* used in the leaf disc assay for response against pathogens causing soft-rot disease. A. Soft-rot disease symptom of an infected leaf (leaf (blackish area with arrow) were used as the source of bacterial pathogen isolation, B. Single colony bacterium isolated from infected leaf sample was used as inoculum in leaf disc assay, C. Representative regenerated plantlet used in leaf disc assay against soft-rot disease, and D. Representative symptoms of susceptible responses in leaf disc assay for resistance against soft-rot disease

PCR primers consisted of three oligonucleotides that were used to generate each of the evaluated SNAP marker loci. Reagents for each PCR amplification (total 12.5 µL) consisted of total genomic DNA (4 µL), each of the three-primer set (0.25 µL), 10× PCR Ready Mix (1.25 µL, KAPA Biosystem), and ddH_2O (6.5 µL). The PCR amplification used the following steps: a pre-denaturation (95ºC for 3 min.); followed by 35 amplification cycles, each cycle consisted of denaturation (95ºC for 15 sec.), primer annealing (47.9-59.8ºC for 15 sec., depending on the appropriate primer Ta), primer extension (72ºC for 1 sec.); and a final primer extension (72ºC for 10 min.) as recommended by the KAPA Biosystem PCR kit.

The PCR amplicons were fractionated in a 1× sodium borate (SB) agarose (2%) gel electrophoresis at 50 V for 35 min. The gel was stained using GelRedTM (Biotium Inc.), visualized on a UV transilluminator and the gel image photographed using a digital camera. Allele scoring was done manually using the gel image and the observed alleles were recorded as genotypes of the evaluated plantlets. The genotype data were analyzed using Cervus 3.0.7 software (Kalinowski et al. 2007) for parentage analysis and calculated the PIC values. Unrooted Weighted Neighbour-Joining the phylogenetic tree was constructed based on the genetic dissimilarity using DARwin 6.0.14 software (Perrier and Jacquemoud-Collet 2006).

RESULTS AND DISCUSSION

In vitro leaf morphological diversity

The identified leaf morphology of the *P. amabilis* in vitro plantlets regenerated from irradiated and non-irradiated protocorms was summarized in Table 1. The plantlets regenerated from non-irradiated protocorms (Table 1) mostly had the lanceolate leaf shape (95%) and only a few were subulate (5%). In contrast, the seven leaf shape types existed among the combined plantlets regenerated from the irradiated protocorms (Table 1). However, most of the plantlets regenerated from irradiated protocorms (Table 1) have a lanceolate leaf shape (67%), while the percentages of other leaf shape types ranged from 1% (oblong and obovate) up to 8% (ovate). The plantlets regenerated from irradiated protocorms exhibited a more variable leaf shape than those of non-irradiated ones (Table 1).

The percentages of leaf apex shape types among the plantlets regenerated from either irradiated or non-irradiated protocorms were dissimilar (Table 1). Leaf apex shape of plantlets regenerated from non-irradiated protocorms was distributed among five different types. On the other hand, the leaf apex shape of those regenerated from irradiated protocorms was distributed among seven different types. The mucronate (11%) and truncate (1%)

leaf apex shapes were only observed in the plantlets regenerated from irradiated protocorms. As for the leaf edge shape, the leaf surface texture, and leaf symmetry characters, plantlets regenerated from irradiated and non-irradiated protocorms were similar (Table 1). There was no variability among leaf thickness character~~,~~ among regenerated plantlets, either from irradiated or non-irradiated protocorms. The average of leaf thickness among plantlets regenerated from irradiated protocorms was 0.09-0.10 mm (ranged: 0.05-0.11 mm), while those from non-irradiated protocorms were 0.10 mm (ranged: 0.09-0.15 mm).

In vitro plantlet stomatal characters

The evaluated stomatal characters of plantlets include stomatal length, width, and density and the observed results were presented in Figure 2.A, B, and C. Meanwhile, Figure 2.D. presented the average number of chloroplasts in each guard cell of plantlets regenerated from the irradiated and non-irradiated protocorms. Plantlets regenerated from irradiated protocorms at 15 and 20 Gy have larger stomatal length and width than those from irradiated protocorms at either 5 or 10 Gy or from non-irradiated protocorms. Moreover, some plantlets regenerated from irradiated protocorms at 15 Gy tended to have the lowest stomatal density (Figure 2.C). The experiments further indicated a negative association among stomatal length and stomatal width to stomatal density.

Meanwhile, the average numbers of chloroplast in each guard cell among the evaluated plantlets were presented in Figure 2.D. Individual plantlets with a high number of chloroplast in each guard cell were those regenerated from irradiated protocorms at 5 and 15 Gy. Guard cells of plantlets regenerated from irradiated protocorms at 10 Gy tend to have the same chloroplast numbers as those of non-irradiated ones (Figure 2.D).

The heatmap cluster gram analysis for all plantlets using the stomatal characters and the chloroplast numbers in the guard cells ~~were~~was presented in Figure 3.A. Based on the heatmap cluster gram analysis, there were two main groups of plantlets. It showed group I of plantlets tended to have low chloroplast numbers, stomatal length, stomatal width and high stomatal density. The group II of plantlets tended to have high chloroplast numbers, stomatal length, stomatal width and low stomatal density (Figure 3.A). Fig 3.B presented only 13 plantlets belonging to group II. Most of the plantlets belonging to group II were regenerated from irradiated protocorms at 15 Gy (9 plantlets), 10 Gy (1 plantlet), 5 Gy (1 plantlet) and from non-irradiation treatment (0 Gy, 2 plantlets), respectively (Figure 3.B). Representative photographs of two contrasting plantlets having different stomatal characters were presented in Figure 4. The IP 0 Gy-19 plantlet was regenerated from non-irradiated protocorms; it has high stomatal density and small stomatal size (Figure 4.A, B). In contrast, the IP 15 Gy-81 plantlet was regenerated from irradiated protocorms at 15 Gy; it has low stomatal density and large stomatal size (Figure 4.C, D).

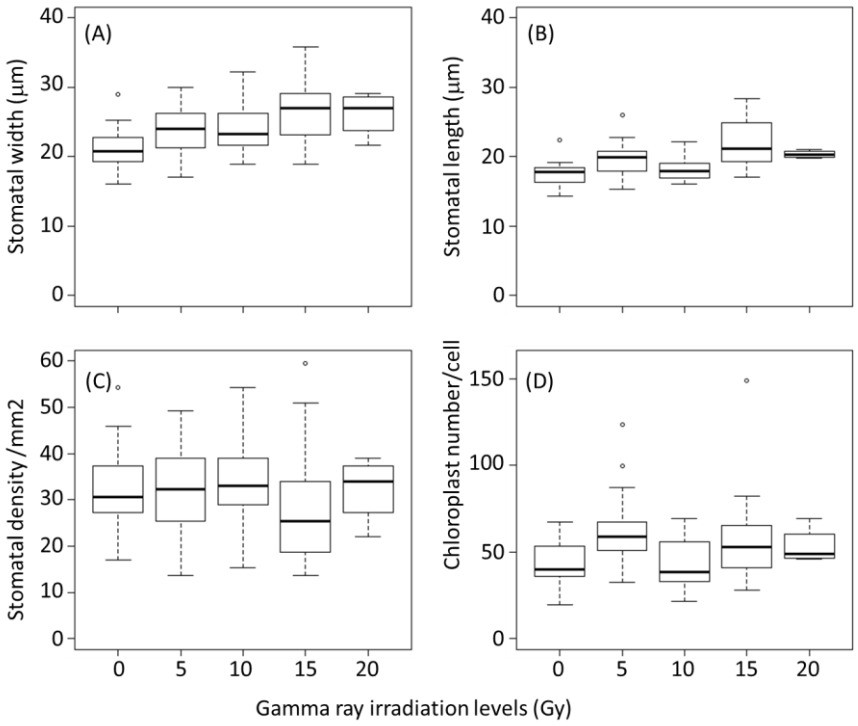
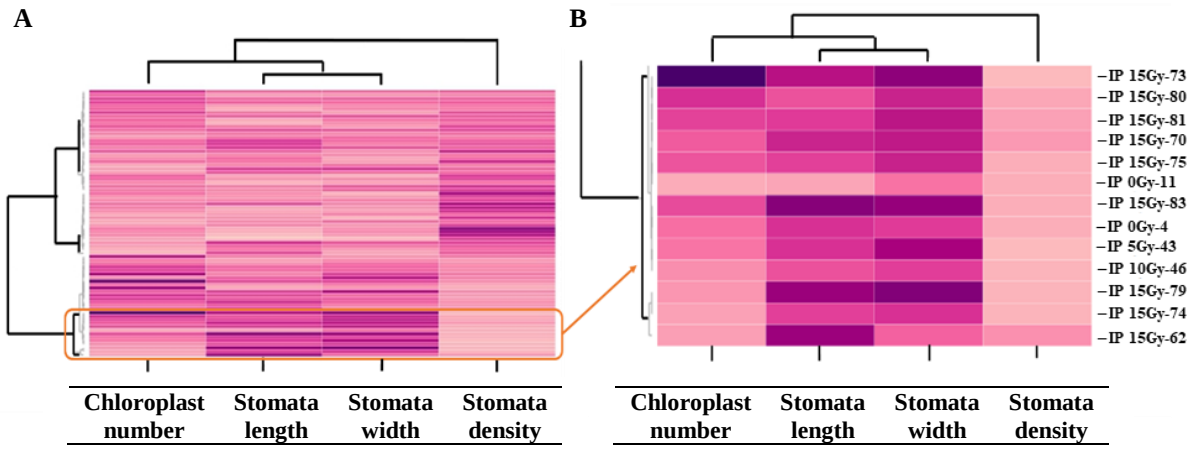


Figure 2. Boxplot analysis of stomatal character (stomatal width, length, stomatal density and chloroplast numbers in guard cells) variability among *Phalaenopsis amabilis* plantlets regenerated from irradiated versus non-irradiated protocorms



Number of chloroplast	Stomata length (µm)	Stomata width (µm)	Stomata density/mm^2
22-40	16-18	14-15	14-17
41-62	19-21	16-18	18-22
63-78	22-25	19-21	23-39
79-100	26-28	22-24	40-51
101-137	29-36	25-28	52-59

Figure 3. Heatmap cluster grams based on the stomatal characters of *Phalaenopsis amabilis* plantlets regenerated from irradiated and non-irradiated protocorms. A. Clustergram of all plantlets. B. Clustergram of 13 plantlets, having a high stomatal size, high chloroplast numbers and low stomatal density.

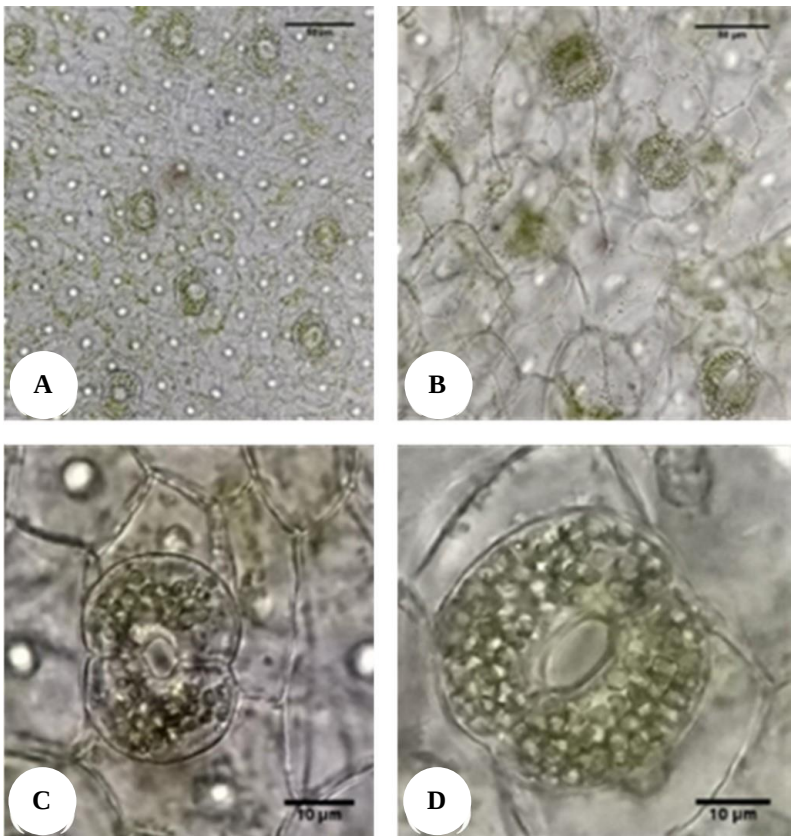


Figure 4. Representative photographs of *Phalaenopsis amabilis* (IP 0 Gy-19 and IP 15 Gy-81) plantlets that have different stomatal characters. The IP 0 Gy-19 plantlets were regenerated from non-irradiated protocorms, with high stomatal density and small stomatal size, while The IP 15 Gy-81 was regenerated from irradiated protocorms at 15 Gy, with low stomatal density and large stomatal size

Plantlet responses to soft-rot disease

A summary of the responses against the soft-rot disease of *P. amabilis* plantlet was presented in Table 2. The responses against the soft-rot disease were grouped into either resistance, moderately resistance, moderately susceptible, susceptible, or very susceptible (Table 2). The total evaluated plantlets regenerated from non-irradiated protocorms were nine plantlets and most of them were very susceptible (56%) (Table 2). The other plantlets were susceptible (22%), moderately susceptible (11%), and resistant (11%) (Table 2). On the other hand, the total evaluated plantlets regenerated from irradiated protocorms were 18 plantlets and most of them were also very susceptible (67%) (Table 2). The other plantlets were susceptible (11%), moderately susceptible (6%), and resistant (17%) (Table 2).

The representative of leaf disc assay for responses against the soft-rot disease was presented in Figure 5. Most of the susceptible plantlets (IP 0 Gy-8, IP 05 Gy-38, IP 15 Gy-71 and IP 15 Gy-83) exhibited soft-rot symptoms within 72 hours after inoculation (Figure 5). On the other hand, the resistance plantlets showed either very limited symptoms (IP 05 Gy-33) or did not show any soft-rot symptom (IP 0 Gy-1, IP 05 Gy-23, and IP 05 Gy-31), even after three consecutive inoculations within 3 × 72 hours (Figure 5). Among the four identified resistant plantlets, one was previously suspected as polyploid based on its stomatal sizes and chloroplast numbers of the guard cell (plantlet no. IP 15 Gy-83).

Plantlet variability based on SNAP marker

Summary of the molecular analysis of 95 putative variants and the original *P. amabilis* mother plant using 11 SNAP marker loci was presented in Table 3. The SNAP loci were developed based on PTO nucleotide sequence variability (Elina et al. 2017; Sukma et al. 2007; Raynalta et al. 2018). The evaluated SNAP marker loci yielded amplicon sizes of approximately 205-294 bp (Table 3). The average PIC of each SNAP marker locus ranged from 0 to 0.4 (Table 3). Pto-181, Pto-37 and Pto-355 loci yielded monomorphic or a low polymorphism across the evaluated samples and had PICs of 0-0.01. The other seven SNAP marker loci exhibited an average PIC of 0.4 (Table 3).

The gel image representative for PCR amplicon generated at the Pto-424 SNAP marker locus was presented in Figure 6. The evaluated plantlets no. 1-22 were regenerated from non-irradiated protocorms. Results in Figure 6 indicated one plantlet (no. 17) could not generate both reference and alternate alleles. The genotype of plantlet no 17 for the Pto-424 SNAP locus could not be determined since both alleles for the Pto-424 SNAP locus were not generated. Four plantlets were only able to amplify either reference or alternate allele, which indicated that genotypes for the Pto-424 SNAP locus were homozygous for either the reference (TT) or the alternate (CC) alleles. Most of the evaluated plantlets were able to generate both reference and alternate alleles. Therefore, the genotype of these plantlets for the Pto-424 SNAP was a heterozygous (CT).

Figure 7. presented the gel image for PCR amplicon generated at the Pto-380 SNAP marker locus. The evaluated plantlets no. 76-95 were regenerated from irradiated and non-irradiated protocorms. Results in Figure 7 indicated one plantlet (no. 86) could not generate the alternate allele. Therefore, the plantlet no 86 genotype for the Pto-380 SNAP marker locus was homozygous for reference (AA) allele. Most of the evaluated plantlets were able to generate both reference and alternate alleles (Figure 7). Therefore, the genotype of these plantlets for the Pto-380 SNAP marker locus was a heterozygous (CT).

Results of the genotyping using 11 SNAP marker loci were subsequently used to construct a phylogenetic tree. The generated phylogenetic tree was presented in Figure 8. Results in Figure 8. indicated that the evaluated plantlets were grouped into three main groups (Group I, II, and III). Group I consisted of only the *P. amabilis* original mother plant and Group II consisted of only two plantlets (no. 14 and 95). Meanwhile, Group III members were divided into Sub-group IIIa and Sub-group IIIb (Figure 8). Only three plantlets belonged to Sub-group IIIa (Plantlet no. 24, 31 and 90). Meanwhile, most of the evaluated plantlets belonged to Sub-group IIIb. The main group in Figure 8 consisted of a mixture of plantlets regenerated from irradiated and non-irradiated protocorms.

Table 2. The responses against soft-rot diseases of *Phalaenopsis amabilis* plantlets regenerated from irradiated and non-irradiated protocorms in the leaf disc assay

Responses against soft-rot disease in leaf disc assay	Percentages of plantlet responses from:	
	Non-irradiated (%)	Irradiated-protocorms (%)
Resistance	11	17
Moderately resistance	0	0
Moderately susceptible	11	6
Susceptible	22	11
Very susceptible	56	67

Note: The total *P.amabilis* plantlet numbers from non-irradiated protocorms were nine, while those from irradiated protocorms were 18

Table 3. The Pto-SNAP marker loci used in the molecular analysis of *Phalaenopsis amabilis* plantlets regenerated from irradiated and non-irradiated protocorms

Loci	Tm (ºC)	PCR product size (bp)	Polymorphic Information Content (PIC)
Pto-79	55.01	252	0.375
Pto-181	55.47	251	0.000
Pto-220	56.12	218	0.374
Pto-223	56.87	205	0.374
Pto-229	57.16	208	0.010
Pto-241	55.97	258	0.375
Pto-292	54.34	261	0.373
Pto-380	55.09	294	0.375
Pto-424	56.29	291	0.375
Pto-37	56.36	265	0.000
Pto-355	55.98	294	0.000

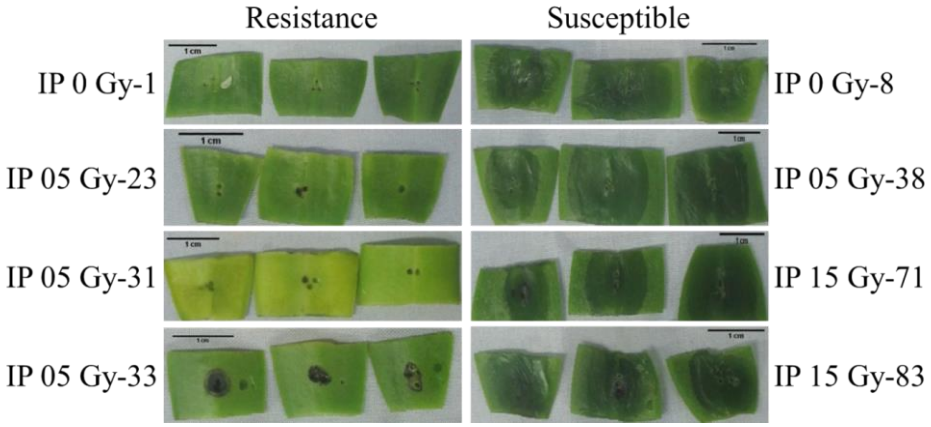


Figure 5. The representative results of soft-rot disease evaluation of *Phalaenopsis amabilis* plantlets regenerated from non-irradiated and irradiated protocorms, showing resistant (left) and susceptible (right) plantlets against soft-rot disease. IP 0 Gy-1, IP 05 Gy-23, IP 05 Gy-31 and IP 05 Gy-33 are representative resistance plantlets while IP 0 Gy-8, IP 05 Gy-38, IP 15 Gy-71 and IP 15 Gy-83 are representative susceptible plantlets. For susceptible plantlets, the photographs were taken 72 hours after inoculation. The resistance ones were inoculated with the bacterial isolates in three consecutive inoculations and the photographs were taken after 3 × 72 hours after initial inoculation.

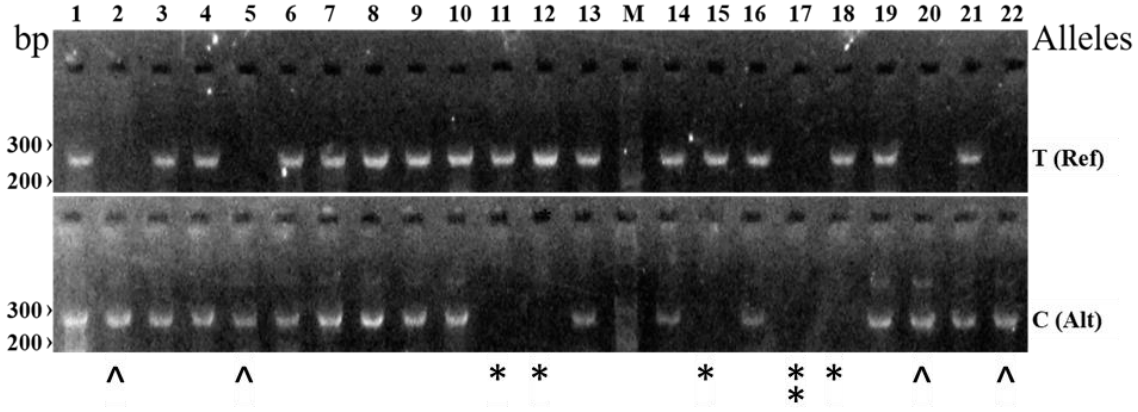


Figure 6. The gel image of PCR amplicons resulted from the Pto-424 SNAP marker analysis of 22 *Phalaenopsis amabilis* plantlet no. 1-22 regenerated from non-irradiated protocorms. M-100 bp DNA ladder. The T (Ref)-reference allele and the C (Alt)-alternate allele. ▵ Plantlets were unable to generate the reference allele. Their genotype for the Pto-424 SNAP marker locus was a homozygous CC. * Plantlets were unable to generate the alternate allele. Their genotype was a homozygous TT. ** Plantlet (no. 17)-unable to generate both the reference and the alternate alleles. Their genotype was unknown

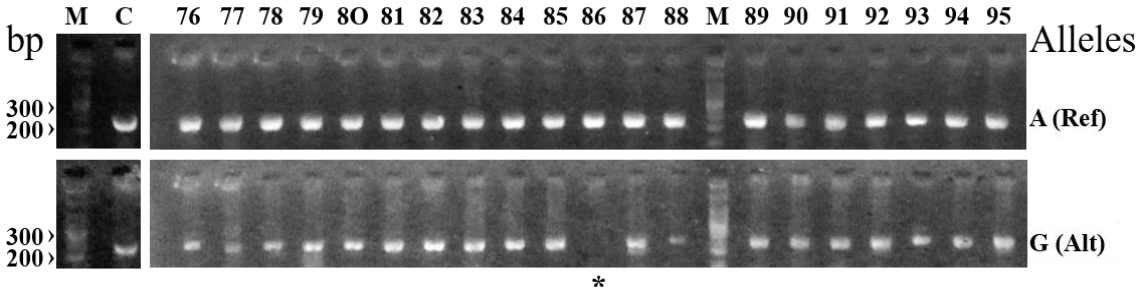


Figure 7. The gel image of PCR amplicons resulted from the Pto-380 SNAP marker analysis of 20 putative *Phalaenopsis amabilis* plantlet variant no. 76-95 regenerated from irradiated and non-irradiated protocorms. M-100 bp DNA ladder. C-amplicon profiles of the *P. amabilis* mother plant. The genotype of the mother plant for this locus was an AC heterozygous. The A (Ref)-reference allele and the G (Alt)-alternate allele. * Sample 86 (IP 20 Gy-86 plantlet) unable to generate alternate alleles. The genotype of the IP 20 Gy-86 plantlet changed into a homozygous AA. Except for sample 86, other sample genotypes for this locus are AC heterozygous

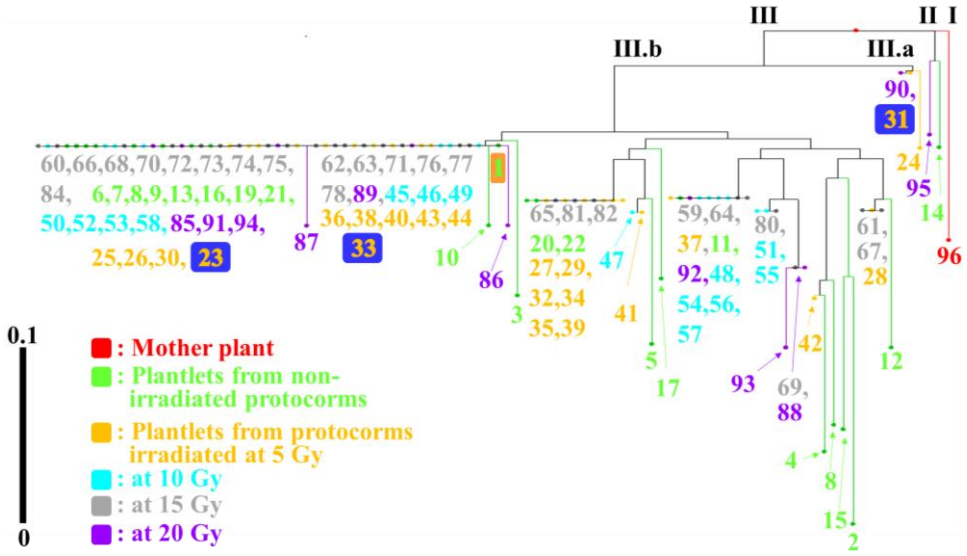


Figure 8. Unrooted Weighted Neighbour-Joining phylogenetic tree constructed based on the genetic dissimilarity as measured using 11 loci of Pto-based single-nucleotide amplified polymorphism (Pto SNAP) markers. The evaluated *Phalaenopsis amabilis* plantlet accessions and the irradiation treatments are indicated in the color labels. Sample no. 23, 31, and 33 were regenerated from 5 Gy irradiated protocorms, while no. 1 was from non-irradiated protocorm. Sample no. 1, 23, 31, and 33 were resistant plantlets, while the others were either susceptible or not tested against soft-rot disease

Discussion

Somaclonal variation may be undesirable in micropropagation through in vitro techniques (Hammerschlag et al. 1992; Taylor et al. 1995; Biswas et al. 2009; Miguel and Marum 2011; Mulanda et al. 2015; Park et al. 2009; Smulders and Klerk 2011; Us-Camas et al. 2014). Therefore, the early indicators for the existence of somaclonal variation are important to commercial plant growers. Fortunately, some of the observed phenotypic variants among the regenerated plantlets in tissue culture propagated planting materials are controlled by epigenetics (Miguel and Marum 2011; Mulanda et al. 2015; Park et al. 2009; Smulders and Klerk 2011; Us-Camas et al. 2014). We found some phenotypic variations such as leaf shape and leaf apex shape in the plantlet from irradiated protocorm in the present results. However, the stability of the phenotypes in the further generation is needed to be evaluated. The epigenetically controlled phenotypic variants eventually disappear as either the plantlet develops into mature plants or subsequent sexual generations (Kaeppler et al. 2000; Widoretno et al. 2003; Jablonka 2012).

However, to generate either somaclonal variants or mutants having certain desirable phenotypes, which is useful for breeding programs, the phenotype changes need to be genetically controlled. A few leaf morphological characters were reported as genetically controlled, such as leaf and leaflet shapes (Hibara et al. 2009; Ikezaki et al. 2010; Moon and Hake 2011). For some ornamentals such as *Begonia x elatior*, *Saintpaulia ionanta* and *Petunia*, leaf shape and morphology changes may be commercially valuable (Jain 1993; Abu-Qaoud et al. 2010). Unfortunately for *Phalaenopsis*, the changes in leaf morphological characters may not have significant commercial values. Therefore, as previously suggested (Bouiamrine et al. 2012) in durum wheat, the existence of leaf morphological changes among *Phalaenopsis* plantlets may only be useful for either somaclonal variation or mutation indicators.

In this research, the evaluated leaf morphological characters include leaf shape, apex shape, edge shape, surface texture, and symmetry. These leaf morphological characters are genetically controlled (Hibara et al. 2009; Ikezaki et al. 2010; Moon and Hake 2011). Leaf shape, leaf edge shape and leaf symmetry variations were similar among the irradiated and non-irradiated plantlet population and there was no variation for these morphological characters among the evaluated plantlets. On the other hand, variations were observed for the leaf and leaf apex shapes among the irradiated and non-irradiated plantlet populations. Plantlets regenerated from irradiated protocorms exhibited more diverse leaf and leaf apex shape than those of non-irradiated ones. Therefore, irradiation might induce more variation for the leaf and leaf apex shapes than that of somaclonal variation. Leaf morphology variation because of irradiation indicates the potential success in inducing variants among regenerated plantlets. However, further evaluation is needed to verify this finding.

Phenotype variants among plantlets may also result from changes in the chromosome numbers, as it has been revealed in *Coffea arabica* and *P.* 'Wedding Promenade' (WP) (Lee et al. 2017). In *C. arabica*, phenotypically normal plants have normal chromosome numbers, while the abnormal ones have abnormal chromosomal numbers (Landey et al. 2015). Stomatal characters are also known to correlate with the ploidy level (Chen et al. 2009; Winarto et al. 2010). Large stomatal size is usually associated with

higher ploidy levels (Yu et al. 2009; Azmi et al. 2015; Grosso et al. 2017). According to Grosso et al. *(*2017), tetraploid plants have a larger stomatal length than the diploid. Moreover, changes in stomatal sizes among plantlet variants or mutants may be used to indicate the ploidy level changes (Murti et al. 2012). The changes in stomatal size may also affect stomatal density (Allario et al. 2011). Leaves with a large stomatal size may have low stomatal density, while a small one may have a high density (Doheny-Adams et al. 2011). Therefore, both increased in stomatal sizes and reduced stomatal density may be used to indicate the occurrence of ploidy level changes, as has been stated by Grouh et al. (2011) and Tomiczak et al. (2015).

In this research, a few plantlets regenerated from irradiated and non-irradiated protocorms exhibited low stomatal density and large stomatal sizes. Those plantlets were probably polyploids. Chromosome counting and flow cytometry are effective methods to assess plant ploidy levels (Ochatt et al. 2011). For many plantlets, the ploidy level screening using flow cytometry is a cost-effective approach (Guo et al. 2012). Subsequently, the suspected polyploid plantlets regenerated from the irradiated and non-irradiated protocorms need further evaluation to confirm their ploidy levels, either by chromosome counting or flow cytometry analysis. However, no further evaluation was conducted in this experiment to confirm the ploidy levels of the regenerated plantlets. They will be done in other experiments once the plantlet acclimatization is completed.

The number of chloroplast organelles in the guard cells is also associated with the stomatal sizes. A large stomatal size is associated with a large guard cell (Franks and Beerling 2009; Taylor et al. 2012) and the large guard cell contains more chloroplast organelles (Ewald et al. 2009). Therefore, changes in chloroplast number in the guard cells may indicate the changes in ploidy level (Omidbaigi et al. 2010; Tang et al. 2010). The chromosomal abnormality may occur due to the endoreduplication, the process of repeated nuclear DNA replication without mitosis (Joubès and Chevalier 2000) or through many other processes (Holland and Cleveland 2009) resulting in various degrees of polyploidy (Tremblay et al.1999; Giorgetti et al. 2011). Endoreduplication has naturally occurred at a higher rate in *Orchidaceae* species than other plant families (Joubès and Chevalier 2000; Lee et al. 2004; Park et al. 2010).

In this research, the plantlets ~~having~~ with low stomatal density and large stomatal sizes also contained high chloroplast numbers in the guard cell, which confirmed they were probably polyploids. The putative polyploid plantlets include IP 15 Gy-73, IP 15 Gy-80, IP 15 Gy-81, IP 15 Gy-70, IP 15 Gy-75, IP 0 Gy-11, IP 15 Gy-83, IP 0 Gy-4, IP 05 Gy-43, IP 10 Gy-46, IP 15 Gy-79, IP 15 Gy-74 and IP 15 Gy-62. Most of those suspected polyploid plantlets of *P. amabilis* were regenerated from irradiated protocorms at 15 Gy. This finding was like those of Kumari et al. (2013), which showed irradiation at 15 Gy increased plantlet ploidy levels in *Chrysanthemum*. Polyploid *Phalaenopsis* species can be used as donor parents to develop commercial varieties (Chen et al. 2009).

The main objective of the induced mutagenesis using gamma irradiation in this experiment is to generate soft-rot disease-resistant mutants of *P. amabilis,* which is originally susceptible to this disease (Raynalta et al. 2018, Sukma et al. 2017; Fu and Huang 2011; Fu et al. 2011). Plantlets regenerated from irradiated and non-irradiated protocorms were mostly either susceptible or very susceptible to soft-rot disease. However, four resistance plantlets of *P. amabilis* were regenerated from our research, one plantlet from non-irradiated (IP 0 Gy-1) and three from irradiated (05 Gy) protocorms (IP 05 Gy-23, IP 05 Gy-31 and IP 05 Gy-33). The leaf discs of the four resistant plantlets showed either limited areas of tissue maceration (IP 05 Gy-33) or no tissue maceration (IP 0 Gy-1, IP 05 Gy-23 and IP 05 Gy-31) after three consecutive periods of inoculation. These resistance plantlets may have acquired certain resistance mechanisms by either inhibiting tissue maceration symptoms commonly associated with a soft-rot disease or inhibiting the bacterial pathogen development or both. Further studies are required to determine which resistance mechanisms existed in the selected putative *P. amabilis* plantlet mutants.

Three of the four resistance plantlets were regenerated from irradiated protocorms (5 Gy) and one from non-irradiated one (0 Gy). These results suggested either irradiation at 5 Gy or non-irradiation treatments of *P. amabilis* protocorms could induce putative soft-rot disease resistance plantlets. The putative soft-rot resistance plantlet regenerated from non-irradiated (0 Gy) protocorms may have originated from somaclonal variation. Finding in our experiments using either irradiated or non-irradiated protocorms further confirms the ability of irradiation or somaclonal variation to generate disease-resistant mutants (Yusnita et al. 2005; Purwati et al. 2007; Hassan et al. 2012; Indrayanti et al. 2018). Induced mutation combined with tissue culture and molecular analysis provides a powerful tool to generate mutants for many asexually propagated crops (Saif-Ur-Rashed et al. 2001) and commercial varieties were developed from such mutants (Ariffin and Basiran 2000; Basiran and Ariffin 2002). Induced mutation and somaclonal variation have also been used to generate disease-resistant mutants of various crops (Yusnita et al. 2005; Purwati et al. 2007; Hassan et al. 2012; Gaswanto et al. 2016; Indrayanti et al. 2018).

Phenotypic variants due to induced mutations may be associated with mutation of certain genes. The peloric mutant was an example of floral morphology and development mutant (Chen et al. 2008) due to changes in the *Dmnt* gene. Changes in the *Mlo* and *Pme 3* gene sequences have also been reported as a mutation indicator in flaxseed (*Linum usitatissimum*) (Kokina et al. 2017). Kim et al. (2010) used SNAP markers based on the *Kti* gene to identify the induced mutation in soybean, while Raynalta et al. (2018) used SNAP markers based on the *Pto* gene to identify somaclonal variant among PLBs regenerated from the leaf of *Phalaenopsis*. The SNAP marker was the simplest marker developed utilizing the abundant SNPs in the plant genomes (Pesik et al. 2017). Therefore, nucleotide sequence variation analysis among

the target genes may differentiate between wild types and their respective mutants.

Nucleotide sequences of the *Pto* gene were characterized using previously developed SNAP markers (eleven loci). The original *P. amabilis* mother plant was used as the wild-type *Pto* sequences source and most of the plantlets from irradiated and non-irradiated protocorms should have the same *Pto* gene sequences. Changes in nucleotide sequences from the wild-type *Pto* should indicate either the results of somaclonal variation (i.e. among plantlets regenerated from non-irradiated [0 Gy] protocorms) or induced mutation (i.e. among plantlets regenerated from irradiated [5-20 Gy] protocorms). Based on the 11 SNAP marker loci analysis in the *Pto* gene of the original *P. amabilis* mother plant, the alleles configurations in the evaluated loci were mostly heterozygous.

A few SNAP loci of the *Pto* gene in the putative plantlet variants regenerated from non-irradiated (0 Gy) protocorms exhibited homozygous allele configurations, indicating the presence of nucleotide substitution mutation due to somaclonal variation. Somaclonal variation may occur among plantlets regenerated from cultured protocorms as early as 20 weeks after the initial culture of *P. gigantea* (Samarfard et al. 2014) or 24 weeks for *P. bellina* (Khoddamzadeh et al. 2010). In this study, protocorms of *P. amabilis* have been maintained for almost a year since the protocorm initiation.

Moreover, a few SNAP loci of the *Pto* gene in the putative plantlet mutants regenerated from irradiated (5-20 Gy) protocorms also exhibited nucleotide substitution mutations. Therefore, these substitution mutations are most probably due to induced mutation. Results in this experiment confirmed the finding of Raynalta et al. (2018) that the SNAP marker based on the Pto gene could detect both mutants and variants among plantlets regenerated from irradiated and non-irradiated *P. amabilis* protocorms.

Subsequently, the observed *Pto* sequence variability among the regenerated plantlets was used to evaluate the association among the resistance responses to *Pto* nucleotide changes. In this study, the variability of *Pto* sequences isolated from plantlets was grouped into I, II, and III groups. Subsequently, the Group III *Pto* sequences were divided into sub-group III.A and III.B. Results of the phylogenetic analysis indicated plantlets regenerated from irradiation and non-irradiation treatments were equally distributed among the different groups.

Based on the phylogenetic analysis results, the *Pto* gene from the soft-rot resistance plantlets belonged to either group III.A (1 resistant plantlet) or group III.B (three resistant plantlets). The *Pto* gene from many susceptible or very susceptible plantlets also belonged to the same groups as the resistance one based on the phylogenetic tree constructed using 11 Pto SNAP marker loci. However, since the *Pto* sequence variability was evaluated at only 11 SNP loci using the associated SNAP markers, nucleotide substitution in other *Pto* gene sites was not tested. Therefore, the possible role of the mutant *Pto* gene in resistance plantlets should not be ruled out.

These results might also suggest that resistance phenotype among the four selected resistant plantlets may not be associated with the mutant *Pto* gene. In addition to *Pto*, there are at least 25 plant resistant genes associated with soft-rot disease infection (Oh and Martin 2011). Those resistant genes include *PaCDPK1* (Tsai et al. 2007), *PaPTP1* (Fu et al. 2011), *CHS*, *ABC*, *GST*, *ACC* and *PR10* (Fu and Huang 2011). Therefore, further studies are required to elucidate the resistance mechanisms against soft-rot disease in the identified resistant *P. amabilis* plantlets. One such study includes developing segregated populations derived from crossing between the regenerated resistant mutants and the susceptible recurrent parents and using the population to conduct linkage analysis among markers and the gene associated with soft-rot disease-resistant phenotypes. Such evaluation will be reported in other studies.

The current study results demonstrated the success of regenerating *P. amabilis* plantlet variants and their characterization based on leaf morphology and stomatal size variations. The study also successfully identified four soft-rot resistant plantlets regenerated from irradiated and non-irradiated *P. amabilis* protocorms. The resistant plantlets were identified after three consecutive periods of inoculations with pathogens causing soft-rot disease using leaf disc assay. The evaluation also confirmed the presence of nucleotide variation among the *Pto* gene isolated from *P. amabilis* plantlet variants. Elucidation of the resistance mechanisms in the identified *P. amabilis* plantlets will be described in other studies.

ACKNOWLEDGEMENTS

Competence grant (*Hibah Kompetensi*) supported by the Ministry of Research and Technology and Higher Education (RISTEK-DIKTI), Republic of Indonesia-Project 083/SP2H/PL/Dit.Litabmas/II/2015, under the coordination of Dewi Sukma supported part of this research.

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