BIODIVERSITAS
Volume 18, Number 1, January 2017
Pages: 20-27

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d180104

Improvement of genetic variability in seedlings of *Spathoglottis plicata* orchids through X-ray irradiation

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Manuscript received: 6 March 2016. Revision accepted: 2 November 2016.

Abstract. *Aloysius S, Purwantoro A, Dewi K, Semiarti E. 2017. Improvement of genetic variability in seedlings of Spathoglottis plicata orchids through X-ray irradiation. Biodiversitas 18*: *20-27.* Developing genetic variability of orchids via mutation is promising for orchid breeding. The objective of this research was to improve genetic variation of *Spathoglottis plicata* orchids through X-ray-irradiation of the orchid seeds. The method involved X-ray irradiation of one month old mature orchid seeds at doses of 0, 6, 12, 18, and 24 rad. The X-ray irradiated seeds were sown on a half strength of MS medium and grown into protocorms (developing orchid embryo). Eight weeks-old protocorms were subcultured onto NP-SIM medium. Five months-old seedlings were then subcultured again into a new flask and morphological observations were recorded. Genetic variability detection was conducted using PCR RAPD based on nine primers, i.e. OPA1, OPA2, OPA11, OPA12, OPA14, OPB1, OPB4, OPD12, OPD14. The data was analyzed using GenAlex 6.1 software to obtain the genetic distance. Moreover, NTSys ver.2 was used to analyze the data for clustering and constructing a dendrogram based on Neighbour Joining model. The result showed that X-ray irradiation with doses of 12-18 rad was able to stimulate morphological variation of seedlings, especially characters of leaf, root and shoot. Interestingly, we found 2 in vitro early flowering plants among one years old X-ray irradiated plants, indicated that somehow X-ray mutation affect flowering time in orchid. Based on the dendrogram of genetic distance that the group of mutants was farther than the WT group and the percentage of polymorphism was bigger than that of wild type group, it is concluded that 12-18 rad doses of X-ray irradiation can be used for induction of genetic variability in Spathoglottis orchid.

Keywords: Genetic variability, seed, seedling phenotype, *Spathoglottis plicata,* X-ray
Abbreviations: BAP = benzylaminopurine; NAA = naphthaleneacetic acid; 2iP = 2 = isopentenyl adenine; NP = new phalaenopsis; SIM = shoot induction medium

INTRODUCTION

Spathoglottis plicata is a common terrestrial orchid in Indonesia. The beauty of purple flowers and ease cultivation made this orchid can be used as a model plant for orchid study. however, the diversity of this species is still low (Effendie et al. 2002). The variation may emerge from breeding result of seeds. Nevertheless, since orchid seeds are very small in size and have no endosperm (Semiarti et al. 2014), so in nature the orchid breeding by the seed is quite difficult. To develop the orchid variability, an *in vitro* culture system equipped with mutation induction worth to be conducted.

The development of plant diversity with X-ray irradiation is the easiest way, but its application to orchids is still very limited. Seeds are very sensitive stage to radiation that precisely used as an irradiation target to obtain new characteristics (Jan et al. 2012; Shu et al. 2012; Kara et al. 2015). Irradiation on orchids is usually directed towards on its pod. The effectiveness of irradiation decrease because of the thickness of the pod coat. Furthermore, the amount of water in the pod certainly inhibit penetration of irradiation to the seed. Gamma ray-irradiation has been done on *Phalaenopsis*, *Dendrobium*, and *Catleya* orchids (Sulistyaningsih 2013), and also to protocorms (Gonzales et al. 2008), and *S. plicata* seedling (Pinmonrat and Suraninpong 2009). But so far, there has been no report especially concerning the effect of X-ray, which is irradiated directly on the seed. X-ray was also reported on it capability to induce mutation in *Arabidopsis* (Shu et al. 2012), *Zinnia elegans* Jacq (Pratiwi 2010; Gultom and Gultom 2015), and other horticulture plants, so that it is expected could be act as a trigger for inducing mutation in orchids, especially *S. plicata*.

Ionizing during ray irradiation such as X-ray and gamma ray through material generates excitation and ionization (Han and Yu 2009). Ionization gives rise to the breaking up of substantial compound chemical bonds such that nucleic acid in biological systems (Whitmore 1995; Han and Yu 2009). The damage extent of the ionizing ray is affected by the type or quality of the ray, irradiation dose, and also genotype factor or organism sensitivity level (Hameed et al. 2008; De Micco et al. 2010; Al-Enezi and Al-Khoyri 2012). Organism sensitivity is affected by age, genotype, metabolism activity level, physiological condition, and tissue complexity (De Micco et al. 2010).

From all of the reasons above, the focus of this research is to scrutinize whether X-ray irradiation upon the seed is capable for (i) inducing the emergence of morphological variation and (ii) bringing out genetic variability on *S. plicata* orchids plantlets. The objective of this research is to determine the genetic variability in the seedling as a result of X-ray irradiation treatment on seeds.

MATERIALS AND METHODS

Plant materials

Plant materials used in this work is 30-32 days old (mature) seeds of *Spathoglottis plicata.* The seeds were obtained from self pollinated *S. plicata* flowers from the orchid collection of Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. Seed irradiation was done in STTN BATAN, Yogyakarta with doses of 0, 6, 12, 18, and 24 rad that decided based on preliminary tests.

In vitro culture

In vitro culture was conducted in Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. The irradiated seeds were cultured in a half strength of MS medium in a petridish until the 8 weeks old protocorms were formed. The protocorms were then sub-cultured on NP-SIM medium (shoot induction medium with New Phalaenopsis basic medium) and maintained for 5 months under continuous white light at 25ºC condition. NP-SIM with addition of 6-benzylaminopurine (BAP)growth regulator and 2iP (0.3 µM) and NAA (0.15 µM) were used for culture medium. The seedlings were then sub-cultured again into a new flash for growing further maintenance. Morphological characters such as number of leaf, length and width of leaf, plant height, number of root, the length and thickness of root were measured from 10 months old seedlings, covering Morphological observation was conducted to detect mutant seedlings and picked up its for further molecular analyses compare to the wild type.

DNA isolation and analysis

DNA analysis was conducted upon genome DNA of wild type seedling and its mutant. DNA isolation was done using CTAB method according to Murray and Tompson (1980) with modification. DNA quantification was carried out using spectrophotometry on the ratio of λ 260 nm and 280 nm. DNA visualization was performed with electrophoresis.

Table 1. Screening results of decamer types oligonucleotides for RAPD

Primer	Nucleotide order 5'—3'	Primer	Nucleotide order 5'—3'
OPA-01	CAGGCCCCTTC	OPB-1	GTTTCGCTCC
OPA-02	TGCCGAGCTG	OPB-4	GGACTGGAGT
OPA-11	CAATCGCCGT	OPD-12	CACCGTATCC
OPA-12	TCGGCGATAG	OPD-14	CTTCCCCAAG
OPA-14	TCTGTGCTGG		

PCR-RAPD analysis

Molecular analysis was done by Polymerase chain reaction (PCR) using Random Amplified Polymorphic DNA (PCR-RAPD). To detect the existence of DNA polymorphism among the independence seedlings DNA to determine the genetic variabilities that could be used to distinguish the wild type seedlings and mutant seedlings. The primer type used for PCR-RAPD was decided based on screening result using three genom DNA samples. From 22 types of decamer (OPA1, OPA2, OPA3, OPA4, OPA8, OPA11, OPA12, OPA14, OPB1, OPB3, OPB4, OPB6, OPB8, OPB11, OPD1, OPD2, OPD3, OPD9, OPD10, OPD12, and OPD14) which were being screened. Nine types of oligonucleotide primers were found that showed the presence of polymorphic DNAs (Table 1)

RAPD PCR reaction was conducted in 200 µL microtube with final volume of 10 µL, containing 5 µL Go Taq Green (Promega, Madison, USA), 0.25 µL (10 µM) primer (decamer), 2.5 µL DNA template (25x dilution), and 2.25 µL nuclease-free water. The PCR reaction started from pre-denaturation step (94^{o}C, 1 minute). Followed by further steps consist of denaturation (94^{o}C, 30 seconds), annealing (37^{o}C, 30 seconds), and elongation (72^{o}C, 1 minute 30 seconds), run in 45 cycles, then elongation (72^{o}C, 7 minutes) and finally terminated with hold it at 4^{o}C.

PCR products were visualized with electrophoresis in 1.5% agarose gel, 1 µL of Florosafe was used for DNA staining (1st Base). For DNA marker, Vivantis DNA Ladder which gives DNA band size in the range of 100-3000 bp was used. Electrophoresis was conducted in 1x TBE solution in 100 volt for 60 minutes. The electrophoresis result is observed under UV transilluminator and photographed using a Canon digital camera.

Data processing

The qualitative data related to the observation of morphological variation of the seedling were descriptively analyzed. The growths of seedling were measured and analyzed statistically by one-way analysis of variance, followed by Duncan test (DMRT). The data resulted from RAPD were converted into binary data by giving scores. Score 1 if there was a DNA band and score 0 if there was no DNA band. The data was processed with GenAlex 6 program (Peakall and Smouse 2006) to obtain genetic distance and its principal coordinates analysis. Clustering was carried out using Numerical Taxonomy and Multivariate Analysis System (NTSys) ver 2 program (Rohlf 1990) employing Sequential, Agglomerative, Hierarchical and Nasted clustering (SAHN) with Unwighted Pair Group Methods with Arithmetic Averages (UPGMA) to obtain the dendrogram. Dendrogram was also constructed based on Neigbour Joining model.

RESULTS AND DISCUSSION

Effects of x-ray irradiation on the growth of embryos and seedlings

The basic requirement for an effective use of mutation induction of terrestrial orchid *S. plicata* via X ray-irradiated

seeds were determined using embryo sensitivity level, based on the growth and survival of embryo to become a plantlet that ready to be planted *ex vitro*. The growth of embryo and stages of embryogenesis, germination rate, and its survival was suppressed by X-ray irradiation at a dose of 24 rad (Figure 1, Table 2).

The growth of embryos were significantly inhibited by x-ray irradiation. At irradiation of 0-24 rad, the embryos from non-irradiated seeds (0 rad) reached an average lengths of 1,382 mm while the embryos from irradiated seeds was only reached less than 0.67 mm. Effect of seed irradiation continues on further growth and development of seedlings. Based on the decrease size of the leaf lengths, plant heights and root lengths (Figure 2), the growth of seedlings from irradiated seeds, it is known that irradiation of seeds at a dose above 18 rad was significantly inhibited the growth and development of *S. plicata* cells and organs.

Growth of embryos (Table 2) and seedlings (Figure 2) were suppressed at the doses of 18 rad. The growth inhibition of embryo development can be evoked by the damage of the cells of embryo and the meristem tissues, also the damage of auxin activity that very sensitive to photo-oxidation or caused by disturbance of physiological activity. Inhibition of growth caused by genome damage and disruption of mitosis, mainly due to disruption of the cell cycle at the G2/ M phase (Jan et al. 2012). Inhibition of growth rate can also be triggered due to the death of meristem cells, not only by the genetic damage. The growth can also be suppressed by physiological disorders (De Micco and Arena 2011; Jan et al. 2012). Gonzales et al. (2008) was also found some inhibition of plantlets growth due to gamma-ray irradiation to the embryos of *S. plicata*. Inhibition of growth due to gamma ray irradiation may be linked to the disruption of auxin and DNA that involved in the synthesis of auxin, or because of the damaged activity of auxin (Jan et al. 2012).

Effect of x-ray towards morphology of *S. plicata* seedling

Almost all seedlings derived from irradiated seed culture, exhibited normal that similar to the wild type (wild type-like phenotype). Only a few seedlings undergo morphological changed predominantly. This changed might be caused by mutation. There were five (5) groups of mutant (mutant pool) of seedling occurred in this work, namely (i) leaf shape mutants, (ii) root mutants, (iii) shoot mutants, (iv) pigment mutants, and (v) early flowering mutants.

Overall, based on the analysis of total number of seedlings, almost all seedlings from x-ray irradiated seed (>98 %) have morphological characteristics similar to the normal seedlings (Table 3). However, upon irradiation with doses of 18 and 24 rad, some mutant seedlings exhibited different morphological characters from that of wild type (< 1.0%). The altered phenotype of the seedlings likely due to the effect of mutation.

X-ray irradiation on *S. plicata* seed produces various phenotypes on leaves (color and shape), roots, stems, and early flowering. This results support the work of Romeida et al. (2013) that also found albino mutant, purple stem, yellow leaf, and flower color mutants of *S. plicata* after pod plant irradiation using gamma ray. Mutants from 18-and 24-rad irradiated plants showed some abnormal leaf pigmentation, which termed as xantha, arboviridis, and viridis mutants. According to Mueller (2012), chlorophyll mutant might caused by deficiency of chlorophyll-a or chlorophyll-b, or low production of pigment. However, the pigment mutations were not permanent. In subsequent stage of the growth of seedling, new emerged leaves showed normal leaf color.

In shoot mutant, the shoot grew very slow, puffy, and formed multi-shoots. This phenomenon allegedly caused by the abnormal activity of some genes that involved in the development of shoot apical meristem, and in turn it affected the process of morphogenesis. Semiarti (2014) stated that overexpression of *KNAT1* gene (class1-*Knox* gene) on *AS2* mutant generated adventive shoot development in leaf explant of orchid similar to the abnormal expression (misexpression) of *KNAT1* gene that initiated the formation of adventive multi-shoots in *Arabidopsis*. Multi-shoots as a consequence of X-ray irradiation on *S. plicata* seedlings might also caused by over expression of *KNAT1* heterologous gene in orchid.

X-ray irradiated seeds have also produced seedlings with much number of adventive roots, stunted roots, swollen roots and horizontal roots with the tip tend upwards (Table 3 and Figure 3). It is similar to the mutant phenotype of *Arabidopsis* as described by Howell (1998), termed as various forms of mutant roots. Root of mutants that fail to grow toward the bottom known as a agravitropic mutant. Other root mutants produced short root, the cortical-loosed roots, enlarged root (Howell 1998) and extensive adventitious root (Aynehband and Afsharinafar 2012). Scale-like leaf mutants that produced very dense leaves might also due to the disruption of shoot meristem activity. Leyser and Day (2003) stated that gamma irradiation caused cell division failure and initiated leaf primordial that occurred only through cell elongation. Without cell division, leaf development grew abnormal and that way it produced a stunted leaf and stunted stem. Since the mutant frequency observed in these research were very low (less than 1%) we suggest that those mutants were caused by X-ray irradiation and had not categorized as neither epigenetic nor somaclonal variation. Mueller (van Harten 1998) mentioned that epigenetic change is marked by very high occurrence frequencies.

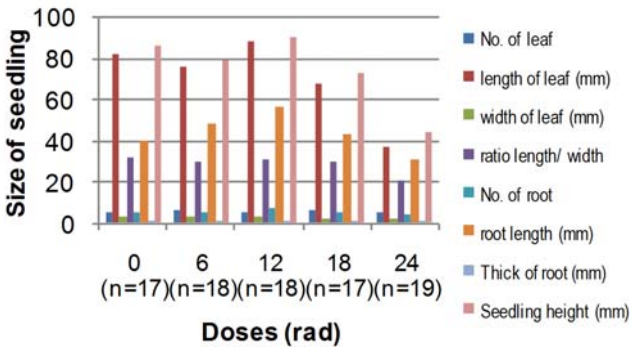

Figure 2. Growth achievement of the ten months old seedlings at various doses of irradiation (0-24 rad)

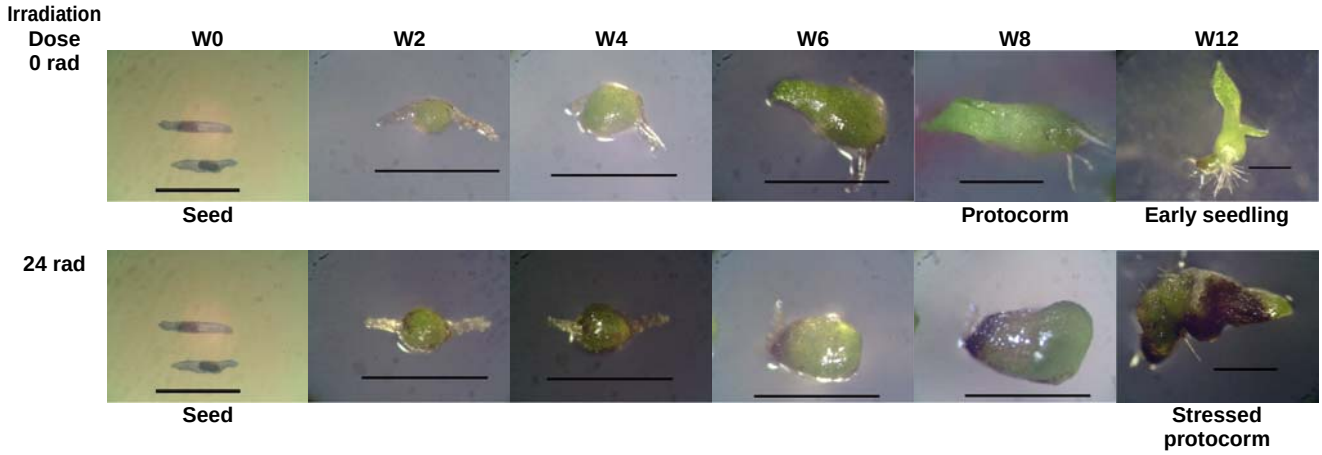

Figure 1. The growth and development of *S. plicata* embryo derived from non-irradiated seed and irradiated seed. w = week after seeds plantation. Bars: 1 mm

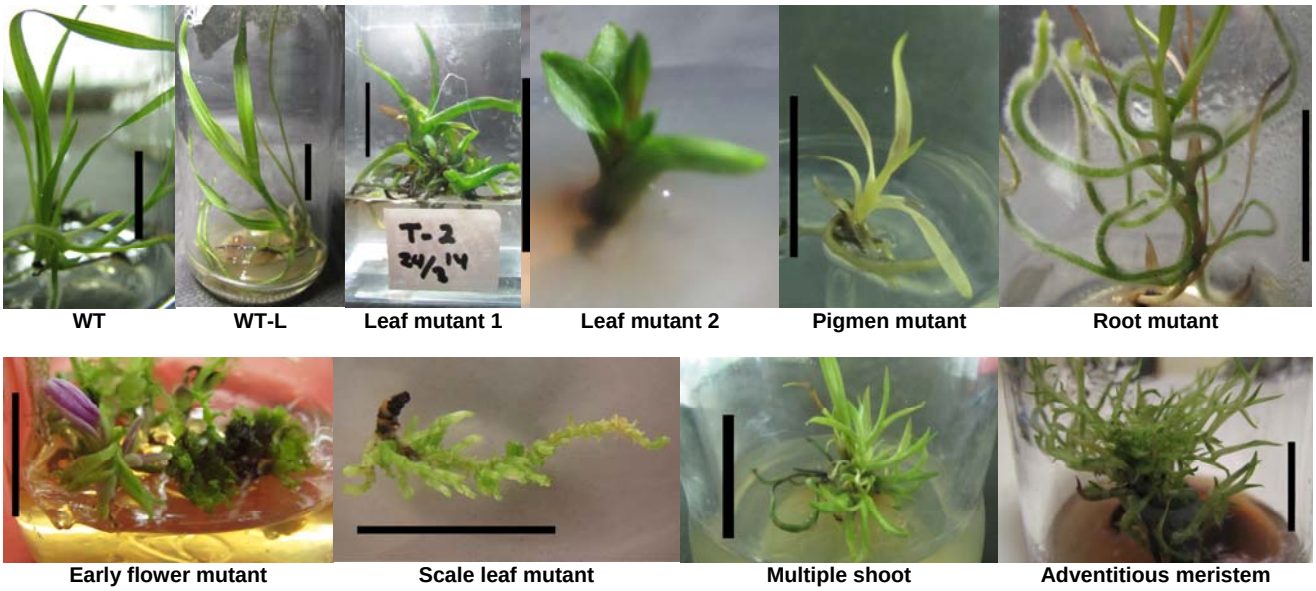

Figure 3. Morphology of wild type seedling and some mutants resulting from the irradiated seed at the doses of 18-24 rad, at 10-15 months old after seed plantation. WT-L = wild type-like. Bars: 2 cm

Table 2. The seed germination and size of embryo at 6 weeks after seed plantation at the dose of irradiation 0-24 rad

Dose (rad)	Germination (%) (ns)	Survival of embryo (%)*	Length (mm) of embryo*	Width (mm) of embryo*
0	94.56 ± 1.99	85.10±1.50 a	1.362± 0.36 a	0.577± 0.10 a
6	95.18 ± 1.81	70.70± 2.78 b	0.664± 0.03 b	0.487± 0.03 b
12	93.78 ± 2.32	72.19±5.23 b	0.645 ± 0.02 b	0.508 ± 0.01 b
18	93.02± 1.61	62.23± 9.82 c	0.616 ± 0.04 b	0.489± 0.01 b
24	93.93± 2.25	40.37± 15.44d	0,58 ± 0,01 b	0,493± 0,01 b

Note: No. of samples: 0 rad = 8, 6 rad = 8, 12 rad = 9, 18 rad = 8, 24 rad = 7. ns = non significant. * = significantly different by one way analysis of variance, with degree of confidence 95 %, Means in each column followed by the same letters are not significantly different by DMRT analysis

Table 3. Phenotype and percentage of *S. plicata* seedlings from X-ray irradiated seed at the dose of 0-24 rad

Organ	Phenotype	Σ total seedlings/ mutation observed (%)				
		0 (WT)	6 rad	12 rad	18 rad	24 rad
leaf, root and stem	Normal	6187 (100)	1334 (100)	2722 (99.92)	917 (95.92)	2380 (98.22)
Leaf	Thick, sleek, leaf does not open	0	0	0	0	3 (0.12)
	Ellipse, rosset-like	0	0	0	8 (0.8)	1 (0.04)
	Scale leaf	0	0	0	0	3 (0.12)
Root	Adventive root, oriented upwards	0	0	0	3 (0.31)	2 (0.08)
	Puffy or swollen root	0	0	0	3 (0.31)	3 (0.12)
Shoot	Multiple shoots	0	0	0	3 (0.31)	6 (0.24)
	Puffy or multiple branch with scale leaf	0	0	0	0	6 (0.24)
Leaf color	Xantha/chlorina (yellow greenish)	0	0	2 (0.07)	17 (1.78)	11 (0.4)
	Arboviridis (the leaf with green tip and yellow base)	0	0	0	3 (0.31)	2 (0.08)
	Viridis (glossy dark green leaves)	0	0	0	2 (0.21)	4 (0.16)
Flower	Early flowering in vitro	0	0	0	0	2 (0.08)
Total of embryo survive		6187	1334	2724	956	2423

Naturally, the flowering time of *S. plicata* are about 30 month. However, we found 2 mutants that start to have flower in the age of 14-15 months in vitro as an impact of X-ray irradiation (Table 3). The frequency of these mutants was very low (<0.1%). Early flowering might be caused by some flowering time genes mutation which concerns vegetative growth control, i.e. *Embryonic Flower1* (*EMF1*) or *Flowering Time* (*FT*). *EMF1* is one of flowering identity genes which is involved in the regulation of flowering transition (Puterill at al. 2011; Kim et al. 2010), that roles in the delay of flowering transition period. *EMF1* gene and *Terminal Flower 1* (*TFL1*) gene reciprocally control the expression of *APETALA* (*AP1*) and *LEAFY* (*LFY*) flowering genes. In emf*1* mutant, the promotor of *AP1* gene will be activated prematurely on shoot meristem and triggers the occurrence of early flowering (Chen *et. al.* 1997; Kim et al. 2010). Stress condition as a consequence of seed irradiation may affected embryo development that triggered early flowering. In this work, the highest doses of X-ray irradiation that can be used for orchid was less than 24 rad, because almost all of protocorms death just after seed plantation after 24 rad x-ray irradiation of the seeds. It can be proposed, that X-ray irradiation at dose 12-18 rad can be used to improve the genetic variability in *S. plicata* orchid.

Molecular analysis using PCR-RAPD

The total genome DNA of some wild type and mutant seedlings (Figure 4) were analyzed to detect the genetic variabilities among the mutants and non-irradiated plants using PCR-RAPD analysis. Amplified DNA using RAPD primers were separated in 1.5 % agarose gel electrophoresis and visualized under uv light resulting in random amplified polymorphic DNA pattern of each mutant analyzed.

The pattern of DNA band of wild type seedling group (Figure 5) is very homogeneous (monomorphism). By using 9 random primers the result showed that we found up to 15 band patterns which is reach 90.9-100% of polymorphism (Table 4). The percentage of DNA polymorphism from mutant seedling group (81.65%) was higher than the wild type seedling group (8.25)%. This shows that a change occurs in the seedling DNA of the mutant group. The genetic distance between the mutant seedlings and the non-irradiated seedlings are quite far away (Table 5). Furthermore, the PCA of them showed exact separation between the mutants and non-irradiated plants (Figure 6). Those results indicated that the changes of DNA were caused by X-ray irradiation.

Induction of mutation in plants using irradiation was also successfully conducted on rose flower by Chakrabarty and Datta (2010) using gamma ray.–Mutants were also produced through irradiation on *Gypsophila paniculata* (Barakat and El-Sammak 2011) and *Moluccella laevis* (Minisi et. al. 2013). In general, X-ray irradiation causes one or some deletion of nucleotides in DNA fragments. X-ray very rarely causes chromosome aberration, except in special cases, such as irradiation on anther of *Tradescantia*. X-ray brings out ring deletion of 0.5-3.0%. Murata *et.al.* (2008) was succeeded in finding mutation of one nucleotide and insertion of some nucleotide on a group of *Angelica acutiloba* herbal plants using RAPD approaches. X-ray irradiation with doses of 12 to 18 rad on *S. plicata* seeds were able to induce morphological alteration of orchid seedlings, as well as genetic variations among orchid mutant's seedlings.

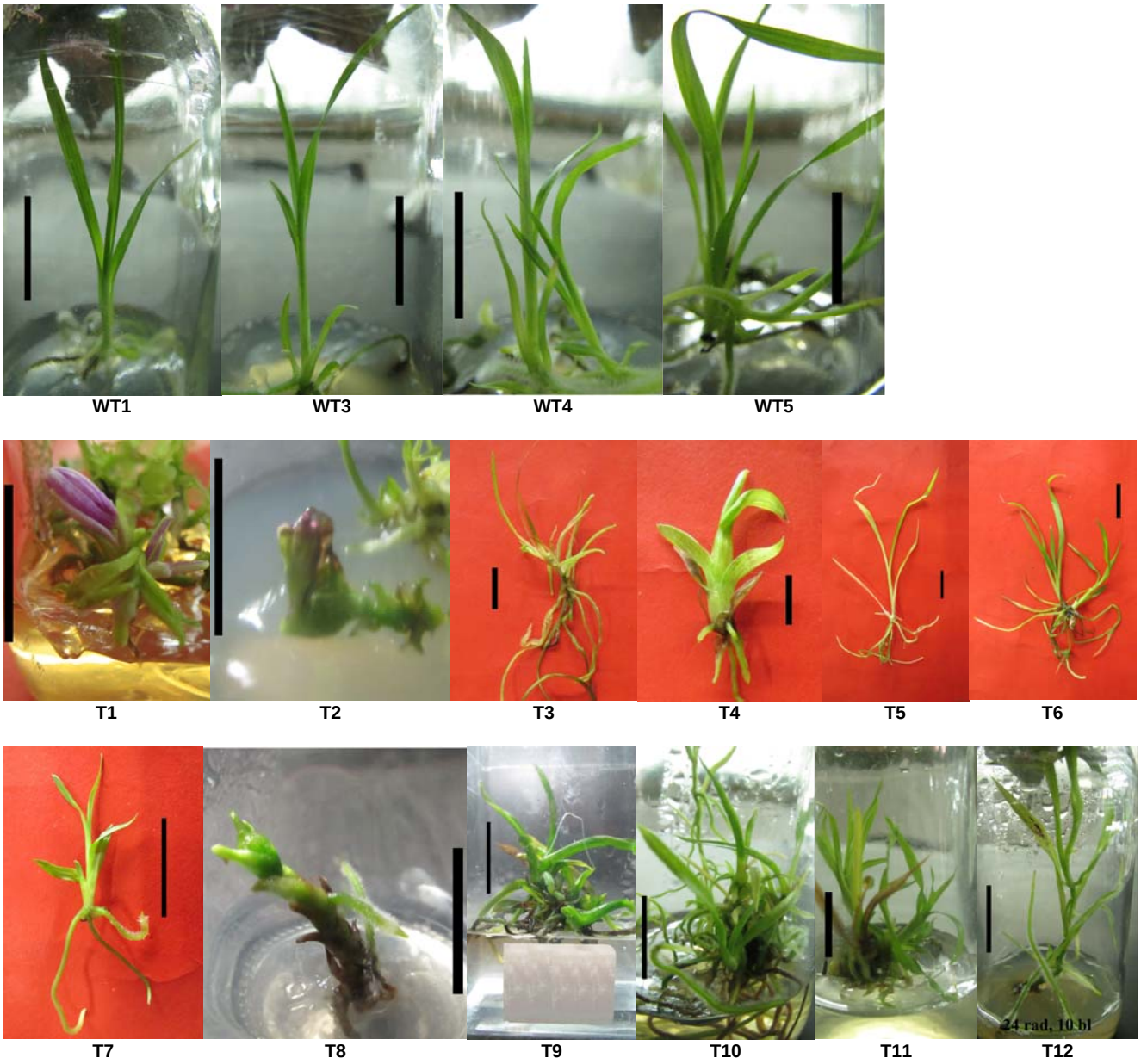

Figure 4. Wild type and mutant of *S. plicata* seedlings which is used for genetic variability analysis. Note: WT = wild type (K); T = Mt (mutant). All of the bar line = 2 cm. T1-T2 = premature flowering mutant. T3 = adventives root mutant, T4 = white spot leaf mutant, T5 = yellow leaf mutant, T6 = wild type-like seedling, T7 = arboviridis, T8 = bloated shoot mutant, T9 = thick leaf, slippery, leaf does not open, T10 = yellowish leaf mutant, T11 = yellow leaf, T12 = yellowish leaf, necrotic, upward stem

Table 4. Random Amplified Polymorphic DNA patterns in X-ray irradiated mutant genomes of *S. plicata* orchids using nine primers

Primer	Sequences	No. of DNA bands	No. of polymorphic DNA bands	No. of monomorphic DNA bands	Polymorphism (%)
OPA-01	CAGGCCCCTTC	11	10	1	90.9
OPA-02	TGCCGAGCTG	14	14	0	100
OPA-11	CAATCGCCGT	13	12	1	92.3
OPA-12	TCGGCGATAG	13	13	0	100
OPA-14	TCTGTGCTGG	10	10	0	100
OPB-1	GTTTCGCTCC	12	12	0	100
OPB-4	GGACTGGAGT	7	7	0	100
OPD-12	CACCGTATCC	15	14	1	93.3
OPD-14	CTTCCCCAAG	14	14	0	100

Note: Based on the GenAlex analysis, DNA polymorphism of mutant group reached 81.65% while the WT only 8.25%

Table 5. The genetic distance of wild type (WT) and X-ray irradiated mutants of *S. plicata* orchids mutant (T) seedlings

	WT1	WT3	WT4	WT5	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
WT1	0															
WT3	6	0														
WT4	6	6	0													
WT5	3	5	3	0												
T1	57	61	59	58	0											
T2	58	60	58	61	17	0										
T3	54	54	52	55	29	20	0									
T4	56	56	54	57	35	28	14	0								
T5	49	49	47	50	42	35	21	23	0							
T6	48	48	48	49	39	30	18	18	15	0						
T7	55	55	53	54	46	39	31	37	26	29	0					
T8	43	43	45	44	44	37	23	25	18	15	34	0				
T9	59	61	57	60	28	21	33	37	46	41	50	40	0			
T10	44	44	46	45	45	38	26	24	27	18	33	15	37	0		
T11	53	53	53	52	46	41	35	33	28	27	30	24	42	15	0	
T12	43	45	41	44	56	47	37	39	28	27	38	24	46	19	28	0

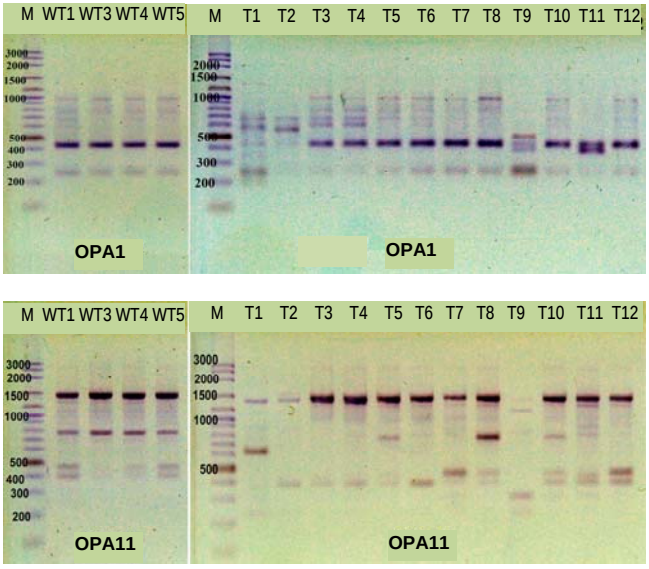

Figure 5. Random Amplified Polymorphic DNA patterns of wild type and mutant *S. plicata* orchid plants using primer OPA-1 and OPA11. WT = Wild type; T1-T12 = mutant lines no. 1 up to no 12; M = DNA marker.

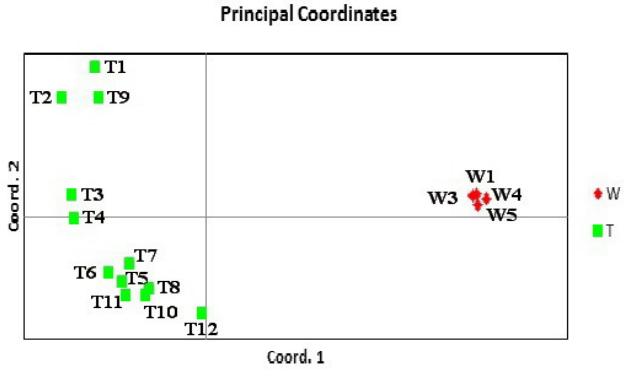

Figure 6. Principal coordinates of molecular characters of wild type and mutant seedlings.

Based on the description above, it can be concluded that X-ray irradiation at the doses of 12-18 rad on seeds was able to induce mutations in *S. plicata* orchid seedlings, which showed morphological variation among some mutant seedlings, that indicated various mutations, i.e. the mutation of leaf shape, root, shoot, chlorophyll, and *in vitro* flowering. Molecular analyses of mutant seedlings showed there were genetic distances among the mutant seedling groups which increasingly going further away from the wild type seedling group. X-ray irradiation on *S. plicata* orchid seeds can be used to improve genetic variabilities in orchids, the increase of polymorphism percentage and the genetic distance of the mutant plants.

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