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In vitro mutagenesis of *Etlingera elatior* (Jack) and early detection of mutation using RAPD markers

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Abstract: Mutation breeding techniques in combination with tissue culture and molecular marker methods provide a powerful tool for improvement of vegetatively propagated plants. The aim of this study was to develop a protocol for shoot regeneration and mutation induction of *Etlingera elatior*. The results of irradiation on in vitro buds of *E. elatior* showed LD_{50} to be 10 Gy, with the survival of explants being sharply reduced at this dosage. All 8 selected gamma irradiated regenerants were differentiated from the untreated control based on the banding patterns obtained using 9 primers, which generated 59 reproducible bands, whereby 35 (55.31%) were found to be polymorphic. Jaccard's coefficient of similarity values ranging from 0.537 to 0.860 were indicative of the level of genetic variation among the mutants studied. For comparison between the potential lines (PL) and the control, a maximum similarity value (0.814) was observed in PL1 mutant, while the minimum value (0.537) was observed in PL7. In summary, a combination of irradiation, regeneration, multiplication, and random amplification of polymorphic DNA (RAPD) analysis for early screening of mutants can speed up the breeding program of *E. elatior*.

Key words: *Etlingera elatior*, LD_{50}, genetic variation, mutation, potential line, RAPD

1. Introduction

For modern and industrialized horticulture, there is always a demand and necessity for new cultivars. Induced mutations have played an important role in the improvement of plants, and more than 3200 mutant cultivars have been developed through this approach (Pierre, 2012). Modern day plant breeding is based on creating variations followed by selection, evaluation, and multiplication of the desired genotypes. In a crop improvement program, plant breeders often combine several techniques in order to increase efficiency and reduce the time needed for the development of a new cultivar. Plant breeders combine tissue culture technique for rapid multiplication of regenerants, mutation induction to enhance variations, and molecular marker methods to detect the genetic variation (Ahloowalia and Maluszynski, 2001). Such a combination of techniques has been exploited for the creation of new and novel plant cultivars, particularly in vegetatively propagated species (Broertjes and Van Harten, 1988; Pinet-Leblay et al., 1992).

E. elatior, also known as torch ginger, is one of the neglected plants in the family Zingiberaceae, and scientific research on its propagation, biotechnology, and ecology is limited. However, this ginger plant offers great scope for the development of a large range of ornamental and cut flower types (Poulsen, 2010). Ismail (2009) reported that *E. elatior* is one of the 30 popular herbs or new industrial crops that are in high demand in Malaysia. It is now cultivated on a commercial scale in places like Australia, Thailand, and Costa Rica for cut flower production (Ismail, 2009; Segalen, 2010). The plant itself creates a great garden landscape, its flowers having an immense ornamental value, and it also has a place in eco-gardens (Wong, 2008). To sustain in the ornamental and cut flower industry, torch ginger requires continuous improvement of certain characters such as flower color, morphology, longevity, size, odor, and decreasing time to flower formation. Unfortunately, conventional breeding of *E. elatior* is handicapped by cross incompatibility, poor fruit set, and low seed production (Marcsik and Hoult, 2010). Due to these factors, alternative approaches for crop improvement of *E. elatior*, such as mutation induction, could be explored.

Previous reports have shown that *E. elatior* has even more potential as a new and promising crop. Studies have

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proven that torch ginger has high antioxidant content (Chan et al., 2009a, 2009b; Andarwulan et al., 2010; Haleagrahara et al., 2010; Wijekoon et al., 2011a), essential oils (Jaafar et al., 2007), antibacterial activity (Chan et al., 2007; Lachumy et al., 2010), beneficial nutrient components (Wijekoon et al., 2011b), phenolic compounds and scavenging activity (Yan and Asmah, 2010), antitumor promoting and cytotoxic activity (Habsah et al., 2005), and antifungal properties (Ficker et al., 2003; Lachumy et al., 2010).

The successful outcome of a mutation depends on the efficient induction of mutation as the well as effective recognition and recovery of the desired mutant plants through repeated subculture (Puchooa, 2005). In this report, a technique has been standardized for the management of chimeric tissues through multiple bud regeneration. Molecular techniques have greatly aided the understanding of plant cell responses to mutation induction. They also provide a better understanding of the potential and limitations of mutation breeding, which can lead to early identification of useful variants. The major advantages of RAPD are the low cost and effort required for its application (Atienzar and Jha, 2002; Dhakshanamoorthy et al., 2011). To our knowledge, this is the first report on in vitro mutagenesis in *E. elatior* and the application of RAPD markers to detect variations.

2. Materials and methods

2.1. Plant materials

Individual buds (±0.5 cm) obtained from 6-month-old axenic cultures (at the multiplication stage) (Yunus et al., 2012) were excised and cultured on MS medium (Murashige and Skoog, 1962) containing 3% sucrose (w/v), 0.4% Gelrite (Duchefa, Haarlem, the Netherlands), 0.1 mg L^{-1} myo-inositol, and devoid of plant growth regulator. The pH of the medium was adjusted to 5.7–5.8 before autoclaving at 121 °C for 15 min. Five buds were cultured in each petri dish. Cultures were incubated for 3 days at 25 ± 1 °C under dark conditions prior to irradiation.

2.2. Gamma irradiation of individual buds

The irradiation process was carried out at the gamma ray laboratory, School of Applied Physics, Faculty of Science, Universiti Kebangsaan Malaysia (UKM), Selangor, Malaysia. The individual buds in petri dishes were irradiated with ^{60}Co source (Gammacell 220 Excel Co-60, Nordion, Canada) (dose rate: 2.8741 Kgy/-h) at different doses: 0 (control), 10, 20, 40, 60, 80, 100, 120, and 140 Gy. One Gy equals the absorption of 1 joule of energy per kilogram of product irradiated. There were 20 replications per dose, and each replication consisted of 1 explant. After irradiation, the irradiated explants were maintained in the same media in the petri dish and incubated at 25 ± 1 °C under dark conditions.

2.3. Postmutagenesis handling

After 3 days of incubation, the irradiated explants (designated M1V0) were transferred to shoot multiplication medium. The irradiated explants were placed in vials containing 10 mL of MS medium supplemented with 13.32 µM BAP, 3% sucrose, 0.4% Gelrite, and 0.1 mg L^{-1} myo-inositol. The cultures were incubated at 25 ± 1 °C under dark conditions. Cultures were maintained on the same medium by regular subculturing at 4-week intervals over a period of 8 weeks. A radiosensitivity test of the plant was determined by measuring the survivability of the explant 8 weeks after irradiation. Other parameters observed were the mean number of shoots and leaves developed from the irradiated explants and morphological changes observed in the plantlets. The morphological changes were recorded visually after 1–8 weeks of culture.

After 8 weeks of culture under dark conditions, each irradiated bud was transferred to fresh medium with the same growth regulator composition. The medium was distributed into jam jars (50 mL each), and a single irradiated bud was placed in each jar. The cultures were incubated under reduced light conditions provided by white fluorescent tubes (21 µmol m^{-2} s^{-1}) at 26 ± 1 °C with a 16/8-h photoperiod (light/dark) for a week. Then the cultures were incubated under full light conditions for 3 subsequent subcultures at 26 ± 1 °C with a 16/8-h photoperiod (light/dark) (30 µmol m^{-2} s^{-1}) provided by white fluorescent tubes.

When the new shoots that emerged (designated M1V1) were 1 cm in length, they were excised from the irradiated shoot clumps (M1V0) and subcultured singly onto fresh medium of the same hormone composition (MS + 13.32 µM of BAP) at 4-week intervals for shoot multiplication through the second and third regeneration cycle (M1V2 and M1V3). Cultures were maintained under full light conditions at 26 ± 1 °C with a 16/8-h photoperiod (light/dark) (30 µmol m^{-2} s^{-1}) provided by white fluorescent tubes.

2.4. Experimental design and statistical analysis

Randomized complete block design (RCBD) was used in all experiments. Data were subjected to a Kruskal–Wallis test (nonparametric test) for the normality test of the treatment means. Data were subjected to analysis by using SPSS version 18.

2.5. Polymerase chain reaction (PCR) and primer screening

A total of 8 regenerants, chosen at random from the healthy growing irradiated explants, were analyzed individually after the third generation (M1V3) in culture. A nonirradiated explant (M1V3) was used as the control. Total DNA was extracted from in vitro young leaves using the method described by Kiefer et al. (2000), with one modification whereby spermidine was omitted. Each

genomic DNA sample was checked for the level of purity and concentration using Nanodrops (Spectrophotometer ND-1000 V3.5, NanoDrop Technologies, Wilmington, DE, USA). DNA was diluted to make a working solution for RAPD marker analysis. The quality of dilution was further checked on 1.0% agarose gel.

2.6. Primer selection

A total of 14 primers were used for preliminary amplification and primer selection. Primers that produced reproducible polymorphic bands were selected. The RAPD primers (OPAW 11, OPAW 17, OPU 10, OPU 13, OPU 16, and OPG 14) were randomly chosen based on the study of *Phalaenopsis* orchids by Goh et al. (2005). Meanwhile, 8 SSR rice primers were tested for a cross amplification study based on the Jatoi et al. (2006) study of the family Zingiberaceae. The primers were RM1, RM117, RM125, RM131, RM135, RM153, RM154, and RM171.

PCR amplification was performed twice for each primer to ensure their reproducibility. The Biometra T1 Thermocycler (Biometra GmbH, Goettingen, Germany) was used for PCR amplification reactions. The final volume of the reaction mixture that was used in the PCR analysis was 25 µL. The solution contains 12.5 µL of DreamTaq Green PCR Master Mix (2X) (Fermentas, First Base Laboratories Ptd. Ltd., Seri Kembangan, Selangor, Malaysia), 0.5 mM of $MgCl_2$ (Fermentas), 0.4 µm of primer (Fermentas), and 50 ng of DNA template. The thermal profile of the reaction was programmed as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min and 30 s. The final extension was at 72 °C for 10 min followed by cooling to 4 °C. PCR products were separated by electrophoresis through 1.2% agarose gels in 1X TAE buffer at 70 V for 75 min. The gels were stained in ethidium bromide and were documented using GeneSnap gel doc system (SynGene, Cambridge, UK).

2.7. Scoring and analysis of RAPD data

DNA fragments showing polymorphism were scored using a binary system of 0 (absent) and 1 (present). Genetic relatedness among the 9 regenerants was estimated using Jaccard's (1908) coefficient of similarity. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). The genetic similarity and dendrogram were computed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS)-pc software, version 2.11a (32).

3. Results

3.1. Effect of gamma irradiation on regeneration of shoots from in vitro buds

The present study was conducted to determine the effects of irradiation on regeneration and morphological changes of *E. elatior* buds. Gamma rays are mainly used to irradiate multicellular layers because of the high penetration rate into tissues. Irradiation of *E. elatior* buds with gamma rays ranging from 10 to 140 Gy showed decreasing survival of the explants with increasing radiation doses. The effects of increasing doses of radiation on regeneration rates of buds after 8 weeks in culture are summarized in Figure 1.

The LD_{50} is defined as the dose corresponding to a 50% decrease in the control regeneration percentage. In this experiment, the 50% lethal dose of explants 8 weeks after irradiation (LD_{50}) was estimated to be 10 Gy, and doses beyond 80 Gy did not show any signs of regeneration (Figure 1). The regeneration of irradiated shoots was strongly influenced by the radiation dose. Higher doses inhibit regeneration, whereby gamma irradiation in excess of a critical dose caused obvious depression

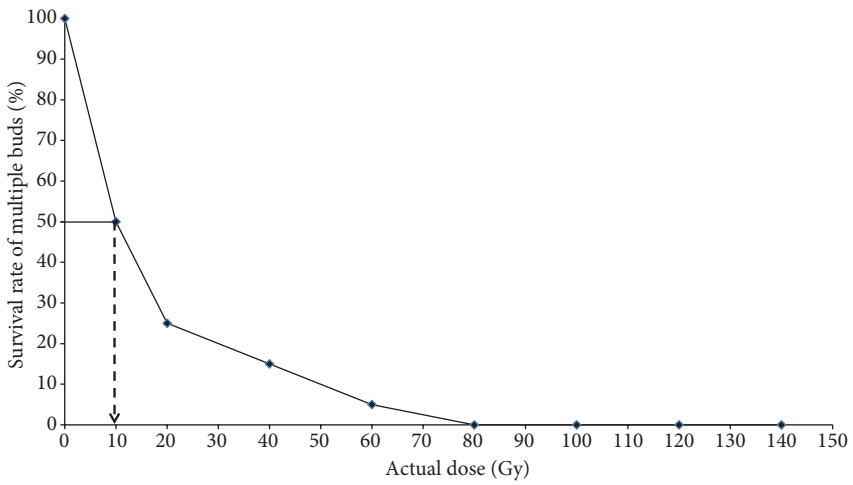


Figure 1. LD_{50} determination in survival rate of irradiated buds of *E. elatior* after 8 weeks of incubation.

in multiple bud growth. Treatments as low as 10 Gy showed irradiation damage; half of the explants turned black, retarded, and finally died. Surviving explants were observed at the lower dose of 10 Gy (Figure 2A) and the control treatment. Morphological abnormality appeared on some of the irradiated explants (Figure 2B). Meanwhile, no abnormality in morphology appeared for the surviving irradiated explants. In addition, all explants irradiated beyond 20 Gy failed to survive after the third subculture (12 weeks of culture) (Figure 2C), and all morphologically defective explants failed to grow. No callus was formed during the 8-week incubation period.

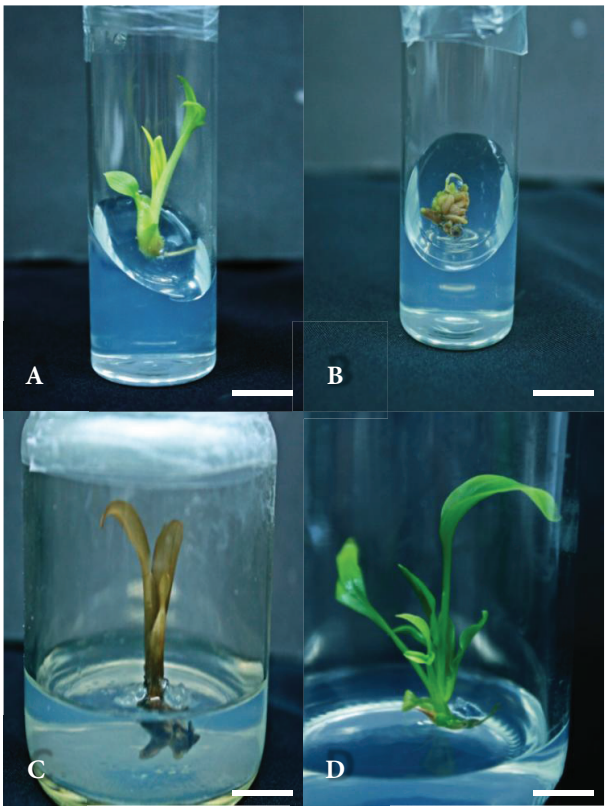


Figure 2. Effect of different levels of gamma irradiation on in vitro buds of *E. elatior*. A) Survival of explants exposed to 10 Gy after 8 weeks of culture. B) Morphological abnormality observed in irradiated explant which later died after M1V1 stage. C) Explant irradiated with 20 Gy failed to survive after the third subculture. D) M1V1 shoots produced from 10 Gy irradiated explant after 12 weeks of culture. Bar = 1 cm.

In this study, the gamma-ray–treated buds were cultured under optimal conditions for shoot regeneration. They were cultured on MS medium supplemented with 13.32 µM of BAP under dark conditions for the 8 weeks. The use of gamma ray extended the days until first shoot emergence from 2 weeks for the untreated explant to 6–12 weeks for the irradiated explant. New multiple shoot regeneration began with visible proliferation of buds from the basal part of the explants. Differences were observed after 8 weeks of irradiation with regard to the mean number of shoots and mean number of leaves produced (Table 1).

Poor regeneration frequency was observed in the postirradiation culture. The shoots showed slow growth but were identical to the control treatment in terms of morphology (Figure 2D). Gamma-ray irradiation did not affect the ability to produce M1V2 and M1V3 shoots by the surviving irradiated buds. When the irradiation dose was more than 10 Gy, the new shoots derived from the gamma-irradiated explants grew slower than the control. A minimum of 12 weeks were needed for new shoots from the irradiated explants (M1V0) to reach 1 cm in length, whereas shoots from untreated explants needed only 4 weeks to reach 1 cm. Gamma-ray irradiation did not affect the ability of the M1V0 shoots to produce adventitious roots.

Attempts to regenerate and multiply irradiated buds (M1V0) under light conditions were unsuccessful (data not shown). All irradiated buds became necrotic and died after a period of 6–8 weeks of incubation under light conditions. The present study indicated that irradiated buds only survived and regenerated shoots if the buds were maintained under dark conditions on a regeneration medium during the 8 weeks.

3.2. RAPD marker analysis of gamma-ray–treated regenerants

Markers were selected based on their ability to generate a visible polymorphism between the samples. For the RAPD analysis performed in this study, 9 of the 14 primers tested gave scorable bands. The 9 primers generated 59 scorable bands ranging from 300 to 3500 bp in size. Of these, 35 bands (55.31%) were polymorphic (Table 2). The number of bands for each primer varied from 3 (OPAW17) to 9 (RM125), with an average of 6.6 bands per primer. The

Table 1. Effects of different doses of gamma irradiation on shoot regeneration from buds of *E. elatior* after 8 weeks of culture.

Irradiation dose (Gy)	Percentage of buds producing shoots	Mean number of shoots	Mean number of leaves
0	100	2.20	3.15
10	20	1.6	1.8

Values are the mean of 20 replicates. Each replicate contained one explant.
Note: 20–140 Gy dosages were lethal to explants after 8 weeks of culture.

Table 2. List of primers, number of amplified products and polymorphic bands, and polymorphism percentages.

Primer	Sequence (5'–3')	Total amplified products	Polymorphic bands	Percent polymorphism
RM117	CGATCCATTCCT GCTGCTCGCG	8	6	75.00
RM125	ATCAGCAGCCAT GGCAGCGACC	9	7	77.78
RM153	GCCTCGAGCATC ATCATCAG	8	6	75.00
OPAW 11	CTGCCACGAG	8	4	25.00
OPAW 17	TGCTGCTGCC	3	1	33.33
OPU 10	ACCTCGGCAC	5	1	20.00
OPU 13	GGCTGGTTCC	6	4	33.33
OPU 16	CTGCGCTGGA	6	2	16.67
OPG 14	GGATGAGACC	6	4	66.67
Total		59	35	
Mean		6.56	3.89	55.31

percentage of polymorphisms per primer ranged from 20% to 77.8% with an average of 55.3%. This indicates that there was considerable RAPD variation among the regenerants. Figure 3 shows the amplification profiles generated by primers OPAW 11, OPU 13, and OPU 16 across the regenerants.

The Jaccard's coefficient of similarity provided similarity values ranging from 0.537 to 0.860, indicative of the level of genetic variation among the potential mutant lines (Table 3). These coefficients reflected the genetic relationship between the samples. The highest level of similarity between 2 potential mutant lines was found between PL2 and PL3, with a similarity coefficient of 0.86 (the shortest genetic distance), while a minimum similarity coefficient (0.628) was observed between PL1 and PL7 (the highest genetic distance). For comparison between the potential mutant lines and the control, the maximum similarity value (0.814) was observed in PL1 mutant while the minimum value (0.537) was observed in PL7. The dendrogram obtained by UPGMA from the 59 markers scored for the 9 regenerants is shown in Figure 4. The cluster analysis separated mutants into 2 well-defined groups, (a): the control, PL1, PL2, PL3, PL4, and PL8 and (b): PL5, PL6, and PL7. Based on these results, RAPD successfully detected variation between the irradiated and nonirradiated clones, which were morphologically indistinguishable. The DNA polymorphism detected by RAPD analysis offers a useful molecular marker for the identification of mutants in gamma-radiation–treated plants of *E. elatior*.

4. Discussion

4.1. Effect of gamma rays on shoot regeneration of in vitro buds

A matter of interest for plant breeders is the use of mutagens, in combination with in vitro cultures, to create genetic variation (Broertjes and Van Harten, 1988). Determination of the radiosensitivity of in vitro buds to gamma rays is the initial step since it focuses on early screening of variants with altered genetic patterns. The critical level of gamma radiation at which mutations are induced must be within the range of tolerance for regeneration. The irradiation dose LD_{50}, which is not highly inhibitory to plant development, is recommended (Zhou et al., 2006).

In this study the gamma ray dose clearly affected shoot development from in vitro buds of *E. elatior*. With an increase in gamma ray dose, the percentage of explants that produced shoots and leaves decreased. The results showed that shoot induction and shoot growth of irradiated explants were reduced sharply upon exposure to 10 Gy, as compared to the nonirradiated explants, indicating that in vitro buds of *E. elatior* are very sensitive to gamma radiation. Lu et al. (2007) found that the LD_{50} of in vitro *Narcissus tazetta* var. chinensis (Chinese narcissus) bulb-scales irradiated by gamma rays was similar to *E. elatior* (10

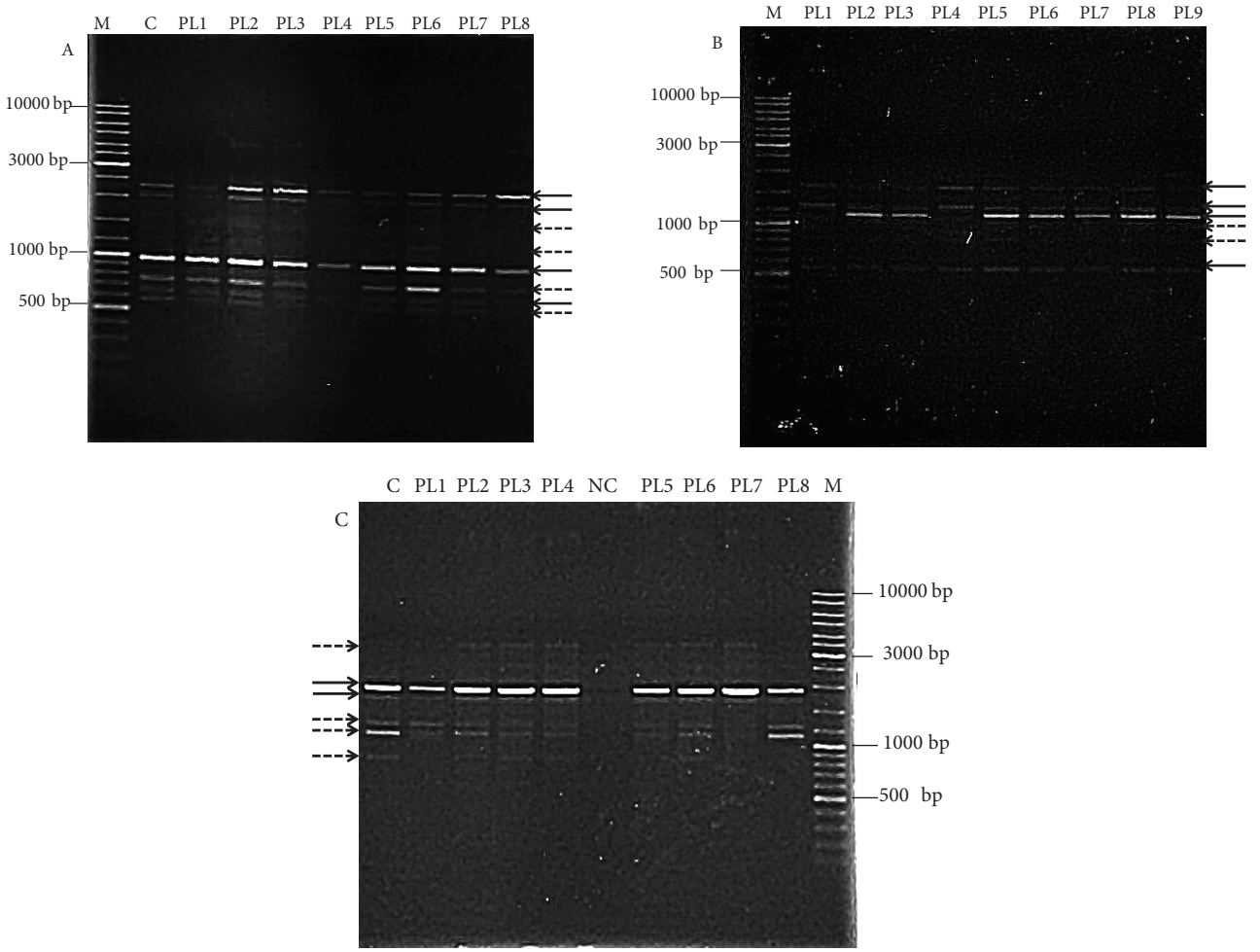


Figure 3. Amplification of genomic DNA from in vitro shoots (M1V3) treated with 10 Gy of gamma ray using various RAPD primers: A) primer OPAW 11, B) primer OPU 16, and C) primer OPU 13. In each of the 3 panels, lane M corresponds to 10 kb DNA ladder; lane C corresponds to the control (non-irradiated plant); lanes 2–10 correspond to DNA from 8 potential regenerant lines; and lane NC on the third panel corresponds to the negative control. Note: solid arrows point to polymorphic bands, while the blank arrows point to monomorphic bands.

Table 3. Jaccard's coefficient of similarity matrix for control and potential mutant lines of *E. elatior* determined from RAPD analysis using 9 different primers and analyzed by UPGMA program.

	Control	PL1	PL2	PL3	PL4	PL5	PL6	PL7	PL8
Control	1.00								
PL1	0.814	1.00							
PL2	0.745	0.846	1.00						
PL3	0.727	0.759	0.860	1.00					
PL4	0.679	0.679	0.813	0.720	1.00				
PL5	0.590	0.653	0.680	0.694	0.604	1.00			
PL6	0.582	0.673	0.700	0.714	0.696	0.829	1.00		
PL7	0.537	0.628	0.687	0.702	0.721	0.690	0.846	1.00	
PL8	0.600	0.692	0.755	0.771	0.681	0.689	0.711	0.698	1.00

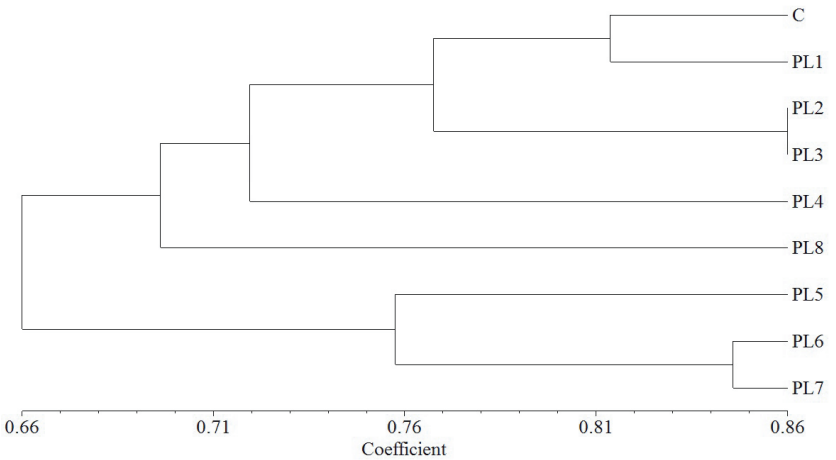


Figure 4. Dendogram constructed from Jaccard's similarity coefficients from RAPD 3 data showing the clustering of the 9 regenerants. C: control and PL1–PL8: potential 4 lines.

Gy). Meanwhile, the dosage of radiation that decreased the regeneration by 50% was 25 Gy for in vitro plant tubers of *Solanum tuberosum* L. (Yaycili and Alikamanoğlu, 2012). However, the dose suggested here was much lower than that reported by Gavidia and Perez-Bermudez (1999), who used a LD_{50} of 60 Gy on in vitro shoot tips of genotype T4 of *Digitalis obscura.* In another report, the LD_{50} for in vitro shoot explants of *Actinidia chinensis* var. hispida and *A. deliciosa* cultivar 'Hayward' reached 50–60 Gy and 80–90 Gy, respectively (Shen et al., 1990). Sensitivity of the multicellular layers to gamma-ray irradiation varied among species and also from genotype to genotype within species.

Some researchers used a low dose of gamma ray to induce mutagenesis. In vitro shoots of pear (*Pyrus communis* L.) cultivars 'Conference', 'Doyenné d'Hiver', 'Passe Crassane', 'Bartlett', 'Abbé Fetel,' and 'Butirra Precoce Morettini' were irradiated with a low dose of gamma rays (3.5 Gy) (Predieri and Zimmerman, 2001). In vitro shoot tip explants of *Rhododendron* varieties 'Alfred' and 'Paars' were irradiated with doses of 5 and 10 Gy of gamma rays (Atak et al., 2011). Puchooa (2005), working with in vitro plantlets of Anthurium variety 'Nitta', stated that the best response was observed with 5 Gy of treatment in terms of regeneration, and 10 Gy was moderately lethal to the explants, while a 15 Gy dose was lethal. Interestingly, some researchers reported that gamma irradiation can increase the quality of the explants. For example, the germination of potato microtubers derived from in-vitro–irradiated microstems (20 and 40 Gy) increased significantly as compared to the nonirradiated microstems (Das et al., 2000).

4.2. Management of chimera and the biological effect of gamma rays

The use of multicellular cultures for mutant induction in vegetatively propagated crops has been suggested, although reports have indicated the difficulties in obtaining solid mutants (Gavidia and Perez-Bermudez, 1999). For mutation breeding, the production of nonchimeric mutants is highly desirable. Gamma radiation treatment of multicellular tissues such as multiple buds often leads to chimeras, depending on the occurrence of mutations in the L1, L2, and L3 meristematic layers (Jain, 2010). Unstable mericlinal chimeras automatically occur when plant parts with multicellular layers are irradiated. Fortunately, chimeras can be converted into solid mutants by repeated multiplication (Yang and Schmidt, 1994). Therefore, it is highly desirable to dissociate chimeras by subsequent subcultures up to M1V3–M1V4 generations (Jain et al., 1998; Mandal et al., 2000). This should be done to maintain the stability of mutant traits and to ensure that the selected mutant lines are solid mutants or stable periclinal chimeras. Such procedures could be done in just 6–7 months by using in vitro techniques, whereas it is estimated that the conventional field process needs more than a year to build up a population for screening gamma-ray–irradiated suckers. Thus, in vitro mutagenesis saves time, equipment, cost, and space. Additionally, in vitro selection allows researchers to control the experimental environment more precisely.

The decline and lower rate of regeneration of irradiated explants at different doses as observed in the present study could be attributed to intrasomatic competition (diplontic selection) in the irradiated multicellular layers. In chimeric tissue, mutated cells are present along with the normal cells. During subsequent cell division, the mutated cells compete with the surrounding normal cells for survival

(Datta et al., 2005). Thus, the low rate of irradiated explant recovery may be due to replacement of mutated sectors with normal cells, reduced competitiveness of mutated cells, and prevention of the appearance of mutated cells (Mandal et al., 2000; Suprasanna et al., 2008). If mutated cells survive in intrasomatic competition, they can potentially be expressed in plants.

Gamma rays have been reported to affect the morphology, anatomy, biochemistry, and physiology of plants, resulting in changes in plant growth and development. The lower regeneration response observed in the present study could be attributed to the toxic effect of gamma radiation (Suprasanna et al., 2008). The ionizing irradiation causes extensive cell nuclear damage and is responsible for the lethality. When a gamma ray is absorbed by biological materials, it will interact with atoms or molecules (particularly water) to produce free radicals in cells, which attack nearby cells. These radicals can modify different important compounds of the plant cell, causing damage to plant cells. This effect of irradiation is critical for vegetative cells, due to the fact that cytoplasm contains around 80% water (Kovacs and Keresztes, 2002).

4.3. Postmutagenesis handling and modification of the culture conditions

It should be taken into consideration that many other factors affect plant responses after irradiation. Therefore, consideration must be given to plant material treatment conditions such as light conditions, regeneration media, and the PGR used in this experiment. Several modifications from previous proliferation conditions (Yunus et al., 2012) that could eliminate the stress were attempted. The full light conditions clearly affected multiple shoot development from the irradiated in vitro buds. Complete absence of light was necessary for shoot induction after irradiation, indicating that irradiated shoots are sensitive to light. The appearance of variation might be related to the stress suffered by the explants postirradiation (Martin et al., 2002). The fact that some mutant shoots died could be due to stress, irreversible injuries, local disruption, and unsuitable conditions for regeneration provoked by gamma irradiation that may exceed the cell's capacity to repair the damage. Intrasomatic competition could be controlled by modifying the light conditions which may result in enhanced competitiveness in irradiated cells.

4.4. Evaluation of genetic variation with RAPD markers

The present study aimed to produce useful variants through gamma irradiation and analyze the variation at a genetic level using RAPD. Traditionally, the identification of variation has been based on morphological characters. However, *E. elatior* has a long flowering cycle (around 1–2 years to flowering). Hence, morphological variation in flowers can only be detected at a late developmental stage. Therefore, early detection of genetic variability is a critical requirement in an *E. elatior* breeding program. The development of PCR-based techniques such as RAPD has allowed analyses based on DNA information (Martin et al., 2002). The PCR profile amplified by RAPD markers successfully detected genetic variations among the induced mutant clones of *E. elatior*. The level of similarity was in all cases less than 90%. The possible genotypic differences might have occurred based on the sensitivity of the irradiated tissue to mutagenic treatments (Ahloowalia, 1998).

The RAPD-marker–generated genetic variation among the control and gamma- ray–treated lines will help distinguish the plants for early selection. The RAPD method has successfully been used to study genetic variability between in vitro *Rhododendron* varieties and mutants, which were exposed to gamma irradiation doses of 5 and 10 Gy (Atak et al., 2011). Barakat et al. (2010) suggested that by using RAPD markers, the newly gamma-irradiated chrysanthemum lines can be easily differentiated from the in vitro *Chrysanthemum morifolium* cultivar 'Delistar White.' The RAPD technique has successfully differentiated *Gypsophila paniculata* variants obtained through in vitro mutagenesis from their parent (Barakat and El-Sammak, 2011). Detection of in-vitro- mutagenesis–induced DNA polymorphisms with RAPD has also been reported in *Saccharum officinarum* (Ahmed Khan et al., 2009; Bibi et al., 2010). These results indicated that the RAPD technique can be a useful tool to detect variation induced through mutation under in vitro conditions.

Induced mutation contributes to genetic variability by increasing DNA polymorphism. Mutagenesis disturbs the normal biological composition, and there are 2 effects of ionizing radiation on the heredity material: gene mutations and chromosome breaks (Atak et al., 2004; Dhakshanamoorthy et al., 2011). Generally, the presence or absence of RAPD bands is used to estimate the diversity of the samples. The disappearance of bands and appearance of new bands from the plants exposed to gamma irradiation could be compared to the control. The disappearance of bands may be related to the occurrence of molecular events such as DNA damage, point mutations, genomic and chromosomal rearrangements, and deletions and insertions of sequences induced by gamma radiation. The appearance of new bands could be attributed to mutations caused by point mutations or by the insertion or deletion of sequences. Thus, both of these result in the alteration of DNA, which leads to greater changes and polymorphism in the RAPD patterns of gamma-ray–treated plants (Atienzar and Jha, 2002; Dhakshanamoorthy et al., 2011).

For the purpose of plant breeding programs, mutagenic treatments with low physiological effects, complete recovery of the desired mutant plants, and strong genetic effects are desirable. The critical level of gamma radiation at which mutations are induced must be within the range of tolerance for regeneration. Hence, we used lower doses of

gamma irradiation (10 Gy) for *E. elatior* improvement. Our results support the view that the RAPD analysis is a highly effective method for detection of DNA polymorphism and early screening of potential mutant lines. This is the first report on in vitro mutagenesis through gamma irradiation and molecular characterization using RAPD marker analysis in *E. elatior*. Thus, this technique could be further exploited to isolate new and novel cultivars for the improvement of *E. elatior*.

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References

Ahloowalia BS (1998). In vitro techniques and mutagenesis for the improvement of vegetatively propagated plants. In: Jain SM, Brar DS, Ahloowalia BS, editors. Somaclonal Variation and Induced Mutations in Crop Improvement. Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 293–309.

Ahloowalia BS, Maluszynski M (2001). Induced mutations—a new paradigm in plant breeding. Euphytica 119: 167–173.

Ahmed Khan I, Dahot MU, Seema N, Yasmin S, Bibi S, Raza S, Khatri A (2009). Genetic variability in sugarcane plantlets developed through in vitro mutagenesis. Pak J Bot 41: 153–166.

Andarwulan N, Batari R, Sandrasari DA, Bolling B, Wijaya H (2010). Flavonoid content and antioxidant activity of vegetables from Indonesia. Food Chem 121: 1231–1235.

Atak C, Alikamanoglu S, Acik L, Canbolat Y (2004). Induced of plastid mutations in soybean plant (*Glycine max* L. Merrill) with gamma radiation and determination with RAPD. Mutat Res 556: 35–44.

Atak C, Celik O, Acik L (2011). Genetic analysis of *Rhododendron* mutants using random amplified polymorphic DNA (RAPD). Pak J Bot 43: 1173–1182.

Atienzar FA, Jha AN (2006). The random amplified polymorphic DNA (RAPD) assay and related techniques applied to genotoxicity and carcinogenesis studies: a critical review. Mutat Res 613: 76–102.

Barakat MN, Abdel Fattah RS, Badr M, El-Torky MG (2010). In vitro mutagenesis and identification of new variants via RAPD markers for improving *Chrysanthemum morifolium*. Afr J Agric Res 5: 748–757.

Barakat MN, El-Sammak H (2011). In vitro mutagenesis, plant regeneration and characterization of mutants via RAPD analysis in baby's breath *Gypsophila paniculata* L. Am J Crop Sci 5: 214–222.

Bibi S, Ahmed Khan I, Khatri A, Yasmin S, Seema N, Afghan S, Arain MA (2010). Screening of mutated population of sugarcane through RAPD. Pak J Bot 42: 3765–3773.

Broertjes C, Van Harten AM (1988). Applied Mutation Breeding for Vegetatively Propagated Crops. Amsterdam, Holland: Elsevier.

Chan EWC, Lim YY, Omar M (2007). Antioxidant and antibacterial activity of leaves of *Etlingera* species (Zingiberaceae) in Peninsular Malaysia. Food Chem 104: 1586–1593.

Chan EWC, Lim YY, Ling SK, Tan SP, Lim KK, Khoo MGH (2009a). Caffeoylquinic acids from leaves of *Etlingera* species (Zingiberaceae). Food Chem 104: 1586–1593.

Chan EWC, Lim YY, Wong SK, Lim KK, Tan SP, Lianto FS, Yong MY (2009b). Effects of different drying methods on the antioxidant properties of leaves and tea of ginger species. Food Chem 113: 166–172.

Das A, Gosal SS, Sidhu JS, Dhaliwal HS (2000). Induction of mutations for heat tolerance in potato by using in vitro culture and radiation. Euphytica 114: 205– 209.

Datta SK, Misra P, Mandal AKA (2005). In vitro mutagenesis—a quick method for establishment of solid mutant in chrysanthemum. Curr Sci 88: 155–158.

Dhakshanamoorthy D, Selvaraj R, Chidambaram ALA (2011). Induced mutagenesis in *Jatropha curcas* L. using gamma rays and detection of DNA polymorphism through RAPD marker. Comptes Rendus Biol 334: 24–30.

Ficker CE, Smith ML, Siti S (2003). Inhibition of human pathogenic fungi by members of Zingiberaceae used by the Kenyah (Indonesian Borneo). J Ethnopharmacol 85: 289–293.

Gavidia I, Perez-Bermudez P (1999). Variants of *Digitalis obscura* from irradiated shoot tips. Euphytica 110: 153–159.

Goh MWK, Kumar PP, Lim SH, Tan HTW (2005). Random amplified polymorphic DNA analysis of the moth orchids, *Phalaenopsis* (Epidendroideae: Orchidaceae). Euphytica 141: 11–22.

Habsah M, Ali AM, Lajis NH, Sukari MA, Yap YH, Kikuzaki H, Nakatani N (2005). Antitumor-promoting and cytotoxic constituents of *Etlingera elatior.* Malaysian J Med Sci 12: 6–12.

Haleagrahara N, Jackie T, Chakravarthi S, Rao M, Pasupathi T (2010). Protective effects of *Etlingera elatior* extract on lead acetate-induced changes in oxidative biomarkers in bone marrow of rats. Food Chem Toxicol 48: 2688–2694.

Ismail NA. Promising accessions of kantan (*Etlingera elatior*) with high antioxidant activity. In: Poster Presentation, National Conference on New Crops and Bioresources, Seremban, Malaysia, 2009 Dec 15–17.

Jaafar MF, Osman CP, Ismail NH, Awang K (2007). Analysis of essential oils of leaves, stems, flowers and rhizomes of *Etlingera elatior* (Jack) R.M. Malaysian J Anal Sci 10: 269–273.

Jaccard P (1908). Nouvelles recherché sur la distribution florale. Bull Soc Vaud Sci Nat 44: 223–270.

Jain SM, Ahloowalia BS, Veilleux RE (1998). Somaclonal variation in crop improvement. In: Mohan Jain S, Brar DS, Ahloowalia BS, editors. Somaclonal Variation and Induced Mutation in Crop Improvement. Dordrecht, the Netherlands: Kluwer Academic Publishers, pp. 203–218.

Jain SM (2010). In vitro mutagenesis in banana (*Musa* spp.) improvement. Acta Hortic 879: 605–614.

Jatoi SA, Kikuchi A, Yi SS, Naing KW, Yamanaka S, Watanabe JA, Watanabe KN (2006). Use of rice SSR markers as RAPD markers for genetic diversity analysis in Zingiberaceae. Breeding Sci 56: 107–111.

Kiefer E, Heller W, Ernst DA (2000). Simple and efficient protocol for isolation of functional RNA from plant tissues rich in secondary metabolites. Plant Mol Biol Rep 18: 33–39.

Kovacs E, Keresztes A (2002). Effects of gamma and UV-B/C radiation on plant cells. Micron 33: 199–210.

Lachumy SJT, Sasidharan S, Sumathy V, Zuraini Z (2010). Pharmacological activity, phytochemical analysis and toxicity of methanol extract of *Etlingera elatior* (torch ginger) flowers. Asian Pac J Trop Med 3: 769–774.

Lu G, Zhang X, Zou Y, Xiang X, Cao J (2007). Effect of radiation on regeneration of Chinese narcissus and analysis of genetic variation with AFLP and RAPD markers. Plant Cell Tissue Organ Cult 88: 319–327.

Mandal AKA, Chakrabarty D, Datta SK (2000). Application of in vitro techniques in mutation breeding of chrysanthemum. Plant Cell Tissue Organ Cult 60: 33–38.

Marcsik D, Hoult M. Tissue culture of tropical ornamentals. Available from: URL: http://www.nt.gov.au/d/Primary_Industry/Content/File/horticulture/cut_flower/PAGES+FROM+TB280-ORNAMENTALS+TC.pdf [accessed 19 February 2010].

Martin C, Uberhuaga E, Perez C (2002). Application of RAPD markers in the characterisation of *Chrysanthemum* varieties and the assessment of somaclonal variation. Euphytica 127: 247–253.

Murashige T, Skoog FA (1962). Revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497.

Pierre JLL. Plant Breeding Genetics Newsletter 28. Available from: URL: http://www-naweb.iaea.org/nafa/pbg/public/pbg-nl-28.pdf [accessed 19 November 2012].

Pinet-Leblay C, Turpin FX, Chevreau E (1992). Effect of gamma and ultraviolet irradiation on adventitious regeneration from in vitro cultured pear leaves. Euphytica 62: 225–233.

Poulsen AD. The ginger genus *Etlingera*. Available from: URL: http://www.dalbergpoulsen.com/ginger_poster.html [accessed 19 February 2010].

Puchooa D (2005). In vitro mutation breeding of Anthurium by gamma radiation. Int J Agric Biol 7: 11–20.

Predieri S, Zimmerman RH (2001). Pear mutagenesis: in vitro treatment with gamma- rays and field selection for productivity and fruit traits. Euphytica 117: 217–227.

Rohlf FJ (2000). NTSYSpc 21 Numerical Taxonomy and Multivariate Analysis System. New York, USA: Exeter Software, Setauket.

Segalen J (2010). The torch ginger. Available from: URL: http://www.rosemarybasil.net/html/modules.php?op=modloadandname=News andfil e=article andsid=829 [accessed 19 February 2010].

Shen XS, Wan JZ, Luol WY, Ding XL (1990). Preliminary results of using in vitro axillary and adventitious buds in mutation breeding of Chinese gooseberry. Euphytica 49: 77–82.

Suprasanna P, Rupali C, Desai NS, Bapat VA (2008). Partial desiccation augments plant regeneration from irradiated embryogenic cultures of sugarcane. Plant Cell Tissue Organ Cult 92: 101–105.

Wijekoon MMJO, Bhat R, Karim AA (2011a). Effect of extraction solvents on the phenolic compounds and antioxidant activities of bunga kantan (*Etlingera elatior* Jack.) inflorescence. J Food Compos Anal 24: 615–619.

Wijekoon MMJO, Karim AA, Bhat R (2011b). Evaluation of nutritional quality of torch ginger (*Etlingera elatior* Jack.) inflorescence. Int Food Res J 18: 1415–1420.

Wong W. Light up your garden with a torch ginger. Available from: URL:www.greenculturesg.com/articles/may08/may08_torchginger.pdf [accessed 19 February 2010].

Yan SW, Asmah R (2010). Comparison of total phenolic contents and antioxidant activities of turmeric leaf, pandan leaf and torch ginger flower. Int Food Res J 17: 417–423.

Yang H, Schmidt H (1994). Selection of a mutant from adventitious shoots formed in X-ray treated cherry leaves and differentiation of standard and mutant with RAPDs. Euphytica 77: 89–92.

Yaycılı O, Alikamanoğlu S (2012). Induction of salt-tolerant potato (*Solanum tuberosum* L.) mutants with gamma irradiation and characterization of genetic variations via RAPD-PCR analysis. Turk J Biol 36: 405–412.

Yunus MF, Aziz MA, Kadir MA, Rashid AA (2012). In vitro propagation of *Etlingera elatior* (Jack) (torch ginger). Sci Hortic 135: 145–150.

Zhou LB, Li WJ, Ma S, Dong XC, Yu LX, Li Q, Zhou GM, Gao QX (2006). Effects of ion beam irradiation on adventitious shoot regeneration from in vitro leaf explants of *Saintpaulia ionahta*. Nucl Instr Meth Phys Res B 244: 349–353.