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IDENTIFICATION OF MORPHOLOGICAL AND MOLECULAR VARIATION INDUCED BY GAMMA IRRADIATION ON *MUSA* CV. PISANG TANDUK (AAB)

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ABSTRACT

The Banana and plantain improvement through conventional breeding method has been very slow mainly due to narrow genetic variability, sterility and polyploid nature of most banana cultivars. Thus, mutation breeding is a promising tool to create new cultivars for the improvement of banana. Therefore, gamma treated banana plantlets were subjected to acclimatization under nursery and several study was conducted. Morphological study on the growth of mutated plantlets showed that, 10 Gy induced a significant stimulation effect on root length, leaf length and leaf width compared to non-treated banana. In the meanwhile, 30 Gy treatment induced dwarf plant. IRAP molecular analysis revealed that, the total of 37 DNA bands position were detected, out of them 20 were polymorphic, producing 54% polymorphism. The highest IRAP bands were detected using primer Sukkula LTR with a maximum number of polymorphic bands, whereas primer LTR 6149, Sukkula LTR/LTR 6149, Sukkula LTR/3'LTR and Sukkula LTR/5'LTR1 yielded monomorphic bands. Hence, in overall application of 10 Gy of γ induced the significant higher phenotypic and genotypic variation. Therefore, the current research was conducted to study the effect of gamma radiation on the morphological and genetic variation in γ irradiated banana plant.

Keywords: IRAP, Morphology, Molecular, *In vitro* mutagenesis

1. INTRODUCTION

The global population is getting bigger and starving. A latest report by United Nation projected that the world population to be increase by one billion over the next 10-12 years, reaching more than 9.6 billion by 2050. In order to feed a bourgeoning world population, the global food production and productivity must be increased. For that reason, most of the existing cultivar need to be improved to meet our subsistence food requirement and mainly to surplus for the growing world population. Banana and plantains improvement through conventional breeding method has been slow mainly, due to narrow genetic variability. As most bananas are propagated vegetative, conventional cross breeding is not possible due to its sterility and ploidy nature of most popular banana cultivars. With this, mutation breeding is a promising tool to improve banana varieties using mutagenic treatment to generate novel cultivars of banana (Govindaraj et al., 2015; Kalwar and Dahot, 2017).

Several studies have been conducted for induction of mutations using γ rays through *in vitro* mutagenesis technique. Gamma rays can be advantageous for the modification of physiology and morphology of mutated plant material. However, the effect of γ rays is built on the interaction of atoms or molecules inside the cell, mainly water, to produce free radicals that can harm or adjust important constituents of the plant cells. The effects include changes in the plant cellular structure and metabolism such as activating enzymes, alteration in photosynthesis, inflection of the anti-oxidative system, and buildup of phenolic compounds (Kovic and Keresztes, 2002; Rajoka et al., 2003; Wi et al., 2005; Majed et al., 2015; Qamar et al., 2016).

Harmful effect of ionization radiation in biological systems is depends on radiolysis of water. The final product of this process is the hydroxyl radical (OH) that can damage the DNA and introduces mutations or changes in genotype. Radiation brought DNA with single and double-strand disruptions may be fixed incorrectly leading to chromosomal readjustments (Spotheim and Davidkova, 2010).

The molecular marker technology offers opportunities to help in detecting alteration by examining both genetic variant within populations and rapid detection of mutants with desired traits (Predieri, and Di Virgilio, 2007). This method is based on the polymerase chain reaction (PCR) by extracting the DNA from the organism in question and a variety of different technique can be used to monitor and detect variation (Muchugi, 2008). As evidence by Yassein and Aly (2016), who studied the effect of gamma radiation on the physiology and molecular characteristic of *Brassica napus* by exposing seeds at different dose of γ dose, the result had revealed that higher dose produced different DNA profile with absence of certain bands in RAPD analysis. Therefore, this investigation was conducted to study the effect of gamma radiation on the morpho-

logy and genetic variation using morphological and molecular (IRAP) markers.

2. Material and Method

2.1. Gamma irradiation procedure, media component and *in vitro* shoot multiplication: The research was conducted at Faculty of Veterinary Medicine, University Malaysia Kelantan, Kelantan, Malaysia. The sword sucker of banana cv. Tanduk with the size of 25-35 cm in height were collected from a banana farm in Pengkalan Hulu, Perak Malaysia (5.7064° N, 100.9998° E). Gamma irradiation was carried out at Malaysian Nuclear Agency in Bangi, Selangor using BIOBEAM gamma irradiation machine, using ^{137} Cesium gamma ray source. Shoot meristem (1.5 x 1.0cm) were placed in the sterile Petri dish wetted with a few drops of sterilized distilled water and exposed to three different gamma doses (10, 20 and 30 Gy). Following, irradiation, treated explants were washed with sterilized distilled water thoroughly and then transferred to initiation media consisted of macro, micro nutrients, iron, organic constituent, 30 g/L sucrose and 2.0 mg-1 N6-benzylaminopurine (Dagnew et al., 2012) and sub-culturing was carried out in four weeks interval up to four cycles using the same proliferation medium and then transferred to rooting media (2 g/1 activated charcoal) and cultured for one month. All cultures were incubated at 26±2 °C with 65% humidity at 17 hours photoperiod under fluorescent light (1000 lux) and 8 hours in the dark.

2.2. Acclimatization of plantlets: After four weeks on rooting medium, well grown plantlets about 7 to 9 cm in length were carefully detached from the culture vessel and their roots wash away carefully under running water to remove the traces of nutrients and potted in (10 x 15cm) polybags. The polybags medium consisted of a mixture of peat moss and cocopeat (2:1 w/w) respectively. Potted banana plantlets were placed in a nursery area with 50% shade and then exposed to full sunlight after 20 Days. Benomyl 0.4g /100 ml diluted with water were sprayed on the plant to prevention of black Sigatoka. A total of 95, 90, 90 and 86 plantlets were potted for control, 10 Gy, 20 Gy and 30 Gy treatments, of these 15, 11, 10 and 9 respectively, died after potting.

2.3. Genomic DNA extraction: Genetic variability of seedlings treated with different γ doses were evaluated using Inter-retrotransposon amplified polymorphism (IRAP) molecular marker analysis. The leaf sample was collected based on morphological variation observed in the nursery (Table 1).

Table 1: The leaf sample collected for molecular study

No	Phenotype variation observed	γ dose
1	Control	Control (0 Gy)
2	Longest leaf length	10 Gy
3	Small leaves	20 Gy
4	Dwarf plant	30 Gy

Genomic DNA was extracted from young leaves of banana seedlings according to the CTAB protocol Gawel and Jarret (1991). 3–4g of young leaf tissue (1st leaf) of 60day old banana seedling were taken from the nursery and grounded under liquid nitrogen to a fine powder using mortar and pestle. The leaf powder was added into 50 ml tube and mixed thoroughly with 40 ml pre-warmed CTAB extraction buffer (2% CTAB, 1.4M NaCl, 100 mM Tris-HCl, 20 mM EDTA, and 0.2% 2-mercaptoethanol). Grinded sample were incubated at 65 ºC for 60 minutes in a water bath and the tubes were inverted by hand at every 10 minutes to mix the contents. Incubated sample were transferred to a new tubes and equal volume of chloroform-isoamyl (24:1) were added and then shacked gently for 15 minutes followed by centrifuging at 2700 rpm for 10 minutes. The aqueous (top) layer were pipetted out and 2/3 volume of cold (-20 ºC) 2-propanol were added and kept overnight in (-20 ºC) for DNA precipitation. Cloudy like solution was transferred into new sterile centrifuge tubes and spanned at 12000 rpm for 10 minutes centrifuge again at 2700 rpm for 10 minutes to pellet down the DNA. Supernatant were poured away and 1 ml of wash buffer (76% ethanol and 10mM ammonium acetate) was added into the tube. After one hour in washing buffer and centrifuged at 12000 rpm for 10 minutes, the supernatant was carefully poured off and pellet allowed to dry overnight. Finally, pellet was dissolved in 50 µl TE buffer (50 mM-Tris-HCl pH 8 and 20 mM DTA). The quality of genomic DNA was measured using Nano Drop and gel electrophoresis.

2.4. PCR condition for IRAP analysis: The genetic variability of mutant banana plant caused by γ treatments along with their control was assessed using IRAP marker following the protocol described by Teo et al., (2005) using LTR primers. The PCR was performed in a 25 µl reaction mixture containing 25 ng/µl DNA concentration, 5X PCR buffer, 25 mM MgCl2, and 10 mM dNTP mix, 5X of Go *Taq* DNA polymerase, 10 µM of primer as in Table 2 and 2 µl (50 ng) of DNA were used. The PCR reaction were conducted using 14 single and combination of primers with the following sequences in Table 2.

Table 2: Primers sequences, sources, combination and annealing temperature for IRAP

No	Name	Retrotransposons source	Sequence	Annealing Temperature (T_a)	References
1	Sukkula LTR	*Sukkula* →	GATAGGGTCGCATCTTGGGCGTGAC	45.5	Teo *et al*., 2005
2	LTR 6149	BARE-1←	CTGGTTCGGCCCATGTCTATGTATCCACA	40.5	''
3	3'LTR	BARE-1→	TGTTTCCCATGCGACGTTCCCCAACA	48.4	''
4	5'LTR1	BARE-1←	TTGCCTCTAGGGCATATTTCCAACA	41.5	''
5	5'LTR2	BARE-1←	ATCATTGCCTCTAGGGCATAATTC	43.2	''
6	Nikita LTR	*Nikita* →	CGCATTTGTTCAAGCCTAAACC	48.4	''
7	Sukkula + LTR 6149	-	-	45.5	''
8	Sukkula + 3'LTR	-	-	47.0	''
9	Sukkula + 5'LTR1	-	-	43.2	''
10	LTR 6149 + 3' LTR	-	-	45.5	''
12	3'LTR + 5' LTR2	-	-	48.4	''
13	5'LTR1 + 5'LTR2	-	-	43.2	''
14	Nikita + 3' LTR	-	-	47.0	''

The PCR was performed using (BIO RAD 100, USA) thermocycler and the PCR machine was programmed to perform an initial denaturation of 95 °C, 2min; 30 cycles of 95 °C, 60 s, annealing at the T_a for 60 s, ramp +0.5 °C s"1 to 72 °C, and 72 °C for 2 min + 3s per cycle; a final extension at 72 °C for 10 min. 3% (w/v) agarose gel was stained with 2 µl and 10 µl PCR product was loaded into well and 100 bp DNA ladder was used to estimate the size. The PCR product were visualized under Gel Documentation System (BIO RAD, USA).

2.5. Data collection and statistical analysis: After 8 weeks, data were collected for morphological characteristics of γ treated and non-treated seedlings based on visual observations. The growth parameters measured included plant height (from ground to leaf tip), number of leaves, leaf length, leaf width, root number, root length measured (by uprooting the plants). The data recorded on morphological characteristics were subjected to one-way analysis of variance (ANOVA) by Duncan Multiple Range Test ($p<0.05$). SPSS version 20.0 statistical software used to carry out the analysis. The percentage of polymorphism was calculated according to (Saraswathi et al., 2011) using the following formula

$$Polymorphism\ (\%) = \frac{Number\ of\ polymorphic\ bands}{Number\ of\ Amplified\ bands} \times 100$$

Equation 1: Polymorphism percentage

3. RESULTS AND DISCUSSION

3.1. Morphological variation assessment of irradiated plantlets

Effect of γ on leaf formation, leaf length and width: Analysis of variance for the leaf number per explants showed no significant difference among the controls, seedlings treated with 10 Gy (T1) and 20 Gy (T2). However, there was a significant difference being observed between seedlings treated with 30 Gy with the controls (Table 3). Leaf number tends to be lower with the increase of γ dose radiation. The maximum number of leaves recorded was 7.98±0.19 in the 10 Gy seedlings, followed by control (0 Gy), 20 Gy (T2) and 30 Gy (T3) recorded 7.83±0.25, 7.44±0.66 and 6.50±0.57 respectively, our experiments is consistent with recent studies by Jala, (2011), who observed significance reduction in number of leaves at 20 and 30 Gy of γ radiation in *Torenia fourmieri* plant. Morphological variation could be from the genetic construction of γ irradiated explants and that brings variations in various cytological and physiological aspects which expresses through morphological changes (Ikram et al., 2010). The result on leaf growth showed that, 10 Gy had significant difference with control in terms of leaf length and width but no significant difference being observed in the leaf number. Among treated banana seedlings, 30 Gy had induced reduction in leaf growth. This finding suggests that the 10 Gy treated plant able to photosynthesize soundly thus resulted in better leaf growth performance.

Table 3: Growth of banana cv. Tanduk seedlings leaves number, length and width after 60 days in the nursery

γ doses (Gy)	Growth parameters		
	Leaf number	Leaf length (cm)	Leaf width (cm)
T0: 0 Gy	7.80±0.25^{a}	14.37±0.57^{b}	6.59±0.25^{b}
T1: 10 Gy	7.98±0.18^{a}	23.23±1.18^{a}	9.76±0.59^{a}
T2: 20 Gy	7.43±0.66^{ab}	12.03±0.90^{bc}	5.97±0.02^{bc}
T3: 30 Gy	6.50±0.57^{b}	10.05±0.26^{c}	5.30±0.20^{c}

*Means with the same letter has no significant difference at P<0.05 after determined by a Duncan test.

Effect of γ on plant height and rooting performance: It is clear from the present study that γ radiation used in this study lower dose (10 Gy) had no significance influence on plant height. However, higher γ dose (20 and 30 Gy) significantly reduced the plant height as shown in Table 4 and Figure 1. The tallest plant observed was 35.03±0.3cm in control, followed by 33.30±0.70cm in 20 Gy, 31.63±0.83cm in 20 Gy and 29.73±0.86cm in 30 Gy seedlings. The result was closely in accordance with Jamil and Khan, (2002), reported that increase in γ radiation dose resulted reduced plant height.

Table 4: Radiation effect on plants height, root number and root length after 60 days

γ doses (Gy)	Plant height (cm)	Root length (cm)	Root number
T0: 0 Gy	35.03±0.31^{a}	24.30 ± 0.20^{b}	18.0 ± 1.7^{ab}
T1: 10 Gy	33.30±0.70^{ab}	32.10 ± 3.70^{a}	22.3 ± 0.8^{a}
T2: 20 Gy	31.63±0.83^{bc}	22.33 ± 0.88^{b}	15.6 ± 1.4^{b}
T3: 30 Gy	29.73±0.86^{c}	22.53 ± 1.03^{b}	16.6 ± 1.2^{b}

*Means with the same letter has no significant difference at P<0.05 after determined by a Duncan test.

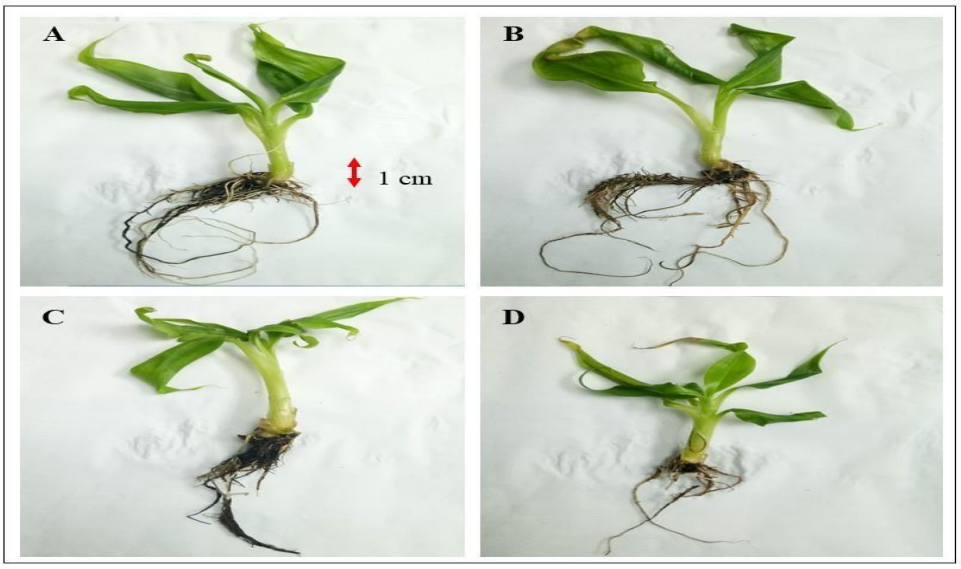

Figure 1: Effects of γ radiation on *in vitro* rooting performance (A: 0 Gy), (B: 10 Gy) (C: 20 Gy) and (D: 30 Gy)

The rooting performance of control and irradiated samples were measured 60 days after plantlets was being transferred to the nursery (Table 4). From the obtained data, it was noticed that γ radiation at 10 Gy had significant impact on root length, however, higher dose 20 and 30 Gy showed no significance difference over control. The longest root was observed in the irradiated plants at 10 Gy (32.10 ± 3.70cm), followed by 24.30±0.20cm in control, 22.53±1.03cm in T2, and 22.33±0.88cm in T3. From the data obtained on root number noticed that no significance difference over control. However, higher γ doses (20 and 30 Gy) showed significance reduction in root length.

Table 5: Analysis of polymorphism obtained with random primers among non-treated and treated γ radiation

Primer	Total number of bands	No of polymorphic bands	Polymorphism (%)
Sukkula LTR	11	8	73
LTR 6149	1	0	-
3'LTR	8	3	37
5'LTR 1	-	-	-
5'LTR 2	-	-	-
Nikita	1	0	-
Sukkula LTR + LTR 6149	1	0	-
Sukkula LTR + 3'LTR	1	0	-
Sukkula LTR + 5'LTR 1	1	0	-
LTR 6149 + 3'LTR	6	3	50
3'LTR + 5'LTR 1	-	-	-
3'LTR + 5'LTR 2	-	-	-
5'LTR 1 + 5'LTR 2	-	-	-
Nikita + 3'LTR	7	6	85
Total	37	20	
Average	3	1.5	18

3.2. Molecular variation analysis using IRAP technique: The IRAP analysis was carried out for detection of DNA profile changes due to γ rays treatments (10, 20 and 30 Gy). The result showed that majority of primers used for analysis resulted in the presence of PCR products with a different number of bands (Figure 2 and 3).

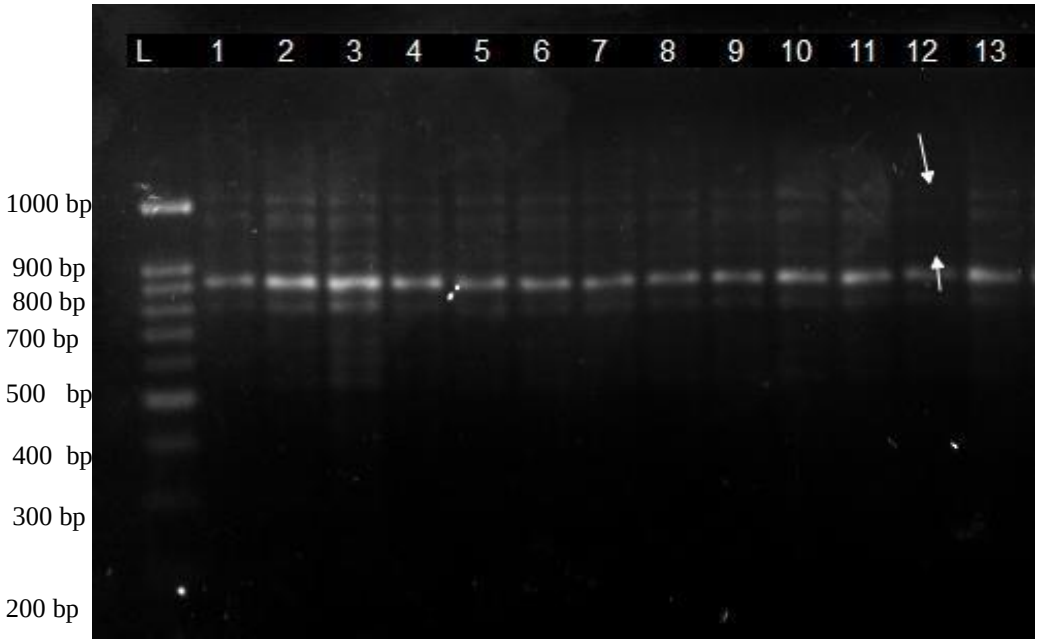

Figure 2: Inter-retrotransposon amplified polymorphism (IRAP) analysis using primer Sukkula LTR, Lane 1: control, Lane 2-6: 10 Gy, Lane 7-11: 20 Gy, Lane 12-16: 30 Gy, L= 1 kb ladder. The arrows in Lane 2 and 3 represent missing bands in γ irradiated banana DNA sample

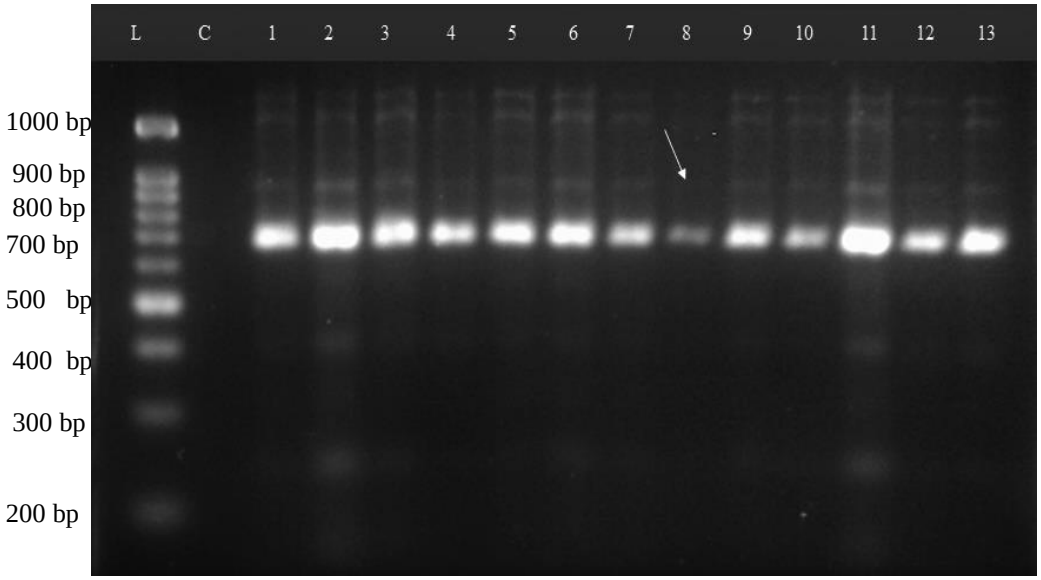

Figure 3: Inter-retrotransposon amplified polymorphism (IRAP) analysis using primer LTR6149 +3 LTR, Lane 1: control, Lane 2-5: 10 Gy, Lane 6-10: 20 Gy, Lane 11-15: 30 Gy, L= 1 kb ladder. The arrows in Lane 2 and 3 represent missing bands in γ irradiated banana DNA sample

The primer Sukkula LTR produced maximum (11) bands and produced the most polymorphic pattern. The highest polymorphic bands recorded was 8 in Sukkula LTR primer, followed by 6 polymorphic bands in primer combination of Nikita + 3'LTR, 3 in primer 3'LTR and 3 in LTR 6149 + 3'LTR poly- morphic band were observed. In this study, the total of 37 DNA bands position were detected, out of them 20 them 20 were polymorphic, producing 54% polymerphism. The average number of bands per primers was about 2, the minimum number of band was zero and the maximum number was 11 band. The IRAP profile generated by primer Sukkula LTR, 3ˈLTR, LTR 6149+3ˈLTR and Nikita+ 3ˈLTR showed a loss of bands in several fragments bp in genomic DNA derived from γ treated line (10, 20 and 30Gy).

Therefore, primer Sukkula LTR, 3ˈLTR, LTR 6149 + 3ˈLTR and Nikita + 3ˈLTR distinguished DNA changes and genetically variable clones with the absence of several fragments (Figure 2). Therefore, those primers are considered as potential markers to identify genetic variability caused by γ rays in banana cultivar Tanduk. In the meanwhile, primer LTR 6149, Sukkula LTR + LTR 6149, Sukkula LTR +3' LTR and Sukkula LTR + 5'LTR1 generated single band. The result of IRAP analysis using fourteen primers revealed the presence and absence of DNA polymorphic bands at all doses of γ rays. The results agreed with Ganapathi et al., (2008) who studied the effect of γ irradiation on banana using RAPD technique. They detected the presence or loss of DNA bands in genomic DNA derived from γ treated population. The absence of bands indicates DNA damage includes single and double-strand breaks, modified bases, point mutations, and/or complex chromosomal rearrangements induced by mutagens that damages the genetic information within a cell causing mutations (Wolf, 2004).

It is clear from the results of IRAP markers characterization of variants in banana cultivars tested in the present study is that, IRAP markers could be assumed as an efficient tool for estimation of genetic variability among gamma treated lines in banana and has been successfully utilized to detect mutation in banana cultivar Tanduk plants induced by γ rays. In general, among, mutant, 10 Gy and 20 Gy treated plants had shown higher genotypic variation by showing several DNA fragments absence and it is identified as somaclonal variants due to gamma ray's treatment. The results of IRAP analysis using primers (Sukkula LTR and LTR 6149 + 3ˈLTR) respectively are illustrated in Figures 2 and 3 respectively.

4. CONCLUSION AND RECOMMENDATION

Result from the experiment concluded that higher γ dosage had caused sever effects on the growth, morphological and molecular variation on banana cultivar Tanduk. Lower dose of γ irradiation had a positive impact on the growth of *in vitro* plants during multiplication phase and rooting stage, whereas higher γ irradiation had caused a negative impact on the growth, in terms of higher mortality rate and reduction in growth. Among the gamma treatment applied in the present study, 10 Gy was found to be the best treatment in the induction of variability. This was demonstrated by higher survivality, leaf growth and higher root growth. Therefore, the most suitable dose of γ radiation for inducing morphological and molecular variation was 10 Gy. While higher γ irradiation (> 30 Gy), showed lower survivality and reduction in growth. Molecular analysis showed that, γ radiation exerted variation in genomic DNA derived from γ treated populartion (10, 20 and 30 Gy) and forming distinct banding pattern (missing and new band) than normal (non-mutant DNA). Among, mutant DNA, 10 Gy had shown higher genotypic variation by showing several DNA fragments absence.

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