

# The effect of gamma rays irradiation on diversity of *Musa paradisiaca* var. *sapientum* as revealed by ISSR molecular marker

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Manuscript received: 7 February 2019. Revision accepted: 28 April 2019.

**Abstract.** Due MS, Susilowati A, Yunus A. 2019. The effect of gamma rays irradiation on diversity of *Musa paradisiaca* var. *sapientum* as revealed by ISSR molecular marker. *Biodiversitas* 20: 1416-1422. Pisang Ambon (*Musa paradisiaca* var. *sapientum* (L.) Kunt.) is one of the horticultural commodities that have high production in Indonesia, making it a superior product. Bananas are propagated vegetatively, which hampers the production of genetic variation and requires a long generation time in the vegetative cycle. This study aimed to determine the diversity of Pisang Ambon induced by gamma radiation of various dosages (10 Gy, 20 Gy and 30 Gy) in vitro based on the ISSR (Inter-Simple Sequence Repeats) marker. The results showed that gamma-ray radiation influenced the morphological properties of plant height, stem diameter, leaf length, leaf width and leaf number based on ANOVA variance analysis. Increased growth occurs in plants with radiation doses of 10 Gy and 20 Gy. The molecular analysis of ISSR uses 6 primers, namely ISSR 835, ISSR 836, ISSR 846, ISSR 847, ISSR 848 and ISSR 855. Of the 6 primers, only 2 primers were able to produce polymorphisms above 50%. A total of 9 bands were produced, in which 8 bands showed polymorphic with a percentage of 90%. The similarity matrix ranged from 0.25-0.93, indicating the level of variation between mutants. The result of dendrogram was divided into two groups with a coefficient distance of 0.47-0.93; plants at a dose of 10 Gy have the highest diversity with the furthest coefficient of diversity distance of 0.47.

**Keywords:** Diversity, gamma ray, irradiation, molecular markers, pisang ambon

## INTRODUCTION

Banana (*Musa* spp.) is an important genus in the family of Musaceae which can be found on the African and Asian continents. Bananas are known to originate from South and Southeast Asia (Abdulhafiz et al. 2018). According to Crouch et al. (1998), bananas are the most widely cultivated fruit trees in tropical areas. Generally, banana plants have herbal habitus and only bear fruit once (monocarpic) and then die. Banana plants ranked fourth as the most important food crop in the world after rice, wheat and corn (Frison et al. 2004; Megia 2005).

Pisang Ambon (*Musa paradisiaca* var. *sapientum* (L.) Kunt.) is a tropical fruit that grows and has become popular in Indonesian. This banana species is a superior commodity, which contributes the most to national fruit production. This plant is popular because it tastes good, contains various nutrients, vitamins, and calories that are beneficial to health (Prahardini et al. 2011). Bananas are known as annual plants with their fruiting times which are not dependent on the season (Bakry et al. 2006).

Pisang Ambon is propagated vegetatively so that they have limitations in obtaining genetic variation and require a long generation time in the vegetative cycle. To obtain new superior varieties, improvements can be made to the nature of plants by increasing their genetic diversity. According to Sari et al. (2014), an increase in genetic diversity of a plant can be done through genetic introduction, hybridization,

induced mutation, and genetic engineering. Among these methods, induced mutation combined with in vitro culture techniques is an optimistic and faster way in an effort to increase plant genetic diversity.

Induced mutation with in vitro culture techniques has been widely used for various purposes such as improving plants through increasing genetic diversity and selecting mutants as potential sources to produce a new cultivar (Orbovic et al. 2008). According to Javed et al. (2004), in vitro mutation breeding can overcome the problems that occur when producing superior new banana cultivars for the sustainability of fruit production without the spread of disease. With the discovery of this technology, a large number of mutant varieties have been developed for commercial cultivation (Pena et al. 2012).

Induced mutation in plants can be done using mutagens, either physical or chemical mutagens. The use of physical mutagens in plants is highly recommended as compared to chemical mutagens since the physical mutagens are more environmentally friendly and have a high frequency of mutations. Among existing physical mutagens, gamma rays are the most commonly used as mutagen agents in in-vitro induction of mutation experiments (Roux et al. 2004). The gamma rays are selected as a mutagenic agent because it has a higher level of accuracy, a strong absorption power to plant's biological material and is able to ionize the molecules it passes (Harten 1998). Kovac and Keresztes (2002) explained that the biological effects of gamma

radiation are very dependent on the interaction of atoms or molecules in cells, especially in water to produce free radicals.

Previous studies on induced mutation using gamma rays with in vitro culture techniques produced positive results where gamma-ray mutagens provided advantages such as the occurrence of physiological and morphological modifications in mutated plant material. Even so, the optimum dose of the radiation agent must still be elucidated. A previous study conducted by Dwimahayani and Widiarsih (2011) showed a change in *Chrysanthemum* plants at a dose of 10 Gy carried out in vitro. The gamma-ray radiation dose of 10 Gy increased the height of Cavendish banana shoots, but the high irradiation dose will give more inhabitation on its growth (Qamar et al. 2016). In addition, Abdulhafiz et al. (2018) showed that radiation doses of 10 Gy and 20 Gy had a significant effect on quantitative and qualitative morphology of pisang tanduk.

Gamma rays radiation which applied to plants will affect the morphological characteristics due to damage to DNA. This damage induces mutations at both the chromosome level and free radical genes. Molecular techniques such as molecular marker offers an opportunity to help detect the changes by examining both genetic variants in the population and detect the desired mutants in a more robust way (Abdulhafiz et al. 2018). This molecular marker can serve as complements for morphological markers. Molecular markers can reveal differences among irradiated and non-irradiated individual plants at the DNA level and, thus, provide a more direct, reliable and efficient tool for germplasm conservation and management (Kumar et al. 2006).

One of the molecular markers used to detect genetic diversity is Inter-Simple Sequence Repeats (ISSR). ISSR is a part of microsatellite that does not encode protein (non-coding region), and it is usually mono, di or trinucleotide (Ng and Tan 2015). Generally, ISSR markers are more sensitive in detecting genetic diversity at low levels, easier and cheaper than other markers such as RAPD. ISSR markers have been widely used, including analysis of the diversity of mangosteen (Widiastuti et al. 2013), genetic polymorphism from coconut crossing (Manimekalai et al. 2003), detection of DNA polymorphism in *Curcuma alismatifolia* (Taheri et al. 2013), and for genetic linkages in plants *Catharanthus roseus* (Gupta et al. 2007). The purpose of this study was to use ISSR molecular markers to reveal the genetic diversity of pisang ambon caused by in vitro gamma-ray irradiation.

## MATERIALS AND METHODS

### Plant material

The plant materials in this study were the aseptic shoots of pisang ambon that were in one-month-old multiplication stage obtained from the Salaman Horticulture Seed Garden, Magelang, Central Java, Indonesia.

### Gamma irradiation

The aseptic shoot plantlets of pisang Ambon were first proliferated on Murashige and Skoog (MS) media in

culture bottles and were labeled according to the treatment. Furthermore, the culture bottles were inserted into the Cobalt<sup>60</sup> gamma chamber for the radiation process with radiation doses of 0 Gy, 10 Gy, 20 Gy, and 30 Gy. Irradiation of plant materials was carried out at the Research and Development Center for Isotope and Radiation Technology, the National Nuclear Energy Agency (BATAN) Pasar Jumat, South Jakarta, Indonesia.

### Subculture of plantlet

The shoots of the banana from the gamma rays irradiation are removed from the old medium, then subcultured to the new medium twice at intervals of 4 weeks in liquid medium containing MS, growth regulators NAA and sucrose. After 8 weeks of age, the buds were transferred and cultured on MS solid media, with the addition of NAA and sucrose, for 4 weeks to induce shoot lengthening and further root formation. Furthermore, the plantlets were ready to be transferred to the planting media.

### Greenhouse transfer and morphological analysis

Rooted shoots were washed in running tap water and acclimatized in hydroponic media for 4 weeks with routine provision of nutrition in every 2 days. After 4 weeks, the plants were moved and planted in polybags containing a mixture of soil, manure and fuel husk of the same ratio. Polybags containing plants are treated with regular weeding and watering every morning and evening using water in the glasshouse of the Faculty of Agriculture, Sebelas Maret University. Morphological observations were carried out on vegetative characteristics such as plant height, stem diameter, leaf length, leaf width and number of leaves per plant.

### ISSR Assay

*Plant material for extraction.* Fresh leaf samples were taken from banana plants which were previously used for quantitative morphological characterization observations. The leaves were young leaves randomly selected from each treatment based on morphological characteristics which showed the most noticeable differences compared to controls.

*Preparation of DNA template.* Leaf samples were extracted using DNA Mini Kit (Plant) provided by Geneaid. The quality and quantity of extracted DNA were examined using a standard biophotometer and electrophoresis.

*DNA amplification.* Amplification was done by PCR (Polymerase Chain Reaction) reaction using a Thermal cycler (Eppendorf Thermal cycler) device. The amplification process used 6 ISSR primers (Table 1). The PCR reaction was carried out at a total volume of 50µL for each PCR tube using MyTaq TM HS Red Mix (Bioline). The PCR reaction consisted of 1 µL DNA template, 1 µL primer, 25 µL MyTaq HS Red Mix and 23 µL water (ddH<sub>2</sub>O). PCR was carried out at an initial denaturation of 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C for 15 seconds, extension at 72°C for 10 seconds and final extension for 10 minutes at 72°C. The PCR results were detected in

electrophoresis using 1% agarose which was made by dissolving 0.5 grams of agarose into 50 ml of 1X TAE buffer. The running process was carried out with an electric current of 85 volts for 45 minutes. Running gel was soaked in EtBR (ethidium bromide) for 15 minutes and rinsed using distilled water. The results of electrophoresis were visualized by using UV light using a *Biodoc Analyze* and photographed using Olympus digital camera. The size of the amplification product was estimated based on 100 bp DNA ladder (Geneaid).

### Data analysis

Quantitative morphological characters were analyzed by analysis of variance (ANOVA) on the level 5%. If the treatment has real effects, the test will be continued by Duncan's Multiple Range Test (DMRT) on the level of 5%. Analysis of molecular data was done by looking at the results of the advent of DNA amplification using a primer. The obtained data in scoring was present (1) or not present (0) ribbon of each individual at a certain size in each primer. From these data, it can be seen the number and percentage of polymorphic loci. Next, cluster analysis (Cluster analysis) used the NTSYS (Numerical Taxonomy and Multivariate Analysis System) program version 16 with the method of Group Unity Pair Arithmetic Method (UPGMA) SIMQUAL (similarity qualitative) function. The genetic similarity matrix was calculated based on the DICE coefficient.

## RESULTS AND DISCUSSION

### Effect of gamma irradiation on morphological characters

In this study, the number of explants living after the advanced root extension stage in vitro that is ready for acclimatization is 28 experimental plants with 7 of them being controls. This plant does not experience death until the end of quantitative vegetative observation (15 MST). However, there are some plants that experience stunting characterized by a decrease in the process of growth and development. The declining process of growth and development of pisang Ambon kuning is caused by exposure to gamma radiation which produces ions and free radicals causing changes in the levels of genomes, chromosomes, and DNA (Herison et al. 2008).

ANOVA results showed that gamma irradiation affected the quantitative morphological traits of pisang ambon such as plant height, stem diameter, leaf length, leaf width and number of leaves.

Based on data (Table 2), it can be seen that plants treated with a radiation dose of 10 Gy have the highest average plant height of 22.69 cm or increased by 13.88% when compared to the control treatment (0 Gy). For plants at radiation doses of 20 Gy and 30 Gy, they are reduced by 4.24% and 15.04% respectively. However, plant height does not show a significant difference between control and radiation-treated plants. Observations on the quantitative character of stem diameter show a significant difference ( $< 0.05$ ) between the control and irradiated plants. The stem diameter ranges from 4.52 mm to 7.13 mm. The radiation dose of 10 Gy is the dose with the largest stem diameter acquisition of 7.13 mm and the lowest is in irradiated plants at a dose of 30 Gy with 4.52 mm (reduced by 23.12%). The treatment on plant with 20 Gy results in average stem diameter of 6.91 mm or an increase in growth of 17.51% compared to the control which has an average of 5.88 mm. The diameter of the plant stem from the treatment of gamma-ray irradiation 10 Gy is not significantly different from each other.

The leaves of pisang ambon differ significantly between control plants with irradiation treatment plants of 20 Gy and 30 Gy, while in the 10 Gy treatment, it does not differ significantly. The treatment of 20 Gy irradiation has the highest leaf length of 12.69 cm or increased by 8.92% compared to the control, whereas the treatment of 30 Gy is the treatment with the smallest leaf length acquisition of 9.06 cm (reduced by 22.23%).

Gamma-ray irradiation does not have a significant effect on the control and the treated plant on leaf width and number of leaflets. The highest leaf width of 5.15 cm and the highest number of leaflets at 5.61 occurred in the treatment of 10 Gy, while the lowest leaf width (3.56 cm) and the number of leaflets (4.61) are observed in the treatment of 30 Gy. Plant height, leaf width and number of leaves of pisang ambon plant decreased at the highest irradiation dose (30 Gy), although it does not reach a significant level.

**Table 1.** List of primers Inter-Simple Sequence Repeat (ISSR) (Tantasawat et al. 2010)

Primers	Sequence	Tm (°C)
ISSR 835	5'-AGA GAG AGA GAG AGA GYC-3'	48.9
ISSR 836	5'-AGA GAG AGA GAG AGA GYA-3'	48.5
ISSR 846	5'-GAG AGA GAG AGA GAG AA-3'	45.7
ISSR 847	5'-CAC ACA CAC ACA CAC ARC-3'	53.1
ISSR 848	5'-CAC ACA CAC ACA CAC ARG-3'	52.7
ISSR 855	5'-ACA CAC ACA CAC ACA CYT-3'	53.1

**Table 2.** The effect of gamma-ray irradiation on the quantitative morphological properties of pisang ambon

Dose (Gy)	Observation parameters of growth				
	Plant height (cm)	Stem diameter (mm)	Leaf length (cm)	Leaf width (cm)	Number of leaves
0 Gy	19.81±6.79 <sup>a</sup>	5.88±1.53 <sup>ab</sup>	11.65±2.92 <sup>ab</sup>	5.04±1.86 <sup>a</sup>	5.57±2.30 <sup>a</sup>
10 Gy	22.56±8.45 <sup>a</sup>	7.13±1.84 <sup>b</sup>	11.72±2.72 <sup>ab</sup>	5.15±1.85 <sup>a</sup>	5.61±2.37 <sup>a</sup>
20 Gy	18.97±6.44 <sup>a</sup>	6.91±1.44 <sup>b</sup>	12.69±3.25 <sup>b</sup>	5.07±1.67 <sup>a</sup>	5.46±2.16 <sup>a</sup>
30 Gy	16.83±6.23 <sup>a</sup>	4.52±1.00 <sup>a</sup>	9.06 ±2.59 <sup>a</sup>	3.56±1.42 <sup>a</sup>	4.61±1.71 <sup>a</sup>

Note: The number followed by the same letter within a column are not significantly different at  $P < 0.05$  of DMRT test.



Janick (2008), an interesting phenomenon of growth stimulation can be found that plants that are given irradiation treatment show higher survival rates and better growth than untreated plants. Increased plant growth from irradiation results also occurred in the study of Suwarno et al. (2013) in which they found that radiation doses of 10 Gy and 20 Gy were optimal doses to stimulate orchid growth in all parameters of observation.

Gamma-ray radiation at the highest dose (30 Gy) shows a reduction in all growth parameters. This result is the same as the research conducted by Dewi and Dwimahyani (2013) which states that giving radiation doses of more than 20 Gy to hibiscus plants causes growth to be slow and can even cause death. Hasbullah et al. (2012) in their study reported that an increase in the dose of gamma irradiation can suppress plant cell activity and influence the morphological characteristics of plants as a result of chromosome aberrations in addition to genetic mutations. Afrasiab and Iqbal (2010) reported that higher doses of gamma radiation reduce the amount of endogenous growth regulators like cytokines. Although there is a reduction in growth in quantitative morphological properties, there is no significant reduction. Radiation of 10 Gy and 20 Gy in this study can be used as alternatives for plant breeding because it shows better survival and growth.

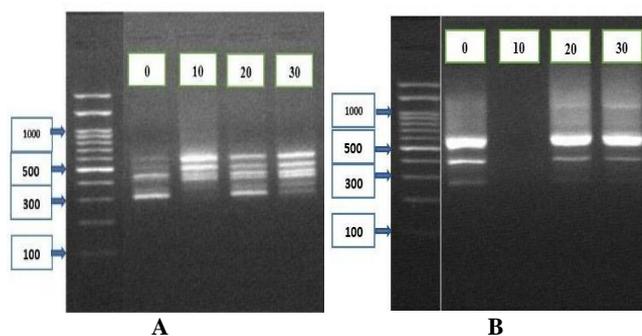
#### Analysis of ISSR molecular markers

Detection of genetic diversity using molecular markers of ISSR has been carried out in several previous studies (Wang et al., 2012; Patel et al., 2015; Taheri et al., 2013; Wang et al., 2017). Single nucleotide polymorphisms are the genetic markers of choice in various genetic, ecological and evolutionary studies (Tang et al. 2016). In this study, the level of polymorphism of pisang ambon DNA bands obtained by gamma irradiation is quite high, namely, at 90%. From the 9 DNA bands produced by two ISSR primers, only 1 monomorphic band is observed while 8 others are polymorphic bands. The polymorphism resulted from the ISSR amplification comes from variations in DNA sequences at the primer attachment site. According to Reddy et al. (2002), the polymorphism may be due to mutation at priming sites and/or insertion/deletion event within the SSR region; and the extent of polymorphism also varies with the nature and the sequence repeat of the primer used.

From 6 selected ISSR primers, 4 primers have not been able to amplify properly because there is no match between the complement of pisang ambon DNA and the primary sequence used. This research is the same as the research conducted by Carsono et al. (2014) which shows that from 10 molecular SSR markers used for polymorphic identification of rice plants, only 6 primers are able to produce polymorphisms above 50%, while 4 other primers do not meet the requirements because they do not produce polymorphism above 50% and some of them cannot be properly amplified. The same results also occur in the study of Lal et al. (2012) in the maximum species. From the 6 ISSR primers, 21 RAPD primers and 6 SSR primers only produced 3 ISSR primers, 11 RAPD primers, and 2 SSR primers which give satisfactory amplification results. According to Ye et al. (2005), in a successful ISSR analysis, the SSRs (*inversely oriented*) pairs must be within an adjacent distance on the same chromosome, which can be amplified by the PCR reaction to produce a band that can be absorbed by agarose gel or polyacrylamide. SSR locus in the genome that is not detected by ISSR analysis because the distance between two SSR motifs with opposite orientation is far apart or even though the motif is clustered at a distance that can be amplified by PCR detection, but SSR motifs are not oriented as required in PCR amplification.

The amplified DNA bands ranged from 275-1250 bp. Total DNA is detected in each primer depends on the base sequence of the primer and the presence or absence of variation in certain genotypes (Upadhyay et al. 2004). The ISSR primers used in this study is included in microsatellite primers, where the primer is designed from the microsatellite region which is a repetitive sequence. According to Udupa and Baun (2001), one of the important characteristics of repetitive sequences is the tendency for mutations that are higher than others (RAPD, AFLP, long primer-RAPD) and usually cause changes in their length arrangement. Microsatellites have spontaneous instability that is based on their vulnerability compared to other regions or the imbalance of the process of replication or recombination that is not the same and the high average of the two processes makes this locus brittle against radiation induction (Bridges, 2001). In general, the produced DNA bands have different thicknesses. DNA bands at base pairs 560 bp ISSR primary 855 control treatment, 20 Gy treatment and 30 Gy treatment appear thicker than the others. This is due to the presence of more recognizable segments of DNA in these base pairs so that they appear thicker (Poerba and Martanti, 2008).

The diversity dendrogram based on the ISSR marker shows that there is no specific pattern between the control and the plants produced by irradiation. Plants in the treatment of 20 Gy and 30 Gy have genetic similarity distance that is quite close to the control plants, while the plant of 10 Gy treatments is at the farthest distance compared to controls and other treatments (Figure 2). In general, the success of mutation induction is very dependent on plant material receiving mutagen treatment (Harten 1998). Besides plant material, primary selection is



**Figure 2.** Amplification of the pisang ambon DNA pattern using ISSR 846 (A) and ISSR 855 primer (B)

also very important. Wang et al. (2017) in his research on the *Sophora davidii* plant stated that the induction of gamma radiation was able to produce genetic diversity between 75-86% based on the ISSR marker. Another study conducted by Taheri et al. (2013) on *Curcuma alismatifolia* plants based on ISSR analysis results of gamma-ray irradiation obtained diversity between 27-100%. In this study, the genetic diversity that occurred ranged from 47-93%, whereas the increase was not too large at 7%.

In conclusion, plants at irradiation doses of 10 Gy and 20 Gy experience increased growth in all morphological parameters that have been analyzed. The genetic diversity of pisang ambon from gamma-ray irradiation by molecular markers of ISSR increases by 7%. The 10 Gy dose has a fairly high diversity from the other doses.

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