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# Genetic dissimilarity of putative gamma-ray-induced 'Preciosa - AAAB-Pome type' banana (*Musa* sp) mutants based on multivariate statistical analysis

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**ABSTRACT.** Bananas are among the most important fruit crops worldwide, being cultivated in more than 120 countries, mainly by small-scale producers. However, short-stature high-yielding bananas presenting good agronomic characteristics are hard to find. Consequently, wind continues to damage a great number of plantations each year, leading to lodging of plants and bunch loss. Development of new cultivars through conventional genetic breeding methods is hindered by female sterility and the low number of seeds. Mutation induction seems to have great potential for the development of new cultivars. We evaluated genetic dissimilarity among putative 'Preciosa' banana mutants generated by gamma-ray irradiation, using morphoagronomic characteristics and ISSR markers. The genetic distances between the putative 'Preciosa' mutants varied from 0.21 to 0.66, with a cophenetic correlation coefficient of 0.8064. We found good variability after

 

irradiation of 'Preciosa' bananas; this procedure could be useful for banana breeding programs aimed at developing short-stature varieties with good agronomic characteristics.

**Key words:** Gamma-rays; Ward-MLM; Mutation induction; Genetic variability; ISSR markers

## INTRODUCTION

Bananas are produced in more than 120 tropical and subtropical countries, mainly by small-scale farmers. According to the FAO (2011), in 2009, the total area harvested and total production was approximately 5 million hectares and 95 million tons, respectively. These numbers demonstrate the importance of bananas as a strong commodity, playing key economic and social roles in many developing countries, and also considered a staple food for millions of people worldwide.

In Brazil, the second largest banana producer in South America, the banana represents the second largest market of fresh fruit consumption after citrus (Silva and Torres Filho, 1997). Although bananas play a key economic and social role in the country and worldwide, there is still low availability of high yielding commercial varieties short in stature and presenting good agronomic characteristics. High stature is one of the factors that lead to great losses in banana productions worldwide, where plant lodging directly leads to bunch loss.

A strategy to overcome this problem is the development of new cultivars through conventional genetic breeding. However, due to many obstacles in conventional banana breeding, such as female sterility and low seed production, the use of induced mutation aimed at the selection of mutants with desirable agronomic characteristics becomes a feasible approach for the development of new high yielding short stature bananas.

Plant height reduction is one of the most frequent mutant characteristics sought out in *Musa* spp when induced (Novak et al., 1990). Therefore, associated with other *in vitro* and molecular technologies, it constitutes an alternative to conventional banana breeding aiming for short stature, not only for broadening genetic variability, but also shortening the time period required to release a new short variety.

Gamma radiation is used to produce new varieties presenting higher yield, early maturation cycle, resistance to disease and shorter stature and the procedure is very well established nowadays (Alves and Lima, 2000; Jain 2005, 2010). According to the FAO/IAEA (2009), 3,100 varieties were obtained by mutation induction and approximately 50% were developed by direct use of mutants.

The use of mutagenic agents to increase variability has been widespread in plant breeding due to its capacity to alter one or a few characteristics of well-established cultivars without altering the desirable ones (Brortjes and Van Harten, 1988) and can be particularly important in the *Musa* genus, which is characterized by asexual reproduction where the genetic variability is naturally reduced (Novak et al., 1986).

Molecular markers are important tools in precisely detecting the effects of gamma radiation since they identify genetic polymorphism at the DNA level and have been used to study genetic dissimilarity in many crop species (Souframanien et al., 2002; Roy et al., 2006; Barakat et al., 2010). ISSR (inter-simple sequence repeat) markers are semi-arbitrary markers

 

amplified by PCR using primers complementary to a target microsatellite. ISSRs have been widely used in *Musa* genetic diversity studies (Racharak and Eiadthong, 2007; Venkatachalam et al., 2008; Pestana et al., 2011) and the pattern quality and reproducibility of these markers have indicated that they are quick, easy to apply and highly reproducible.

In genetic breeding, multivariate data regarding continuous and categorical traits are gathered with the objective of selecting genotypes and accessions which best represent the entire population or gene collection with the minimum loss of genetic diversity (Crossa and Franco, 2004). The Ward-Modified Location Model strategy (1998) is a useful method for analyzing genetic variability (Barbé et al., 2009) and the selection of genotypes and accessions that best represent the entire population with minimum loss of genetic diversity (Crossa and Franco 2004). This procedure has been used in many crop species such as snap beans (Barbé et al., 2009), *Capsicum* spp (Sudré et al., 2010), heirloom tomato (Gonçalves et al., 2008), kale (Padilla et al., 2007) and bananas (Pestana et al., 2011).

Multicategoric and quantitative data for the second cycle of putative mutant "Preciosa" banana production were used in this study. "Preciosa" is a Pome-type banana cultivar with resistance to yellow Sigatoka and *Fusarium*, the latter considered one of the most devastating banana diseases worldwide. The possibility of combining multicategoric, quantitative and molecular data opens new perspectives in dissimilarity studies. Therefore, the objective of the present work was to select short banana "Preciosa - AAAB" putative mutants generated by gamma-ray irradiation and estimate the genetic variability using morphoagronomic and ISSR molecular marker data via a multivariate statistical algorithm.

## MATERIAL AND METHODS

### Plant material

Healthy *in vitro* banana plants from the Preciosa (AAAB, subgroup Pome) cultivar, approximately five centimeters in length and 4-5 leaf primordia, supplied by the Campo Biotecnologia Vegetal Ltda. Company from Cruz das Almas, Bahia, were used for the induction of mutation by gamma-rays. Meristems of Preciosa banana plants in the field at Embrapa-CNPMF were used for the mutation induction.

### Gamma-ray induction of *in vitro* buds

Approximately 200 *in vitro* buds of the "Preciosa" banana cultivar were irradiated at the Centro de Energia Nuclear na Agricultura (CENA), at the University of São Paulo (USP), using $Co^{60}$ at a dose of 30 Gy, with rates of 1.322 kGy/h. This dose was selected from a sensitivity test carried out previously by Resende (2005), which indicated 30 Gy as the best dose for the "Pacovan Ken" (AAAB, subgroup Silk) cultivar. Ten "Preciosa" buds were used as controls, without exposure to $Co^{60}$.

The irradiated buds were transferred to basic MS medium, solidified with 2.2 g/L Phytagel, supplemented with 30 g of sucrose, 3.0 mg/L 6-bezilaminopurine (BAP), pH 5.8 and maintained in the growth chamber (27 ± 2°C and 16 h of light photoperiod). Plants were submitted to subcultures at 30-day intervals and after 4 subcultures, were rooted in MS medium supplemented with 0.25 mg/L of naphthalene acetic acid (NAA) and 8 g/L of agar with

 

pH adjusted to 5.8 and taken to the growth chamber with 16 h photoperiod and light intensity of 40 µmol $m^{-2}$ $s^{-1}$ and 26 ± 2ºC for 35-30 days. Approximately 3,200 rooted plants were taken to a screen house (50% screen shade) and acclimated in dibble tubes containing Plantmax.

## Morphoagronomic characterization

The experiment was carried out on the experimental field at Embrapa-CNPMF located at Cruz das Almas-Bahia, Brazil, without any previous statistical design. Each plant was considered a replicate. Spacings were 3 m x 4 m and plants were fertilized according to technical recommendations.

A pre-selection of plants was carried out in the screen house using as the criteria plants with at least 10% smaller stature in comparison to the controls. One hundred and ninety plants were selected among the 3,200 irradiated "Preciosa" banana plants using this criteria (Resende, 2005).

The 190 selected "Preciosa" irradiated plants and 17 controls were evaluated in two production cycles, whereas for this study, the data used were from the second production cycle and the following agronomic variables were evaluated: 7 multicategoric (color of pseudostem-CPS; color of mid-rib-CMR; shape of roseta-SR; shoot color-SC; opening of the base of the petiole-OBP; color of petiole border-CPB and position of leaves-PL) and 21 quantitative variables (number of days from planting until flowering-NDPF; number of days from planting until harvest-NDPH; number of days from flowering until harvest-NDFH; diameter of pseudostem-DPS (cm); plant height-PH (m); number of leaves until flowering-NLF;. number of suckers-S; bunch weight-BW (kg), hand weight-HW (kg); number of fruits-NF; average weight of fruit-AWF (g); length of fruit of the second hand-LFSH (cm); length of fruit of penultimate hand-LFPH (cm); diameter of the fruit of the second hand-DFSH (mm); diameter of fruit of next to the penultimate hand-DFPH (mm); number of hands-NH; number of live leaves at harvest-NLLH; length of peduncle-LE (cm); diameter of peduncle-DP(cm); presence of yellow Sigatoka during flowering-PYSF; and presence of yellow Sigatoka during harvesting-PYSH).

## Selection of short stature mutants based on morphoagronomic data

In order to select 10% of plants with shorter stature, the methodology proposed by Resende (2005) was used. Initially, an individual classification of plants in the population was carried out for the characteristics considered important in the selection of banana genotypes with short stature, early flowering period and heavier bunches. Data obtained for these characteristics was organized in increasing order (for plant height and number of days until bunch emission) and decreasing order (for bunch weight) obtaining the classification number of each plant (Resende, 2005).

The classification number obtained for each plant was multiplied by the ´weight´ corresponding to each variable. A weight of six was given for plant height, for being considered the most relevant variable in this study and 2 for number of days from planting to flowering and weight of bunch. At the end of this process the final punctuation of each plant was obtained using the following formula: y = [0.6 x (height classification) + 0.2 x (classification of bunch emission) + 0.2 x (classification of bunch weight)] (Resende, 2005). At the end of the production cycle, the final punctuation was organized in increasing order, obtaining the final classifica-

tion. Plants classified in the top 10% at the end of the second production cycle were selected.

Seventy-four putative "Preciosa" mutants were sampled. Sampling was carried out at the end of the second production cycle using short stature and good bunches as the main criteria.

## DNA extraction and amplification using ISSR markers

Genomic DNA was extracted from young leaves of the 74 putative "Preciosa" mutants and 5 controls (non-irradiated *in vitro* plants) using the CTAB method (Doyle and Doyle, 1990). DNA quantity and quality was performed by comparative analysis of the samples on 0.8% agarose gels and ethidium bromide. The samples were diluted in ultrapure water and the concentration adjusted to 10 ng/μL.

For the amplification via ISSRs a final volume of 15 μL was used, containing: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 100 μM of each dNTPs (dATP, dTTP, dGTP, dCTP), 0.4 μM of each primer, 20 ng genomic DNA and 1 U *Taq* DNA polymerase (Pharmacia Biotech, USA).

Amplifications were carried out in the Perkin Elmer 9700 model thermocycler with the following amplification steps: 94°C for 4 min. followed by 35 cycles of 94°C for 40 s, 48ºC, for 40 s, 72°C for 1 min., with a final extension of 72°C for 2 min. The amplification products were separated on 2% gel eletrophoresis. Information regarding the ISSR markers is shown in Table 1.

**Table 1.** ISSR primers used in the amplification of putative "Preciosa" banana mutants with their respective sequences, annealing temperatures (Ta).

| Primer | Sequence | Ta (°C) | TNB |
|---|---|---|---|
| DiGA3'C | $(GA)_8C$ | 48 | 12 |
| DiGA3'RC | $(GA)_8RC$ | 48 | 12 |
| TriGTA3'RC | $(GTA)_5RC$ | 48 | 12 |
| DiGT3'RC | $(GT)_8RG$ | 48 | 6 |
| TriTAG3'RC | $(TAG)_5RC$ | 48 | 12 |
| TriTGG3'RC | $(TGG)_5RC$ | 48 | 11 |
| TriCTC3'RC | $(CTC)_5RC$ | 48 | 8 |
| TriGAT3'RC | $(GAT)_5RC$ | 48 | 9 |
| TriAGA3'RC | $(AGA)_5RC$ | 48 | 10 |
| TriCAG5'RC | $CR(CAG)_5$ | 48 | 10 |
| TriAAG3'RC | $(AAG)_5RC$ | 48 | 15 |
| TriTGA3'RC | $(TGA)_5RC$ | 48 | 14 |
| TriCAA3'RC | $(CAA)_5RC$ | 48 | 13 |
| TriCTG3'RC | $(CTG)_5RC$ | 48 | 11 |
| DiGA3'YC | $(GA)_8YC$ | 48 | 08 |

R = A, G; Y = C, T; TNB = total number of bands.

## Statistical analysis

Seventy-four putative "Preciosa" mutants and 5 controls (non-irradiated *in vitro* plants) in the second production cycle were evaluated. Morphoagronomic and molecular data from the second production cycle were submitted to the Ward-MLM statistical algorithm (Franco et al., 1998) using the CLUSTER and MLM procedure in the SAS statistic software package (SAS Institute, 2001), considering a total of 7 multicategoric characteristics, 21 quantitative characteristics and 15 ISSR primers (161 polymorphic bands) in order to evaluate the genetic

variability. The amplified fragments were evaluated as present (1) or absent (0) bands.

Cluster analysis was carried out by the UPGMA (unweighted pair-group method with arithmetic mean) method using Mega4 software (Tamura et al., 2007), based on the distance matrix by the Gower algorithm (Gower, 1971). The cophenetic correlation coefficient between the dissimilarity matrix and the cluster matrix was calculated using the GENES software (Cruz, 2003). The ideal number of groups was defined according to the pseudo-F and pseudo-$t^2$ criteria (SAS Institute, 2001).

## RESULTS

Seventy-four putative "Preciosa" mutants were sampled. Sampling was carried out at the end of the second production cycle using short stature and good bunches as the main criteria (Figure 1).

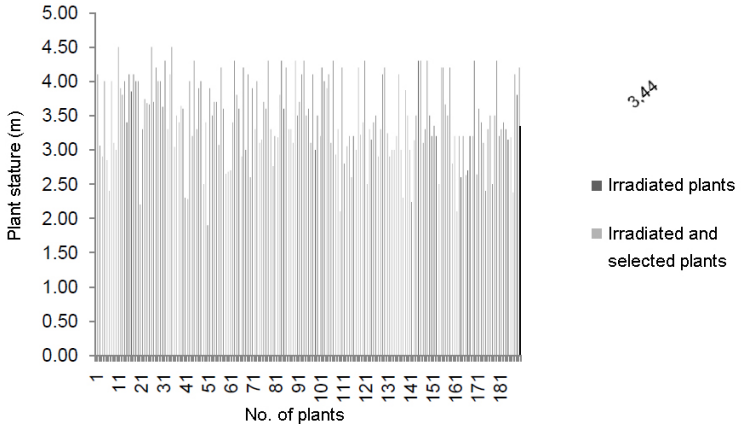


**Figure 1.** Plants chosen based on short stature in comparison to average height of the control plants.

The multicategoric and quantitative data for the second cycle of putative mutant "Preciosa" banana production was used. The genetic variability of 74 putative "Preciosa" mutants and 5 controls was analyzed using 21 quantitative, 7 multicategoric and 161 polymorphic bands originating from 15 ISSR markers using Ward-MLM method (Figure 2).

For the ISSR analysis with 15 primers for the irradiated "Preciosa" cultivars, a total of 161 polymorphic bands were obtained whereas 75 were monomorphic. The highest number of bands was identified for the primer TriAAG3'RC (15 bands) and the lowest (6 bands) with primer TriTGA3'RC, with an average of 12.8 bands per primer.

The distance between the putative "Preciosa" mutants and controls varied from 0.21 to 0.66, with an average distance of 0.43 and cophenetic correlation coefficient of 0.8064** ($P < 0.01$), which according to Vas Patto et al. (2004) is considered highly acceptable.

The pseudo-F and pseudo-$t^2$ criteria showed that the optimum number of groups is 3, with the largest drop difference from 16.8405 to 5.0011. The closest putative "Preciosa" mutants were Preciosa-50 and Preciosa-51, with a genetic distance of 0.21, and the most dissimilar were the Control-75 and Preciosa-34, with a distance of 0.66.

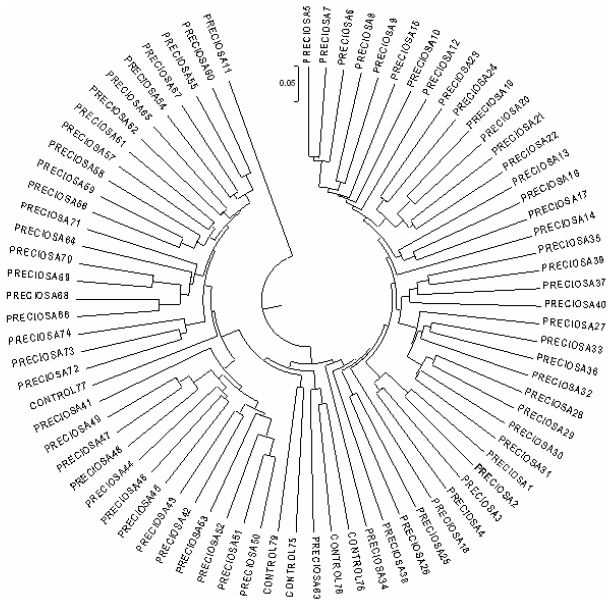


**Figure 2.** Dendrogram constructed with 74 putative banana "Preciosa" mutants and 5 controls using 21 quantitative, 7 multicategoric and 161 binary data (ISSR markers) by the Gower algorithm. The dendrogram was constructed using the UPGMA method and the MEGA-4 software package.

Four putative "Preciosa" mutants were selected (Preciosa-5, Preciosa-6, Preciosa-18 and Preciosa-64) from the 10% best classified for the following characteristics evaluated at the end of the first production cycle: plant height, bunch weight and number of days from planting until flowering. Regarding these mutants, Preciosa-5 and Preciosa-6 were clustered together in a subgroup in group 3.

As for the number of days until bunch emission, Preciosa-5, -6 and -64 presented lower values when compared to the controls for the first production cycle. Preciosa-5 was the most precocious among those selected with a difference of 71 days in comparison to the average of the controls, which was 341 days. The remaining plants selected presented higher values for this characteristic, but closer to the controls. For the second cycle, however, the plants selected presented values similar to the average of the controls for this characteristic except for plant number 6 and 64, which emitted the inflorescence at 86 and 96 days before the average of the controls, 683 days**,** respectively. In combination with plant height, this characteristic allowed for the selection of these two plants as the best of the 10%.

In regard to weight of bunch and hand, in the first cycle, plant 18 presented bunch and hand weight higher than the average of the controls and of the irradiated clones (highlighted). As for number of fruits, values similar to those observed in the controls in both cycles evaluated were observed in the selected plants, except for Preciosa-9 in the first cycle, which presented lower values for this characteristic when compared to the controls.

For average fruit weight, in the first cycle, Preciosa-18 presented a greater value in comparison to the controls. As for the second cycle, similar averages between the selected plants and the controls were observed.

## DISCUSSION

Mutation induction via gamma-rays has been applied to *Musa* spp. for improving many desirable traits such as early flowering (Novak et al., 1990) and tolerance to aluminum (Matsumoto and Yamaguchi, 1990), salinity (Alves, 2000) and *Fusarium* (Ho et al., 2001; Chai et al., 2004).

In regard to the obtainment of short stature bananas through gamma-ray mutation induction, also aiming for other desirable agronomic characteristics, such as in our study, some studies can be found in the literature. López et al. (2004) identified a promising banana cultivar irradiated with gamma-rays. A total of 900 irradiated plants were evaluated in the field. According to the authors, in the first cycle, 58% of potential induced mutants showed phenotypic variations and no selections were carried out in the first cycle. However, material was propagated for further evaluation and in the second cycle eight irradiated clones showed better performance regarding height and yield. Two of the better performing clones (V6-32 and V6-44) also showed tolerance to black Sigatoka and were further evaluated in the third cycle.

Bermudez-Caraballoso et al. (2010) also obtained interesting results. Multiple buds were irradiated with gamma-rays ($^{60}$Co sources at dose of 25 Gy). In the clonal study, 98 mutant clones with lower pseudostem height compared with the original cultivar were selected. Four mutants were selected whereas mutant IBP 14-23 presented interesting characteristics. The mutants are now undergoing field trials.

Pestana et al. (2011) also presented interesting results regarding the use of gamma irradiated buds of the "Pacovan  Pomme type" banana cultivar for short stature and other interesting agronomic characteristics. Four promising putative mutants were identified: Pacovan-28, Pacovan-35, Pacovan-38 and Pacovan-40 and are now also in field trials.

The combination of irradiation with gamma-rays and the use of molecular markers has also been widely used to investigate and determine differences in radiation-induced mutants in many species (Roy et al., 2006; Lu et al., 2007; Barakat et al., 2010; Pestana et al., 2011), bringing a new dimension to gene technologies.

Hautea et al. (2005) used RAPD, SSR and AFLP markers to detect variation in profiles of gamma-ray and fast neutron induced mutant banana clones. The authors emphasize the successful use of mutation and molecular marker techniques to improve bananas in the Philippines.

Somaclonal variation, regarded as the genetic variability that may occur during *in vitro* cultivation (Larkin and Scowcroft, 1981), is a highly discussed topic in the literature (Hwang and Ko, 1988; Álvares and Caldas, 2002; Sahijram et al., 2003; Santos and Rodrigues, 2004). Hwang and Ko (1988) also reported greater frequency of variants occurring in bananas of the AAA subgroup (Cavendish). Álvares and Caldas (2002) identified 4.6% of somaclonal variants in micropropagated plants of the Nanicão cultivar (AAA group) and 1.5% of the Prata-Anã cultivar (AAB group).

The increase in the rate of somaclonal variation in subcultivations of *in vitro* banana cultivars was investigated by Santos and Rodrigues (2004). The authors studied the effect of the increase in the number of *in vitro* subcultivations in the increase in somaclonal variation in commercial bananas being micropropagated. The authors observed that only after the fifth *in vitro* subcultivation of micropropagated Pacovan (Pomme type) bananas somaclonal variation started to occur and that after 9 subcultivations 5.8% of somaclonal variation was observed. The authors also suggested that specific micropropagation protocols should be used for each commercial banana cultivar.

In our study, we believe that most of the variation found may be attributed to the gamma-ray irradiation, mainly due to the fact that very few subcultivations were carried out, only four, and also, a specific micropropagation protocol for the "Preciosa" cultivar was used.

Mutations are considered rare, random events and mutants are generally recessive and deleterious, therefore mutation breeding requires the screening of a large sample of mutagen-treated populations in order to identify desirable individuals (Chai et al., 2004). Taking this statement into consideration, we believe that our threshold of 10% in the selection process will lead to very interesting results.

The use of the Ward-MLM, a combined data approach, enabled the obtainment of reliable results regarding the genetic variability of irradiated "Preciosa" bananas using gamma-rays. These results show that there is enough genetic variability that can be used in the banana breeding program aiming at the obtainment of short, precocious high yield banana plants.

In our study not only the use of molecular data, but also a combined analysis with morphoagronomic data was used to enhance diversity studies regarding the development of new banana varieties with good agronomic characteristics. Although the controls were separated in the combined cluster analysis, when considering only the molecular data, the distances between the controls varied from 0.18 to 0.20, with an average distance of 0.19. This shows that genetically they are closely related and therefore could serve as a good basis to be used in comparison to the irradiated material. Also, in the combined analysis, although they were somewhat separated spatially, they were still placed in the same group, G2. Similarly to the works mentioned above, our studies show that it is possible to generate new individuals through induced mutation using gamma-rays.

The use of combined data enables the study of genetic variability in a more reliable approach. The four promising putative banana mutants of the "Preciosa" cultivar selected, based on best agronomical characteristics and short stature (Preciosa-5, Preciosa-6, Preciosa-18 and Preciosa-64), are now undergoing field evaluations.

## CONCLUSION

The ISSR markers were able to show variability among the irradiated "Pacovan" banana putative mutants. The use of mutation induction (gamma-rays) combined with *in vitro* and molecular marker techniques is an interesting approach to generate information for obtaining promising banana cultivars with short stature that present good agronomic characteristics.

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