Mutation Breeding of Banana cv. ‘Grand-Nain’ for Resistance to Some Banana Viruses Using Biotechnology and Physical Mutagens

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ABSTRACT

Banana is one of the most important fruit crops in Egypt both for domestic and export markets. At present, the industry is beset with low productivity due to Banana bunchy top virus (BBTV) and Banana mosaic virus (BMV). Banana cultivars are not appropriate for sexual breeding because both male and female are sterile. Thus, BBTV and BMV resistance cannot be incorporated into the banana by conventional breeding. Use of biotechnology techniques combined with mutagenesis using physical mutagens in Musa spp. allows genetic variability to be increased to improve important agronomic characters. The objective of this study was to develop mutant clones of Musa cv. ‘Grand-Nain’ with reduced height, good agronomical traits and resistance to both of BBTV and BMV viruses. Multiple buds obtained by in vitro culture were subjected to gamma radiation from 60Co sources at 20, 40 and 60 Gy. Irradiated buds were multiplied, elongated, rooted, hardened and acclimatized in the glasshouse and were transplanted to the field. The selection of mutant plants is based on morphological and ISSR-PCR markers. DNA-based markers were reliable and reproducible for mutant selection of BBTV- and BMV-resistant banana plants used in this study. Field screening was carried out against two different isolates of BBTV and BMV using a syringe method of inoculation. Only three banana plants treated with 60 Gy were resistant to the BMV isolate. No BBTV-resistant banana plants were obtained.

Keywords: banana improvement, molecular markers, ISSR-PCR, DAS-ELISA

INTRODUCTION

Banana is one of the most important fruit crops in Egypt both for domestic and export markets. Banana plantations are subjected to various natural calamities, but diseases, in particular, viral diseases constitute a major setback to this crop worldwide. Among viral infections the Banana bunchy top nanavirus (BBTV) (Islam et al. 2010) and Banana mosaic cucumovirus (BMV) (Swennen and Vuylsteke 2001). The pathenocarpic nature of cultivated banana makes it difficult to breed for resistance to diseases (Jones 2000). Resistance can be introduced into banana by conventional and unconventional improvement methods (Crouch et al. 1998). Some improvement methods, such as a long-term breeding program, have many limitations due to sterility of cultivated banana, long growth cycles, low seed set and hybrids that are often not accepted by local consumers (Sagi 2000). Improving disease resistance is vital for the future survival of banana (Pearca 2003). Smith et al. (2006) have also tried to generate disease resistance in banana plants by using tissue culture based techniques such as in vitro mutagenesis. Mutations for banana plants improvement can further be induced by gamma irradiation and by somaclonal variants (SVs).

During gamma irradiation the mutation rate is based on the time the cells are subjected to the gamma as well as the dosage. The survival rate of banana plants, however, decreases inversely (Bhagwat and Duncan 1998). There are three different techniques to apply gamma irradiation to banana plants. The first technique is to irradiate the sucker before the meristem-tip is isolated (Novak et al. 1990). In the second technique, in vitro banana plantlets from shoot-tip culture are irradiated (Novak et al. 1990). Thirdly, embryogenic cell suspensions can be exposed to gamma irradiation. By using embryogenic cell suspensions, chimeric plants can be avoided (Xu et al. 2005).

In vitro mutagenesis can be lead to variability in banana clones that are generated from a single mother plant. This process, called SVs, can be the result of nuclear chromosomal re-arrangement, gene amplification, non-reciprocal mitotic recombination, transposable element activation of silent genes (Jain 2001; Karamura et al. 2010). Somaclonal in banana are induced as the number of multiplication cycles is increased (Sahijram et al. 2003). Once the number of multiplication in banana by shoot-tip culture exceeds 12 cycles. Subsequently, the number of SVs increases substantially (Ko et al. 1991).

The development of simple sequence repeat markers, also known as microsatellites, with 2-5 nucleotide repeats for Banana, has recently been undertaken (Garcia et al. 2010); however, genetic diversity or population structure studies using more robust DNA markers are only at the early stages and more DNA markers are still needed.

The primary obective of this study was to find new mutations from banana plants resistance of BBTV and BMV using physical mutations and SVs. In addition to, evaluate the genetic stability of banana plants obtained through exposure of gamma irradiation and in vitro multiplication via apical shoot culture through 11 cycles of micropropagation.
MATERIALS AND METHODS

Induction of mutation through gamma rays

One hundred and five shoot apices from side-suckers were tested for potential infection with BBTV and BMV by DAS-ELISA technique and were used as the source materials for the establishment of in vitro shoot-tip cultures. The suckers collected from the field growing banana plants (Bechira governorate, Egypt). The shoot apices (trimmed to a size of 1.0–1.5 cm, with minimum basal corn tissue) were cultured on 20 ml of solid MS medium (Murashige and Skoog, 1962) containing 5 mg/l 6-benzylaminopurine (6-BA) (Duchefa Biochemie, The Netherlands) in culture tubes and exposed to Gamma rays ranging from 20, 40, and 60 Gy (60Co gamma, Indian Cell, 10 Kgy/h) after seven days from culture. Following irradiation, the shoot-tips were immediately transferred to fresh MS medium (20 ml/tube). Then, individual shoots can be separated from the shoot-tip multiples and rooted by sub-culture on MS-medium containing 1 mg/l 1-naphthaleneacetic acid (NAA). The cultures were incubated at 25 ± 2°C under a 16-h photoperiod. Light intensity was 42 μmol m-2 s-1 with white fluorescent tubes (Al-Nasr Co., Cairo, Egypt).

Induction of mutation through SV

After 21 days of initiation, the cultured shoot tips were sectioned vertically and sub-cultured on MS-medium containing 5 mg/l 6-BA. Multiple shoots formed need to be maintained as stock cultures by regular sub-culturing at an interval of about four weeks, on MS-medium containing 3 mg/l 6-BA for 11 generations (gene-ration period 21 d). Individual shoots can be separated from the shoot-tip multiples and rooted by sub-culture medium containing 1 mg/l NAA.

DNA extraction

After 21 days of initiation young leaves of banana plants obtained through exposure of gamma irradiation and in vitro multiplication via apical shoot culture through 7th, 9th and 11th sub-cultures were collected and soaked in liquid nitrogen for DNA extraction using the 2% CTAB method modified by Agrawal et al. (1992).

Inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR)

A total of five primers (Table 1) were used to amplify DNA (Lakshmanan et al. 2007; Racharak and Eiadthong 2007; Life Technologies Inc, Gaithersburg, USA). The total reaction mixture was 15 μl contained 10× PCR buffer, 2 mM MgCl2, 0.2 mM dNTP mixed, 10 μl primer, 1.25 U Taq polymerase and about 150 ng genomic DNA. DNA amplification was obtained through 40 cycles in a DNA thermal cycler. The temperature profile was as follow: denature temperature 94°C for 1 min; annealing temperature 52°C for 1 min; and extension at 72°C for 8 min. After completion of the amplification, the PCR product were separated on a 1% agarose gel in 0.5×TBE buffer (1×TBE buffer: 0.045 M Tris-borate, 0.001 M EDTA) and 0.5 μg/ml ethidium bromide for 45 min at 90 V. The size of each fragment was estimated with reference to a size marker of 100 bp DNA ladder (BIORON GmbH, Ludwigshafen, Germany).

Gel analysis

The gel analysis was applied by a programme (UVI Geltec version 12.4, city, USA).

Greenhouse hardening of plantlets

After four weeks from the 11th generation, plantlets with well developed root-system were transferred to perforated polythene bags filled with autoclaved mixture of soil of mix and a suitable commercial hardening mixture such as peat moss: sand: vermiculite (v/v/v, 1: 2: 1). The plantlets were maintained in the greenhouse for three months (depending on the growth), under natural light with relative humidity of 90-100% and an ambient temperature of 25 ± 2°C. Evaluation morphological characterizations of banana plants were recorded such as (plantlet growth, leaf area, No. of roots, roots length, fresh weight, dry weight and chlorophyll).

Response of banana to mixed infection (BBTV and BMV)

Two-months-old hardened banana plants with similar sizes were inoculated artificially by a syringe method. Infected leaves from young shoots of infected banana BBTV and BMV were crushed with 0.1 M phosphate buffer pH 7.2 (w/v) and macerated using sterilized mortar and pestle. The crude sap was filtered through two layers of cheese cloth. The healthy banana resulted from tissue culture virus-free plants (2-months-old plantlets) were injected with clarified infected sap by the needle of sterilized plastic syringes and inverted vertically in petioles to 1 cm depth and 30°C slope in the lower part of the stem and the piston was pushed to inject 0.1 ml of the inoculums (Hussain and Afghan 2001). The inoculated plants and control were maintained in an insect-proof greenhouse at 25 ± 2°C and a 12-h photoperiod. The plants were fertilized with a 19: 19: 19 N-P-K solution and insecticides were applied to ensure vigorous growth and freedom from insects. Plants were observed daily at two months for visible symptoms.

Detection of BBTV and BMV by DAS-ELISA

All the samples were tested for the presence of BBTV and BMV by the double antibody sandwich enzyme linked immunosorbent assay technique (DAS-ELISA) as described by (Clark and Adams 1977), BBTV and BMV ELISA (kits provided from Sanofi Co. Sante Animal Paris, France). Polystyrene plates were coated with IgGs diluted in coating buffer (pH: 9.6) and incubated at 37°C for 4 h. The plate then washed three times with washing buffer for 3 min intervals. 100 μl samples were loaded duplicate wells of polystyrene microtitre plate. After loading the diluted extracts of 100 μl the plates were incubated overnight at 4°C. Following washing, 100 μl of conjugated antibodies were added to each well and the plate was incubated at 37°C for 4 h. After 3 additional washes, freshly prepared p-nitrophenylphosphate in substrate buffer (1 mg/ml) were loaded to each well. The plate was incubated at room temperature and photometric measurement was done at 405 nm after 2 h. Samples were considered as positive if their absorbance values were more than 2.5 times the negative control. ELISA test was carried out with four repetitions including positive and negative controls.

Statistical analysis

All experiments were arranged in factorial completely randomize design and data were compared according to method described by (Snedecor and Cochran 1972). Analysis of variance (ANOVA) for all measured variables was performed using the software new MSTAT-C (version 2.1). The level of significance was measured using Duncan’s multiple range test; P ≤ 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Effect of different treatment of gamma radiation on plant regeneration

The rate of survival for shoots treated with 20 and 40 Gy was 26.66 and 0.266%, respectively compared with the control 100% (Table 2). On the other hand, banana plants treated with 40 Gy showed a significant increased in leaf area and chlorophyll contents compared to the control. On

Table 2 Effect of different treatment of gamma radiation on plant regeneration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaf area (cm²)</th>
<th>Chlorophyll (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.2</td>
<td>42</td>
</tr>
<tr>
<td>20 Gy</td>
<td>12.4</td>
<td>48</td>
</tr>
<tr>
<td>40 Gy</td>
<td>15.6</td>
<td>54</td>
</tr>
<tr>
<td>60 Gy</td>
<td>17.8</td>
<td>62</td>
</tr>
</tbody>
</table>

The rate of survival for shoots treated with 20 and 40 Gy was 26.66 and 0.266%, respectively compared with the control 100% (Table 2). On the other hand, banana plants treated with 40 Gy showed a significant increased in leaf area and chlorophyll contents compared to the control. On
the contrary, abnormal changes were found only in plants treated with 60 Gy such as dwarf plant type, yellow, white leaves and easy to necrosis. To obtain the LD₅₀ (50% lethal dose) the data in Table 2 was plotted.

### Induction of SV in banana plants by multiplication cycles

The rate of survival decreased as multiplication cycles increased. The obtained results showed that the rate of survival of the 7th, 9th and 11th sub-cultures were 100, 92 and 79%, respectively compared with 100% in the control (Table 4). On the other hand, there were significantly differences in some morphological characters such as root length and chlorophyll contents in the 9th subculture and length of plantlets, leaf area, length of roots and chlorophyll contents in the 11th sub-culture compared with the control (Table 4). These results were with similar with those of Hwang and Ko (2004), who showed that one of the sources for inducing genetic variability in crop plants, including banana, is SV. Plant tissue culture leading to SV has been considered as a rapid and reliable approach for improvement of plants as the generated variation can be used either directly or indirectly in a breeding program aimed at crop improvement and has been used in banana cultivars to obtain superior quality banana clones. SV is used to describe the occurrence of genetic variants derived from in vitro procedures; it is also called tissue or culture-induced variation. Such variation arises in tissue culture as a manifestation of epigenetic influence or a change in the genome of different types of variations obtained after gamma ray treatment in the present study, mainly chlorophyll variants e.g., albino plants were predominant in 40 and 60 Gy treatments. Although other variants such as plants with thin and dwarf plants, hyperhydric and plants with comparatively smaller and narrow leaves were also observed. These results were similar to those of Khan et al. (1998), who also reported more chlorophyll variants in sugarcane (Saccharum officinarum) after 20 Gy treatment. Although other variants such as plants with thin and curled leaves, hyperhydric and plants with comparatively smaller and narrow leaves were also observed. Lethal and mutagenic effects of ionizing radiation result principally from incompletely or incorrectly repaired DNA lesions. Lethal and mutagenic effects of ionizing radiation result principally from incompletely or incorrectly repaired DNA lesions. Among these lesions, strand breaks are considered to be most important as they interrupt the continuity and integrity of the double helix. An unpaired single stranded break (SSB) in ssDNA; an unrepair double strand break (DSBs) in dsDNA and crosslinking of DNA to itself or proteins has been shown to be responsible for the lethal effects of ionising radiations. Radiation-induced mutagenesis is due to the involvement of multiple damaged sites (such as double-strand breaks) (Ali et al. 2007).

### Table 2

<table>
<thead>
<tr>
<th>Radiation doses</th>
<th>Plantlet characters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survival %</td>
</tr>
<tr>
<td>20 Gy</td>
<td>26.66</td>
</tr>
<tr>
<td>40 Gy</td>
<td>0.266</td>
</tr>
<tr>
<td>60 Gy</td>
<td>0.133</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
</tbody>
</table>

Means followed by the same letters are not significantly different from each other at the 5% level.

### Table 3

<table>
<thead>
<tr>
<th>Radiation doses</th>
<th>Plantlet characters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survival %</td>
</tr>
<tr>
<td>20 Gy</td>
<td>26.66</td>
</tr>
<tr>
<td>40 Gy</td>
<td>0.266</td>
</tr>
<tr>
<td>60 Gy</td>
<td>0.08</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
</tbody>
</table>

Means followed by the same letters are not significantly different from each other at the 5% level.

### Table 4

<table>
<thead>
<tr>
<th>Subculture</th>
<th>Plantlet characters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survival %</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>92.5</td>
</tr>
<tr>
<td>11</td>
<td>79</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
</tbody>
</table>

Means followed by the same letters are not significantly different from each other at the 5% level.
and thus it is non-directional; besides, it is a non-predictable phenomenon and cannot be controlled. The main advantage of SV is that the selection procedure of the useful somaclonal variants is simple and straightforward; this is especially true for vegetative propagated crops such as banana (Tang 2005).

**Multiplication cycles effect on transplanted banana plants after 90 days in a greenhouse**

Rooted banana plants were transplanted to a greenhouse. The rate of survival of the 7th, 9th and 11th sub-cultures was 100, 92.5 and 75%, respectively (Table 5). Growth of banana plants in the greenhouse was assessed by three traits namely average height, plant diameter and leaf area (Table 6). Significantly variants in height plant and leaf area were found in the 11th sub-culture compared with the control (Table 5). Significantly differences were also observed in some morphological characters in the 9th and 11th sub-cultures compared with the control (Table 5). The results were similar to those of Hwang and Ko (2004), who found that one of the sources for inducing genetic variability in banana plants is SV. Plant tissue culture leading to SV has been considered as a rapid and reliable approach for improvement of plants as the generated variation can be used either directly or indirectly in a breeding program aimed at crop improvement (Jain 2000) and has been used in banana cultivars to obtain superior quality banana clones (Hwang and Ko 2004). SV by multiplication cycles is used to describe the occurrence of genetic variants derived from in vitro procedures; it is also called tissue or culture-induced variation. Such variation arises in tissue culture as a manifestation of epigenetic influence or a change in the genome of differentiating vegetative cells induced by tissue culture and is expected to generate stable Banana plants carrying interesting heritable traits (Soniy et al. 2001). Four critical variables for SV: genotype, explant origin, cultivation period and the cultural condition in which the culture is made. Roux (2004) explained that use of in vitro mutagenesis and tissue culture has been found to make induction and selection of induced somatic mutations more effective. According to Novak et al. (1990), the frequency of phenotypic and morphological variation: (plant height, leaf color), physiological variations (growth and sucker multiplication, duration of flowering, fruit ripening), and agronomic variations (bunch qualities) varies from 3 to 40% in the first generation, depending on the genotype.

**ISSR analysis**

Changes in DNA caused by mutagens result in genetic variation detected by ISSR analysis were performed using five random primers; three primers UBC-814, UBC-817 and UBC-835 appeared polymorphism among the controls and banana plants treatment with the physical mutagens and SV.

Primer UBC-814 revealed nine amplified fragments with sizes ranged from 3060 to 180 bp, six amplified fragments were polymorphic with 66.67% polymorphism and three were commonly bands detected in all banana explants exposed of Gamma rays 20, 40 and 60 Gy doses and the control. In addition, five bands with molecular weights 3060, 300, 270, 220 and 180 bp existed in the control and disappeared in all physical mutagens. One specific band of 650 bp appeared uniquely in 60 Gy-treated plants; however, primer UBC-817 generated six amplified fragments with sizes ranging from 3060 to 280 bp, whereas three fragments were polymorphic with 50% polymorphism. The other three fragments with molecular sizes 370, 350 and 280 bp were commonly detected among three doses from irradiations and the control. On the other hand, primer UBC-835 revealed seven amplified fragments with sizes ranging between 140 and 1500 bp whereas six fragments were polymorphic with 85.71% polymorphism. One band with molecular weight 140 bp was commonly detected among three doses from irradiations and the control, three specific bands with molecular weights 1500, 1100, 720 bp revealed in banana plants exposed of 20 Gy and disappeared in 40 and 60 Gy doses and the control.

In according to, SV in banana plants by multiplication cycles, it was observed that, primer UBC-814 showed nine amplified fragments with sizes ranged from 3060 to 180 bp, six amplified fragments were polymorphic with 66.67% polymorphism (Fig. 1). The three bands with molecular sizes (600, 300 and 370 bp) revealed in three subcultures the 7th, 9th and 11th and the control. One band with 650 bp showed in three subcultures and it has not showed in the control. In the contrast, five bands with 3060, 300, 270, 220 and 180 bp were existed in the control and disappeared in the three sub-cultures.

**Table 5** Analysis of variance for quantitative characters of banana plants treated with different subcultures under greenhouse condition.

<table>
<thead>
<tr>
<th>Subculture</th>
<th>Survival %</th>
<th>Plant height (cm)</th>
<th>Plant diameter (cm)</th>
<th>Leaf area (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(7)</td>
<td>100</td>
<td>24.67 ab</td>
<td>4.833 a</td>
<td>101.5 a</td>
</tr>
<tr>
<td>(9)</td>
<td>92.5</td>
<td>20.00 bc</td>
<td>4.333 a</td>
<td>71.87 ab</td>
</tr>
<tr>
<td>(11)</td>
<td>75</td>
<td>18.33 c</td>
<td>3.667 a</td>
<td>44.93 b</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>26.67 a</td>
<td>5.167 a</td>
<td>109.4 a</td>
</tr>
</tbody>
</table>

Means followed by the same letters are not significantly different from each other at the 5% level.

**Table 6** ISSR amplified bands, polymorphic bands and markers for irradiation doses treated using three primers in banana plants.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Polymorphism</th>
<th>No. of markers</th>
<th>20 Gy</th>
<th>40 Gy</th>
<th>60 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBC-814</td>
<td>66.67%</td>
<td>1</td>
<td>650</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>UBC-817</td>
<td>50%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UBC-835</td>
<td>85.71%</td>
<td>42.86%</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
breakage, single base changes, changes in copy number of repeated sequences and alteration in DNA methylation patterns (Munthali et al. 1996). Traditional methods for mutant plant selection based on morphological and biochemical markers, but these markers are less reproducible due to influenced by environmental conditions. The mutation detection based on PCR and non-PCR techniques are reliable and reproducible and have been used in various mutant crops for screening. The simplest use of the PCR in mutation analysis determines the presence or absence of a particular region of DNA (Salim et al. 2009). It could be concluded that ISSR can be successfully used to detect SVs among in vitro regenerated banana plants. Numerous researchers proved that the sensitivity of ISSR was sufficient enough to detect genetic change in banana plants (Racharak and Eiadthong 2007).

Field screening for BBTV and BMV resistance

Banana plants were hardened in the green house and a total of three plants were remaining from 60 Gy treatment were shifted in to the field for R1 generation. On maturity, three plants showing BMV resistance responded. No selected lines were found resistance against BBTV tested individually by syringe method of inoculation. These results were in agreement with Pearce (2003) who mentioned that improving disease resistance is vital for the future survival of banana. Researchers have also tried to generate disease resistance in banana plants by using tissue culture-based techniques such as in vitro mutagenesis. Mutations for banana plants improvement can further be induced by gamma irradiation and by SV (Smith et al. 2006).

CONCLUSION

Development of BBTV and BMV resistant banana crop is necessary to reduce the environmental pollution caused by hazardous and non-hazardous materials. The conventional breeding method takes several years to develop new cultivars from wild species. Physical mutagens (gamma irradiation) and SVs are potential tools and being highly used in crops to improve their quality and yield traits. These mutations are easy to apply on crops and inexpensive to develop resistant varieties. Gamma irradiation, a potent mutagen in plants, and create point mutation easily. The mutant plant species can be easily selected from banana plants by ISSR-PCR. This marker is reproducible and consistent as compared to morphological. The mutant plants produced by gamma irradiation and SVs are capable to tolerate various biotic and stress conditions and avoid application of hazardous pesticides against harmful pathogens. Therefore, it should be apply on various crops, which are susceptible to harmful pathogens and create resistance to them against these pathogens.

ACKNOWLEDGEMENTS

The authors thank Dr. Jaime A. Teixeira da Silva for linguistic improvement.

REFERENCES


Clark MF, Adams AN (1977) Characteristics of the microplate method of
enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34, 475-483


Garcia SAL, van der Lee TAJ, Hekkert BTL, Zapater MF, Goodwin SB, Guzmán M, Kema GIL, Souza MT (2010) Variable number of tandem repeat markers in the genome sequence of *Mycosphaerella fijiensis*, the causal agent of black leaf streak disease of banana (*Musa* spp.). *Genetics and Molecular Research* 9 (4), 2207-2212


Snedecor GW, Cochran WG (1972) *Statistical Methods* (6th Edn), The Iowa State University Press, Ames, Iowa, USA, 593 pp


