The African Journal of Plant Science and Biotechnology ©2011 Global Science Books

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

Eman El-Sayed[1] • Sherin Mahfouze[2*] • Assam Shaltout[3] • Khalid El-Dougdoug[4] • Ramadan Sayed[1]

[1] Horticulture Research Institute, Agriculture Research Center, Giza, Egypt
[2] Genetic Engineering and Biotechnology Division, Genetic and Cytology Department, National Research Center, Dokki, 12622, Egypt
[3] Horticulture Department, Agriculture Faculty, Ain Shams University, Cairo, Egypt
[4] Agriculture Microbiology Department (Virology Laboratory), Agriculture Faculty, Ain Shams University, Cairo, Egypt

***Corresponding author*: * Sherinmahfouze@yahoo.com**

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 ^{60}Co 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 parthenocarpic 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 amplication, non-reciprocal mitotic recombination, transposable element activation of silent genes (Jain 2001; Karamura1 *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 objective 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.

Received: 27 March, 2011. Accepted: 22 June, 2011.

Original Research Paper

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 plants (Beehira governorate, Egypt). The shoot apices (trimmed to a size of 1.0–1.5 cm, with minimum basal corm 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 (^{60}Co 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 (generation 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 $MgCl_2$, 0.2 mM dNTP mixed, 10 *p*mol 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 containing 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).

Table 1 Base sequence of reliable ISSR primers used in this study.

Primer	Sequence (5′-3′)
UBC-811	GA GA GA GA GA GA GA GA C
UBC-814	CTC TCT CTC TCT CTC TA
UBC-817	CA CA CA CA CA CA CA CA A
UBC-820	GT GT GT GT GT GT GT GT T
UBC-835	AGA GAG AGA GAG AGA GYC

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 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 (plantlets length, 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 2:1(w/v) and macerated using sterilized mortar and pestle. The crude sap was filtrated 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 were 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 Cochron 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; $P \leq 0.05$ 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 Analysis of variance for quantitative characters of banana plantlets treated with different irradiation doses (*in vitro*).

Radiation doses	Plantlet characters							
	Survival %	Length of plantlets (cm)	Leaf area (cm^2)	No. of roots	Length of root (cm)	Fresh weight (g)	Dry weight (g)	Chlorophyll (SPAD)
20 Gy	26.66	5.500 ab	7.727 b	11.00 ab	2.167 bc	26.07 ab	5.517 a	22.33 c
40 Gy	0.266	3.667 b	16.35 a	7.333 bc	3.167 b	18.29 bc	5.56 a	38.33 a
60 Gy	0.133	1.167 c	2.003 c	3.333 c	1.667 c	7.49 c	4.15 b	11.67 d
Control	100	6.000 a	9.027 b	14.67 a	6.833 a	34.01 a	5.74 a	29.33 b

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

Table 3 Analysis of variance for quantitative characters of banana plants treated with different irradiation doses under greenhouse conditions.

Radiation doses	Plantlet characters			
	Survival %	Plant height (cm)	plant diameter (cm)	Leaf area (cm^2)
20 Gy	26.66	22.66 a	5.050 a	115.9 a
40 Gy	0.266	25.17 a	5.333 a	102.4 a
60 Gy	0.08	7.503 b	2.334 b	23.20 b
Control	100	26.67 a	5.167 a	109.4 a

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

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 LD_{40} (50% lethal dose) the data in **Table 2** was plotted.

Radiation effect on transplanted banana plants after 90 days in greenhouse

When all rooted plants were transplanted to the greenhouse, only the controls and those plants treated with 20 and 40 Gy were able to survive. The percentage survival of the controls and treated plants was 26.66 and 0.26%, respectively, as shown in (**Table 3**). Growth of banana plants in the greenhouse was assessed by three traits (average height, plant diameter, leaf area), as shown in (**Table 3**). Banana plants treated with 60 Gy were dwarf compared with the control (**Table 3**).

These results were in an agreement with those of Ziv (1991), who used mutation breeding as a methodology for crop improvement based on the possibility of altering genes by exposing their vegetative parts, cells, tissues, gametes or seeds to physical and chemical mutagens. Mutagenesis of *in vitro* cultures avoids the need for large-scale facilities and allows better control of treatment, as hyperhydric tissues may be more permeable to mutagens. The irradiation of callus cultures which are capable of embryogenesis and organogenesis can be used to obtain mutants quickly and in large numbers (Ahloowalia 1995). Radioactive materials like ^{60}Co emit high energy photons which are called as gamma radiation. These radiations can alter the structure of chromosome in two ways (Ali *et al.* 2007) directly by quanta of energy which hit the chromosomes like bullets hitting a target and indirectly by ionization which produces free radicals.

Among 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 unrepaired single stranded break (SSB) in ssDNA; an unrepaired 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).

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 differentiating vegetative cells induced by tissue culture and is expected to generate stable plants carrying interesting heritable traits (Soniya *et al.* 2001). Four variables are critical for SV: genotype, explant origin, cultivation period, and the cultural condition in which the culture is made. One of the disadvantages is the fact that SV is a random process

Table 4 Analysis of variance for quantitative characters of banana plantlets treated with different subculture (*in vitro*).

Subculture	Plantlet characters							
	Survival %	Length of plantlets (cm)	Leaf area (cm^2)	No. of roots	Length of root (cm)	Fresh weight (g)	Dry weight (g)	Chlorophyll (SPAD)
7	100	5.500 a	9.060 a	15.67 a	5.167 a	26.34 ab	5.200 a	25.67 ab
9	92.5	5.167 a	6.400 b	15.33 a	2.500 b	25.07 ab	5.683 a	19.33 b
11	79	2.167 b	2.547 c	17.33 a	1.833 b	22.32 b	5.447 a	10.00 c
Control	100	5.833 a	9.027 a	14.67 a	6.167 a	34.01 a	5.740 a	29.33 a

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

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

Subculture	Plantlet characters			
	Survival %	Plant height (cm)	plant diameter (cm)	Leaf area (cm^2)
(7)	100	24.67 ab	4.833 a	101.5 a
(9)	92.5	20.00 bc	4.333 a	71.87 ab
(11)	75	18.33 c	3.667 a	44.93 b
Control	100	26.67 a	5.167 a	109.4 a

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 straight-forward; 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 (Soniya *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 variations (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.

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

Primer name	Polymorphism		No. of markers With pb		20 Gy	40 Gy	60 Gy
	Total	P %					
UBC-814	9	6	1	650			+
	66.67%		11.11%		0	0	1
UBC-817	6	3	0	0			
	50%		0%		0	0	0
UBC-835	7	6	3	1500	+		
				1100	+		
				720	+		
	85.71%		42.86%		3	0	0
Total =	22	15	4		3	0	1
Polymorphic = 68.18%			18.18%				

* P = Number of polymorphic bands with polymorphic percentages.
** Total = Total number of amplified fragments. + = Presence of marker band.

Total number of 22 scorable amplified DNA fragment ranging from 3060 to 140 bp was observed using the three primers, whereas 15 fragments were polymorphic and the other amplified were commonly detected among banana plants treatment with three doses of Gamma irradiation. The three primers UBC-814, UBC-817 and UBC-835 showed mean polymorphic percentage of 68.18%. The polymorphic percentage of primer UBC-835 recorded the highest percentage (85.71%), whereas primer UBC-817 displayed the lowest percentage (50%). Among the 15 polymorphic bands, four bands were specific markers with a total average of 18.18%. The banana plants treatment with gamma irradiation were varied considerably using the three primers, whereas the 20 Gy dose revealed the highest number with three markers, followed by 60 Gy dose at one marker, however dose 40 Gy dose have not induced any markers (**Table 6**).

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, 500 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.

Primer UBC-817 appeared to six amplified fragments with sizes ranged from 3060 to 280 bp, three bands were polymorphic with 50% polymorphism, three bands were commonly revealed in the 7th, 9th and 11th sub-cultures with (370, 350 and 280 bp) and the control (the first subculture). One amplified fragment with molecular size 420 bp appeared in the 11th sub-culture and the control and disappeared in the 7th and the 9th sub-cultures. In addition to, two amplified fragments with 3060 and 470 bp showed only in the control. Finally, primer UBC-835 revealed four ampli-

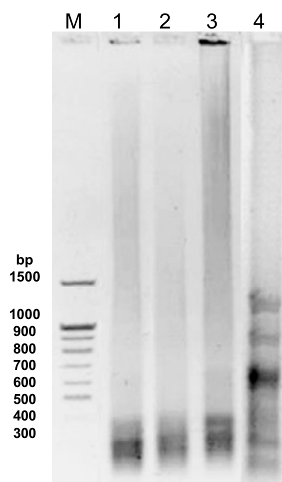

Fig. 1 ISSR pattern bands generated by primers UBC-814. Lane M = 100 bp DNA ladder plus, lane 1, 2 and 3 banana plants treated with different three doses from gamma irradiation. 1 = 20 Gy dose; 2 = 40 Gy dose; 3 = 60 Gy dose; 4 = Control (no gamma irradiation).

fied fragments with sizes ranged from 380 to 140 bp, three amplified fragments were polymorphic with 75% polymorphism. One band with molecular size 140 bp was commonly detected among three sub-cultures and the control. One band with molecular weight 270 bp was showed in the 9th sub-culture and the control and disappeared in the 7th and the 11th sub-cultures. Also, one band with 380 bp appeared uniquely in the control. On the contrary, one amplified fragment with 220 bp induced in three subcultures and disappeared in the control.

Total number of 19 scorable amplified DNA fragment ranging from 3060 to 140 bp was observed using the three primers, whereas 12 fragments were polymorphic and the other amplified were commonly detected among three sub-cultures the 7th, 9th and 11th. The three primers UBC-814, UBC-817 and UBC-835 showed mean polymorphic percentage 63.16%. The polymorphic percentage of primer UBC-835 recorded the highest percentage (75%), whereas primer UBC-817 displayed the lowest percentage (50%). On the other hand, it has not found any markers induced in three sub-cultures. Consequently, variations observed in total number of ISSR bands as well as the number of specific bands among the parental plants (the control) and regenerated plants of different sub-cultures show genetic differences of the genotypes due to tissue culture and SV induced. The presence of specific band/locus in the parental plants and loss of it in the regenerated plants of different sub-cultures show the loss of certain loci during tissue culture due to SV in banana plants, while the occurrence of specific bands/locus in the regenerated plants of different sub-cultures and their absence in mother plants may indicate the occurrence of genetic changes leading to formation of new binding sites in these plants. Such specific loci are of high importance in the genetic identification of the genotypes or somaclones from one another. These results were in an agreement with (Shepherd and dos Santos 1996) who found that tissue-culture instability of *in vitro* propagated plants is a common event in *Musa* genus. Some growth regulators as cytokinins and auxins can change the chromosome number of *Musa* spp. grown *in vitro*, which affect the nuclear DNA content of *in vitro* derived CIEN BTA-03 plants. The use of cytokinin 6-BA in *Musa* multiplication has been proved to generate ascendant and descendant aneuploid cells. Rani *et al*. (1995) found that polymorphic amplification products which represent one allele per locus can result from changes in either the sequence of the primer binding site, such as point mutations, or from changes altering the size or preventing successful amplification of a target DNA such as insertions, deletions, and inversions. The molecular mechanism underlying SVs have been attributed to chromosome 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 researches 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 R_1 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 mutagens 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 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

Agrawal GK, Pandey RN, Agrawal VP (1992) Isolation of DNA from *Cheorospondias axillaris* leaves. *Biotechnology Letters* **2**, 19-24

Ahloowalia BS (1995) *In vitro* mutagenesis for improvement of vegetatively propagated plants. In: *Proceedings: Induced Mutation and Molecular Techniques for Crop Improvement*, International Symposium, IAEA and Food Agriculture Organization of the United Nations, IAEA SM-340, Vienna, pp 531-541

Ali A, Naz S, Alam SS, Iqbal J (2007) *In vitro* induced mutation for screening of red rot (*Colletotrichum falcatum*) resistance in sugarcane (*Saccharum officinarum*). *Pakistan Journal of Botany* **39 (6)**, 1979-1994

Bhagwat B, Duncan EJ (1998) Mutation breeding of banana cv. Highgate (*Musa* spp., AAA Group) for tolerance to *Fusarium oxysporum* f.sp. *cubense* using chemical mutagens. *Scientia Hortculturae* **73**, 11-22

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

Crouch JH, Vuylsteke D, Ortiz R (1998) Perspectives on the application of biotechnology to assist the genetic enhancement of plantain and banana (*Musa* spp.). *Electronic Journal of Biotechnology* **1**, 11-22

Garcia SAL, van der Lee TAJ, Ferreira CF, Hekkert BTL, Zapater MF, Goodwin SB, Guzmán M, Kema GHJ, 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

Hussain Z, Afghan S (2001) A comparative study on artificial inoculation methods for red rot (*Colletotrichum falcatum* (went) screening. *Pakistan Sugar Journal* **16 (2)**, 3-5

Hwang SC, Ko WH (2004) Cavendish banana cultivars resistant to Fusarium wilt acquired through SV in Taiwan. *Plant Diseases* **88**, 580-588

Islam NMd, Naqvi AR, Jan AT, Mohd Q, Haq R (2010) Genetic diversity and possible evidence of recombination among *Banana bunchy top virus* (BBTV) isolates. *International Research Journal of Microbiology* **1 (1)**, 1-12

Jain MS (2001) Tissue culture-derived variation in crop improvement. *Euphytica* **118**, 153-166

Jones DA (2000) Banana breeding for disease resistance: History of banana breeding. In: Jones DR (Ed) *Diseases of Banana, Abaca and Enset*, CAB International, London, UK, pp 425-434

Khan IA, Khatri A, Ahmad M, Siddiqui SH, Nizamani GS, Khanzada MH, Dahar NA, Khan R (1998) *In vitro* mutagenesis in sugarcane. *Pakistan Journal of Botany* **30 (2)**, 253-261

Karamura D, Karamura E, Tushemereirwe W, Rubaihayo PR, Markham R (2010) Somatic mutations and their implications to the conservation strategies of the east African highland bananas (*Musa* spp.). *Acta Horticulturae* **879**, 615-622

Ko WH, Hwang SC, Ku FM (1991) A new technique for storage of meristem tip cultures of Cavendish banana. *Plant Cell, Tissue and Organ Culture* **25**, 179-183

Lakshmanan V, Venkataramareddy SR, Neelwarne B (2007) Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers. *Electronic Journal of Biotechnology* **10 (1)**, 106-113

Munthali TM, Newbury JH, Fordloyd VB (1996) The detection of somaclonal variants of beet using RAPD. *Plant Cell Reports* **15**, 474-478

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-487

Novak FJ, Afza R, Van Duren M, Omar MS (1990) Mutation induction by gamma irradiation of *in vitro* cultured shoot tips of banana and plantain (*Musa* cvs.). *Tropical Agriculture* **67**, 21-28

Pearce F (2003) Going bananas. *New Scientist* **177 (2378)**, 26-29

Racharak P, Eiadthong W (2007) Genetic relationship among subspecies of *Musa acuminata* colla and A-genome consisting edible cultivated banana assayed with ISSR markers. *Songklanakarin Journal Science Technology* **29 (61)**, 1439-1489

Rani V, Parida A, Raina SN (1995) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. *Plant Cell Reports* **14**, 459-462

Roux NS (2004) Mutation induction in Musa-review. In: Jain SM, Swennen R (Eds) *Banana Improvement: Cellular, Molecular Biology, and Induced Mutations*, Science, Enfield, USA, pp 23-32

Sagi L (2000) Genetic engineering of banana of banana for disease resistance-future possibilities. In: Jones DR (Ed) *Diseases of Banana, Abaca and Enset*, CAB International, London, UK, pp 465-515

Sahijram L, Soneji JR, Bollamma KT (2003) Analyzing SV in micropropagated banana (*Musa* spp.). *In Vitro Cellular and Developmental Biology – Plant* **39**, 551-556

Salim K, Al-Qurainy F, Anwar F (2009) Sodium azide: A chemical mutagen for enhancement of agronomic traits of crop plants. *Environment and We: An International Journal of Science and Technology* **4**, 1-21

Shepherd K, dos Santos JA (1996) Mitotic instability in banana varieties. I - Plants from callus and shoot tip cultures. *Fruits* **51**, 5-11

Soniya EV, Banerjee NS, Das MR (2001) Genetic analysis of SV among callus-derived plants of tomato. *Current Science* **80**, 1213-1215

Smith MK, Hamill SD, Langdon PW, Giles JE, Doogan VJ, Pegg KG (2006) Towards the development of a Cavendish banana resistant plant to race 4 of Fusarium wilt: Gamma irradiation of micropropagated dwarf Parfitt (*Musa* spp.) AAA group, Cavendish subgroup. *Australian Journal of Experimental Agriculture* **46**, 107-113

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

Swennen R, Vuylsteke D (2001) Banana. In: Raemaekers RH (Ed) *Crop Production in Tropical Africa*, Directorate General for International Cooperation, Brussels, Belgium, pp 530-552

Tang CY (2005) Seasonal variation: A tool for the improvement of Cavendish banana cultivars. *Acta Horticulturae* **692**, 61-65

Xu CX, Panis B, Stross H, Li HP, Xiao HG, Fan HZ, Swennen R (2005) Establishment of embryogenic cell suspensions and plant regeneration of the dessert banana Williams (*Musa* AAA group). *Journal of Horticultural Science and Biotechnology* **80**, 523-528

Ziv M (1991) Vitrification: Morphological and physiological disorders of *in vitro* plants. In: Debergh PC, Zimmerman RH (Eds) *Mocropropagation: Technology and Application*, Kluwer Academic Publishers, Dordrecht, pp 45-69