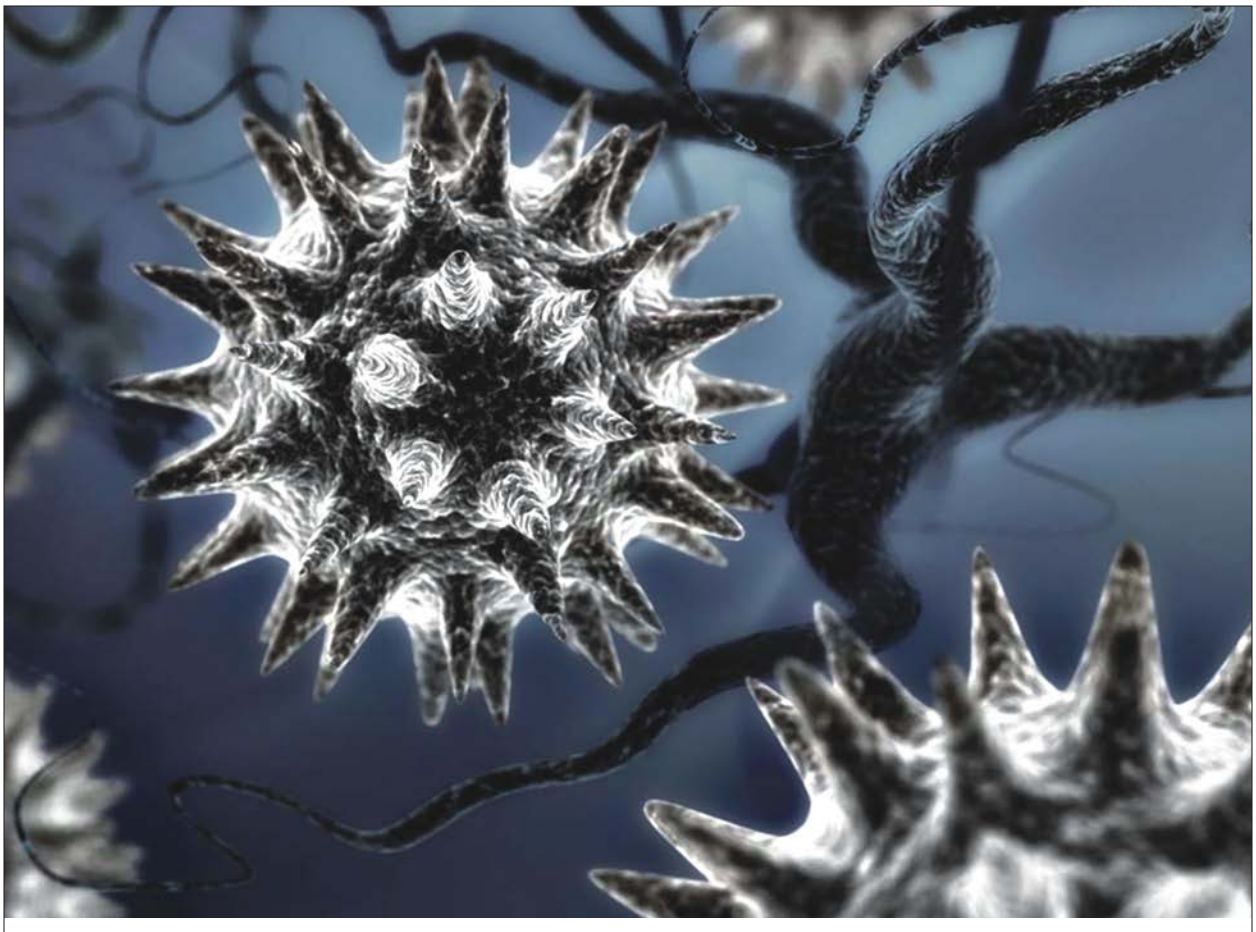

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Chemical Mutation Induction *in vitro* Cultured Shoot Tip of Banana Cv. Grand Nain for Resistance some Virus Diseases

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ABSTRACT

The chemical mutagens have become important tool to enhance agronomic traits of banana crop. It is being used to produce virus resistance in various susceptible banana crop to improve it yield and quality traits against BBTV and BMV viruses. There are several mutagens available for banana crop improvement and each mutagen has its important role as positive or negative effects on banana plants. The chemical mutagens such as create mutations in the genome of plants. Selection of plant mutants is based on morphological and ISSR-PCR markers. The DNA based marker is reliable and reproducible for mutant selection for BBTV and BMV resistance banana plants used in the study. Explants from shoot apical meristem were cultured on MS-medium supplemented with different concentrations of 2, 4-D (2, 4 and 6 mg L^{-1}) 6-Benzylaminopurine (6, 7 and 8 mg L^{-1}) and Sodium Azide ranging from (1, 2 and 3 mg L^{-1}) for induction of mutation. *In vitro* screened plants after rooting were hardened and acclimatized in the glass house and were shifted into the field. Screening banana plants resistance of BBTV and BMV was carried out by using syringe method of inoculation. It was observed that, only two banana plants of the concentration 6 mg L^{-1} from 2, 4-D was found to be resistant against BMV isolate. In the same time, it is not BBTV resistance banana plants.

Key words: Banana improvement, 2, 4-D; 6-benzylaminopurine, sodium azide, ISSR-PCR

INTRODUCTION

Banana (*Musa* sp.) is considered the fourth most important tropical fruit crops in the world (Amar, 2000). At present, the industry is beset with low productivity due to Banana bunchy top virus (BBTV) and Banana mosaic virus (BMV) (Swennen and Vuylsteke, 2001). The parthenocarpic nature of cultivated banana makes it difficult to breed for resistance to diseases (Jones, 2000). Diseases resistant hybrids have been developed by means of classical breeding and although good resistance has been obtained, most of these hybrids are not acceptable to local markets. 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 *in vitro* chemical treatment (Smith *et al*., 2006). A number of chemical are able toinduce

mutation in banana plants such as sodium azide, 2,4-D (2,4 dichlorophenoxy acetic acid) and 6-Benzylaminopurine (6-BA) (Bhagwat and Duncan, 1998). Many of these chemicals have clastogenic (chromosome damaging) effects on plants via reactive oxygen-derived radicals (Yuan and Zhang, 1993). These effects can occur both spontaneously and artificially following induction by mutagens. Chemical mutagen generally produce induced mutation which lead to base pair substitutions, especially GC---AT resulting in amino acid change which change the function of proteins but do not abolish their functions as deletions or frame shift mutations mostly do. These chemomutagens induce a broad variation of morphological and yield structure parameters in comparison to normal plants. Many researchers compared the mutagenic efficiencies of different mutagens on different crops and their results seem to be entirely specific for particular species and even varieties, while many researchers found chemical mutagens are to be more effective than physical ones (Bhat *et al*., 2005). A number of workers (Montalvan and Ando, 1998) have reported the role of chemical mutagens in enhancing genetic variability in higher plants because it is the fundamental characteristics to successful breeding programs in vegetatively and sexually propagated plants. This variation can occur naturally or can be induced through mutations, using physical, biological or chemical mutagens and has attracted the interest of plant breeders for many decades. The mutants so produced facilitate the isolation, identification and cloning of genes used in designing crops with improved yield and quality traits (Ahloowalia and Maluszynski, 2001).

The primary objectives of this study were to find new mutations from banana plants resistance of BBTV and BMV using chemical mutations. In addition to, determine the potential of ISSR-PCR to differentiate chemical mutagens in banana plants.

MATERIALS AND METHODS

Shoot-tip culture

Initiation of aseptic shoot-tip cultures: One hundred and fifty BBTV and BMV-free shoot apices from side-suckers of banana Grand Nain cv. were used as the source materials for the establishment of *in vitro* shoot-tip cultures. The suckers collected from the field growing plants (El-Behira governorate) were thoroughly washed with tap water to remove adhering soil, after removing leafy top and the roots. These were trimmed to a size of approximately 3-4 cm in length and 2 cm in diameter. The shoot apices were transferred to a 250 ml capacity jar and washed with a standard liquid detergent followed by several washes with distilled water. The explants were sequentially treated with 70% Sodium hypochlorite for 15 minutes, 70% ethanol (8 min) and 0.1% $HgCl_2$ (7 min), under aseptic conditions. Each of these treatments was followed by minimum of four rinses with sterile distilled water and excision of few sheathing leaves and some corm tissue. Finally, the shoot apices (trimmed to a size of 1.0-1.5 cm, with minimum basal corm tissue) were cultured on SM1 Murashige and Skoog (1962) medium (Table 1). The cultures were incubated at 24±2 C under photo period cycle of 16/8 h. as light/dark. Light intensity was used at 3000 lux. with white fluorescent tubes.

Proliferation of multiple shoots and rooting of individual shoots: After 10 days of initiation, the cultured shoot tips are sectioned vertically and subcultured on SM2 medium (Table 1). Multiple shoots formed need to be maintained as stock cultures by regular subculturing at an interval of about four weeks, on SM2 medium. The stock cultures are freshly initiated as and when necessary. Individual shoots can be separated from the shoot-tip multiples and rooted by culturing on SM3 or SM4 medium (Table 1).

Table 1: Media compositions for banana micropropagation stages and different concentrations of chemical mutagens

Code	Culture phase	Medium composition	Growth condition
SM1,2	Shoot-tip culturing	MS + BAP (5) + Suc (3%), pH 5.7, Gelrite (0.2%)	Dark
SM3,4	Multiplication	MS + BAP (3) + Suc (3%), pH 5.7, Gelrite (0.2%).	Light
SM5	Rooting	MS + Suc (3%), pH 5.7, Agar-0.70%	Light
SM6	Rooting	MS + NAA (1), Suc (1%), pH 5.7, Agar (0.7%)	Light

BAP: 6-Benzylaminopurine, NAA: Naphthaleneacetic acid, Suc: Sucrose.

Induction of mutation through chemical mutagens (2,4-D, 6-benzylaminopurine and sodium azide): In the present investigation, banana *in vitro* cultures (subculture 2) were treated with different concentrations of 2,4-D (2, 4 and 6 mg L^{-1}), 6-Benzylaminopurine (6, 7 and 8 mg L^{-1}) and Sodium azide (1, 2 and 3 mg L^{-1}) for 21 days and after treatment, shoots were transferred to MS- medium without chemical mutagens.

DNA extraction: Young leaves of all banana samples 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 2) were used to amplify DNA (Life Technologies, Gaithersburg, Md.). The total reaction mixture was 15 μL contained 10x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP mixed, 10 pmol primer, 1.25 U *Taq* polymerase and approximately 150 ng genomic DNA. DNA amplification was obtained through 40 cycles in a DNA thermal cycler. The temperature profile was as follows: Denature temperature 94°C for 1 min annealing temperature 52°C for 1 min and extension temperature 72 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^{-1} ethidium bromide for 45 min. at 90 V. The sizes of each fragment were estimated with reference to a size marker of 100 bp DNA ladder.

Gel analysis: The gel analysis was applied by programme (UVI geltec version 12.4, 1999-2005, USA).

Green house hardening of plantlets: After four weeks, plantlets with well developed root-system were carefully removed from the culture vessel and gently washed in running tap water to remove the entire gelled medium. A paint brush with smooth bristles can be used to remove adhering gel pieces. The plantlets shall then transferred to perforated polythene bags, filled with autoclaved mixture of soil and a suitable commercial hardening mixture such as peat moss: sand: Vermiculite (1:2:1). The plantlets were maintained in the greenhouse for 2-4 months (depending on the growth) under natural light with relative humidity of 90–100% and an ambient temperature of 24±2°C. Approximately 40 cm tall plants can be field planted for evaluation.

Response of banana to mixed infection (BBTV and BMV): Hardened banana plants with similar size were inoculated artificially by a syringe method (Hussnain and Afghan, 2001) both of BBTV and BMV isolates using 0.1 M phosphate buffer pH 7.4 (1:2 w/v). The inoculated plants and control were maintained in insect-proof greenhouse at 24±2°C supplemented with (photoperiod 12 h). 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 2 mon for visible symptoms.

Table 2: Base sequence of reliable ISSR primers used in this study

Primer	Sequence	Reference
UBC-811	- GA GA GA GA GA GA GA GA C-	Lakshmanan *et al.* (2007)
UBC-814	-CTC TCT CTC TCT CTC TA-	Racharak and Eiadthong (2007)
UBC-817	-CA CA CA CA CA CA CA CA A-	Lakshmanan *et al.* (2007)
UBC-820	-GT GT GT GT GT GT GT GT T-	Lakshmanan *et al.* (2007)
UBC-835	-AGA GAG AGA GAG AGA GYC-	Racharak and Eiadthong (2007)

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 Company Sante Animal Paris, France.

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 (Snedecor and Cochran, 1972).

RESULTS AND DISCUSSION

Effect of different treatment of 2,4-D, 6-Benzylaminopurine and Sodium azide on plant regeneration: The effect of 2,4-D treatment on survival are depicted in Table 3, It is obvious that in the control cultures, incorporation of 2,4-D into the medium adversely affected the rate of plant survival which was found to be decreased with the concentration of 2,4-D, when the concentration of 2,4-D was increased from 2 and 4 mg L^{-1} to 6 mg L^{-1} survival rate were 86.5, 83 and 57%, respectively. Maximum number of morphological variants was obtained at the concentration of 6 mg L^{-1} from 2,4-D (Table 3). At this concentration showed the morphologically abnormal plants produced such as albino and yellowing of leaves. It was found that, significantly differences in chlorophyll content in concentration 6 mg L^{-1} of 2,4-D (17.67 SPAD) compared to the control (29 SPAD) due to mutagenic treatment. These results were agreement with Sikka and Sharma (1976) who mentioned that 2,4-D is widely used in culture media as an auxin analog. Some authors have reported that 2,4-D at high (herbicidal) concentrations (higher than 200 μM) induced polyploidization, disturbances of mitotic spindle, chromosome stickiness and chromosome breaks. Such effects were not observed if low concentrations of 2,4-D (5-50 μM) were applied to garlic root meristem cells (Dolezel and Novak, 1984). The effect of 2,4-D on the mutation induction is unclear, while some authors report induction of heritable changes (Pohlheim *et al.*, 1977) other reports are negative (Ali and Amer, 1974) or rather unclear of (Seiler, 1978).

On the other hand, BAP affected the rate of survival and chlorophyll content, it was found that rate of survival was decreased as the concentration increment of BAP. Thus, survival rate of 7, 8 and 9 mg L^{-1} concentrations from BAP was 98, 67 and 60%, respectively (Table 3). However, two concentrations 7 and 8 mg L^{-1} from BAP caused dwarf banana plants (Table 3). On the contrary, chlorophyll content was increased (40.00 and 38.67 SPAD) with two concentrations (7 and 8 mg L^{-1}), respectively compared to the control (29.00 SPAD). These results were agreement with Vuylsteke *et al.* (1991) who observed 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 plants (Shepherd and Da Silva, 1996). The use of cytokinin 6-BA in *Musa*

Table 3: Analysis of variance for quantitative characters of grand nain cv. banana plantlets treated with different chemical concentrations (*In vitro*)

		Plantlets characters						
Treatments (Mg L^{-1})	Survival %	Length of plantlets (cm)	Leaf area (cm^2)	No. of roots	Length of root (cm)	Fresh weight (g)	Dry weight (g)	Chlorophyll SPAD
2,4-D								
2	86.5	5.167ab	14.33a	7.00c	4.333b	14.43cde	5.067e	26.33cd
4	83	4.667ab	5.853cd	6.667cd	3.667bc	18.37c	5.613bc	25.33d
6	57	2.667cd	4.717cd	4.333de	2.50cd	15.60cd	5.933a	17.67e
Means		4.167b	8.301a	6.00bc	3.50b	16.14c	5.538b	23.11c
6-Benzylaminop urine								
6	98	5.167ab	13.19a	10.00b	3.833b	12.68def	5.84ab	40.00a
7	67	3.333cd	7.413bc	5.667cde	2.50cd	10.63ef	5.753ab	38.67a
8	60	2.667cd	4.100d	3.333e	1.667d	9.243f	4.933e	34.00bc
Means		3.722bc	8.234a	6.333b	2.667c	10.85d	5.509b	37.56a
Sodium azide								
1	54	4.167abc	12.70a	5.667cde	2.50cd	23.83b	5.437c	26.67bcd
2	32	2.833cd	6.67bcd	5.00cde	1.833d	14.66cde	5.353cd	26.00d
2	10	1.833d	4.933cd	3.667e	1.500d	18.60c	5.137de	34.33ab
Means		2.944c	8.102a	4.778c	1.944d	19.03b	5.409c	29.00b
Control	100	6.000a	9.030b	15.00a	6.200a	34.01a	5.740ab	29.00bcd
Means		6.000a	9.030a	15.00a	6.200a	34.01a	5.740a	29.00b

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

multiplication has been proved to generate ascendant and descendant aneuploid cells (Sandoval *et al*., 1996). Also, Shirani *et al*. (2009) mentioned that using 6-BA above 33.3 μM significantly caused higher gross of abnormal shoot regeneration. Matsumoto *et al*. (2006) reported that Grand naine cultivar however, increasing the concentration of BAP from 3 to 10 mg L^{-1} significantly increased the mean number of shoots produced by dwarf lines, moreover the dwarf lines grown on the medium with 10 mg L^{-1} BAP, also produced 40% more shoots than true-to-type plants.

Incorporation of Sodium azide into culture medium affected the rate of plant survival which was found to be decreased with the concentration of Sodium azide, when the concentration of Sodium azide was increased 1, 2 and 3 mg L^{-1} number of plants survived were 54, 32 and 10%, respectively (Table 3). Concentration of 3 mg L^{-1} from sodium azide significantly increased in number of dwarf banana plants (Table 3). Also, chlorophyll content was increased (34.33 SPAD) in concentration of 3 mg L^{-1} compared to the control (29.00 SPAD). In the contrast, chlorophyll content was decreased (26.67 and 26.00 SPAD) of two concentrations (1 and 2 mg L^{-1}) from sodium azide, respectively. These results were agreement with those of Ziv (1991) that who found mutation breeding as a methodology for crop improvement is 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 vitrified tissues may be more permeable to mutagens. In this study Sodium azide was used as chemical mutagen. Bhagwat and Duncan (1998) used sodium azide to induce mutation against *Fusarium oxysporium* in banana. Maniu and Mihailescu (1998) demonstrated that sodium azide acts through a promutagen or organic metabolite. This metabolite has been isolated and identified as azidoalanine. Sodium azide also produce DNA single strand breaks (Veleminsky *et al*., 1985), it readily induces base substitution but not "frameshifts" or deletions. It preferentially

induced G:C → A:T transitions at the second codon position (Koch *et al*., 1994). Although, sodium azide causes mutation at gene level but few chromosme aberrations have also been detected (El-Den, 1993). Del Campo *et al*. (1999) also reported the induction of chromosomal aberrations during replication phase (S) by Sodium azide. Mendhulkar (2002) found mitotic chromosomal aberrations (laggards, bridges, chromosome stickiness) by single treatment with Sodium azide. The immediate effect of Sodium azide on meristematic cells appears to be a blocking action at the beginning of the genome separation reducing its velocity of the progression in the cell cycle; which it acts as the respiratory chain inhibitor. Johnsen *et al*. (2005) also mentioned Sodium azide as electron transport blocker.

Effect of different treatment of 2,4-D, 6-benzylaminopurine and sodium azide on transplanted banana plants after 90 days: When all rooted plantlets were transplanted to the greenhouse, it was observed that, rate of survival was decreased as the concentration of all chemical mutagens (Table 4, Fig. 1). On the other hand, data cleared significant differences in plant height between different three concentrations of 2,4-D, BAP and sodium Azide compared with the control (Table 4 and Fig. 1). These results were agreement with 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 between the controls and banana plants treatment with the chemical mutagens.

Primer UBC-814 revealed 17 amplified fragments with sizes ranged from 3000 to 180 bp, whereas 14 fragments were polymorphic with 82.35% polymorphism. The other three fragments with molecular sizes (600, 500 and 370 bp) were commonly detected between all chemical mutagens and the control (Fig. 2a). On the Contrary, one band with 180 bp showed in the control and it has not showed in all chemical mutagens. Also, two bands with 3000 and 280 bp induced in two concentrations 4 and 6 mg L^{-1} of 2,4-D and the control but disappeared in concentration of 2 mg L^{-1}. In addition, one amplified fragment at 1250 bp revealed in the two concentrations of 4 and 6 mg L^{-1} from 2,4-D and they have not existed in 2 mg L^{-1} of 2,4-D. On the other hand, one band with 720 bp showed two concentrations 2 and 6 mg L^{-1} of 2,4-D, respectively (Fig. 2a). In according to, BAP primer-814 appeared to two amplified fragments with 300 and 280 bp in concentration of 7 mg L^{-1} from BAP and the control, however it has not existed in the other two concentrations, in addition to one band with molecular size 720 bp induced uniquely in concentration of 7 mg L^{-1} from BAP (Fig. 2a). One the other hand, primer-814 revealed one band with 720 bp in two concentrations of 1 and 2 mg L^{-1}. from sodium azide and it has not showed in concentration of 3 mg L^{-1} from sodium azide and the control. Also, one amplified fragments at 220 bp is showed on concentration of 3 mg L^{-1} from sodium azide and control and it has not showed in the other two concentrations. Two markers with molecular sizes 360 and 350 bp appeared in concentration of 2 mg L^{-1} from sodium azide (Fig. 2a).

Primer UBC-817 revealed 11 amplified fragments with sizes ranged from 3060 to 240 bp, whereas nine fragments were polymorphic with 81.82% polymorphism. The other two fragments

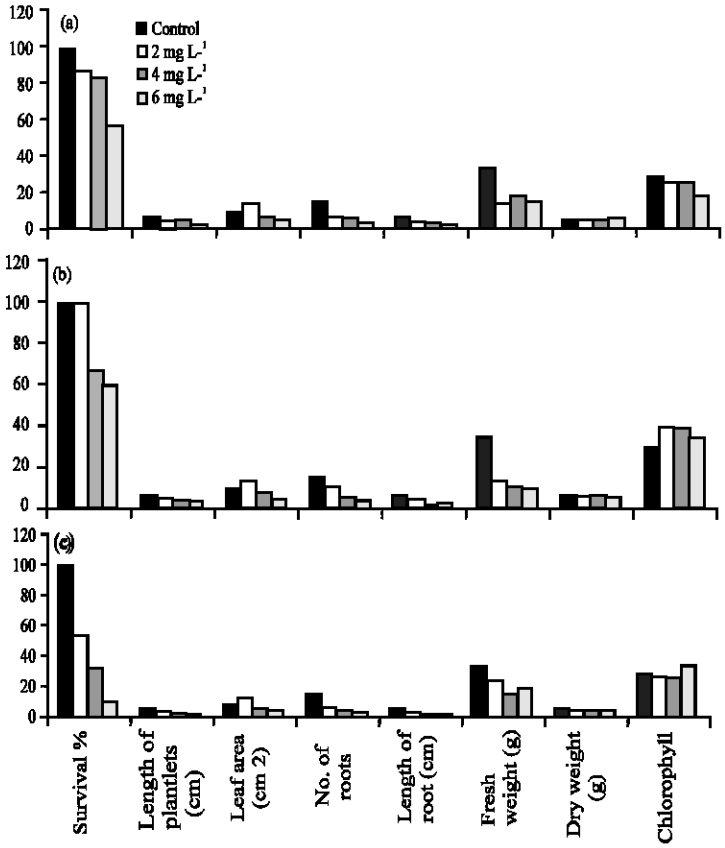

Fig. 1: Histogram showing effect chemical mutagens on banana plants under greenhouse; 2,4-S, 6-Benzylami-npurine and sodium azide

with molecular sizes 370 and 350 bp were commonly detected among three chemical mutagens. On the other hand, one amplified fragment with 610 bp revealed in two concentrations of 4 and 6 mg L^{-1} from 2,4-D and they has not existed in concentration 2 mg L^{-1} and the control. Also, one marker with molecular weight 720 bp appeared in concentration of 6 mg L^{-1} from 2.4-D and absent in the control. Primer UBC-817 revealed two amplified fragments with molecular sizes 3060 and 280 bp showed in two concentrations of 7 and 8 mg L^{-1} from BAP and control and were disappeared in concentration 6 mg L^{-1}. On the contrary, one band with 270 bp was revealed in two concentrations of 7 and 8 mg L^{-1} from BAP and disappeared in concentration of 6 mg L^{-1} and control. One marker with 610 bp was appeared in banana plants mutated with 7 mg L^{-1} (Fig. 2b).PrimerUBC-817 showed three fragments with molecular weights 3060, 470 and 420 bp in two concentrations of 1 and 2 mg L^{-1} from sodium azide and the control and they have not existed in concentration of 3 mg L^{-1} .Whereas, one band with 240 bp appeared in the concentration of 1 mg L^{-1} from Sodium azide and disappeared from the other two concentrations and the control. Two markers were appeared in two concentrations of 1 and 2 mg L^{-1} from sodium azide with molecular weights 720 and 270 bp and 610 and 400 bp, respectively (Fig. 2b).

PrimerUBC-835 revealed seven amplified fragments with sizes ranged from 1500 to 140 bp whereas 6 fragments were polymorphic with 85.71% polymorphism. One amplified fragment with 270 bp revealed in concentration of 6 mg L^{-1} from 2,4-D and control and disappeared in the other two concentrations. One marker with molecular size showed in banana plants mutated with

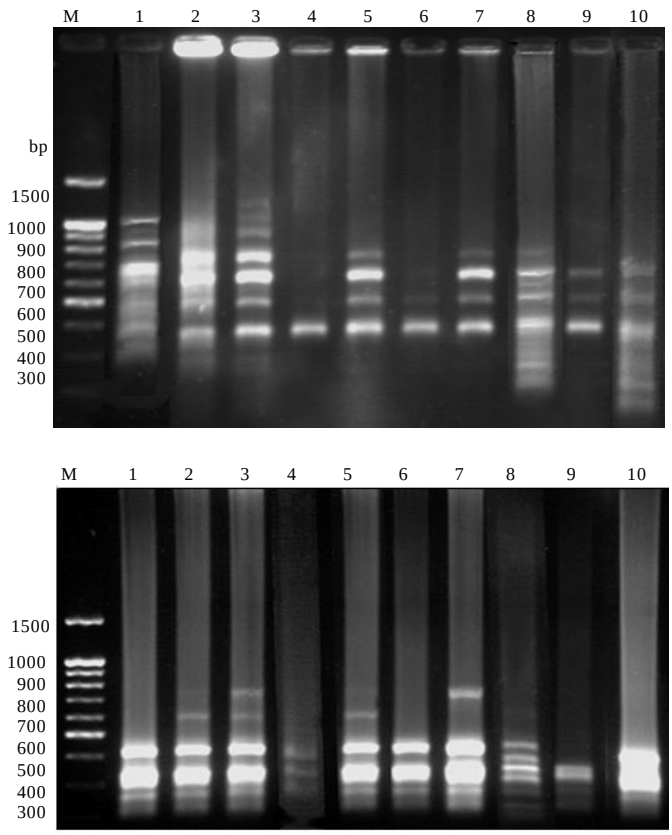

Fig. 2(a-b): ISSR-PCR pattern bands generated by primers (A) UBC-814 and (B) UBC-817, Lane M = 100 bp DNA ladder plus, lane 1-9 banana plants treated with different three concentrations from chemical mutagens compared with the control (Lane 10). Lane 1: 2 mg L^{-1} of 2,4-D, lane 2: 4 mg L^{-1} of 2,4-D, lane 3: 6 mg L^{-1} of 2,4-D, lane 4: 6 mg L^{-1} of 6-BA, lane 5: 7 mg L^{-1} of 6-BA, lane 6: 8 mg L^{-1} of 6-BA, lane 7: 1 mg L^{-1} of sodium azide, lane 8: 2 mg L^{-1} of sodium azide, lane 9: 3 mg L^{-1} of sodium azide

2 mg L^{-1} from 2,4-D. One amplified fragment with 380 bp appeared in two concentrations of 7 and 8 mg L^{-1} from BAP and control and disappeared in concentration of 6 mg L^{-1}. Two fragments with 1400 and 530 bp were induced in concentration of 7 mg L^{-1} from BAP and it was considered as a marker, whereas such fragment was not existed in the control. Two amplified fragments with 380 and 270 bp were shown in concentration 3 mg L^{-1} of sodium azide and the control and disappeared in the other two concentrations. One fragment with 1500 bp was uniquely in concentration 3 mg L^{-1} of sodium azide and it was considered as a marker.

Total number of 35 scorable amplified DNA fragment ranging from 3060 to 140 bp was observed using the three primers, whereas 29 fragments were polymorphic and the other amplified were commonly detected among banana plants treatment with three chemical mutagens (Table 5). The three primers UBC-814, UBC-817 and UBC-835 showed mean polymorphic percentage of 82.86%. The polymorphic percentage of primer UBC-835 recorded the highest percentage (85.71%), whereas primer UBC-814 displayed the lowest percentage (82.35%). Among the 29 polymorphic

Table 4: Analysis of variance for quantitative characters of Grand Nain cv. banana plants treated with deferent chemicals concentrations under Greenhouse

		Plantlets characters		
Treatments	Survival (%)	Plant height (cm)	Plant diameter (cm)	Leaf area (cm^2)
2,4-D mg L^{-1}				
2	78	18.00bc	4.167abc	68.27a
4	83	18.17bc	4.667abc	80.64ab
6	30.5	20.50b	4.333abc	76.40ab
Means		18.89b	4.389b	75.10b
6-Benzylaminopurine mg L^{-1}				
6	98	17.00bc	d4.667abc	86.93ab
7	6716.00bcd	5.00ab	86.83ab	
8	60	15.67bcd	3.333cde	78.40ab
Means		16.22b	4.333b	84.06ab
Sodium azide mg L^{-1}				
1	46	14.33cd	3.667bcd	84.37ab
2	28	12.67cd	2.667de	53.73ab
3	3	11.67d	2.333e	47.80b
Means		12.89c	2.889c	61.97b
Control	100	26.66a	5.160a	109.4a
Means		26.66a	5.160a	109.4a

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

bands, 13 bands were specific markers to the resistance for BBTV and BMV with a total average of 37.14%. Banana plants treatment with three chemical mutagens were varied considerably in their resistant makers using the three primers, whereas the concentration of 7 mg L^{-1} from BAP revealed the highest number with 5 markers thus the, followed by concentration of 2 mg L^{-1} from sodium azide at 4 markers, whenever the two concentrations of 2 mg L^{-1} from 2,4-D and 1 mg L^{-1} from sodium azide were equally in number the markers (Two) and the concentrations 4 and 6 mg L^{-1} from 2,4-D, 8 mg L^{-1} of BAP and 3 mg L^{-1} of sodium azide were also equal in markers number (one) (Table 5). These results are in agreement with 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 somaclonal variations 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 Polymerase Chain Reaction (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 (Khan *et al.*, 2009).

Field screening according to BBTV and BMV resistance: Banana plants were hardened in the green house and a total of 15 plants were remaining from treatment 2,4- D 6 mg L^{-1} were shifted in to the field for R generation. On maturity, 2 out of 15 selected resistant lines showed BMV resistance response. No selected lines were found resistance against BBTV tested individually

Table 5: ISSR amplified bands, polymorphic bands and markers for different chemicals concentrations treated using three primers in banana plantlets

	Polymorphism				2,4-D mg L^{-1}			6-Benzyl aminoadenine mg L^{-1}			Sodium azid mg L^{-1}		
Primer name	Total	P%	No. of markers with pb		2	4	6	6	7	8	1	2	3
UBC -814	17	14	5	830		+							
				720					+				
				640	+								
				360								+	
				350								+	
	82.35 %			29.41%	1	1	0	0	1	0	0	2	0
UBC -817	11	9	4	720			+				+		
				610					+			+	
				400								+	
				270					+	+	+		
	81.82 %			36.36 %	0	0	1	0	2	1	2	2	0
UBC -835	7	6	4	1500									+
				1400					+				
				530					+				
				220	+								
	85.7 1%			57.1 %	1	0	0	0	2	0	0	0	1
Total	35	29	13		2	1	1	0	5	1	2	4	1
Polymorphic	82.86 %		37.14 %										

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

by syringe method of inoculation. In term of percentage, 13.33% BMV resistant plants were obtained from field trials of R1 generation. These results are in agreement with Jones (2000) who mentioned the parthenocarpic nature of cultivated banana makes it difficult to breed for resistance to diseases. Diseases resistant hybrids have been developed by means of classical breeding and although good resistance has been obtained, most of these hybrids are not acceptable to local markets. Researchers have also tried to generate disease resistance in banana plants by using tissue culture based techniques such as *in vitro* mutagenesis.

CONCLUSION

Development of BBTV and BMV resistant banana crops are 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. Chemical mutagens 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. The Chemical mutagens, a potent mutagen in plants and create point mutation easily. The mutant plant species can be easily selected from wild plants by ISSR-PCR. This marker is reproducible and consistent as compared to morphological. The mutant plants produced by Chemical mutagens 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.

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