Analysis of induced mutants of salinity resistant banana (*Musa acuminata* cv. Dwarf Cavendish) using morphological and molecular markers

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Abstract
Genetic diversity amongst 21 induced mutant clones tolerant to salinity along with one non-irradiated sensitive clone of banana (*Musa acuminata* cv. Dwarf Cavendish (AAA)) were studied using morphological and random amplified polymorphic DNA (RAPD) markers. Out of the 30 phenotypic indices screened, 23 were polymorph and two traits, leaf habit and blotches color, were differentiated by non-irradiated clone. RAPDs established 106 major amplified products using 14 primers. Out of 106 markers, eight were monomorph, and the remaining (98) were polymorph. The extent of polymorphism indicated the existence of considerable variation DNA level within induced mutant clones. Primer OPA-02 revealed banding patterns specific to salinity resistant clones. Both morphological and RAPD analyses successfully detected genetic variation within induced mutant clones, RAPD also detected variation between the irradiated and non-irradiated clones, which were morphologically indistinguishable. Results were indicative that induced mutations bear a great potential in improving banana for salinity resistant.
Keywords: Genetic diversity; banana; mutation; salinity; morphology; RAPDs

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INTRODUCTON

Banana is an important fruit crop in the world in terms of production and consumption. Its production in Iran was confined to certain areas along southern regions, especially at Sistan and Baloochestan province. One of the most serious problems limiting the extension of banana plantation in Iran is the salinity of soil and water. Therefore, to extend banana cultivation it is important to improve salinity resistance in this crop (Miri *et al*., 2003).

The breeding of most banana cultivars is made difficult due to genetic sterility and triploidy, hence, producing hardly any seeds. In addition, it is further complicated by low seed germination of hybrid banana plants (Javed *et al*., 2004). Induced mutation has been utilized as a tool to generate variation and breeding in a number of vegetatively propagated crops such as banana (Hautea *et al*., 2004).

Genetic improvement of banana is to some degree limited by poor knowledge of genetic diversity. To make the collection useful for breeders, morphological and molecular characterization of the germplasms is necessary. Differentiation of genomes through morphological characteristics is unreliable. The disadvantages of phenotype-based assays can be overcome by direct identification of genotypes with DNA-based

markers (Nsabimana and Staden, 2007). Molecular markers have been employed in *Musa* genotypes to assess ploidy (Oselebe *et al*., 2006), phylogenetic relationships (Jain *et al*, 2007; Nsabimana and Staden, 2007; Uma *et al*., 2006) and genetic diversity due to somaclonal variation (Lakshmanan *et al*., 2007; Bairu *et al*., 2006; Ray *et al*., 2006) or mutation induction (Hautea *et al*., 2004; Finalet *et al*., 2000; Toruan-Mathius and Haris, 1999). Random amplified polymorphic DNA (RAPD) analysis is a polymerase chain reaction (PCR)-based technique, which uses random primers to generate DNA fragments. Polymorphism generated by RAPD analysis has been used for fingerprinting and classification of the *Musa* genotypes. Linkage of RAPD markers to specific traits such as disease resistance has been possible (Damasco *et al*., 1996). Because RAPD polymorphisms result from either a nucleotide base change that alters the primers binding site or from an insertion or deletion within the amplified region, polymorphisms usually result in the presence or absence of an amplification product from a single locus. The products of these amplifications can be polymorphic and are useful as genetic markers (Vidal and de Garcia, 2000).

The objective of this study was primarily to assess the level of genetic diversity in mutated plants and to compare RAPDs and morphological dendrogram schemes of the plants. This study also helps identification of RAPD markers that would differentiate salinity resistance from susceptible clones.

MARERIALS AND METHODS

Plant materials: Plant materials (listed in Table 1) were generated in Nuclear Research Center for Agriculture and Medicine, Atomic Energy Organization of Iran (AEOI), Karaj, Iran. This experiment containing 3350 irradiated tissue culture shoot tips of banana (*Musa acuminata* cv. Dwarf Cavendish (AAA)) irradiated with gamma ray doses of 0, 25, 35, and 45 Gy, were produced after three times of subcultures. Explants were cultured on MS medium (Murashige and Skoog, 1962) containing 2.5 mg/l BAP and NaCl in concentrations of 0, 6, 7, 8, 9 g/l for 60 days. Green buds were then transferred to a similar medium without salt. After 30 days, buds were transferred to a medium similar to the initial stage and incubated for 60 days. Thereafter, all viable buds were rooted and shifted to the pots. Acclimatized plants were irrigated weekly with the solutions containing 0, 6, 7, 8, 9 g/l NaCl for 2 months. Research was conducted in a factorial experiment as randomized complete block design (Data not presented). Finally, 22 greenhouse-grown plants including a non-irradiated clone sensitive to salinity as control (sample No. 1) and twenty one irradiated clones resistance to salinity (No. 2 to 22) were collected for morphological and RAPD analyses.

Table 1. Clones of banana used in the study.

Code no.	Treatment[a]	
	Irradiation (Gy)	Salinity[b] (g/l)
1	0	0
2	35	6
3	45	6
4	25	6
5	25	6
6	25	6
7	35	7
8	35	7
9	25	7
10	25	7
11	25	8
12	25	7
13	25	6
14	25	7
15	45	8
16	25	6
17	25	7
18	25	6
19	45	7
20	25	6
21	25	7
22	35	7

a: Shoot tips of banana were irradiated with gamma ray and then treated under salinity. b: Salt concentration that each clone was able to tolerate during three stages.

Morphological characteristics: Morphological characterization of greenhouse-grown plants was carried out using the banana descriptor (IPGRI-INIBAP/CIRAD, 1996). Each clone was characterized using 30 traits taken at the vegetative stage.

DNA extraction: Total DNA was isolated by a modified cetyl-trimethyl ammonium bromide (CTAB) extraction technique (IAEA, 2002). Approximately 0.2 g of fresh leaf tissue was ground in liquid nitrogen and added to 700 µl of extraction buffer (2% CTAB, 100

mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol, pH=8) and incubated at 60ºC for 30 min. The aqueous solution was extracted with 600 µl of chloroform:isoamyl alcohol (24:1) and centrifuged for 20 min at 10000 rpm. The extraction was repeated and 1 ml 96% cold ethanol was added to aqueous phase and left at -20ºC for 20 min to precipitate the nucleic acid. The samples were then centrifuged for 10 min at 10000 rpm. The precipitated nucleic acid was washed with one ml 70% ethanol, left to air dry, rehydrated in 100 µl TE buffer and digested with 1 µl RNase (10 mg/ml, Fermentas, Germany) for 45 min at 37ºC. The quality of genomic DNA was examined by agarose (1%) gel electrophoresis and quantified spectrophotometically at A_{260} and A_{280} nm. Each sample was diluted to 50 ng/µl in TE buffer and stored at -20ºC.

DNA amplification: The PCR reaction contained 50 ng of template DNA, 1 U *Taq* polymerase (Fermentas), 2.5 µl 10X PCR buffer, 0.2 mM dNTP, 4 mM $MgCl_2$ and sterile distilled water to 25 µl. Fourteen arbitrary decamer primers (Metabion, Germany) were used for the PCR. The reactions were incubated in a thermocycler (Biothermal, Germany) programmed for 3 min at 93ºC, followed by 25 cycles of 1 min denaturation at 93ºC, 40s annealing at 36ºC and 1 min extension at 72ºC. After the 25th cycle, the extension was continued for 4 min at 72ºC. The products were held at 4ºC until analyzed. Amplified samples were run on a 1% agarose gel stained with ethidium bromide at 100 V for up to 90 min with 1X TAE as running buffer and gels were visualized under UV light and photographed with Polaroid film. Duplicate reactions were performed to ensure reproducibility.

Data analysis: Amplification product profiles were scored for the presence (1) or absence (0) of bands. Molecular size of the amplified fragments was estimated using 100 bp DNA ladder (Fermentas). RAPD assays generating weak or ambiguous amplification products were repeated to confirm the consistency of these markers. The NTSYS-pc software was used to estimate genetic similarities with the Jaccard's similarity coefficient. The generated matrix of similarities was analyzed by the unweighted pair-group method with arithmetic average (UPGMA), using the sequential hierarchical agglomerative and nested clustering (SHAN) module. Morphological traits were also analyzed using the same software.

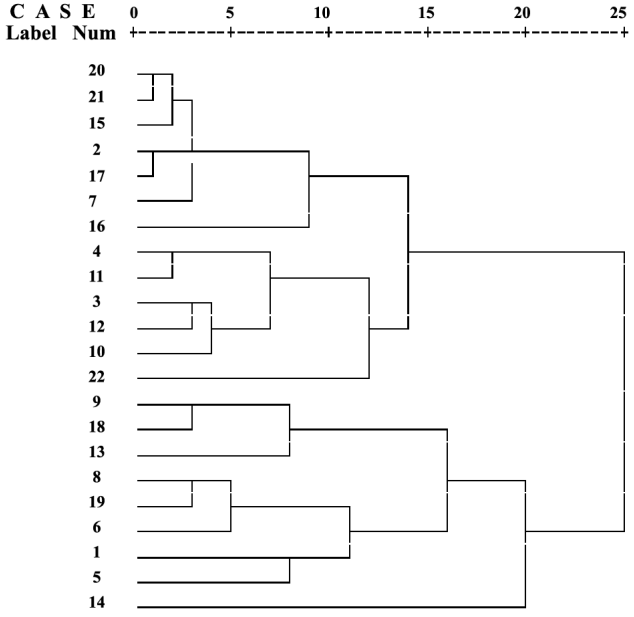

Figure 1. Dendrogram generated by UPGMA cluster analysis for 22 banana clones based on morphological analysis. Code numbers are as those listed in Table 1.

RESULTS

Morphological characterization of the 22 clones using 30 traits was carried out. Of these, 22 distinctive traits were considered for diversity evaluation, which exhibited a total of 69 classifications. Traits of drooping leaf habit and black-purple blotches were only characterized in non-irradiated clone, while the irradiated clones were shown other classifications (Table 2). The dendrogram and clustering pattern are observed in Figure 1. The UPGMA dendrogram based on morphological characterization indicated that all clones were clustered into two major groups: the first group included 13 irradiated clones and the second group contained other irradiated clones plus non-irradiated clone.

Amplification patterns are performed twice in order to evaluate if the RAPD patterns can be reproduced. The results indicate that reproducible RAPD patterns can be obtained under the same amplification conditions for two replicates.

The fourteen primers resulted in 106 scorable bands, ranging from 150-3100 bp in size. Of these, 98 (92.4%) bands were polymorphic (Table 3). The number of bands for each primer varied from 2 (OPH-07

Table 2. Morphological characterization and abundance of classifications among banana clones.

No.	Trait	Classification types	
		Non-irradiated	Irradiated
1	Leaf habit	Drooping	Erect (23.8)*, Intermediate (76.2)
2	Dwarfism	Normal	Normal (95.2), Dwarf type (4.8)
3	Pseudostem color	Red-purple	Green-yellow (4.8), Medium green (38.2), Green (9.5), Green-red (19.0), Red-purple (9.5), Chimerical (19.0)
4	Predominant underlying color of the pseudostem	Red-purple	Watery green (4.8), Light green (66.7), Green (4.8), Cream (4.8), Pink-purple (14.3), Red-purple (4.7)
5	Pigmentation of the underlying pseudostem	Purple	Pink-purple (33.3), Purple (4.8), Other (61.9)
6	Sap color	Milky	Watery (57.1), Milky (42.9)
7	Blotches at the petiol base	Extensive pigmentation	Sparse blotching (19), Small blotches (9.6), Large blotches (19.0), Extensive pigmentation (19.0), Without pigmentation (33.4)
8	Blotches color	Black-purple	Brown (28.6), Dark brown (33.3), Brown-black (4.8), Other (33.3)
9	Petiole canal leaf III	Open with margins spreading	Open with margins spreading (71.4), Wide with erect margins (28.6)
10	Petiole margins	Winged and undulating	Winged and undulating (33.3), Winged and not clasping the pseudostem (42.9), Winged and clasping the pseudostem (23.8)
11	Leaf ratio	2.1-2.3	≤ 2 (61.9), 2.1-2.3 (33.3), 2.4-2.6 (4.8)
12	Color of leaf upper surface	Dark green with red-purple	Medium green (4.8), Green (14.2), Dark green (4.8), Dark green with red-purple (76.2)
13	Color of leaf lower surface	Medium green	Green-yellow (66.6), Medium green (23.8), Green (4.8), Red-purple (4.8)
14	Appearance of leaf lower surface	Shiny	Dull (81.0), Shiny (19.0)
15	Wax on leaves	Very little or no visible sign of wax	Very little or no visible sign of wax (19.0), Few wax (81.0)
16	Insertion point of leaf blades on petiole	Symmetric	Symmetric (33.3), Asymmetric (66.7)
17	Shape of leaf blade base	Both sides pointed	Both sides rounded (47.6), One side rounded, one pointed (33.3), Both sides pointed (19.1)
18	Color of midrib dorsal surface	Yellow	Yellow (90.5), Light green (9.5)
19	Color of midrib ventral surface	Green	Yellow (9.5), Light green (57.1), Green (28.6), Pink-purple (4.8)
20	Pigmentation in the pseudostem	Brown/rusty brown	None (38.1), Brown/rusty brown (61.9)
21	Sap dripping on the cutting the leaf's petiole	No dripping	No dripping (42.9), Drips (57.1)
22	Leaf tips	Twisted	Not twisted (14.3), Twisted (85.7)

*Abundance of classification is mentioned in parenthesis in front of each class.

and OPJ-09) to 14 (OPA-04) with an average of 7.5 bands per primer. The average polymorphism exhibited by all 14 primers was 92.1% and the OPAA-14 was the only primer generating an amplification product in less than 70% of the accession tested. A 240 bp band derived from the primer OPA-02 was observed to be present only in the resistant clones (Fig. 2).

To estimate the similarities among clones, the simple matching coefficient provided similarity values ranging from 0.30 to 0.79. According to the UPGMA dendrogram, the twenty-two clones were clustered into two major clusters: the irradiated clones were grouped together and separate from the non-irradiated clone (Fig. 3). Clones No. 20 and 21 at a similarity coefficient of 0.79 were close to each other. Minimum similarity coefficient (0.30) was observed between clones No. 1 and 17.

DISCUSSION

This study was conducted to determine the extent of genetic diversity among irradiated (resistance to salinity) and non-irradiated (sensitive to salinity) 'dwarf

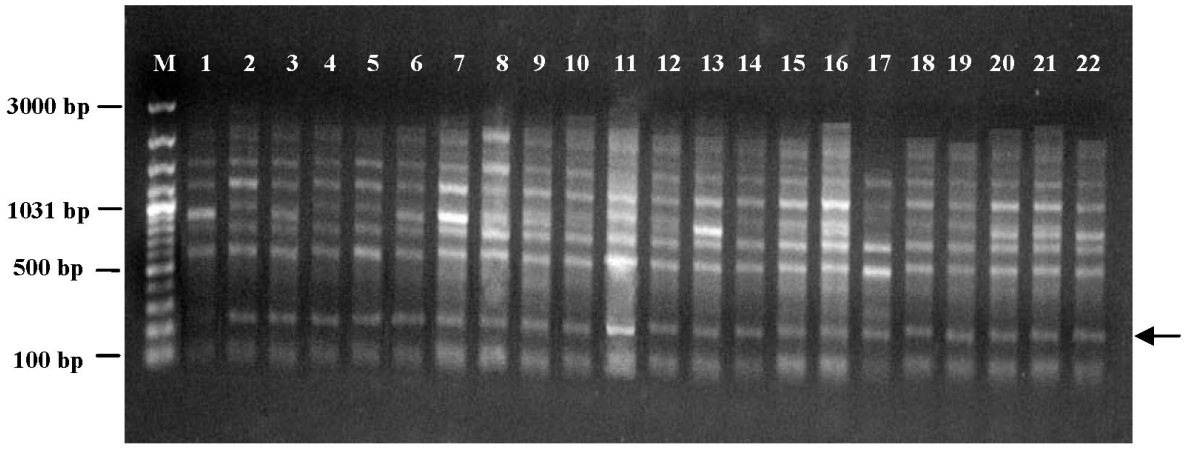

Figure 2. Banding pattern of banana clones with primer OPA-02. Ten major bands ranging from 150 to 2500 bp were observed. A major band of 240 bp was observed in all resistant clones but absent in sensitive clone. Lane 1 is non-irradiated clone sensitive to salinity and lanes 2-22 are irradiated ones resistant to salinity as those listed in Table 1. M, 100 bp DNA ladder (Fermentas). Arrow indicates the OPA-02_{240} band.

Cavendish' banana based on the morphological and RAPD systems.

In plant breeding, genetic variation is essential for the creation of plants with superior traits. Conventional banana breeding is complicated by low seed fertility, triploidy, slow and vegetative propagation, time (2 years from seed to seed) and space (6 m^2/plant) requirements. Consequently, alternative approaches such as mutation have also been considered (Okole *et al*., 2000; Vuylsteke, 2000). The mutation is a powerful technique, which may well produce desired variation in well established clones (Hautea *et al*., 2004). The frequency of polymorphism obtained by RAPD technique was 98 markers/14 selected primers. This is much higher than the ratio obtained in banana by Javed *et al*. (2004) of 84/15 and Muhammad and Othman (2005) of 44/15. This high polymorphism indicates that there is considerable variation at the DNA level within radiation-induced mutants. Furthermore, the data obtained from both

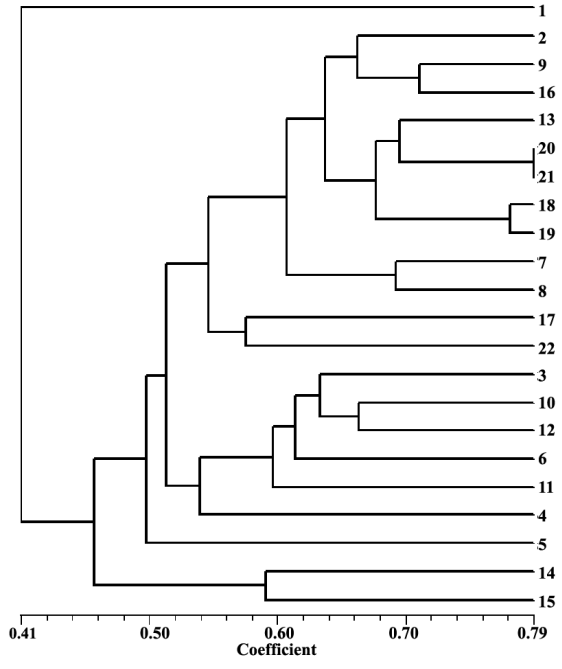

Fig 3. Dendrogram generated by UPGMA cluster analysis from the similarity matrix obtained by Jaccard's genetic distance for 22 banana clones based on RAPD markers. Code numbers are as those listed in Table 1.

Table 3. Primer information and the results of genome analysis of 22 banana clones by RAPD markers.

No.	Primer code	Sequence (5 → 3)	Total bands	Polymorphic bands	Polymorphism (%)
1	OPAA-14	AACGGGCCAA	3	2	66.7
2	OPA-01	CAGGCCCTTC	11	10	90.9
3	OPA-02	TGCCGAGCTG	10	9	90
4	OPA-03	AGTCAGCCAC	10	9	90
5	OPA-04	AATCGGGCTG	14	14	100
6	OPA-05	AGGGGTCTTG	4	4	100
7	OPA-08	GTGACGTAGG	10	8	80
8	OPA-09	GGGTAACGCC	9	8	88.8
9	OPA-10	GTGATCGCAG	12	10	83.3
10	OPA-11	CAATCGCCGT	4	4	100
11	OPA-13	CAGCACCCAC	10	10	100
12	OPD-07	TTGGCACGGG	6	6	100
13	OPH-07	CTGCATCGTG	2	2	100
14	OPJ-09	TGAGCCTCAC	2	2	100

morphological and molecular analyses indicate related to genome alterations in these accessions. Our study supports the earlier reports that mutation causes genetic variation in banana (Chun Hai *et al*., 2000; Toruan-Mathius and Haris, 1999; Domingues *et al*., 1994).

All irradiated tolerant clones were clustered separately from non-irradiated sensitive clone based on RAPD analyses. This result is in agreement with Gomes *et al*. (2005) who reported that the varieties of higher tolerance to saline stress were genetically distant compared to most of the salt sensitive ones.

Morphology markers have been used extremely to determine the relationships among banana populations (Uma *et al*., 2006; Noyer *et al*., 2005; Hautea *et al*., 2004; Pillay *et al*., 2001, 2000; Crouch *et al*., 2000, Newbury *et al*., 2000; Sanchez *et al*., 2000; Howell *et al*., 1994). Based on the results of UPGMA cluster analysis, non-irradiated and irradiated clones share a large number of morphological characterizations and cannot be distinguished on the basis of morphology (Fig. 1). Leaf habit and blotches color were the only traits that could be used to differentiate between mutants and wild type (Table 2). Nevertheless, tracking the mutants even using these two traits was near to impossible. Although induced mutants are usually differentiated from the original plant by phenotypic analysis, but it can be severely limited by the large size of mutagenized populations, particularly in the case of banana, and developmental and environmental effects. The application of molecular markers may overcome this limitation (Hautea *et al*., 2004). DNA amplification of polymorphic markers (presence and absence of bands) could be the result of base deletion or insertions at priming sites (Muhammad and Othman, 2005). In this study, RAPDs were chosen because it amplify different region of the genome allowing better analysis of genetic stability/variation of clones, as well as their simplicity and cost-effectiveness (Ray *et al*., 2006). Similar to earlier studies that were implemented RAPD in analyzing irradiated banana genotypes (Hautea *et al*, 2004; Finalet *et al*, 2000; Toruan-Mathius and Haris, 1999), we were able to efficiently examine and differentiate genotypic changes in gamma irradiated banana clones.

Primer OPA-02_{240} was only observed to be present in the resistant clones and it can be used as a salinity resistance marker. Damasco *et al*. (1996) and Bairu *et al*. (2006) successfully demonstrated the use of RAPD markers and detected a marker linked with dwarfness in bananas. Vidal and de Garcia (2000) and Javed *et al*. (2004) have also detected similar results with a resistant marker to yellow Sigatoka and Fusarium wilt diseases in bananas, respectively.

CONCLUSION

Our results suggest that RAPD could be considered as an alternative molecular marker tool for rapid and inexpensive evaluation of genetic variability in the bananas obtained by mutation. Sequencing of the marker band present in tolerant clones determines its base composition and it can be used to study the molecular basis and mechanisms that might be related to the resistance to salinity, which may be beneficial to the crop improvement.

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