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Genetic Diversity in Haploid *Nicotiana alata* Induced by Gamma Irradiation, Salt Tolerance and Detection of These Differences by RAPD

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Abstract

Haploid plants of *Nicotiana alata* were cultured *in vitro* on MS medium with IAA + KIN. The resulting plantlets were irradiated using gamma radiation doses of 10, 15, 20 and 25 Gy. Single node pieces were cut and transferred onto fresh MS medium. Gamma radiation doses caused the death of 9% and up to 28% of explants. NaCl concentrations caused the death of 8% up to 36% of explants, while the combined effect between gamma radiation doses and salinity had an impact suffused on the percentage of survival. The combined effect of gamma radiation doses 20 Gy and 25 Gy on NaCl concentrations of 100, 150 and 200 mM were deadly. Even more, the combined effect of gamma radiation doses and salinity had a severe negative impact on both the proline content and total soluble protein. Random amplified polymorphic DNA (RAPD) analysis was used to determine the degree of genetic variation in treated haploid *Nicotiana alata* plants. Total genomic DNAs from different haploid plantlets treated were amplified using five arbitrary primers. Two hundred and seventy bands were detected from plantlets irradiated with doses of 15, 20 and 25 Gy, with polymorphic band number 226 (83.7%). The total number of bands resulted from plant grew on 150 mM and 200 mM NaCl were 260 bands with polymorphic bands 185 (85.6%). However, the total number of bands produced from combined effects between gamma rays and salinity (20 Gy X 50 mM NaCl, 20 Gy X 100 mM NaCl and 25 Gy X 50 mM NaCl) were 270, with polymorphic band number 231 (85.5%). High similarity between treatments was revealed. Treatments relationships were estimated through cluster analysis (UPGMA) based on RAPD data.

Keywords: microcutting, *Nicotiana alata*, radiation, RAPD, salinity, stress

Introduction

Nicotiana genus is one of the five major genera of the *Solanacae* family. *Nicotiana* sp. has been cultivated for thousands of years and served as a medicinal herb, trade commodity and crop plant by different cultivars. Nowadays, it became one of the most important commercial crops in the world. Within the past several decades, it was found yet another use of the genus, serving as a widely utilized model system in plant cell culture and genetic engineering research (Zhang *et al.,* 2007). Because of its economic importance and the value as biological research tool, numerous investigations have been undertaken to examine its evolutionary origin and genome structure and organization.

Salinity in soil or water is one of the major abiotic stresses that reduce plant growth and crop productivity worldwide. More than 800 million hectares of land throughout the world are salt-affected (including both saline and sodic soils), equating to more than 6% of the world's total land area (FAO, 2008). Some of the most serious examples of salinity occur in the arid and semiarid regions. For example, in Iran, Pakistan, Egypt and Argentina, out of the total land area of 162.2, 77.1, 99.5 and 237.7 million hectares, about 23.8, 10, 8.7 and 33.1 million hectares are salt-affected, respectively (FAO, 2008). Low rainfall, high evaporation, native rocks, saline irrigation water and poor water management increasingly cause salinity problems in agricultural areas. It is estimated that of the current 230 million hectares of land under irrigation, 45 million hectares are salt-affected (20%) and of the 1,500 million hectares of dry land agriculture, 32 million hectares (2%) are salt-affected (FAO, 2008). Overall, it was estimated that the world is losing at least 3 ha of arable land every minute because of soil salinity (FAO, 2008).

Salinity is one of the most severe environmental factors limiting the productivity of agricultural crops. Most crops are sensitive to salinity caused by high concentrations of salts in the soil. The cost of salinity to agriculture is estimated to high values and is expected to increase as soils are further affected (Ghassemi *et al.*, 1995). In addition, to this enormous financial cost of production, there are other serious impacts of salinity on infrastructure, water supplies and on social structure and stability of communities.

Responses to salinization have been of two general kinds; engineering the environment to manage increased salt in the soil by irrigation and drainage management, or by "engineering" the plants to increase their salt tolerance. Salt tolerant plants may also ameliorate the environment by lowering the water table in salt affected soils.

Gamma rays belong to ionizing radiation and interact to atoms or molecules to produce free radicals inside treated cells.

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These radicals can damage or modify important components of plant cells and have been reported to affect differentially the morphology, anatomy, biochemistry and physiology of plants, depending on the irradiation level. These effects include changes in the plant cellular structure and metabolism, e.g. dilation of thylakoid membranes, alteration in photosynthesis, modulation of the antioxidative system and accumulation of phenolic compounds (Kovacs and Keresztes, 2002; Kim *et al*., 2004; Wi *et al*., 2005).

Molecular genetic markers have become useful tools in providing a relatively unbiased estimation of genetic diversity and phylogeny in plants (Clegg, 1990). Several different PCR techniques for DNA fingerprinting have been developed during the last decades, each one with specific advantages and disadvantages. Random amplified polymorphic DNA (RAPD) is the simplest and fastest of DNA-based techniques in genetic similarity studies (Gwanama *et al*., 2002). A number of scientists have used RAPD markers to study polymorphism in various plants (Ortiz *et al*., 1997; Ranade *et al*., 2002; Rout and Das, 2002; Samal *et al*., 2003). In tobacco, RAPD has been used mainly to identify markers linked to genes for resistance to pathogens (Bai *et al*., 1995; Rufty, *et al*., 1997; Yi *et al*., 1998).

The aim of the present study was to investigate the induction of genetic variability using gamma radiation and selection for salt tolerance based on the similarity of the RAPD technique data, undertaken to distinguish *Nicotiana alata* diploid and haploid plants treated with gamma radiation and/ or salinity and to detect genetic diversity among them.

Materials and Methods

Nicotiana alata haploid plants obtained from anther culture (El-Fiki *et al*., 2015) were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 0.2 mgl^{-1} IAA + 0.5 mgl^{-1} KIN. Micropropagation began after 4-5 weeks, when the plantlets reached 5 cm height. The culture was maintained by cutting into single nodes. The pH of the culture medium was adjusted to 5.7 before autoclaving and incubated inside a growth chamber at 25 ± 2 °C under photoperiod of 16 h.

Irradiation was carried out with a ^{60}Co source at National Centre for Radiation Research and Technology, Atomic Energy Authority, Cairo, Egypt. Mass cultures of *in vitro* grown plantlets derived from single nodes were irradiated with different doses of gamma rays (10, 15, 20 and 25 Gy). Thus, 800 *in vitro* grown plantlets were irradiated with gamma rays at each dose.

For the selection of salt tolerance after irradiation, the single node pieces were transferred onto MS medium supplemented with 50, 100, 150 and 200 mM NaCl. The survival of single node cuttings was recorded after 40 days of culture.

The proline content

Proline content was estimated according to Batels *et al*. (1973) for plantlets grown on saline medium, irradiated plantlets and the plantlets grown under combined effects between gamma radiation and salinity.

The total soluble protein

The plantlets grown on saline medium, irradiated plantlets and the plantlets grown under combined effects between gamma radiation and salinity were estimated according to Bradford (1976).

Extraction of genomic DNA

Thirty days old plantlets of each diploid and haploid plants were collected, bulked and frozen in liquid nitrogen. Plantlets were ground to fine powder and then bulked DNA extraction (QIAGEN) was under taken and quantified on 0.8% agarose gel.

RAPD amplification

Five different primers were chosen arbitrarily. The primers used in the current experiment were 10-mer synthesized by Operon biotechnologies (Inc. Germany). Primers sequences (5' – 3') were as follows: Op-C13: AAGCCTCGTC, Op-Do7: GGACCCAACC, Op-I15: AAGAGAGGGG, Op-L12: GGGCGGTACT, Op-M20: GTTGGTGGCT. Amplification reactions were performed in a 25 µl volume, containing: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM each of dNTPs, 1 µM primer, 30 ng of genomic DNA, 1.5 U of *Taq* DNA polymerase. The reaction mixture was overlaid with two drops of mineral oil, incubated for 5 min at 95 °C for initial denaturation, and then amplified for 35 cycles consisting of 30 s at 94 °C, 30 s at 36 °C and 60 s at 72 °C, followed by 7 min incubation at 72 °C. Amplification products were separated by gel electrophoresis on precast 0.8% agarose and visualized under UV illumination after staining with ethidiam bromide and photographed.

Data analysis

The size of RAPD fragments were estimated by comparison with the marker. RAPD fingerprints were recorded in the binary form (1 = presence of a band and 0 = absence of a band). All data were scored twice by two independent scorings. A simple matching coefficient was calculated to construct a similarity matrix and the UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram by using NTSYS-pc package (Rohlf, 1990).

Results and Discussion

Data in Table 1 summarizes the effect of gamma radiation, NaCl concentrations and the combined effect between them on the percentage of microcuttings survival and shoot length.

Gamma irradiation doses of 10, 15, 20 and 25 Gy caused a decrease in the survival percent of micropropagated buds to 87, 81, 74 and 68% respectively. The irradiation doses had a negative impact on shoot length as shown in Fig. 1, where the shoot length decreased
with increasing gamma radiation dose (5.1, 4.5, 3.9 and 3.2 cm respectively). The results showed by the radiation sensitivity test, based on survival percentage of irradiated and non-irradiated plantlets, showed a significant reduction in survival percentage as observed with increasing gamma dosage. These results were in accordance with radiation sensitivity test done by Hasegawa *et al*. (1995) for tobacco, El-Fiki (1997) for potato, El-Fiki *et al*. (2005a and b) for alfalfa, Norfadzrin *et al*. (2007) for tomato and okra and Kiong *et al*. (2008) for *Orthosiphon stamineus*.

Table 1. Effect of gamma irradiation and salinity concentration on *Nicotiana alata* buds survival

NaCl conc/mM	Radiation dose/Gy.									
	0		10		15		20		25	
	Bud survival (%)	Shoot length (cm)	Bud survival (%)	Shoot length (cm)	Bud survival (%)	Shoot length (cm)	Bud survival (%)	Shoot length (cm)	Bud survival (%)	Shoot length (cm)
0.0	96	10.2	87	5.1	81	4.5	74	3.9	68	3.2
50	88	6.6	80	5.1	64	4.7	52	3.5	36	2.0
100	76	5.3	72	4.3	56	3.8	44	2.6	0	0
150	68	4.6	60	3.8	48	3.1	0	0	0	0
200	60	3.9	48	3.2	40	2.8	0	0	0	0
LSD (5%)		0.76		0.39		0.62		0.34		0.11

Fig. 1. Effect of gamma radiation doses on *Nicotiana alata* haploid plants survival in *Nicotiana alata*

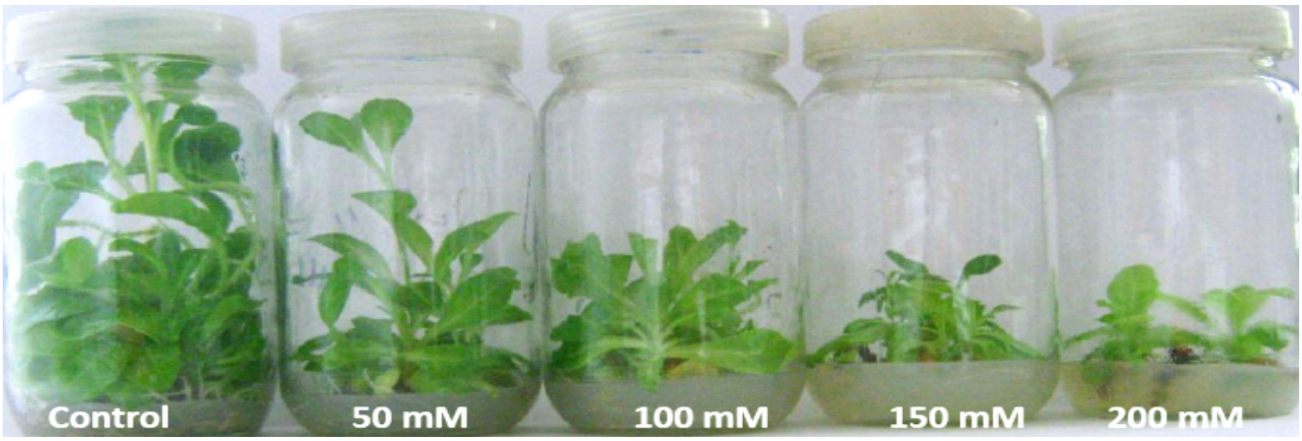

Fig. 2. Effect of NaCl concentrations on *Nicotiana alata* haploid plants survival in *Nicotiana alata*

The survival percent of single node buds grown on a medium containing different concentrations of NaCl (50, 100, 150 and 200 mM) decreased to 88, 76, 68 and 60% respectively. As shown in Fig. 2, the NaCl caused decreasing in shoot length with increasing NaCl concentrations (6.6, 5.3, 4.6 and 3.9 cm respectively). The results showed by the salinity sensitivity test on survival percentage of plantlets grown on saline medium and control ones emphasize a significant reduction in survival percentage as observed with increasing NaCl concentration.

Environmental abiotic stresses, such as drought, extreme temperatures, or high salinity, severely compromise plant growth and development. Water availability is one of the major factors affecting crop-yield worldwide. Many irrigated areas are poorly drained so that additionally, serious problems of waterlogging, alkalinization and soil salinity are frequent. Research on the two major abiotic stresses, drought and salinity, have much in common. Salinity reduces the ability of plants to take up water, which quickly causes a reductions in growth rate along with metabolic changes identical to those caused by water stress. Unfortunately, the complexity and polygenic nature of drought and salt stress tolerance make it difficult to select these characters in conventional breeding programs. Abiotic stresses induce morphological, biochemical and physiological changes in plants during the acquisition of stress tolerance. At the cellular level, water deficit may cause cellular damage or initiate adaptive responses (Cellier *et al*., 1998). The products of stress-inducible genes can be classified into two groups (Bray, 1997; Hasegawa *et al*., 2000): (i) genes that directly protect against stress, (ii) genes that regulate gene expression and signal transduction in the stress response. A large number of genes have been linked to stress response pathways although their precise functions often remain unclear (Zhu, 2000). Many salt-responsive genes do not increase tolerance, but induce stress damage and genes important for salt tolerance may not be expressed during salt stress. However,

the genomic drought and salt stress responses both reflect the necessity for cellular protection by free-radical scavengers, chaperonins and regulators of redox and osmotic potential (Hasegawa *et al*., 2000).

The combined effect of different gamma radiation doses and NaCl concentrations on the percentage of microcutting survival and shoot length were decreased within all combinations. As shown in Table 1 the gamma radiation dose of 20 Gy had a fatal effect on survival of buds grown on medium containing 150 and 200 mM NaCl. The effect of gamma radiation dose 25 Gy caused mortality on microcutting survival buds grown on medium containing 100, 150 and 200 mM NaCl.

Low and high temperatures, salinity and water availability severely reduced grain yields in agricultural systems (Grant *et al*., 1989). Stress tolerance in plants is a complex trait and direct selection for grain yield under stress conditions has been hampered by low heritability, polygenic control, epistasis and high genotype-by-environment (G x E) interactions. Early plant responses to environmental stresses include gens involved in perception of the environmental change and signal transduction to initiate biochemical and physiological responses, together with expression of the genes responsible for these responses. These results could be explained by the existence of a high interaction between the genotype and environmental conditions.

Radiation treatment increased the variability in the genetic background of the variety rather than changing the gene expression. The variability in the presence of NaCl may be due to the interaction between the mutagen and the selection medium.

Proline content

Data in Table 2 illustrated that the *Nicotiana alata* haploid plants have been influenced by exposure to different doses of gamma rays, as proline content increased with increasing gamma radiation dose. As well, proline content increased with increasing NaCl concentration. However, the combined effect between gamma radiation doses and NaCl concentrations on proline content had a negative impact.

Total soluble protein

Total soluble protein was estimated in *Nicotiana alata* haploid plant as shown in Table 3. The irradiated plantlets with gamma radiation doses had the total soluble protein increased with increasing gamma radiation dose. As well, plants that have been exposed to different concentrations of salinity were found to have increased total soluble protein content with increasing NaCl concentration. However, the combined effect of gamma radiation doses and salinity had a negative impact on plants' total soluble protein.

The physiological and biochemical changes in plant tissue in response to different types of osmotic stresses are not completely understood. Stress effects on plant cells and tissues were investigated and the stress inducing compounds used in different experiments were both ionic and penetrating (e.g. NaCl), non-ionic and penetrating (e.g. mannitol, sorbitol etc.) or non-ionic and non-penetrating (e.g. polyethylene glycol). Results of such experiments, however, have shown the following general trends in the plant tissues: (1) retardation of growth (Binzel *et al*., 1985; Rains, 1989; Kumar and Sharma, 1989; Thomas *et al*., 1992); (2) acquisition of the ability to adapt to stressful environments (Lerner, 1985; Binzel *et al*., 1985; Handa *et al*., 1986; Fallon and Phillips, 1989); and (3) accumulation of proline at a high level (Yancey *et al*., 1982; Watad *et al*., 1983; Rudulier *et al*., 1984; Chandler and Thorpe, 1987; Paek *et al*., 1988; Kumar and Sharma, 1989; Jain *et al*., 1991b; Thomas *et al*., 1992; Verbruggen *et al*., 1993). Although investigated by many researchers, proline status of plant organs and cell cultures still continues to be an active area of research in stress physiology (Jain *et al*., 1991a). Effects of different osmotic, each with different physico-chemical properties, are yet to be critically addressed or compared. These compounds when treated in high concentrations produce shock-effects on tissues and are responsible for tissue damage, either permanent or temporary (Leone *et al*., 1994), depending on the tissues, adapted or unadapted. Damage may be due to leakage of osmotically active substances or due to loss of membrane functionality (Harrington and Alm, 1988). Proline, an osmotically active substance (Rains, 1989), may also be released from the cells due to shock. In the shock-treated tissues however, proline may be retained in certain cases and protect the cell viability (Fallon and Phillips, 1989). Implications related to proline production and its retention may be a point of physiological significance in experiments with stress-shocks. The most crucial function of irradiated plant cell is to respond to gamma stress by developing defines mechanisms. This defines may be affected by alteration in the pattern of gene expression (Corthals *et al*., 2000), which may led to modulation of certain metabolic and defensive pathways (Zolla *et al*., 2003). Owing to gene expression altered under gamma stress, qualitative and quantitative changes in total soluble protein contents were obvious in the study of Corthals *et al*. (2000). These proteins play an important role in signal transduction, antioxidative defines, antifreezing, heat shock, metal binding, antipathogenesis or osmolyte synthesis, which are essential to a plant's function and growth (Gygi *et al*., 1999; El-Fiki *et al*., 2003; El-Fiki *et al*., 2004).

Total genomic DNA from ten *Nicotiana alata* treatments were used as templates within RAPD finger printing. Five random decamer primers were used in the current study. The number of fragments amplified by each primer ranged between

Table 2. The combined effects of gamma radiation doses and NaCl concentrations on proline contents (mg/100 gm fresh wt.) in haploid *Nicotiana alata* plants

NaCl	Radiation dose/Gy				
conc/mM	0	10	15	20	25
0.0	0.18	1.09	1.13	1.23	1.25
50	1.55	0.55	0.89	0.99	1.05
100	1.61	0.71	0.95	1.06	0
150	1.71	0.77	0.99	0	0
200	1.88	0.83	1.08	0	0

Table 3. The combined effects of gamma radiation doses and NaCl concentrations on total soluble protein (mg/100 g fresh wt) in haploid *Nicotiana alata* plants

NaCl	Radiation dose/Gy				
conc/mM	0	10	15	20	25
0.0	0.98	1.72	1.98	2.16	2.95
50	1.13	1.81	0.89	1.92	1.14
100	1.65	1.99	0.77	1.68	0.0
150	2.11	2.17	0.62	0.0	0.0
200	2.24	2.12	0.56	0.0	0.0

1-13, with an average of 8.9 fragments per primer. Maximum numbers of bands (13) was produced by the primer OP-L12 (Fig. 3. D), whereas the minimum number of bands (1) was produced by the primer OP-I15 (Fig. 3. C). A representative RAPD profile was obtained with primers OP-I15 and OP-M20 (Fig. 3. C and E). The primer OP-L12 produced the maximum number of bands (13) in the salinity test and within the combined effect of salinity and gamma radiation; even more, among radiation treatments the same primer produced the maximum number of bands (12). The minimum number of bands was observed with primer OP-I15 (3, 3 and 1) in the irradiated, salt stress and combined effect of gamma radiation and salt stress plants respectively.

All tested primers were polymorphic. A total of 270 bands were amplified of which 226 (83.7 %) were polymorphic across the radiation treatments. The level of polymorphism among the salinity stress was 85.6%, whereas, the polymorphism among the combined effects of gamma radiation and salinity was 85.5 % (Table 4).

Treatments Specific Markers

All tested primers except OP-I12 gave specific markers. A total of 11 treatments specific markers were generated. Radiation and salinity gave 4 specific markers each. However, 3 specific markers in the study of the combined effect between gamma irradiation and salinity were generated (Table 5). Among the 10 treatments were clearly differentiated by producing one or two specific bands with all the five primers used.

Genetic relationships in treatments

Common bands were scored as present, ambiguous, or absent and the data were used to calculate values of genetic distance between all the studied treatments. The results are given

Table 4. RAPD patterns generated for haploid *Nicotiana alata* under different treatments

Primer No.	Treatments					
	Radiation		Salinity		Salinity x Radiation	
	Total No. band	No. polymorphic band	Total No. band	No. polymorphic band	Total No. band	No. polymorphic band
OP-C13	60	49(81.7%)	48	40(83.3%)	60	53(88.3%)
OP-D07	55	51(92.77%)	44	41(93.2%)	55	50(90.9%)
OP-I15	30	24(80%)	24	20(83.3%)	30	16(53.3%)
OP-L12	65	57(88.77%)	52	47(90.4%)	65	62(95.4%)
OP-M20	60	45(75%)	48	37(77.1%)	60	50(83.3%)
Total	270	226(83.7%)	216	185(85.6%)	270	231(85.51%)

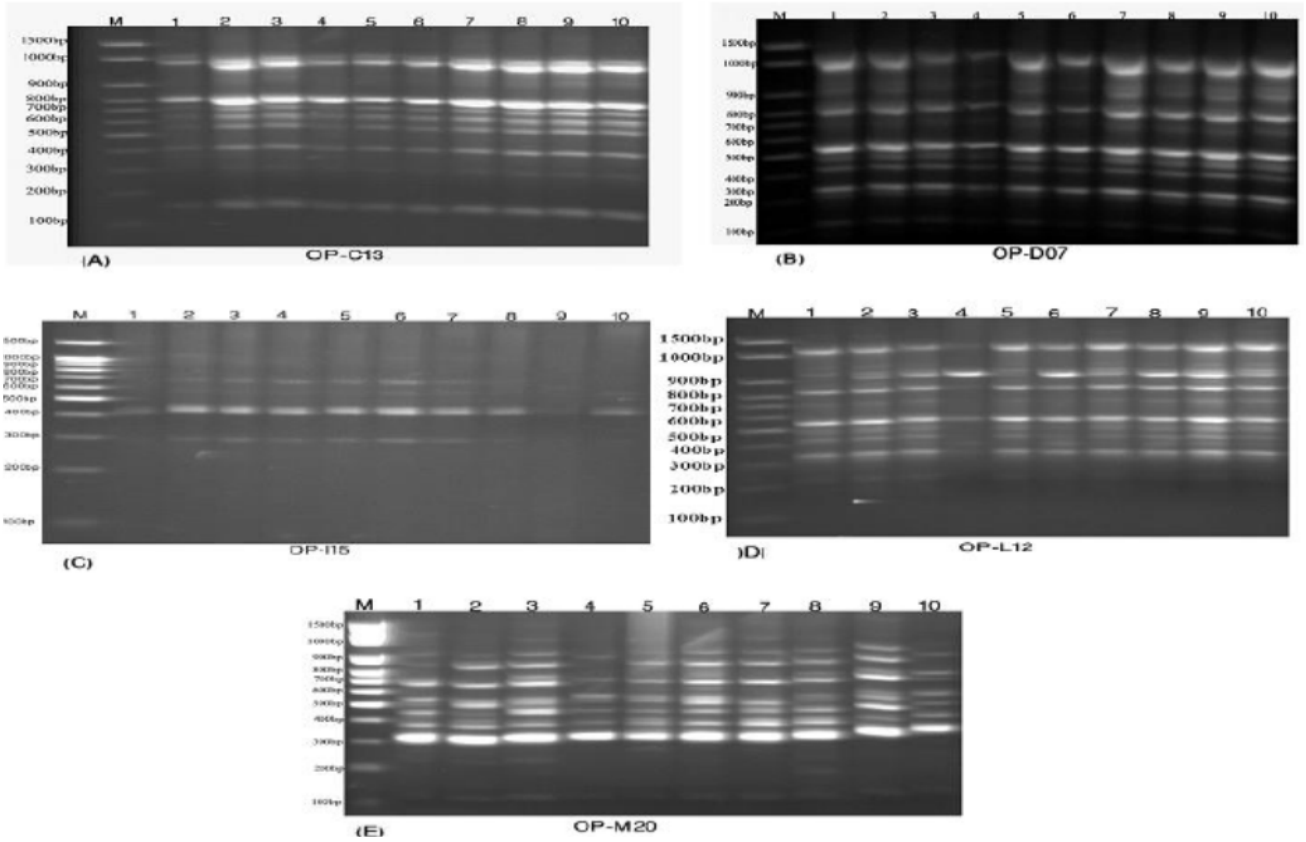

Fig. 3. Representative RAPD profile of treated haploid *Nicotiana alata*. Lane 1 Cont. diploid; Lane 2 Cont. haploid; Lane 3 Irradiated haploid 15 Gy; Lane 4 Irradiated haploid 20 Gy; Lane 5 Irradiated haploid 25 Gy; Lane 6 haploid Grew on 150 mM; Lane 7 haploid grew on 200 mM NaCl; Lane 8 haploid 20 Gy X 50 mM NaCl; Lane 9 haploid 20 Gy X 100 mM NaCl; Lane 10 haploid 25 Gy 50 X 50 mM NaCl.

Table 5. Used primers and the specific markers generated for salinity, radiation and combined treatments

Primer no.	Specific marker
OP-C13	1140 bp-Radiation, 1120bp-salinity, 1140bp-combind effect
OP-D07	980bp-salinity
OP-I15	1120bp-Radiation, 1120bp, 680bp-combind effect
OP-L12	None
OP-M20	800,290bp-Radiation, 800bp, 525bp-salinity

in Table (4). The genetic distance scale run from 0 (identical) to 100 (different for all criteria studied hereby).The similarity index as shown in Table (6) revealed the maximum similarity of diploid with irradiated haploid with dose 20 Gy (similarity indices 1.0) while distantly related treatments were (20Gy×50 mM NaCl) with (20Gy×100 mM NaCl) (similarity indices 0.0). The relationships within radiation treatments were 54%, however these relationships within salinity treatments were 49%, while in the combined effects between salinity and gamma radiation were 17%.

RAPD based genetic relationships

All treatments of haploid *Nicotiana alata* have been separated into three main clusters; it was revealed that one cluster consisted of combined effects between salinity and gamma radiation and salinity treatments, the second one grouped gamma radiation treatments, with diploid and haploid plants, and the third one consisted of irradiated plants with 20 Gy and the haploid plants grown on 200 mM NaCl. The treatments and the similarity between them in the first cluster were (0.11), in the second cluster were (0.56) and in third cluster were (0.57) (Fig. 4).

The arbitrary nucleotide sequence, RAPD finger printing, is a frequently used technique for investigating genetic polymorphisms (Versalovic *et al*., 1994; Teaumroong and Boonkerd, 1998). RAPD markers have been used for numerous applications in plant molecular genetics research, despite having specific disadvantages of poor reproducibility and not generally being associated with gene regions (Welsh and McClelland, 1990; Williams *et al*., 1990). RAPD, being a multi locus marker (Karp *et al*., 1997) with the simplest and fastest detection technology, have been successfully employed for determination of intra species genetic diversity. Characterization and quantification of genetic diversity has long been a major goal in plant breeding as information on the genetic diversity within and among closely related crop species is essential for a rational use of genetic resources. The results showed that RAPD assay discriminated those flue-cure tobacco cultivars with similar genotypes. It seems that RAPD is an effective tool for flue-cured tobacco germplasm management, cultivar protection and cultivar improvement. The fragment polymorphism was higher than the one reported in the case of eggplant (10.28 bands per primer), which also belongs to the *Solanaeceae* family (Singh *et al*., 2005). The number of fragments amplified in *N. tabacum* was significantly higher than that reported earlier by Del Piano *et al*. (2000). A high degree of genetic polymorphism among 7 species of *Nicotiana* was also reported using AFLP (Ren and Timko, 2002). On the contrast, genetic diversity analysis by RAPD markers revealed 79% polymorphism among the species of *L. peruvianum* and very low (9%) in the species *L. peruvianum* of the genus *Lycopersicon* (Kochieva *et al*., 2002). Species specific RAPD patterns have been developed and used to confirm hybridity in potato (Takemori *et al*., 1994) and intergeneric

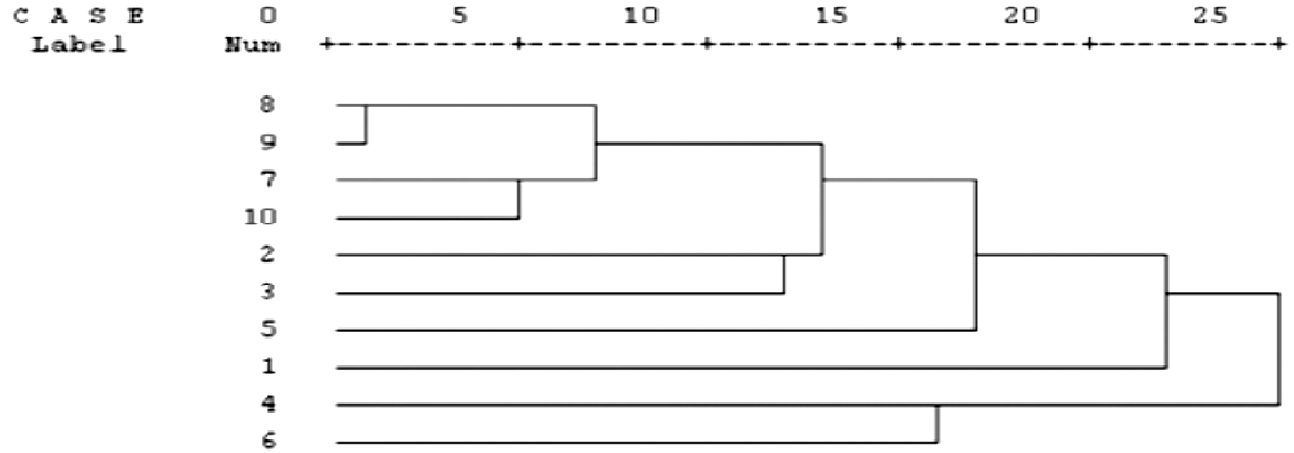

Fig. 4. A dendogram showing the genetic distance among ten treatments using RAPD data

Table 6. Similarity index (pairwise comparison) among the ten treatments based on RAPD analysis

Treatments	1	2	3	4	5	6	7	8	9
Haploid	0.46								
15 Gy	0.72	0.30							
20 Gy	1.0	0.79	0.66						
25 Gy	0.47	0.31	0.56	0.54					
150 mM NaCl	0.73	0.43	0.31	0.41	0.57				
200 mM NaCl	0.51	0.11	0.24	0.59	0.24	0.49			
20 Gy x 50 mM NaCl	0.53	0.37	.037	0.87	0.50	0.63	0.18		
20 Gy x 100 mM NaCl	0.60	0.43	.043	095	0.57	0.70	0.24	0.0	
25 Gy x 50 mM NaCl	0.59	0.30	0.30	0.66	0.43	0.56	0.11	0.12	0.17

hybrids of *Saccharum* and *Erianthus* (Nair *et al*., 2002). Goodspeed (1954) had postulated that the present day assemblage of species was derived from a pregeneric genetic reservoir with three major components that had been designated pre- *Nicotiana*, pre-*Cestrum* and pre-*Petunia*. The *Cestroid* complex was thought to be ancestral to the *Rustica* whereas *petunioides* complex ancestral to the subgenus *petunioides*. Grouping of these species was clearly in accordance with the earlier classification based on traditional analysis of cytological and morphological characteristics.

Conclusions

The exposed haploid *Nicotiana alata* plants to radiation and salinity had a negative effect on the growth rate and the length of plants. While the combined effect between gamma rays and salinity was suffused on the growth rate and the length of the plants until the impact of certain treatments caused plant death. The present study would be useful for establishing molecular phylogeny in the species of *Nicotiana*. RAPD assay was also found effective in analyzing the polymorphism among treatments. The species specific markers identified would be utilized in future introgression breeding programs.

Acknowledgement

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