In vitro, induction of salt tolerant potato (*solanum tuberosum* l.) Plants with gamma irradiation and characterization of genetic variations through sds-page and issr-pcr analysis

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ABSTRACT

Salt tolerant mutants of potato (*Solanum tuberosum* L. 'Sponta') were obtained via gamma irradiation. bud explants of two strains of potato (Sponta and lady rosetta were treated with various dosages of gamma irradiation, and the clonal generations were developed. Selection of salt-tolerant mutants was accomplished by in vitro selection media containing 30, 60, 90 and 120 mM NaCl. Molecular-level differences between the control and mutant plants were elucidated using ISSR technique, and the polymorphism rate according to the selected primers was calculated as 89.66%. Genetic distances between the controls and mutants were also calculated, and related dendrograms were produced. On average the mutants were genetically 27.5% different from the control plants. The greatest difference encountered between the control and mutants was 47%, which was detected in mutant plants produced by 20 or 30 Gy gamma irradiation and regenerated in selection medium containing 100 mM NaCl.

Key words: Gamma radiation, in vitro mutagenesis, mutation breeding, potato, ISSR, salt stress

Introduction

Salinity is an environmental stress factor that found in nearly 25% of agricultural land. It has become a serious problem especially in agricultural regions with the greatest crop yield potential such as the Mediterranean Basin, California, and Southeast Asia. Unless measures are taken it is estimated that by the year 2050 about 50% of agricultural lands could be suffering from excessive salinity, causing considerable damage to plant growth (**Pareek et al.**, 2007; **Blumwald and Grover** 2006 and **Leblebici et al.,** 2011). In addition to preventing growth, salt stress can decrease yield and quality, eventually causing abrupt plant death. It is particularly crucial to assess the maximum salinity tolerance of economically important crops grown in the regions where salinity levels cannot be lowered significantly (**Munns and Richards**, 2007; **Mba et al.,** 2007; **Muft uoğlu et al.,** 2006; **Sen and Alikamanoglu** 2011 and **Sairam and Tyagi**, 2004). Improving plants via mutation can lead to the development of varieties that are more tolerant of or resistant to environmental stress factors such as salinity. In particular, somatic mutations are highly valuable for mutant production in vegetative plants. There are several researches about somatic mutation induction to produce desired mutants, and thus new varieties (**Saleem** et al., 2005, **Das** et al., 2000). Another approach in vegetative plant development is to combine mutagenesis and in vitro methods. This combination has proven effective in increasing plant variation. In addition, the desired genotype was produced in a shorter time and in smaller fields; consequently, selection procedures were facilitated (**Saleem et al.,** 2005; **Das et al.,** 2000; **Lu et al.,** 2007; **Szarejko and Forster**, 2007; **El-Sayed et al.,** 2007; **Hewawasam et al.,** 2004; **Lee et al., Saif-ur-Rasheed, et al.**, 2001, **Ahloowalia and Maluszynski**, 2001, **Alikamanoğlu,** 2002). A search of the literature demonstrates that, in direct and indirect mutation studies conducted in several species, great number of mutant varieties have been obtained. Among them, some mutant varieties were produced via gamma irradiation while others were produced by gamma irradiation combined with in vitro techniques. Mutant individuals with the desired characteristics are easily detected via stability tests (**Saif-ur-Rasheed** et al, 2001, **Gulsen** et al, 2007, **Zhen**, 2001). Potato (*Solanum tuberosum* L.), a vegetative plant grown for its starch-rich tubers, is the fourth most important agricultural crop after rice, wheat, and corn, with a yearly production of 300 million tons (**Byun et al.,** 2007 and **Nhut et al.,** 2006). Economically, it is the most important tuberous plant, and potato plant varieties are usually very sensitive to environmental stresses such as temperature changes, drought, and salinity due to their sparse and short root systems. There is significant loss in plant growth and product yields when potato is grown in soil that contains 20-35 mM concentrations of NaCl. When compared to other agricultural plants such as pepper and corn, the potato plant is more resistant to salinity; however, it is less resistant than tomato, rice, soy, and barley (**Byun et al.,** 2007, **Manrique,** 2000). In the current work, the aim was to induce mutations in vegetatively growing potato plants via gamma irradiation and to demonstrate the molecular-level differences among mutants using protein electrophoresis and the inter simple sequence repeats (ISSR) method.

Materials and methods

Materials

Potato plant tubers from Sponta and Lady rosetta (*Solanum tuberosum* L.) were obtained from the Horticultural Research Institute, Agricultural Research Centre, Doki, Giza, Egypt. The tubers stored at 4 °C. These tubers were then incubated in the dark

at room temperature for 2 weeks until 5-6 cm-long shoots appeared (**Sharabash** (2001)).

Explant production

Shoots formed by the tubers were surface sterilized by placing them in 70% ethanol for 2 min and 5% hypochloride solution for 10 min. Then they were rinsed 3 times with distilled water, dried with sterile drying paper, and planted in MS (**Murashige and Skoog**, 1962) medium containing 30 g/L saccharose. The shoots were incubated for 10 days at 26 °C in growth chambers with 16 h light/8 h dark periods, and the node explants used in the study were obtained (**Sharabash**, 2001).

Potato tissue culture and irradiation of the explants

Node explants from Sponta and Lady rosetta potato varieties were planted in MS medium containing 0.5 mg/L ZR and 1.5mg/L IAA. Explants were irradiated with 0, 5, 10, 15, 20, 25, 30, or 50 Gy gamma radiation by a cesium-137 (Cs137) gamma source with an activity of 6.5 Gy/min (**Saif-ur-Rasheed** et al, 2001, **Sharabash**, 2001, **Gosal et al**., 2001.).

Generation of the M1V2 and M1V3 plants

In order to form large populations from which to select mutants with the desired characteristics, individuals of the M1V1 generation were vegetatively reproduced, and M1V2 and M1V3 generations were created.

Treatment with NaCl concentrations

In order to determine the sensitivity of Sponta potato variety against NaCl and choose the selection medium to be used in the study, explants were planted in regeneration media containing 0, 50, 100, 125, 150,175, or 200 mM of NaCl. The regeneration ratios of the 28-day-old cultures were then evaluated (Table1), and growth media containing 50, 100, or 125 mM NaCl were chosen as selective media for selection of the plants with salinity tolerance.

Table 1. Physiological parameters of the 28-day old cultures of mutated bud explants of Sponta.

Irradiation level 5 Gray	Average shoot length	Branch No. per plant	Number of nodes	Root dry weight	Shoot dry weight
Control	3.34	4.0	16.0	0.00074	0.00708
30 mM Nacl	3.82	1.8	08.2	0.00066	0.00438
60 mM Nacl	5.48	1.8	07.2	0.00066	0.00338
90 mM Nacl	3.62	2.0	08.4	0.00062	0.00344
120 mM Nacl	2.82	1.6	07.2	0.00080	0.00614
10 Gray					
30 mM Nacl	5.42	1.6	11.0	0.00074	0.00270
60 mM Nacl	4.48	1.8	09.0	0.00064	0.00636
90 mM Nacl	4.26	1.6	09.2	0.00068	0.00462
120 mM Nacl	4.30	1.6	08.0	0.00054	0.00364
20 Gray					
30 mM Nacl	3.38	1.8	10.2	0.00060	0.00422
60 mM Nacl	4.52	1.6	10.8	0.00064	0.00454
90 mM Nacl	3.34	1.4	08.2	0.00050	0.00364
120 mM Nacl	3.26	1.4	08.4	0.00060	0.00702
30 Gray					
30 mM Nacl	3.84	1.6	09.8	0.00064	0.00356
60 mM Nacl	4.16	1.8	09.4	0.00060	0.00692
90 mM Nacl	3.16	1.6	06.4	0.00070	0.00642
120 mM Nacl	0.56	1.6	08.8	0.00046	0.00466
40 Gray					
30 mM Nacl	3.10	1.6	08.0	0.00052	0.00280
60 mM Nacl	3.40	1.4	06.0	0.00058	0.00376
90 mM Nacl	0.00	1.4	08.6	0.00054	0.00510
120 mM Nacl	0.00	1.4	04.4	0.00062	0.00452

Molecular analysis

Molecular differences between the control and salt tolerant mutants of Sponta potato variety were demonstrated using the PCR-based RAPD technique.

Genomic DNA isolation and analysis

The plant DNA extraction kit from Fujifilm (Quick-Gene DNA tissue kit S) was used for genomic DNA isolation from the leaf samples of control and salinity-tolerant individuals of Sponta potato variety.

Amplification conditions

For PCR amplification, randomly selected ISSR primers were used (Table 2). A PCR experiment was set up using 50 ng genomic DNA, 2.5 mM MgCl2, 0.1 mM dNTP, 0.4 µM primer, and 0.5 U Taq DNA polymerase in a total volume of 50 µL. The PCR was designed as 40 cycles of 1.5 min at 94 °C, 1 min at 36 °C, and 3 min at 72 °C. PCR products were then run on a 1.7% (w/v) agarose gel in TBE buff er at 90 V. Each PCR amplification was repeated at least 3 times. After separation, ISSR bands were examined and documented under UV.

Table 2. Physiological parameters of the 28-day old cultures of mutated bud explants of Lady rosetta.

Irradiation level 5 Gray	Average shoot length	Branch No. per plant	Number of nodes	Root dry weight	Shoot dry weight
Control	4.40	5.0	15.0	0.00066	0.00841
30 mM Nacl	4.58	2.2	09.6	0.00074	0.00532
60 mM Nacl	4.70	1.8	09.2	0.00072	0.00356
90 mM Nacl	5.50	2.0	09.4	0.00088	0.00582
120 mM Nacl	5.54	1.6	10.6	0.00084	0.00698
10 Gray					
30 mM Nacl	5.50	2.4	10.0	0.00070	0.00534
60 mM Nacl	٦.40	1.6	08.6	0.00062	0.00476
90 mM Nacl	5.54	2.0	06.2	0.00050	0.00380
120 mM Nacl	6.92	1.6	07.4	0.00056	0.00448
20 Gray					
30 mM Nacl	3.80	1.6	10.2	0.00070	0.00742
60 mM Nacl	4.90	1.6	10.0	0.00058	0.00676
90 mM Nacl	5.38	1.8	09.0	0.00054	0.00616
120 mM Nacl	5.68	1.8	09.8	0.00060	0.00720
30 Gray					
30 mM Nacl	4.10	2.2	07.2	0.00062	0.00456
60 mM Nacl	3.32	1.6	09.2	0.00054	0.00610
90 mM Nacl	3.00	1.4	08.8	0.00060	0.00706
120 mM Nacl	3.28	2.0	08.8	0.00076	0.00828
40 Gray					
30 mM Nacl	5.56	1.8	10.6	0.00070	0.00310
60 mM Nacl	4.76	1.4	10.2	0.00060	0.00400
90 mM Nacl	1.74	1.6	05.8	0.00066	0.00516
120 mM Nacl	0.00	1.6	09.0	0.00056	0.00546

Statistical analysis and determination of genetic distance

Shoot length, Number of braches per plant, number of nodes per plant, Shoot dry weight and root dry weight data of the gamma irradiated or non-irradiated (control) 28-day-old cultures of Sponta and Lady rosetta potato plants were produced. The data were analyzed by one-way ANOVA, and statistically significant data were compared (**Zar, 1984.**). In order to determine the genetic distance between the control variety Hermis and mutant plants of Sponta and Lady rosetta potato varieties, during ISSR-PCR analysis numerical values of 1 and 0 were assigned to the amplified and non amplified ISSR bands, respectively. These values were then used in clustering analysis to form a dendrogram demonstrating the genetic distance among the three varieties (**Abbas et al.**, 2008; **Atak et al.,** 2004; **Babaoğlu et al.,** 2004 and **Wolf and Rijini** 1993).

Results

Effect of gamma irradiation on tissue cultures

Sensitivity of the Sponta potato plant variety towards irradiation was demonstrated with respect to the average shoot length, average branch number, average node number and dry weight of shoot and root. (Tables 1 and 2). The dosage of radiation that decreased average shoot length (zero) was 40 Gy, while the dosage that decreased average number of branches/plant was 20 and 40 Gy. The average number of nodes/plant decreased when irradiated with 40 Gy, while it decreased by 50% when irradiated with 20 Gy. Evaluation of the root formation ratio revealed that the radiation dosages that decreased root formation by 30% and 50% were 18 Gy and 23 Gy, respectively.

Table 3. Primers used in ISSR analysis and number of total and polymorphic bands in the amplified primers.

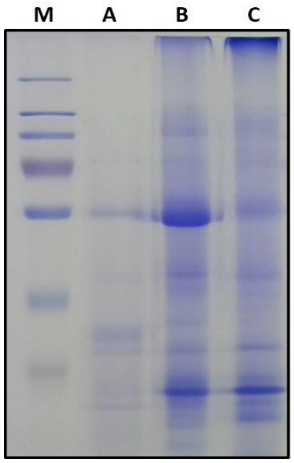
Primer		Primer sequence	Total bands	Unique bands	Polymorphic bands
1	ISSR 1	5'-AGAGAGAGAGAGAGAGYC-3'	25	5	4
2	ISSR 2	5'-AGAGAGAGAGAGAGAGYG-3'	20	4	4
3	ISSR 3	5'-ACACACACACACACACYT-3'	19	0	2
4	ISSR 4	5'-ACACACACACACACACYG-3'	27	3	3
5	ISSR 5	5'-GTGTGTGTGTGTGTGTYG-3'	20	1	2
6	ISSR 8	5'-AGACAGACAGACAGACGC-3'	21	5	5
7	ISSR 9	5'-GATAGATAGATAGATAGC-3'	41	5	4
8	ISSR 10	5'-GACAGACAGACAGACAAT-3'	28	2	4
9	ISSR 11	5'-ACACACACACACACACYA-3'	25	8	1
10	ISSR 12	5'-ACACACACACACACACYC-3'	22	4	6
	Total		248	37	35

Induction of salt-tolerant potato (*Solanum tuberosum* L.) mutants with gamma irradiation and characterization of genetic variations via ISSR analysis.

Production of salt-tolerant somatic plants

In order to detect some characteristics of the salt-tolerant plants, following plantation of the explants into selection media with 30, 60, 90, and 120 mM concentrations of NaCl, Shoot length rates on day 28 were recorded; they were 5.48 in the sponta variety irradiated with 5 Gy, 6.92 in the Lady rosetta irradiated with 10 Gy, respectively. They were 2 branches/plant in the sponta variety irradiated with 5 Gy, Meanwhile, 2.4 branches/plant in the Lady rosetta irradiated with 10 GY. % in the 15, 20, and 30 Gy gamma irradiated groups, respectively (Table 4). Number of nodes/plant were 11 in the sponta variety irradiated with 10 Gy, 10.6 in the Lady rosetta irradiated with 40 Gy, respectively. (Table 4). Non significant effects were detected in case of shoot dry weight and root dry weight.

Determination of genetic differences via Protein electrophoresis



A: Hermis variety B: Sponta C: Lady rosetta

Fig. 1. SDS-PAGE of soluble proteins. In comparison to control variety, Hermis (lane A), lane B for the Sponta variety and Lane C for the Lady rosetta Lane M: protein markers.

Table 4. Number of monomorphic bands, polymorphic bands and unique bands obtained from protein electrophoresis for three varieties of potato under investigation

Bands	Hermis	Sponta	Lady rosetta
Monomorphic bands	**3**	**3**	**3**
Unique bands	**2**	**3**	**4**
Polymorphic bands	**0**	**3**	**3**
Total	**5**	**9**	**10**

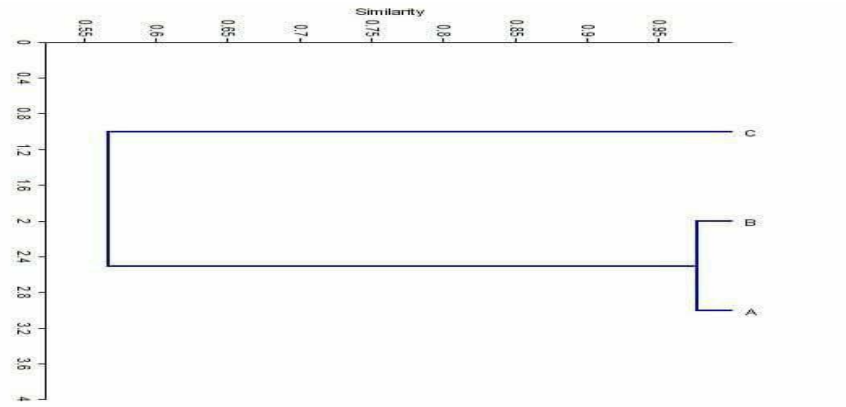
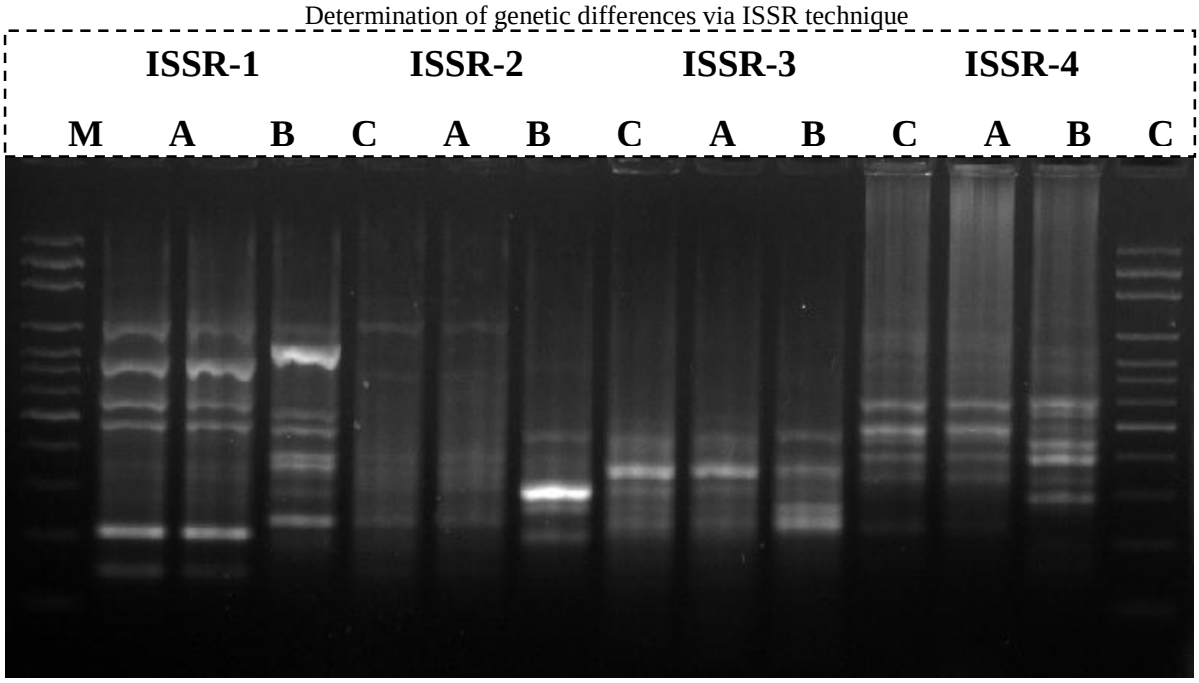


Fig. 2. Phylogenetic tree (dendrogram) of studied *solanum tuberosum* varieties based on the analysis of protein electrophoresis banding patterns after using molecular marker



Among the 10 ISSR primers examined in this study, all the primers were used for amplification of the samples belonging to Hermis, Sponta and Lady rosetta potato varieties (Table 3). The highest number of amplified bands was found in Lady rosetta (15 bands) and ISSR 9 (41 bands), while in the control variety Hermis 13 bands were observed. The number of polymorphic bands of the 10 primers used was estimated as 35 band (Table 3) while the number of unique bands was 37 band.

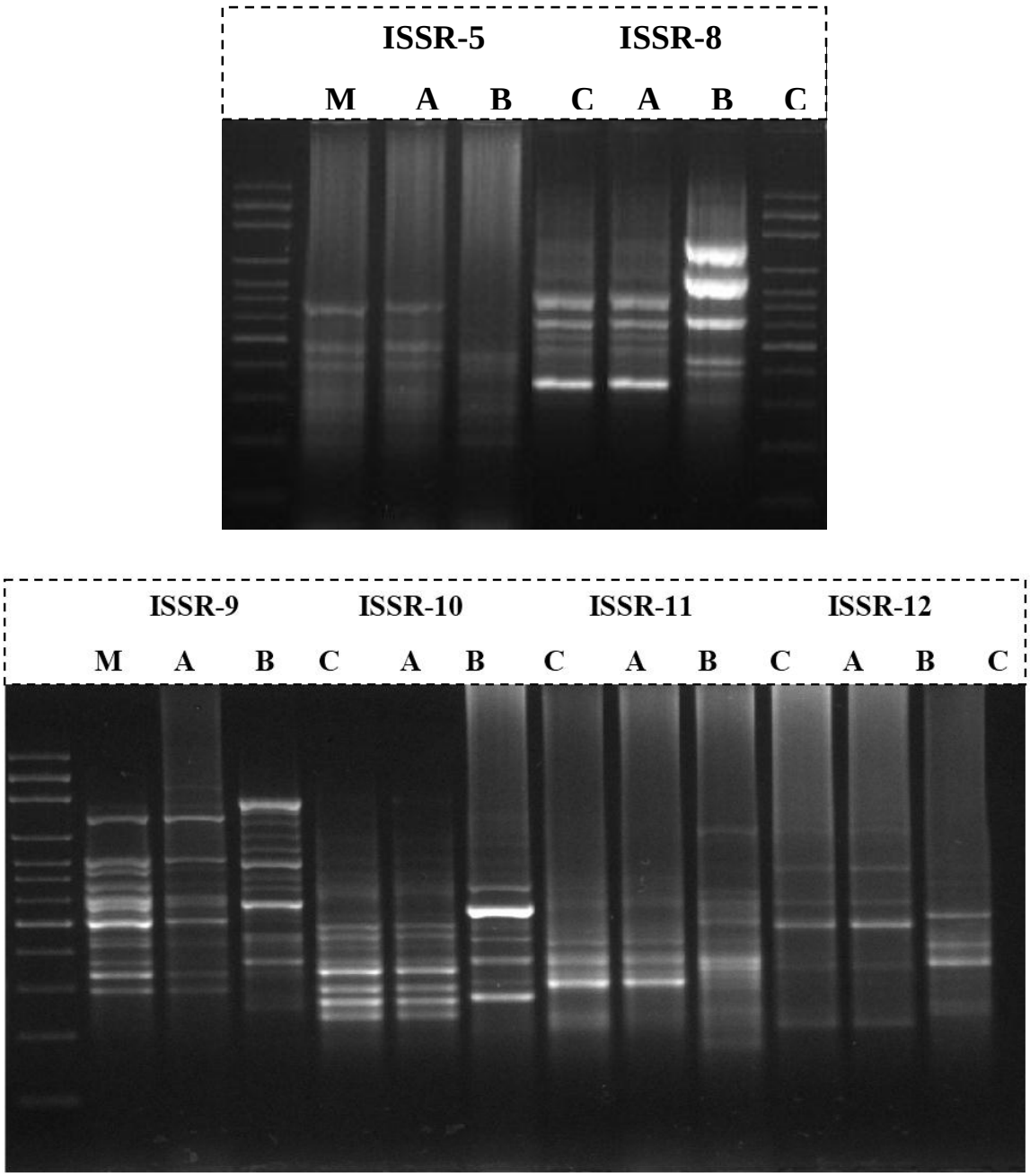


Fig. 2. ISSR banding patterns of *Solanum tuberosum* varieties after using 10 ISSR primers.

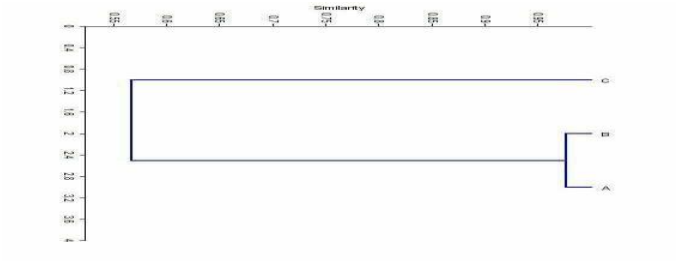


Fig. 3. Phylogenetic tree (dendrogram) of studied *solanum tuberosum* varieties based on the analysis of ISSR banding patterns after using 10 ISSR primers.

	A	B	C
A	100		
B	97	100	
C	60	59	100

Table 5. Similarity coefficients (Dice Similarity Measure) among the studied *solanum tuberosum* varieties based on the analysis of ISSR banding patterns after using 10 ISSR primers.

Upon evaluation of the ISSR results from all primers used, the genetic distance of the control variety Hermis and the sponta variety was 97%, while the distance of the control variety Hermis and the lady rosetta variety was 60 (Table 5). Upon evaluation of the dendrograms demonstrating genetic distances, the control variety Hermis separated with a subcluster consisted of Sponta and Lady rosetta varieties. The Sponta potato variety appeared to be very close the Lady rosetta variety and the control variety hermis was very distant.

Discussion

Due to their genotypic differences, plants respond differently to irradiation dosages. Higher doses of radiation cause chromosomal damage in plant meristematic cells, deceleration of the cell cycle, and delay of mitosis, which significantly affect overall plant regeneration and development. While an increase in radiation doses boosts mutation frequency, it also increases damage to the plant (**Hewawasam** et al., 2004, **Alikamanoğlu,** 2002; **Gulsen** et al., 2007, **Zhen**, 2001, **Sharabash**, 2001, **Toker** et al., 2007). Therefore, selection of the correct dosages in mutation studies is very important. In mutation studies with vegetative plants, Semi lethal Dose is usually taken as the upper limit, while in plant improvement studies Induction of salt-tolerant potato (*Solanum tuberosum* L.) mutants with gamma irradiation and characterization of genetic variations via ISSR analysis dosages around Dose 30 are preferred (**Alikamanoğlu,** 2002, **Predieri and Di Virgilio** 2007). In this study the salt-tolerant plants were also obtained with an irradiation dosage around 20 to30 Gy. Regardless of genetic differences among plants, for somatic mutation induction radiation doses applied to plant cells and tissues for in vitro tissue culture studies must be around 20 Gy (**Donini and Sanino (1998)**). In various in vitro mutation studies with potato plants the effective dose was 20 Gy, and it was noted that higher doses could be lethal (**Saif-ur-Rasheed** et al., 2001, **Sharabash**, 2001).

During somatic mutation studies in tissue cultures using micropropagation techniques, late generations are formed, and mutant plants with the desired characteristics can be successfully selected in vitro (**Hewawasam** et al., 2004, **Ahloowalia and Maluszynski** 2001, **Alikamanoğlu,** 2002). In order to induce somatic mutations in potato plant in the current study, tissue cultures were formed using bud explants, and these cultures were then gamma-irradiated. Various studies reported that stability tests of salinity tolerant mutants were generally conducted on plants of the third generation (**Das** et al., 2000, **Sharabash**,2001). A total of 51 salt-tolerant mutants (**Gulsen** et al., 2007, **Ahloowalia and Maluszynski** 2001, and 14 mutant plants created by 15 Gy, 20 Gy, and 30 Gy gamma irradiation, respectively) were detected in selection media; these mutant plants grew significantly better than the controls. Nevertheless, salt-tolerant mutants could not be induced in the experimental group exposed to 20 Gy gamma irradiation. It can be assumed that the gene mutations in this group of plants occurred in the regulatory regions responsible for suppressing genes that play a role in salinity tolerance by preventing or increasing transcription and/or translation (**Luleyap**, 2008). Evaluation demonstrated that the salt tolerant plants resulted by induction of somatic mutations via gamma irradiation exhibited low percentage of genetic difference from control plants. While the physical damage caused by irradiation can be evaluated by studying physiological parameters in the M1 generation, hereditary changes in living organisms can only be assessed in later generations. Genetic changes in organisms exposed to irradiation may vary from one cell to another. These changes may consist of differences in DNA repair mechanisms (pre-replicative or during replication) as well as changes in the regulation of gene expression (transcriptional, posttranscriptional, or translational) (**Luleyap**, 2008, **Kumar and Kumar Rai**, 2009.). Thus, even within a group of the same type of plant irradiated with a given dosage, the formation of different genotypic and phenotypic characters can be expected.

In conclusion, salt-tolerant potato plants were successfully created for in vitro tissue cultures via mutation induction using 5, 10, 20, 30 and 40 Gy gamma irradiation. The genetic distances between the two varieties and the control variety were demonstrated using ISSR analysis. The data produced are valuable for selection, plant development, and characterization of gene sources in future studies of this plant.

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