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, SDS-PAGE, salt stress

Introduction

Saltiness is an ecological pressure discovered in almost 25% of horticultural land. It has turned into a significant issue particularly in agrarian areas with the best product yield potential, for example, the Mediterranean Basin, California, and Southeast Asia. Except if measures are taken it is evaluated that constantly 2050 around half of agrarian grounds could be experiencing exorbitant saltiness, making impressive harm plant development. Notwithstanding avoiding development, salt pressure can diminish yield and quality, in the end causing sudden plant demise. It is especially critical to survey the most extreme saltiness resistance of monetarily imperative harvests developed in the areas where saltiness levels can't be brought down fundamentally. Enhancing plants by means of change can prompt the advancement of assortments that are more tolerant of or impervious to natural pressure factors, for example, saltiness. Specifically, somatic mutations are exceptionally profitable for mutant generation in vegetative plants. There are a few looks into about somatic mutations acceptance to deliver wanted mutants, and along these lines new assortments. Another approach in vegetative plant improvement is to consolidate mutagenesis and in vitro strategies. This blend has demonstrated compelling in expanding plant variety. Also, the coveted genotype was created in a shorter time and in littler fields; therefore, determination techniques were encouraged. A hunt of the writing shows that, in immediate and aberrant transformation considers led in a few animal categories, extraordinary number of mutant assortments have been acquired. Among them, some mutant assortments were delivered by means of gamma light while others were created by gamma illumination joined with in vitro methods. Mutant people with the coveted qualities are effortlessly recognized by means of stability tests. Potato (*Solanum tuberosum* L.), a vegetative plant developed for its starch-rich tubers, is the fourth most essential farming harvest after rice, wheat, and corn, with a yearly creation of 300 million tons (**Byun, *et al.*, 2007 and Nhut, *et al.*, 2006**).

Monetarily, it is the most essential tuberous plant, and potato plant assortments are generally extremely touchy to natural burdens, for example, temperature changes, dry spell, and saltiness due to their scanty and short root frameworks. There is noteworthy misfortune in plant development and item yields when potato is developed in soil that contains 20-35 mM concentrations of NaCl. At the point when contrasted with other rural plants, for example, pepper and corn, the potato plant is more impervious to saltiness; in any case, it is less safe than tomato, rice, soy, and grain (**Byun, *et al.*, 2007 and Manrique, 2000**). In the present work, the point was to prompt transformations in vegetatively developing potato plants by means of gamma light and to show the sub-atomic level contrasts among mutants utilizing protein electrophoresis and the bury basic arrangement rehashes (ISSR) Inter Simple Sequence Repeats technique.

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 sucrose. 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 (Indole acetic acid**)**. 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 M_1V_1 generation were vegetatively reproduced, and M_1V_2 and M_1V_3 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, 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. 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.

Molecular and Biochemical analysis:

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

Extraction of PROTEIN for SDS-PAGE Analysis:

Protein SDS-PAGE analysis was carried out for three potato cultivars. Extraction of proteins for gel electrophoresis according to **Laemmli (1970)** and Molecular weights of the different protein bands were determined in respect to standard protein markers (Bioline Hyper Page prestained protein marker, 10_200 kDa) with the KodakMI software after documentation of the gel slab with Gel-Doc system (Biostep GmbH, Germany).

Genomic DNA isolation and analysis:

The plant DNA extraction kit from Fujifilm (Quick-Gene DNA tissue kits) 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 3). 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 buffer at 90 V. Each PCR amplification was repeated at least 3 times. After separation, ISSR bands were examined and documented under UV.

Statistical analysis and determination of genetic distance:

Shoot length, Number of branches 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 and SDS-PAGE analysis numerical values of 1 and 0 were assigned to the amplified and non-amplified ISSR and SDS-PAGE 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 assortment towards illumination was shown as for the normal shoot length, normal branch number, normal hub number and dry weight of shoot and root. (Table 2). The dosage of radiation that diminished normal shoot length (zero) was 40 Gy, while the dose that diminished normal number of branches/plant was 20 and 40 Gy. The average number of nodes/plant diminished when illuminated with 40 Gy, while it diminished by half when lighted with 20 Gy. Assessment of the root development proportion uncovered that the radiation measurements that diminished root arrangement by 30% and half were 18 Gy and 23 Gy, individually. Generation of salt-tolerant substantial plants keeping in mind the end goal to distinguish a few attributes of the salt-tolerant plants, following manor of the explants into determination media with 30, 60, 90, and 120 mM centralizations of NaCl, Shoot length rates on day 28 were recorded; they were 5.48 in the Sponta assortment lighted with 5 Gy, 6.92 in the Lady Rosetta illuminated with 10 Gy, respectively.

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

Irradiation level 5 0 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
LSD 0.05	**0.091**	**0.139**	**0.580**	**0.110**	**0.420**

Table 3. Physiological parameters of the 28-day old cultures of mutated bud explants of Lady Rosetta.

Irradiation level 5 0 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	6.40	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
LSD 0.05	**0.091**	**0.139**	**0.580**	**0.110**	**0.420**

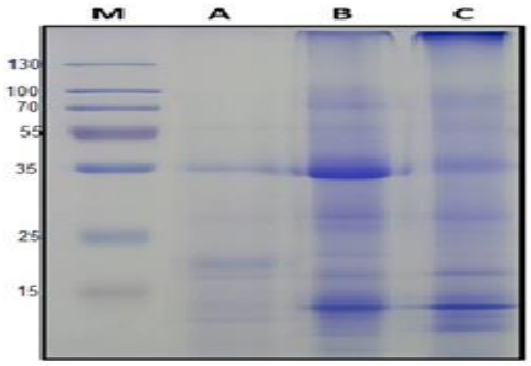
Production of salt-tolerant somatic plants:

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 3). 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 3). Non-significant effects were detected in case of shoot dry weight and root dry weight.

Determination of Genetic Differences Via Protein Electrophoresis:

The analysis of electrophoretic pattern of soluble proteins showed that NaCl induced the appearance of a new polypeptide with a molecular mass of about 17 kDa, whose synthesis was increased in 90 mM-tolerant calli (Table 4 and Fig. 1,2).

Moreover, it was possible to identify a set of polypeptides whose synthesis was up-regulated in NaCl tolerant calli. In this group, the synthesis of polypeptides with a molecular mass of about 32 and 34 kDa was found to be enhanced in calli lines grown on medium with NaCl. In addition, the synthesis of two polypeptides with a molecular mass of about 22 and 24 kDa appeared increased in 90 mM NaCl-tolerant calli compared to control, whereas the synthesis of 22 kDa polypeptide was not affected by the higher NaCl concentration ((Table 4 and Fig. 1,2).



A: Hermis 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(A)		Sponta(B)		Lady Rosetta(C)	
	No. of Bands	MW	No. of Bands	MW	No. of Bands	MW
Monomorphic bands	0	--	1	16.8 K_{Da}	1	16.8 K_{Da}
Unique bands	0	--	5	82.9,63.1,49.2,34.6 and 22.8K_{Da}	2	86.5 and 56.1 K_{Da}
Polymorphic bands	4	38.8, 17.9, 16.5 and 13.9 K_{Da}	3	17.1, 14.6 and 13.3K_{Da}	5	39.6,23.4,14.5,14.1 and13.5K_{Da}
Total	4		9		10	

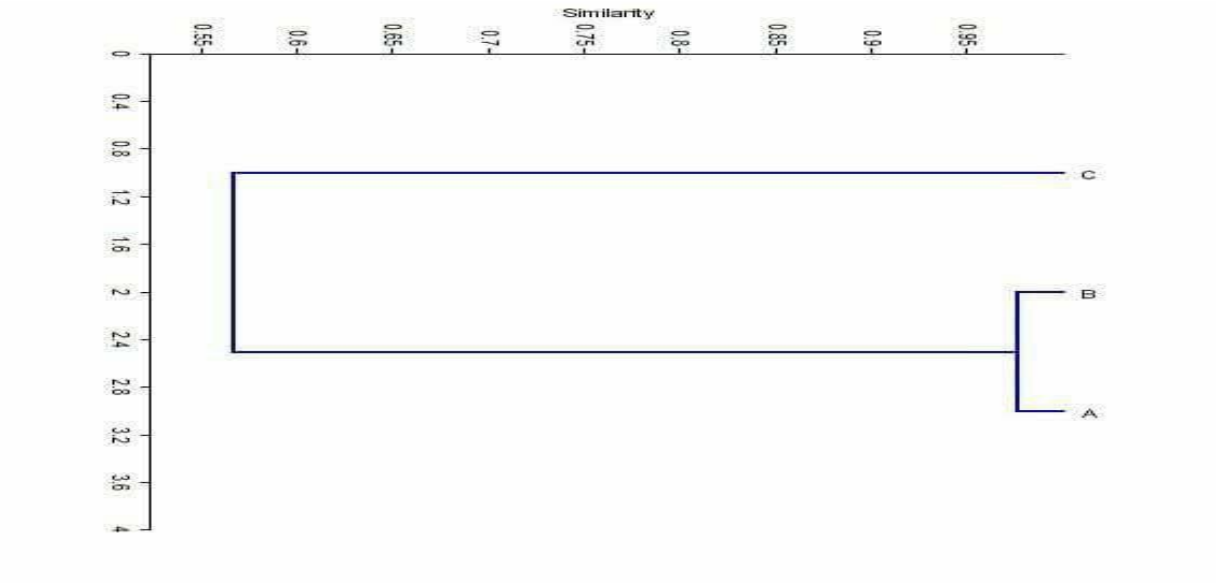


Fig. 2. Phylogenetic tree (dendrogram) of studied *Solanum tuberosum* varieties based on the analysis of protein electrophoresis banding patterns after using molecular marker.

Determination of genetic differences via ISSR technique:

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 and Fig.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 1 and Fig.3) while the number of unique bands was 37 band.

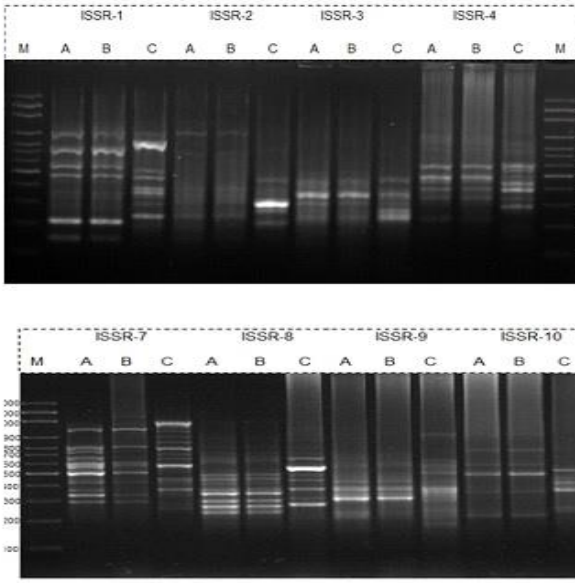


Fig. 3. ISSR banding patterns of Solanum tubero varieties (A: Hermis, B: Sponta and C: Lady rosetta a using 10 ISSR primers.

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.

	Hermis	Sponta	Lady rosetta
Hermis	**100**		
Sponta	**97**	**100**	
Lady rosetta	**60**	**59**	**100**

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 and Fig.4). 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) 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**, 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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