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Original Research Article

Salinity Tolerance and Stevioside Improvement of *In Vitro* Selected *Stevia* (*Stevia rebaudiana*) Mutants

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Abstract

This work aimed to induce genetic variations in *Stevia* using gamma rays and *in vitro* selection, which might be useful for tolerance to a biotic stresses such as salinity and increase the bioactive material (stevioside). Stem nods and shoot tips of micropropagated plants were treated with four (0, 750, 1500 and 2250 rad) doses of gamma rays to obtain the suitable explants for bud multiplication and callus induction. The induced calluses were transferred to regeneration media supplemented with 0, 2000, 4000 and 6000 ppm of NaCl. Gamma ray doses of 0.0 and 750 rad gave the highest values for both shoot tip and stem nod explants (100%), followed by 95 and 67.5% for shoot tips and stem nods at 1500 rad of gamma rays respectively. the highest numbers of regenerated shoots were observed at 0.0 rad gamma rays dose (control) under all NaCl concentrations and the number of shoots decreased with the increase of gamma ray doses. The combined effects of gamma rays and salinity severely affected the number of shoots rather than the effect of salinity or gamma rays alone. The dose of 750 rad enhanced all characters of propagated shoots produced from both shoot tip and node explants. *Stevia* leaves which produced from propagated clones at 0.0 rad gave nearly duplicated amount of stevioside (505.51 mg/100g), compared with the original plant (278.9 mg/100g) .The amount of stevioside decreased dramatically as the dose of gamma rays increased. RAPD analysis revealed genetic variations between the regenerated plants and their original.

Keywords

Gamma rays
HPLC analysis
RAPD
Salinity
Stevia rebaudiana

Introduction

Stevia rebaudiana Bertoni is a sweet herb indigenous to the elevated terrain of northeastern Paraguay near its borders with Brazil (Soejarto et al., 1983). The leaves of stevia are the source diterpene glycodides, *viz.*, stevioside and rebaudioside (Bhosle, 2004). Stevioside is 300 times sweeter than sucrose at 0.4% sucrose

concentration (Soejarto et al. 1983; Liu and Li 1995). Stevioside is chemically stable and occurs in the dried leaves of *S. rebaudiana* at about 42% (w/w). *S. rebaudiana* essential oil and extracts possess high antioxidant, anti-inflammation and antimicrobial properties (Muanda et al., 2011). The propagation through seeds is not adequate leading to a very low seed germination percentage (Taware et al., 2010).

Somaclonal variation is considered to be a useful source of variation in stevia (Kuntal et al., 2005; Moktaduzzaman and Rahman, 2009). Mutations can be induced by chemicals, or various types of ionizing radiation (X-rays, gamma rays, neutrons, ultraviolet light, etc.). For induction of mutations in vegetatively propagated plants, chemical mutagens are not usually considered, mainly because they are not very successful, and this could be attributed to the poor uptake and penetration of the chemical compound. Salinity of the soil either natural or caused by irrigation in arid environment and excessive use of fertilizers is a great problem in agriculture. DNA fingerprints as revealed by randomly amplified polymorphic DNA (RAPD) have been found to be useful in identification of genotype (Chunwonges et al. 1993) and genetic differentiation of populations (Russell et al. 1993). Mathius et al. (1995) induced some useful mutants in *Stevia* and produced plants with high stevioside using gamma radiation with 1500 rad. They used the differences in RAPD banding patterns among the studied plants as markers for somaclonal variation. In this paper we sought to produce proper *Stevia* plant through somaclonal variation and/ or micropropagation and gamma irradiation treatments tolerant to salinity conditions and enhancement the bioactive component, stevioside.

Materials and methods

Seedlings and seeds of *Stevia rebaudiana* Bertoni var. spanti used in the present study were provided by Sugar Crops Research Institute, Agric. Research Center, Ministry of Agriculture, Giza, Egypt. Shoot-tip and node explants taken from micropropagated plants were treated with 0.0, 750, 1500 and 2250 rad of gamma ray. These explants were transferred to callus induction medium (MS supplemented with 1.5mg/l NAA and 1mg/l BA). All cultures were incubated at 25±2^{o}C and the responded explants for callus induction were recorded after two weeks. The induced calli were transferred to regeneration MS medium supplemented with1.8 mg/l BA and 0.120 mg/L NAA) and containing different concentration of NaCl (0.0, 2.0, 4.0, 6.0) mg/L and incubated in the culture room at 25±2^{o}C under 16:8 light/dark photoperiods with light intensity of about 2500 lux. After twenty days of incubation, cultures were evaluated in the basis of regeneration rate calculated as percentage of explants produced shoot and the mean number of regenerated shoots per explant. In addition, shoot-tip and node explants taken from micropropagated plants (six weeks old) were treated with 0.0, 750, 1500 and 2250 rad doses of gamma ray. The treated shoot tip and node explants were transferred to MS medium supplemented with 0.5 mg BA / l for bud multiplication.

Genomic DNA extraction and RAPD-PCR analysis

DNA extracted from fresh leaves of the regenerated mutants and their original plants by Cetyltrimethyl Ammonium Bromide (CTAB) according to Doyle and Doyle (1990). RAPD was performed using eight random decamer primers (Table 1). Polymerase Chain Reaction (PCR) was carried out in presence of 1X Taq DNA polymerase buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 100 μM dNTPs, 5 picomole single random primers, 30 ng template DNA, 0.5 unit of Taq DNA polymerase in a total volume of 25 μl. PCR amplification was performed in automated thermal cycler (MJ-Mini, Bio Rad) programmed as follow, 95°C for 4 min followed by 40 cycles of 1 min for denaturation at 94°C, 30 sec for annealing at 37°C and 3 min for polymerization at 72°C, followed by a final extension step at 72°C for 7 min. The amplification products were resolved by electrophoresis in 1.5 % agarose gels in 0.5 X TBE buffer and documented on Gel Documentation UVITEC, UK.

Table 1. List of the used primers for detecting polymorphisms of induced *Stevia* mutants and somaclones.

Primer	Sequences
BB1	5'- GGTGCGGGAA-3'
BB3	5'-GTAGACCCGT-3'
BB5	5'- AACGCGCAAC-3'
CA-1	5'-AGGTCACTGA-3'
OPD07	5'-TTGGCACGGG-3'
OPA19	5'-CAAACGTCGG-3'
OPB08	5'-GTCCACACGG-3'
OPV07	5'-GAAGCCAGCC-3'

Statistical analyses

The analysis of variance (ANOVA) was used to test the differences between the performances of gamma irradiation doses, explants and different media (Snedecor, 1962).

Stevioside content

High-performance liquid chromatography (HPLC) analysis was done to evaluate the amount of the bioactive component stevioside in the original and mutants.

Results and discussion

Effects of gamma rays on callus induction and plant regeneration

In addition to the traditional breeding approaches, genetic variability in *Stevia* can be induced also by mutagenesis and tissue culture technique. The effect of various doses of gamma rays on the *in vitro* shoot formation from shoot tip and nod explants of Stevia was studied. the mean percentage of callus induction and regeneration rate decreased gradually with the increase of gamma ray doses (Table 2 and Fig. 1).

Gamma ray doses of 0.0 and 750 rad gave the highest values for both shoot tip and stem nod explants (100%), followed by 95 and 67.5% for shoot tips and stem nods at 1500 rad of gamma rays respectively. While, gamma rays at 2250 rad gave the lowest value in both explants (35%). The average value of callus induction percentage over all gamma rays doses indicated that the shoot tip explant gave the highest value (82.5%) for callus induction trait compared with node explant (75.6). shoot formation percentages from shoot tip and stem nod explants at 0.0 and 750 rad reached 100, 90% and 95, 90 % respectively, these values reduced drastically with the increment of gamma ray doses (Table 2).

Table 2. Mean percentage of callus induction and shoot formation at four gamma irradiation doses and two explants (node and shoot tip).

Dose (Rad)	Type of explant	No. of explants	Callus induction%	Shoot formation%
0	Node Shoot tip	5 5	100 100	95 100
750	Node Shoot tip	5 5	100 100	90 95
1500	Node Shoot tip	5 5	67.5 95	40 70
2250	Node Shoot tip	5 5	35 35	25 35
Average	Node Shoot tip	5 5	75.6 82.5	62.5 75
Mean			79	68.75
LSD 0.05			0.073	0.133
0.01			0.102	0.186

In vitro selection for salinity tolerance

The results in Table 3 showed that the highest numbers of regenerated shoots were observed at 0.0 rad gamma rays dose (control) under all NaCl concentrations with the mean value of 4.7 and the number of shoots decreased with the increase of gamma ray doses (Fig. 2). The highest number of shoots per callus with the mean value of 7.9 was obtained at 0.0 rad. These values decreased with the increase of gamma ray doses (2.8 at 750 rad, 2.5 at 1500 rad and 2.25 at 2250 rad). In spite of the lowest number of shoots (0.5) was obtained under NaCl concentration of 6000 at 1500 and 2250 rad, 31 salt tolerant plants with percentage of 2.58% was recorded at 0.0 rad under this concentration (6000 ppm) . On the other hand, the salinity stresses showed values of 66 and 34 tolerant plants under 2000 and 4000 ppm NaCl, respectively. Under 2000 ppm NaCl, the highest number of shoots per callus were observed at 0.0 rad (5.5) followed by 750 rad (1.66) while, the lowest value achieved at 2250 rad of gamma rays (0.83). NaCl concentration of 4000 ppm generated the highest number

of shoots per callus (2.83) at 0.0 followed by 1.8 at 750 rad and 1.6 at 1500 rad meanwhile, 2250 rad showed the lowest number of shoot per callus (0.66). The combined effects of gamma rays and salinity severely affected the number of shoots rather than the effect of salinity or gamma rays alone.

Fig. 1: Callus induction of (A=node, B=shoot tip) explants treated with four gamma ray doses.

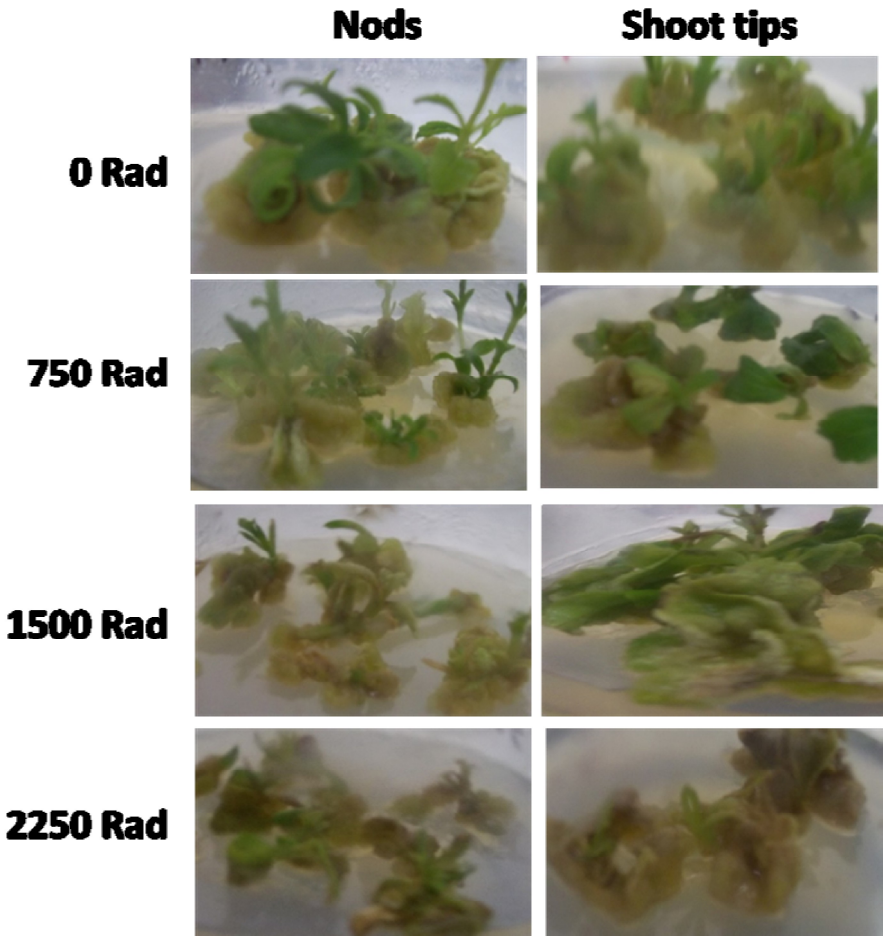
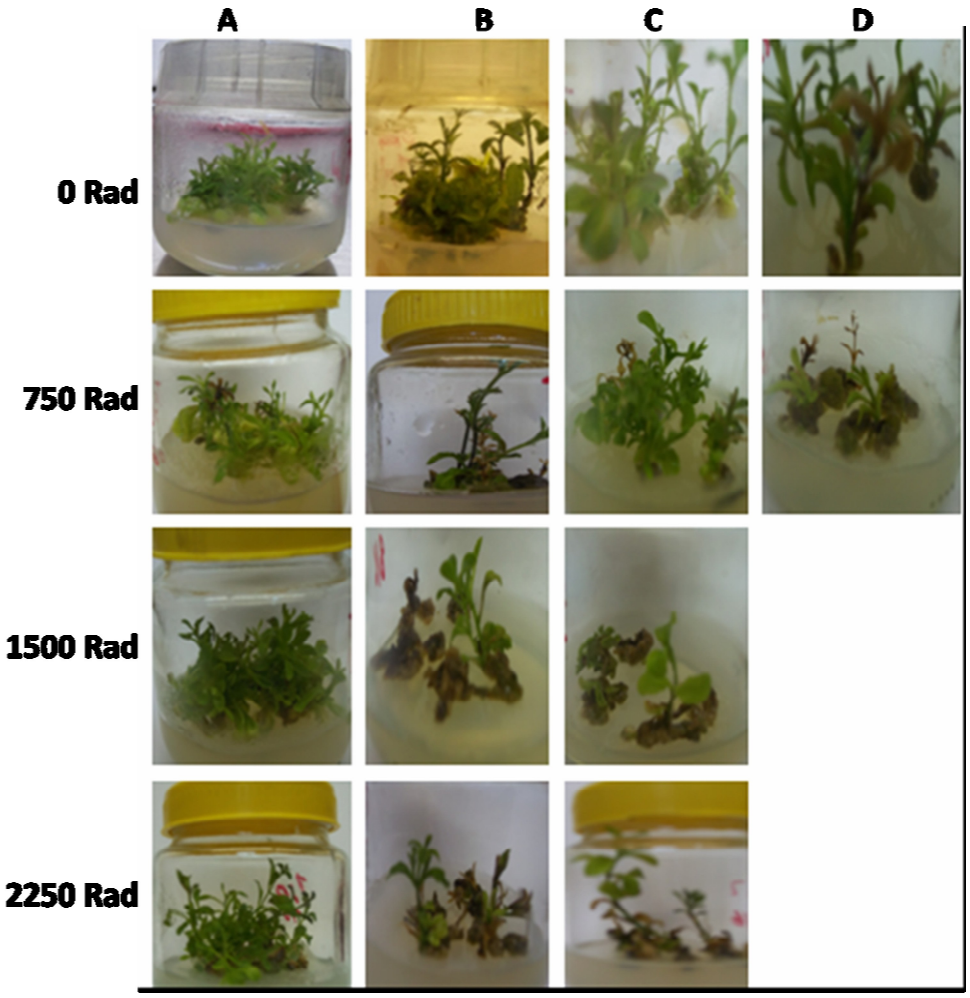


Table 2. Mean percentage of callus induction and shoot formation at four gamma irradiation doses and two explants (node and shoot tip).

Dose (Rad)	Type of explant	No. of explants	Callus induction%	Shoot formation%
0	Node Shoot tip	5 5	100 100	95 100
750	Node Shoot tip	5 5	100 100	90 95
1500	Node Shoot tip	5 5	67.5 95	40 70
2250	Node Shoot tip	5 5	35 35	25 35
Average	Node Shoot tip	5 5	75.6 82.5	62.5 75
Mean			79	68.75
LSD 0.05			0.073	0.133
0.01			0.102	0.186

Fig. 2: Shoots regenerated from callus of four gamma ray doses and four NaCl concentrations.
{A (0 ppm), B (2000 ppm), C (4000 ppm), and D (6000 ppm)}.



The aforementioned results showed that at 0.0 ppm of NaCl, the highest number of shoots per callus with the mean value of 7.9 was recorded, 2.8 at 750 rad, 2.5 at 1500 rad and 2.25 at 2250 rad. While, 6000 ppm of NaCl showed the lowest number of shoots at 1500 and 2250 rad (0.5). Similar findings were obtained by (Epstein et al. (1980); Francois et al. (1986) and Sharp et al. (1990) that plant response to salt stress varies at different developmental stages. Increasing salinity during plant development would delay germination, vegetative growth reduction and formation of thinner roots. It is well known that one of the first plant responses to salinity stress is a reduction in leaf growth rate with associated reduction in leaf area available for photosynthesis. Subsequently, the excessive accumulation of salt can lead to death of tissues, organs and whole plants (Munns and Termaat, 1986; Munns, 1993). Salinization can inhibit both cell division and cell expansion in growing tissues of roots, stems and leaves (Zidan et al., 1990).

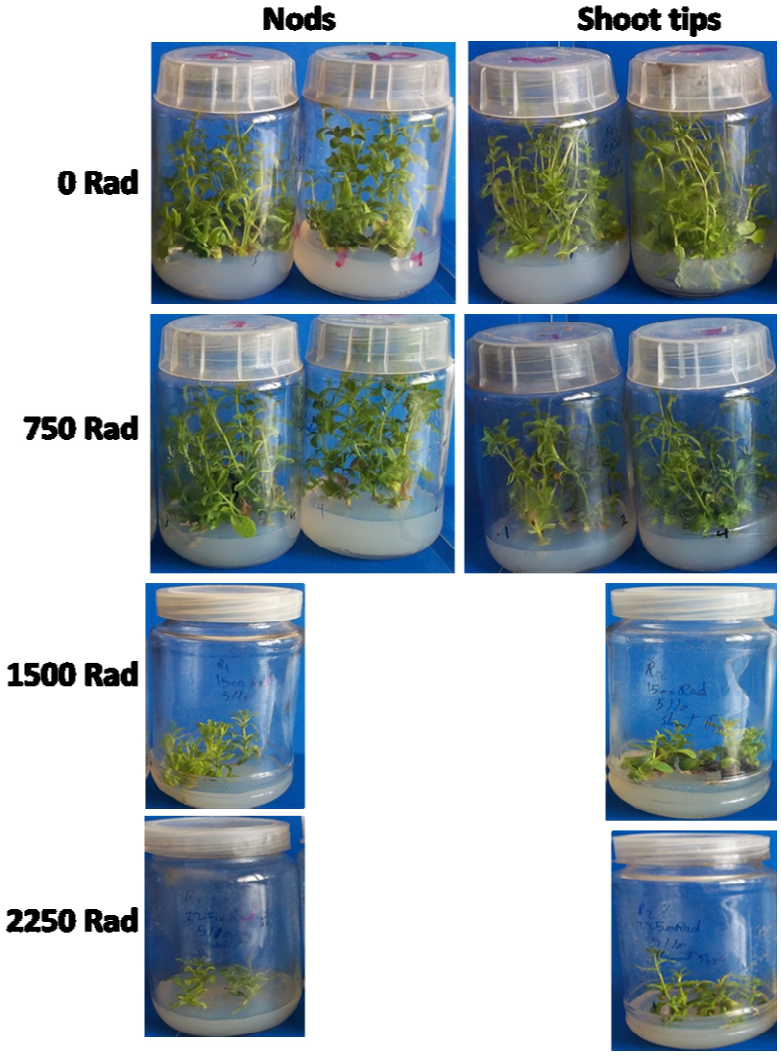
Effect of gamma rays on the growth traits of micropropagated plant

The results in Table 4 that supported with Fig. 3, revealed that the dose of 750 rad was the activated dose and enhanced all characters of propagated shoots produced from both shoot tip and node explants. While, the other two doses (1500 and 2250 rad) gave very close effects for all traits in both explants, except the length of inter node trait was longer (1 cm) at 2250 rad compared with 1500 rad in both explants (0.5 and 0.3 cm) respectively. The obtained data revealed that, highly significant differences between gamma ray doses were recorded.

Table 4. Effect of Gamma rays on the growth traits of micropropagated plants obtained from two explants resources at four gamma ray doses.

Doses (rad)	Explants	Responded explants	Plant length (cm)	No. of shoots	No. of leaves	Length of inter node (cm)	Average	Mean
0	Shoot tip Node	5 5	3.5 5.4	3 2.9	48.5 50	1.1 1.4	12.22 12.94	12.58
Mean		5	4.45	2.95	49.25	1.25		
750	Shoot tip Node	5 5	5 6.1	3.1 3.2	68.3 55	1.6 1.8	16.6 14.22	15.41
Mean		5	5.55	3.15	61.65	1.7		
1500	Shoot tip Node	4.7 4.7	1.4 1.78	1.6 1.9	18.75 26.9	0.5 0.3	5.39 7.11	6.25
Mean		4.7	1.59	1.75	22.82	0.4		
2250	Shoot tip Node	4.3 3.3	1.8 1.9	1.9 2.1	18.5 19	1 1	5.5 5.46	5.48
Mean		3.8	1.85	2	18.75	1		
Mean	Shoot tip Node	4.8 4.5	2.9 3.8	2.4 2.5	38.5 37.7	1 1.1	9.92 9.92	9.92

Fig. 3: propagated *Stevia* plants obtained from explants.
{A (Node) and B (Shoot tip)} treated with four gamma ray doses.



The dose of 750 rad was that activated dose and gave the highest values of plant length, number of shoots, number of leaves, length of inter node and normal morphology in both explants. While, higher doses of gamma ray (1500 and 2250 rad) caused plant dwarfism. The data obtained by Mathius et al. (1995) showed significant differences between the clones and between irradiation doses within each clone**.** They found that the highest growth rate with normal morphology was obtained with a 1500 rad gamma ray dose.

Effects of gamma rays on steviosides production in micropropagated plants

Stevioside is major low-calorie diterpene steviol glycosides in the leaves of *Stevia rebaudiana*. It the widely used as natural sweeteners for diabetic patients, but the long extraction procedures required and the optimization of product yield present challenging problems. The data presented in Fig. 4 revealed that *Stevia* leaves which produced from propagated clones at 0.0 rad gave nearly duplicated amount of stevioside (505.51 mg/100g with 0.5 %), compared with the original plant (278.9 mg/100g with 0.28 %).

The amount of stevioside decreased dramatically as the dose of gamma rays increased. There are no available references about bioactive components in *Stevia* plants produced through tissue culture (micropropagated and regenerated plants) or gamma rays irradiated plants. Under field conditions Kolb et al. (2001) used 30 samples of *S. rebaudiana* from Misiones (Northeastern Argentina), and the stevioside content found ranged between 3.78 and 9.75% (weight) whereas, the Rebaudiside A content ranged between 1.62 and 7.27% (weight). These results are in agreement with those obtained by Ali (1998) in garlic who found that tissue culture change the content of bioactive component profiles in somaclonal variants. Badria and Ali (1999) also found that allicin bioactive component in garlic was increased and decreased on somaclonal variants using HPLC analysis.

Molecular analysis of polymorphism based on RAPD marker

Eight random primers were used to identify the genetic variations between some selected salt tolerant mutants and high stevioside production somaclonal variants. Three primers BB1, BB3 and BB5 were not informative and produced monomorphic bands. On the other hand, five primers (CA-1, OPD-07, OPA-19, OPB-08 and OPV-07) gave scorable fragments (Table 5 and Fig. 5 A). The results of four responded random primers showed that there were genetic variations between the selected mutants under different concentrations of NaCl. The total number of amplified DNA fragments varied between four (CA-1 primer) and eight (OPA-19 and OPB-08 primers). The polymorphism percentage ranged from 33.3% (OPD-07 primer) to 100% (CA-1 primer).

Fig. 4: The amount of stevioside in propagated plants produced from explants treated with four doses gamma rays.

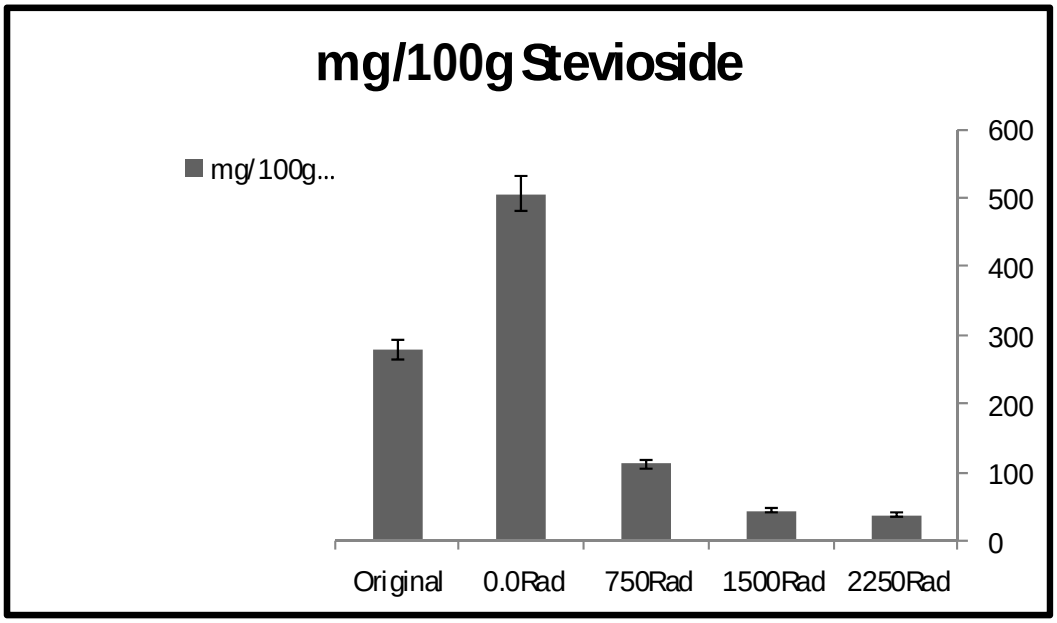
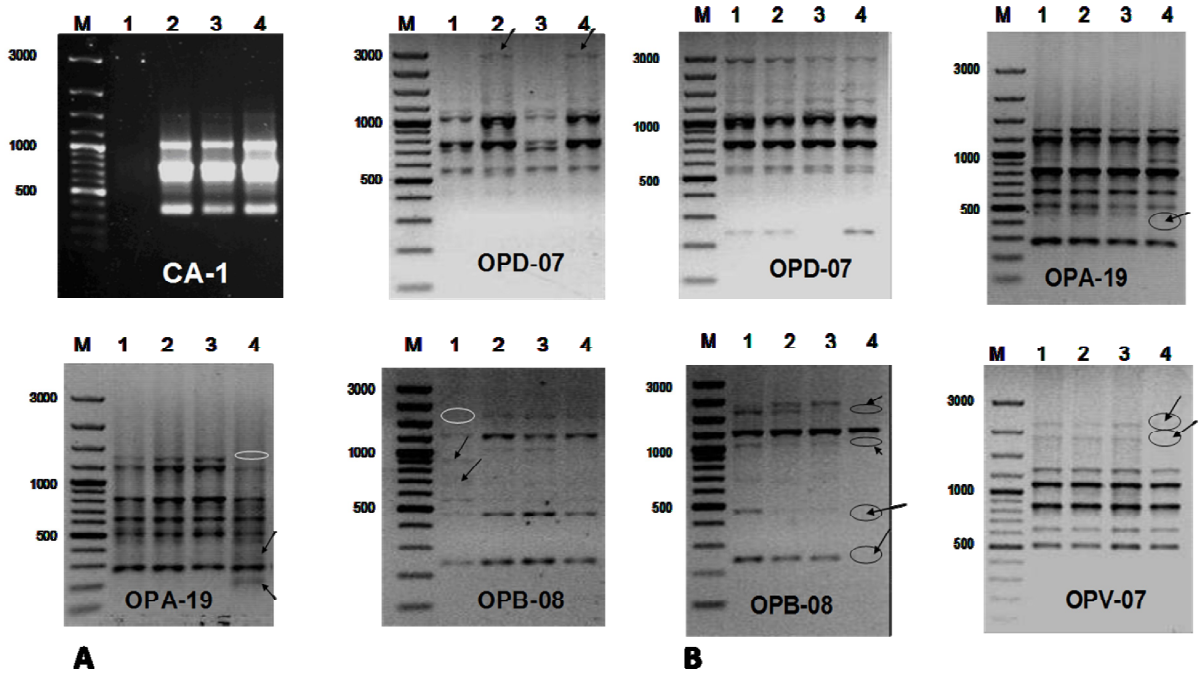


Table 5. Distribution of RAPD markers among the induced mutants and somaclones.

Primer	Total bands	Monomorphic bands	Polymorphic bands	Polymorphism percentage
Salt tolerant mutants				
CA-1	4	0	4	100%
OPD-07	4	3	1	33.3%
OPA-19	8	5	3	37.5%
OPB-08	8	4	4	50.0%
OPV-07	7	7	0	00.0%
Total	31	19	12	38.7%
Stevioside produced somaclones				
OPD-07	6	5	1	16.6%
OPA-19	8	6	2	25.0%
OPB-08	6	1	5	83.3%
OPV-07	7	5	2	28.5%
Total	27	17	10	37.0%

Fig. 5: RAPD banding pattern of four Stevia mutants.

(A) produced under salinity stress, (1) 0.0 ppm NaCl concentration, (2) 2000 ppm, (3) 4000 ppm and (4) 6000 ppm. (B), Stevioside produced somaclones (M) 100bp DNA ladder, (1) original plant, (2-4) Stevia plants propagated from 0.0 rad treated explants. Arrows and circles indicated some positive and /or negative markers.



Results of the RAPD analysis showed that there were two out of 12 polymorphic fragments were unique which detected by OPA-19 primer may be related to the highest salinity stress (6000 ppm). As well as, there were positive and negative polymorphic fragments related to salinity stresses (2000, 4000 and 6000 ppm) produced by the primer OPB-08. Somaclonal variations among selected propagated plants and their original plant were tested by RAPD analysis. Among the primers used to identify the genetic variation in the selected micropropagated plants (0.0 rad). The primer OPA-19 amplified 8 bands followed by primer OPV-07 (7) whereas, the primers OPD-07 and OPB-08 amplified six DNA fragments (Table 5 and Fig. 5 B).The polymorphism percentage ranged from 16.6% (OPD-07 primer) to 83.3% (OPB-08 primer).

Molecular techniques such as Random Amplified Polymorphic DNA (RAPD) is often favored over

traditional phenotypic, cytological and biochemical analysis, and generally assess even small variations in the genome. Detection of somaclonal variations using RAPD markers has several advantages, since RAPD markers are technically simple, quick to perform with small amount of DNA and do not require previous information about genome or radioactive labeling (Michelmore et al., 1991).

The obtained results are in correspondence of Mathius et al. (1995) who reported that some useful mutants induced in *Stevia* by using gamma radiation. They used the differences in RAPD banding patterns among the studied plants as markers for variations. Polymorphisms due to RAPD-PCR analysis could be caused by differences in nucleotide sequences at the primer sites (such as point mutations), or by structural rearrangements within the amplified sequence, (e.g., insertions, deletions, inversions, Welsh and McClelland, 1990). It could be concluded that RAPD can be successfully used to detect somaclonal variations among *in vitro* regenerated *Stevia* plants. Numerous researches proved that the sensitivity of RAPD was sufficient enough to detect genetic changes in many of tissue culture derived plants; for instance, Taylor et al. (1995) in sugarcane and Rani et al. (1995) in populous, Saker and Sawahel (1998) and Al-Zahim et al. (1999) in garlic.

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