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Effect of Chemical Mutagen on Some Biochemical Properties of *Stevia rebaudiana* Bertoni

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Abstract

Induced mutagenesis causes an increase variation of some products with limited genetic resources. In this study, the antioxidant enzymes activity, biochemical properties and glycoside content of Stevia were assessed by EMS (chemical mutagen) based on two experiments as factorial with completely randomized design with three replications. The results of the first experiment showed that some properties of regenerated calli were influenced by different concentrations of EMS, different times of exposure and interactions of these two factors. Therefore, more EMS concentration and its duration of exposure indicated fewer regeneration rates and indices of the calcified masses. According to the second experiment, our data showed that EMS mutant activity was related to enzymatic activity and some biochemical properties of regenerated plants at a 1% significance level. Among the mutants, M_{10}, M_6, and M_{19} had the highest enzyme activity and M3 and M10 mutants with + 41.4 and +26.12% revealed the higher percentage of incremental changes in proline accumulation than the control sample. In addition, the highest amount of total protein was allocated to M16, M14 and M3 mutants (values of 0.69, 0.67 and 0.58 mg/g tissue texture, respectively) over the control sample (0.27 mg/ gram of tissue) and M8 mutant had the highest percentage of changes in the amount of stevioside (87.3%) and rebaudioside A (58.3%), respectively. Overall, significant changes were observed in the antioxidant activity and biochemical properties and the amount of sugary glycosides of regenerated plants that could be used to create plants with higher quality traits.

Key words: *Stevia rebaudiana*; EMS; HPLC; Glycoside; Proline

Introduction

Stevia is an herb of the *Asteraceae* family that grows on high latitudes with long days. The genus of *Stevia* has more than 200 species, the LS *rebaudiana* species has a sweetener essence among all species and is considered as a natural sweetener and dietary sugar, which is an appropriate substitute for all kinds of candy, drinks, and sweets. Stevia extract has antioxidant and antiviral properties. This plant propagates in nature through seeds, but the power of seed survival is very low. Seeds hardly sprout, and many seeds are often absent and sterile due to their self-incompatibility phenomenon, and therefore cannot be cultivated. For this reason, in most cases, cuttings and tissue culture are used to propagate it (Lemus-Mondaca *et al*., 2012).

Although *stevia* is one of the most important sources of medicine, its natural habitat is limited, and its accumulation is difficult due to environmental and geographical conditions of plant growth. For this reason, recently, researchers have been attracted to use of biotechnology techniques to increase production of this plant as renewable sources for drug production because of restrictions on natural resources of medicinal plants, as well as difficulties in domestication and cropping of these plants. Utilization of induced mutagenesis especially in plant tissue culture and plant breeding can be a helpful tool to create variance

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among different plant species (Jain & Spencer, 2006).
Two types of mutagens, physical and chemical, have been used to enhance desired metabolic profile in both vegetative grown crops as well as seeded crops. For instance, the effect of various concentrations of EMS on different vinegar leaves of African violets was investigated under in vitro environment. The results showed that survival and formation of aerial parts of plants decreased by increasing EMS concentration and time of exposure (Fang *et al*., 2011). In another study, the effect of gamma radiation was evaluated on *Raphanus sativus* L. showing 8% increase of antioxidant compounds (Jang *et al*., 2005). In the previous study, the effect of gamma radiation on antioxidant enzyme activity of peanut resulted in modification of phenolic, flavonoids, tannin contents and antioxidant activity (de Camargo *et al*., 2012).
In general, chemical mutagenesis is more likely to be present because that they are easier to use, do not require specialized equipment and they are able to provide high frequency of mutations. Compared to radiation methods, chemical mutations lead to single nucleotide polymorphism (SNPs) instead of deletion and substitution. Among the chemical mutagens, EMS is the most widely used agents which alkylates guanine residues and causes DNA polymerase to place thymine preferentially in place of cytosine against o-6-ethyl guanine during DNA replication, resulting in point mutations. The majority of changes (70 to 90%) in the mutated populations with EMS are GC-to-AT substitution (Waugh *et al*., 2006).
The objective of this study was 1) to mutate stevia plant by different concentration of EMS mutagen under various times, 2) then, measure some antioxidant enzyme activity, biochemical and glycoside contents of regenerated stevia with probable desirable characteristics for high proliferation and exploitation of optimal plants.

Materials and methods

Plant material

For EMS treatments, straw explants were cut from each seedling and sterilized by 70% alcohol for 30 s and 2% sodium hypochlorite solution for 20 min, then washed with deionized distilled water. The sterile explants were placed on MS medium without hormones including 3% sucrose apnd 0.8% agarose (pH 5.8) to isolate examined explants for callus induction. The leaf explants isolated from one-month old seedlings were cultivated on MS medium supplemented with 0.1 mg/l TDZ. After six weeks, callus was formed and subculture of each explant was carried out in the same medium every three weeks. Then the calli were cut into small pieces in a size of five millimeters in order to expose to different concentration of EMS mutagen.

EMS mutagen treatments

The calli were dipped in the solution of 0.1, 0.2, 0.5 % v/v EMS with the exposure time of 30, 60 and 120 s, respectively. After that, calli were washed with sterile water twice and placed on regeneration medium for direct shoot bud organogenesis. The treated calli were incubated at 25 ± 2ºC under 8/16 h photoperiod with 2500-3000 light intensity and 75-85 % of relative humidity.

Antioxidant enzyme activity

Catalase enzyme activity (CAT)

20 µl of total protein extracts were combined with 980 µl of phosphate buffer containing 2 mM of H_2O_2, and their absorbance was calculated at 240 nm using the spectrophotometer. The enzyme activity was calculated based on Beyer-Lambert law and quenching factor of 40 $\mu M^{-1} cm^{-1}$ (Luck, 1974).

Poly phenol peroxidase activity

In order to analyze poly phenol peroxidase activity, 2 ml of extract from each sample were inoculated by 2 M phosphate buffer, pH 6.0, and 0.2 M pirogalol, then their absorbance was calculated at 430 nm by the spectrophotometer (Raymond *et al.,* 1993).

Peroxidase enzyme activity (POD)

To measure POD activity, 20 µl of total protein extracts was added to the solution of 490 µl of 225 mM H_2O_2 and 490 µl of 45 mM guaiacol solution at low temperature (on ice). The

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absorbance difference was analyzed at 470 nm by the spectrophotometer. The enzyme unit was on µm per gram of fresh tissue per minute (µm/g/m) (In *et al*., 2007).

Proline assay

Proline assay was carried out based on Bates *et al*. (1973) procedure. The fresh aerial organs were converted to powder by liquid nitrogen and then 10 ml of 3% sulfosalicylic acid was added to the mixture. 2 ml of the above extract, 2 ml ninhydrin and 2 ml acetic acid were mixed in one test tube and boiled for one hour. To stop the reaction, the samples were quickly transferred to a mixture of water and ice for 20 min. Then, 4 ml toluene was added to each sample. The amount of proline was investigated by the reaction of proline amino acid with ninhydrin in acidic solution which results in the appearance of red color in the mixture. Absorbance analysis of each sample was performed at 520 nm by spectrophotometer and proline concentration was analyzed based on proline standard curve in µg/g fresh weigh.

Phenylalanine ammonia-lyase enzyme activity (PAL)

The 0.3 g weighed of plant tissue was homogenized in 6.5 ml of Tris-HCl buffer (pH 8.8) containing 15 mM beta-mercaptoethanol and the obtained mixture was centrifuged 5000 g for 30 min. Then, the extraction buffer (1 ml), 10 mM phenylalanine (0.5 ml), distilled water (0.4 ml), and enzyme extract (0.1 ml) were incubated at 37 ºC for 1 hour. The reaction was stopped by addition of 0.5 ml of 6 M chloride acid and then added to a solution of 1.5 ml ethyl acetate. The ethyl acetate was collected and evaporated at room temperature. The remaining phase, which is the same as cinnamic acid, was dissolved in 3 ml of 0.05 M NaOH and cinnamic acid concentration was measured at 290 nm using an extinction coefficient of 9500 $M^{-1}cm^{-1}$.
The activity of this enzyme is determined by the rate of conversion of phenylalanine to trans-cinnamic acid. An activity unit of PAL was defined as 1 micromole of cinnamic acid produced per minute per gram of protein (Wang *et al*. 2006).

Total protein assay

The 2 µl extract of each sample was mixed with 970 µl of Bradford reagent and distilled water to a volume of 1 ml in a 1.5 ml tube (Bradford, 1976). Then, the absorbance was calculated at 595 nm using the spectrophotometer.

Data analysis in total protein assay

The average light absorption for each sample was calculated based on standard curve of total protein content for each sample. The experiments above mentioned were performed in three replications on the basis of completely randomized design. Our data was analyzed by SAS software and comparison of mean treatments was done in accordance with Duncan multiple range tests at the level of 5% difference using MSTAT-C software.

Extraction and HPLC analysis of stevioside and rebaudioside A

Amount of 100 mg of dry leaves were mixed in 10 ml of pure methanol for 15 minutes. Then the methanol was evaporated at 45°C and then added to n-hexane (20 ml) to be neutralized. After evaporation of the solvent, 5 milliliters of solution (including acetonitrile and water (80:20)) added and filtered to subjected to HPLC analysis (Rahi *et al*., 2010).
Amount of 10 µl of the extract was injected into chromatography column with specimens of Cosmosil NH2-MS with a length of 15 cm, a diameter of 4.6 mm and a diameter of 5 micrometers attached to the HPLC device of the Unicam-crystal-200 model. The mobile phase consisted of distilled water and acetonitrile with isocratic conditions, which passed through the column with a ratio of 20% water and 80% of acetonitrile at a rate of 1 ml/min. A diode array detector was used at a wavelength of 210 nm. The pump pressure was set at 800 psi and the amount of each substance was compared to standard courier by comparing the inhibition time of the output courier and the surface area under their curve.

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Results and Discussion

First experiment

Callus explants were treated with different doses of 0.1, 0.2 and 0.5 % v/v EMS and placed on regeneration medium i.e., MS + 1 mg/l BAP + 1 mg/l NAA. Among the different EMS concentrations, 0.1 % v/v was the dose on which explants showed the highest regeneration frequency in 30 min, while 0.5 doses showed the lowest regeneration frequency level (Fig. 1). The highest regeneration was observed in the control treatment and the level of regeneration decreased significantly by time passed. One of the important issues in the field of in vitro mutation is the penetration of mutagen into vegetative tissue, which can increase the effect of mutagen. Mutation creation at in vitro culture can increase the penetration rate of mutagen and results in productivity and improvement of products (van Harten, 1998). Lee *et al*. (2003) suggested that the regeneration rate of callus under EMS treatments was strongly influenced by concentration and exposure time. In the previous study, the effects of different concentrations of EMS (0.2, 0.4 and 0.6%) were investigated at different times (30, 60, 120 and 240 minutes) on African violet leaves in MS medium. The results showed that the survival rate and the formation of aerial parts of plants at 0.6% EMS concentration were zero at 120 and 240 minutes. The highest percentage of regeneration and formation of aerial parts of samples exposed to EMS was 0.2% EMS treatment (Fang & Traore, 2011).

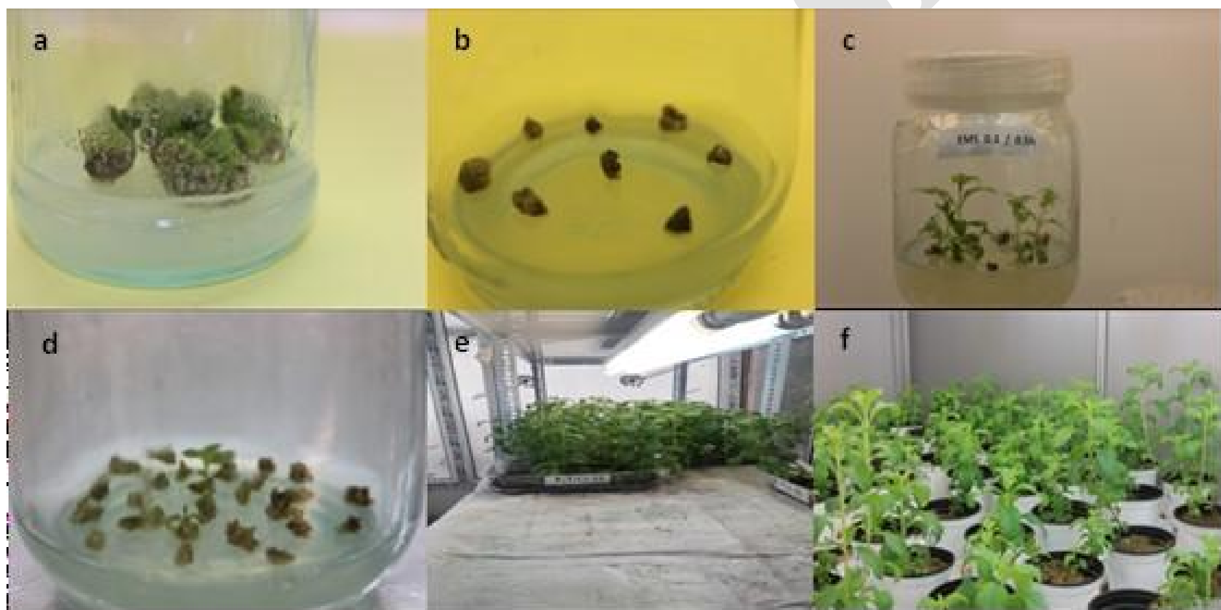

Fig. 1. Different steps of *Stevia rebaudiana* regeneration from EMS treated explants: (a), calli induced from leaf explants, (b), No regeneration from calli treated with 0.2% EMS for 120 minutes. (c), shoot regeneration from calli treated with 0.1% EMS for 30 minutes. (d), elongation of shoots regenerated from calli treated with 0.2% EMS for 30 minutes. (e), Acclimated plantlets (f), transfer plantlets into vases under salt stress.

Second experiment

The results of variance analysis showed that EMS mutation treatment had a significant effect on some enzymatic activities such as catalase, peroxidase, polyphenol oxidase and free radical inhibitor (Table 1). The comparison between average amount of catalase enzyme showed that among the 18 samples exposed to EMS, 15 samples had a higher catalase enzyme than control, as M10, M5 and M11 mutants (with values of 1.57, 1.23 and 1.22 enzyme unit/mg protein/min) showed the highest amount of enzyme compared to the control (0.26 units/mg

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protein/min), and the lowest amount of enzyme was related to M2 mutant with 0.24 enzyme unit/mg protein/min (Table 2).
The comparison between mean value of peroxidase showed that among the mutants studied, 12 mutants exhibited the highest activity of this enzyme, as M13 (30.5%), M15 (28.6%) and M19 (28.1%) mutants showed the ighest amount of peroxidase in comparison with control, and M3 mutant (with -40.8%) had the highest percentage of reduction. Also, the results showed that 7 mutants had a higher activity of polyphenol oxidase than controls, the highest amount of this enzyme was assigned to M10, M6, M19 mutants (with values of 0.1, 0.094 and 0.094, respectively) compared to the control sample (0.06), and the lowest activity of polyphenol oxidase enzyme was related to M3 (0.036) and M9 (0.037) mutants. The mean comparison of free radicals showed that 11 mutants had a higher inhibition than the control sample, in terms of M6, M19 and M4 mutants exhibited the highest percentage of incremental changes (39.5, 38.8 and 38.4%), and M15, M7, M5 mutants had the highest percentage of reduction (-18.9, -13.3 and -13.2%, respectively) compared to the control sample.
The obtained data from this research was consistent with the study of Khalil *et al*. (2015) reported on effects of different doses of gamma rays (5, 10, 15, 20 grays) on regenerated callus of stevia leaves. The results indicated that different doses of gamma rays increased the amount of antioxidant enzymes (DPPH) at all levels, which had a significant difference at 5% level with the control, however, the group treated by 20 grays of gamma radiation showed the highest antioxidant enzyme compared with the remaining doses of gamma rays (Khalil *et al*., 2015).
In another report, the use of different doses of gamma rays on almonds increased the antioxidant capacity of this plant (Harrison & Were, 2007). The results of this study showed that the amount of antioxidant enzymes (superoxide dismutase, peroxidase, ascorbate) was investigated aiming at evaluating the effect of gamma radiation on *Citrus limon* L. protoplast in order to increase resistance to salt stress. The results showed that the amount of peroxidase, catalase, and glutathione reductase enhanced by increasing levels of gamma-ray doses at different levels of salinity. In a similar study, the effects of gamma rays (doses of 100, 200, 300 and 400 grays) were investigated on some biochemical properties of two wheat genotypes (*Triticum aestivum* L.) with names as bright and mutant T-65-58-8 in greenhouse conditions (Borzouei *et al*., 2013). The results revealed that the peroxidase activity of mutant line at doses of 100, 200, 300 and 400 grays was about twice as high as non-irradiation conditions, so that the reaction of the plant in response to some doses of the pathogamy made changes in metabolic pathways and synthesis of biological molecules contained in plant cells (Borzouei *et al*., 2013). Since peroxidase enzyme is necessary for a number of intracellular tasks such as ligninization, tissue build-up, prolongation of cells, growth and regulation and cell wall synthesis, cell exposure to mutagens causes changes in the cell (Wi *et al*., 2007). There is an evidence that the activity of peroxidase, catalase, and glutathione reductase enzymes is increased by degradation of reactive oxygen species through environmental stresses or irradiation with gamma rays (Sgherri *et al*., 2000).
The results of variance analysis showed that the effect of EMS mutation was significant on some biochemical properties including electrolyte leakage, relative humidity, proline, ammonilase enzyme and total protein in regenerated stevia plants at 1% level (Table 3).
The mean of traits showed that among samples studied, 6 mutants had relative leaf moisture content more than the control sample. So that the M14 and M3 mutants with +31.46 and +13.15% had the highest percentage of changes, and the M15 mutant with 25.05% showed the highest percentage of reduction. Moreover, the percentage of leakage of electrolyte in mutants showed that the highest percentage of leakage belonged to M9 mutant (+28.2% leakage) and the lowest belonged to M11 mutant (14.86% leakage) compared to the control sample (23.62% leakage). Since one of the most important factors in maintaining of plant cell survival is the ability to maintain cellular water, so measurement of relative humidity in the leaf can be considered as an indicator for evaluation of cellular stability. To identify tolerant

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cultivars, cultivars with more relative water content than susceptible cultivars have a higher content of relative humidity (Sánchez-Rodríguez *et al*., 2010). Akhtar (2014) reported the application of different concentrations of EMS (4, 8, 16, 24 and 32 mM) on tomato plants (*Lycopersicon esculentum* L.) under thermal conditions, which resulted in increasing the relative humidity of leaves, in terms that 16 and 24 mM of EMS concentrations caused the highest relative humidity, which implicated that the EMS mutation causes more water to be absorbed in plant samples (Akhtar, 2014).

Based on proline results, 14 mutants had a higher proline concentration than the control sample, as M3 (+41.4 %) and M10 (+26.12%) mutants showed the highest percentage of changes and M4 (-29.48 %) and M7 (-19.40%) mutants showed the highest percentage of declining changes.

Table 1. Variance analysis of antioxidant enzymes in samples treated by EMS based on randomized complete design.

Source of variance	df	Catalase	Peroxidase	Polyphenoloxidase	DPPH
Treatment	18	0.504**	258.45**	0.0012**	294.27**
Error	38	0.00029	10.37	0.00002	4.91
Coefficient of variation	-	2.64	6.01	8.34	3.94

*Significance difference in 1 %

Table 2. Mean comparison of some antioxidant enzymes in samples treated by EMS

Treatment	Code	Catalase	Change (%)	Peroxidase	Change (%)	Polyphenoloxidase	Change (%)	DPPH	Change (%)
E0T0	Control	0.26 m	0	51.18 fgh	0	0.060 de	0	51.69 def	0
E1T1	M2	0.24 m	-7.69	52.81 efg	+3.18	0.044 ghi	-26.6	50.83 ef	-1.6
E1T1	M3	0.26 m	0	30.26 j	-40.8	0.036 j	-40	55.28 d	+6.9
E1T1	M4	0.98 d	+277	49.74 gh	-2.81	0.071 c	+18.3	71.44 a	+38.2
E1T1	M5	1.23 b	+373	54.81 defg	+7.09	0.085 b	+41.6	44.83 gh	-13.2
E1T1	M6	0.39 k	+50	39.35 i	-23.1	0.094 a	+56.6	72.15 a	+39.5
E1T1	M7	0.50 h	+92.3	57.34 cde	+12	0.050 fg	-16.6	44.83 gh	-13.3
E1T1	M8	1.14 c	+338	57.71 cde	+12.7	0.039 hij	-35	60.62 c	+17.2
E1T1	M9	0.67 f	+158	54.89 defg	+7.25	0.037 ij	-38.3	46.34 g	-10.3
E1T2	M10	1.57 a	+504	54.23 efg	+5.96	0.100 a	+66.6	66.38 b	+28.4
E1T2	M11	1.22 b	+369	60.41 bc	+18	0.068 cd	+13.3	63.15 bc	+22.1
E1T2	M12	0.33 l	+26.9	41.77 i	-18.3	0.058 ef	-3.33	48.42 fg	-6.3
E1T2	M13	0.48 i	+84.6	66.79 a	+30.5	0.051 efg	-15	53.29 de	+3.1
E1T2	M14	0.16 n	-38.4	47.45 h	-7.29	0.057 ef	-5	47.12 g	-8.8
E1T2	M15	0.42 j	+61.5	65.85 a	+28.6	0.048 g	-20	41.90 h	-18.9
E2T1	M16	0.91 e	+250	50.34 gh	-1.64	0.045 gh	-25	52.45 de	+1.4
E2T1	M17	0.53 g	+103	59.74 cd	+16.7	0.051 fg	-15	61.87 c	+19.6
E2T1	M18	0.56 g	+115	56.24 cdef	+9.89	0.071 c	+18.3	63.75 bc	+23.3
E3T1	M19	0.34 l	+30.7	65.59 ab	+28.1	0.094 a	+56.6	71.78 a	+38.8

In each column, means that have at least one letter in common didn't show any significance difference. + and – indicates percentage of increase and decrease than control.

Table 3. Variance analysis of biochemical properties in samples treated by EMS based on randomized enzyme.

Source of variance	df	Leakage percentage	Content of relative humidity	Proline	PAL	Total protein
Treatment	18	33.78**	173.86**	0.006**	5.458**	0.077**
Error	38	0.73	0.153	0.0001	0.0032	0.00005
Coefficient of variance		3.91	0.766	4.32	1.422	1.772

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In a study reported on resistance to the salinity of soy mutated plants, it was found that mutated samples showed more proline accumulation under 90 mM salinity than the control sample, so that the mutated sample (150-2/504-05) had the highest transcription of the Gm P5Cs gene encoding proline-5-carboxylase synthase enzyme. The accumulation of this gene resulted in the increase of proline concentration. Ma et al. (2008) suggested that expression of P5Cs (proline-5-carboxylic synthase) promotes conversion of proline 5-carboxylic to proline (Ma *et al*., 2008). In another study, high doses of gamma rays (500 grays) increased the amount of proline by 16 percent (Borzouei *et al*., 2013).

Proline is a compound that has a higher efficiency for tensile protection compared to other conventional adaptive osmolites, especially conventional sugars and alcoholic beverages. Proline has a direct protective effect on the stabilization of macromolecules and their hydration layers indirectly due to their antioxidant properties (Shevyakova *et al*., 2009). These compounds interact directly or indirectly with macromolecules and help maintain their natural shape and structure under stress conditions. The role of proline antioxidants is on its ability to disable hydroxyl radicals and other high activity compounds that are produced under stress conditions and disturb electron transport in chloroplasts and mitochondria, to protect proteins and membranes against damage (Bohnert *et al*., 1995).

According to the mean of phenyl ammonialis (PAL) activity, 15 mutants had more enzymatic activity than the control sample, with the highest activity of this enzyme in M7, M13 and M10 mutants (Table 4). This enzyme is the first enzyme responsible for phenolic compounds synthesis, which converts L-phenylalanine into trans-cinnamic acid, by activation the phenylpropanoid pathway and increases production of phenolic compounds. This stage is an important biochemical reaction in development and defense stages of plants (Chang *et al*., 2008). Also, among the mutants studied, 15 mutants had higher total protein content than the control sample, with the highest amount assigned to M16, M14 and M3 mutants (155.5, 148.18, 114.8% and, respectively). The use of mutagenic agents can affect the amount of protein in plants. So that, in carrot genotype, a single-dose gamma-gamma mutation reduces the amount of protein (Kiong *et al*., 2008). Increasing the amount of proteins is considered as a protective mechanism in plants (Al-Rumaih & Al-Rumaih, 2008).

Physical and chemical mutations induced growth or alteration of plant characteristics by inducing cytological, biochemical, physiological, morphological and genetic changes in cells and tissues such as thylacoid membrane dilatation, photosynthetic changes, and system changes in antioxidant responses of the plant (El Sherif *et al*., 2011). The mutagenesis factor can act as an energetic process in the form of ATP influence on photosynthesis process in plants, which results in enhancement of photosynthesis process in plants, for example, gamma rays activate proton pump membranes, followed by conversion of ADP to ATP, which affects chlorophyll content (Maslobrod *et al*., 2010).

In one study, researchers investigated the effect of 0.1 and 0.12% EMS on chickpea which showed that the highest amount of protein was present in 0.1% EMS treatments and total protein content in treated plants was less than control by 0.12% EMS. It could be suggested that mutation can affect changing the amount of protein. In addition, EMS mutation is capable to modify nucleotides and substitution of AT nucleotides instead of CG in the genome, resulting in the creation of mutant plants with different protein contents relative to each other and the control plant (Arulbalachandran & Mullainathan, 2009; Wattoo *et al*., 2012).

Table 5 shows the variance analysis of sugar glycosides under EMS concentration. The mean of stevioside rate indicated 13 mutants which had a higher steviosidic sugar content than control sample. The highest amount of this sugar belonged to mutants of M8 (55.60 mg/g dry tissue), M18 (53.27 mg/g dry tissue) and M4 (94.47 mg/g dry tissue), compared to control as 19.59 mg/g dry tissue, while the lowest amount belonged to M9 (19.34 mg /g dry tissue), M11 (24.22 mg/g dry tissue) and M7 (24.18 mg/g dry tissue) mutants. The amount of stevioside and rebaudioside A was

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determined based on the calculation of the sub-curved surface (Table 6). On the basis of the result, regenerated plants from calli exposed to 0.1% EMS in a 30 minute time period (M8) showed the highest amounts of aveozide and rebaudioside A among all samples (Table 6).

Table 4. Mean comparison of phytochemical properties of some enzymes in samples treated by EMS

Treatment	Code	Relative Humidity	Change (%)	Leakage Percent	Change (%)	Proline	Change (%)	PAL	Change (%)	Total Protein	Change (%)
E0T0	Control	54.76 e	0	23.62 bcd	0	0.268 ef	0	2.58m	0	0.27 l	0
E1T1	M2	51.76 f	-5.48	21.49 ef	-9.02	0.305 c	+13.81	3.16j	+22.48	0.33 k	+22.22
E1T1	M3	61.96 b	+13.15	22.53de	-4.61	0.378 a	+41.04	4.46e	+72.87	0.58 c	+114.8
E1T1	M4	43.68 l	-20.23	18.79 hi	-20.45	0.189 h	-29.48	2.34n	-9.30	0.39 j	+44.44
E1T1	M5	48.98 h	-10.56	21.86 ef	-7.45	0.320 bc	+19.40	4.89d	+89.53	0.52 e	+92.59
E1T1	M6	44.90 k	-18.01	23.08 fg	-2.29	0.314 c	+17.16	2.23o	-13.57	0.47 gh	+74.07
E1T1	M7	44.78 k	-18.22	26.75 a	+13.25	0.216 g	-19.40	6.48a	+151.1	0.28 l	+3.70
E1T1	M8	55.09 e	+0.60	24.99 b	+5.80	0.324 bc	+20.90	4.08g	+58.14	0.46 hi	+70.37
E1T1	M9	54.78 e	+0.04	28.02 a	+18.63	0.251 f	-6.34	4.5e	+74.42	0.56 d	+107.4
E1T2	M10	46.81 i	-14.52	19.73 gh	-16.47	0.338 b	+26.12	5.72b	+121.7	0.53 e	+96.30
E1T2	M11	55.76 d	+1.83	14.86 k	-37.09	0.308 c	+14.93	4.91d	+90.31	0.50 f	+85.19
E1T2	M12	46.55 ij	-14.99	23.43 cd	-0.80	0.284 de	+5.97	2.56m	-0.78	0.45 i	+66.67
E1T2	M13	49.84 g	-8.98	22.63 de	-4.19	0.275 e	+2.61	6.48a	+151.1	0.39 j	+44.44
E1T2	M14	71.99 a	+31.46	17.77 ij	-24.77	0.313 c	+16.79	3.05k	+18.22	0.67 b	+148.1
E1T2	M15	41.04 m	-25.05	16.62 j	-29.64	0.309 c	+15.30	5.15c	+99.61	0.19 m	-29.63
E2T1	M16	59.18 c	+8.07	24.36 bc	+3.13	0.222 g	-17.16	4.28f	+65.89	0.69 a	+155.5
E2T1	M17	45.98 j	-16.03	24.75 bc	+4.78	0.320 bc	+19.40	3.27i	+26.74	0.48 g	+77.78
E2T1	M18	44.37 k	-18.97	21.03 fg	-10.97	0.305 c	+13.81	2.77l	+7.36	0.19 m	-29.63
E3T1	M19	48.43 h	-11.56	20.70 fg	-12.36	0.304 cd	+13.43	3.4h	+31.78	0.13 n	-51.85

In each column, means that have at least one letter in common didn't show any significance difference. + and – indicates percentage of increase and decrease than control.

Table 5. Variance analysis of glycoside sugar content (stevioside and ribodioside) of samples treated by EMS

Source of variance	Df	Stevioside	Rebaudioside A
Treatment	18	346.72**	85.54**
Error	38	1.70	0.386
Coefficient of variance		3.64	3.61

Table 6. Mean comparison of glycoside sugar content (stevioside and rebaudioside A) in samples treated by EMS

Treatment	Code	Stevioside	Change (%)	Rebaudioside A	Change (%)
E_0T_0	M_1	29.6 gh	-	19.5 ef	-
E_1T_1	M_2	37.4 e	+26.2	15.8 j	-18.8
E_1T_1	M_3	41.2 d	+38.8	20.3 de	+3.78
E_1T_1	M_4	47.9 c	+61.5	21.5 d	+9.85
E_1T_1	M_5	30.8 g	+3.87	16.3 ij	-16.7
E_1T_1	M_6	28 hi	-5.56	17.2 hij	-12.2
E_1T_1	M_7	24.1 j	-18.5	12 lm	-38/7
E_1T_1	M_8	55.6 a	+87.3	31 a	+58.3
E_1T_1	M_9	19.3 k	-34.8	9.78 n	-50
E_1T_2	M_{10}	35.1 f	+18.3	14.1 k	-27.6
E_1T_2	M_{11}	22.2 j	-25	11.1 mn	-42.9
E_1T_2	M_{12}	43.2 d	+45.6	24 bc	+22.9
E_1T_2	M_{13}	26.6 i	-10.1	14/1 k	-27.9
E_1T_2	M_{14}	30.2 g	+1.89	17.7 ghi	-9.39
E_1T_2	M_{15}	52.6 b	+77.4	24.9 b	+27.4
E_2T_1	M_{16}	36.9 ef	+24.5	18.5 fgh	-5.56
E_2T_1	M_{17}	29.8 gh	+0.57	13.1 kl	-32.9
E_2T_1	M_{18}	53.2 b	+79.4	23.1 c	+18
E_3T_1	M_{19}	36.2 ef	+22.2	19 efg	-2.76

In each column, means that have at least one letter in common didn't show any significance difference. + and – indicates percentage of increase and decrease than control.

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The results of this study were in parallel with (Khan *et al*., 2007) which released the highest amount of stevioside and rebaudioside A in stevia leaf under 0.4 % EMS concentration and at lower concentrations of this mutagen, more stimulatory effects were observed on growth parameters and enzymes activation. In one study, the effects of gamma rays with doses of (2.5, 5, 7.5 and 10 grays) were evaluated on the amount of stevia sugars (Khalil *et al*., 2014). The results showed that the highest amount of stevioside and rebaudioside A was assigned to samples treated with the mutagenic agent. Moreover, the amount of secondary metabolites increased with lower doses of gamma rays. In conclusion, it can be stated that lower doses of EMS treatments have been found effective in altering the biochemical state, antioxidant enzyme activity and glycoside content of *S. rebaudiana* plants, and further generation-wise study of the raised variants may lead to new variety development with desirable traits.

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