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RESEARCH ARTICLE

Chemical Mutagen Effect on Physiological Properties of *Stevia rebaudiana* Bertoni under Salt Stress

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ABSTRACT: The present study was performed to evaluate the effects of different concentrations of ethyl methane sulfonate (0, 0.1, 0.2 and 0.5%) on some physiological characteristics of regenerated plants from calli of stevia at 30, 60 and 120 min under various levels of salinity stress (0, 50 and 100 mM of NaCl). This experiment was carried out based on completely randomized two-factorial designs with three replications. With respect to the result, the regenerated calli became dark and hidden in the medium under exposure time of 120 min, the length of stem regenerated calli was increased under exposure times of 30 and 60 min. Moreover, our data showed that EMS mutagenesis had a significant effect on physiological traits of regenerated stevia under salinity stress at the probability level of 1%. Consequently, the stevia mutants of M10, M11, and M19 showed the highest resistance to different levels of salinity which can be considered as potential samples for further breeding programs.

KEYWORDS: Abiotic stress, EMS, Medicinal plant

INTRODUCTION

Stevia rebaudiana (Bertoni) with approximately more than 200 species belongs to Asteraceae family. Stevia has major natural sweetener and dietary sugar called stevioside in its leaf and stem tissue which could be an appropriate substitute for all kinds of sweets [26]. In addition, stevioside possesses antimicrobial and antifungal properties for medicinal applications.

Stevia has limited natural habitat and its collection is not easy due to its environmental and geographical growth conditions. Although stevia can propagate by seeds in nature, its propagation involves numerous difficulties such as 1) seeds don't have enough power to survive for a long time, 2) seeds hardly sprout, and 3) most seeds are often absent and sterile because of their self-incompatibility phenomenon. However, cuttings and tissue culture methods are known as appropriate tools for propagation of this plant [20]. Currently, the use of biotechnology techniques has been increased aiming at

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high production of stevia for pharmaceutical and industrial applications. Among different types of biotechnology techniques, induced mutagenesis could be a useful tool for the production of genetic variation among plant species which can be conducted through two ways as physical and chemical [11, 13].

Among all types of mutagenesis agents, chemical mutagenesis has many advantages due to being easy to use, high frequency of mutation, simple equipment requirement, and creation of SNP instead of huge chromosomal abnormalities (deletion or substitution) [2]. Moreover, the most common alkylating agents used in mutation induction is ethyl methanesulfonate (EMS) which results in point mutation since thymine substitutes with cytosine against o-6-ethyl guanine by DNA polymerase during DNA replication [10].

In one study, it is proved that EMS causes 70% to 90% of changes in mutated populations by GC-to-AT substitution [24]. In another study, the growth, physiological responses, and steviol glycoside composition of *Stevia rebaudiana(*Bertoni) were assessed under three different concentrations of NaCl (60, 90, and 120 mM) for 4 weeks [28]. Results showed the indirect relationships between chlorophyll contents and salt concentration, as more salt concentration caused decrease in chlorophyll contents (10-70%). However, the enzyme activities of superoxide dismutase, peroxidase, and catalase were increased by 1.0–1.6, 1.2–1.3, and 2.0–4.0 times, respectively. In addition, salt-treated plants showed higher proline contents (17-42 times) than the control sample [28].

In another similar research, the new generation of *Stevia rebaudiana* was produced for higher UGT expression and glycoside profile on the basis of physical and chemical mutagenesis [12]. Based on phytochemical results, the rebaudioside A became two-fold and stevioside content was decreased as (3.2 ± 0.22 % dry wt.) in G plants, while the above glycosides were increased more than two-fold in E plants than control samples. Furthermore, the morphological and physiological traits of stevia were evaluated using EMS mutagen, exhibiting the highest variation of three mutants as M_{10}, M_{11}, M_6 compared to the control sample [4].

Considering the importance of Stevia as a medicinal plant and the ease of EMS utilization for induced mutagenesis, this study was performed to assess physiological properties of regenerated stevia by different doses of EMS mutagen under salt stress to create a new generation of stevia with desirable traits for optimization of its yield.

MATERIALS AND METHODS

Plant material

For EMS treatments, straw explants were cut from each seedling and sterilized by 70% alcohol for the 30s as well as 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 and 0.8% agarose (pH=5.8) to isolate examined explants for callus induction. The 75 leaf-explants isolated from one-month-old seedlings were cultivated on MS medium including 0.1 mg/L TDZ (Three leaves of stevia per glass bottle) After six weeks, 20 calli obtained from differentiation was formed and each callus were cut into 10 pieces forming 200 pieces of callus. Then, 50 calli were selected based on their size (2 × 2 × 2 cm) and appearance characteristics (such as being brittle and fragile) and were sub-cultured in the same medium every three weeks. Then, the calli were divided into small pieces of five millimeters and exposed to different concentration of EMS mutagen.

EMS mutagen treatments

The calli were dipped in EMS solutions (0, 0.1, 0.2, 0.5 % v/v concentrations) under 30, 60 and 120 min of exposure time. 50 calli were considered for each treatment. Three plantlets with the same size were chosen for each treatment. Then, 50 calli washed twice with sterile water and added to regeneration medium in order to direct shoot bud organogenesis. After the calli treatment, the calli were incubated under specific conditions as temperature of 25 ± 2ºC, photoperiod of 8/16 h, light intensity of 2500–3000, and relative humidity of 75–85%. In table 1, the E0-E3 indicated the concentration of EMS as 0-0.5% and T0-T3 showed the exposure time of 0-120 min.

Screening of regenerated plant from callus treated by EMS

Each regenerated plant obtained from the treated callus by EMS mutagen expressed a mutation phenomenon which is followed by creation of colony population with the same age from each individual seedling. The suitable growth of regenerated seedlings was considered as

appropriate criteria in order to label/give a unique code to each individual seedling.

Adaptation of seedlings and their exposure to salinity stress

After *in vitro* growth of seedlings, the root washed with distilled water and then transferred to transparent plastic containers, containing peat moss and perlite (50:50) to preserve moisture of seedling. Cultivars were kept at 25°C and white fluorescence was used to regulate photoperiod (16 hours of light and 8 hours of darkness). When one-month-old seedlings reached to adaptation stage, the adapted plants were transferred to plastic pots. Then, 20 ml of Hoagland solution mixed with 20 ml of the desired salinity treatments and added to samples for two days. The obtained data was calculated based on completely randomized factorial design with 18 mutated specimens (mutants) and three levels of salinity stress (0, 50 and 100 mM) with three replications. Based on the number of mutants and different salt stress levels, the physical slicing method was used for each level of salt stress.

Estimation of total chlorophyll content and leaf carotenoids

Leaf chlorophyll content as well as carotenoids was assessed according to Arnon method [1]. The absorbance of light was determined by spectrophotometer (Shimadzu UV 1800) at 663 and 645 nm wavelengths. The total chlorophyll (mg/g) and carotenoid concentrations were obtained by the following formulas:

$$\text{Total Chl} = \{20.2(A_{645}) + 8.02(A_{663})\} * V/W*1000$$

$$\text{Carotenoid} = \{(1000*A_{480})–1.82\ \text{chla}–85.02\ \text{chl b}/198)\} * V/W*1000$$

In the above formulas, V is the final volume of the sample extracted, W is the weight of sample, and A is the optical absorption of extract.

Total anthocyanin assay

To measure the amount of total anthocyanin, 0.20 g of plant dry tissue washed with 4 ml of 1% methanol chloride solution in a Chinese moss. The solution stored at refrigerator for 24 hours. Then, the solution was centrifuged at 13000 rpm. Absorption of solutions was measured at 530 and 657 nm wavelengths. The following equation was utilized to calculate the anthocyanin level for each extract.

$$A = A_{530} – (0.25*A_{657})$$

Determination of membrane lipids peroxidation

Peroxidation of membrane lipids was calculated by Heath and Packer method [7]. Initially, 0.5 g of fresh leaf tissue blended with liquid nitrogen in a Chinese masonry mill which is followed by addition of one ml of trichloroacetic acid (TCA). The obtained extract was centrifuged at 14000 rpm at 4°C for 20 min. Afterwards, five ml of 20% TCA solution including 0.5% thiobarbituricccid (TBA) mixed with one ml of aqueous solution. The resulting mixture was incubated at 95°C for 30 min and cooled on ice immediately, following by centrifugation at 10000 rpm for five min. The red phosphorus (MDA-TBA) of malondialdehyde was measured by spectrophotometer at a wavelength of 532 nm and its concentration was calculated in grams of nanofibers [7].

Measurement of soluble sugar

To measure the amount of soluble sugar, 0.1 g of fresh tissue blended with 5 ml of 80% ethanol in Chinese moss and put them in bin Mari for 15 min. The lower part of the solution, with 5 mM ethanol 80%, transferred again to a boiling water bath for replication the extraction step for four times. To remove chlorophyll, the extract mixed with chloroform in a ratio of 1 to 5, and then vortex for 50 min. The extraction phase was centrifuged at 10,000 rpm for 10 min. The upper clear part was separated and used to measure the amount of soluble sugar. Measurement of soluble sugar was carried out by intratron according to McCready and his colleagues [18]. Three ml of inverted solution was added to 200 µl of the extract and heated in boiling water for 20 min. After cooling the samples, their absorbance was measured at 620 nm by spectrophotometer [18].

Measurement of total flavonoids

The aluminum chloride colorimetric method was performed for total flavonoids evaluation [21]. Each methanolic extracts (0.5 ml of 1:10 g ml^{-1}) was individually mixed with 1.5 ml of methanol, 0.1 ml of aluminum chloride (10% methanol), 0.1 ml potassium acetate (M1) and 2.8 ml distilled water. The absorbance of each reaction mixture was measured at 415 nm by spectrophotometer.

DPPH free radical inhibitory activity

Different concentrations of extract were mixed with 2 ml methanolic solution of DPPH (0.004%) and then incubated for 30 min in darkness at room temperature. Absorption of samples was read at 517 nm (containing 2 ml of DPPH and 2 ml of methanol). The percentage of free radicals inhibitory (% I) of each extract was calculated using the following formula [19]:

$$\% I = (A_{control} - A_{sample})/A_{control}*100$$

Data analysis

All physiological traits studied as total chlorophyll and carotenoid contents, total anthocyanin, membrane lipid peroxidation, total flavonoid, soluble sugar and the DPPH free radical inhibitory activity were examined using SAS software version 9.1 and the mean comparison of treatments was performed according to Duncan's multi-domain test at %1 difference level using MSTAT-C software version 8.

RESULTS

Effect of EMS on regeneration percentage

Based on the ANOVA results, all of EMS concentration and of exposure duration time had a significant effect ($P<0.01$) on some traits of the plants regenerated from treated calli (Table 1). The results showed that regeneration percentage and number of stems produced as well as the time needed for stems regeneration were affected by different concentrations of EMS (0, 0.1, 0.2 and 0.5%) and various exposure times as 30, 60 and 120 min. All calli treated with different concentrations of EMS were darkened and disappeared by EMS exposure time of 120 min, suggesting the indirect relationship of stem regeneration percentage and increase of EMS exposure time (Table 1). In this case, the all treatments with 120 min of exposure time was removed from the further analysis of this experiment. The highest regeneration percentage (90%) was obtained in (E0T0), while the lowest (0%) was indicated in E2T2 and E3T2 (Table 2). The highest and lowest number of shoots/callus were indicated in E0T0 (18) and E3T2 (0) and E2T2 (0). In addition, the highest number of regeneration days was belonged to E1T2 (67.36) and the lowest one was related to E2T2 (0) and E3T2 (0). Based on the obtained results, the E1T1 showed the most regeneration percentage (66.67%) and number of shoots produced/callus (9) after control treatment. Also, the number of produced shoot from calli was affected by different concentrations of EMS in that way the increase in concentration of EMS treatment as well as the length of EMS exposure enhanced the number of days for regeneration (Table 2).

Table 1. Variance analysis of the effect of different levels of EMS and exposure time on some traits of regenerated calli

Source of variation	df	Regeneration (%)	No. of shoots	No. of regeneration day
Mutant (M)	2	40.99**	5.47**	6.51**
Time (T)	1	47.72**	3.44**	71.93**
M × T	2	4.43**	0.61**	24.63**
Error	14	0.49	0.08	0.04
CV (%)		15.14	14.07	4.62

**Significant difference on probability level of 1%.

Table 2. Mean comparison of some traits such as regeneration percentage, number of shoot produced and number of regeneration days from regenerated callus under EMS treatment

Treatment	Regeneration (%)	No. of shoot produced/callus	No. of regeneration days
E0T0	90[a]	18[a]	16[ab]
E1T1	66.67[a]	9[ab]	30.33[ab]
E1T2	26.67[abc]	6[bc]	67/36[ab]
E2T1	33.33[ab]	5[bc]	44[a]
E2T2	0[c]	0[c]	0[b]
E3T1	6.67[bc]	1[cd]	55.67[a]
E3T2	0[c]	0[c]	0[b]

Means in each column followed by at least one letter in common are not significantly different at the 1% level of probability.

Evaluation of some physiological properties under salt stress

The results of variance analysis showed that the salinity had significant effect on all traits studied at probability level of 1% (Table 3). Moreover, the salt × mutant effect was significant on all traits studied at probability level of 1% as well (Table 3). With respect to mean comparison of some physiologic traits under salinity stress (50 mM level of NaCl), the highest total chlorophyll content was belonged to M10 (1.29 mg/g FW) and M11 (1.37 mg/g FW), while the lowest was related to M9 (0.140 mg/g FW) compared to control sample (0.417) (Table 4). The M10 and M2 treatments showed the highest and lowest

Table 3. Variance analysis of the effect of different levels of salt stress and EMS on some physiological traits of samples

Source of variation	df	Total chlorophyll content (mg/g FW)	Carotenoid (mg/g FW)	Anthocyanin (mg/g FW)	Membrane peroxidation (µmol/g FW)	Soluble sugar (mg/gFW)	Flavonoid (mg/gDW)	Free radical inhibition (%)
Salt (S)	2	2.132**	0.063**	0.900**	6402.8**	0.002**	4010.1**	8750.8**
Mutant (M)	18	1.33**	0.064**	0.026**	131.7**	0.00**	900.1**	840.2**
S × M	36	0.026**	0.002**	0.013**	41.74**	0.000**	1392.2**	40.63**
Error	114	0.000	0.000	0.000	1.13	0.000	1.793	3.197
CV (%)		1.48	6.50	2.57	5.09	6.014	2.22	4.10

**Significant difference on probability level of 1%.

amount of carotenoid as 0.283 mg/g FW and 0.008 mg/g FW, respectively with comparison to control sample (0.050). The anthocyanin content in M13 and M3 had the highest and lowest level as 0.374 mg/g FW and 0.168 mg/g FW, respectively compared to control sample (0.067). With comparison to control sample of membrane peroxidation (12.77), the highest amount of membrane peroxidation was assigned to M9 (31.80 µmol/g FW), while the lowest amount was allocated to M2 (13.30 µmol/g FW). The mutant of M10 exhibited the highest amount of soluble sugar as 0.058 mg/g FW, while the mutants of M1 and M5 showed the lowest amount of this trait as 0.031 mg/g/FW compared to control sample (0.0275) Flavonoid contents reached the highest level in M4 (171.2 mg/g DW), but it indicated the lowest level in M16 (45.86 mg/g DW) which was in contrast to control sample (45.13). The highest free radical scavenging percentage was 60.92% in M19 and the lowest was 30.24% in M14 which was different with control sample as (51.69) (Table 4).

In 100 mM level of NaCl, the highest and lowest total chlorophyll content were allotted to M11 (1.105 mg/g FW) and M9 (0.060 mg/g FW) compared to control treatment as (0.190) (Table 4). In case of carotenoid, the maximum and minimum its amount was assigned to M10 (0.229 mg/g FW) and M2 (0.006 mg/g FW) in comparison with control sample (0.028). The highest amount of anthocyanin was indicated in M19 (0.540 mg/g FW), while the lowest amount was observed in M11 (0.191 mg/g FW) (Table 4) in contrast with control sample (0.406). The maximum level of membrane peroxidation was related to M17 (46.45 µmol/g FW) and the minimum level of this trait was exhibited in M12 (20.81 µmol/g FW) (Table 4) which was different with control sample (30.24). The result of soluable sugar in control sample showed as (0.0332). However, the highest and lowest soluble sugar was indicated in M10 (0.060 mg/g FW) and M14 (0.0312 mg/g FW), respectively (Table 4). For flavonoid content, the mutant of M3 displayed the highest amount as 116.8 mg/g DW and M9 mentioned the lowest amount as 34.33 mg/g DW compared to contro treatment as (90.93). The highest and lowest free radical scavenging percentage was shown in M19 (53.57%) and M5 (14.13%), respectively (Table 4). However, the result of control sample was indicated as 17.48 in the trait studied.

DISCUSSION

Recently, researchers have focused on stevia plant because of its profitable characteristics for human health as medicinal drug. In this case, chemical mutagenesis in an in vitro medium could be an effective tool in order to penetrate mutagenic material into vegetative tissues [25], especially mutation induced by EMS which depends on EMS concentration and EMS exposure time [16].

In this case, Zhu et al. (1995) reported that high concentration of EMS (more than 0.9%) caused reduction in desired effects of mutation in soybean (*Glycine max* L.) [30]. In another study, various concentrations of EMS (0.2, 0.4 and 0.6%) at different times (30, 60, 120 and 240 min) were investigated on leaf samples of African violet plant in MS medium [3]. The results showed lack of survival rate and formation of aerial parts of plants by concentration of 0.6% EMS for 120 and 240 min [3]. The present results showed that the exposure time of EMS for 120 min was injured most calli and removed from the all examination which was in parallel with the results of Zhu et al. (1995).

Based on the results of our study, the mutant of E1T2 (0.1% EMS, 60 min of exposure time) showed the highest amount of photosynthesis pigments (total chlorophyll, carotenoid and anthocyanin contents) which

Table 4. Mean comparison of some physiological traits in stevia sample treated by different variations of EMS and exposure time under 0, 50 and 100 mM salt stress.

Traits	Total chlorophyll content (mg/g FW)			Carotenoid (mg/g FW)			Anthocyanin (mg/g FW)			Membrane peroxidation (µmol/g FW)			Soluble sugar (mg/g FW)			Flavonoid (mg/g DW)			Free radical inhibition (%)		
Salt levels	0	50	100	0	50	100	0	50	100	0	50	100	0	50	100	0	50	100	0	50	100
(M1) E0T0	0.417 lm	0.331 j	0.190 k	0.050 l	0.038 i	0.028 h	0.067 m	0.269 g	0.406 h	12.77 bcde	16.72 fgh	30.24 ef	0.0275 j	0.031 l	0.0332 j	45.13 g	64.73 e	90.93 b	51.69 def	36.83 hi	17.48 i
(M2) E1T1	0.308 n	0.270 lm	0.170 l	0.011 2	0.008 j	0.006 j	0.181 e	0.201 i	0.338 k	9.49 k	13.30 l	25.67 hi	0.0249 k	0.043 d	0.0410 g	43.66 g	50.13 jkl	41.86 m	50.83 ef	39.77 fg	24.08 gh
(M3) E1T1	0.617 j	0.568 i	0.469 h	0.030 m	0.018 j	0.012 ij	0.087 l	0.168 j	0.386 i	12.32 defg	20.80 c	33.41 d	0.0340 fg	0.045 c	0.0460 f	44.33 g	50.66 jk	116.8 a	55.28 d	37.48 ghi	14.61 kj
(M4) E1T1	0.811 i	0.669 g	0.487 g	0.168 f	0.133 e	0.097 e	0.192 d	0.214 h	0.429 g	9.98 jk	14.81 ijkl	28.30 fg	0.0395 c	0.031 l	0.059 a	40.80 h	171.2 a	48.80 k	71.44 a	54.14 b	45.13 b
(M5)E1T1	1.14 f	0.964 e	0.688 e	0.263 c	0.193 c	0.148 c	0.268 b	0.286 f	0.498 d	11.65 efghi	14.66 ijkl	27.67 gh	0.0387 c	0.031 l	0.054 b	62.40 a	49.20 kl	73.46 e	44.83 gh	35.70 i	14.13 k
(M6) E1T1	1.35 d	0.987 d	0.711 d	0.355 b	0.247 b	0.186 b	0.136 h	0.301 e	0.454 f	10.82 hijk	14.50 jkl	24.90 i	0.0301 i	0.038 gh	0.0422 g	52.53 de	53.26 hi	86.33 c	72.15 a	52.45 c	42.37 c
(M7) E1T1	0.98 g	0.751 f	0.529 f	0.094 j	0.074 g	0.039 g	0.112 jk	0.221 h	0.319 l	14.15 b	24.14 b	35.72 c	0.0324 h	0.036 ij	0.0498 e	48.86 f	79.53 c	45.86 l	44.83 gh	35.93 hi	30.04 e
(M8) E1T1	0.428 l	0.295 k	0.146 m	0.234 d	0.171 d	0.112 d	0.111 jk	0.215 h	0.353 j	12.48 cdef	19.51 cd	45.50 a	0.0364 de	0.039 g	0.040 g	62.06 a	48.20 l	64.66 g	60.62 c	47.90 d	40.47 c
(M9) E1T1	0.277 o	0.140 o	0.060 o	0.051 l	0.031 i	0.009 j	0.126 i	0.198 i	0.252 m	17.014 a	31.80 c	40.74 b	0.0368 d	0.039 g	0.0351 i	40.93 h	62.33 f	34.33 n	46.34 g	38.16 gh	25.96 fg

Table 4 Continued. Mean comparison of some physiological traits in stevia sample treated by different variations of EMS and exposure time under 0, 50 and 100 mM salt stress.

(M10) E1T2	1.50 b	1.29 b	0.951 b	0.379 a	0.283 a	0.229 a	0.156 g	0.314 cd	0.527 b	10.02 jk	15.43 hijk	24.65 i	0.0385 c	0.058 a	0.060 a	59.60 b	51.26 ijk	73.13 e	66.38 b	49.22 d	36.60 d
(M11) E1T2	1.58 a	1.37 a	1.105 a	0.105 i	0.077 g	0.025 h	0.118 ij	0.342 b	0.191 kl	11.32 fghij	20.83 c	32.66 d	0.0327 gh	0.054 b	0.0453 f	54.06 d	57.60 g	70.60 f	63.15 bc	43.85 e	37.94 d
(M12) E1T2	0.499 k	0.282 l	0.189 k	0.025 m	0.015 j	0.008 j	0.193 d	0.301 e	0.474 e	12.15 efgh	16.98 fgh	20.81 j	0.0385 c	0.042 de	0.052 bc	34.40 i	51.80 ij	54.33 j	48.42 fg	35.06 i	27.67 ef
(M13) E1T2	0.89 h	0.651 h	0.393 i	0.182 e	0.129 ef	0.088 f	0.317 a	0.374 a	0.483 e	13.98 b	18.81 de	33.46 d	0.0440 b	0.034 k	0.0515 d	56.86 c	65.20 e	78.20 d	53.29 de	42.13 ef	28.44 e
(M14) E1T2	0.40 m	0.268 m	0.164 l	0.033 m	0.016 j	0.008 j	0.251 c	0.289 f	0.308 l	10.32 ijk	16.14 ghij	33.01 d	0.0259 k	0.035 kj	0.0312 k	52.66 de	58.33 g	57.93 i	47.12 g	30.24 j	23.35 h
(M15) E1T2	0.29 no	0.164 n	0.088 n	0.131 h	0.119 f	0.093 ef	0.138 h	0.306 de	0.513 c	10.98 ghij	17.64 efg	36.99 c	0.0497 a	0.0351 kj	0.0456 f	51.80 e	63.34 ef	68.73 f	41.90 h	30.44 j	16.99 ij
(M16) E2T1	0.51 k	0.302 k	0.210 j	0.071 k	0.055 h	0.020 hi	0.264 b	0.306 de	0.314 l	13.65 bcd	16.31 ghi	40.45 b	0.0230 l	0.037 hi	0.0379 h	40.46 h	45.86 m	59.06 h	52.45 de	41.90 ef	36.15 d
(M17) E2T1	1.33 e	1.11 c	0.798 c	0.191 e	0.168 d	0.115 d	0.171 f	0.261 g	0.261 m	13.81 bc	18.48 def	46.45 a	0.0301 i	0.041 f	0.051 cd	57.33 c	83.13 b	45.53 l	61.87 c	52.73 c	40.71 c
(M18) E2T1	0.99 g	0.759 f	0.458 h	0.152 g	0.121 f	0.095 ef	0.107 k	0.352 b	0.507 cd	11.07 fghij	20.14 cd	30.46 e	0.0352 ef	0.041 ef	0.053 b	51.06 e	54.53 h	60.40 h	63.75 bc	53.29 c	41.89 c
(M19) E3T1	1.46 c	1.11 c	0.684 e	0.152 g	0.132 e	0.099 e	0.106 k	0.323 c	0.540 a	10.32 ijk	14.13 kl	29.42 efg	0.0316 h	0.036 ij	0.0390 h	62.73 a	69.80 d	73.80 e	71.78 a	60.92 a	53.57 a

In each column, means that have at least one letter in common don't show any significance difference. E0T0 = EMS (0%)-Time (0 min); E1T1 = EMS (0.1%)-Time (30 min); E1T2 = EMS (0.1%)-Time (60 min); E2T1 = EMS (0.2%)-Time (30 min); E2T2 = EMS (0.2%)-Time (60 min); E3T1 = EMS (0.5%)-Time (30 min); E3T2 = EMS (0.5%)-Time (60 min).

was in consistent with the result of previous study on Al-samma varieties of wheat plant (*Triticum aestivum* L.) resulting in increase of chlorophyll contents by application of EMS [15]. The effect of EMS was assessed on pepper (*Capsicum annuum* L.) showing the lowest amount of chlorophyll at concentration of 2% for 15 min which was in parallel with our data [22]. Moreover, the total chlorophyll content of sunflower was decreased within seven hours under EMS treatment [22]. It could be assumed that lack or increase in the amount of chlorophyll could be related to role of EMS inhibition in chlorophyll biosynthesis [22]. The reason of chlorophyll changes at various concentrations of EMS can be due to DNA changes in the chloroplast. DNA chloroplast is rich in guanine and cytosine in some plants, and EMS reacts favorably with guanine. Therefore, chlorophyll changes can be attributed to changes in alkylation of chloroplast DNA. However, different concentration and duration of EMS treatment which result in a change of chlorophyll would be different for each plant and depends on types of plant and test conditions [9].

In this study, the soluble sugar content, membrane peroxidation and free radical inhibition percentage were decreased by increase of EMS concentration, while flavonoid content was increased with increase of EMS concentration. Hamid et al (2015) reported on the effect of EMS on physio-biochemical traits of *Cichoriumintybus* L. resulting in increase of lipid peroxidation by increase of EMS concentration [5] which was not in relation to our data, while it was shown the increase of sugar content with lower concentration of EMS which was similar to this present result.

With respect to salinity stress, in vitro culture accompanied by chemical mutagenesis is a practical plant breeding tool in developing plant resistant to different abiotic stresses [23]. Numerous studies have been carried out to determine the most tolerant species to salt stress such as petunia [14], *Chrysanthemum morifolium* Ramat. [8], sweet potato [6, 17], sugarcane [12], potato [27] and peanut [29], but no reports on stevia. Therefore, it would be necessary to understand the mechanisms of salt adaptation and tolerance of *S. rebaudiana*. In this study, the mutants of M10, M11 and M19 obtained from 0.1% EMS mutagen showed the highest physiological parameters including total chlorophyll content, carotenoid, anthocyanin, membrane peroxidation, soluble sugar content, flavonoid and free radical inhibition percentage under 50 and 100 mM level of NaCl suggesting as the most potential salt-tolerant samples in stevia compared to non-mutated plant.

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تاثیر ماده جهش‌زای شیمیایی بر ویژگی‌های فیزیولوژیکی گیاه استویا (*Stevia rebaudiana* B.) در شرایط تنش شوری

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چکیده

استفاده از راهکارهای فناوری زیستی جهت افزایش تولید گیاهان دارویی با استفاده از راهکارهای کشت سلول‌ها، اندام‌ها، بافت‌ها و مهندسی ژنتیک یکی از اهداف مهم افزایش بهره‌وری این گیاهان می‌باشد. در این پژوهش به منظور بررسی اثرات ماده جهش‌زای اتیل متان سولفونات (EMS) بر برخی خصوصیات فیزیولوژیکی درگیاهان باززایی شده از کالوس گیاه استویا در شرایط شوری، دو آزمایش فاکتوریل درقالب طرح کاملاً تصادفی با سه تکرار اجرا گردید. دراین آزمایش ریزنمونه‌های برگی به محیط کشت MS حاوی ۰/۱ میلی گرم در لیتر تیدیازرون (TDZ) منتقل شدند. توده‌های کالوسی با غلظت‌های مختلف EMS (۰/۱، ۰/۲ و ۰/۵ درصد) در زمان‌های (۳۰ ، ۶۰ و ۱۲۰ دقیقه) تیمار دهی و بر روی محیط کشت MS واکشت شدند. نتایج آزمایش اول نشان داد که برخی از خصوصیات کالوس های باززایی شده تحت تأثیر غلظت‌های مختلف EMS و زمان‌های متفاوت در معرض قرار گیری و اثرات متقابل این دو فاکتور قرار گرفتند. در آزمایش دوم هرگیاه باززایی شده از کالوس‌های تحت تیمارEMS، به عنوان یک رویداد جهش درنظرگرفته شده وجمعیت کلونی با سن مشابه از هرگیاهچه منحصربه فرد تهیه و بعد از دو ماه، برخی ازخصوصیات گیاهان اندازه‌گیری شد. نتایج نشان داد که عامل جهش‌زا اثرکاملاً معنی‌داری بر صفات فیزیولوژیکی در سطح یک درصد داشت. بررسی صفات فیزیولوژیکی حاکی ازآن بود که بیشترین مقاومت به سطوح مختلف شوری اختصاص به موتانت‌هایM6، M10، M19 و M18 داشتند.

کلمات کلیدی: استویا، صفات فیزیولوژیک، تنش شوری