Induction of Salt Tolerant Mutants of *Foeniculum vulgare* by Dimethyl Sulphate and Their Identification Using Protein Pattern and ISSR Markers

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ABSTRACT

This work aimed to study the effect of dimethyl sulphate (DMS) at 0, 1000 and 2000 ppm. and salt stress at 0, 50 and 100 mM NaCl on the growth, essential oils percentage and composition and antioxidant enzyme of *Foeniculum vulgare* (fennel). In addition, to study the ability of DMS in induction salt tolerant mutant and evaluate the genetic variation using protein patterns and six ISSR markers. Salinity decreased fennel growth and productivity chlorophyll a and b and peroxidase (POD) activity. It induced a noticeable increase in carotenoids, catalase (CAT) activity, Na^+ and lipid peroxidation. Conversely, DMS alleviated the lethal effect of salt stress on fennel plants especially on the M_2 generation. Two mutants were found to be superior to the control plant grown under salt stress and differed in their essential oil composition. The dendrogram grouped the two tolerant mutants into one cluster.

Key words: Antioxidant; Dimethyl sulphate; *Foeniculum vulgare*; salinity; Mutant; ISSR markers.

INTRODUCTION

Fennel (*Foeniculum vulgare* Mill) plant belongs to the family Apiaceae is one of the oldest herbs and possesses beneficial medicinal effects. Fennel used for various purposes in the food, cosmetic and medical industries. The essential oil of fennel has a valuable antioxidant, antibacterial, anticancer and antifungal activity (Bahmani *et al*., 2012 and EL- Awadi and Esmat, 2010). The increasing commercial value of fennel necessitates the need to develop salt tolerant varieties.

Salinity is one of the major abiotic stresses that adversely affect crop productivity and quality, about 20 % of irrigated agricultural land adversely affected by salinity. The problem of soil salinity is further increasing because poor quality of the irrigation water and the poor drainage (Chinnusamy *et al*., 2005). About 40 % of all arabian lands cannot be used because of the potential salinity problems. High salinity causes ion imbalance due to elevated toxic levels of the cytoplasmic sodium and drought stress (Town and Mahamed, 2008). Moreover, under different abiotic stresses, plants accumulate reactive oxygen species (ROS) as reported by Shou *et al*. (2004).

ROS include superoxide(O_2-), hydrogen peroxide(H_2O_2), and hydroxyl radical OH (Hernandez *et al*., 2001). These ROS are cytotoxic and can seriously disrupt normal metabolism through oxidative damage of lipids, proteins, chlorophyll, nucleic acids and other important macromolecules, fatally affect in plant metabolism and, ultimately, growth and yield. To reduce the deleterious effects of oxidative stress, plant cells posses an antioxidative system consisting of low molecular weight antioxidants such as ascorbate, α tocopherol, glutathione and carotenoids as well as antioxidative enzymes (Mohamed *et al.* 2007).

Antioxidant enzymes such as peroxidases (PODs), ascorbate peroxidase(APX), glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD) are scavengers of ROS. Plants with high levels of antioxidants either constitutive or induced have been reported to have greater resistance to this oxidative damage (Uddin *et al*., 2007).

Chemical mutagenesis is a simple approach to create mutation in plants for their potential agronomic improvement. Mutation methodology has been used to produce many cultivars with improved economic value and to study the genetics and plant developmental phenomena (Aruna *et al*., 2010). Dimethyl sulphate (DMS) is a chemical compound with the formula of $(CH_3O)_2SO_2$ which is monofunctional alkylating agent that have been shown to induce mutations, chromosomal aberrations and other genetic alterations in a diversity of organisms (Jain and Mathur, 2006; Mostafa, 2009 and Gad, 2012).

Inter simple sequence repeat (ISSR) markers have been used with success to identify the mutants and study the genetic diversity of different medicinal plant species and crops

(Farajpour *et al*. 2011).

This study aimed to study the effect of dimethyl sulphate at 0, 1000 and 2000 ppm and salt stress at 0, 50 and 100 mM NaCl on the growth, essential oils and antioxidant enzyme of *Foeniculum vulgare*. In addition, to study the ability of DMS in inducing salt tolerant mutant and evaluate the genetic variation using protein patterns and six ISSR markers.

MATERIALS AND METHODS

This study was carried out at the Nursery of Ornamental Plants, Faculty of Agriculture, South Valley University, Qena, Egypt, during the two successive generations of 2012/2013 and 2013/2014.

1. Plant treatments

Seeds of sweet fennel (*Foeniculum vulgare var. vulgare*), Local cultivar were used. The seeds were soaked in dimethy sulphate solution (DMS) at the concentrations of 0, 1000 and 2000 ppm for 14 h. After that, seeds were washed in distilled water and sown in 25 cm plastic pots containing a soil mixture of clay and sand (1:1 v/v) on November 1, 2012. One hundred and fifty seeds were sown for each treatment. The seeds were sown in three replications; each replication contained five pots (ten seeds in each pot). After 45 days from sowing seeds, seedlings were transplanted into a 20 cm. plastic pots containing the soil mixture of clay and sand (3:1 v/v). Plants were irrigated by saline water after one week from transplanting using three concentrations of NaCl (0, 50, 100 mM). Seeds for the M_2 -generation were sown on November 15 th 2013. The procedure of sowing and transplanting were made likewise the M_1- generation

The experimental layout was a randomized complete block design containing three replications (Steel and Torrie, 1982). Each replication contained nine treatments (three concentrations of DMS X three concentration of NaCl and every treatment consisted of 12 plants.

2. Collected data:

Parameters of vegetative growth for one-half of the plants were calculated at the beginning of flowering. In addition, young leaves were excised for protein pattern and ISSR analysis, and also for determination of the photosynthetic pigments (chl.a and b and carotenoids), catalase (CAT), ascorbate peroxidase (APX), peroxidases (PODs), Na^+ and malondialdehyde (MDA). Plants were then dried in an oven at 80◦C for 72 h, and root and shoot dry weights were determined. The other half plants were left for maturation. Then, No. of inflorescence and florets, 20 seeds weight, total seed weight/plant, oil percentage and seed oil/ plant were calculated.

3. Essential oil percentage:

The essential oil percentage of each treatment of fennel plants was determined in the fruits (seeds) using water distillation methods according to British pharmacopeia (1963).

4. Essential oil yield (ml)/plant:

The essential oil yield (ml)/plant were calculated in proportion to fruit weight as described by Selim *et al*. (2013)

$$\text{Oil yield/ plant} = \frac{\text{Fruit yield X Oil content (\%)}}{100} = \text{ml/ plant}$$

5. Essential oil constituents

Gas Chromatography- Mass Spectrometry (GC-MS) analysis of the essential oil was carried out as described by Selim *et al.* (2013) with some modification using Thermo Scientific Apparatus. The GC-MS system was equipped with trace gold (TG) 0.5 MS, 30 m x 0.32 mm capillary column with 0.25 µm film thickness. Five µl of essential oil were added to one ml of dichloromethane for analysis. Analysis was carried out using helium as the carrier gas at a flow rate of 1 ml/ min. The GC oven temperature was programmed at an initial temperature of 45 ºC for 2 min. Then rising to 165 ºC with rate 4 ºC/min and hold for 5 min and heated up to 300 ºC with rate 15 ºC/min and hold at this degree for 5 min. Injector temperature was set at 250 ºC with splitters injection mode for 1 min at split flow with 1:10 ratio. Using mass transfer line 300 ºC, the mass spectrometry was run in the electron ionization (EI) mode at 220 ºC ion source temperature, and the scanning was begun after 5 min with mass range between 40-500amu(atomic mass unit). The separated components of the essential oil were identified according to National Institute of Standards and Technology (NIST) spectral library data. The ratios of the component per each other were carried out based on peak area.

6. Photosynthetic pigments determination

The photosynthetic pigments, chl. a, chl.b and carotenoids were determined using the spectrophotometric method recommended by Metzner *et al*. (1965).

7. Antioxidant enzymes activity

Plant samples were prepared as described by Mukherjee and Choudhuri **(**1983**)**. A fresh sample (250 mg) was frozen in liquid nitrogen and finely ground by pestle in a chilled motor. The frozen powder was added to 10 ml of 100 mM phosphate buffer (KH_2PO_4 /K2HPO_4, pH 7.0) containing 0.1 mM Na_2EDTA and 0.1 g of polyvinyl pyrrolidone (PVP). The homogenate was filtered through cheesecloth then centrifuged at 15000 g for 10 min. The supernatant was recentrifuged at 18000 g for 10 min; the resulted supernatant was collected and stored at 4 ◦C for assay of catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX).

7.1. CAT (EC 1.11.1.6) activity

CAT activity is measured by the decrease of absorbance at 240 nm as a consequence of H_2O_2

consumption and was expressed according to Havir and Mellate **(**1987**)**

7.2. POD (EC 1.11.1.7) activity

POD activity is determined according to Maehly and Chance **(**1954**)**.

7.3. APX (EC 1.11.1.11) activity

APX activity is determined from the decrease in absorbance ascorbic at 290 nm as ascorbic acid oxidized (Asada and Chen 1992).

7.4. Determination of malondialdehyde (MDA)

A level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using the method of Heath and Packer (1968). To 2.0 ml aliquot of the supernatant 4.0 ml of 0.5% thiobarbituric acid (TBA) in 20 % TCA was added. The mixture was heated at 95 ^{o}C for 30 min and then quickly cooled in an ice bath centrifugation at 10000 g for 10 min. The absorbance of supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The MDA content was calculated using its absorption coefficient of 155 $nmol^{-1}$ cm^{-1} and expressed as nmol (MDA) g^{-1} fresh weight.

8. Sodium determination

A known weight (0.2 g) of the dried leaves was ground into a fine powder by a micro mill and assayed for mineral determinations. The wet digestion method (Humphries, 1956) using perchloric acid was used. After complete charring by one ml conc. Sulphuric acid, one ml of sulphuric-perchloric mixure (1:1 by volume) of 50% perchloric was added. The flask was further left on a hot plate until the sample become colorless and the majority of fumes developed. After cooling, the solution was filtered and then diluted to a definite volume.

A tomic absorption (Spectra Varian 55) was employed for sodium determination.

9. Protein pattern and SDS-PAGE

For extraction of protein, 2g fresh weight of plant leaves and 10 ml of extraction buffer (0.5 M Tris-HCl (pH 6.8), 10% sucrose, 2% SDS and 5% 2-mercaptoethanol) were grinded together in mortar. The slurry was centrifuged at 5000 rpm for 20 min. Three milliliters of ammonium sulphate solution were added 1 ml^{-1} of the supernatant to precipitate the proteins then kept over night in a refrigerator. It was then centrifuged at 5000 rpm for 20 min and the pellet was washed two or three times in 70% acetone. SDS-PAGE was performed by the method described by Laemmli (1970). Proteins were analyzed on 1.5 mm thick and 15 cm long gels run in a dual vertical slab unit (Hoefer Scientific Instruments, san Francisco, CA, USA, MODEL SE 600 Series Hoefer, Pharmacia Biotech). From each sample, 50 ul of extract was loaded into a polyacrylamide gel. The separation gel (12%) and staking (4%) were prepared from an acrylamide monomer solution. Electrophoresis was carried out at constant current of 35 mA through the stacking gel, and at 90 mA through the separation gel at 4°C. After electrophoresis the gel was stained by Coomassie Brilliant Blue R-250 and the molecular weight (MW) of protein corresponding to each band was calculated by protein marker.

10. DNA Extraction and ISSR analysis

Genomic DNA from young leaves was extracted using DNeasy mini plant kit according to manufacturer's instructions (Omega Co.USA). ISSR analysis of DNA was performed using six primers Table (1). The amplification was done in volume 25 µl consists of 2.5 µl Taq polymerase buffer, 0.2 mM each of dATP, dTTP, dCTP, dGTP , 5.0 p moles of ISSR primers, 25 ng of genomic DNA and 1.0 U *Taq* DNA polymerase (Promega). The amplification was performed by including the reaction mixture for 40 cycles in a thermocycler Gene Amp 9700. Each cycle consisted of denaturation at 94°C for 1 min. followed by annealing at 48-53°C for 1 min. nd extension at 72°C for 2 min. with initial delay for 4 min. at 95°C at the beginning of the first cycle and post extension step for 5 min. at 72°C after the end of the last cycle (Bahmani *et al*., 2012). PCR products were then separated on agarose gel electrophoresis using 1.5% (w/v) agarose in 0.5x TBE buffer. The size of each band was estimated by using DNA molecular weight marker.Finally, the gel was photographed by using gel documentation system.

RESULTS AND DISCUSSION.

Values of all traits decreased gradually with increasing the concentration of salinity for both generations of plants treated with zero and 1000 ppm DMS as shown in Tables 2, 3 and 4. The same results were obtained for the treatment of 2000 ppm in the second generation. With respect to M_1 generation, plant treated with 2000 ppm DMS posses a gradually decrease in plant height with increasing irrigation water salinity.

Table 1: Sequence, annealing temperature and GC content % of the used six ISSR primers

No	Sequence	annealing temperature (°C)	GC Content %
1	5'-(CT)8RG-3'	48	50
2	5'-(AG)8T-3'	49	44
3	5'-(CA)8G-3'	52	53
4	5'-(AG)8RC-3'	53	47
5	5'-(GA)8T-3'	48	47
6	5'-(AG)8YT-3'	52	50

These slightly decrease in traits values with increasing the salinity concentrations were supported by the results of Semiz *et al*., 2012 and Omer *et al.*, 2014 on *Foeniculum vulgare*. Salinity can inhibit plant growth by three major ways: 1) water deficit arising from the more negative water potential (elevated osmotic pressure) of the soil solution; 2) specific ion toxicity usually associated with either excessive chloride or sodium uptake; and 3) Nutrient ion imbalance when the excess of Na^+ or Cl^- leads to a diminished uptake of K^+, Ca^+, NO_3- or P or to impaired internal distribution of one or another of these ions (Assem *et al*. 2009).

Plants treated with 2000 ppm DMS and irrigated with 100 mM NaCl gave the highest values of total seed weight/plant, number of leaves and branches/ plant, fresh and dry weights of vegetative growth, weight of 20 seeds, No. of florets and inflorescence in the M_1- generation compared to plants irrigated with zero or 50 mM NaCl. These values did not differ significantly from the control plants (untreated plants either with mutagen or with saline water). However, in some cases increased significantly from the control as found by the number of leaves, branches and inflorescences.

No significant differences were found with respect to root length in the M_2- generation and the weight of 20 seeds in the M_1- generation. Plants treated with 2000 ppm DMS and irrigated with 100 mM NaCl gave higher values of total seed weight per plant. These values did not differ significantly from the control in the M_1- generation (19.0, 12.0 for treated and untreated plants, respectively).

These results refers to the ability of dimethyl sulphate as a chemical mutages to alleviate salt stress.

Chlorophyll a and b decreased under the effect of increasing salinity as shown in Table 5. Tammam *et al*. (2008) and Turan *et al*. (2009) found that salt stress decreased chlorophyll content (a and b). Several scientists have suggested that there is a positive correlation between decrease in chlorophyll content and salt-induced weakening of protein-pigment-lipid complex (Strogonov *et al*. 1970) or increased chlorophyllase enzyme activity (Stivsev *et al.* 1973).

DMS induced some stimulating effect. This stimulating effect may be attributed to the activation of plant metabolism resulted from the using of the mutagen. Both concentrations of DMS increased chlorophyll a, b and carotenoids significantly in compare with the control. The estimated amounts of Chl.a were 14.31 and 14.64 mg, Chl. b were 5.28 and 4.51 and carotenoids were 6.03 and 6.45 in the1000 and 2000 ppm DMS treated plants and was 12.6 mg (Chl. a), 3.86 mg(Chl. b), and 3.31 (carotenoids) in the control.. Plants treated with DMS increased considerably their content of Ch.la, Chl.b and carotenoids compared with non-mutagenic salt stressed plants.

CAT activity increased significantly under the effect of DMS up to 130.5% in both non-salinized and salinized plants, compared to the control (Table 5). DMS did not exhibit any action to stop the POD activity reduction resulted from the salinity. APX activity not affected significantly by salt stress and/ or DMS treatments. The increase in CAT activity might be explained to counter the toxicity generated by salt stress, mediated through the generation of reactiveoxygen species, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet-}$). This confirmed earlier by Chai *et al*. (2005), Kukreja *et al*.(2005) and Seckin *et al*., (2010).

The level of lipid peroxidation has been used as an indicator of cell membrane damage by free radicals under stress conditions (Abou Alhamd and Shebany 2012). Our results (Table 5) showed that the salt stress induced a noticeably amounts of MDA. MDA increased up to 18% at 50 mM NaCl and 29 % at 100 mM NaCl compared with control. This indicates that the presence of NaCl may be enhancing the membrane lipid peroxidation. Consequently, causes an increase in permeability of plasma membrane or loss membrane integrity (Chai *et al*. ,2005).

Under salt stress, DMS treatment can decrease the accumulation of Na^+ content inside plants. On the other hand, salinity (without DMS treatment) increased Na^+ content in both root and shoot compared with control. This confirmed by Sperling *et al*. (2014).

Studies carried out on DMS in plants treated with salinity are scarce. In the present study, the increase in most traits especially in the M_2-generation, in the presence of DMS compared to untreated plants could possibly provide an adaptive mechanism in maintaining favorable potential under salt toxicity.

Induction of variation:

Two salt tolerant mutants were obtained in the M_2 -generations. The mutant 1 (Mu_1) was obtained from the treatment of 1000 ppm DMS and 100 mM NaCl. The mutant 2 (Mu_2) was obtained from the treatments of 2000 ppm DMS and 100 mM NaCl. The two mutants were superior to the control for plant height, no. of branches and leaves, stem diameter, total seeds weight, oil percentage and seed oil/ plant as shown in Table (6) and Figure (1). Uddin et al. (2007) on rice also generated salt tolerant lines through mutagenesis. The stimulatory effect of the mutagen may be attributed to the increase in the rate of cell division or cell elongation as reported by Joshi *et al*. (2011) and Mostafa *et al*. (2014).

Table 2: Plant height (cm), Stem diameter(cm), Number of leaves and branches / plant, Fresh and dry weights of vegetative growth (g) of fennel (*Foeniculum vulgare*) plants treated with dimethyl sulphate and/or salt stress.

Treatment		Plant height (cm)		Stem diameter(cm)		Number of leaves/ plant		Number of branches/ plant		Fresh weight of vegetative growth (g)		Dry weight of vegetative growth(g)	
NaCl (mM)	DMS (ppm)	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2
0.0	0 (T1)	97.8 cde	101.5 a	0.70 a	0.73 abc	47.1 b	83.6 ab	24.3 b	35.0 b	80.3 ab	104.4 ab	20.0 a	26.6 a
	50(T2)	93.5 cde	82.8 cd	0.70 a	0.65 c	44.6 b	63.5 c	23.3 b	30.6 b	75.8 abc	102.7 ab	16.9 abc	25.2 bc
	100(T3)	88.8 e	79.6 d	0.68 ab	0.63 c	42.6 b	37.8 d	17.5 de	17.5 c	68.7 bcd	63.7 c	14.1 bc	18.4 cd
1000	0 (T4)	109.8 a	99.3 ab	0.70 a	0.76 ab	44.6 b	88.5 a	21.1 bc	44.3 a	87.1 a	120.0 ab	18.9 ab	31.0 ab
	50 (T5)	101.6 abc	89.3 bcd	0.58 abc	0.71 abc	41.3 b	64.3 c	19.0 cd	30.8 b	77.4 abc	100.8 b	18.9 ab	31.0 ab
	100(T6)	99.0 bcd	80.0 d	0.53 bc	0.63 c	40.0 b	65.1 c	17.0 de	32.0 b	54.4 d	92.8 bc	12.7 c	29.0 b
2000	0 (T7)	109.1 ab	103.5 a	0.56 abc	0.78 a	39.6 b	90.1 a	15.1 e	45.0 a	63.7 cd	135.3 a	16.0 abc	37.8 a
	50 (T8)	98.8 cde	90.8 bc	0.45 c	0.76 ab	40.0 b	88.3 a	18.3 cde	31.5 b	56.1 d	114.9 ab	13.9 c	29.4 b
	100(T9)	89.8 de	81.3 cd	0.61 ab	0.66 bc	61.3 a	69.6 bc	31.1 a	34.5 b	78.7 abc	100.2 b	19.4 a	16.7 d
$LSD_{0.05}$		10.2**	10.3**	2.72*	0.11*	2.54*	15.2**	3.6**	7.5**	15.3**	33.1*	4.9*	8.1 **

Values in the same column not followed by the same letter are significantly different at the 5 % level of probability. *and ** =significant at p= 0.05 and 0.01 ,respectively.

Table 3: Fresh and dry weight of root (g plant^{-1}), root length (cm), 20 seeds weight, number of florets and inflorescence in the two generations of fennel (*Foeniculum vulgare*) plants treated with dimethyl sulphate and/or salt stress.

Treatment		Fresh weight of root (g)		Dry weight of root (g)		Root length (cm)		20 seeds weight		Number of florets		Number of inflorescence	
NaCl (mM)	DMS (ppm)	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2
0.0	0 (T1)	16.4 a	16.5 ab	6.4 a	4.8 ab	46.8 a	32.0 a	0.201 a	0.228 abc	16.3 ab	15.6 ab	14.6 b	34.8 ab
	50(T2)	13.9 ab	13.0 bcd	4.0 bc	4.5 ab	35.1 b	28.5 a	0.183 a	0.172 d	15.3 bc	12.0 cd	13.3 b	30.1 ab
	100(T3)	13.9 ab	9.6 d	3.7 bc	2.8 c	30.0 bc	23.0 a	0.151 a	0.168 d	14.6 bcd	11.8 d	11.8 b	17.5 c
1000	0 (T4)	14.4 ab	16.5 ab	5.0 ab	4.9 ab	37.0 ab	32.6 a	0.185 a	0.233 ab	19.0 a	16.1 a	13.0 b	35.5 ab
	50 (T5)	12.5 abc	10.5 cd	4.2 bc	3.6 bc	35.8 b	28.8 a	0.179 a	0.193 bcd	15.5 b	13.1 cd	12.0 b	30.3 ab
	100(T6)	9.3 c	14.9 cd	3.2 c	3.6 bc	34.1 bc	27.5 a	0.165 a	0.184 cd	11.5 e	13.3 cd	7.1 c	28.5 b
2000	0 (T7)	11.2 bc	18.4 a	4.8 b	5.5 a	32.6 bc	35.5 a	0.155 a	0.242 a	12.0 de	16.3 a	12.5 b	39.3 a
	50 (T8)	8.7 c	15.5 abc	2.9 c	4.9 ab	28.3 bc	28.6 a	0.209 a	0.221 abc	12.5 cde	13.5 cd	13.3 b	30.3 ab
	100(T9)	10.9 bc	14.9 abc	3.7 bc	4.4 ab	24.8 c	30.0 a	0.251 a	0.204 abcd	14.5 bcd	14.0 bc	22.0 a	32.0 ab
	$LSD_{0.05}$	4.0*	5.3*	1.6*	1.4**	10.1	NS	N S	0.047	3.0**	2.1**	3.6**	10.5*

Values in the same column not followed by the same letter are significantly different at the 5 % level of probability.
NS, *and ** =non significant, significant at p= 0.05 and 0.01 ,respectively

Table 4: Total seed weight/plant, Oil % and Seed oil/plant of fennel (*Foeniculum vulgare*) plants treated with dimethyl sulphate and/or salt stress.

Treatment		Total seed weight/plant(g)		Oil %	Seed oil/plant(ml)
NaCl (mM)	DMS (ppm)	M_1	M_2	M_2	M_2
0.0	0 (T1)	12.0 abc	34.6 bcd	1.2 ab	0.397 bc
	50(T2)	9.0 b	25.3 ef	0.71 d	0.242 de
	100(T3)	6.1 c	12.6 b	0.71 d	0.114 f
1000	0 (T4)	14.0 ab	41.5 b	1.1 bc	0.498 b
	50 (T5)	10.4 bc	28.4 def	1.0 bc	0.210 ef
	100(T6)	8.5 bc	20.0 fg	1.0 bc	0.300 cde
2000	0 (T7)	9.0 bc	61.4 a	1.4 a	0.683 a
	50 (T8)	9.5 bc	38.4 bc	1.0 bc	0.349 cd
	100(T9)	19.0 a	30.0 cde	0.9 cd	0.357 c
$LSD_{0.05}$		8.0*	8.5**	0.26**	0.112**

Values in the same column not followed by the same letter are significantly different at the 5 % level of probability.
*and ** =significant at p= 0.05 and 0.01, respectively.

Comparing the protein profiles of control plants versus the NaCl salinized and DMS treated plants showed that NaCl or/and DMS induced some changes in the pattern of proteins. It was found that the intensity of certain bands was increased in salt and/or DMS treated plants and others decreased noticeably (Figure 2).

The comparison between the control plant (untreated either by DMS or by NaCl) and sample 2 that treated with 100 mM NaCl only showed that the bands 42, 75, 80 and 93 were more intensity in the plant grown under salt stress. On the other hand salt stress decreased the intensity of the bands 30 and 70 KD compared to control (Figure 2).

The bands of 93 and 99 KD disappeared from the sample 4, the salt tolerant mutant that treated with 1000 ppm DMS and 100mM NaCl, compared with the control. The intensity of the bands 30, 42 and 93 KD were increased in the sample 4. The salt tolerant mutant (sample 6) produced the band 15 KD obtained from 2000 ppm DMS and 100mM NaCl compared with the control. The mutagenesis treatments seemed to activate expression of some genes that resulted in the increased intensity and or density of SDS electrophoresis bands of protein as reported by Mahmoud and Nada (2006) and Tripathy *et al*., 2010. The increased intensity of certain protein bands in fennel plants may increase the tolerance against salinity.

The six ISSR primers yielded 48 amplified fragments. 45 band of them were polymorphic; the total polymorphic rate was 91.65% of the total fragments (Table 7). The primers 2, 3 and 6 showed 100 % polymorphism. The primer 6 showed the high number of bands (16 bands) with molecular weight ranged between 150 and 1100 pb. The lower molecular weight (90-370 pb) appeared in primer 1 (Figure 3).

It has to be noted that direct acting alkylating agents such as DMS or EMS primarily cause point mutations, which are the result of single base deletion, addition, or substation (Schy and Plewa 1989). The polymorphism revealed by ISSR may be due to deletion and/or addition, resulting in amino acid changes that change the function of proteins. These polymorphic bands could be very valuable for DNA identification between salt tolerant and sensitive plants. Arulbalachandran *et al.* (2009) reported the same results using gamma rays and EMS.

Genetic distance of DNA of mutants and control is presented in Table (8). The dendrogram grouped the six samples into three clusters. The two tolerant mutants (sample 4 and 6) were grouped into cluster A with zero genetic distance between each other. Sample 2 which treated with 0.0 DMS and 100 mM NaCl and sample 5 which treated with 2000 ppm DMS without salt stress grouped into cluster B. Non salinized plant that treated with 1000 ppm DMS were groued with the control plant into cluster C. (Figure 4).

Similarity between the two tolerant mutants and increase genetic distance between them and the control plants indicates the ability of DMS on the occurrence of genetic differences makes the plant more tolerant to salinity.

It is vital for any breeding program to have sufficient diversity available to allow the production of new varieties with improved crop productivity and tolerance to biotic and abiotic stress. Salt tolerant fennel plants were generated through mutagenesis using dimethyl sulphate (DMS) treatment. Selection program must take place including salt tolerant plants Mu_1 and Mu_2 to produce new salt tolerant cultivar.

Table 5: Chl a, Chl b and carotenoids (mg g^{-1} fresh weight), Catalase (CAT), Peroxidase (POD) and Ascrobate peroxidase (APX) (unit $min^{-1}g^{-1}$ fresh weight), Malondialdehyde (MDA) (nmol g^{-1} fresh weight) and Na^+ (mg g^{-1} dry weight) in the M_2 generation of fennel (*Foeniculum vulgare*) plants treated with dimethyl sulphate and/or salt stress.

Treament		Chl. a	Chl. b	carotenoids	CAT	APX	POD	MDA	Na^+ in root	Na^+ in shoot
DMS (ppm)	NaCl (mM)									
0.0	0	12.60 bc	3.86 bc	3.31 e	3.31 e	0.13 a	2.16 a	110.0 bc	22.75 c	33.25 bc
	50	10.86 d	3.51 bcd	4.01 de	4.01 de	0.15 a	1.50 b	129.6 ab	29.75 ab	33.25 bc
	100	8.85 e	2.48 d	4.95 cd	4.95 cd	0.15 a	1.33 b	141.8 a	35.00 a	38.50 a
1000	0	14.31 a	5.28 a	6.03 bc	6.03 bc	0.13 a	1.50 b	104.8 bc	21.00 c	26.25 d
	50	12.06 bcd	3.73 bc	6.31 ab	6.31 ab	0.15 a	1.16 b	112.6 bc	24.50 bc	31.50 bc
	100	11.21 cd	3.08 cd	6.75 ab	6.75 ab	0.16 a	1.16 b	127.8 ab	33.25 a	35.00 ab
2000	0	14.64 a	4.51 ab	6.45 ab	6.45 ab	0.15 a	1.50 b	98.9 c	21.00 c	29.75 cd
	50	13.31 ab	3.83 bc	6.28 abc	6.28 abc	0.16 a	1.33 b	117.2 abc	24.50 bc	31.50 bc
	100	11.02 cd	4.39 ab	7.63 a	7.63 a	0.16 a	1.16 b	127.0 ab	29.75 ab	35.00 ab
$LSD_{0.05}$		1.67**	1.18**	1.36**	1.36**	NS	0.46**	26.7*	5.39**	3.81 **

Values in the same column not followed by the same letter are significantly different at the 5 % level of probability.
NS, *and ** =non significant, significant at p= 0.05 and 0.01 ,respectively.

Table 6: Comparable between untreated plants and the two mutants of fennel (*Foeniculum vulgare*) plants treated with dimethyl sulphate and salt stress.

Traits	Control	Mutant 1	Mutant 2
Plant height (cm.)	103.5	120	130
No. of branches	45.0	80	112
No. of leaves	83.6	208	245
Stem diameter(cm)	0.76	2.0	2.3
Total seeds weight/ plant(g)	41.5	96.7	130.5
Oil %	1.2	1.1	1.8
Seed oil/ plant (ml/plant)	0.498	1.063	2.349

Fig.1: Photograph showing control plant, mutant1 and mutant 2 (from right to left) of fennel plants (*Foeniculum vulgare*) obtained after dimethyl sulphate treatments.

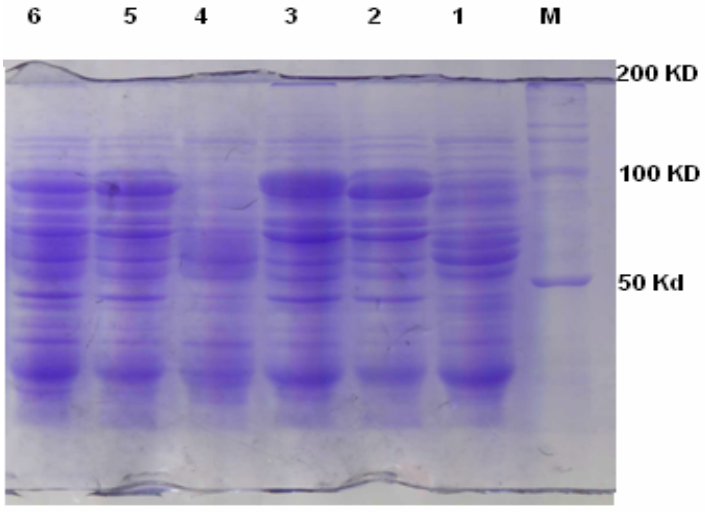

Fig. 2: SDS- electrophoresis of soluble protein of M_2 -generation of Fennel (*Foeniculum vulgare*) grown under salt stress; Lan 1: control, Lan 2: DMS(0ppm) NaCl(100mM), Lan 3: DMS (1000ppm) NaCl(0.0mM),Lan 4: DMS (1000ppm) NaCl(100mM), Lan 5: DMS(2000ppm) NaCl(0.0mM), Lan 6: DMS(2000) NaCl(100mM).

Table 7: Number of bands, number of polymorphic bands and polymorphism % detected by ISSR marker in the M_2 generation of fennel(*Foeniculum vulgare*) plants treated by dimethyl sulphate and/or salt stress.

No of ISSR primer	Sequence of ISSR primer	Total Number of bands	Number of polymorphic bands	% polymorphism
1	5'-(CT)8RG-3'	6	5	83.3
2	5'-(AG)8T-3'	8	8	100
3	5'-(CA)8G-3'	6	6	100
4	5'-(AG)8RC-3'	6	5	83.3
5	5'-(GA)8T-3'	6	5	83.3
6	5'-(AG)8YT-3'	16	16	100
Total		48	45	
Average		8	7.5	91.65

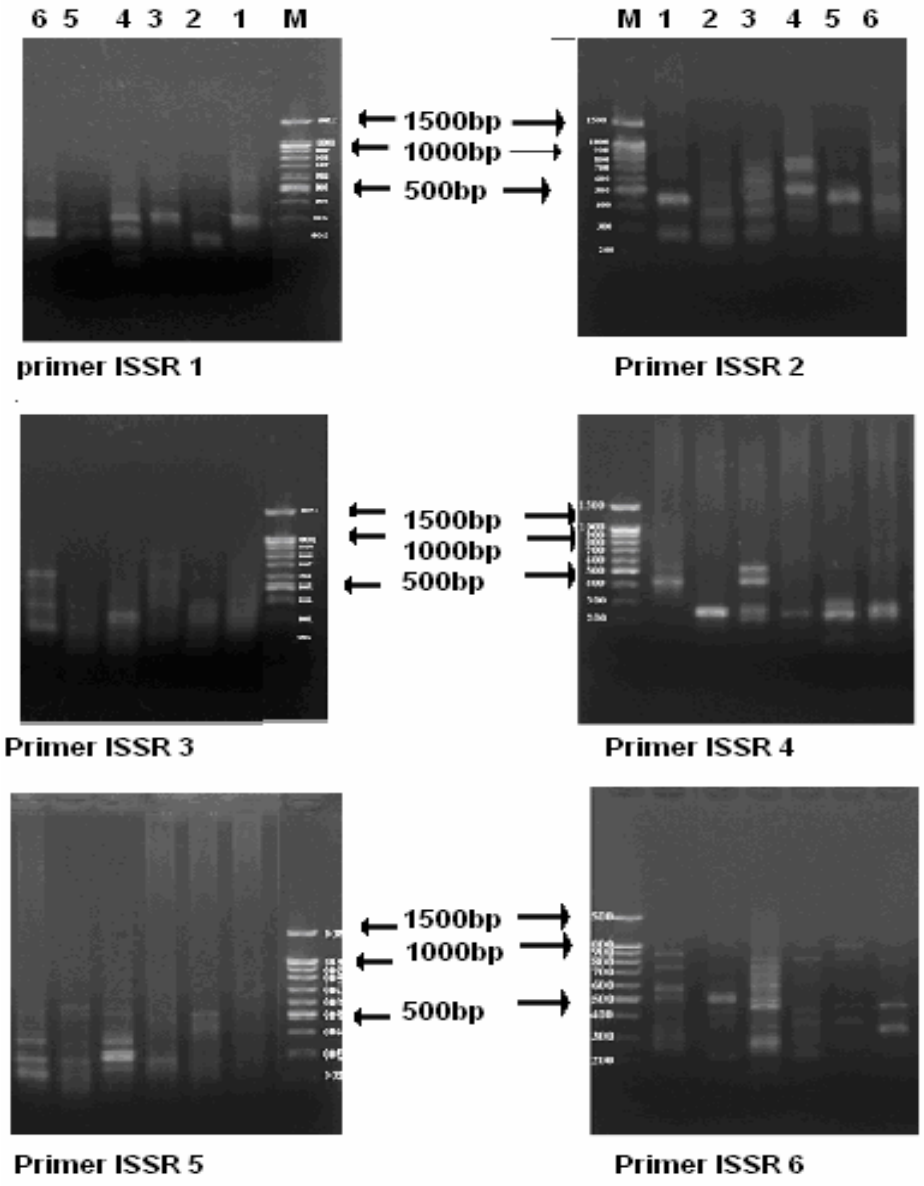

Fig 3: ISSR- PCR using primers 1- 6 for M_2 -generation of Fennel (*Foeniculum vulgare*) grown under salt stress; Lan 1: control, Lan 2: DMS at 0ppm and NaCl at100mM, Lan 3: DMS at 1000ppm and NaCl at 0.0mM, Lan 4: Mutant 1(DMS at1000ppm and NaCl at100mM), Lan 5: DMS at 2000ppm and NaCl at 0.0mM); and Lan 6: Mutant 2 (DMS at2000 and NaCl at100mM).

Table 8: Genetic distance of DNA among M_2 generation of fennel (*Foeniculum vulgare*) plants treated by dimethyl sulphate and/or salt stress using ISSR marker.

	1	2	3	4	5	6
1	0.0					
2	67.1	0.0				
3	44.1	47.9	0.0			
4	55.5	37.3	63.8	0.0		
5	44.2	27.8	48.0	50.1	0.0	
6	80	36.7	49.5	0.0	50.0	0.0

Sample 1: control, sample 2: DMS(0ppm) NaCl(100mM), Sample 3: DMS (1000ppm) NaCl(0.0mM), Sample 4: DMS (1000ppm) NaCl(100mM), Sample 5: DMS(2000ppm) NaCl(0.0mM), Sample 6: DMS(2000) NaCl(100mM),

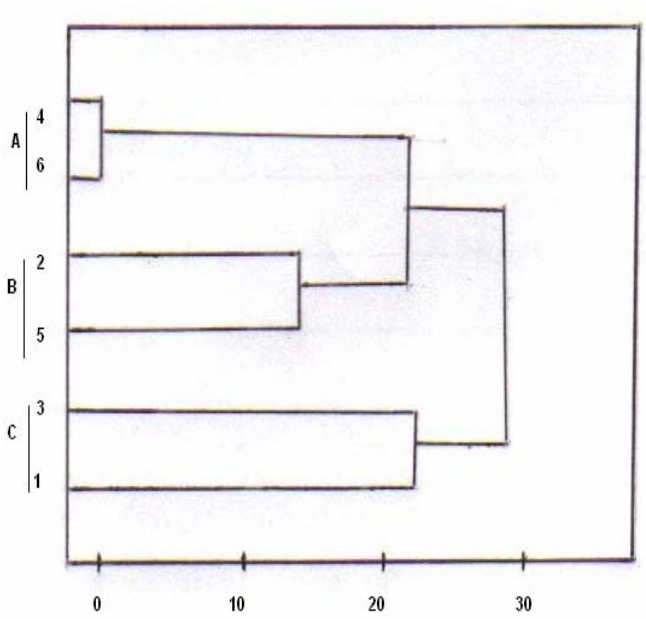

Fig 4: Tree diagram for M_2 generation of fennel (*Foeniculum vulgare*) plants treated by dimethyl sulphate and grown under salt stress on the basis of ISSR profile using six ISSR primers.

Essential oil composition:

Results of GC-MS investigations show that the main constituents of fennel essential oil were estragole (76.66-92.05) and D-Limonene (4.81-15.98) as shown in Table 9. These results agree with the findings of Shalaby and Hendawy(2011), Abd-Elaaty *et al.*(2011) and Selim *et al*.(2013) on fennel. Estragole percentage was increased in the mutants 1 and 2 than the control (88.13, 87.52 and 81.28, respectively), while the component of D-Lemonene were decreased (10.24, 9.9 and 14.03, respectively)

Fenchon, Fenchyl acetate and 3-Methoxy cinnamaldehyde concentrations increased gradually with increasing salt irrigation for untreated plants with mutagene. The two mutants gave the lowest value of fenchon comparable to all treatments. Fenchyl acetate was disappeared from mutant 2 only. The concentration of 2, 4, 6-Octatriene, 2,6-dimethyl,-(E,Z)- was increased gradually with increasing the salt irrigation(0.03, 0.19 and 0.36), and the mutant 1 and 2 gave percentage value over than of the control plants (0.06, 0.34 and 0.03, respectively). Benzene, 1-methoxy-4-(1-propenyl) - was found only in the mutant2 while the component 1-perillaldehyde was found only in the control. On the other hand, δ-cadinene was disappeared from control. Isocaryophillene was found only in the two mutants with percentage 0.01 for each. Essential oil composition depends upon internal, environmental, and agricultural practices as well as factors affecting the plant such as genetic and ecological conditions (Selim *et al*., 2013).

CONCLUSION

It was found that salinity decreased fennel plant growth, productivity, chlorophyll a and b and peroxidase (POD) activity. Soaking of seeds with DMS at the range 1000 – 2000 ppm for 14 hours alleviated the lethal effect of salt stress on fennel plants. Two mutants were found to be superior to the control plant grown under salt stress and differed in their essential oil composition, protein pattern, and ISSR markers.

Table 9: Chemical constituents (%) of the essential oil of fennel as affected by dimethyl sulphate and or salt stress and the two mutants

Treatment / Compound name	RT	T1	T2	T3	T4	T5	T6	T7	T8	T9	M1	M2
1R-a-pinene	6.88	0.27	0.16	0.38	0.47	0.39	0.2	0.08	0.15	0.25	0.08	0.29
á-phellandrene	8.19	0.13	0.07	0.16	0.22	0.18	0.09	0.05	0.09	0.15	0.05	0.17
á-myrcene	8.84	0.18	0.12	0.26	0.24	0.24	0.13	0.07	0.16	0.13	0.08	0.14
o-cymene	9.94	0.02				0.01					-----	
D-limonen	10.15	14.03	9.19	15.13	15.98	15.47	13.54	4.81	10.55	10.0	10.24	9.9
á-pinene	10.49	0.30	0.12	0.37	0.32	0.29	0.11	0.11	0.2	0.2	0.04	0.33
á-cis-Ocimene	10.84			0.04		0.04			0.02	0.02		0.03
3,careen	10.85	0.03			0.03						---	
ç-Terpinen	11.17	0.23	0.04	0.12	0.23	0.19	0.09	0.13	0.05	0.05	---	0.04
Terpinen	11.18										---	
Fenchone	12.22	1.51	1.7	5.39	2.41	3.13	1.01	1.48	4.29	0.55	0.37	0.5
Nonanal	12.92						0.03					
Trans-p-mentha-2,4,-dienol	13.41	0.03		0.03	0.04	0.03			0.02	0.03	0.07	0.03
2,4,6-octatriene,2,6-dimrthyl,-(E,Z)-	13.82	0.03	0.14	0.36	0.3	0.32	0.14	0.14	0.21	0.26	0.06	0.34
Limonene oxid	14.04	0.03	0.04	0.05	0.03	0.02	0.07	0.04	0.03	0.04	0.04	0.04
Camphor	14.27	0.04		0.1	0.06	0.07	0.04	0.02	0.06	0.02	----	0.02
P-Menth-1-en-4-01	15.49	0.02		0.04	0.03	0.05		0.02	0.02	0.03	0.02	0.04
estragole	16.87	81.28	87.21	76.66	78.79	78.97	83.2	92.05	83.07	86.86	88.13	87.52
Carveol acetate	17.16	0.05		0.06	0.07	0.04	0.04	0.04		0.05	0.11	0.06
p-Mentha-1,8-dien-6-ol	17.49			0.02	0.02							0.02
Fenchyl acetate	17.62	0.04	0.16	0.17	0.04	0.05	0.14	0.15	0.33	0.02	0.03	
Karvon	17.93	0.06	0.04	0.08	0.06	0.04	0.07	0.07	0.05	0.06	0.07	0.06
Benzene, 1-methoxy-4-(1-propenyl)-	18.24											0.02
Anisaldehyde	18.25	0.03	0.03	0.02	0.03	0.03	0.03		0.02	0.03	0.02	
1-perillaldehyde	18.95	0.01									---	
Anisol,p-propenyl	19.37	0.21	0.27	0.2	0.2	0.17	0.25	0.29	0.21	0.2	0.22	0.21
Copaen	22.46	0.03	0.02	0.04	0.04	0.03	0.08	0.04	0.03		0.03	0.03
Cubebene	22.94	0.02		0.02	0.02	0.02	0.04	0.02	0.02	0.02	0.02	0.01
Benzene, 1,2-dimethoxy-4-(2-propenyl)	23.41								0.02			
Dodecana	23.54	---	0.02								----	
Caryophyllene	23.87	0.04	0.03	0.04	0.05	0.05	0.05	0.04	0.03	0.04	0.04	0.04
Isocaryophillene	24.95										0.01	0.01
á-Farnesene	25.08								0.01	0.05		
Germacrene D	25.83	0.06	0.05	0.09	0.08	0.06	0.15	0.07	0.06		0.06	0.04

Con.Table 9: Chemical constituents (%) of the essential oil of fennel as affected by dimethyl sulphate and or salt stress and the two mutants

beta-Bisabolene	26.68					0.02			0.02			
δ-cadinene	27.14		0.03	0.03	0.03	0.02	0.06	0.03	0.02	0.1	0.03	0.01
3-Methoxycinnam aldehyde	28.38	0.41	0.07	0.02	0.1	0.05	0.22	0.07	0.15	0.02	0.06	0.04
Caryophyllene oxide	28.87	----	0.04	0.02	0.01			0.03		0.03	0.01	0.02
Apiol	30.07	0.04	0.13	0.05	0.04	0.02	0.06	0.06	0.05		0.06	0.03
Tricyclo[5.2.2.0(1.6)]undecan-3-ol,	31.77	----	0.02							0.01	---	
1-nonadecene	33.9	0.02	0.04					0.02			0.02	0.01
2-pentadecanone, 6,10,14-trimethyle	33.61	0.02	0.11		0.02		0.08	0.05	0.03		0.01	
Heneicosane	35.24	----	0.24								----	
n-Hexadecanoic acid	35.86	----	0.06				0.04	0.03			----	
n-Heptadecanol-1	36.9						0.03					
Pentacosane	39.53	-----	0.03									
10-Nonadecanone	42.74	-----	0.03									

RT= retention time, T1-T3=DMS treatment without stress, T4-T6=DMS treatments at 50 mM NaCl, T7-T9= DMS treatments at 100 mM NaCl, M1= the mutant that obtain from 1000 ppm DMS and 100 mM NaCl, M2= the mutant that obtain from 2000 ppm DMS and 100 mM NaCl.

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الملخص العربى

إنتاج طفرات فى نبات الشمر مقاومة للملوحة بالمعاملة بكبريتات ثنائى كحول الميثايل وتقييم الاختلافات الوراثية بتفريد البروتين والمعلمات الجزيئية ISSR

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أجريت هذه الدراسة بهدف دراسة تأثير كبريتات ثنائى كحول الميثايل (٠ و١٠٠٠ و٢٠٠٠ جزء فى المليـون) والملوحة(٠ و٥٠ و ١٠٠ ملليمول كلوريد الصوديوم) على النمو والنسبة المئوية للزيـت وتركيبـه وكـذلك علـى الانزيمات المضادة للاكسدة فى نبات الشمر. درست أيضا قدرة كبريتات ثنائى كحول الميثايل على إنتـاج طفـرات مقاومة للملوحة وتقييم الاختلافات الوراثية الناتجة بإستخدام تفريد البروتين والمعلمات الجزيئية ISSR.

أظهرت النتائج تأثير الملوحة فى خفض كل من النمو والإنتاجية وكـذلك كلوروفيـل أ و ب ونـشاط انـزيم البيروكسيديز. بينما زادت الملوحة من الكاروتينويدات ونشاط انزيم الكتاليز ومحتوى الاوراق من أيون الـصوديوم وكذلك إنزيم أكسدة الدهون.

أدى إستخدام كبريتات ثنائى كحول الميثايل لتقليل الأثر الضار للملوحة على نبات الشمر وخاصة فـى الجيـل الثانى. ظهر من خلال الدراسة نباتين متفوقين فى نموهم بالمقارنة بالكونترول تحت ظروف الرى بالماء المالح مـع وجود اختلافات بينهم وبين الكونترول فى مكونات الزيت وتفريد البروتين والمعلمات الجزيئيـة ISSR . وأوضـح تقدير القرابة الوراثية الى تشابه الطفرتين وانضمامهم لمجموعة واحدة مختلفة عن الكونترول.