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PHYSIOLOGICAL STUDIES ON INCREASING THE SALT TOLERANCE OF WHEAT PLANTS THROUGH CALLI TREATMENT WITH LASER RAYS AND ETHYLMETHANE-SULPHONATE

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ABSTRACT

The present study was designed to compare the effectiveness of helium-neon laser rays (0.0, 60, 120 and 180 minutes) and Ethylmethansulphonate (EMS) (0.0, 5, 15 and 25 mM), each alone or in combination, in improving the tolerance to salinity in salt-sensitive Giza 168 wheat cultivar. The salt-tolerant Sakha 93 cultivar, was used to be compared with the sensitive one under the same salinity levels (0.0, 15%, 30% and 45% sea salted water). For this reason preliminary experiment was conducting during 1999/2000 season, and the main experiments during 2000/2001 and 2001/2002 seasons. The results of the main experiments confirmed the previous conditions drawn from the data obtained from the preliminary one. The most promising three treatments were, 120 min. Laser rays, 15 mM EMS and 120 min. laser rays + 15 mM EMS. The obtained results of the main experiments clearly confirmed the effectiveness of all selected treatments with an absolute superiority of combined treatment (120 min. laser rays + 15 mM EMS) which yielded significant (and at the meantime the highest) increases in the calli fresh weight over the untreated calli either which the derived from the tolerant and sensitive cultivars. Meanwhile, the other two treatments, i.e. 120 min. laser rays or 15 mM EMS recorded their significant increments over the respective values of the sensitive control only (control II). Also, the calli derived from grains of the combined treatment exhibited more tolerance to salinity and was able to produce the significant increment over both controls up to 30% sea salted water level , and only up to 15% sea salted water level for the other treatments. The obtained wheat plantlets derived from calli treated with the combination of laser rays + EMS were tolerance up to 30% sea salted water level and were able to continue their growth in sand culture till maturity and till grain production which yielded as grains / plant more than two folds of the control of the tolerant cultivars *Sakha 93* and more than four folds that of the sensitive one *Giza 168*. The data also revealed that tolerance which was more pronounced as a result of the double treatment exist as well as for the single treatment of each and was associated with high accumulation of much more quantities inorganic osmotica, i.e. N, P, K, Mg, Ca and the highest K/Na ratio as well as lowest quantities of Na and lowest Na / Ca ratio , in addition to considerable accumulation of organic protective osmolytes, i.e. sucrose, proline and amino acids into calli and shoot tissues. This was accompanied with an accumulation of endogenous hormones, i.e. IAA, GA_3 and ABA in the stressed shoots, in addition to the lowest invertase activity in the stressed leaves in favor of accumulation more non-reducing sugars. Such accumulation increased as the salinity level was increased in order to adjust the ratio between the protective and toxic intermediates of metabolism in favour of more tolerance to salinity. Such behaviour seems to induce more ability for wheat plants to continue their growth till maturity and production of grains even under 30% sea salted water. The produced wheat grains from the different treatments especially the double one, were characterized with increased accumulation of protein, sugars and mineral nutrients by increasing salinity level in the medium. Sodium level gradually decreased from the calli

and shoot tissues to attain minimum value in the produced grains. The obtained data suggested the possibility of successful application of the combination of laser rays and EMS to improve salinity tolerance of economic crops such as wheat.

INTRODUCTION

Wheat (*Triticum aestivum L.*) is the most important growing cereal crops in Egypt . The amount needed is greater than that locally produced . Due to restricted resources of fresh water from the River Nile., the use of less quality and saline water or even diluted sea water became an important source of irrigation water especially in the newly cultivated areas. Therefore, the production of salinity tolerant lines of wheat is highly recommended in order to fulfill the great need from this crop by the majority of the Egyptian population.

Biotechnology and plant tissue culture technique are effective tools for producing salt tolerant cell lines., tissues and plants. Irradiation with fast neutrons, laser and gamma rays may provide insight into the mechanism of action of the radiation in physiological and genetic variability, thus have been directly used to produce useful variation in quantitatively inherited characters, such as quality and maturity time (Cholakov, 1995) . This in addition to the other mutagen agents including Ethylmethanesulphonate [EMS] as a chemical mutagen which is an effective tool for inducing genetical changes . The induced gene mutations might involve the polygene of the qualitative traits, or the major genes controlling the qualitative (Ahmed, 1998). Therefore, the results previously obtained by several authors suggested the possibility of successful applications of gamma rays (Whan *et al.*, 1991 ; El-Shihy *et al.*,1994 a and b and Ghallab and Neslem, 1999), fast neutrons (El-Shafey *et al.*, 1994) and EMS (Wong *et al.*, 1986) to improve salinity tolerance of the sensitive wheat and rice cultivars, since these mutagen agents are considered the most effective tools for inducing the useful genetical changes in the treated plant materials.

Moreover, Whan, *et al.*, (1991) mentioned that two salt resistant mutants of citrus were obtained after treating callus with gamma rays [5-8 KR] and EMS [0.3-o.5 %] and screening with sodium chloride. Also, Wang-lunshun *et al.*, (1995) found that the original wild type of sterile culture of *Lycim barbarum* ceased to grow at 1 % NaCl, but the mutant which were treated with 0.34 % EMS continued their growth up to 1.5 % NaCl. Recently, Harb *et al.*, (2002) suggested the possibility of successful application of EMS to improving salinity tolerance of banana which associated by the accumulation of sugars, free amino acids, proline and soluble phenol in banana tissues in response to increasing salinity level. El-shafey *et al.*, (2003) suggested the possibility of successful application of gamma rays, putrescine and abscisic acid to improve salinity tolerance of wheat.

Therefore, the present work was conducted to effectiveness of both single and combined treatments with laser rays and chemical mutagen substance EMS (Ethylmethanesulphonate) in improving salinity tolerance of the cultured wheat calli as well as the produced plants grown under different levels of salinity, aiming to induce [*in vitro*] salt tolerant cell lines, tissue, plantlets and finally plants. In the present experiments, the salt tolerant cultivar of wheat was

used, to be compared with the sensitive one under the same experimental conditions in order to evaluate the improving extent in such sensitive cultivar.

MATERIALS AND METHODS

The present work was carried out in the Biotechnology and Bioengineering Lab. and greenhouse of Plant Physiology Department, Faculty of Agriculture, Cairo University, as well as the National Institute of Laser Enhanced Sciences, Cairo Univr., during three successive seasons. During 1999 / 2000 season as a preliminary experiment and 2000 / 2001 as well as 2001 / 2002 seasons as the main experiment. In the present work, two cultivars were used, the salt tolerant Sakha 93 cultivar and the susceptible one ; Giza 168. Wheat grains of both cultivars were obtained from the Department of Wheat Research, Ministry of Agric., Giza, Egypt. On the other hand, it seems logic to investigate the effect of helium-neon laser irradiation [HN]and EMS treatments only on the sensitive cultivar aiming to approach nearly similar degree of tolerance of the comparable one.

The preliminary experiment done in the first growing season, was carried out in order to select the promising treatments for the main experiments in the following two successive growing seasons. Therefore, the preliminary experiment included 17 treatments, while the main one included only 5. The treatments were carried out during the preliminary experiment as follows:
[1] Control I (The tolerant cultivar) , [2] Control II (The sensitive cultivar) , [3] 60 minutes HN (Helium-Neon Laser irradiation), [4] 120 min. HN, [5] 180 min. HN, [6] 5 mM EMS , [7] 10 mM EMS , [8] 15 EMS , [9] 60 min. HN + 5 mM EMS , [10] 60 min. HN + 10 mM EMS , [11] 60 min. HN + 15 mM EMS, [12] 120 min. HN + 5 mM EMS, [13] 120 min. HN + 10 mM EMS, [14] 120 min. HN + 15 mM EMS, [15] 180 min. HN + 5 mM EMS, [16] 180 min. HN + 10 mM EMS , [17] 180 min. HN + 15 mM EMS.

I- Laser rays treatments :
Mature wheat grains of the sensitive cultivars (Giza 168) were soaked for 12 hourse in distilled water, then exposed to the different doses of helium-neon laser irradiation [0.0 , 60 min., 120 min. and 180 min. in the preliminary experiment and only to 120 min. in the main one] from " Helium- Neon Laser beam at 632 nm wavelength and 20 mwt. Output power " provided by the National Institute of Laser Enhanced Sciences, Cairo Univ., Giza , Egypt.

II- Embryos isolation and callus initiation :
The unirradiated grains (control treatments, which were only soaked for 12 hours in distilled water) and the irradiated ones , were surface sterilized for 30 min. with sodium hypochlorite [clorax] and rinsed 5 times with distilled water. The embryos were excised aseptically under sterilized conditions and then were cultured in 100 ml jars, containing 30 ml of agar solidified MS medium (Murashige and Skooge, 1962) supplemented with 2 mg / L 2,4-D [2,4 dichlorophenoxy acetic acid] , 100 mg / L myo-inositol, 3 % sucrose and

adjusted to a pH of 5.7. The embryos were cultured and incubated in a growth room at 25 ± 2 °C. day / night cycle, controlled by means of air conditioned system under 16 hours light and 8 hours darkness, using day light florescent lamp (110 cm long) and supported by (40 µE / cm^2 / S) light intensity.

III- Ethylmethansulphonate [EMS] treatments :

Embryos initiated from unirradiated grains were obtained at the same previously mentioned conditions in [II] , then were cultured in 100 ml jars containing 30 ml agar solidified MS (Murashige and Skoog, 1962), supplemented with 2 mg / L 2.4-D, 100 mg / L myo-inositol, 3 % sucrose and 5 ml / L EMS at different concentrations (0.0, 5 , 10 and 15 mM in the preliminary experiment and 10 mM in the main one) were also supplemented and adjusted to pH 5.7 .

IV- The combined treatments of laser rays and EMS :

Embryos initiated from irradiated grains with different doses of laser rays were obtained at the previously mentioned conditions in [II], then were cultured under the different levels of EMS. In the preliminary experiment all possible combinations between laser rays and EMS were made , while the main experiment only combined treatment 120 min. + 10 mM EMS was investigated.

V- Salinity treatments for callus :

The produced calli from each treatment (6 weeks after culture) either in the preliminary experiment or in the main one were subdivided into 100 mg inoculums pieces and subcultered on a solidified MS medium + the same concentration of 2.4-D , myo-inossitol and sucrose were supplemented to the different levels of sea salt , i.e. 0.0, 15 % , 30 % and 45 % by using sea salted water [Sea salt obtained from Sigma Co. USA] where 40 gm / L from the salt gave EC = 33000 ppm. . Each treatment included 20 replicates. The cultures were incubated for 6 weeks under the same previously mentioned conditions in [II] . After 6 weeks in culture under different levels of salinity, the fresh weights of growing calli produced from each treatment were recorded .

For each treatment 10 replicates of the obtained calli were dried in an oven at 70 °C for 48 hours and then the crude dry weights were determined. The dried calli were powdered and prepared for chemical determinations. Only small part of the calli was kept fresh for proline estimation.

The rest of the calli produced from all investigated treatments [10 replicates for each] were divided into 100 mg incoulum pices and sub-cultured on the plant regeneration media " [the same culture medium but supplemented with 2 mg / L BAP [N^6-benzyl-aminopurine]. The 300 ml jars , containing 50 ml of agar solidified MS medium were used. After 8 weeks on plant regeneration media supplemented with different levels of salinity, the calli were transferred to the rooting media [50 ml of agar solidified MS medium supplemented with 2 mg / L NAA + 3 % sucrose + 100 mg myo-inositol + the different levels of salinity] .

VI- Plant regeneration and grain production :

After 4 weeks on rooting media, the root formation was realized and the produce plantlets were transferred to grown in sand culture under the green house conditions. The complete nutrient solution as described by Hewitt (1952) was used. The nutrient solution was applied twice a week either alone for the controls or mixed with the same previously mentioned levels of salinity through a subirrigation device permitting drainage of excess solution. Washing with tap water followed with distilled water was made at two-weeks interval immediately before solution addition to prevent salt accumulation In both preliminary as well as the main experiment, a sample of 5 replicates ; 15 plants, from each treatment was taken at 45-days old, each treatment was obtained. Plant height, number of leaves / plant and dry weight of shoot were recorded. The shoots were dried in an oven at 70 °C for 48 hours and then the crude dry weight were determined. The dried shoots of each treatment were powdered and prepared for chemical analysis. For proline , invertase activity and endogenous phytohormones estimation, part of the shoot system was kept fresh, and 5 replicates from each treatment were left to grow till harvest.

At harvesting stage, after two months in sand culture, grain yield was recorded. The grains were dried in an oven at 70 °C for 48 hours and the crude dry weights were determined. The dried grains were powdered and prepared for chemical analyses.

It is worth to be mentioned that all experiments in the three successive seasons were repeated 4 times.

The mean values of calli fresh weights, growth and yield of the preliminary as well as the main experiments were statistically analyzed and compared using New L.S.D. at 5 % levels (Gomez and Gomez , 1984).

Chemical analysis :

For the determination of total nitrogen, the modified " Micro Kjeldahl" apparatus of Pamas and Wagner as described by Jones *et al.* (1991) was used. Crude protein was estimated by multiplying total N% by 6.25, then the protein content of grains were determined. For total P, K, Ca, Na, and Mg determination, the wet digestion of plant material was carried out as recommended by Piper (1947). Phosphorus was estimated colorimetrically using the stannous chloride reduced molydophosphoric blue color method in sulphoric acid system as described by Jackson (1973). K, Na, Ca and Mg were determined by the Atomic Absorption Sepctrophotometer (GBC, 932 AA).

Hot ethanol extract was used for determination of sugars (reducing, non-reducing and total) using the phosphomolybdic acid method (A. O. A. C., 1975). Total free amino acids were determined using ninhydrin reagent (Moor and Stein, 1954). Free proline concentration in the fresh material was determined colormetrically using sulphosalicylic acid ninhydrin methods as described by Bates *et al.* (1973). Extraction of invertase enzyme from the fresh leaves was carried out according to Rathert (1982). Invertase activity was expressed in terms of µ mol glucose liberated during 1 min. per (g) fresh weight of the pellet (105°C) obtained from centrifugation of crude extract, according to Sumner and Howell (1935).

Extraction of plant hormones was carried out according to Sadeghian (1971). Methanolic extract of the fresh leaves were used for endogenous hormones estimation by Gas-liquied chromatography (GLC) [Ati-Unicam-610 Series) according to the method described by Vogel (1975). The glass Column (1.5 X 4 mm) was packed with 1% OV-17. Temperature: Injector 260°C, detector 300 °C and column initially for 3 min. at 200 °C then increased to 220 °C (rate 20 °C /min.) for 4 min., then increased again to 240 °C (rate 20 °C/min.) flow rates; carrier gas (N_2 special) 30 ml / min., hydrogen special 33 ml/min. and synthetic air 330 ml/min. and the chart speed 1 cm/min.

RESULTS AND DISCUSSION

Callus Fresh Weight:

The results in Table 1 clearly revealed that the fresh weight of wheat calli (Mean S) gradually decreased as the salinity level increased when compared with the control treatment of either the tolerant or the sensitive cultivar (control I and II respectively).

Decreasing the wheat calli growth as salinity level was increased in the medium were previously reported by Barakat and Abdel-Latief (1995), El-Hennaway (1996), Ahmed (1998), Quraishi *et al.*, (2000) and El-Shafey *et al.* (2003).

Table (1): Calli fresh weight (g) derived from wheat embryos exposed to different laser rays and EMS treatments grown under different levels of salinity (% sea salted water) for 6 weeks, the inoculum weight was 100 mg (combined analysis for two seasons)

Treatments	% sea salted water				Mean (T)
	Control	15	30	45	
Control I	3.89	3.18	2.58	1.99	2.88
Control II	3.40	2.45	1.91	1.24	2.25
120 min.	5.14	3.88	1.27	0.39	2.67
15 mM EMS	5.09	3.82	1.35	0.37	2.66
120 min. + 15 mM EMS	5.88	4.62	3.55	0.56	3.65
Mean (S)	4.68	3.59	2.13	0.88	

New L. S. D. value at 5%

Salinity (S)	0.17
Treatment (T)	0.25
(S) X (T)	0.54

When consider the mean values of calli fresh weights due to each treatment (Mean T) regardless the salinity level, it could be noticed that, the superiority was confirmed for the combined treatment of laser rays and EMS which produced the significant yield of callus fresh weight over the untreated calli either which the derived from the tolerant and sensitive cultivars.

Meanwhile, the other two treatments, i.e 120 min. laser rays or 15 mM EMS recorded their significant increments over the respective values of the sensitive control only (Control II).

The percentage of increment in the callus fresh weight induced by the combined treatment over its control (control II) was 62.22 % and over the salt-tolerant control (control I) was 26.74 % . Comparing the effect of the interaction between treatments and salinity level clearly reveal that, the calli derived from grains of the combined treatment exhibited more tolerance to salinity and was able to produce the significant increment over both control up to 30% sea salted water level, and only up to 15% sea salted water level for the other two treatments. Similar results were reported by El-Shihy *et al.* (1994 a) on the faba bean calli as affected by fast neutron and gamma rays treatments, as well as El-Shafey *et al.* (1998) on wheat calli as affected by the combination between 15 KR gamma rays + 5 mM sodium azide.

The all derived calli either from the both controls or the all other treatments were able to continue their growth even at the highest level of salinity, though with differential degrees, and were also able to produce shoots under the all salinity levels.

Chemical analyses of callus tissues:

Comparing the results obtained in Table 2, clearly revealed that increasing salinity levels caused high accumulation of the protective compounds (Mean S), i.e. sugars and proline as well as free amino acids. As with calli fresh weights (Table 1), the Mean T of the combined treatment between laser rays and EMS which recorded considerably increased the accumulation of the previous protective compounds over the respective Mean T of the untreated calli of the tolerant cultivar; meanwhile, the single treatment of either laser rays and EMS exceeded only the salt-sensitive control in this regard. Comparing the effect of the interaction between treatments and salinity level clearly reveal that, the combined treatment accumulated much more concentrations of sugars and proline as well as free amino acids which greatly exceeded both controls up to 30% sea salted water level, and only 15% sea salted water level for the other two treatments. In this connection, Lone *et al.* (1987) found that the external addition of proline and glycine betaine to the culture of barely embryos increased shoot elongation under saline condition. Barakat and Abdel-Latief (1995) and El-Shafey *et al.* (1998) reported that proline accumulation increased several folds in salt stressed wheat callus.

Also, many reportes proved the rapid increase in synthesis and accumulation of sugar under saline conditions. Kirst (1990) described sucrose as one of the compatible solutes that increase during salt stress. Moreover, Pessaraki (2002) concluded that the plants that fail to increase soluble sugars biosynthesis could not tolerate salt. Also, El-Shafey *et al.* (2003) found that high accumulation of the protective substances, i.e. sucrose and proline in wheat calli as the salt level was increased.

Concerning amino acid accumulation with increasing salinity levels. Cano *et al.*, (1996) reported that amino acid concentrations increased in tomato callus with increasing salinity levels. Also, Fatouh Youssef (2003) who indicated that leaves of tolerant wheat plants showed high degree of osmotic

adjustment by the accumulation of more K^+ , proline, sugars and amino acids. As for the efficiency of EMS in this regard. Harb *et al.* (2002) suggested the possibility of successful application of EMS as a chemical mutagen to improve salinity tolerance of banana, which was associated with increasing accumulation of the protective substances such as total sugars, free amino acids, proline and soluble phenols in plant banana tissues with increasing salinity levels in MS media on which the shoot tip of banana plants were cultured.

Also, the obtained data in Table 2 clearly show that, although the concentrations of Na,Ca and Mg gradually increased in the calli tissues of the untreated control treatments, i.e. control I and control II as the salt level increased in the medium, the concentrations of N, P and K exhibited the opposite trend. Moreover, when detecting the effect of different treatments on N, P, K, Na, Mg and Ca levels, it could be realized that Mean T of calli treated with combination between laser rays and EMS confirmed the superiority of the previous nutrients accumulation over the respective Mean T of the salt-tolerant control; meanwhile the other two treatments could only exceeded the Mean T of the salt-sensitive control. As to the calli fresh weight (Table 1) as well as the accumulation of the protective compounds (Table 2), the data of nutrients accumulation recorded in Table 2, strongly confirmed the superiority of the calli derived from grains exposed to the combined treatment of laser rays and EMS regarding the clear positive response between salinity levels up to 30% sea salted water level and nutrient accumulation; meanwhile the single treatment of either laser rays and EMS exceeded only up to 15% sea salted water level.

Excess salt in the root medium impaired N uptake, the competitive relationship between NO_3^- and Cl^- are well documented in Zarate (1990). Wilkinson (1994) recorded an increase of Na^+ and Mg^{++} absorption at high exchangeable sodium percentage values in wheat plants grown under salinity conditions; while N, P, Ca and K absorption decreased. Also, Marschner (1995) indicated that Ca^{++} is strongly competitive with Mg^{++} and binding sites on the root plasma membrane appear to have less affinity for the highly hydrated Mg^{++} than for Ca^{++}. Moreover, Trivedi et al, (1991) and El-Shafey *et al.* (2003) working on wheat callus and they found that total N, P, K and Ca decreased with increasing salinity level.

On the other hand, the ratio of K/Na in the calli tissues either untreated or treated with laser rays and EMS each alone or in combination was highly influenced by increasing Na concentration as the salinity levels increased in the medium even in the treated calli. Therefore, K/Na ratio was gradually decreased as the salinity levels increased in the medium.

In saline soil, the Na^+ / K^+ ratio is very high. Therefore, it seems logically to expect that plants which tolerate salinity such as halophytes must develop a mechanism for the preferential uptake of K^+ from mixture rich in Na^+. These plants must have a very developed absorption system (Rengel, 1999). Protein synthesizing system from wheat germ is sensitive to K^+ / Na^+ , its activity was substantially reduced when K^+ / Na^+ 1 or less (Pessarakli, 2002).

The foregoing callus data (Tables 1 and 2), draw the attention to the most promising effect of the combination between 120 min laser rays + 15 mM EMS, which induced the maximum significant increases in calli fresh weight. This

combined treatment induced the higher accumulation of sugars, proline, free amino acids and nutrient elements in the calli tissues up to 30% sea salted water level. The only significant increment in calli fresh weight and the only increments in the accumulation of different components even over the tolerant control were also recorded as a result of this combined treatment, unlike the other single ones.

Table 2: **Effect of laser rays and EMS treatments on reducing sugars, non-reducing sugars, total sugars as mg glucose/g. D. W., free amino acids as mg/g. D. W., Proline as mg/g. F. W., and concentrations of N, P, K, Na, Ca and Mg as mg/g. D. W. of wheat calli grow under different levels of salinity (% sea salted water) for 6 weeks (combined analysis for two seasons).**

Treatments	Reducing sugars					Non-reducing sugars					Total sugars-				
	% sea salted water														
	0.0	15	30	45	Mean T	0.0	15	30	45	Mean T	0.0	15	30	45	Mean T
Control I	24.13	26.12	34.68	37.19	30.53	27.64	33.61	45.79	47.29	38.58	51.77	59.73	80.47	84.48	69.11
Control II	18.19	22.18	31.58	34.02	26.49	24.12	27.72	40.27	43.33	33.86	42.31	49.9	71.85	77.35	60.35
120 min.	26.48	28.01	29.17	30.42	28.52	31.72	37.52	38.45	39.81	36.88	58.20	65.53	67.62	70.23	65.40
15 mM EMS	25.85	27.87	28.87	30.33	28.23	30.99	36.59	37.27	38.76	35.90	56.84	64.46	66.14	69.09	64.13
120 min. + 15 mM EMS	36.19	51.99	70.36	71.44	57.49	49.19	69.83	88.41	89.51	74.24	85.38	121.82	158.77	160.95	131.73
Mean (S)	26.17	31.23	38.93	40.68		32.73	41.05	50.04	51.74		58.9	72.28	88.97	92.42	
	Free amino acids					Proline					N				
Control I	4.27	6.12	8.71	9.67	7.19	2.43	2.64	4.62	4.78	3.62	22.72	21.16	17.73	12.92	18.65
Control II	3.58	5.28	7.97	8.89	6.43	1.98	2.35	3.88	4.22	3.11	20.42	17.89	15.83	9.71	15.96
120 min.	5.26	7.15	7.31	8.13	6.96	2.64	3.11	3.52	3.71	3.25	26.13	24.31	10.97	9.08	17.62
15 mM EMS	5.05	5.96	7.14	7.95	6.78	2.58	2.94	3.44	3.65	3.15	26.04	24.01	10.41	9.04	17.38
120 min. + 15 mM EMS	9.04	12.56	16.68	17.24	13.88	4.71	6.42	8.79	9.32	7.31	32.53	27.81	22.81	9.18	23.08
Mean (S)	5.44	7.61	9.56	10.38		2.87	3.49	4.85	5.14		25.59	23.04	15.55	9.99	
	P					K					Na				
Control I	2.72	2.49	2.20	1.43	2.21	25.89	21.89	20.22	16.24	21.06	2.12	2.57	2.64	2.75	2.52
Control II	2.26	2.09	1.89	1.05	1.82	23.28	19.29	17.78	12.89	18.31	2.29	2.62	3.31	4.02	3.06
120 min.	3.09	2.75	1.15	0.98	1.99	28.15	26.01	14.22	10.14	19.63	1.94	2.25	4.39	5.68	3.57
15 mM EMS	3.10	2.69	1.12	0.95	1.97	28.21	25.89	13.31	10.03	19.36	1.99	2.31	4.71	5.58	3.65
120 min. + 15 mM EMS	3.32	3.19	2.98	1.02	2.63	34.94	30.06	26.94	11.24	25.79	1.89	2.04	2.38	3.67	2.49
Mean (S)	2.89	2.64	1.87	1.09		28.09	24.63	18.49	12.49		2.05	2.36	3.49	4.34	
	Ca					Mg					K/Na ratio				
Control I	4.96	6.93	11.43	13.35	9.17	2.78	3.67	5.79	7.78	5.01	12.21	8.52	7.66	5.91	8.36
Control II	3.19	4.85	9.63	12.55	7.56	1.98	2.55	4.91	7.14	4.15	10.17	7.36	5.37	3.21	5.98
120 min.	6.09	8.69	8.93	9.45	8.29	3.77	4.44	4.55	5.41	4.54	14.51	11.56	3.24	1.78	5.49
15 mM EMS	6.05	8.59	8.82	9.38	8.21	3.59	4.27	4.52	5.29	4.42	14.18	11.21	2.83	1.79	5.30
120 min. + 15 mM EMS	6.76	9.66	12.04	12.24	10.18	3.85	4.69	6.83	6.97	5.59	18.49	14.74	11.32	3.06	10.36
Mean (S)	5.41	7.74	10.17	11.39		3.19	3.92	5.32	6.52		13.70	10.44	5.29	2.68	

Growth and Yield of the regenerated plants:

The results in Table 3 reported the different values of plant height, number of leaves, shoot dry weight as well as the grain yield (g./plant) after 45 days in sand culture irrigated with the different levels of salinity (% sea salted water). The obtained results clearly revealed the same expected negative correlation between salinity levels and growth of wheat plants (Mean S), especially, in the untreated treatments (control I and II). These results are in agreement with these reported by Singh *et al.* (2000), Hanafey Ahmed *et al.* (2002) and El-Shafey *et al.* (2003) on wheat plants.

It is important to mention that the reduction in wheat grain yield due to salinity may be attributed to the inhibitor effects of salinity on most growth characters, may be through its effects on photosynthesis and transpiration. In this respect, many workers suggested that the reduction in plant growth and yield due to salinity may be attributed to the effect of salinity on many metabolic processes including protein, nucleic acids and polyamine synthesis (Reggiani *et al.*, 1994), activity of the mitochondria and chloroplasts (Singh and Dubey, 1995), decreasing transpiration, stomatal conductance and photosynthesis (Ashraf and O'leary, 1996 and Adams *et al.*, 2004), restricts the absorption of water by plant roots and water use efficiency (Rengel, 1999), the toxic effects of certain ions present in soil solution (Pessarakli, 2002) and /or imbalance in phytohormone levels through its effect on either the biosynthesis or the destruction of the plant hormones (Dunlap and Binzel, 1996).

Also, the obtained data in Table 3 clearly show the significant stimulative effects of laser rays and EMS either each alone or in combination on the growth, dry matter accumulation and grain yield / plant of the regenerated wheat plants. The growth and yield values reached their maximum under 15% sea salted water level for all treatments followed by a sharp decrease under 30% and 45% for 120 min. Laser rays and 15 mM EMS treatments; while sharp decrease was found under 45% sea salted water level for the combined treatment. Hence, the clear superiority of the combination between laser rays and EMS in inducing the highest degree of salt tolerance of the regenerated wheat plants could be clearly seen and which even surpassed over both untreated controls; meanwhile the other two treatments recorded the significant increases in the mean values (Mean T) of the above growth and yield of the regenerated wheat plants only over the respective values of the salt-sensitive control. As a result of the simulative effects of the combination treatment, the grain yield per plant of the treated wheat was more than two folds of the respective yield of the tolerant control (Sakha 93) and more four folds than that of the sensitive control (Giza 168) under 30% sea salted water level. This result needs further investigation in a broad scale of pot experiments and in the field. Similar results were reported by El-Shafey *et al.* (1998) with wheat as affected by the combination between 15 KR gamma rays + 5 mM sodium azide and with rice by 5 KR gamma rays +1 mM sodium azide. Also, Whan *et al.* (1991) successfully produced two salt-resistant citrus mutants using gamma rays and EMS. Recently, Harb *et al.* (2002) suggested the possibility of successful application of EMS in improving salinity tolerance of banana plants.

Table 3: Effect of laser rays and EMS treatments on growth characters of regenerated w heat p lants (45 days-old) grown ls sand culture under different levels of salinity (% sea salted water) (Combined analysis for two seasons).

Treatments	Plant height (cm.)					No. of leaves / Plant				
	% sea salted water									
	0.0	15	30	45	Mean (T)	0.0	15	30	45	Mean (T)
Control I	31.48	28.21	23.11	15.69	24.62	8.35	7.84	7.32	6.86	7.59
Control II	27.69	22.66	17.78	12.85	20.25	7.24	7.02	6.34	5.88	6.62
120 min.	36.33	32.05	13.39	9.61	22.85	9.88	9.31	5.35	3.89	7.12
15 mN EMS	33.96	31.29	12.96	9.42	21.91	9.69	9.19	5.28	3.75	6.98
120 min. + 15 mM EMS	41.41	36.42	32.31	10.82	30.24	10.32	9.24	9.06	5.10	8.43
Mean (S)	34.17	30.13	19.91	11.68		9.09	8.52	6.67	5.09	
New L.S.D. at 5%										
Salinity (S)			0.65				0.24			
Treatment (T)			0.74				0.33			
(S) x (T)			1.49				0.66			
	Shoot dry weight (g/plant)					Grain yield (g. / plant)				
Control I	2.89	2.43	2.11	1.88	2.33	2.59	1.81	1.56	1.42	1.85
Control II	2.39	1.90	1.69	1.43	1.85	1.92	1.24	1.02	0.84	1.26
120 min.	3.27	2.73	1.39	1.11	2.13	3.45	2.70	0.55	0.25	1.74
15 mM EMS	3.11	2.69	1.25	1.16	2.05	3.39	2.64	0.49	0.21	1.68
120 min. + 15 mM EMS	3.68	3.05	2.73	1.22	2.67	5.17	4.81	4.28	0.39	3.74
Mean (S)	3.07	2.56	1.83	1.36		3.30	2.64	1.58	0.62	
New L.S.D. at 5%										
Salinity (S)			0.13				0.34			
Treatment (T)			0.18				0.38			
(S) x (T)			0.38				0.79			

Chemical analyses of the regenerated plants:

A-Sugars, proline, total free amino acids and invertase activity:

As previously revealed with the calli tissues (Table 2) increasing salinity levels (% sea salted water) caused high accumulation of the protective compounds, i.e. sugars, proline and free amino acids (Mean S) in the regenerated (45 days – old) plants (Table 4). The present results strongly confirm the previous conclusion drawn from the callus data, regarding the superiority of the combination treatment of laser rays and EMS. The regenerated wheat plants from grains exposed to laser rays + EMS exhibited the highest degree of salt tolerant, i.e. the positive correlation between such treatments and improving of salinity tolerance. Since under 30% and 45% sea salted w ater l evels o f salinity, the wheat plants with the combination of laser rays + EMS accumulated nearly 2 folds of that of the tolerant control I and more than 2 folds of the sensitive one (control II). On the contrary, the other two treatments (laser rays and EMS each alone) caused noticeable reduction in the accumulation of the protective compounds when compared with the tolerant control (control I) and sensitive control (control II).

Also, the absolute superiority was confirmed here the combined treatment of laser rays and EMS in inducing the highest increases in the Mean T of concentrations of the protective compounds in the produced shoot tissues

over the respective Mean T of the salt-tolerant control; meanwhile the other two single treatments either with laser rays or EMS exceeded only the salt – sensitive control in this regard. These findings are in great agreement with the opinion of Binzel and Reuveni (1994) reported that under saline conditions the accumulation of non toxic substances such as sucrose, proline, organic acids, pigments, nucleic acids and protein is considered to be protective adaptation and the survival of plants under saline conditions depends upon the regulation of m etabolic processes and the quantitative ratio between the protective and toxic metabolic intermediale.

Table 4: Effect of laser rays and EMS treatments on reducing sugars, non-reducing sugars, total sugars as mg glucose/g. D. W., free amino acids as mg/g. D. W., Proline as mg/g. F. W., and Invertase activity as μ mol / glucose / min. / g. F. W. in the shoots of regenerated plants grown in sand culture under different levels of salinity (% sea salted water) for 45 days – old (combined analysis for two seasons).

Treatments	Reducing sugars					Non-reducing sugars					Total sugars				
	% sea salted water														
	0.0	15	30	45	Mean T	0.0	15	30	45	Mean T	0.0	15	30	45	Mean T
Control I	29.13	31.85	42.29	46.35	37.20	33.71	40.99	55.85	57.68	47.06	63.02	72.84	98.14	103.03	84.26
Control II	22.19	27.05	38.51	41.49	32.31	29.41	33.81	48.11	52.84	41.29	51.59	60.86	87.62	94.33	73.60
120 min.	32.29	34.15	32.57	37.99	34.78	38.68	45.78	46.89	48.58	44.97	70.97	79.91	82.46	86.64	79.75
15 mM EMS	31.62	33.99	35.21	36.99	34.43	37.79	44.63	45.45	47.27	43.79	69.31	78.62	80.66	84.26	78.22
120 min. + 15 mM EMS	44.13	63.41	85.81	87.12	70.12	59.87	85.16	107.81	109.15	90.49	104.0	148.57	193.62	196.27	160.61
Mean (S)	31.89	38.09	47.48	49.81		39.89	50.07	61.02	63.09		71.78	88.18	108.50	112.70	
	Free amino acids					Proline					Invertase activity				
Control I	5.21	7.35	10.62	11.79	8.74	2.90	3.22	5.64	5.81	4.41	68.33	42.28	31.16	18.86	40.16
Control II	4.36	6.44	9.72	10.85	7.84	2.41	2.87	4.73	5.15	3.79	72.84	55.99	45.89	29.32	51.02
120 min.	6.41	8.59	8.92	9.92	8.46	3.22	3.79	4.29	4.52	3.96	63.28	50.77	40.05	28.18	45.72
15 mM EMS	6.16	8.23	8.71	9.12	8.06	3.15	3.58	4.19	4.45	3.84	65.13	47.73	39.81	27.01	44.92
120 min. + 15 mM EMS	11.03	15.32	20.34	21.59	17.07	5.74	7.83	10.72	11.36	8.91	49.75	32.95	23.89	19.51	31.53
Mean (S)	6.83	9.19	11.66	12.25		3.49	4.26	5.91	6.26		63.38	45.94	36.24	24.60	

Moreover, it has been suggested that the high concentration of organic solutions in the cytoplasm could have the following roles: a – e contribution to the osmotic balance when electrolytes are lower in the cytoplasm than the vacuole, b- a protective effect of enzymes in the presence of high electrolytes in the cytoplasm (Marschner, 1995). The sugars as osmolytes can enable plants to keep better water relations under stress conditions. Also, sucrose protected isolated chloroplasts against desiccation (Rengel, 1999). More recently, Pessarakli (2002) concluded that plant use soluble sugars as an osmoticum under saline conditions. Hence, the plants that fail to increased soluble sugars biosynthesis could not tolerate salt stress. El-Shafey *et al.* (2003) reported that salt-tolerant Sakha 8 wheat cultivar showed much higher degree of osmotic adjustment through the accumulation of considerable quantities of organic protective osmolytes, i.e. sugars (especially non-reducing ones), proline and free amino acids in their shoots and roots, which greatly exceeded that in the salt susceptible Giza 167 wheat cultivar.

The endogenous concentration of free proline in plants can be used as an indicateor of salt tolerance. For each plant, it appears that there is an external salt c oncentration a bovewhich t he p lant's p roline l evel s harply r ises. This critical point is directly to the ability of plant to tolerate salt. Thus, measurements of condition can be used to determine salt resistance of plants

(Pessarakli, 2002). Moreover, proline and other compatible solutes are believed to case the minimal inhibition of metabolism. Also, proline is organic osmolytes solute with an amphiphilic molecule protects the hydrophobic parts of proteins which suffer first when water potential is lowered. By forming association with the hydrophobic proteins of macromolecules, proline converts them into hydrophilic parts (Binzel and Reuveni, 1994). In addition, Good and Zaplachinski (1994) reported that, the concentration of free amino acids (particularly proline) often increases markedly in the leaves or other plant tissues with exposure to many biotic or abiotic stress. Recently, Salem *et al.* (2002) with faba bean and Fatouh Youssef (2003) with wheat found that proline, sugars and free amino acids increased with increasing salinity level.

New class of genes, called "Osm" (Osmotic tolerance) genes that is used for protection against osmotic stress and may work in a similar manner in plants, bacteria and animals now attracted the attention of physiologists, through their action following salinity. The over produced proline may be explained on the basis that osmogenes govern the production of a class of molecules such as betaine and proline that protect the cell and its constituents against " dehydration Osm" (Pessarakli, 2002).

Also, many reports proved the rapid increase in synthesis and accumulation of sugar under saline conditions. Nasir *et al.* (2000) reported that leaves of salt tolerant line sugarcane showed high degree of osmotic adjustment by the accumulation of more K^+, free proline and sugar contents. Cordoba *et al.* (2001) found that roots from salt-treated of *Chloris gayana* plants accumulated higher concentrations of soluble sugars.

Concerning accumulations of amino acids with increasing salinity levels, El-Shafey *et al.* (1998) and Fatouh Youssef (2003) reported that amino acid concentration increased in wheat callus with increasing salinity levels.

As for the efficiency of EMS in this regard. Harb *et al.* (2002) suggested the possibility of successful application of EMS as a chemical mutagen to improve salinity tolerance of banana, which was associated with increasing accumulation of the protective substances such as total sugars, free amino acids, proline and soluble phenols in plant banana tissues with increasing salinity levels in MS media on which the shoots tip of banana plants were cultured.

Concerning the invertase activity in the regenerated shoots of 45 days – old, the obtained results in Table 4 clearly showed the negative correlation between increasing salinity level and invertase activity, was also more pronounced as a result of combined treatment (15 mM EMS + 120 min. laser rays) which recorded much lower activities as compared with that in the salt-tolerant control at the all applied levels of salinity as well as when calculating the mean value of this treatment (Mean T), the only exception is that, at 45% sea salted water when the enzyme activity in the treated plants was nearly equal to its activity in the salt-tolerant control. On the contrary, the other two treatments, i.e. 120 min laser rays and 15 mM EMS increased invertase activity over that in the salt-tolerant control at 15%, 30% and 45% sea salted water and consequently the mean values of these treatments (Mean T). This finding added another supportive evidence for the superiority of the combined treatment in increasing salinity tolerance of the regenerations brought about by

inhibiting invertase activity in favour of accumulations of more quantities of non-reducing sugars, which play the vital role in improving salinity tolerance of the regenerated *in vitro* plant. This finding also strongly indicating that the high degree of tolerance to salinity in the treated-sensitive wheat plants, was transmissible to the regenerations under *in vitro* conditions.

Supportive evidence for this finding is found in the results by Dubey and Sing (1999) who reported that invertase activity decrease in rice shoots of the salt-tolerant cultivars, wheares increased in the salt-sensitive ones. Moreover, Fatouh Youssef (2003) who disclosed that the invertase activity in the leaves of 75 days – old wheat plants of the salt-tolerant Sakha 8 cultivar showed much lower activities than that of the leaves of the salt-sensitive Giza 167 cultivar at the all applied salinity level (0.0, 15.0, 30.0 and 45.0 % sea water). Moreover, the same auther added that, the physiological treatments (ABA, gamma rays and puterscine) that induced more tolerance to salinity in the salt-sensitive Giza 167 wheat cultivar, resulted in much more reduction in the activity of invertase enzyme in the treated leaves.

8- Minerals:

The nutrient elements concentration in the shoot tissues of growing wheat plants (45 days – old) are shown in Table 5. The obtained results exhibited the same previous trend drawn from the obtained data of calli analysis (Table 2) either for the untreated or treated plants. The concentrations of N, P and K in the regenerated shoots tended to decreased gradually by increasing salinity level in the medium (Mean S) to reach their lowest values at highest level of salinity, i.e. 45 % sea water level, the concentrations of Na, Ca and Mg contrary increased.

Comparing the nutrient elements concentrations of the treated plants shows that all treatments, accumulated different nutrients over both control treatments under zero and 15% sea salted water salinity level. Such accumulation was decreased under higher levels of salinity 30% and 45% sea salted water levels as a result of 120 min. laser rays and 15 mM EMS and 45% sea water level with combined treatment (laser rays + EMS).

The combined treatment could exceeded accumulation of the nutrients over the respective values of both controls (Mean T), meanwhile the other two treatments (120 min laser rays and 15 mM EMS) could only exceeded the Mean T of the salt-sensitive control. The results confirm the previous findings drawn with calli tissues (Table 2). The favorable effects of the combined treatment were reflected on the growth and yield of the regenerated plants (Table 3), protective compounds (Table 4) and nutrient uptake (Table 5). These effects may be as a result of plant adaptation to stress conditions. Increasing nutrient accumulation induced by gamma ray + sodium azide under saline conditions was previously recorded by El-Shafey *et al.* (1998) with rice and wheat, Ghallab and Nesiem (1999) with 5.0 KR gamma rays with wheat, Harb *et al.* (2002) with EMS with banana and El-Shafey *et al.* (2003) with 15.0 KR gamma rays with wheat.

The ratios K / Na and Na / Ca were gradually decreased as the salinity levels increased in the medium. This was also true even in the treated plants

with laser rays and EMS either alone or in combination, because the rate of Na increases was higher than that of K or Ca as the salinity levels increased.

Pessarakli (2002) stated that reduction in N under saline conditions may be due to reduction in water absorbed and a decrease in root permeability. The high concentration of Na in the high salt level suggested that under saline conditions sodium influx across the plasmalemma to the vacuole may play a major role in permitting turgor maintenance. Some crops show marked beneficial effects of Na especially if the K supply is limited. These crops take up large amounts of Na which contribute to the osmotic potential of the leaves and increases resistance to water stress. In the wheat plants, the yield response to Na often exceeds that of K (Ghallab and Nesiem, 1999). Also, under saline conditions Na has important specific effects. Both Na and K will move along the electrochemical gradients of tissue but because of discrimination of the cell membranes or Na excursion the ultimate concentration ratio may be 20 K to 1.0 Na (Wilkinson, 1994).

Table 5: Effect of laser rays and EMS treatments on N, P, K, Na, Ca and Mg concentrations (mg/g. D. W.) as well as K/Na and Na/Ca ratios in the shoots of regenerated wheat plants grown in sand culture under different levels of salinity (% sea salted water) for 45 days-old (Combined analysis for two seasons).

Treatments	N					P				
	% sea salted water									
	0.0	15	30	45	Mean (T)	0.0	15	30	45	Mean (T)
Control I	27.42	25.47	21.34	15.56	22.45	3.27	3.01	2.65	1.72	2.66
Control II	24.82	23.75	19.24	11.70	19.40	2.51	2.47	2.31	1.28	2.14
120 min.	31.04	29.19	14.19	9.69	21.03	3.79	3.58	1.69	0.91	2.49
15 mM EMS	30.94	28.98	13.86	9.45	20.81	3.65	3.49	1.58	0.88	2.40
120 min. + 15 mM EMS	39.16	33.61	27.47	10.16	27.60	3.96	3.84	3.58	0.99	3.09
Mean (S)	30.68	27.80	19.22	11.33		3.44	3.28	2.36	1.16	
	K					Na				
Control I	31.17	26.35	24.34	19.56	25.35	2.72	3.29	3.38	3.91	3.33
Control II	28.29	23.45	21.61	15.67	22.26	2.94	3.38	4.24	5.15	3.92
120 min.	33.79	29.89	19.66	12.78	24.03	2.49	2.89	5.64	7.28	4.58
15 mM EMS	32.69	29.17	19.11	12.53	23.38	2.58	2.95	8.04	7.15	4.68
120 min. + 15 mM EMS	42.16	38.11	32.44	13.54	31.06	2.43	2.61	3.05	5.97	3.27
Mean (S)	33.62	28.99	23.43	14.81		2.63	3.02	4.47	5.69	
	Ca					Mg				
Control I	6.37	8.89	14.88	17.11	11.76	3.57	4.71	7.43	9.97	6.42
Control II	4.10	6.22	12.34	16.09	9.69	2.54	3.27	6.29	9.15	5.31
120 min.	7.82	11.15	11.45	12.11	10.63	4.83	5.69	5.83	6.93	5.82
15 mM EMS	7.75	11.01	11.31	12.02	10.52	4.61	5.48	5.79	6.78	5.67
120 min. + 15 mM EMS	8.67	12.19	15.44	15.69	13.05	4.94	6.01	8.75	8.93	7.16
Mean (S)	6.94	9.93	13.04	14.60		4.09	5.03	6.82	8.35	
	K / Na					Na / Ca				
Control I	11.46	8.01	7.18	5.00	7.81	0.43	0.37	0.23	0.22	0.28
Control II	9.62	6.98	5.09	3.04	5.68	0.72	0.54	0.34	0.32	0.40
120 min.	13.57	10.34	3.48	1.76	5.25	0.31	0.26	0.49	0.60	0.43
15 mM EMS	12.77	9.89	3.16	1.75	4.99	0.33	0.27	0.53	0.59	0.44
120 min. + 15 mM EMS	17.35	13.64	10.64	2.72	9.49	0.28	0.21	0.19	0.32	0.25
Mean (S)	12.78	9.59	5.24	2.60		0.38	0.30	0.34	0.39	

The specific effects of Na / Ca ratio was recorded by several works. It is evidenced that the adequate level of Ca in the plant has beneficial effect for reduction Na uptake; thus plants with high Ca / Na ratio had more ability to exclude Na under saline conditions (Kent and Lauchi, 1985). Also, the low Na / Ca ratio is important in maintaining membrane function as reported by Greenway and Munnus (1980) who added that , growth of beans was markedly influenced by the Na / Ca ratio , at high external Na Cl. Growth of beans decreased and Na increased in the leaves onl when Na / Ca exceeded 17. Moreover, Nuttall *et al.* (2003) found that in wheat plants when K/Na ratio was 2.5, adverse effects of salinity could be expected. K/Na ratio 1.5 is corresponding to 50% reduction in growth.

Taiz and Zeiger (1991) postulated that Mg concentration in chloroplasts may influence photosynthesis during water stress through its role in coupling electron transport to ATP production. The plants with the lower tissue Mg concentrations maintained higher photosynthetic rates as leaves became hydrated.

C- Phytohormones:

The obtained data in Table 6 regarding hormonal analyses of the shoot tissues of growing wheat plants (45 days-old) clearly reveal that the concentrations of inolde-3-acetic acid (IAA) and gibberelic acid (GA_3) (µ/g. F.W.) were decreasing by increasing salinity levels (Mean S) to reach their lowest values at highest level of salinity, i.e. 45% sea salted water level, the concentration of absclsic acid (ABA) contrary increased. Similar results were reported by El-Antably *et al.* (1994), Amer *et al.* (1995), Ibrahim and Shehata (2000) and El-Shafey *et al.* (2003).

Table 6: Effect of laser rays and EMS treatments on Indol-3-acetic acid (IAA), gibberellic acid (GA_3) and absclsic acid (ABA) concentrations (µg / g. F. W.) in the shoots of regenerated plants grown in sand culture under different levels of salinity (% sea salted water) for 45 days – old (combined analysis for two seasons).

Treatments	IAA					GA_3					ABA				
	% sea salted water														
	0.0	15	30	45	Mean (T)	0.0	15	30	45	Mean (T)	0.0	15	30	45	Mean (T)
Control I	22.82	19.44	16.11	12.47	17.71	24.86	22.46	20.43	12.36	20.03	2.55	3.19	4.35	5.05	3.79
Control II	19.62	16.89	13.93	11.32	15.44	20.07	17.33	14.79	9.39	15.39	2.67	2.88	4.11	4.89	3.64
120 min.	33.34	28.41	11.62	9.52	20.72	29.52	27.41	12.59	8.18	19.43	2.71	3.76	3.85	3.96	3.57
15 mM EMS	32.51	27.83	11.44	9.39	20.29	29.19	27.23	12.45	8.09	19.24	2.66	3.71	3.82	3.92	3.53
120 min. + 15 mM EMS	37.19	33.46	28.28	10.06	27.25	35.84	30.54	27.25	8.21	25.46	3.29	4.48	4.65	4.71	4.28
Mean (S)	29.09	25.21	16.28	10.55		27.89	24.99	17.50	9.25		2.78	3.60	4.16	4.51	

Comparing the concentrations of the all estimated hormones of the treated plants shows that all treatments, greatly exceeded the comparable concentrations of all estimated hormones over both control treatments under 15% sea water level. Such accumulation was decreased under higher levels of salinity 30% and 45% sea water levels as a result of single treatments (120 min laser rays and 15 mM EMS) and 45% sea water level with combined treatment (laser rays + EMS). The combined treatment could exceeded accumulation of

IAA, GA_3 and ABA over the respective values of both controls (Mean T), meanwhile the other two treatments (120 min laser rays and 15 mM EMS) could only exceeded the Mean T of the salt-sensitive control. In this concern, Wilkinson (1994) elucidated the effects of radiation on plant metabolic sites which are included the synthesis of DNA, enzymes, amino acids, proteins and auxins, in addition to photosynthesis but he indicated that auxin synthesis seems to be the most sensitive non-genetical process affected by irradiation, thus the resultant altered concentration of auxim would be logical cause of many secondary effects. Accordingly, it could be postulated that the combined treatment seems the most suitable one for enhancing plant growth and development through stimulation of auxin biosynthesis.

Moreover, Zeinab and Sallam (1996) reported that with increasing Na concentration, the tryptophan synthesis ∝- monomers were gradually dissociated from the oligomers producing less active isoenzyme. This reduced the biosynthesis of L-tryptophan and consequently that of IAA, so that wheat growth was retarded or even stopped. Also, the same authors added that at higher salinity there was an accumulation of gibberellin inhibitors and no gibberellin activity was found in wheat plants. El-Desoky and Atwai (1998) stated that in sour orange the biological activities of cytokinins, gibberellins and auxins were significantly reduced by excess salinity (5000 ppm) in the irrigation water.

For ABA, Maslenkova *et al.* (1993) reported that ABA level increased with salinity stress, and that this level correlated with plant resistance to the salt stress. Also, Wang-Yongyin *et al.* (2001) and Hatung (2004)considered ABA is the primary hormone that mediates plant responses to stress such as cold, drought and salinity; thus its endogenous level increased with salinity stress.

Chemical analyses of the produced grains:

Comparing the sugar concentration in wheat grains (Table 7) as affected by different treatments under salinity levels (% sea salted water), clearly revealed that the same observed trend with calli tissues or the growing shoot plants; as to sugar accumulation, it was recorded the same especially in case of the grains either treated or that of the untreated control treatments. The present results show that the produced grains of 120 min. laser rays and 15 mM EMS accumulates more sugars, mainly sucrose, over both controls till 15% sea salted water level.

While for the combined treatment (laser rays + EMS) till 30% sea salted water level; however sugar concentrations in the grains produced from these treated plants were nearly equal to that in the grains of untreated salt-tolerant control at 45% sea salted water level. Thus, the combined treatment greatly exceeded both controls when the Mean T was considered, for reducing, non-reduced and total sugars. Meanwhile, the other two treatments (120 min. laser rays and 15 mM EMS) greatly exceeded only the Mean T of the salt-sensitive control. The amount of sucrose were 5-6 folds that of reducing sugars.

The crude protein concentration in the wheat grains of treated plants showed the same observed trend drawn with sugars. Regarding the control

treatments, i.e. control I and II the crude protein adversely affected by increasing salinity levels in the medium.

The results in Table 8 represent the percentage (on D. Wt. bases) of P, K, Ca, Mg and Na as well as K/Na ratio.

Table 7 : Sugar concentrations (mg glucose / g. D. W.) and crude protein (mg/g. D. W.) in wheat grains produced from treated calli with laser rays and EMS and plantlets grown in sand culture under different level of salinity. (Combined analysis for two seasons).

Treatments	Reducing sugars					Non-reducing sugars				
	% sea salted water									
	0.0	15	30	45	Mean (T)	0.0	15	30	45	Mean (T)
Control I	2.95	4.28	8.35	10.52	6.53	24.71	28.93	35.53	40.62	32.45
Control II	2.49	3.21	6.31	7.49	4.88	20.75	24.84	32.16	35.94	28.42
120 min.	4.11	5.35	6.06	6.25	5.44	27.32	31.11	31.69	33.17	30.82
15 mM EMS	3.99	5.27	5.99	6.19	5.36	26.82	31.03	31.53	33.04	30.61
120 min. + 15 mM EMS	4.42	6.29	9.79	9.99	7.62	31.56	33.92	37.39	40.19	35.77
Mean (S)	3.59	4.88	7.30	8.09		32.79	27.97	33.66	36.59	
	Total sugars					Crude protein				
Control I	27.66	33.21	43.88	51.14	38.98	125.59	97.15	86.72	72.74	95.55
Control II	23.24	28.05	38.47	43.43	33.30	105.83	79.11	67.71	47.24	74.97
120 min.	31.43	36.46	37.75	39.42	36.26	144.18	122.53	62.35	36.91	91.49
15 mM EMS	30.81	36.30	37.52	39.23	35.97	143.44	118.75	62.11	36.79	90.27
120 min. + 15 mM EMS	35.98	40.21	47.18	50.18	43.39	160.65	136.13	113.32	42.91	113.25
Mean (S)	36.38	34.85	40.96	44.68		135.94	110.73	78.44	47.32	

Table 8: The percent of P, K, Ca, Mg and Na concentrations as well as K/Na ratio in wheat grains produced from treated calli with laser rays and EMS and planetlets grown in sand culture under different levels of salinity (combined analysis for two seasons).

Treatments	P					K					Ca				
	% sea salted water														
	0.0	15	30	45	Mean (T)	0.0	15	30	45	Mean (T)	0.0	15	30	45	Mean (T)
Control I	0.16	0.14	0.12	0.09	0.13	0.41	0.32	0.27	0.24	0.31	0.17	0.24	0.47	0.65	0.38
Control II	0.13	0.11	0.09	0.07	0.10	0.32	0.28	0.19	0.14	0.23	0.11	0.15	0.32	0.58	0.26
120 min.	0.21	0.17	0.07	0.04	0.12	0.51	0.45	0.11	1.01	0.52	0.22	0.28	0.30	0.44	0.31
15 mM EMS	0.19	0.16	0.06	0.04	0.11	0.53	0.41	0.13	1.08	0.54	0.21	0.27	0.29	0.41	0.29
120 min. + 15 mM EMS	0.25	0.21	0.19	0.05	0.18	0.55	0.48	0.36	1.19	0.65	0.29	0.32	0.54	0.65	0.43
Mean (S)	0.19	0.16	0.11	0.06		0.46	0.39	0.21	0.73		0.20	0.25	0.38	0.53	
	Mg					Na					K/Na				
Control I	0.28	0.30	0.42	0.62	0.41	0.08	0.12	0.13	0.15	0.12	5.13	2.67	2.08	1.60	2.58
Control II	0.11	0.25	0.37	0.44	0.29	0.09	0.13	0.15	0.16	0.13	3.56	2.15	1.27	0.88	1.77
120 min.	0.32	0.34	0.35	0.37	0.35	0.07	0.11	0.18	0.19	0.14	7.29	4.09	0.61	5.32	3.71
15 mM EMS	0.30	0.33	0.34	0.36	0.33	0.06	0.11	0.19	0.20	0.14	8.83	3.73	0.68	5.40	3.86
120 min. + 15 mM EMS	0.35	0.37	0.39	0.42	0.38	0.04	0.09	0.11	0.13	0.09	13.75	5.33	3.27	9.15	7.22
Mean (S)	0.27	0.32	0.37	0.44		0.07	0.11	0.15	0.17		6.57	3.55	1.40	4.29	

In the control treatments of the tolerant (Sakha 93) or sensitive (Giza 168) cultivars, P and K concentrations in the wheat grains decreased as the

salinity levels increased, while Ca, Mg and Na concentrations exhibited the opposite trend. As regard the nutrient concentration affected by laser rays and EMS treatments under the different levels of salinity, the obtained data showed nearly the same trend of the sugar concentration in the grains with special superiority of the combined treatments. Comparing Na concentration in calli, shoot tissues as well as in the grains (Tables 2, 5 and 8), clearly shows that its level declined from calli and shoot tissues to attain its minimum values in the grains (2.5%, 2.1% and 0.13%) for 120 min. laser rays + 15 mM EMS under 45% sea salted water level. In this concern, Marschner (1995) stated that fruit are very low in Na and Cl and high in K even when grown at high NaCl concentration. In these results, the concentrations of K and Na in wheat grown under 45% sea water level for the same combined treatment was 1.19% and 0.13% respectively.

Therefore, it might be expected that K/Na ratio must be decreased in their response to decreasing salt concentration even under the different laser rays and EMS treatments. In this respect, Marschner (1995) stated that, in salin soil the Na/K ratio is very high. Thus, the plants which are tolerant to salinity such as halophytes must develop a mechanism for preferential uptake of K from mixture rich in Na. These plants must have a very developed "absorption system".

CONCLUSION

A wide survey of all foregoing results in the present study, clearly reveal that the main experiments (during the 2nd and 3rd growing seasons) confirm the previous conclusion drawn from the results obtained from the preliminary ones (in the 1st season) as regards the most promising treatments. Moreover, the results obtained during the two growing seasons of the main experiment confirm the absolute superiority of the combination of laser rays + EMS compared with the single treatment of each alone. The results emphasized its superiority in inducing the higher degree of salt tolerance and consequently growth of the calli and induction of plant regeneration, tolerant up to 30% sea water level and which were able to continue their growth till maturing and even attained grain production and yielded grains/plant more than two folds that of the control of the tolerant cultivar Sakha 93 and more than four fold that of the sensitive one Giza 168 under the 30% sea water level. Such high degree of tolerance exhibited by combined treatment (120 min laser rays + 15 mM EMS) was positively associated with higher accumulation of the endogenous hormonal status (IAA, GA_3 and ABA) protective substances, i.e. sucrose, proline and amino acids in the shoot tissues. This accumulation was positively correlated with the increase in salinity levels in the medium. This in addition to the considerable reduction in invertase activity in the treated shoot tissues in favor of more accumulation of non-reducing sugars. This is also applied to the considerable accumulation of much more quantities of inorganic osmolica, i.e. N, P, K, Ca and Ca as well as highest K/Na ratio, as will as to the lowest quantities of Na in their calli and shoots.

The obtained data of combined treatment during the two growth seasons of the main experiment offered strong evidence for the absolute superiority of such treatment in inducing higher degree of tolerance to salinity through the accumulation more quantities compared even with the tolerance control, of the protective solutes in order to adjust the ratio between the protective and toxic intermediates of metabolism in favor of more tolerance to salinity. Moreover, such behavior in the treated plants of the combined treatment evidently increased their ability to counteract salinity stress, thus were able to keep better performance against salinity till harvest. This was reflected in a significant increments in the grain yield/plant over the respective yield of all other treatments including even the salt-tolerant control up to 30% sea salted water level.

The obtained data suggested that the combination between laser rays and EMS may be successfully applied to improve salinity tolerance of economic crops such as wheat, but it must be applied widely and after precise study with each crop to approach its optimal effectiveness in improving tolerance to salinity.

Further genetical as well as physiological studies are needed at the cell level to disclose whether, the role of laser rays and EMS, in regulating the uptake and accumulation of different solutes, is attributed to some alternation in the properties of the cell membranes or to any other genetic changes or somatic mutation.

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