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EFFECT OF JASMONIC ACID ON INCREASING SALINITY TOLERANT OF MICROPROPAGATED STEVIA PLANTS.

Ghallab, A. M.*; M. I. Kobasy; Hanaa F. Y. Mohamed* and A. E. Nafie*****

* **Plant Physiology Division, Agricultural Botany Department, Faculty of Agriculture, Cairo University , Giza, Egypt**

** **Biochemistry Dept., Fac. of Agriculture, Cairo University , Giza, Egypt**

*** **Sugar Crops Research Institute, Agric. Research Center, Giza, Egypt**

ABSTRACT

The effect of jasmonic acid (0.0, 10.0, 20.0, and 30.0µM/l) in improving salinity tolerance of the micropropagated stevia plants grown under different levels of salinity (0.0 10.0, 20.0, 30.0 and 40.0 % sea salted water) was studied. Experiments were conducted during 2003 and 2005 seasons. The obtained results clearly confirmed the absolute superiority of lower concentration (10µM/l) of jasmonic acid treatment, which significantly increased the growth parameters of stevia grown *in vitro* (survival percentage, shootlet length, no. of leaves / shootlet. no. of roots / plantlet and fresh as well as dry weight of shootlet) and *ex vitro* (shoot height, no, of leaves / plant and shoot dry weight) , yield components (leaves, stem and shoot / plant) and over the untreated control treatment. Also, the stevia plantlets derived from shoot tip treated with the lower concentration (10 µM/l) of jasmonic acid were tolerant up to 20 % salinity level and were able to continue their growth under glasshouse conditions till maturity and stevia yield production. The data also revealed that tolerance which was more pronounced as a result of phenylalanine amonialyase (PAL) activity the lower rate of jasmonic acid and was associated with high accumulation of much more quantities inorganic osmotica,i.e. N, P , K, Mg and Ca as well as lowest quantities of Na, in addition to considerable accumulation of organic protective osmolytes (sucrose, proline, amino acids and total soluble phenols), stevioside (%), photosynthetic pigments (carotenoids and chlorophylls) and endogenous hormones (IAA, GA_3 and ABA) , in addition to the lowest invertase activity and higher phenylalanine in the stressed leaves in favor of accumulation more non-reducing sugars in the stevia grown *in vitro* and *ex vitro* . Such accumulation increased as the salinity level was increased. Such behavior seems to induce more ability for stevia plants to continue their growth till maturity and production of stevia yield even under 20 % salinity level. The obtained data suggested the possibility of successful application of the jasmonic acid to improve salinity tolerance of economic crops such as stevia.

Keywords: Jasmonic acid, stevia, salinity tolerance, tissues plantlets, *in vitro*, *ex vitro*.

INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni) is a sweet herb indigenous to the elevated terrain of northeastern Paraguay near its borders with Brazil. The principles responsible for the intense sweetness of this herb are a group of ent-kaurene glycosides of which the most abundant is stevioside. The sweet component (stevioside) of the stevia plant is 300 times sweeter than sucrose and has similar tasteful properties (Sociar o *et al.*, 1983).

Stevioside is chemically stable and occurs in the dried leaves of *Stevia rebaudiana* at about 42% (w/w). It was proposed as a suitable substitute for

saccharin. Currently, about 750-1000 tons of stevia are used annually by Japan, Brazil and other nations in a variety of foods including soft drinks, sea foods and pickled vegetables ..... etc. (Richard, 1996).

Reduction of the extremely high sugar consumption and its substitution with natural sweeteners are quite important problems, especially artificial substances used in present day food industry such as saccharine, sorbine, aspartame. Hence, they have not always met the producers and consumers requests. Also, in the Arab World up till the year 1999, sugar gab reached 4.3 million ton that equal 1.323 millar American Dollar representing 11.31 % of the total value of the main food gab (Allam, 2001 and El-Kholi, 2003). Thus, increasing its productivity and the cultivated areas are highly demanded in Egypt and the Arab World.

Also, 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 culyivatedareas.

Biotechnology and plant tissue culture technique are effective tools for producing salinity tolerant cell lines, tissues and plants. The micropropagation of shoot tip *in vitro* is the most common application of biotechnology in agriculture (Dole, 1990). Also, it has been proposed on a useful, quick and economical to evaluate stress as well as a better system for testing and selecting for stress tolerance (Jose *et al.*, 2000, Shin *et al.*, 2000 and Abd-Eltawab, 2001).

Maslenkova *et al.*, (1992) reported that some of the phytohormones, such as abscisic acid (ABA), ethylene, or jasmonates, may act as stress modulators by suppressing or enhancing the stress responses of plants. Applied exogenously, they can induce physiological changes identical with characteristic parts of the stress responses. Lehmann *et al.*, (1995) stated that application of (ABA) or methyl ester of jasmonic acid (JA-ME) to barley segments or exposure to osmotic stress led to the synthesis of novel proteins that were identical with respect to immunological properties and molecular masses. Moreover, Tsonev *et al.* (1998) showed that barley seedling treated with a daily of 25 and 100µM/l NaCl or pretreated with jasmonic acid (25µM/l) before salinization had little decline in the growth and much lower inhibition of photosynthesis, indicating that barley plants are capable of tolerating a relatively high level of salinity (100µM/l NaCl), and probably (JA) is partially involved in the process of adaptation.

Therefore, the present work was conducted to study the effectiveness of jasmonic acid (JA) treatment in improving salinity tolerance of the microporpagated stevia as well as the produced plants grown under different levels of salinity, aiming to induce (*in vitro*) salinity tolerant cell lines, tissues, plantlets and finally plants.

MATERIALS AND METHODS

The present work was carried out in the Plant Physiology Division, Agricultural Botany Dept., Biochemistry Dept, Faculty of Agric. Cairo Univ. and the laboratory of plant tissue culture, Sugar Crops Research Institute, Agricultural Research Center (ARC), Giza, during two years (2003 – 2005). Seeds of stevia var, spanti were imported from spain.

Preparation of the material and isolation of meristem

Actively growing shoots of stevia plants (var. Spanti) were collected from (6-9) months old crop or from traditional cuttings grown in the field. Shoot tips with the growing apices were taken and the meristematic apex together with a base of tissue approx. 0.5 cm × 0.2 cm was cultured. The shoots are then washed with soap water for about 2-3 minutes followed by several changes of water for assuring the removal of most external contamination. The shoot tip explants were thoroughly rinsed in 7o % (ethanol) for 1 minute. Rinse with sterile distilled water 4-5 times till alcohol was completely washed off. Then the shoot tips immersed for 15 minutes in sodium hypochlorite (1.7-3.4%) and few drops of tween 20 were added as a wetting agent, then rinsed 10 minutes in 3 times in sterilized distilled water to remove all traces of the disinfectant. All steps of sterilization have been done under aseptic conditions inside the culture cabinet (laminar airflow) and by using sterilized instruments.

Established Shoot tip explants:

The following experiment was conducted with Murashige and Skoog (1962) (MS) basal medium. The pH of the prepared medium was adjusted at 5.7±0.1 prior to addition of agar at 7 g l^{-1}. The medium was distributed into the culture jars where each jar contained 50 ml of the medium. The jars were immediately capped with polypropylene closer, and then were autoclaved at 121°C for 20 min. The MS basal medium supplemented with 0.5 mg l^{-1} benzyl adinine (BA) +1mgl^{1} benzyl amino purine (BAP) + 30 g l^{-1} sucrose + 7 g l^{-1} agar was used as initiation medium for culturing sterilized shoot tip explants. The medium was distributed into culture jars (150 ml) where each jar contained 30 ml of medium. Shoot tip explants were incubated at day and night temperature of 27± 2 °C. Light was provided by fluorescent lamps giving intensity of 1500 Lux for 16 hrs per day.

Jasmonic acid (JA) treatments:

Established shoot tip explants were transferred and cultured individually on shoot multiplication MS basal medium supplemented with100 mg 1^{-1} myo-inositol + 30 g 1^{-1} sucrose + 0.5 mg 1^{-1} nicotinic acid + 0.5 mg 1^{-1} pyrodoxine + 2 mg 1^{-1} glycine + 0.2 mg 1^{-1} BA + 2.5 mg 1^{-1} Kin. + 7 g 1^{-1} agar + 10 ml 1^{-1} JA at different concentrations [0.0, 10.0, 20.0 and 30.0 µM]. The explants were repeatedly subculture 3 times at 4 weeks intervals until obtaining cluster explants, each one containing 2-4 developed buds.

Salinity treatments:

The explants transferred and cultured on multiplication basal MS medium as described before. Explanted were treated with sea salt at levels of 0, 10, 20 and 30 % by using sea salted water [sea salt obtained from Sigma Co. USA] where 40 g l^{-1} from the sea salt gave EC = 33000 ppm. Subculturing was done 7 times at 4 weeks intervals into corresponding multiplication fresh media.

Adventious growing shoots were then separated *in vitro* and transferred to rooting ½ MS basal medium supplemented with 2.0 mg l^{-1} IBA + 80 g l^{-1}

sucrose + 3.0 g l^{1} activated charcoal + 6 g l^{-1} agar. The same levels of sea salts were added to the culture medium. In all *in vitro* experiments each treatment consists of 20 replicates, each replicate consists of 12 jars where each jar contain one plantlet.

For each treatment 10 replicates, the morphological parameters (survival percentage, shoot height, leaves and roots number per plantlet, fresh and dry weights of the shoots) were recorded. The shootlets were dried in an oven at 70 °C for 48 hrs and then crude dry weight were determined. The dried shootlets of each treatment were powdered and prepared for chemical analysis at the end of this period (6- weeks after culturing on rooting media). For proline, total soluble phenols, sugars and total free amino acids. Invertase and phenylalanine ammonialyase activities, endogenous phytohormones and photosynthetic pigments estimation, part of the shootlet was kept fresh

Acclimatization of the growing plants and Stevia production:

The produced plantlets were washed with tap water three times to remove all traces of agar; then immersed in vitafax (0.1% for 3 min.) and cultured individually in plastic pots containing a mixture of peatmoss and sand 1:1 (w/w); covered with white transparent plastic sheets (which were punched up 3 cm from two sides) under glasshouse conditions,i.e. light intensity of about 1500 Lux for 16 hrs per day provided by white fluorescent lamps, the temperature of about 28±2°C and the relative humidity was adjusted to 85-90% by adding water for half hour every three hrs through the mist during the nursery stage (21-30 days after transplanting). The white transparent plastic sheets were completely removed at the end of this stage. After two months, the acclimatized plants were transplanted to plastic pots, 40 cm. diameter, containing a mixture of clay and sand at a ratio of 2:1 by weight (Ghallab and Nesiem, 1999). The chemical analysis of the used soil was carried out as described by Page *et al.* (1982) were as follows:

PH = 7.63, EC = 0.69 ds/m, $HCO_3 + CO_3$ = 2.3 me/L , Cl^- = 10.9 me/L , SO_4 = 45.8 me/L , Ca = 7.9 me/L , Mg = 4,2 me/L , Na = 9,6 me/L and K = 2.4 me/L. The complete nutrient solution as described by Hewitt (1952) was used. The plants were irrigated at three days intervals with nutrient solution either alone for the control or mixed with the same previously mentioned levels of salinity throughout the whole growth stages. Washing with tap water was made at two-weeks interval immediately before solution addition to prevent salt accumulation. The plants were incubated at the glasshouse under the same experimental conditions for 8 months. In all pot experiments, each treatment consists of 10 replicates with 15 plants for each replicate (one plant for pot). During the growth period, one sample of 5 replicates; 15 plants from each treatment was taken at 90 days old. Shoot height (cm), number of leaves / plant, and dry weight of shoot were recorded. The shoots were dried in an oven at 70 °C for 48 hrs and then the crude dry weight were determined. The dried shoots of each treatment were powdered and prepared for chemical analysis. For proline, sugars, steviosi de,total soluble phenols, total free amino acids, endogenous phytohormones , photosynthetic pigments, invertase and (PAL) activities estimation, part of the shoot system

was kept fresh, and 5 replicates from each treatments were left to grow till harvest.

At harvesting stage, after 8 months in pots, weight of leaves (g) / plant, weight of stem (g) / plant and dry weight of shoot (g) /plant were recorded. Stem, leaves and shoot were dried in shade under natural condition. Cutting was carried out at 3-5 cm above soil surface.

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

Statistical analysis:

Data of morphological characters and Chemical analysis of stevia grown *in vitro* and *ex vitro* as well as yield components of stevia grown in *ex vitro* were statistically analyzed and the mean values were compared using L.S.D. values at 5 % levels (Gomez and Gomez, 1984).

Chemical analysis:

1- Photosynthetic pigments:

Nornai (1982) method was employed to determine the photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids).

2- Total sugars and stevioside content:

The ethanol extracts of shoots were used to determinate reducing, non-reducing and total soluble sugars, stevioside, total free amino acids and total soluble phenols. Reducing, non-reducing and total soluble sugars were determined by using phosphomolybdic acid reagent as described in A.O.A.C. (2000). Moreover, stevioside was calculated according to Nishiyama *et al.* (1991) formula: which used was

TS = 7.56 + 0.96 St Where,

TS = total sugars

St = Stevioside content

3- Total free amino acid and proline content:

Total free amino acids were determined by using ninhydrin reagent according to Moore and Stein (1954).

Free proline concentration was measured calorimetically in the extraction of fresh shoots using ninhydrin reagent according to Bates *et al.* (1973).

4- Extraction and determination of invertase activity:

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 obtained from centrifugation of crude extract, according to Sumner and Howell (1935).

5- Total soluble phenols:

The colorimetric method of Folin-Denis as described by Swain and Hillis (1959) was employed for determination the total soluble phenols.

6- Determination of minerals:

The determination of N, P, K, Ca, Na, and Mg were carried out on the shoots ground dry material. Dry sample was digested by using sulphoric and perchloric acids according to Piper (1947). Nitrogen was determined using the micro kejeldahl apparatus of Parnas-Wagner as described by Jones *et al.*

(1991). Phosphorus was estimated calorimetrically by using chlorostannous reduced molybdophosphoric, blue color method according to Jackson (1973). Sodium, magnesium, potassium and calcium were determined by using atomic absorption spectrophotometer (GBC,932 AA).

7- Extraction and determination of some plant hormones:

Extraction and determination of some 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.

8- Extraction and determination of phenylalanine ammonialyase (PAL) activity:

Assay of phenylalanine ammonialyase (PAL) wer done according to Edwards and Kessmann (1992).

One gram of fresh tissue sample was ground with 2ml of extraction buffer containing 50 mM Tris-Hcl (pH 8.5), 14 mM 2-mercaptoethanol and 5% (w/v) polyvinyl pyrrolidone. The homogenate was immediately centrifuged at 10.000 g for 10 min at 4 °C and the supernatant was immediately taken for enzyme assay.

For assay of PAL activity, 100 µl of the supernatant was incubated at 40 °C with 0.9 ml of 50 mM Tris-Hcl (pH 8.5) containing 12.1 mM L-phenylalanine with parallel incubation of 100 µl of the supernatant with 0.9 ml of 50 mM Tris-HCl (pH 8.5) containing 12.1 mM D-phenylalanine working as control. The formation of cinnamic acid was monitored using spectrophotometer by reading the absorbance at 30 min intervals and up to 2 hr at 290 nm. PAL activity was calculated on the basis of soluble proteins (n kat /g protein) according to the following equation:

$$\text{PAL activity (n kt/g protein)} = \frac{27780 \times (\Delta A_{290}\ \text{L-Phe/60min} - \Delta A_{290}\ \text{D-Phe/60min}}{\mu\text{g protein per incubation}}$$

RESULTS AND DISCUSSION

Growth characters :

The results in Tables (1 and 2) generally indicated that all growth characters of both stevia plantlets grown *in vitro*; i.e. survival percentage, shootlet length, no. of leaves / shootlet, no. of roots / plantlet, fresh and dry weight of shootlet (g/shootlet) and stevia plants grown in *ex vitro*; i.e. shoot height (cm.), no. of leaves/plant and dry weight of shoot (g/plant) gradually and significantly decreased as the salinity levels increased (Mean S). These results are in agreement with those previously reported by Robertson *et al.* (1999), Wiedenfeld (2000) and Ghallab *et al.* (2004) on sugarcane plants.

Concerning the effect of the salinity on decreasing plant growth characters, Taiz and Zeiger (2003) reported that, the decrease in survival

percentage under stress-condition may be due to the energy spent to maintain turgor pressure at the expense of growth or the decrease in the availability of water to plants (Hopkins, 2003). Moreover, the disturbance in water uptake results in severely reduction in plant growth and maintenance (Rengel, 1999).

Table 1: Effect of jasmonic acid treatments on growth characters of stevia plantlets grown under different levels of salinity (% sea salted water) for 6 weeks after culturing on rooting media (Combined analysis for two seasons).

Jasmonic acid (µM/l) Treatments	Growth characters											
	Survival percentage						Shootlet length (cm)					
	% sea salted water					Mean (T)	% sea salted water					Mean (T)
	0	10	20	30	40		0	10	20	30	40	
Control	100	81.78	79.46	72.92	31.16	73.06	7.19	6.18	6.01	5.25	4.25	5.78
10	97.81	95.62	92.87	65.45	23.67	75.08	8.57	7.95	7.49	4.19	3.13	6.27
20	83.54	75.26	7o.56	61.64	19.82	62.16	5.72	5.43	4.69	3.77	2.85	4.49
30	65.35	56.67	54.18	43.81	13.65	46.73	5.61	4.52	4.28	3.54	2.54	4.10
Mean (S)	86.52	77.33	74.27	60.96	22.08		6.77	6.02	5.62	4.19	3.19	
L.S.D. at 5%	T = 2.87 S=3.45 T*S= 6.51						T = 0.44 S = 0.51 T*S= 1.14					
	No. of leaves / shootlet						No. of roots / plantlet					
Control	8.55	7.42	6.05	5.09	4.11	6.24	6.75	5.66	5.19	4.25	3.55	5.08
10	9.96	9.34	8.74	4.09	3.45	7.12	7.83	7.22	6.89	3.23	2.18	5.47
20	6.55	5.64	5.31	3.85	3.29	4.93	5.46	4.92	4.56	3.02	1.95	3.59
30	6.01	5.16	4.93	3.51	3.01	4.52	5.13	4.21	4.01	2.67	1.59	3.52
Mean (S)	7.77	6.89	6.26	4.14			6.29	5.50	5.16	3.29	2.31	
L. S. D. at 5%	T = 0.38 S=0.54 T*S= 1.01						T = 0.37 S=0.44 T*S= 0.83					
	Shootlet fresh weight (g)						Shootlet dry weight (g)					
Control	9.81	8.79	8.12	6.35	5.63	7.74	0.49	0.45	0.42	0.33	0.27	0.39
10	11.35	10.89	9.67	5.36	4.48	8.35	0.70	0.63	0.58	0.21	0.15	o.45
20	8.68	7.89	6.89	5.31	4.17	6.59	0.35	0.29	0.24	0.16	0.12	0.23
30	6.89	5.75	4.72	4.18	3.66	5.04	0.28	0.23	0.18	0.13	0.09	0.18
Mean (S)	9.18	8.33	7.35	5.30	4.48		0.46	0.40	0.35	0.21	0.16	
L. S. D. at 5%	T = 0.51 S=0.69 T*S= 1.29						T = 0.046 S=0.065 T*S= 0.121					

T= Treatments with jasmonic acis (µm/l)
S= Salinity%
T*S= Interaction between treatments with jasmonic acid and Salinity%.

Also, it is important to mention that the reduction in all growth characters of both stevia plantelets and plants grown *in* and *ex vitro* 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 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 (Pessarakl, 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).

The retardant in plant growth may be also explained by the great portion of energy will be used for salinity stress tolerance rather than for growth and biomass production of the organism. In addition, high metabolic activity is necessary for transformation ions from the aerial parts to the roots (Cornillon and Palloix, 1995). ATP is an important regulator of cell metabolism and UDPG could often be limiting factor for cell wall synthesis and growth (Marschner, 1995). In this respect, (Pessaraki, 2002) indicated that salinity stress condition reduced the synthesis of uridine nucleotides or the production of uridine triphosphate (UTP) and uridine diphosphate glucose (UDPG).

Table 2:Effect of jasmonic acid treatments on growth characters of stevia plants grown in glass house conditions under the same different levels of salinity for 90-days old after acclimatization (Combined analysis for two seasons).

Jasmonic Acid (µM/l) Treatments	Growth characters					
	Shoot height (cm.)					
	% sea salted water					Mean (T)
	0	10	20	30	40	
Control	53.15	42.84	31.66	22.55	15.43	33.13
10	65.06	53.85	42.69	15.91	11.79	37.86
20	32.47	28.16	22.99	11.85	9.66	21.03
30	21.17	19.09	14.81	8.77	6.59	14.09
Mean (S)	42.96	35.98	28.04	14.77	10.87	
L.S.D. at 5%	T = 0.24 S= 0.33 T*S= 0.62					
	No. of leaves / plant					
Control	48.20	36.93	25.69	19.83	10.99	28.33
10	61.94	50.52	38.73	11.12	8.25	34.11
20	35.42	23.13	19.69	10.55	7.32	19.22
30	22.83	18.42	11.81	9.72	6.89	13.93
Mean (S)	42.10	32.25	23.98	12.80	8.36	
L. S. D. at 5%	T = 0.75 S=1.43 T*S= 2.68					
	Shoot dry weight / plant (g)					
Control	51.13	42.41	35.63	27.39	18.79	35.07
10	76.36	62.68	55.84	21.18	16.88	46.59
20	43.95	31.84	26.73	17.52	15.58	27.12
30	31.83	25.21	19.24	14.26	11.13	20.33
Mean (S)	50.82	40.53	43.36	20.09	15.59	
L. S. D. at 5%	T = 1.08 S=1.90 T*S= 2.75					

T= Treatments with jasmonic acis (µm/l)
S= Salinity%
T*S= Interaction between treatments with jasmonic acid and Salinity%.

When consider the mean value of all growth characters of stevia grown *in vitro* and *ex vitro* due to each treatment (Mean T) regardless of the salinity level (Tables 1 and 2), it could be noticed that as the concentration of jasmonic acid (JA) increased, all growth characters gradually and significantly decreased when compared with the control treatment (0.0 concentration) except for the lower concentration (10 µM/l) which induced an opposite trend. The percentage of increment in the dry weight of stevia grown *in vitro* was

15.38% and *ex vitro* was 32.85% induced by the lower concentration of JA over the control treatment. This increase of all growth characters might be due to stimulative effects of JA on the growth and dry matter accumulation. In this respect, Maslenkova *et al.* (1990) and Tsonev *et al.* (1998) suggested that JA application to the growth medium of barley seedling can be used successfully to alleviate the NaCl stress injuries and consequently improved their growth to be cosideriable extent .

Comparing the effect of the interaction between JA treatments and salinity levels clearly reveal that, at 10 and 20% salinity levels, treatment with 10 µM/l JA produced significantly higher values of the studied growth parameters when compared with the control treatment, while at 30% and 40% salinity levels, a negative trend were elicited. The same lower concentration (10 µM/l) of JA treatment recorded the highest considerable increases in the dry weight of stevia grown *in vitro* (38.09%) and *ex vitro* (56.72%) as compared with the control treatment at 20% salinity level. Also, at the higher concentrations (20 and 30 µM/l) of JA under all salinity levels, all growth characters of stevia grown *in vitro* and *ex vitro* were not significantly changed as compared with the control treatment.

This clearly indicates that the highest concentrations of JA have an inhibitory effect on the growth characters of stevia grown *in vitro* and *ex vitro*. Hence, the clear superiority of the lower concentration (10 µM/l) of JA in inducing the highest degree of salinity tolerance of the stevia plants grown in *ex vitro* could be clearly seen and which even surpassed over untreated control. Similar results were obtained by Maslenkova *et al.* (1992) and Epstein and Bloom(2004).

2: Chemical Composition:

a- Sugar, proline, total free amino acids, total soluble phenols, invertase and PAL activities and stevioside content:

Sugars (reducing, non-reducing and total soluble), proline, total free amino acids , total soluble phenols contents, and phenylalanine ammonialyase (PAL) activity concentrations as well as stevioside (%) in the stevia grown *in vitro* and *ex vitro* significantly increased dramatically (Mean S) by increasing salinity levels while invertase activity exhibited an opposite trend (Table 3). As with growth characters of stevia grown *in vitro* and *ex vitro* (Tables 1 and 2), the Mean T of the lower concentration (10 µM/l) of JA which recorded considerable significantly increase the accumulation of the protective compounds (sugars, proline, total free amino acids and total soluble phenols PAL activity), and stevioside (%) over the respective (Mean T) of the control treatment, except invertase activity exhibited an opposite trend. Meanwhile both jasmonic acid treatments (20 and 30 µM/l) caused noticeable significantly reduction in the accumulation of the protective compounds and stevioside (%) when compared with the control, except invertase activity exhibited an opposite trend in the stevia comparing the effect of the interaction between JA acid treatments and salinity levels clearly reveal that, the lower concentration of JA significantly accumulated much more concentrations of the protective compounds and stevioside (%), except

invertase activity exhibited an opposite trend, in the stevia grown *in vitro* and *ex vitro* which greatly exceeded control treatment up to 20% salinity .

Table 3: Effect of jasmonic acid treatments on sugars (reducing, non-reducing and total soluble) as mg glucose / g. F. W., proline, total free amino acids , total soluble phenols as mg/g F.W. , stevioside (%), Invertase activity as µ mol / glucose/min per g fresh weight and PAL activity n-kat/g protein of stevia grown *in vitro* for 6 weeks after culturing on rooting media and *ex vitro* for 90-days old after acclimatization (Combined analysis for two seasons).

Jasmonic Acid (µM/l) Treatments	*In vitro* plants											
	Reducing sugars						Non-reducing sugars					
	% sea salted water					Mean	% sea salted water					Mean
	0	10	20	30	40	(T)	0	10	20	30	40	(T)
0	3.28	4.18	5.17	7.15	7.25	5.41	12.35	15.71	16.65	20.48	21.03	17.24
10	5.01	6.11	6.67	6.81	6.96	6.32	16.67	18.22	19.15	19.64	19.99	18.73
20	3.01	3.88	4.75	5.99	6.04	4.73	11.16	14.29	15.31	18.64	19.11	15.74
30	2.83	3.61	4.35	5.21	5.52	4.30	10.01	13.43	14.25	17.33	18.58	14.72
Mean (S)	3.57	4.45	5.24	6.29	6.45		12.55	15.41	16.34	19.07	19.86	
L.S.D at 5%	S= 0.37		T= 0.43		S*T= 0.95		S= 0.54		T= 0.57		S*T= 1.31	
	Total sugars						Total free amino acids					
0	15.63	19.89	21.82	27.63	28.28	22.65	4.64	5.21	6.29	7.78	7.99	6.38
10	21.68	24.33	25.82	26.45	26.95	25.05	5.89	6.92	7.46	7.61	7.79	7.13
20	14.17	18.17	20.06	24.63	25.15	20.44	4.24	4.76	5.78	7.16	7.29	5.85
30	12.84	17.04	18.60	22.54	24.10	19.02	3.11	4.48	5.42	6.64	6.93	5.32
Mean (S)	16.08	19.86	21.58	25.31	26.12		4.47	5.34	6.24	7.29	7.5	
L.S.D at 5%	S= 0.65		T= 0.71		S*T= 1.59		S= 0.26		T= 0.29		S*T= 0.65	
	Proline						Total soluble phenols					
0	1.18	1.49	2.19	2.86	2.97	2.14	2.23	2.38	3.41	5.56	5.67	3.85
10	1.99	2.46	2.68	2.74	2.76	2.53	4.42	4.74	5.28	5.34	5.45	5.05
20	1.08	1.37	1.95	2.53	2.66	1.92	1.98	2.18	3.23	4.74	4.83	3.39
30	0.96	1.25	1.87	2.34	2.47	1.78	1.79	2.09	2.96	4.36	4.72	3.16
Mean (S)	1.30	1.64	2.17	2.62	2.72		2.61	2.85	3.69	5.00	5.17	
L.S.D at 5%	S= 0.14		T=0.17		S*T= 0.35		S= 0.21		T= 0.45		S*T= 0.97	
	Acclimatized plants *(ex vitro)*											
	Reducing sugars						Non-reducing sugars					
0	3.67	4.72	5.84	8.07	8.19	6.09	13.79	16.65	17.84	21.71	22.29	18.46
10	5.61	6.91	7.54	7.69	7.86	7.12	17.65	19.31	20.31	20.82	21.19	19.86
20	3.42	4.38	5.37	6.77	6.83	5.35	11.83	15.15	16.23	19.76	20.26	16.65
30	3.19	4.07	4.92	5.89	6.24	4.86	10.61	14.24	15.11	18.37	19.69	15.60
Mean (S)	3.97	5.02	5.92	7.61	7.28		13.47	16.34	17.37	20.17	20.86	
L.S.D at 5%	S= 0.49		T= 0.52		S*T= 1.17		S= 0.34		T= 0.36		S*T= 0.83	
	Total sugars						Total free amino acids					
0	17.46	21.37	23.68	29.78	30.48	24.55	5.21	5.87	7.12	8.89	9.16	7.25
10	23.26	26.22	27.85	28.51	29.05	26.98	6.88	7.99	8.49	8.49	8.71	8.11
20	15.25	19.53	21.60	26.53	27.09	22.00	4.64	5.45	6.62	8.14	8.36	6.64
30	13.80	18.31	20.03	24.26	25.93	20.47	4.35	5.09	6.18	7.63	7.79	6.21
Mean (S)	17.44	21.36	23.29	27.27	28.14		5.27	6.10	7.10	8.29	8.51	
L.S.D at 5%	S= 0.42		T= 0.45		S*T= 0.93		S= 0.44		T= 0.47		S*T= 0.98	

Table (3) cont.

	Proline						Total soluble phenols					
0	1.64	1.99	2.95	3.83	3.95	2.87	2.82	2.96	4.28	6.89	6.97	4.78
10	2.72	3.27	3.58	3.64	3.78	3.39	5.47	5.74	6.67	6.47	6.67	6.20
20	1.51	1.68	2.47	3.17	3.39	2.44	2.39	2.67	3.95	5.63	6.05	4.14
30	1.23	1.57	2.32	3.02	3.11	2.25	2.29	2.68	3.58	5.34	5.67	3.91
Mean (S)	1.78	2.13	2.83	3.42	3.56		3.24	3.51	4.62	6.08	6.34	
L.S.D at 5%	S= 0.29		T= 0.31		S*T= 0.61		S= 0.24		T= 0.46		S*T= 1.01	
	Stevioside (%)											
	In vitro plants						Acclimatized plants *(ex vitro)*					
0	0.84	1.28	1.49	2.09	2.16	1.57	1.03	1.44	1.68	2.31	2.39	1.77
10	1.47	1.75	1.90	1.97	2.02	1.82	1.64	1.94	2.11	2.18	2.24	2.02
20	0.69	1.11	1.30	1.78	1.83	1.34	0.89	1.25	1.46	1.98	2.03	1.52
30	0.55	0.99	1.15	1.56	1.72	1.19	0.65	1.12	1.29	1.74	1.91	1.34
Mean (S)	0.89	1.28	1.46	1.85	1.93		1.05	1.44	1.64	2.06	2.14	
L.S.D at 5%	S= 0.19		T= 0.22		S*T= 0.38		S= 0.20		T= 0.23		S*T= 0.41	
	Invertase activity											
	In vitro plants						Acclimatized plants *(ex vitro)*					
0	69.33	41.21	27.65	13.56	9.96	32.34	75.59	46.35	33.49	19.98	15.88	38.26
10	39.21	30.67	22.49	18.76	14.38	25.10	42.69	34.49	27.64	22.55	18.36	29.15
20	56.55	49.37	38.58	29.12	19.43	38.61	61.58	52.79	44.39	31.47	24.28	42.90
30	59.72	53.46	44.82	31.42	23.71	42.63	66.97	58.81	49.78	37.31	28.89	48.35
Mean (S)	56.20	43.68	33.39	23.22	16.87		61.71	48.11	38.83	27.83	21.85	
L.S.D at 5%	S= 2.18		T= 2.44		S*T= 7.05		S= 3.57		T= 3.92		S*T= 8.85	
	PAL activity											
	in vitro plants						Acclimatization plants (*ex vitro*)					
0	5.85	14.25	21.40	34.01	38.22	22.75	7.22	19.18	24.53	38.12	41.83	26.18
10	12.12	25.13	29.34	31.25	35.63	26.69	19.69	28.43	32.72	34.69	37.22	30.55
20	3.20	11.17	16.43	25.16	29.49	17.09	4.99	15.38	20.11	29.89	33.16	20.71
30	1.99	6 93	11.69	19.55	24.34	12.90	3.29	9.71	15.44	24.61	28.35	16.28
Mean (S)	5.79	14.37	19.72	27.49	31.92		8.79	18.18	23.20	31.83	35.14	
L.S.D at 5%	S= 1.25		T= 2.14		S*T= 3.22		S= 1.83		T= 2.91		S*T= 4.03	

T= Treatments with jasmonic acis (µm/l)
S= Salinity%
T*S= Interaction between treatments with jasmonic acid and Salinity%.

On the contrary both JA (20 and 30 µM/l) treatments under all salinity levels and 30% as well as 40 % of salinity levels for lower concentration of JA caused noticeable significant reduction in the accumulation of the protective compounds and stevioside (%)when compared with control, except invertase activity exhibited an opposite trend. Hence, the regenerated stevia plants from cluster explants exposed to 10 µM/l of JA exhibited the highest degree of salinity tolerant, i.e. the positive correlation between such treatment and improving of salinity tolerance. In this respect, Buchana and Jones (2000) discussed the cell adaptation to salinity stress by additional accumulation of sugars, amino acids and other metabolically protective osmolites. Also, the same authors suggested that starch and polysaccharides are converted to simple sugars to maintain more negative water potential values inside the plant, the sugars as osmolytes enable plants to keep better water relations under water stress conditions.

Furthermore, Fitter and Hay (2001) stated that under saline conditions, the accumulation of non toxic substances such as sucrose, proline, organic acids, pigments, nucleic acids and protein are considered to be protective adaptation and the survival of plants under water stress and saline conditions depends upon the regulation of metabolic processes and the quantitative ratio between the protective and toxic metabolic intermediates. Moreover, it has been suggested that the high concentration of organic solutions in the cytoplasm could have the following roles: a- a 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 (Hirt and Shinozaki, 2003).

The sugars as osmolytes can enable plants to keep better water relations under stress conditions. Also, sucrose protected isolated chloroplasts against desiccation (Luttge and Lauchi, 2002). Moreover, Pessaraki (2002) concluded that plant use soluble sugars as an osmoticum under water stress and 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 to Giza 167 wheat cultivar. Moreover, Ghallab *et al* (2004) with sugarcane and Harb *et al.* (2005) with banana grown *in vitro* and *ex vitro* found that proline and sugars increased with increasing salinity Levels.

Concerning accumulation of phenols and amino acids with increasing water stress levels, Hanafy (1991) noticed that the increasing in sugars, total free amino acids and total soluble phenols concentrations when plants subjected to stress conditions could be explained on the assumption that such plants might have less efficiency to condensate simple organic compounds into more complex one. In addition, the same author assumed that the higher level of total soluble phenols and total free amino acids concentrations might be due to the increase in the metabolic activity to synthesis shikimic acid. Moreover, Mengel *et al.* (2001) mentioned that, the accumulation of amino acids under salinity stress condition was due to the synthesis of organic acids and not from the hydrolysis of proteins or from preexisting of amino acids. It has been suggested that organic acids (citric, malic....etc) served as a C source in the synthesis of amino acids, i.e. proline *via* glutamate dehydrogenase. Recently, Ghallab *et al* (2004) with sugarcane found phenols and amino acid increased with increasing polyethylene glycol (PEG) level. Also, El-Shihy *et al.* (2004) with wheat and Harb *et al* (2005) with banana found that amino acids increased with increasing salinity level.

The endogenous concentration of free proline in plants can be used as an indicator of salt and water stress tolerance. For each plant, it appears that there is an external salt concentration above, which the plant's proline level sharply rises. This critical point is directly to the ability of plant to tolerante salt. Thus, measurements of condition can be used to determine salt resistance of plants (Larcher, 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 hydrophilic parts (Binzell 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, Fatouh Youssef (2003) with wheat, Ghallab *et al.* (2004) with sugarcane and Harb *et al.* (2005) with banana 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" (Pessaraki, 2002). Also, many reports proved the rapid increase in synthesis and accumulation of sugar under water stress and saline conditions. Nasir *et al.* (2000) reported that leaves of salt tolerant line in 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 salt-treated of *Chloris gayana* plants accumulated higher concentrations of soluble sugars.

Supportive evidence for the finding of invertase activity 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 increase 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.

Generally the increase in total soluble phenols content may be due to cinnamic acid is the product of phenylalanine ammonialyase (PAL) activity. This enzyme is a key regulator of the phenyl propanoid pathway that yield a diversity of phenolics with structural and defense-related function (Alexander *et al.*, 1992). Also treatment of plant cells with Me-JA leads to an increase in the expression of genes associated with synthesis of compounds related to Me-JA to soybean cell suspension cultures increased the level of detectable PAL poly (A) + RNA and this was followed by increased PAL activity (Gundlach *et al.*, 1992).

As for the efficiency of JA in this regard, Maslenkova *et al.* (1990) and Tsonev *et al.*, (1998) suggested the possibility of successful application of JA to improve salinity tolerance of barely seedling, which was associated with increasing accumulation of the protective substances such as total sugars,

free amino acids, proline and soluble phenols in the barely seedling with increasing salinity levels.

b- Photosynthetic Pigments

Results obtained in Table 4, clearly revealed that photosynthetic Pigments concentration, i.e. chlorophylls (a, b and total) and carotenoids significantly decreased in stevia grown *in vitro* and *ex vitro* with increasing salinity level (Mean S). As with growth characters (Tables 1 and 2) and protective compounds (Table 3) of stevia grown *in vitro* and *ex vitro*, the (Mean T) of the lower rate (10 µM/l) of JA which recorded considerable significant increase in accumulation of the photosynthetic pigments over the respective (Mean T) of the control treatment; meanwhile both JA treatments (20 and 30µM/l) exhibited an opposite trend.

Table 4: Effect of jasmonic acid treatments on photosynthetic pigments as mg / g. F. W., of stevia grown *in vitro* for 6 weeks after culturing on rooting media and *ex vitro* for 90-days old after acclimatization (Combined analysis for two seasons).

Jasmonic Acid (µM/l) Treatments	*In vitro* plants											
	Chlorophyll a						Chlorophyll b					
	% sea salted water					Mean (T)	% sea salted water					Mean (T)
	0	10	20	30	40		0	10	20	30	40	
0	1.53	1.48	1.35	1.28	0.97	1.32	0.78	0.63	0.58	0.46	0.37	0.56
10	1.86	1.75	1.68	1.01	0.77	1.41	0.94	0.86	0.74	0.32	0.27	0.63
20	1.36	1.15	1.02	0.86	0.59	0.99	0.68	0.51	0.42	0.25	0.19	0.41
30	1.26	1.02	0.87	0.78	0.48	0.88	0.53	0.41	0.31	0.20	0.15	0.32
Mean (S)	1.50	1.35	1.23	0.98	0.70		0.73	0.60	0.51	0.31	0.25	
L.S.D at 5%	S= 0.05		T= 0.09		S*T= 0.17		S= 0.04		T= 0.06		S*T= 0.13	
	Carotenoids						Total chlorophylls					
0	0.68	0.43	0.37	0.32	0.28	0.42	2.31	2.11	1.93	1.74	1.34	1.89
10	0.87	0.59	0.53	0.29	0.24	0.50	2.80	2.61	2.42	1.33	1.04	2.04
20	0.59	0.36	0.31	0.24	0.22	0.34	2.04	1.66	1.44	1.11	0.78	1.41
30	0.48	0.28	0.25	0.21	0.17	0.28	1.79	1.43	1.18	0.98	0.63	1.20
Mean (S)	0.66	0.42	0.37	0.27	0.23		2.24	1.95	1.74	1.29	0.95	
L.S.D at 5%	S= 0.03		T= 0.05		S*T= 0.11		S= 0.11		T= 0.12		S*T= 0.29	
	Acclimatized plants *(ex vitro)*											
	Chlorophyll a						Chlorophyll b					
0	1.91	1.86	1.68	1.57	1.15	1.63	0.95	0.72	0.64	0.59	0.41	0.66
10	2.32	2.23	2.08	1.41	0.98	1.80	1.12	1.05	0.98	0.41	0.35	0.78
20	1.54	1.46	1.32	1.20	0.87	1.28	0.73	0.61	0.49	0.36	0.29	0.49
30	1.41	1.31	1.22	1.13	0.74	1.16	0.61	0.42	0.38	0.27	0.19	0.37
Mean (S)	1.79	1.72	1.58	1.33	0.94		0.85	0.70	0.62	0.41	0.31	
L.S.D at 5%	S= 0.06		T= 0.15		S*T= 0.32		S= 0.05		T= 0.08		S*T= 0.19	
	Carotenoids						Total Chlorophylls					
0	0.89	0.67	0.57	0.43	0.37	0.59	2.86	2.58	2.32	2.16	1.56	2.29
10	1.12	0.92	0.84	0.36	0.31	0.71	3.44	3.28	3.06	1.82	1.33	2.59
20	0.64	0.56	0.42	0.31	0.29	0.44	2.27	2.07	1.81	1.56	1.16	1.77
30	0.59	0.42	0.39	0.26	0.24	0.38	2.02	1.73	1.60	1.40	0.93	1.54
Mean (S)	0.81	0.64	0.56	0.34	0.30		2.65	2.42	2.19	1.74	1.25	
L.S.D at 5%	S= 0.08		T= 0.11		S*T= 0.23		S= 0.15		T= 0.25		S*T= 0.71	

T= Treatments with jasmonic acis (µm/l)
S= Salinity%
T*S= Interaction between treatments with jasmonic acid and Salinity%.

Comparing the effect of the interaction between JA treatments and salinity levels clearly reveal that, the lower rate (10 µM/l) of JA significantly accumulated much more concentrations of the photosynthetic pigments in the stevia grown *in vitro* and *ex vitro* which greatly exceeded control treatment up to 20% salinity level. On the contrary, both JA (20 and 30 µM/l) treatments under all salinity levels and 20% as well as 40% salinity levels for lower rate (10 µM/l) of JA caused noticeable significant reduction in the accumulation of the photosynthetic pigments when compared with control treatment.

In this respect Hirt and Shinozaki (2003) indicated that the depressive effect of salinity stress conditions on the absorption of some ions which was involved in the chloroplast formation such as Mg and Fe could be expected as reason for chlorophyll suppression in leaves, and / or increase in growth inhibitors such as ethylene or abscisic acid production which enhanced senescence under stress conditions. In addition, un-available uptake of specific ions by the plants, and the accumulation of some ions in the leaves are widely assumed to result in the inhibition of photosynthesis. However, biosynthesis of chlorophyll and subsequently CO_2 fixation were inhibited under salinity stress conditions (Luttge and Lauchi, 2002).

Moreover, Maslenkova *et al.* (1990) and Tsonev *et al.* (1998) found that application of JA to barely seedling increased photosynthetic pigments concentration with increasing salinity levels.

C- Minerals:

The obtained data in Table 5 clearly show that the concentrations of Na, Mg and Ca gradually increased in the stevia grown *in vitro* and *ex vitro* as salinity levels increased. The concentrations of N, P and K exhibited an opposite trend (Mean S). Also, the (Mean T) of the lower concentrations (10 µM/l) of JA recorded significant increase the accumulation of nutrient elements concentrations over the respective (Mean T) of the control treatment; meanwhile both JA treatments (20 and 30 µM/l) exhibited an opposite trend. Comparing the nutrient elements concentrations of the treated plants shows that the accumulations of N, P, K, Na, Ca and Mg uptake into stevia grown *in vitro* and *ex vitro* treated with 10 µM/l. of JA as the salinity level increased up to 20%, while Jasmonic acid treatments (20 and 30 µM/l) under all salinity levels and 30% as well as 40% salinity levels for 10 µM/l of JA treatment greatly significantly decreased such uptake and accumulations when compared to the respective values of the untreated control treatment. This strongly emphasized the superiority of JA at lower concentration (10 µM/l) in stimulating the uptake and accumulations of various nutrient elements as previously reported for sugars, proline, total free amino acids and total soluble phenols (Table 3), photosynthetic pigments (Table 4) and simulative effects on growth characters (Tables 1 and 2) of stevia grown *in vitro* and *ex vitro* under different levels of salinity up to 20% salinity level.

The favorable effects of the low concentration of JA (10 µM/l) were reflected on the growth (Tables 1 and 2), protective compounds (Table 3) and Photosynthetic pigments (Table 4). These effects may be as a result of plant adaptation to stress conditions. Increasing nutrient accumulation induced by JA under salinity stress conditions was previously recorded by Tsonev *et al.* (1998) with barely.

Table 5: Effect of jasmonic acid treatments on nitrogen, phosphorus, potassium, calcium, sodium and magnesium concentrations (mg/g D.W.) of stevia grown *in vitro* for 6 weeks after culturing on rooting media and *ex vitro* for 90-days old after acclimatization (Combined analysis for two seasons).

Jasmonic Acid (µM/l) Treatments	*In vitro* plants											
	Nitrogen						Phosphorus					
	% sea salted water					Mean	% sea salted water					Mean
	0	10	20	30	40	(T)	0	10	20	30	40	(T)
0	28.52	25.19	21.17	16.19	14.74	21.16	2.46	2.24	2.05	1.85	1.62	2.04
10	42.35	39.47	34.28	14.68	13.85	28.93	2.85	2.78	2.63	1.67	1.45	2.28
20	24.95	22.33	18.38	13.43	12.67	18.35	2.25	2.06	1.87	1.56	1.34	2.27
30	22.19	19.49	16.94	12.37	10.73	16.34	1.95	1.87	1.72	1.44	1.25	1.65
Mean (S)	29.50	26.62	22.69	14.17	12.99		2.38	2.24	2.07	1.66	1.42	
L.S.D at 5%	S= 2.09		T= 2.54		S*T= 4.71		S= 0.11		T= 0.19		S*T= 0.32	
	Potassium						Calcium					
0	38.21	36.73	32.86	23.49	20.15	30.29	10.87	11.54	12.46	21.52	22.51	15.78
10	49.82	47.12	44.92	21.85	18.74	36.49	15.56	17.75	19.22	20.35	21.11	18.79
20	35.15	33.42	28.92	20.31	17.61	27.07	9.89	10.51	11.33	17.11	18.81	13.53
30	31.92	29.56	26.72	18.69	16.92	24.76	9.27	9.76	10.66	15.04	16.34	12.21
Mean (S)	38.78	36.71	33.36	21.09	18.36		11.39	12.39	13.42	18.51	19.69	
L.S.D at 5%	S= 1.99		T= 3.02		S*T= 5.11		S= 0.73		T= 1.15		S*T= 2.32	
	Sodium						Magnesium					
0	1.65	1.83	2.01	2.21	2.59	2.06	2.22	2.63	3.01	4.39	4.51	3.35
10	1.01	1.25	1.46	2.35	2.73	1.76	3.49	3.75	3.98	4.02	4.23	3.89
20	1.77	1.96	2.19	2.49	2.92	2.27	2.01	2.39	2.73	3.69	3.81	2.93
30	1.91	2.07	2.32	2.65	3.05	2.40	1.92	2.21	2.51	3.21	3.55	2.68
Mean (S)	1.59	1.78	1.99	2.43	2.82		2.41	2.75	3.06	3.83	4.03	
L.S.D at 5%	S= 0.14		T= 0.26		S*T= 0.49		S= 0.29		T= 0.36		S*T= 0.69	
	Acclimatized plants (*ex vitro*)											
	Nitrogen						Phosphorus					
0	39.84	35.34	29.96	23.14	21.28	29.91	2.65	2.41	2.19	1.99	1.75	2.19
10	55.78	51.85	44.92	21.16	20.01	38.74	3.01	2.89	2.74	1.83	1.61	2.42
20	35.14	31.46	26.16	19.45	18.39	26.12	2.44	2.22	2.02	1.69	1.47	1.97
30	31.25	27.63	24.23	18.02	15.82	23.39	2.15	2.07	1.86	1.59	1.39	1.81
Mean (S)	40.50	36.57	31.32	20.44	18.88		2.56	2.39	2.[illegible]	1.78	1.56	
L.S.D at 5%	S= 3.10		T= 4.61		S*T= 8.21		S= 0.14		T= 0.21		S*T= 0.44	
	Potassium						Calcium					
0	46.23	44.44	39.76	28.42	24.38	36.65	12.61	13.39	14.45	24.96	26.11	18.30
10	60.28	57.11	54.35	26.44	22.67	44.17	18.05	20.59	22.29	23.61	24.49	21.81
20	42.53	40.43	34.99	24.58	21.31	32.77	11.47	12.19	13.14	19.85	21.82	15.69
30	38.62	35.77	32.33	26.61	20.47	30.76	10.75	11.32	12.37	17.45	18.95	14.17
Mean (S)	46.92	44.44	40.36	26.51	22.21		13.22	14.37	15.56	21.47	22.84	
L.S.D at 5%	S= 2.18		T= 5.61		S*T= 9.67		S= 1.09		T= 2.51		S*T= 4.89	
	Sodium						Magnesium					
0	2.13	2.36	2.59	2.85	3.34	2.65	3.39	2.84	3.25	4.74	4.87	3.82
10	1.30	1.61	1.88	3.03	3.52	2.27	3.77	4.05	4.29	4.34	4.57	4.20
20	2.28	2.53	2.83	3.21	3.77	2.92	2.19	2.58	2.95	3.99	4.11	3.16
30	2.46	2.67	2.99	3.42	3.93	3.09	2.08	2.39	2.72	3.47	3.83	2.89
Mean (S)	2.04	2.29	2.57	3.13	3.64		2.86	2.97	3.30	4.14	4.35	
L.S.D at 5%	S= 0.21		T= 0.32		S*T= 0.58		S= 0.08		T= 0.34		S*T= 0.71	

T= Treatments with jasmonic acis (µm/l)

S= Salinity%

T*S= Interaction between treatments with jasmonic acid and Salinity%.

The reduction in N under water stress and saline conditions may be due to reduction in water absorbed and a decrease in root permeability (Pessaraki, 2002). Under stress conditions, Na 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 limiting. These crops take up large amount of Na which contributes to the osmotic potentials of the leaves and increase resistance to water stress. The damage effect of Na, however, may be attributed to that Na is capable of disturbing the fine structure of plant cell causing swelling of chloroplast which may result in chlorosis and necrosis (Marschner, 1995).

Furthermore, Mengel *et al* (2001) 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. Also, Epstein and Bloom (2004) 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, El-Shafey *et al.* (2003) and El-Shihy *et al.*, (2004) working on wheat callus and they found that total N, P, K and Ca decreased with increasing salinity level. Also, Samarah *et al.* (2004) working on soyabean and Harb *et al.* (2005) working on banana, found that N, P and K concentrations decreased with increasing salinity levels.

D- Phytohormones:

The obtained data in Table 6 regarding hormonal analysis of the stevia grown *in vitro* and *ex vitro* clearly reveal that the concentrations of indole – 3 – acetic acid (IAA) and gibberelic acid (GA_3) (µg/g F. W.) were significantlly decreased by increasing salinity levels (Mean S) to reach their lowest values at the highest level of salinity, i.e. 40% salinity level, meanwhile the concentrations of abscisic acid (ABA) contrary significantly increased. Similar results were reported by Ibrahim and Shehata (2000), El-Shafey *et al.* (2003), Ghallab and El-Ghadban (2003) and Harb *et al.* (2005) under saline conditions.

Comparing the concentrations of the all estimated hormones of the treated plants shows that the treatment with lower rate (10 µM/l) of JA considerably significantly increased IAA, GA_3 and ABA concentrations over control treatment under normal and salinity conditions up to 20% salinity level. Such accumulations were decreased under all levels of salinity as a result of higher JA treatments (20 and 30 µM/l) and 30% as well as 40% salinity levels with lower rate of JA treatment. The treatment of 10 µM/l JA could significantly exceed accumulations of IAA, GA_3 and ABA over the respective values of control treatment (Mean T); meanwhile the other JA treatments exhibited an opposite trend. In this concern, Roberts and Tucker (2000) elucidated the effects of JA on plant metabolic sites which are included the synthesis of DNA, enzymes, amino acids, proteins and auxins, in addition to photosynthesis.

Accordingly, it could be postulated that the lower concentration (10 µM/l) of JA treatment seems the most suitable one for enhancing plant

growth and development through stimulation of auxin biosynthesis. For ABA, Hirt and Shinozaki (2003) stated that ABA level increased with salinity stress, and that this level correlated with plant resistance to the salt stress. Also, Hatung (2004) considered that ABA is the primary hormone that mediates plant responses to stress such as cold, drought and salinity; thus is endogenous level increased with water stress.

Table 6: Effect of jasmonic acid treatments on indole-3- acetic acid (IAA), gibberelic acid (GA_3) and abscisic acid (ABA) concentrations (µg/g F.W.) in the leaves of stevia grown *in vitro* for 6 weeks after culturing on rooting media and *ex vitro* for 90-days old after acclimatization (Combined analysis for two seasons).

Jasmonic Acid (µM/l) Treatments	*In vitro* plants											
	IAA						GA_3					
	% sea salted water					Mean (T)	% sea salted water					Mean (T)
	0	4	8	12	16		0	4	8	12	16	
0	19.02	15.47	13.66	11.65	10.98	14.16	20.99	18.98	16.69	14.49	11.73	16.58
10	30.12	27.66	22.97	10.99	9.84	20.32	28.69	25.48	23.35	12.66	8.39	19.71
20	16.69	13.35	12.44	9.67	7.65	11.96	15.36	13.85	11.65	9.33	6.54	11.35
300	15.78	11.87	9.91	7.33	6.46	10.27	12.76	11.39	10.81	8.21	5.95	9.82
Mean (S)	20.40	17.09	14.75	9.91	8.73		19.45	17.43	15.63	11.17	8.15	
L.S.D at 5%	S= 2.13		T= 3.24		S*T= 6.59		S= 1.34		T= 2.16		S*T= 4.48	
	Acclimatized plants (*ex vitro*)											
	IAA						GA_3					
0	22.38	18.52	17.29	15.34	11.88	17.08	24.67	21.41	19.64	17.58	13.78	19.42
10	35.44	32.67	26.95	14.97	10.81	24.17	34.48	29.98	25.96	15.11	11.18	23.34
20	19.96	16.11	14.88	13.17	8.75	14.57	21.99	19.34	16.14	13.56	9.45	16.09
300	18.68	13.87	11.67	8.95	8.31	12.29	19.75	16.88	13.71	9.19	7.61	13.43
Mean (S)	24.12	20.29	17.69	13.11	9.94		25.22	21.90	18.86	13.86	10.51	
L.S.D at 5%	S= 3.45		T= 4.67		S*T= 8.39		S= 2.41		T= 3.13		S*T= 5.93	
	ABA											
	In vitro plants						Acclimatized plants (*ex vitro*)					
0	2.04	2.46	2.62	3.36	3.49	2.79	2.43	3.04	3.19	4.1	4.21	3.39
10	2.91	3.03	3.18	3.24	3.36	3.14	2.65	3.69	3.83	3.99	4.11	3.65
20	1.82	2.09	2.31	2.85	2.97	2.41	2.14	2.89	3.05	3.71	3.92	3.14
30	1.66	1.84	2.14	2.59	2.66	2.18	2.05	2.71	2.91	3.59	3.74	3.00
Mean (S)	2.11	2.36	2.56	3.01	3.12		2.32	3.08	3.25	3.85	3.99	
L.S.D at 5%	S= 0.14		T= 0.21		S*T= 0.39		S= 0.19		T= 0.22		S*T= 0.45	

T= Treatments with jasmonic acis (µm/l)
S= Salinity%
T*S= Interaction between treatments with jasmonic acid and Salinity%.

3- Yield Components:

The results in Table 7 represent the different values of yield components; dry weight of leaves, stem and shoot / plant (g) of regenerated stevia plants (8-monthes old) grown in glasshouse conditions under the same different levels of salinity. The obtained data clearly show the dramatically reduced yield components by increasing salinity levels (Mean S).

These results are in agreement with those obtained by Robertson *et al.* (1999), Wiedenfeld (2000) and Ghallab *et al.* (2004) on sugarcane. The reductions in the number of leaves per plant (Table 2) and photosynthetic pigments concentration (Table 4) due to salinity should have affected the photosynthetic capacity of the plant and thus reflect upon the great reduction obtained in yield components. Moreover, declines in the activities of endogenous auxins and gibberellins would account much for reduction in the yield components (Table 7). In this respect, many workers suggested that the reduction in plant yield due to salinity stress may be attributed to restricts the absorption of water by plant roots and water use efficiency (Rengel, 1999) and / or imbalance in phytohormone levels through its effect on either the biosynthesis or the destruction of the plant hormones (Larcher, 2002).

Table 7: Effect of jasmonic acid treatments on yield components of stvia plants (8 months old) grown in glasshouse conditions under the same different levels of salinity (Combined analysis for two seasons).

Jasmonic acid (µM/l) Treatments	Yield components											
	Leaves dry weight / plant (g)						Stem dry weight / plant (g)					
	% sea salted water					Mean	% sea salted water					Mean
	0	10	20	30	40	(T)	0	10	20	30	40	(T)
Control	71.35	63.78	56.46	42.92	31.16	53.13	88.53	75.64	64.75	52.49	40.87	64.45
10	97.81	91.62	79.87	35.45	24.67	65.88	115.21	98.65	85.84	44.32	32.65	75.33
20	63.54	55.26	4o.56	29.64	19.82	41.76	77.24	63.73	51.98	35.71	25.84	50.90
30	52.35	46.67	34.18	23.81	13.65	34.13	65.48	50.99	43.57	28.69	20.99	41.94
Mean (S)	71.26	64.33	56.84	32.96	22.33		86.62	72.25	61.54	40.30	30.09	
L.S.D. at 5%	S=3.67		T = 2.79		T*S= 5.61		S = 4.01		T = 3.10		T*S= 6.34	
	Shoot dry weight / plant (g)											
Control	159.88	139.42	121.21	95.41	72.03	117.59						
10	213.02	190.27	165.71	79.77	57.32	141.22						
20	140.78	118.99	92.54	65.35	45.66	92.66						
30	117.83	97.66	77.74	52.5	34.64	76.07						
Mean (S)	157.88	136.58	114.30	73.26	52.41							
L. S. D. at 5%	S=4.54		T = 3.88		T*S= 7.01							

T= Treatments with jasmonic acis (µm/l)
S= Salinity%
T*S= Interaction between treatments with jasmonic acid and Salinity%.

Also, the results in (Table 7) show the significant effect of low concentration (10 µM/l) of JA in increasing yield components of regenerated stevia plants over control under normal or salinity conditions up to 20% salinity level; meanwhile completely opposite trends were obtained with other JA (20 and 30 µM/l) treatments under all salinity levels and 30% as well as 40% salinity levels for 10 µM/l treatment. Hence, the clear superiority of low concentration of JA in inducing the highest degree of salinity consequently salinity tolerance of the regenerated stevia plants could be clearly seen and which even surpassed over untreated control (Mean T); meanwhile the other JA treatments exhibited an opposite trend. As for the highly promoting effects of the low concentration (10 µM/l) of JA on productivity of regenerated stevia plants and endogenous growth substances (Table 6), this treatment showed

the greatest growth (Tables 1 and 2), protective substances, invertase, PAL activities, and stevioside percentage (Table 3), photosynthetic pigments (Table 4), and yield (Table 7). This result needs further investigation in a broad scale of pot experiments and in the field.

CONCLUSION

A wide survey of all foregoing results in the present study clearly revealed that the results obtained during the two seasons confirmed the absolute superiority of the low concentration of JA (10 µM/l) compared either with untreated control treatment or with the other JA treatments (20 and 30 µM/l). These results emphasized its superiority in inducing the higher degree of salinity tolerance and consequently growth of stevia grown *in vitro* and *ex vitro* tolerant up to 20% salinity level and which were able to continue their growth till maturing and even attained stevia production. Such high degree of tolerance exhibited by low concentration of JA (10 µM/l) treatment was positively associated with higher accumulation of endogenous hormonal status (IAA, GA_3 and ABA), protective substances (sugars, proline, amino acids and total soluble phenols), stevioside (%) and photosynthetic pigments, i.e. chlorophylls (a, b and total) and carotenoids in addition to the lowest invertase activity in the stressed leaves in favor of accumulation more non-reducing sugars in the stevia grown *in vitro* and *ex vitro*. These accumulations were positively correlated with the increase in salinity levels in the medium. This is also applied to the considerable accumulations of much more quantities of inorganic osmotica, i.e. N, P, K, Ca and Mg in the stevia grown *in vitro* and *ex vitro*.

The obtained data of low concentration of JA (10 µM/l) treatment during the two seasons offered strong evidence for the absolute superiority of such treatment in inducing higher degree of tolerance to salinity through the accumulations more quantities of the protective solutes compared even with the untreated control. Moreover, such behavior in the treated plants of the low concentration of JA treatment evidently increased their ability to counteract salinity stress, thus were able to keep better performance against salinity until harvest. This was reflected on a significant increment in the stevia yield / plant over the respective yield of all other treatments up to 20% salinity level.

The obtained data suggested that the lower concentration of JA (10 µM/l) may be successfully applied to improve salinity tolerance of economic crops such as stevia, but it must be applied widely and after precise study with each crop to approach its optimal effectiveness in improving tolerance to salinity.

Further physiological studies are needed at the cell level to disclose whether, the role of JA in regulating the uptake and accumulations of different solutes, is attributed to some alteration in the properties of the cell membranes .

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دور حمض الجاسمونيك فى زيادة تحمل نباتات الاستيفيا المكاثرة معمليا للاجهاد الملحي

عبد الرحمن مرسى غلاب*، محمد إبراهيم قبيصى، هناء فتوح يوسف محمد* و عبد الله إبراهيم نافع*****

* كلية الزراعة – جامعة القاهرة – قسم النبات الزراعى – فرع فسيولوجيا النبات – الجيزة – مصر

** كلية الزراعة – جامعة القاهرة – قسم الكمياء الحيوية – الجيزة – مصر

*** معهد بحوث المحاصيل السكرية – مركز البحوث الزراعية – الجيزة – مصر

فى هذا البحث تم دراسة تأثير إضافة حمض الجاسمونيك بتركيزات مختلفة (صفر، ١٠، ٢٠، ٣٠ ميكومول/لتر) الى بيئة زراعة الانسجة لنباتات الاستيفيا الناتجة من القمة الخضرية فى محاولة لتحسين صفة مقاومة الملوحة لنباتات الاستيفيا المكاثرة معمليا وذلك تحت تاثير اضافة مستويات مختلفة من املاح البحر المخلقة (صفر، ١٠، ٢٠، ٣٠، ٤٠٪) كمصدر للملوحة وقد اوضحت النتائج المتحصل عليها الآتى

أن معاملة ١٠ ميكرومول/لتر حمض جاسمونيك أدت للحصول على زيادة معنوية فى صفات النمو لنباتات الاستيفيا الصغيرة النامية فى المعمل (النسبة المئوية للمتبقى ، طول الافرع ، عدد الاوراق والجذور ، الوزن الطازج والجاف للمجموع الخضرى) وصفات النمو لنباتات الاستيفيا المكاثرة معمليا والنامية فى الصوبة (طول النبات ، عدد الاوراق والوزن الجاف للمجموع الخضرى) والمحصول (محصول الأوراق و السيقان و المجموع الخضرى لكل نبات) مقارنة بالكنترول الغير معامل وحتى تركيز ٢٠٪ من أملاح البحر المخلقة .وأيضا المعاملة بالجرعة المنخفضة (١٠ميكرومول/لتر) من حمض الجاسمونيك قد مكنت نباتات الأستيفيا الصغيرة والناتجة من القمة الخضرية المعاملة من مقاومة الاجهاد الملحى حتى مستوى ٢٠٪ من أملاح البحر المخلقة فظلت قادرة على الاستمرار فى النمو فى الصوبة حتى مرحلة النضج وإنتاج المحصول.

وقد أظهرت أيضا نتائج التحليل الكيماوى لأنسجة نباتات الاستيفيا الصغيرة النامية فى المعمل والمجموع الخضرى لنباتات الاستيفيا المكاثرة معمليا والنامية فى الصوبة أن تحمل الاجهاد الملحى والذى ظهر بدرجات عالية فى المعاملة ١٠ميكرومول/لتر حمض الجاسمونيك قد ارتبط ارتباطا موجبا بتراكم كميات كبيرة ومتزايدة من عناصر النيتروجين والفوسفور والبوتاسيوم والمغنيسيوم والكالسيوم وتركيز منخفض من الصوديوم، وذلك بالإضافة إلى تراكم كميات كبيرة من السكروز والبرولين والاحماض الامينية والفينولات الذائبة الكلية و النسبة المئوية للاستيفويد وصبغات البناء الضوئى و الهرمونات النباتية (الجبريللينات والاوكسينات وحمض الابسيسيك) بمستويات تزايدت طرديا بزيادة مستوى الأملاح المخلقة فى البيئة. هذا مع تقليل نشاط انزيم الانفرتيز الى أقل مستوى له مما أدى لتراكم المزيد من السكريات غير المختزلة. وزيادة نشاط انزيم الفينايل الانين امونيالياز مما ادى الى تراكم الفينولات الذائبة.

ومن النتائج المتحصل عليها يتضح امكانية استخدام حمض الجاسمونيك بتركيز منخفض (١٠ ميكرومول/لتر) بنجاح فى انتاج نباتات استيفيا معمليا لها القدرة العالية على تحمل الملوحة حتى تركيز ٢٠٪.