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Combined Effects between Genotypes and Salinity on Sweet Orange during the Developmental Stages of its Micropropagation

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ABSTRACT

The present investigation aimed to study the effects of NaCl salinity on immature ovule regeneration of some *Citrus* genotypes. The relative combination effects of NaCl salinity with different sweet orange genotypes were evaluated during the developmental stages of their micropropagation. Somatic embryogenesis was achieved in callus media of nucellus derived from undeveloped ovules of 6 immature fruits of sweet orange genotypes [*Citrus sinensis* (L.) Osb] using modified MS media supplemented with malt and Ascorbic Acid (AsA) under different NaCl salinity levels. Complete plantlets were developed from the somatic embryos by further subculturing in the same specific media depending on the genotypes used and the level of NaCl. The combined data show that the values of genotype variance were positive but less than environmental media variance for the studied levels of salinity. *In vitro* seedlings of sweet orange genotypes accumulated more organic solutes (total amino acids, proline, glybet, total sugars and total organic acids) than those of the corresponding genotypes *in vivo* seedling having the same physiological age under different salinity levels. Moreover, *in vitro* seedlings scored lower level of lipid peroxidation and higher activities of Superoxide Dismutase (SOD), Peroxidase (POX), Ascorbate Peroxidase (APOX), Catalase (CAT), Glutathione Reductase (GR) than *in vivo* seedlings. Thus, *in vitro* seedling exhibited more salt tolerant than the relatively sensitive *in vivo* plants of sweet orange *Citrus*. It could be concluded that Tissue Culture Technique (TCT) in the presence of MS media supplemented with malt and/or AsA improved salt tolerance of *Citrus*; sweet orange genotypes by protecting the protein turnover machinery against stress damage and up-regulating stress protective proteins.

Key words: Somatic embryogenesis, *Citrus sinensis* (L.) Osb, genotypes, nucellus, Organic solutes, lipid peroxidation, enzymatic antioxidants

INTRODUCTION

Citrus has first position in Egypt because of its favorable growing conditions including adequate water supply. Sweet oranges have mostly exotic varieties. It is important for *Citrus* industry to have superior genotypes adaptable to different agro-climatic conditions which should increasingly meet the demands of international markets. *Citrus* cultivated area in Egypt reached to about 375000 feddans (feddan = 4200 m^2) (FAO, 2004).

Salinity problem considered a significant limiting factor affecting *Citrus* production. Differences in salinity tolerance have been reported among number of *Citrus* genes. Na^+ and Cl^- ions are the

dominant ions and usually they exceed by far the plant demand necessity. The excess of these ions in the media alter the aqueous and ionic thermodynamic equilibrium which results in hyper-osmotic stress, ionic imbalance and toxicity (Munns, 2002). As a result of these changes, the activities of various enzymes and the plant metabolism are affected (Lacerda *et al.*, 2003). Many plant species are able to tolerate salt stress by reducing the cellular osmotic potential as a consequence of a net increase in solute accumulation, in a process called osmotic adjustment (Bor *et al.*, 2003). It is accepted that during osmotic adjustment the cells tend to compartmentalize most of the absorbed ions in vacuoles at the same time that they synthesize and accumulate compatible organic solutes in the cytoplasm in order to maintain the osmotic equilibrium between these two compartments (Hasegawa *et al.*, 2000).

The accumulation of osmoprotectant in cytoplasmic compartments are osmotically significant because they have pivotal roles in maintaining cell turgor and the driving gradient for water uptake under stress (Rontein *et al.*, 2002) helping the maintenance of ionic homeostasis and C/N ratio, removal of free radicals and stabilization of macromolecules and organelles, such as proteins, protein complexes and membranes (Bray *et al.*, 2000).

In *Citrus*, it was found that salt stress decreased net CO_2 assimilation, stomatal conductance and water potential in leaves and these effects were associated with an accumulation of excessive concentrations of chloride or sodium (Lloyd *et al.*, 1990). Plants have evolved different defensive systems against environmental stresses. Special attention has been given different plant species which face these extreme condition on their natural environmental because most of their tolerance mechanism have not been well understood, they could be very important tools for genetically engineered stress tolerant plants (Bor *et al.*, 2003).

Citrus species propagated conventionally by means of seeds, grafting and budding methods. Due to present demand of this fruit trees in both local and foreign markets it is necessary to develop a suitable protocol for mass propagation from existing elite cultivars. The process of somatic embryogenesis is a suitable method of micropropagation of tolerant crops and has the potential of mass propagation commercially at low per unit. In *Citrus*, somatic embryogenesis has been widely studied from ovule of cv. Dancy (Coelho *et al.*, 1998). Anther of *C. aurantium* and *C. reticulate* undeveloped ovules and stigma style explants of sweet orange novel group were also used (Carimi *et al.*, 1998). Undeveloped ovules of orange cultivar Xinhue were accepted (Rong *et al.*, 1998). Nucellus embryogenesis in Valencia and Rough lemon has been reported (Obukosia and Waithaka, 2000). It is mostly the nucellus in the ovule which regenerates into embryos because it is least differentiated and has inherited competence for regeneration in culture (Niedz, 2006). Singh *et al.* (2006) raised embryogenic cultures of Kinnow mandarin from unfertilized ovules.

Therefore, the present investigation aimed to study the effects of NaCl salinity on immature ovule regeneration of some *Citrus* cultivars. The relative combination effects of NaCl salinity with different sweet orange genotypes were evaluated during the developmental stages of their micropropagation.

MATERIALS AND METHODS

Nucellus explants (inside masses) from 6 genotypes of sweet oranges novel group [*Citrus sinensis* (L) Osb.] was used to observe the embryogenesis calli induction and plantlets regenerations under three different levels of NaCl salinity. The experiments were carried out at Tissue Culture Laboratory, Horticulture Research Institute; ARC Egypt during the period from

2009-2010. Four to five weeks old immature fruits were collected from the genotypes cultivated and located at the *Citrus* Research Station, El-Kanater El-Khiria Experimental Farm, ARC Egypt. The genotypes used for the micropropagation were Blood red, Valencia. Succari, Washington novel, Moro blood and Jaffa having orange ovules number 9, 14, 13, 9, 12 and 18, respectively.

Fruits were washed thoroughly under running tap water to reduce dust and surface contaminates. They were dipped in 90% alcohol for one minute followed by 5% sodium hypochlorite solution for 10 mins and finally rinsed 4 times with sterile distilled water under laminar airflow cabinet. The fruits were then placed on an autoclaved ceramic tile, cut open by sharp sterilized knife and the undeveloped ovules and immature seeds were separated. For somatic embryogenic callus induction and examined the effect of salinity, nucellus halves were separated .They were cultured on a semi-solid modified MS medium supplemented with 500 mg L^{-1} malt extract and ascorbic acid; AsA at 100 mg L^{-1}. This medium is called Somatic Embryogenic Medium (SEM) as described previously (Tisseret and Murashige, 1977). In the present investigation, the SEM was supplemented with 0 (control), 3000, 6000 mg NaCl/l to examine the effect of salinity. The nucellus halves were taken from each genotype. Thereafter, transferred to a conical flask, six samples in each, with 4 replicates for each treatment. Four conical flasks represented each replication, 5 explants in each and were considered as experimental unit.

Calli initiation was formed within 3 months and the average number of embryos per nucellus was recorded. The responded nucelus after 3 months were further subcultured for another 2 months on the same specific media and simultaneously into hormone free MS specific medium supplemented with or without ascorbic acid AsA; at 100 mg L^{-1}. The normal healthy of the successful embryogenesis was developed to plantlets on the same specific media. The pH of all media was adjusted to 5.7 before addition of agar; 10 g L^{-1} (BDH) and sterilized by autoclaving for 20 mins at 1.05 kg cm^{-2} pressure at 121°C. The flasks containing explants were incubated on culture racks and maintained at 25±2°C under white fluorescent light at 1500 Lux for 16/8 h (s) photoperiod.

The plantlets formed from each treatment have the same physiological age and that having single primary root growth and 18 mm in height which obtained through somatic embryogenesis were transferred and planted individually in plastic pots; tyrpido (5.5×6.5 cm) containing media for hardening (peatmoss: sand: perlite; 1:1:1 v/v). The pots containing plantlets were arranged in the acclimatization room under 25±1°C and 1500 Lux light intensity for 16/8 h, photoperiod and about 80% relative humidity and fertilized with a solution of half strength MS. The pots were covered, enveloped and tightly closed with transparent polyethylene sheets to provide high humidity atmosphere and light intensity around the plantlets. Acclimatization was achieved by gradual reduction of relative humidity around the plantlets by removing the plastic sheets along with four weeks. Then, they were irrigated when even required with half strength MS supplemented with 0 (control), 3000, 6000, mg NaCl/l. After 2 months the survival percentage were recorded.

To confirm the effectiveness of tissue culture technique as a tool for salt tolerance induction, a comparative physiological study were carried out between *in vivo* seedling and *in vitro* regenerated one having the same physiological age and grown under the same conditions. They were chemically analyzed for certain organic osmolytes components that possible involvement of the antioxidant system in relation to salt tolerance. These included Total free Amino Acids (TAA), proline, Glycine Betaine (GlyBet), Total Sugars (TS) and Total water-soluble Organic Acids (TOA). In addition, lipid peroxidation as well as the activities of Super Oxide Dismutase (SOD) Peroxidase (POX), Ascorbate Peroxidase (APOX), Catalase (CAT) and Glutathione Redactase (GR) were examined.

Total free Amino Acids (TAA) and proline as well as total sugars and GlyBet were extracted from the plant materials by 80% ethanol. TAA and proline were determined spectrophotometrically by the methods of Dubey and Rani (1989a, b) and Bates *et al.* (1973), respectively .Total sugars were determined by phenol-sulphoric acid method as described by Sadasivam and Manickam (1996).

Glycine Betaine; GlyBet estimation was performed as described by the method of Grieve and Grattan (1983) spectrophotometrically. Total water soluble Organic Acids (TOA) were extracted by the method of Huang and Redmann (1995) by titration with NaOH using bromothymol blue as an indicator. TOA was calculated as mg g^{-1} fresh weight.

Total carbohydrates as glucose were determined in a well-known volume from the acidic-isopropanol extranet after protein precipitation and neutralization according to Amberger (1954).

Lipid peroxidation was determined according to Rao and Sresty (2000) by estimating the MalonDiAldehyde (MDA) content.

Peroxidase (EC number 1.11.1.x) activity (POX) was assayed based upon the method described by Herzog and Fahimi (1973). One enzyme unit is defined as µmoL mL^{-1} destroyed H_2O_2 per min.

Catalase (EC 1.11.1.6) activity (CAT) was determined according to Bergmeyer (1970) and µ moL H_2O_2 destroyed per min was defined as one unit CAT.

Glutathione Reductase (EC 1.8.1.7) (GR) activity was measured according to Foyer and Halliwell (1976). One enzyme unit is defined as µmoL mL^{-1} oxidized Glutathione per min.

Ascorbate Peroxidase (EC.1.11.1.11); (APOX) activity was assayed according to Nakano and Asada (1981). One enzyme unit is defined as µmoL mL^{-1} oxidized ascorbate per min.

Superoxide Dismutase (EC 1.15.1.1) (SOD) activity was assayed based on the method of Beauchamp and Fridovich (1971) and the specific enzyme activity was expressed as units mg^{-1} protein g FW.

Statistical analysis: The experimental design was randomized complete blocks design with four replications. A combined analysis of variances, two-way type, for the 6 genotype over the three NaCl salinity level was made for the studied trails according to Steel and Torrie (1980). Subsequently, partitions of phenotypic variance to its components were made. Heritability in broad sense was estimated according to the following equation:

$$Hb\% = \sigma^2 g/\sigma^2 ph \times 100$$

Where:

Hb = Heritability $\sigma^2 g$ = genetic variance $\sigma^2 ph$ = phenotypic variance

For the comparative physiological analysis between the *in vivo* and *in vitro* seedling, Duncan's multiple range test for Propagation type (P), Salinity level (L), Genotype (G) and their interactions were employed using SAS Institute (2003).

RESULTS AND DISCUSSION

Time required for the responding regeneration: Table 1 shows that time for callus initiation (responded nucellus) was varied from 8 to 12 weeks in all genotypes developing on the SEM-saline

Table 1: Days for responding nucellus, embryogenic callus formation and plantlets regeneration in the sweet orange genotypes examined as affected by NaCl salinity

		Genotyps (G)						
NaCl levels mg L^{-1} (L)	Developmental stages	Jaffa	Washington novel	Valencia	Moroblood	Blood red	Succari	Mean
0 (control)	C.I	56	56	59	63	68	70	62
	E.C	112	115	120	120	126	129	120.3
	Plantlets	144	148	152	150	162	164	153.3
	Mean	104	106.33	110.33	111	118.66	121	111.87
3000	C.I	65	68	68	70	75	75	70.17
	E.C	120	122	126	125	134	138	127.5
	Plantlets	145	145	148	146	159	164	151.2
	Mean	110	111.67	114	113.67	122.67	125.67	116.29
6000	C.I	84	85	88	88	94	96	89.17
	E.C	135	135	138	140	148	152	141.3
	Plantlets	155	157	162	162	168	171	162.5
	Mean	124.67	125.67	129.33	130	126.67	139.67	130.99
Mean	C.I	65.3	69.7	71.7	73.7	79	80.3	73.8
	E.C	122 3	124	128	128.3	136	139.7	129.7
	Plantlets	148	150	154	152.7	163	166.3	155.7
LSD at 5% for:								
G		1.11	0.96	1.33	1.00	1.66	0.93	
L		1.96	2.01	1.09	1,66	2,00	1.76	
G×L		4.31	4.33	3.63	2.99	4.03	3.96	

CI: Responded nucellus callus induction, EC: Embryogenic callus formation, L: Sanity level, G: Genotype

media. The presence of salinity delayed swelling by about 7-17 days compared with the control and depending on salinity level. Callus initiation was noticed after 62 days from the nucellus incubation whereas, delayed to about 70 and 89 days in salinized media at 3000 and 6000 mg NaCl/l, respectively.

As for the effect of genotypes, overall salinity level, the data in the Table 1 show that Jaffa genotype was responded for callus initiation faster than the other genotypes examined whereas Succari genotypes responded too late. Callus initiation was performed after about 65 and 80 days for Jaffa and Succari genotypes, respectively. Similar trend was found with the interaction treatment (G×L). Succari genotype under normal and salinized media gave best results for callus initiation compared with the other genotype examined.

On the other hand, it was found that calli developed in salt-free (control) media were only embryogenic in nature during this period. It was loose, light green and having good growth. *Calli* obtained in salinized media were compact and initially green but with the lapse of time, they turned brown only in free-AsA media at the high level of salinity in most genotypes. The presence of AsA minimized browning and induced formation of embryogenic calli. Browning noticed in the AsA free SEM salinized media may be due to the oxidation of polyphenols and the formation of quinines which are highly reactive and toxic to the plant tissues (Helaly *et al*., 2008). El-Meskaoui and Tremblay (2001) suggested that ethylene could cause tissue browning under stress, it activate the synthesis of oxidative enzymes. The alleviating effects of AsA may be due to its enhancing role on cell division and synthesis of hydroxyl proline-rich protein (Alqurainy, 2007), regulation of the gene expression and being the precursor of phytochelatins (Wingate *et al*., 1988).

Embryogenic calli when transferred to hormone-free specific media, somatic embryos were developed. Under salinized media, the regeneration to plantlets was delayed and the delaying was a concentration dependent. Embryogenic calli in the control media was achieved after about 120 days whereas delayed significantly to 127 and 141 days under 3000 and 6000 Nacl/l, respectively. Moreover, Jaffa embryogenic calli was developed well and more faster than the other genotypes. The least embryogenic calli was developed with Succari. These results are true under salinixed and non-salinized conditions. In addition, complete plantlets were developed from these somatic embryos by further sub-culturing in the same specific media. However, the least response values for somatic embryos were recorded at the high level of salinity without AsA supplementation in all genotype examined. The somatic embryogenic callus and plantlets were obtained from nucellus of many *Citrus* species on malt supplemented SEM media as reported by Tisseret and Murashige (1977). They reported that the presence of malt in the MS media was suitable to develop a protocol for somatic embryogenesis from nucellus of *Citrus*. However, no data was available regarding sweet oranges *Citrus* nucellus development and its regeneration in the presence of NaCl salinity.

The importance of AsA on preventing browning was previously mentioned by Helaly *et al.* (2008) on date palm regeneration. The delaying effects of salinity on the regeneration may be due to its effects on either colloidal inhibition of water occurred by the explants and/or imbalanced osmotic water uptake occurred by the tissues (Helaly *et al.*, 2009a). Altering in hormonal balances and decreasing endogenous cytokinins biosynthesis and auxin production were also reported under stress (Schmidt, 2005). Helaly *et al.* (2009b) found that stress decreased the accumulation of reducing sugars within the plant tissues which decreased wilting resistance. Gadalla (2009) added that salinity did not induce an increase of AsA which not only quenches reactive oxygen but also regenerates tocopherol. Demir *et al.* (2003) attributed the delaying effects of salinity on the regeneration to the suppression of plant tissues metabolism. Tajdoost *et al.* (2007) reported that salt stress adversely affect the physico-chmical properties of the protoplasts and cell membrane. Inhibition of cytokinins and hormonal unbalances, reducing water content and some nutrients uptake as well as biosyntheses of tocopherol, AsA and high respiration rate were also reported by Tripathi *et al.* (2007) under stress condition.

The beneficial effects of AsA addition to MS basal media with salt affected *Citrus* explants may be associated with the maintenance of cell membrane integrity, reducing Na^+ and Cl^- contents and favoring K^+, Ca^{2+} and Mg^{2+} absorption which is reflected by a reduction in senescing (Gadalla, 2009) as indicated by browning noticed in the present investigation. Several reports indicated that the beneficial effects of additional antioxidants on plant survival under different salt stress are associated with the partial inhibition of ROS formation and its effects (Mayer *et al.*, 2002). Antioxidants decreased lipid peroxidation and increased the activates of CAT and POD enzymes (Shalata and Neumann 2001) which are involved in the detoxification of H_2O_2 in the plant tissues. Moreover, antioxidants increased carotenoids and total phenols which lowering, as an adaptive tools, the generation of free radicals and thus reduces the lipid peroxidation and inhibit the oxidation of lipids, fats and proteins by the donation of a phenolic hydrogen atom to the free radical (Halliwell *et al.*, 1995). Phenolics are able to act as radical scavenger or radical (Gadalla, 2009) chain breakers, so extinguishing strongly oxidative free radicals. Mayer *et al.* (2002) reported that the reaction mechanisms by which the hydrogen atoms of phenols are transferred to a radical could be in two distinct pathways; hydrogen atoms transfer and proton-coupled electron transfer. Similarly, carotenoids can directly deactivate singlet oxygen, reducing the formation of singlet

oxygen species (O_2^-) and protecting the photosynthetic apparatus against photo-inhibitory damage (Foyer *et al.*, 1994).

Analysis of variance for responded nucellus, embryogenic callus formation and plantlets percentages: Data presented in Table 2 show that the mean squares of the replication indicates the absences of significant differences between them regarding callus+embryogenic callus formation and regenerated plantlets.

As shown in the Table, there is a highly significant difference between the replication in case of responded nucellus (431.6). The data also show highly significant differences between the genotype examined for responded nucellus, calli + embryogenic callus formation and the formed plantlet percentages. The values recorded in this respect 912.9**, 1.33** and 803.3** for these criteria, respectively. These results indicated that the nucellus explants which collected from each genotype are different in their ability for culturing purposes. In addition, these findings indicate the presence of real differences among these genotypes with respect to the ability of nucellus to response for culturing purposes. Thus, the partition phenotypic variance to its components and the comparison between the genotypes means are valid.

On the other hand, the mean squares of the salinity were significant for the percentage of responded nucellus, Moreover, the mean squares of salinity levels and the combined between G×L were highly significant for the percentages of both calli+EC formation. The mean squares of salinity levels reached to 1.02** and 1007.6** with callus+EC formation and for S×L reached 1.17** and 711.9** for them respectively and produced plantlets. These results indicate that genotypes responded differently at different salinity levels supplemented to the basal MS media. Similar result was reported by Khatun *et al.* (2006) on eggplants who found that the performance of genotypes was different over the different media. It could be concluded that these results may be one possible way of avoiding the genotypic influence of regeneration ability by using various media depending on genotypic selection.

Genetic parameters and the physiological responses: The total variance of the studied traits, through the developmental stages of sweet orange were partitioned to its components: genetic variance; σ^2_g, environmental variance, σ^2_e, levels of salinity; σ^2_L and levels of salinity by genotypes interaction; σ^2g L variance in the case of callus initiation, embryogenetic calli formation and regenerated plantlets percentages. Moreover, these parameters were used for estimation heritability in broad scenes for these traits.

Data in Table 3 show a positive relation between all genotype parameters and salinity levels interaction (σ^2 g×L) in the three levels. The positive relation of ranged from 0.35 in case of embryogenic callus formation to 171.6 in the 0.35 to 9.91 in the responded nucellus. These finding may explain the different magnitudes with different salinity levels. Moreover, the combined data show that the values of genotype variance were positive. It ranged from 0.09 in the embryogenic callus formation to 7.86 in case of responded nucellus. In the regenerated plantlets, it scored 11.71%. However, these results were less than environmental media variance for the studied levels of salinity. The positive environmental variances ranged from 0.12 to 100.11 for the embryogenic callus formation and responded nucellus, respectively. In the regenerated plantlets, it reached to 162.1%.

On the other hand, the heritability estimated were less than 50% for all the studied traits. It reached about 35.4, 43.9 and 20.2% for responded nucellus, embryogenic callus formation and

Table 2: Variance combined analysis of sweet orange genotypes studied over the three levels of salinity for the percentages (%) of responded nucellus, callus+ embryogenic callus formation; EC and plantlets regeneration

SOV	d.f	Responded nucellus	Callus + EC formation	Regenerated plantlets
Replication	3	431.6**	0.15	171.6
Genotypes (G)	5	912.9**	1.33**	803.3**
Salinity levels (L)	2	301.6*	1.02**	1007.6**
G×L	10	77.6	1.17**	711.9**
Error	52	56.3	0.09	111.3

*and** significant at 5 and 1% level of probability, respectively

Table 3: Genetic parameter for the combined data of sweet orange genotypes under different salinity levels

Genetic parameter	Responded nucellus (C.I) (%)	Embryogenic callus formation (%)	Regenerated plantlets (%)
σ^2 ph	207.22	0.42	411.69
σ^2 g	78.60	0.09	11.71
σ^2 L	7.11	0.05	57.11
σ^2 g×L	9.91	0.35	171.60
σ^2 e	100.11	0.12	162.10
Hb%	38.36	43.93	20.17
C.V	17.21%	13.20%	20.11%

σ^2_{ph}: Phenotypic variance. σ^2g: Genetic variance. σ^2L: Levels of salinity. σ^2g×L: Levels by genotypes interaction. σ^2e: Environmental variance. Hb%: Heritability

the regenerated plantlets, respectively. These findings indicate that all treatments studied are mainly affected by the environmental factors rather than genetic factors. Similar results were reported by Altaf (2006) who reported that *Citrus* has several natural factors (gamma radiation levels) cause of variability. However, Helaly *et al*. (2011) on some wheat varieties found that calli initiation was mainly controlled by genetic factors while emberyogenic callus formation and its regeneration to plantlets were mainly affected by the environmental factors (supplementation of NaCl to the MS media).

Responded nucellus (callus initiation. CI): Table 4 shows that the mean values of the responded nucellus percentage of the six genotypes are specific with the plant genotype and the culture media depending on NaCl level. In NaCl free media (control), Jaffa genotype scored highest values than other genotypes with mean of 72.9 whereas Succari recorded lowest value with means 32.7%. The low salinity level (3000 mg NaCl/l) increased the mean values of all genotypes studied except that of Jaffa and Washington which showed a decrease in this respect. Mean performance of the Jaffa and Washington was decreased overall salinity level as well as their combined and the decreased was a concentration dependent. Other genotypes showed an increase in their mean performance in case of responding nucellus percent due to the low salinity level and thereafter decreased. Blood Red and Jaffe genotypes had highest mean values in their response to 3000 mg NaCl/l with means of 68.8 and 64.2%, respectively. They also scored the highest mean values with respect to the high salinity level (6000 mg L^{-1}) which were significantly higher than the other genotypes with means of 63.3 and 55.3%, respectively. Succari genotype scored the lowest mean value in comparison with other genotypes in all salinity levels with means of 32.7, 40.6 and 34.6% with respect to 0, 3000 and 6000 mg NaCl/l in a descending order.

The significant differences between the studied genotypes emphasized that calli initiation are controlled by the genotypes of donor plants (Galibo and Yamada, 1988; Helaly *et al*., 2011). The

Table 4: Mean values percentages of the responding nucellus. C.I., embryogenic callus formation and plantlet regeneration for the six genotypes studied of sweet orange over the three levels of salinity . Responding nucellus % (swelling and callus induction; CI)

	NaCl levels (L) (mg L^{-1})			
Genotype (G)	0	3000	6000	Mean
Responded nucellus (C.I) %				
Jaffa	72.9	64.2	63.3	66.8
Washington novel	70.2	61.5	50.4	60.7
Valencia	59.9	60.3	50.2	56.8
Moroblood	56.1	60.7	52.1	56.3
Blood red	54.3	68.8	55.3	59.47
Succari	32.7	40.6	34.6	35.97
Mean	57.68	59.35	50.98	56
LSD at 5% for: G = 1.2, L = 1.9 and G×L = 2.6				
Callus and embryogenic callus formation % (EC)				
Jaffa	62.4	51.2	44.2	52.6
Washington novel	51.2	45.2	40.4	45.6
Valencia	52.1	43.3	38.4	44.6
Moroblood	46.1	42.7	32.8	40.53
Blood red	44.3	41.8	30.2	38.77
Succari	22.4	20.2	18.6	20.4
Mean	46.42	40.73	34.1	40.42
LSD at 5% for: G = 2.3, L = 2.5 and G×L = 3.3				
Plantlets regeneration %				
Jaffa	44.2	36.4	28.6	36.4
Washington novel	38.6	31.4	24.5	31.6
Valencia	40.0	32.6	21.2	30.8
Moroblood	34.8	28.8	19.4	27.7
Blood red	36.8	30.2	18.4	28.47
Succari	28.5	18.6	11.2	19.43
Mean	37.15	29.67	20.53	29.06
LSD at 5% for: G = 2.6, L = 2.9 and G×L = 3.3				

decreasing effect of high salinity level (6000 mg L^{-1}) on responding nucleolus percentage may be due to its effect on inhibition of cytokinin biosynthesis and hormonal unbalances (Bartles and Sunkar, 2005). Several investigators reported that callus initiation was varying in frequency from 0 to 100% depending on the growth substances level (Khatun *et al*., 2006; Niedz, 2006), plant genotype (Helaly *et al*., 2011) and the explant used (Tisserat, 1979a). Roy and Banerjee (2003) found that the hormonal balance between Benzyl Amino Purine (BAP) and NAA auxin at the narrow range was important for callus induction. They added that the highest frequency of callusing occurred in the medium supplied with 0.5 mg L^{-1} NAA and 1 mg L^{-1} BAP for *Dendrobium fimbriatun* Lindl var. oculatum HK.F. The requirement of exogenous in addition to the endogenous hormones was previously supported early by many investigators for callus induction (Tisserat, 1979b).

Plantlets regeneration: Data in Table 4 show also that Jaffa genotypes followed with Washington were highest ability for regeneration to form plantlets, 23 mm in height with single primary root growth on saline media especially at the low level. The values recorded were 44.2, 38.6, 36.4, 31.4 and 28.6, 24.5% under 0, 3000 and 6000 mg NaCl/l, respectively. Succari genotype

and Blood red scored the lowest mean values under salinity at 3000 and 6000 mg NaCl/l in comparison with other genotype with means of 18.6, 30.2 and 11.2, 18.4% for the first and of 28.5, 36.8% for the second, respectively. The combined data show that Jaffa and Washington scored the highest mean value whereas Succari and Moroblood Red had the lowest mean values overall genotypes studied. Chen and Murata (2002) previously found differences in the response of each genotype with different salinity level on some wheat varieties and Altaf (2006) on *Citrus*. The latter author found that *Citrus* has several natural factors as cause of variability.

It could be concluded that callus induction, embryogenic callus formation and plantlet regeneration were affected significantly depending on the genotype and the level of salinity. The beneficial effects of salinity at low level on plantlets regeneration may be due to its effects on cell division, differentiation and cell elongation (Kaymakanova and Stoeva, 2008).

Reduced tissue growth in salt stressed media is a usual phenomenon under high levels. Several investigators recorded an inhibition of growth due to salinity more than 300 mg L^{-1} and attributed this inhibition to the hormonal unbalanced (Schmidt, 2005), insufficient supply of nutrients and other osmotic solutes needed for growth (Gadalla, 2009). In addition, it was found that salt stress adversely affect the physico-chemical properties of the protoplasm and cell membranes (Tajdoost *et al*., 2007). Inhibition of cytokinin biosynthesis and hormonal unbalances reducing water content and nutrients uptake as well as biosynthesis of tocopherol, ascorbic acid and net photosynthetic rate accompanied with high respiration rate were also reported under stress condition (Tripathi *et al*., 2007).

Number of embryos per nucellus: Table 5 shows that sweet orange genotypes studied of *Citrus* plants had different embryogenic potential to form embryos from their nuecllus. The maximum average embryo per nucellus was in Jaffa and the minimum was found in genotype Moroblood which recorded 6.0 and 2.35, respectively under no salinity level. The maximum healthy embryo weight (20 mg) was recorded in Washington novel followed by Blood red, Succari and Moro blood genotypes and the least one for Jaffa. Similar trend to that of CI, EC and plantlets regeneration was noticed regarding the effects of salinity.

Table 5: Number of embryos per nucellus after 4 months of Sweet oranges genotype culture under the different levels of salinity

			Average embryos/nucellus			
			NaCl salinity level (mg)			
Genotypes	Shrunken nucellus (%)	F. Wt mg/nucellus	0	3000	6000	Mean
Jaffa	81	11	6.0	4.5	3.7	3.7
Washington novel	20	20	3.8	3.1	2.0	2.9
Valencia	88	13	5.5	5.0	3.6	4.7
Moroblood	31	14	2.35	2.25	1.8	2.12
Blood red	22.2	15	3.4	3.8	2.7	3.3
Succari	23	15	4.0	3.8	3.1	3.63
Mean	44.2	14.67	4.18	3.73	2.82	3.39
LSD at 5% for						
G	3.6	2.3	0.06			
L	ND	ND	0.06			
G×L	ND	ND	0.09			

ND: Not detected

COMPARATIVE PHYSIOLOGICAL ANALYSES BETWEEN THE *IN VITRO* AND *IN VIVO* SEEDLINGS

Organic solutes: Data in Table 6 show that *in vitro* seedlings of sweet orange genotypes accumulated more significant organic solutes represented by Total free Amino Acids (TAA), proline, Glaycinebetain (GlyBet), Total Sugars (TS) and Total Organic Acids (TOA) than those of the corresponding of *in vivo* seedling having the same physiological age. These results are true overall salinity levels and genotypes examined. Moreover, all interaction treatments (between salinity, genotypes and/or propagation type) showed a significant effects in this respect. The accumulation of the organic osmolytes in the *in vitro* plants more than *in vivo* under normal and salinized condition (Table 6) indicate that using tissue culture technique; TCT helped in maintaining the osmotic balance and thus helped in enhanced salt tolerance.

Regarding the effects of salinity, data in Table 6 show that increasing salinity level up to 6000 ppm led to a significant increase in TAA, proline, GlyBet and TS. However, fewer decreases were detected for TOA compared with the control.

Compared with the genotypes, Table 6 shows that the maximum accumulation of organic solutes was differ greatly between them according to its kind. Jaffa accumulated TAA more than the others whereas, Moroblood accumulated proline. In addition, Blood red accumulated more Glycine Betain and TS than the other genotypes and Washington has highest TOA.

The interaction treatments indicated that TAA was accumulated more in Jaffa *in vitro* seedling grown under 3000 ppm NaCl salinity whereas *in vivo* Succari grown under no salinity level recorded lowest level. In addition Proline was accumulated more *in vitro* Washington, Valencia and Moroblood seedlings grown under the highest level of salinity (6000 ppm NaCl) and the lowest values were detected in *in vivo* Jaffa and Blood red seedlings grown under the control. Moreover *in vitro* Jaffa and Blood Red seedling have highest values of Glycine betain whereas, *in vivo* Washington seedlings grown in the control have lowest one.

As for the effects of P×L×G on total sugars, Table 6 shows that *in vitro* Moro blood seedlings grown under 6000 ppm NaCl recorded highest value whereas *in vivo* Valencia grown under the normal condition detected least values in this respect.

Total organic acids showed highest valued *in vitro* Moro blood grown under 3000 ppm NaCl and lowest values *in vivo* Succari seedlings grown under control and 3000 ppm NaCl.

The accumulation of TAA in stressed plants may be due to inhibition of protein synthesis and/or protein degradation (Yadav *et al*., 1999) needed for synthesis of new proteins for growth or survival under the modified condition.

The increasing of organic osmolytes specially that of proline due to NaCl supplementation to the MS media during the regeneration more than *in vivo* seedling suggested that using micro propagation technique (TCT) may alleviate the toxic effects of NaCl. In the present investigation, proline accumulated more *in vitro* seedling than *in vivo* one due to salinity. Proline acts as an osmoticum, a protective agent of enzyme and cellular structure and storage compound of reducing nitrogen for rapid re-growth after stress are relieved. In this study also an increase in free amino acids have been observed in all *in vitro* plants with increasing NaCl in the medium. These results are in agreement with Misra and Saxena (2009) in seedling and callus cultures who found that *in vitro* or seedlings in natural conditions showed a simultaneous decrease in the protein content. The present study on *Citrus* genotypes indicated that the increase in the proline content in stressed seedlings may be due to enhanced proline synthesis and/or stress induced decreases in corporation of proline to other macro-molecules synthesis such as protein. Moreover, it degraded by the

induction of rapid turn-over processes with an increase in proteolytic enzymes. In this connection, it was reported that the increase in proline content under stress condition could be due to enhanced activity of ornithine amino transferase and Pyrroline 5-carboxilate reductase, the enzyme involved in proline biosynthesis as well as due to the inhibition of proline oxidase, proline catabolizing enzymes (Debnath, 2008).

It could be concluded that TCT in the presence of AsA improved salt tolerance of *Citrus*; sweet orange genotypes by increasing organic osmolytes estimated specially that of proline and protecting the protein turn-over machinery against stress damage and up-regulating stress protective proteins. In this context, Tajdoost *et al.* (2007) and Kholova *et al.* (2009) found that AsA may minimize deleterious effect of salinity on plant growth of wheat and increased its adaptation and its content total free amino acids. Tripathi *et al.* (2007) added that proline accumulation may contribute to osmotic adjustment at the cellular level.

Data in the present investigation concluded also that there is a correlation between total sugars accumulation and proline level. Heineke *et al.* (1992) attributed this to the stimulation of proline enzymes synthesis. The regulation of proline enzymes metabolism in response to salt stress or antioxidants has been demonstrated in many plant species (Misra and Saxena, 2009). The accumulation of sugars was a result of an enhanced efficiency in the use of carbon coupled to a reduction in cellular metabolism, to support the osmotic adjustment required to survive saline media (Schnapp *et al.*, 1990).

The role of proline on induction salt tolerance may be due to its multiple functions on regulate osmotic potential and protect membrane integrity was studied by Tripathi *et al.* (2007) as well as Kaymakanova and Stoeva (2008). A stabilization of enzymes proteins, maintain appropriate $NADP^+$/NADPH ratios and scavengering of free radicals were also reported by Misra and Saxena (2009). The increase in proline accumulation more *in vitro* seedlings than *in vivo* plants may be due to its importance as a major source of energy and nitrogen during immediate post stress metabolism (Jain *et al.*, 2001). They added that the accumulated proline apparently supplies energy for growth and survival thereby inducing salt tolerance. The accumulation of proline under salt stress especially in the plants that micro propagated with tissue culture technique may be attributed to the strategies adapted by these plants to cope up stresses conditions (Alqurainy, 2007). Misra and Gupta (2006) as well as Hare and Cress (1996) found that mRNA transcript encoding Δ^1 pyrroline carboxylate synthetase; and pyrroline 5 carboxylate reductase (P5CR). P5CR were increased in phloem tissue in response to water depletion.

Glycinebetaine; GlyBet, a quaternary ammonium compound, is regarded as one of the most effective osmoprotectants owing to its many advantages besides its efficiency as a compatible solute. It has been reported by Rhodes and Hanson (1993) that GlyBet protects the cells from stresses by maintaining an osmotic balance between the intracellular and extracellular environments. They added that GlyBet stabilized the quaternary structures of proteins complex like antioxidants enzymes as well as biomembranes and other functional units like oxygen-involving photosystem II complex. The increase in GlyBet noticed *in vitro* plants more than *in vivo* (Table 6) may be attributed to the fact that the adapted plants having more betaine accumulation as a result of its stimulation biosynthesis. The molecular features of GlyBet enable its interaction with both hydrophobic and hydrophilic domains of macromolecules without perturbing the cellular functions (Sakamoto and Murata 2002).

The increment in total sugars noticed in salinized seedlings, especially that micropropagated by TCT; tissue culture technique (*in vitro* seedlings) may act as osmotica and/or protect specific

Table 6: Concentrations (mg g^{-1} F.Wt) of total free amino acids, proline, Glycinebetaine; GlyBet, total sugars and total water soluble organic acids of *in vitro* and *in vivo* plants having a same physiological age of *Citrus* sweet orange genotypes studied

Propagation type (P)	NaCl Salinity level (L)	Genotype (G)	Total Amino acids (TAA)	Proline	Glycine Betain	Total Sugars (TS)	Total Organic Acids (TOA)
In vivo	0 (control)	Jaffa	79.6r	9.9m	1.08k	15.84p	41.2e
		Washington	76.4s	12.4l	1.02l	16.84o	42.1d
		Valencia	67.4u	11.4l	1.04l	12.64r	40.0f
		Moro blood	61.4w	13.4k	1.12k	14.86q	38.2h
		Blood Red	62.6w	10.4m	1.08k	16.84o	36.8j
		Succary	56.4y	12.6l	1.14t	15.84p	34.4l
	3000	Jaffa	89.4p	13.6k	2.80g	22.68n	44.1b
		Washington	77.6s	16.6j	2.20h	23.62m	43.8c
		Valencia	70.2t	14.8k	2.04i	24.60m	40.6f
		Moro blood	64.6v	16.8j	2.60g	28.68k	41.5e
		Blood Red	68.2u	12.6l	2.78g	28.28k	38.1h
		Succary	70.2t	16.2j	2.04i	18.80o	36.5j
	6000	Jaffa	82.5q	17.8i	4.68d	28.24k	40.5f
		Washington	72.2t	19.5h	3.48f	26.20l	41.20e
		Valencia	65.4v	18.5i	3.63f	22.26n	36.8j
		Moro blood	61.4w	19.4ih	4.58e	32.24i	39.8g
		Blood Red	60.4x	16.8j	4.84d	30.20j	35.20k
		Succary	65.4v	19.3ih	3.28f	22.28n	34.20l
In vitro	0 (control)	Jaffa	152.4d	16.8j	2.06i	28.59k	43.2c
		Washington	148.96e	23.10g	1.86j	31.14i	42.4b
		Valencia	136.42h	21.86h	2.24h	25.21l	41.5e
		Moro blood	118.68n	26.41e	2.02i	26.84i	39.4g
		Blood Red	130.42k	20.02h	2.12i	32.85i	39.4g
		Succary	108.86o	23.64g	4.08e	30.08j	33.6m
	3000	Jaffa	174.2a	25.2f	5.24d	41.74g	45.2a
		Washington	162.42b	30.42d	4.38e	44.65f	44.9b
		Valencia	135.84i	27.62e	4.02e	46.89f	42.6d
		Moro blood	129.82k	31.62c	4.64d	54.86d	45.5a
		Blood Red	136.98h	23.61g	4.86d	51.86e	38.8h
		Succary	137.58g	32.64b	4.02e	36.02h	37.5i
	6000	Jaffa	156.6c	32.5b	9.14a	56.82c	41.6e
		Washington	140.12f	36.94a	6.48c	51.12e	44.2b
		Valencia	128.86l	36.21a	6.82c	42.94f	38.9h
		Moro blood	120.24m	37.12a	8.96b	61.94a	43.7c
		Blood Red	118.84n	31.74c	9.85a	58.96b	36.5j
		Succary	132.58j	37.20a	6.26c	41.82g	35.8t
Means							
Propagation type (P)		*In vivo*	69.52B	15.11B	2.53B	24.78	39.1B
		In vitro	137.2A	28.59A	4.95A	42.47A	40.8A
NaCl salinity level ppm (L)		0 (Control)	99.96C	16.83C	1.73C	26.05	39.3B
		3000	109.74A	21.81B	3.47B	35.23B	3.90C
		6000	100.37B	26.91A	6.01A	39.59A	39.0C
Genotype (G)		Jaffa	122.45A	19.30E	4.18B	32.32C	42.6B
		Washington	112.94B	23.15C	3.25E	32.24D	43.1A

Table 6: Continued

Propagation type (P)	NaCl Salinity level (L)	Genotype (G)	Total Amino acids (TAA)	Proline	Glycine Betain	Total Sugars (TS)	Total Organic Acids (TOA)
		Valencia	100.68C	21.73D	3.26E	29.10E	40.1D
		Moro blood	92.68F	24.12A	3.99C	36.59B	41.3C
		Blood Red	96.22D	19.19F	4.27A	44.02A	37.5E
		Succary	95.18E	23.61B	3.48D	27.47F	35.3F

Means with the same letter are not significantly different at 5% level within columns. Capital letters expressed the main effects and small letters for the interaction effects (salinity×genotype)

macromolecules and contribute to the stabilization of membrane structure (Bartles and Sunkar, 2005). Similarly, the increases in TOA accumulation noticed in salinized stressed seedlings, especially that regenerated from TCT may be due to its important role on increasing the OP of the cytoplasm. No available data were recorded in this respect. However, this accumulation can give information about respiratory activities and the equilibrium of any cation excess. In this respect, Ortiz *et al.* (1994) on beans found that organic acids (citrate, malate, all linked to oxidation) have a role as osmotic in response to stress condition since, hydrolysis of insoluble reserves mechanisms are the first reactive to be activated by water passive absorption.

Lipid peroxidation and Antioxidative enzymes: Table 7 shows the mean values of MDA as an indicator of lipid peroxidation and the activities of antioxidative enzymes as affected by Propagation type (P), Genotypes (G), Salinity Levels (L) and their interactions of *Citrus* seedlings having same physiological age.

Generally, it was found that *in vivo* seedlings, overall salinity levels and genotypes examined, showed higher values of MDA and CAT activities than the *in vitro* one. However, *in vivo* seedlings showed lower SOD and GR activities compared with that of *in vitro* one. Table 7 shows also that there are no significant differences between both propagation types regarding POX and APOX enzymes activity.

As for the effects of salinity levels, data in the same table show that MDA concentration was increased due to an increase in salinity level up to 3000 ppm NaCl and thereafter decreased significantly. SOD, POX and GR activities were increased significantly whereas APOX and CAT values were decreased with an increase in salinity level up to the highest level (6000 ppm NaCl).

Compared the genotypes, it was found a different between them regarding the MDA concentration and the antioxidative enzymes activity. Jaffa genotype has more MDA concentration and lower activities of SOD, CAT and GR than the other genotypes whereas Succari recorded the least values in this respect. Data in Table 7 show also that Jaffa genotype recorded the highest activity of BOX whereas Valencia contained least activity. Moroblood genotype showed the highest values of APOX whereas Jaffa contained lower values in this respect.

Since the statistical analysis showed a significant differences between all the interaction treatments, only the combined analyses of P×L×G were considered. Table 7 shows differences between the genotypes regarding the MDA concentration and the activities of antioxidant enzymes depending on the propagation type and/or salinity levels. *In vivo* Jaffa seedlings grown under 3000 ppm NaCl has highest MDA concentration whereas, *in vitro* Succari seedlings grown under the highest level of salinity contained lowest values. These results indicated that Succari genotype has a higher hereditary and increased capability than the other genotypes overall salinity level which provide to its better production from oxidative damage.

Table 7: Concentration (μ moL g^{-1} F.Wt) of lipid peroxidation; (MDA), as well as the activities (units g^{-1} F.wt) of Superoxide Dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APOX), catalase (CAT), glutathionen reductase (GR) of *in vitro* and *in vivo* seedlings having same physiological age of *Citrus* sweet orange genotypes studied

Propagation type (P)	NaCl salinity level ppm (L)	Genotype (G)	MDA	SOD	POX	APOX	CAT	GR
In vivo	0 (control)	Jaffa	780d	54.5c	27.1h	4.5f	8.2a	52g
		Washington	750d	40.3i	28.3g	5.6e	7.3b	50i
		Valencia	600g	38.2k	27.1h	5.2e	7.2b	48k
		Moro blood	600g	30.1p	28.2g	5.4e	6.5c	52g
		Blood Red	500i	28.4r	28.3g	5.5e	6.1c	47l
		Succary	400k	27.2s	29.4f	5.6e	6.0c	45n
	3000	Jaffa	950a	52.6b	27.5h	3.8g	6.8c	68b
		Washington	900b	46.1f	29.6f	4.5f	6.4c	58e
		Valencia	750d	41.4i	27.5h	4.3f	6.0c	54f
		Moro blood	750d	34.6l	29.8f	4.8f	5.8d	50i
		Blood Red	550h	30.5p	29.6f	4.8f	5.4d	50i
		Succary	450j	28.1r	29.8f	4.8f	5.2d	48k
	6000	Jaffa	850c	48.2e	48.2a	29.0d	5.9d	66c
		Washington	750d	42.4i	42.4b	31.6c	5.6d	57e
		Valencia	700e	39.3j	39.3c	29.3d	5.4d	52g
		Moro blood	680f	32.5n	32.5d	32.4b	4.5e	48k
		Blood Red	550h	27.2s	27.2h	33.1a	4.2e	51h
		Succary	420k	26.3t	26.3i	32.2b	4.0e	46m
In vitro	0 (control)	Jaffa	750d	51.0d	29.0f	4.8f	8.5a	54f
		Washington	700e	43.0h	27.0h	5.0e	7.8b	51h
		Valencia	500i	40.0i	28.0g	5.2e	7.5b	49j
		Moro blood	550h	34.0m	27.0h	5.2e	6.8c	52g
		Blood Red	400k	30.0p	26.0i	5.0e	6.5c	49j
		Succary	350l	29.0q	28.0g	4.5f	6.2c	47l
	3000	Jaffa	800b	57.0b	30.0f	4.2f	6.0c	69a
		Washington	850c	48.0e	29.0f	4.6f	5.4d	61d
		Valencia	550h	44.0g	28.0g	5.0e	3.6f	58e
		Moro blood	550h	35.0l	29.0f	4.6f	5.4d	54f
		Blood Red	450j	32.0n	28.0g	4.8f	5.6d	50i
		Succary	400k	30.0p	29.0f	4.3f	5.4d	48k
	6000	Jaffa	600g	60.0a	32.0d	3.6g	5.4d	69a
		Washington	550h	54.0c	31.0e	4.5f	5.2d	67c
		Valencia	450j	47.0e	27.0h	4.8f	5.0d	60d
		Moro blood	500i	39.0j	33.0d	4.5f	4.8e	58e
		Blood Red	400k	34.0m	31.0e	4.3f	4.3e	52g
		Succary	300m	31.0o	33.0d	4.0f	4.1e	50i
Means								
Propagation type (P)		*In vivo*	657. A	37.1B	29.4 A	4.6 A	5.9 A	52.3B
		In vitro	536.1B	41.0A	29.2 A	4.6 A	5.7 B	55.4A
NaCl salinity level ppm(L)		0 (Control)	573.3B	37.1B	27.8 C	5.1 A	7.0 A	49.7B
		3000	654.2A	39.9A	28.9 B	4.5 B	5.6 B	55.6A
		6000	562.5C	40.0A	31.2 A	4.1 C	4.9 C	56.3A
Genotype (G)		Jaffa	788.3A	53.8A	29.1 C	4.07D	6.80A	63.0A
		Washington	750.0B	45.6B	29.4BC	4.70 B	6.28B	57.3B

Table 7: Continued

Propagation type (P)	NaCl salinity level ppm (L)	Genotype (G)	MDA	SOD	POX	APOX	CAT	GR
		Valencia	591.6C	41.6C	27.8 D	4.72 B	5.78C	53.5C
		Moro blood	588.3D	34.2D	29.9AB	4.83 A	5.65D	52.3D
		Blood Red	475.0E	30.3E	29.3BC	4.78AB	5.35E	49.8E
		Succary	386.6F	28.6F	30.2 A	4.53 C	5.15F	47.3F

Means with the same letter are not significantly different at 5%level within columns. Capital letters expressed the main effects and small letters for the interaction effects

The increase in MDA (a decomposition product of poly unsaturated fatty hydroperoxidase) noticed in the present investigation, due to salinity was previously reports by Hernandez *et al.* (1994) who found that salt stress increased lipid peroxidation in leaves and mezophyll protoplast from *Vigna anguiculatea*.

According to Seckin *et al.* (2010), MDA has been frequently described as a suitable biomarker for lipid peroxidation under salt stress condition which can be initiated by ROS, severely affects functionality and integrity of cell membranes. Bor *et al.* (2003) attributed the increase in MDA to the increase in membrane permeability and/or losses of membrane integrity which leading to an increase in solute leakage and hence decreasing resistance to stress condition.

The changes noticed in SOD activity (Catalizes the conversion of the super oxide anion to H_2O_2), with *in vitro* seedlings than that of *in vivo* one was similarly found with POX and APOX activities (which decomposed the H_2O_2 produced by SOD) depending on salinity levels and genotypes examined.

The increase in SOD, POX and APOX activities noticed in the present investigation due to increasing salinity level was supported by Subbarao *et al.* (1999) on tobacco cell culture who reported that drought enhanced SOD activity by increasing H_2O_2.

On the other hand, it may be suggested that SOD, POX and APOX are working more efficiency in concern to decompose oxidants such as O_2 and H_2O_2 which might possibly be produced during stress condition caused by NaCl supplementation. Moreover, present results lead the *in vitro* seedlings to resist the potential oxidative damage, without the requirement to significant increases of SOD, POX and APOX activities more. In addition, it was found a positive correlation between the increase in SOD activity and that of POX and APOX, similar to that found by Bor *et al.* (2003) on sugar beet.

According to Asada and Takahashi (1987), POX is among the enzymes that scavenge H_2O_2 in chloroplast which is produced through dissimulation of O_2 catalized by SOD). Moreover, POX is the enzyme that decomposes H_2O_2 produced by SOD. Therefore, the increased in POX and APOX activities noticed in the present investigation were expected in both Jaffa and Succari genotypes as well as others. These data may be considered as an indication that *in vitro* seedlings had higher capacity to decompose H_2O_2 more rapidly compared with *in vivo* seedlings. In this connection, Gadalla (2009) found a higher constitutive and induce level of SOD in more tolerant wheat under drought stress.

Dionisio-Sese and Tobita (1998) attributed the increases in POX and APOX under stress to an increase in the activity of their encoding genes or to an increase in the activation of already enzymes. Wang *et al.* (1999) showed that over expression of APOX gene in plants increases protection against oxidative stresses. APOX uses ascorbate as the electron donor for the reduction of H_2O_2 and is well known to be important in the detoxification of H_2O_2 (Asada and Takahashi, 1987).

As for the effects of P×L×G on CAT activities, Table 7 shows that CAT (another scavenger of H_2O_2) recorded highest activity *in vivo* Jaffa seedlings grown under no salinity level whereas, GR (another enzyme in Acada-Halliwell pathway) activity showed highest values *in vitro* Jaffa seedlings compared with the other genotyped examined.

Unlike Jaffa, *in vitro* Valincia grown under 3000 ppm NaCl and *in vivo* Succari grown under no salinity level showed lowest activities from CAT and GR, respectively.

Unyayar *et al*. (2004) attributed the increase in GR activity under drought stress to the increase in APOX activity which would increase the demand for ascorbate generation mediated through increased GR activity.

It could be concluded, in general, that the important compounds of the protective system are enzymatic defenses and predominantly responsible for controlling free radical depending on the method of propagation and environmental condition. Osmoregulators as well as antioxidative enzymes were changed with response to genotypes and salinity level. Hardening with NaCl which supplementation through the micropropagation induced salinity tolerant more than that of *in vivo* seedlings. Enzymatic activities of the Asada –Halliwell pathway have been separately correlated with different stress situation. Moreover, *in vitro* seedlings scored lower level of lipid peroxidation and higher activities of Superoxide Dismutase (SOD), Peroxidase (POX), Ascorbate Peroxidase (APOX), Catalase (CAT), Glutathione Reductase (GR), than *in vivo* seedlings. There are a significant difference between them regarding Total free Amino Acids (TAA), proline and Glycinebetaine (GlyBet), Total Sugars; (TS), Total water soluble Organic Acids (TOA), lipid peroxidation, as well as the activities of SOD, POX, APOX, CAT and GR.

REFERENCES

Alqurainy, F., 2007. Responses of bean and pea to vitamin C under salinity stress. Res. J. Agric. Biol. Sci., 3: 714-722.

Altaf, N., 2006. Embryogenesis in undeveloped ovules of *Citrus* cultivares in response to gamma radiation. Pak. J. Bot., 38: 589-595.

Amberger, A., 1954. Einfluß von kalium und stickstoff auf ferment and kolenhydrathaus halt von grunland pflanzen. J. Plant Nutr. Soil Sci., 66: 211-222.

Asada, K. and M. Takahashi, 1987. Production and Scavenging of Active Oxygen in Photosynthesis. In: Photoinhibition, Kyle, D.J., C.D. Osmond and C.J. Arntzen (Eds.). Elsevier Science Publishers, Amsterdam, pp: 227-287.

Bartels, D. and R. Sunkar, 2005. Drought and salt tolerance in plants. Crit. Rev. Plant. Sci., 24: 23-58.

Bates, L.S., R.P. Waldren and I.D. Teare, 1973. Rapid determination of free proline for water-stress studies. Plant Soil, 39: 205-207.

Beauchamp, C. and I. Fridovich, 1971. Superoxide dismutase: Improved assays and assay applicable to acrylamide gels. Anal. Biochem., 44: 276-287.

Bergmeyer, H.U., 1970. Methods of Enzymatic Analysis. Verlag Chemie, Weinheim/Bergstr, German.

Bor, M., F. Ozdemir and I. Turkan, 2003. The effect of salt stress on lipid peroxidation and antioxidants in leaves of suger beet *Beta vulgaris* L. and wild beet *Beta maritime* L. Plant Sci., 164: 77-84.

Bray, P.R.S., M. Bailey-Serres and E. Weretilnyk, 2000. Responses to a Biotic Stresses. In: Biochemistry and Molecular Biology of Plants, Buchanan, B.B., W. Gruissen and R.L. Jones (Eds.). ASPP, Rockville, USA., pp: 1158-1203.

Carimi, F., M.C. Tortorici, F.D. Pasquala and F. Giulio, 1998. Somatic embryogenesis and plant regeneration from undeveloped ovules and stigma/style explant of sweet orange novel group [*Citrus sinensis* (L.) Qsb.]. Plant Cell Tissue Org. Cult., 54: 183-189.

Chen, T.H.H. and N. Murata, 2002. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr. Opin. Plant Biol., 5: 250-257.

Coelho, A.F.D.S., D.L. De Siqueira, C. Bruckner, A.B.D Oliveira and A.A. Cardoso, 1998. Plants regeneration from ovule culture of *Citrus reticulata* blanco CV dancy. Revista Pab., Vol. 33.

Debnath, M., 2008. Responses of *Bacopa monnieri* to salinity and drought stress *in vitro*. J. Med. Plants Res., 2: 347-351.

Demir, I., K. Mavi, M. Ozcoban and G. Okeu, 2003. Effects of salt stress on germination and seedling growth in serially harvested aubergine (*Solanum melongena* L.) seeds during development. Israel J. Plant Sci., 51: 125-131.

Dionisio-Sese, M.L. and S. Tobita, 1998. Antioxidant responses of rice seedlings to salinity stress. Plant Sci., 135: 1-9.

Dubey, R.S. and M. Rani, 1989a. Influence of NaCl salinity on growth and metabolic status of protein and amino acids in rice seedlings. J. Agron. Crop Sci., 162: 97-106.

Dubey, R.S. and M. Rani, 1989b. Salinity induces accumulation of free amino acids in germinating rice seeds differing in salt tolerance. J. Agron. Crop Sci., 163: 236-247.

El-Meskaoui, A. and F.M. Tremblay, 2001. Involvement of ethylene in the maturation of black spruce embryogenic cell lines with different maturation capacities. J. Exp. Bot., 52: 761-769.

FAO, 2004. Production Year Book. Vol. 52, Food and Agricultural Organization, Rome, Italy.

Foyer, C.H. and B. Halliwell, 1976. Presence of glutathione and glutathione reductase in chloroplasts: A proposed tool in ascorbic acid metabolism. Planta, 133: 21-25.

Foyer, C.H., P. Descourvieres and K.J. Kunert, 1994. Protection against oxygen radicals: An important defense mechanism studied in transgenic plants. Plant Cell Environ., 17: 507-523.

Gadalla, S.F., 2009. The role of antioxidants in inducing wheat flag leaf osmotic adjustment under salinity stress. J. Agric. Sci. Mansoura Univ., 34: 10663-10685.

Galibo, G. and Y. Yamada, 1988. A novel method for increasing the frequency of somatic embryogenesis in wheat tissue culture by NaCl and KCl supplementation. Plant Cell Rep., 7: 55-58.

Grieve, C.M. and S.R. Grattan, 1983. Rapid assay for determination of water soluble quaternary ammonium compounds. Plant Soil, 70: 303-307.

Halliwell, B., R. Aeschbach, J. Loliger and O.I. Aruoma, 1995. The characterization of antioxidants. Food Chem. Toxicol., 33: 601-617.

Hare, P.D. and W.A. Cress, 1996. Tissue-specific accumulation of transcript encoding-pyrroline-5-carboxylate reductase in *Arabidopsis thalians*. Plant Growth Regul., 19: 249-256.

Hasegawa, P.M., R.A. Bressan, J.K. Zhu and H.J. Bohnert, 2000. Plant cellular and molecular responses to high salinity. Annul. Rev. Plant Physiol. Plant Mol. Biol., 51: 463-499.

Heineke D., U. Sonnewald, D. Bussis, G.G. Gunter and K. Leidreiter *et al*., 1992. Apoplastic expression of yeast-derived invertase in potato. Plant Physiol., 100: 301-308.

Helaly, M.N., M.M. Darwish, Z.A. Mohamed, A.A. Hegazy and A.H. El-Hosieny, 2008. Botanical studies on the of date palm (*Phoenix dactylifera*, L.) using tissue culture technique: ²- Effects of antioxidants; AOS on browning control and related compounds. J. Agric. Sci. Mansoura Univ., 33: 7919-7929.

Helaly, M.N., A.A. Arafa, R.A. Fouda and H.F. Esmail, 2009a. Response of two newly development corn genotype (*Zea mays* L.) to some trace elements under salt stress throughout their growth life spane. J. Agric. Sci. Mansoura Univ., 34: 9015-9036.

Helaly, M.N., M.T. Sakr, Z.E. Mohamed, A.E. Allam and M.A. Ghaly, 2009b. Effect of biostimulants remidiation substances (BRS) on seed germination and seedling growth of some sugar beet cultivars under stress conditions. J. Agric. Sci. Mansoura Univ., 34: 11355-11372.

Helaly, M.N., M.A. El-Maksoud, Z. Mohamed and A.A. Saly, 2011. Micropropagation studies on somatic regeneration of wheat plants under salt stress. J. Agric. Sci. Plant Prod., Vol. 35, No. 1, (In Press).

Hernandez, J.A., L.A. Del-Rio and F. Sevilla, 1994. Salt stress induced changes in SOD isozyme in leaves and mesophyll protoplasts from (*Vigna unguiculata* L.) Walp. New Physiol., 126: 37-44.

Herzog, V. and H. Fahimi, 1973. Determination of the activity of peroxidase. Anal. Biochem., 55: 554-562.

Huang, J. and R.E. Redmann, 1995. Solute adjustment to salinity and calcium supply in wild and cultivated barley. J. Plant Nutr., 18: 1371-1380.

Jain, M., G. Mathur, S. Koul and N.B. Sarin, 2001. Ameliorating effects of proline on salt stress lipid peroxidation in cell lines of groundnut (*Arachis hypogea* L.). Plant Cell Rep., 20: 463-468.

Kaymakanova, M. and N. Stoeva, 2008. Physiological relations of bean plants (*Phaseolus culg* L.) to salt stress. Gen. Applied Plant Physiol., 34: 177-188.

Khatun, F., M.B. Meah and K.M. Nasiruddin, 2006. Regeneration of eggplant through anther culture. Pak. J. Biol. Sci., 9: 48-53.

Kholova, J., R.K. Sairam, R.C. Meana and G.C. Srivastava, 2009. Response of maize genotypes to salinity stress in relation to osmolytes and metal ions contents, oxidative stress and antoxidant enzymes activity. Biol. Plant., 53: 249-256.

Lacerda, C.F., J. Cambraia, M.A. Oliva, H.A. Ruiz and J.T. Prisco, 2003. Solute accumulation and distribution during shoot and leaf development in two sorghum genotypes under salt stress. Environ. Exp. Bot., 49: 107-120.

Lloyd, J., P.E. Kriedemann and D. Aspinall, 1990. Contrasts between *Citrus* species in response to salinization: An analysis of photosynthesis and water relations for different rootstock-scion combinations. Physiol. Plantarum, 78: 236-246.

Mayer, J.M., D.A. Hrovat, J.L. Thomas and W. Bordon, 2002. Protein-coupled electron transfer vs. hydrogen atom transfer in enz/tolune, methoxyl/methanol and phenoxyl/phenol self exvhange reactions. J. Am. Chem. Soc., 124: 11142-11147.

Misra, N. and A.K. Gupta, 2006. Interactive effects of sodium and calcium on proline metabolism in salt tolerant green gram cultivar. Am. J. Plant Physiol., 1: 1-12.

Misra, N. and P. Saxena, 2009. Effect of salicylic acid on proline metabolism in lentil grown under salinity stress. Plant Sci., 177: 181-189.

Munns, R., 2002. Comparative physiology of salt and water stress. Plant Cell Environ., 25: 239-250.

Nakano, Y. and K. Asada, 1981. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplast. Plant Cell Physiol., 22: 867-880.

Niedz, R.P., 2006. Regeneration of somatic embryos from sweet orange (*Citrus sinensis*) protoplasts using semi-permeable membranes. Plant Cell Tissue Organ Culture, 84: 353-357.

Obukosia, S.D. and K. Waithaka, 2000. Nucellar embryo culture of *Citrus sinensis* L. and *Citrus limon* L. African Crop Sci. J., 8: 109-116.

Ortiz, A., V. Martinez and A. Cerda, 1994. Effects of osmotic shock and calcium on growth and solute composition of *Phaseolus vulgaris* plants. Physiol. Plant., 91: 468-476.

Rao, K.V.M. and T.V.S. Sresty, 2000. Antioxidative parameters in the seedlings of pigeon pea (*Cajanus cajan* L. Millspaugh) in response to Zn and Ni stresses. Plant Sci., 157: 113-128.

Rhodes, D. and A.D. Hanson, 1993. Quaternary ammonium and tertiary sulfonium compounds in higher plants. Ann. Rev. Plant Physiol. Plant Mol. Biol., 44: 357-384.

Rong, H., R.J. He, S.C. Chen and J.R. Zhang, 1998. Study of *Citrus* somatic embryogenesis with high frequency and plant regeneration. South China Fruits, 27: 9-10.

Rontein, D., G. Basset and A.D. Hanson, 2002. Metabolic engineering of osmoprotectants accumulation in plants. Metab. Eng., 4: 49-56.

Roy, J. and N. Banerjee, 2003. Induction of callus and plant regeneration from shoot-tip explants of *Dendrobium fimbriatum* Lindl. var. *oculatum* Hk. f. Sci. Hortic., 97: 333-340.

SAS, 2003. SAS/STAT User's Guide: Statistics. Version 9.1 Edn., SAS Institute, Carey, NC.

Sadasivam, S. and A. Manickam, 1996. Biochemical Methods. New Age International Publishers, New Delhi, pp: 1-255.

Sakamoto, A. and N. Murata, 2002. The role of glycine betaine in the protection of plants from stress: Clues from transgenic plants. Plant Cell Envion., 25: 163-171.

Schmidt, R.E., 2005. Biostimulants function in turfgrass nutrition. Ph.D. Emeritus Virginia Tech.

Schnapp, S.R., R.A. Bressan and P.M. Hasegawa, 1990. Carbon use efficiency and cell expansion of NaCl-adapted tobacco cells. Plant Physiol., 93: 384-388.

Seckin, B., I. Turkan, A.H. Sekmen and C. Ozfidan, 2010. The role of antioxidant defense systems at differential salt tolerance of *Hordeum marinum* Huds. (sea barleygrass) and *Hordeum vulgare* L. (cultivated barley). Environ. Exp. Bot., 69: 76-85.

Shalata, A. and P.M. Neumann, 2001. Exogenous ascorbic acid (Vitamin C) increases resistance to salt stress and reduces lipid peroxidation. J. Exp. Bot., 52: 2207-2211.

Singh, D.V., G.C. Srivastava and M.Z. Abdin, 2006. Amelioration of negative effect of water stress in *Cassia angustifolia* by benezyladenine and/or ascorbic acid. Biol. Plant, 44: 141-143.

Steel, R.G.D. and J.H. Torrie, 1980. Principles and Procedures of Statistics: A Biometrical Approach. 2nd Edn., McGraw Hill, New York, USA., ISBN-13: 978-0070609259.

Subbarao, G.V., R.M. Wheeler, G.V. Stutte and L.H. Levine, 1999. How far can sodium substitute for potassium in red beet. J. Plant Natr., 22: 1745-1761.

Tajdoost, S., T. Farboodnia and R. Heidari, 2007. Salt pretreatment enhance salt tolerance in *Zea mays* L. seedlings. Pak. J. Biol. Sci., 10: 2086-2090.

Tisserat, B., 1979a. Propagation of date palm (*Phoenix dactylifera* L.) *in vitro*. J. Exp. Bot., 30: 1275-1283.

Tisserat, B., 1979b. Tissue culture of the date palm. J. Heredity, 70: 221-222.

Tisseret, B. and T. Murashige, 1977. Probable identity of substances in *citrus* that repress asexual embryogenesis. In vitro, 13: 785-789.

Tripathi, S.B., K. Gurumurthi, A.K. Panigahi and B.P. Shaw, 2007. Salinity induced changes in proline and betaine contents and synthesis in two aquatic macrophytes differing in salt tolerance. Biol. Plant., 51: 110-115.

Unyayar, S., Y. Keles and E. Unal, 2004. Proline and ABA levels in two sunflower genotypes subjected to water stress. Bulg. J. Plant Physiol., 30: 34-47.

Wang, L., Q. Su, T.T. Kang, M.X. Xu and Z. Chen, 1999. A biochemical marker for resistance to powdery mildew in wheat-peroxidase isozyme Band P 16.1. Acta Agric. Bareoli Sininca, 10: 6-9.

Wingate, V.P.M., M.A. Lawton and C.J. Lamb, 1988. Glutathione causes a massive and selective induction of plant defense genes. Plant Physiol., 31: 206-210.

Yadav, V.K., V. Gupta and Y. Neflam, 1999. Hormonal regeneration of nitrate in gram (*Cicer arietinum*) genotypes under drought. Indian J. Agric. Sci., 69: 59-85.