American Journal of Plant Physiology

ISSN 1557-4539

Academic Journals Inc.

www.academicjournals.com

American Journal of Plant Physiology 6 (4): 190-208, 2011
ISSN 1557-4539 / DOI: 10.3923/ajpp.2011.190.208

Effectiveness of Gamma Irradiated Protoplasts on Improving Salt Tolerance of Lemon (*Citrus limon* L. Burm.f.)

[1]M.N.M. Helaly and [2]A.M.R. Hanan El-Hosieny
[1]Department of Agriculture Botany, Faculty of Agriculture, Mansoura University, Egypt
[2]Hortacture Research, Institute Agriculture, Research Center, Egypt

Corresponding Author: M.N.M. Helaly, Department of Agriculture Botany, Faculty of Agriculture, Mansoura University, Egypt

ABSTRACT

Improvement in salt tolerance requires research focused on physiological processes. There is no major report stating the effectiveness of gamma irradiation, as a physical mutagen, to improve salt tolerance and alter the morphogenetic potential as well as physiological characteristics of *Citrus limon*. In the present investigation, *in vitro* mutagenesis techniques were applied to tackle this issue. Freshly isolated protoplasts were exposed to different doses of gamma rays. They were cultured and incubated for shooting regeneration. Protoplast viability and its growth criteria were estimated. Moreover, the ability of the regenerated shoots to tolerant salinity and its relation with the antioxidative system were evaluated. The data revealed that irradiated protoplast has an ability to continue their growth even at the highest level of NaCl salinity (8000 mg L^{-1}). Radiation sensitivity (LD) was achieved only at 20 krad. However, irradiation improved the formed tissues to tolerate more salinity level and alleviated the detrimental effects of high salinity level on embryogenic callus and shoot growth. Similarly, irradiation counteracted the depressing effects of salinity on total chls, TS and TSP concentrations whereas increased carotenoids and all osmoregulators studied (proline, TSPh, Gly Bet and K). Moreover, irradiation prevented cellular damage as expressed by decreases in lipid peroxidation, membrane leakage, H_2O_2 values as well as Na and Cl accumulatin. The activities of Super Oxide Dismutase (SOD), Peroxidase (POX), Ascorbate Peroxidase (APOX), Catalase (CAT) and Glutathione Reductase (GR) enzymes were increased in irradiated shoots compared with non-irradiated under salinized and non-salinized condition. It could be concluded that, irradiated shoots had a higher hereditary and induced capability under salt stress which provide to it a better protection from oxidative and cellular damage caused by NaCl salinity.

Key words: *Citrus limon*, NaCl salinity, protoplast viability, proline, gamma rays, enzyme activities

INTRODUCTION

Lemon (*Citrus limon* L. Brum.f.) is a member of Rutaceae family distributed in Egypt. Economically, fresh fruit is one of the major export crops. The cultivated area reached to 420.7 thousand feddan (feddan = 4200 m^2) produced about 3.233.448 ton (FAO, 2004). Growers and exporters need to meet local and international market as well as consumers demand according to the new international standards and regulation (EL-Magraby and Saleh, 2010). As a secondary

crop cultivation became restricted only to the newly reclaimed less fertile and saline soils (Ismail, 2007). This mandated a demand for production salt tolerant plants of Citrus in order to fulfill the great need from this crop. Moreover, due to restricted resources of fresh water from the River Nile, the use of less water quality and saline water or even diluted sea water became important sources of irrigation water especially in the newly cultivated areas (Afaf-Salem *et al*., 2009). Therefore, introducing new genotypes that can produce sufficient production under these conditions with good fruit quality are highly appreciated.

Citrus genus is a plant have a long juvenility period and the breeding or sexual hybridization in most Citrus species by conventional method are restricted by the complication of their genetic system (Kayim and Koe, 2006) sexual incompatibility (Louzada *et al*., 2002), heterozygosity (Ollitrault *et al*., 2000) and nucellar embryony (Louzada *et al*., 2002).

Gamma irradiation has been used in the biological studies from low doses stimulation to high-doses inhibition (Ribeiro and Machado, 2007). The relatively low doses ionizing irradiation on plants as well as *in vitro* studies are manifested as accelerated cell proliferation, cell growth, enzyme activity, stress resistance and mutation induction (Chakravarty and Sen, 2001). De Bona *et al*. (2009) added that, the mutation induction may provides the opportunity to increase variability of an economically importable cultivars or used in plants to developing genotypes have high productivity potential. Induced mutation technique is a valuable tool but not yet fully exploited in fruit breeding (Predieri and Gatti, 2003). Tissue culture technique makes it more efficient by allowing the handling of large populations and by increasing mutation induction efficiency, possibility of mutant recovery and speediness of cloning selected variants (Predieri and Gatti, 2000). In addition, tissue culture techniques are effective tools for producing salt tolerant cell lines, tissues and plants. Tissue culture is an efficient means to study the effect of abiotic salt stress on the cell metabolism (Abu *et al*., 2006). Cellular acclimation to salt environments can facilitate lemon plant to continued survival and growth. One such mechanism that is ubiquitous in plants is the accumulation of certain organic metabolites of low molecular weight that are known collectively as compatible solutes. These components are reported to play a pivotal role in cellular osmotic adjustment in response to osmotic and salt stresses (Gadalla, 2009). Irradiation with gamma rays may provide insight into the mechanism of action of the radiation in producing physiological and genetic variability. Thus, it has been used directly to produce useful variation in quantitatively inherited characters (Louzada *et al*., 2002). Somatic hybridization *via* protoplast fusion on the other hand, is a powerful tool in genetic breeding because it circumvents such sexual restraints (Grosser and Gmitter, 2005). Somatic hybridization has contributed tremendously to Citrus improvement and many Citrus somatic hybrids have been reported to be in use in various breeding programs (Calixto *et al*., 2004). Even though symmetric somatic hybrids have great potential for rootstock improvement and as tetraploid breeding parents in interploid crosses, they may not have direct application as scion cultivars as they may present complex genetic constitution. Asymmetric somatic hybridization (donor-recipient fusion) using X- or γ-irradiation, on the other hand, has great potential for scion improvement because it allows partial genomic transfer (Rasmussen *et al*., 2000) as chromosome elimination is induced by high radiation doses. Furthermore, colony formation of irradiated cells tends to be avoided (Predieri and Gatti, 2003). However, no available data were reported regarding the use of gamma irradiation as a physical mutagen to alter the physiological characteristics of lemon under salt stress condition. Therefore, the effects of gamma rays on improving salt tolerance of cultured calli regenerated from

Citrus limon irradiated protoplasts was examined. Certain physiological aspects as well as the activities of the possible enzymes that involvement of the antioxidant system in relation the tolerance of salt stress were investigated.

MATERIALS AND METHODS

The present investigation was carried out at the plant tissue culture laboratory, Horticultural Research Institute, Agriculture Research Center, A.R.C and the Middle Eastern Regional Radio-isotope Center for Arab countries Dokki, Cairo, Egypt. During the periods elapsed from February 2008 to December 2010.

Callus initiation: Ovules as explants taken from eight-year old lemon (*Citrus limon,* L. Burm. f.) trees, cv. Feminello were separated carefully, surface sterilized with 70% ethanol for 1 min followed by soaking in 50% commercial disinfectant Clorox 5.25%; NaOCl for 20 min with two drops of Tween 20 as a wetting agent. The explants were washed three times with sterilized distilled water to remove all traces of the disinfectant. The explants were cultured on MS (Murashige and Skoog, 1962) basal nutrient medium. The media was supplemented with sucrose (20 g L^{-1}), solidified with (6 g L^{-1}) agar (Defco India), adjusted to pH 5.8, dispensed in jars (350 mL) at the rate of 50 mL and autoclaved at 121°C, 15 lbs $inch^{-2}$ for 20 min. The cultures were incubated at 25±2°C, in growth room under a 16/8 h day/night photoperiod with low intensity at about 500 Lux for 12 weeks with 3 subculturing, 4 weeks intervals. The produced embryogenic callus was used for protoplast isolation.

Protoplast isolation: All chemicals were used from Sigma Chemical Comp., Dallas, TX (USA). Suspension callus from *Citrus limon* ovules derived embryogenic callus were maintained in a two-week subculture cycle in liquid half strength H+H basal nutrient medium (Grosser and Gmitter Jr., 2005). The basal media was supplemented with sucrose (20 g L^{-1}) and 2.0 mg L^{-1} benzyl adenine (BA), 0.2 mg L^{-1} α-naphthalene acetic acid; NAA under constant agitation on a horizontal gyratory shaker at 130 rpm at room temperature and under about 500 Lux constant illumination.

Protoplast isolation was carried out according to the protocol of Grosser and Gmitter Jr. (2005) with modifications of De Bona *et al.* (2009).

Protoplasts culture technique for cell wall regeneration and cell division: The protoplast was cultured on an optimum environmental and nutrimental condition for cell wall regeneration and cell division (El-Shihy and Monem, 1994). The culture was took place at density of 5×10^4 protoplast mL^{-1} and incubated in the darkness at 25±2°C for 2 weeks then transferred to a fresh media without NAA addition and incubated for further 4 weeks at a low light intensity at 500 Lux.

Gamma irradiation: Freshly isolated protoplast was exposed to a different doses of gamma rays at the Middle Eastern Regional Radio-isotope Center for Arab countries, Dokki, Cairo Egypt. These doses denoted 0, 5, 10 and 20 krad from Co-60 gamma cell source. The irradiated and non-irradiated (control) protoplast were transferred and re-cultured in a fresh MS basal medium and maintained at the same culture for 12 weeks with 4 subcultures with a photoperiod of 16/8 h day/night in a culture room.

The following determinations were recorded as growth criteria of the protoplasts:

- Radiation sensitivity test using lethal doses; LD determining the gamma doses that killed half (50%) of the cells (Ling *et al*., 2008)
- Cell viability (Widholm, 1972) using Fluorescein Diacetate (FDA)
- Cell wall regeneration (Nagata and Takeba, 1970) using calcofluor white
- Cell division percentage of the total cells using light microscope and hemacytometer slide
- Small and macroscopic colonies formation using light microscope and micrometer slide

Callus induction, embryogenic callus formation and salinity treatments: Pieces from formed colonies (about 1 g in weight and 3 cm^3 in size) were initially cultured in jars (325 mL) on MS basal nutrient media supplemented with 170 mg L^{-1} $NaH_2PO_4.2H_2O$, 200 mg L^{-1} glutamine, 40 mg L^{-1} adenine sulphate and 0.4 mg L^{-1} thiamine-HCl. Each jar contained 50 mL nutrient media and all culture jars were maintained in the complete darkness in the controlled growth room at 25±2°C for 12 weeks with four sub-cultured, three-week intervals.

At the end of incubation period, the produced callus were divided into 100 mg inoculums pieces and sub-cultured on fresh MS media solidified with 6 g L^{-1} agar supplemented with different levels of NaCl salinity denoted 0 (control) 2000, 4000, 6000 and 8000 mg L^{-1}. Each treatment was replicated 6 times. The cultures were incubated for 8 weeks with two subcultures (12 replicates for each treatment) under 500 Lux light intensity at 25±2°C to form developed embryogenesis callus. At the end the of embryogenesis callus formation, three replicates from each treatment was used for fresh weight estimation.

Shoot regeneration: The normal developed embryos were used as an explants material for shooting under the specific MS media. Each treatment was replicated 6 times and all cultured jars were incubated under 16/8 h photoperiod under 1500 Lux intensity. The explants were sub-cultured three times, three weeks intervals, on fresh specific media. Shoot multiplication was took place till forming shoots having 2-3 foliage leaves. At the end of shooting stage, fresh and dry weights as well as certain biochemical constituents were evaluated.

Biochemical analyses: Three replicates from each treatment were used for the biometric parameters and the experiments were repeated twice.

Photosynthetic pigments were extracted and determined as mg/g F.Wt. following the method of Inskeep and Bloom (1985).

Lipid peroxidation was determined according to Rao and Sresty (2000) by estimating Malondialdehyde (MDA) concentration (mg/g F.Wt.) at 532 nm absorbance using extinction coefficient of 1555 mM^{-1} cm^{-1}. The correction was done by subtracting the absorbance at 660 nm for unspecific turbidity.

Membrane leakage (%) was estimated following the method of Leopold *et al*. (1981). Hydrogen peroxide concentration (mg/g F.Wt.) was estimated by forming a titanium hydroperoxide complex *via* methods outlined by Rao *et al*. (1997).

Antioxidant enzymes determination: Antioxidant enzymes activity (unit/mg protein) were extracted from the plant material in a phosphate buffer (Inskeep and Bloom, 1985) and assayed

using the methods of Beauchamp and Fridovich (1971), Herzog and Fahimi (1973), Nakano and Asada (1981) and Foyer and Halliwell (1976) for Superoxide Dismutase; SOD (EC No. 1.15.1.1), Peroxidase; POX (EC No. 1.11.1. x), Ascorbate Peroxidase; APOX(EC No. 1.11.1.11), Catalase; CAT (EC No. 1.11.1.6) and Glutathione Reductase; GR (EC No. 1.8.1.7), respectively.

Compatible osmoregulators determination: Total Soluble Protein (TSP), proline, Total Sugars (TS), Total Soluble Phenols (PSPh) and Glycine Betain (Gly Bet) were extracted from the plant material by 80% ethanol. Concentrations (mg/g F.Wt.) of TSP and proline were determined spectrophotometrically by the methods described by Bradford (1976) and Bates *et al.* (1973) in a descending order.

Glycine Betain (Gly Bet) was also estimated spectrophotometrically by the method of Grieve and Grattan (1983).

Sample extract was also used for total phenolic compounds determination according to the method of Singleton and Rossi Jr. (1965) using Folin-Ciocalteau reagent (mg catecol/g F.wt.) and for total sugars (mg glucose/g F.wt.) using the method of Dubois *et al.* (1956).

Determination of mineral constituents: Dried samples from the regenerated shoots were digested with $HClO_3/H_2SO_4$ solution, cooled and brought to volume at 50 mL using deionized water. Potassium and sodium concentrations were determined flamephotometerically. Calcium and magnesium were determined using versenate methods (Richards, 1954).

Statistical analysis: The experimental design was randomized complete blocks design with six replications. Results of the effects of gamma ray doses on protoplast growth criteria are expressed as Mean±SD. Analysis of variance, one way type was employed using Duncan's multiple range tests. For the statistical analysis of the regenerated shoots, a combined analysis of variances, two-way type, for gamma doses, NaCl salinity levels and their interactions was made for the studied trails according to Steel and Torrie (1980). Duncan's multiple range test was employed using SAS (2003).

RESULTS

Protoplast growth criteria: Table 1 shows that gamma rays decreased percentages of *Citrus limon* protoplast viability, cell wall regeneration and cell division. The decreased was a dose dependent. The least values were recorded at 20 KR. Moreover, callus formation and radiation sensitivity using lethal doses; LD showed its harmful effects only at 20 KR, since this dose found to produce callus but produce 10-15% cell colons and LD was cleared.

Table 1: Mean±SD of the protoplast growth criteria examined of Cirus *limon* as affected by gamma rays

Gamma rays dose (krad)	Viability (%)	Cell wall regeneration (%)	Cell division (%)	Callus formation	Radiation sensitivity; lethal doses (LD)
0	60.0±4.300^a	48.0±2.603^a	8.7±0.302^a	+++	-
5	54.0±2.701^b	36.8±2.501^b	8.0±0.201^b	+++	-
10	50.9±1.203^c	28.4±1.300^c	5.0±0.100^c	++	-
20	20.8±0.701^d	20.0±1.001^d	2.0±0.100^d	10-15 cell colones	+++

Data are the means of six separate experiment±SD. Means of each column with the same letter are not significant different at 5% level. Callus formation was detected well (+++) up to 5 krad dose of gamma rays. It thereafter decreased (++) with an increase in its dose. Radiation sensitivity was achieved only (+++) at 20 krad whereas not detected (-) at the other doses

Embryogenic calli growth: Data in Table 2 show fresh weight (F.Wt) of the embryogenic calli derived from the irradiated protoplasts as affected by different NaCl salinity levels. The data indicated that, regeneration of the irradiated callus under NaCl salinity was affected significantly depending on salinity level and/or gamma dose. Generally increasing salinity level decreased embryogenic calli growth represented by their F.Wt. The reduction was a concentration dependent regardless gamma doses. However, a beneficial effect was detected at the low salinity level (2000 mg NaCl/L) the least value (51.5 g/loculum) achieved at 8000 mg L^{-1} compared with the control (185.75 g). Less increase was detected at 2000 mg NaCl/L. Irradiation, in general increased F.Wt of the embryogenic callus up to 10 KR and thereafter decreased but still higher than the control. Irradiation over all salinity level at 10 KR recorded highest F.Wt. reached to more than two fold of the embryogenic callus (191.8 g) of the non irradiated protoplast (85 g) .The interaction treatments (salinity×gamma) show that, gamma rays induced the protoplast ability to continue their growth and tolerate salinity even at the highest level (8000 mg L^{-1}). These results are true in salinized and non-salinized media compared to the corresponding control. All irradiation doses not only counteractive the depressing effects of salinity on embryogenic callus growth but also, increased this parameter. The best treatment resulted highest F.Wt. value (234 g) was found with irradiated protoplast at 10 KR and salinized with 6000 mg NaCl/L whereas the lowest value (6.1 g/loculum) was achieved from non-irradiated protoplast interacted with NaCl at 8000 mg L^{-1}. Rate of the promoting effects of irradiation on embryogenic callus growth was more pronounced at the two higher salinity levels. Irradiation at 10 KR recorded F.Wt. increase more than about 14 fold compared with non-irradiated protoplast at 8000 mg NaCl/L.

Biometric studies of the regenerated shoots

Shoot growth: Shoot growth expressed as fresh weight (g/loculum) was significantly inhibited due to NaCl salinity treatments (Table 3). The lowest value (61.7g/loculum) was achieved at 8000 mg NaCl/L compared with the control (210.85 g). The low salinity level showed an insignificant decrease in this respect. Conversely, it was found that, gamma irradiation increased significantly F.Wt. of the regenerated shoots over all salinity level. The highest F.Wt. values were recorded at 10 and 20 KR doses without significant differences between them. They recorded about 182 g/loculum compared with about 156 g in the control.

The interaction treatments (salinity×gamma) show that, there was an irregular distribution of shoot F.Wt. in irradiated shoots depending on gamma dose and salinity level. Irradiated shoots at

Table 2: Growth of the embryogenic callus, expressed as fresh weight; F.Wt. (g loculum) in *Citrus limon* at the end of incubation period as affected by NaCl salinity, gamma rays and their interaction (Loculum weight at the beginning = 100 mg)

	NaCl salinity levels (mg L^{-1})					
Gamma rays KR	0	2000	4000	6000	8000	Mean
0	120^{i}	118^{i}	92^{j}	89^{k}	6.1^{o}	85.0^{D}
5	218^{c}	226^{b}	186^{f}	196^{e}	50^{n}	175.2^{B}
10	225^{b}	234^{a}	220^{c}	200^{d}	80^{l}	191.8^{A}
20	180^{g}	178^{g}	198^{d}	170^{h}	70^{m}	159.2^{C}
Mean	185.75^{B}	189^{A}	174^{C}	163.75^{D}	51.53^{E}	

Means with the same letter are not significantly different at 5% level. The capital letters in the columns are comparing the gamma doses overall salinity levels. The capital letters in the rows are comparing the effects of salinity levels overall gamma doses. The small letters refer to the interaction (Salinity level×gamma rays)

Table 3: Shoots F.Wt. (g/loculum) of *Citrus limon* at the end of shooting stage as affected by gamma rays, NaCl salinity levels and their interactions

	NaCl levels (mg L^{-1})					
Gamma rays KR	0	2000	4000	6000	8000	Mean
0	204.4^{e}	204.8^{e}	202.1^{f}	170.0^{i}	10.20^{l}	156.26^{C}
5	211.2^{c}	$211.^{c}$	202.0^{f}	180.3^{h}	69.5^{k}	174.96^{B}
10	219.2^{a}	214.9^{b}	205.3^{e}	188.2^{g}	82.1^{j}	181.94^{A}
20	208.6^{d}	207.7^{d}	208.4^{d}	200.4^{f}	84.847^{j}	181.96^{A}
Mean	210.85^{A}	209.80^{A}	204.20^{B}	184.70^{C}	61.70^{D}	

Means with the same letter are not significantly different at 5% level. The capital letters in the columns are comparing the gamma doses overall salinity levels. The capital letters in the rows are comparing the effects of salinity overall gamma doses. The small letters refer to the interaction (Salinity level×gamma rays)

low doses (5 and 10 KR) caused higher F.Wt. compared to their irradiated at 20 KR under non salinized media and low salinity level (2000 mg NaCl/L). Conversely it was observed that, irradiated shoots at 20 KR displayed a higher F.Wt. compared to that irradiated at low doses under salinity levels more than 2000 NaCl/L. Gamma irradiation treatments up to 10 KR could alleviate the detrimental effects of salinity especially at the two higher levels. The highest growth values were observed in both 0 and 2000 mg L^{-1} NaCl interacted with gamma dose at 10 KR. Moreover, irradiated and non-irradiated shoots were found to be more sensitive to salinity at 8000 mg L^{-1} than at 6000 mg L^{-1}. Their fresh weight recorded least values compared with the other salinity levels. The non-irradiated embryogenic calli produced only 10 g F.Wt. of the regenerated shoots at 8000 mg L^{-1}. Whereas that irradiated at 10 KR produced about 80 g at the same level. These results indicated a possibility of production mutant of *Citrus limon* has ability to salt resistant, by gamma rays application up to 10 KR.

Comparing F.Wt. values of irradiated and non-irradiated shoots, data in the same table show that irradiated shoots at 10 and 20 KR exhibited significantly greater F.Wt. accumulation than those of the corresponding control under each salinity level. Irradiated shoots at 10 KR regenerated under non salinized media exhibited highest F.Wt. value reached to 219.2 g whereas, non irradiated one which regenerated under the highest level of salinity (8000 mg NaCl/L) displayed a lower value (10.2 g).

Photosynthetic pigments: Table 4 shows that salinity regardless gamma doses decreased chlorophyll a, b and total chlorophyll whereas increased carotenoids. Chl a/b ratio was not affected significantly (data not presented). The effect was more pronounced under the high level (8000 mg L^{-1}). The corresponding values obtained under 8000 mg NaCl/L for chl a, b total and carotenoids were 2.31, 0,75, 3.06 and 2.96 mg g^{-1} F. Wt. compared with the control (5.72, 1.85, 7.57 and 2.53 mg g^{-1} F.Wt.) in a descending order. Irradiation up to 10 KR overall salinity levels increased chlorophyll a, b and their total (5.62,1.85 and 7.47 mg g^{-1} F.Wt.) compared to the non-irradiated shoots (2.98, 0.82 and 3.4 mg g^{-1} F.Wt.) there after decreased but still higher than the control. Similarly chl a/b ratio was not affected significantly. Chlorophyll a was found to be higher than Chl b in both irradiated and non irradiated shoots under either salinized or non-salinized media.

The interaction treatments (salinity×gamma) indicated that irradiation counteracted the inhibiting effects of salinity on chlorophylls concentration even at the highest level of salinity. In addition, it showed an additive effect to salinity on increasing carotenoids. Gamma rays at 10 KR

Table 4: Photosynthetic pigments concentration (mg g^{-1} F.Wt.) of *Citrus limon* shoots as affected by gamma rays, NaCl salinity and their interactions

	NaCl levels mg L^{-1}					
Gamma rays KR	0	2000	4000	6000	8000	Mean
Chlorophyll a						
0	3.30^{j}	3.00^{k}	2.66^{l}	2.03^{p}	1.90^{q}	2.58^{D}
5	5.66^{d}	4.67^{e}	3.90^{h}	2.98^{k}	2.33^{o}	3.91^{C}
10	7.89^{a}	7.33^{B}	6.03^{c}	4.27^{g}	2.59^{m}	5.62^{A}
20	6.03^{c}	5.63^{d}	4.33^{f}	3.60^{i}	2.40^{n}	4.40^{B}
Mean	5.72^{A}	5.16^{B}	4.23^{C}	3.22^{D}	2.31^{E}	
Chlorophyll b						
0	1.00^{h}	0.91^{i}	0.90^{i}	0.70^{k}	0.60^{l}	0.82^{D}
5	1.82^{d}	1.50^{e}	1.32^{g}	0.92^{i}	0.75^{k}	1.26^{C}
10	2.56^{a}	2.40^{B}	2.00^{c}	1.45^{f}	0.88^{j}	1.85^{A}
20	2.00^{c}	1.89^{d}	1.42^{f}	1.10^{h}	0.80^{j}	1.40^{B}
Mean	1.85^{A}	1.68^{B}	1.41^{C}	1.04^{D}	0.75^{E}	
Total chlorophylls						
0	4.30^{j}	3.91^{k}	3.56^{l}	2.73^{q}	$2.50r$	3.32^{D}
5	7.48^{d}	6.17^{f}	5.22^{h}	3.90^{k}	3.08^{p}	5.17^{C}
10	10.45^{a}	9.93^{B}	8.03^{c}	5.72^{g}	3.42^{m}	7.47^{A}
20	8.03^{c}	6.52^{e}	5.75^{g}	4.70^{i}	3.20^{o}	5.84^{B}
Mean	7.57^{A}	6.83^{B}	5.64^{C}	4.26^{D}	3.05^{E}	
Carotenoids						
0	1.50^{m}	1.80^{l}	2.20^{k}	2.90^{f}	1.8^{1}	2.04^{C}
5	2.50^{j}	2.60^{i}	2.71^{h}	2.97^{e}	3.03^{e}	2.76^{B}
10	3.50^{c}	3.10^{d}	3.65^{B}	3.86^{a}	3.11^{d}	3.84^{A}
20	2.63^{i}	2.80^{g}	2.90^{f}	2.95^{e}	3.10^{d}	2.88^{B}
Mean	2.53^{C}	2.58^{D}	2.87^{B}	3.17^{A}	2.96^{C}	

Means with the same letter are not significantly different at 5% level. For each trait, the capital letters in the columns are comparing the effects of gamma doses overall salinity levels. The capital letters in the rows are comparing the effects of salinity overall gamma doses. The small letters refer to the interaction (Salinity level×gamma rays)

interacted with non-salinized media recorded highest total chlorophylls concentration (8.03 mg g^{-1} F. Wt.) compared with the control (4.3 mg g^{-1} F. Wt.). The effect of irradiation on increasing chlorophylls may be due to its effects on stimulation chlorophyll its biosynthesis and/or delaying of its degradation. Regarding carotenoids data in the same table show that irradiation at 10 KR interacted with NaCl salinity level at 6000 mg L^{-1} give highest values (3.86 mg g^{-1} F. Wt.) compared with the control (1.5 mg g^{-1} F. Wt.).

Lipid peroxidation, membrane leakage and H_2O_2: Table 5 shows the mean values of lipid peroxidation, membrane leakage and H_2O_2 concentrations in *Citrus limon* shoots as affected by NaCl salinity, gamma irradiation and their interactions.

Generally, it has been demonstrated that NaCl salinity regardless gamma doses induced oxidative stress in *Citrus limon* shoots expressed by an increase in lipid peroxidation (MDA). The lowest MDA value (a decomposition product of poly unsaturated fatty acid hydro peroxidase) was found at zero level of salinity (624 mg g^{-1} F.Wt.) where as the highest value (804.8 mg g^{-1} F.Wt.) was recorded under 8000 mg NaCl/L salinity level.

Table 5: Levels of lipid peroxidation (MDA mg g^{-1} F.Wt.), membrane leakage (%) and H_2O_2 (mg g^{-1} F.Wt) of *Citrus limon* shoots as affected by NaCl salinity, gamma rays and their interactions

	NaCl levels mg L^{-1}					
Gamma rays KR	0	2000	4000	6000	8000	Mean
MDA mg g^{-1} F.Wt.						
0	700^{i}	761^{h}	810^{g}	890^{a}	888^{a}	809.8^{A}
5	666^{l}	700^{j}	719^{i}	781^{b}	805^{c}	734.2^{B}
10	580p	610^{n}	686^{k}	765^{d}	766^{d}	681.4^{C}
20	550^{q}	600^{o}	651^{m}	740^{f}	760^{e}	660.2^{D}
Mean	6240^{D}	677.8^{C}	716.5^{B}	794.0^{A}	804.8^{A}	
Membrane leakage (%)						
0	20^{j}	31^{h}	63^{b}	80^{a}	80^{a}	54.8^{A}
5	16^{k}	27^{i}	41^{e}	50^{c}	49^{c}	36.6^{B}
10	12^{l}	22^{j}	35^{f}	47^{d}	47^{d}	32.6^{C}
20	13^{l}	15^{k}	33^{g}	40^{e}	39^{e}	28.0^{D}
Mean	15.3^{D}	23.8^{C}	43.0^{B}	54.3^{A}	53.8^{A}	
H_2O_2 mg g^{-1} F.Wt.						
0	17.64^{h}	18.86^{g}	20.76^{d}	$24 .00^{a}$	23.96^{a}	21.04^{A}
5	14.81^{l}	16.03^{j}	19.30^{f}	22.22^{b}	22.06^{b}	18.88^{B}
10	13.76^{n}	15.60^{k}	17.06^{i}	19.76^{e}	21.11^{c}	17.46^{C}
20	13.65^{o}	14.33^{m}	16.05^{j}	18.33^{h}	19.66^{e}	16.89^{D}
Mean	14.97^{E}	16.21^{D}	18.41^{C}	21.08^{B}	21.70^{A}	

Means with the same letter are not significantly different at 5% level. For each trait, the capital letters in the columns are comparing the effects of gamma doses overall salinity levels. The capital letters in the rows are comparing the effects of salinity levels overall gamma doses. The small letters refer to the interaction (Salinity level×gamma rays)

Data in the same table show that an increase in MDA due to salinity was accompanied with increases in membrane leakage and H_2O_2 concentrations. Membrane damage (membrane leakage) and H_2O_2 concentrations were increased continuously with an increase in NaCl salinity level overall gamma dose. The lower levels of Lipid peroxidation, membrane leakage and H_2O_2 concentrations may be resulted from elevated activities of antioxidants under salt stress conditions. The lowest values under non salinized conditions showed 15.3 and 14.97% mg g^{-1} F.Wt. for membrane leakage and H_2O_2 respectively. However the highest value of membrane leakage (about 54%) and H_2O_2 concentration (21.7 mg g^{-1} F. Wt.) were obtained under the highest level of salinity (8000 mg NaCl L).

As for the effect of irradiation treatments overall salinity levels, data in the same table show that irradiated shoots had lower levels of lipid peroxidation, membrane leakage and H_2O_2 concentrations than non-irradiated shoots. These effects were a concentration dependent. Non irradiated plants had 809.8 mg g^{-1} F.Wt., 54.81% and 21 mg g^{-1} F.Wt. for MDA, membrane leakage and H_2O_2 in a descending order while that irradiated at 20 KR registered 660.2, 28% and 16.89 mg g^{-1} F. Wt., respectively.

The interaction treatments (salinity×gamma) indicated that irradiated shoots had lower values of lipid peroxidation, membrane leakage and H_2O_2 concentrations under both salinized and non-salinized condition. These values in all irradiation doses were lower under non salinized conditions than under salinized one. The highest values of MDA, membrane leakage and H_2O_2 were recorded on non-irradiated shoots that grown under highest level of salinity. The lowest values were found in irradiated shoots at 20 KR which regenerated under non salinized condition.

Antioxidant enzymes: Data in Table 6 show that increasing NaCl level regardless gamma doses increased the activities of Super Oxide Dismutase (SOD) Peroxidase (POX) Ascorbate Peroxidase; (APOX), Catalase; (CAT) and Glutathione Reductase (GR) enzymes and the increase was a concentration dependent. Therefore, the highest activities of those enzymes were obtained under 8000 mg NaCl/L (52, 29, 3, 5.6, 5.7, 9.4 and 54.8 unit/g F.Wt. in a descending order).

Similarly, gamma rays up to 20 KR overall salinity levels increased the activities of SOD, POX, APOX, CAT and GR enzymes. These activities registered 51.6, 29.2, 5.8, 9.3 and 56.6 unit/g F.Wt. compared with the corresponding controls (33.6, 26.4, 4.8, 8.1 and 48.8 unit/g F.Wt.). The interaction treatments indicated that irradiation showed an additive effect to salinity on increasing the activities of the possible enzymes that involved on antioxidant system in relation to the tolerance of salt stress. SOD and CAT are the most antioxidant enzymes affected significantly by (salinity×gamma) rays interactions preventing cellular damage.

Table 6: Specific activities (units/g F.Wt) of SOD, POX, APOX, CAT and GR enzymes in *Citrus lemon* shoots as affected by NaCl salinity, gamma rays and their interactions

	NaCl levels mg L^{-1}					
Gamma rays KR	0	2000	4000	6000	8000	Mean
Superoxide dismutase (SOD)						
0	30^{k}	31^{j}	34^{i}	36^{h}	37^{g}	33.6^{C}
5	38^{g}	39^{g}	46^{f}	51^{d}	53^{c}	45.4^{B}
10	46^{f}	48^{e}	52^{d}	55^{b}	59^{a}	52.0^{A}
20	45^{f}	46^{f}	52^{d}	56^{b}	59^{a}	51.6^{A}
Mean	39.8^{E}	41.0^{D}	46.0^{C}	49.5^{B}	52.0^{A}	
Peroxidase (POX)						
0	25^{g}	25^{g}	27^{e}	28^{d}	27^{e}	26.4^{D}
5	25^{g}	26^{f}	27^{e}	29^{c}	29^{c}	27.2^{C}
10	26^{f}	27^{e}	28^{d}	30^{b}	30^{b}	28.2^{B}
20	28^{d}	28^{d}	28^{d}	31^{a}	31^{a}	29.2^{A}
Mean	26.0^{C}	26.5^{C}	27.5^{B}	29.5^{B}	29.3^{A}	
Ascorbate peroxidase (APOX)						
0	4.5^{h}	4.6^{h}	4.9^{f}	5.0^{f}	5.1^{e}	4.8^{D}
5	4.8^{g}	4.9^{f}	5.0^{f}	5.4^{d}	5.3^{d}	5.1^{C}
10	4.8^{g}	5.3^{d}	5.5^{d}	6.0^{a}	5.6^{c}	5.5^{B}
20	5.2^{e}	5.7^{c}	5.9^{b}	6.1^{a}	5.6^{c}	5.8^{A}
Mean	4.8^{C}	5.1^{B}	5.3^{B}	5.6^{A}	5.7^{A}	
Catalase (CAT)						
0	6.2^{l}	7.9^{j}	8.6^{g}	8.9^{f}	9.0^{f}	8.1^{D}
5	7.1^{k}	8.3^{i}	8.9^{f}	9.2^{e}	9.1^{e}	8.5^{C}
10	7.9^{j}	8.6^{g}	9.4^{d}	9.6^{b}	9.7^{b}	9.0^{B}
20	8.8^{f}	8.5^{h}	9.5^{c}	9.7^{b}	9.8^{a}	9.3^{A}
Mean	7.5^{D}	8.3^{C}	9.1^{B}	9.4^{A}	9.4^{A}	
Glutathione reductase (GR)						
0	48^{g}	48^{g}	49^{f}	49^{f}	50^{f}	48.8^{D}
5	49^{f}	52^{e}	53^{d}	55^{c}	54^{c}	52.6
10	53^{d}	54^{d}	56^{c}	57^{b}	57^{b}	55.4^{B}
20	54^{d}	56^{c}	57^{b}	58^{a}	58^{a}	56.6^{A}
Mean	51.0^{D}	52.5^{C}	53.8^{B}	54.8^{A}	54.8^{A}	

Means with the same letter are not significantly different at 5% level. For each trait, the capital letters in the columns are comparing the effects of gamma doses overall salinity levels. The capital letters in the rows are comparing the effects of salinity levels overall gamma doses. The small letters refer to the interaction (Salinity level×gamma rays)

SOD (catalyzes the conversion of the super oxide anion to H_2O_2) activity increased due to increasing salinity level in the irradiated and non irradiated shoots of Citrus *limon*. The rate of increase was more pronounced at the higher levels of salinity than lower one. As compared to non-irradiated shoots, irradiated shoots has high activity level of SOD. Another scavenger of H_2O_2, CAT activity increased due to salinity and/or gamma treatments. However, irradiated shoots contained higher activity than non-irradiated under salinized and non-salinized conditions.

POX (is among the enzymes that scavenges and decomposes H_2O_2 in chloroplasts which is produced through dismutation of O_2^- catalyzed by SOD) changed also with respect to irradiation×salinity interaction treatments. Unlike SOD, POX activity was increased slightly with increasing salinity and/or gamma dose. However irradiated shoots has higher activity than non-irradiated ones. APOX (uses ascorbate as the electron donor for the reduction of H_2O_2 and is will known to be important in the H_2O_2 detoxification) was also higher in irradiated shoots than non irradiated under salinized and non-salinized conditions. These effects were more pronounced under salinity. Insignificant increases were noticed in POX activity between 0 and 2000 mg NaCl/L.

GR activity (another enzyme in Asada-Halliwell pathway) was increased significantly due to the effects of salinity and/or gamma irradiation. The induction of GR was higher in irradiated shoots than non-irradiated which grown under salinized and non-salinized conditions. The differences in GR activity was more clear under the two higher salinity levels (6000 and 8000 mg NaCl/L).

Compatible Osmoregulators

Organic osmolytes solutes: Table 7 shows the effects of NaCl salinity, irradiation and their interactions on the concentrations of Total Soluble Protein (TSP), proline, Total Sugars (TS), Total Soluble Phenols (TSPh) and Glycine Betain (Gly Bet) of Citrus *limon*. Data, in general, indicate that concentrations of TSP and TS were decreased whereas that of proline, TSPh and Gly Bet were increased in the shoots due to an increase in salinity level overall gamma doses. However, a beneficial effect was detected for TS under low salinity level (2000 mg NaCl/L).

As for the effect of gamma rays regardless salinity level it was found that irradiation increased the accumulation of all these organic solutes compared with the control. The highest values were achieved at 10 KR and thereafter decreased but still higher than the control.

The interaction treatments (salinity×gamma) indicated that irradiation counteracted and nullified the depressing effect of salinity on TSP and TS accumulation. In addition, it showed an additive effects to salinity on increasing other organic solutes examined (proline, TSPh and Gly Bet) specially that of proline compared with the control. The increase in proline concentration reached to about 2-3 fold that of the control. These results were more pronounced under salinized condition which helped enhancing salt tolerance. The most effective treatment on increasing organic solutes was found at 10 KR interacted with 6000 mg NaCl/L. Less increases were recorded at 10 and 20 KR interacted with 8000 mg NaCl/L.

The increase in total soluble protein and total sugars due to the irradiation overall salinity level indicated that irradiation can affect protein and sugar synthesis directly or indirectly. The differences were connected with the biochemical differentiation based on the irradiation and salinity levels. The highest concentration of protein and sugars was found in the present

Table 7: Organic osmolytes solutes of *Citrus limon* shoots as affected by NaCl salinity, gamma rays and their interactions

	NaCl levels mg L^{-1}					
Gamma rays KR	0	2000	4000	6000	8000	Mean
Total soluble protein (TSP) mg g^{-1} F.Wt.						
0	12.24^{e}	11.48^{f}	11.24	9.22^{g}	0.65^{j}	8.97^{C}
5	14.26^{d}	14.24^{d}	12.24^{e}	11.32^{f}	7.32^{h}	12.08^{B}
10	18.64^{a}	15.42^{c}	14.92^{d}	11.49^{f}	7.29^{h}	13.55^{A}
20	16.10^{b}	14.82	14.18^{d}	9.64^{g}	6.10^{i}	12.71^{B}
Mean	15.65^{A}	13.99^{B}	14.51^{B}	10.42^{C}	5.34^{D}	
Proline µM g^{-1} F.Wt.						
0	10.50^{p}	10.80^{o}	12.00^{m}	14.03^{l}	13.20^{l}	12.11^{D}
5	11.80^{o}	14.52^{k}	15.26^{i}	29.07^{c}	19.97^{e}	18.12^{C}
10	12.02^{m}	14.99^{j}	15.80^{h}	36.56^{b}	22.03^{d}	20.28^{B}
20	13.25^{l}	17.01^{g}	17.85^{f}	39.99^{a}	29.21^{c}	23.46^{A}
Mean	11.89^{E}	14.33^{D}	15.23^{C}	29.91^{A}	21.10^{B}	
Total sugars mg g^{-1} F.Wt.						
0	120.6^{h}	140.1^{f}	137.6^{g}	111.6^{j}	90.3^{l}	120.04^{D}
5	140.0^{f}	143.2^{e}	140.7^{f}	119.3^{j}	101.0^{k}	128.84^{C}
10	156.3^{c}	180.7^{a}	167.1^{b}	146.0^{d}	120.0^{h}	154.02^{A}
20	139.1^{F}	146.4^{d}	143.9^{e}	120.7^{h}	100.4^{k}	130.10^{B}
Mean	139.0^{C}	152.6^{A}	147.3^{B}	124.4^{D}	102.9^{E}	
Total soluble phenols mg g^{-1} F.Wt.						
0	12.40^{j}	12.80^{j}	13.70^{i}	13.99^{g}	11.92^{n}	12.96^{C}
5	12.84^{j}	13.87^{h}	14.27^{f}	14.87^{d}	12.27^{m}	13.63^{B}
10	14.63^{a}	16.21^{b}	16.28^{b}	17.20^{a}	14.04^{g}	15.67^{A}
20	12.70^{k}	13.70^{i}	14.90^{d}	15.02^{c}	12.62^{k}	13.79^{B}
Mean	13.14^{D}	14.15^{C}	14.79^{B}	15.27^{A}	12.71^{E}	
Gly bet mg g^{-1} F.Wt.						
0	1.00^{q}	2.01^{n}	3.99^{i}	5.03^{c}	4.23^{h}	3.25^{C}
5	1.18^{p}	2.36^{m}	4.36^{g}	5.77^{b}	4.59^{e}	3.65^{B}
10	2.71^{l}	3.55^{j}	4.99^{c}	5.96^{a}	5.03^{c}	4.45^{A}
20	1.49^{o}	3.06^{k}	4.51^{p}	4.99^{f}	4.66^{d}	3.73^{B}
Mean	1.60^{E}	2.73^{D}	4.46^{C}	5.44^{A}	4.63^{B}	

Means with the same letter are not significantly different at 5% level. For each trait, the capital letters in the columns are comparing the effects of gamma doses overall salinity levels. The capital letters in the rows are comparing the effects of salinity levels overall gamma doses. The small letters refer to the interaction (Salinity level×gamma rays)

investigation at 10 KR interacted with salinity up to 4000 mg NaCl/L whereas the lowest concentration was detected at 20 KR under 6000 and 8000 mg NaCl/L compared with the control. Unlike TSP and TS, proline, TSPh and Gly Bet were increased due to NaCl salinity and the irradiation showed an additive effects to salinity on increasing these parameters. The highest concentration of TSPh and Gly Bet were found at 10 KR interacted with 6000 mg NaCl/L compared with the control. However,proline recorded highest value at 20 KR interacted with salinity at 6000 mg NaCl/L.

Mineral constituents: Table 8 shows that, NaCl salinity increased the concentrations of Na^{+} and Cl^{-} accompanied with a decrease in K^{+}, Ca^{2+}, Mg^{2+} and K/Na ratio. The highest levels of Na (15.4 mg g^{-1} D.Wt.) and Cl (18.4 mg g^{-1} D.Wt.) were obtained at the highest level of salinity

Table 8: Mineral concentrations (mg g^{-1} D.Wt) of *Citrus limon* shoots as affected by gamma rays, NaCl levels and their interactions

	NaCl levels mg L^{-1}					
Gamma rays KR	0	2000	4000	6000	8000	Mean
Potassium (K)						
0	18.1^{f}	18.0^{f}	15.7^{i}	14.6^{k}	10.9^{o}	15.5^{D}
5	18.6^{d}	18.3^{e}	17.0^{g}	15.3^{j}	11.6^{n}	16.2^{C}
10	20.3^{a}	20.0^{b}	18.6^{d}	17.0^{g}	13.6^{m}	17.9^{B}
20	20.3^{a}	19.9^{b}	18.8^{c}	16.9^{h}	14.0^{l}	18.0^{A}
Mean	19.33^{A}	19.1^{B}	17.5^{C}	16.0^{D}	12.5^{E}	
Calcium (Ca)						
0	17.7^{e}	16.3^{i}	15.0^{k}	13.6^{o}	11.9^{q}	14.9^{C}
5	18.9^{c}	16.9^{g}	15.6^{j}	14.3^{n}	12.3^{p}	15.6^{B}
10	21.0^{a}	18.3^{d}	17.3^{f}	16.6^{h}	14.6^{m}	17.6^{A}
20	20.9^{b}	18.3^{d}	17.4^{f}	16.3^{i}	14.8^{l}	17.5^{A}
Mean	19.63^{A}	17.5^{B}	16.3^{C}	15.2^{D}	13.4^{E}	
Magnesium (Mg)						
0	4.7^{f}	4.0^{h}	3.3^{j}	3.0^{k}	2.7^{m}	3.5^{C}
5	5.4^{d}	4.7^{f}	3.9^{h}	2.8^{l}	2.8^{l}	3.9^{B}
10	7.7^{a}	5.6^{c}	5.0^{e}	4.3^{g}	3.6^{i}	5.2^{A}
20	7.8^{a}	5.9^{b}	4.9^{e}	4.3^{g}	3.6^{i}	5.3^{A}
Mean	6.4^{A}	5.1^{B}	4.3^{C}	3.6^{D}	3.2^{E}	
Sodium (Na)						
0	9.3^{j}	10.6^{g}	12.0^{f}	15.4^{c}	20.6^{a}	13.6^{A}
5	8.9^{j}	10.0^{h}	10.6^{g}	12.3^{e}	17.3^{b}	11.8^{B}
10	6.5^{m}	8.2^{k}	9.9^{h}	10.6^{g}	12.9^{d}	9.6^{C}
20	6.0^{n}	7.0^{l}	9.0^{i}	9.8^{h}	10.6^{g}	8.5^{D}
Mean	7.7^{E}	9.0^{D}	10.4^{C}	12.0^{B}	15.4^{A}	
Chloride (Cl)						
0	12.4^{h}	12.9^{g}	15.6^{d}	19.5^{c}	23.2^{a}	16.7^{A}
5	10.6^{n}	11.3^{k}	14.0^{e}	15.6^{d}	20.6^{b}	14.4^{B}
10	9.0^{p}	11.0^{i}	11.9^{j}	12.9^{g}	16.6^{c}	12.2^{C}
20	7.5^{q}	9.9^{o}	10.9^{m}	12.0^{j}	13.6^{f}	10.8^{D}
Mean	9.9^{E}	11.3^{D}	13.1^{C}	15.0^{B}	18.4^{A}	

Means with the same letter are not significantly different at 5% level. For each trait, the capital letters in the columns are comparing the effects of gamma doses overall salinity levels. The capital letters in the rows are comparing the effects of salinity levels overall gamma doses. The small letters refer to the interaction (Salinity level×gamma rays)

(8000 mg NaCl/L) compared with the control (7.7 and 9.9 mg g^{-1} D.Wt.), respectively due to their presence in the media. Unlike salinity, gamma irradiation decreased the accumulations of Na and Cl whereas increased that of K^+, Ca^{2+} and Mg^{2+} in lemon shoots. Thus K/Na ratio was increased due to gamma irradiation decreased the accumulations of Na and Cl whereas, increased that of K^+, Ca^{2+} and Mg^{2+} in lemon shoots. Thus K/Na ratio was increased due to gamma irradiation (data not presented).

As for the interaction treatments, the data in the same table show that irradiation with gamma rays alleviated the harmful effect of salinity on K^+, Ca^{2+} and Mg^{2+} ions concentration and reduced due to reduced sodium and chloride accumulation. The increase of calcium, potassium and magnesium lead to an increase in K^+/Na^+ Ca^{2+}/Na^+ and Mg^{2+}/Na^+ ratios when compared with non-saline condition (data not presented).

The most effective treatments on increasing accumulation of K, Ca and Mg were found in shoots irradiated at 10 and 20 KR which regenerated under non-salinized media. It recorded about 20, 21 and 7.8 mg g^{-1} D.Wt. Compared with non-irradiated shoots grown under 8000 mg NaCl/L which registered 10.9, 11.9 and 2.7 mg g^{-1} D.Wt. for K, Ca and Mg, respectively. The same treatments achieved least significant values from Na and Cl accumulation.

DISCUSSION

It has been demonstrated that gamma rays decreased protoplast viability, cell wall regeneration, cell division and callus formation. However, radiation sensitivity showed its harmful effects only at 20 krad (Table 1). Similar results were previously reported with red pepper at 16 Gray Kim *et al.* (2004) and Altaf *et al.* (2009) on kinnow Citrus at 15 Gray for the embryogenic tissues and 100 Gray for the seeds. There is not much variation between these studies. This implies that irradiation increases plant tissue sensitivity to gamma rays. Omar (1988) attributed this sensitivity to a reduction in endogenous growth substances especially the cytokines as a result of break down or lack of syntheses due to irradiation.

As for the ability of irradiated protoplast to grow under salinity, data in the present investigation show that gamma rays alleviated the harmful effects of salinity on protoplast regeneration and its growth. All examined doses not only counteracted the depressing effects of salinity on the embryogenic callus growth but also induced the ability of callus to grow under even the two highest salinity levels (6000 and 8000 mg NaCl/L). The irradiated callus at 10 krad produced embryogenic callus F.Wt. more than 8 fold of non-irradiated one which cultured at 8000 mg NaCl/L (Table 2). Irradiation improved salt tolerance and altered the morphological potential of lemon shoots (Table 3). The inhibiting effects of salinity on the growth of the embryogenic calli and the regenerated shoots of lemon are compatible with those obtained on strawberry by Kaya *et al.* (2003) and on *Eucalyptus* Rain tree and Thai neem by Cha-Um and Kirdmanee (2008). Tester and Devenport (2003) attributed the deleterios effects of salinity on growth to reduction of water absorption, ion toxicity and disturbed ionic and hormonal balances. The beneficial effects of gamma rays on improving growth are supported by Afrasiab (2006) who stated that stressed plants altered protein metabolism and synthesized several stress proteins and accumulated it in their tissues. The stress proteins may play an important role in signal translocation, antioxidative defense or osmolyte synthesis which were essential to a plant's function and growth (Gygi *et al.*, 1999). El-Shihy and Monem (1994) on *Vicia faba* succeeded in producing two salt resistant mutant after treating callus with gamma rays at 5-8 KR.

As for the physiological character, data in the present investigation show that irradiated lemon shoots were responded to salt stress by developing defences mechanisms to improve salt tolerance and induce capability under salt stress. The defense mechanism was brought about by qualitative and quantitative changes in physiological status which provide better protection from oxidative and cellular damage caused by salinity. Irradiation counteracted the depressing effects of salinity on T chl (s), TS, TSP and increased carotenoids and all osmoregulators studied (proline, TSPh, Gly Bet and K). Moreover, irradiation prevented cellular damage as expressed by decreases in lipid peroxidation, membrane leakage, H_2O_2 as well as Na and Cl accumulations. The ionic balance within lemon tissues was improved to tolerate more salinity levels. The activity of SOD, POX, APOX, CAT and GR enzymes were also increased in irradiated shoots compared with non-irradiated ones. Corthals *et al.* (2000) attributed most of these changes to irreversible alteration in protein conformation and in the pattern on gene expression which altered under gamma stress. This led to modulation of certain metabolic and defensive pathways (Zolla *et al.*, 2003).

The effectiveness of gamma rays at low doses on scavenging of ROS that have destroyed the chlorophyll pigments, TSP and TS noticed in the present investigation was supported by several workers on other plant species (Kim *et al*., 2004; Hung *et al*., 2005; Abu *et al*., 2006). Zabalza *et al*. (2006) attributed the stimulating effect of irradiation on chlorophyll to stabilizing the enzyme active site and photosynthetic reactions. There is a cycle of interconversion between Chl a and Chl b that is particularly significant under stress (Matile *et al*., 1999). In this context, Jaleel *et al*. (2007) found that stress enhances the activity of chlorophyllase and interferase with the *de-novo* synthesis of protein, such as those that bind chlorophyll. The noticed increase in carotenoids due to stress conditions caused by either salinity and/or gamma rays was expected. Foyer and Harbinson (1994) reported that carotenoids are involved protein of the photosynthetic apparatus against photo-inhibitory damage by singlet oxygen (O_2^-) that is produced by the excited triplet state of chlorophyll. The reduction of TSP and TS under salinization, may be due to the effects of ROS toxic (reaction oxygen species) caused by salinity on oxidative proteins, lipids and DNA when they react certain threshold level associated with nutritional relocation to the developing tissues resulting in lipid peroxidation, cellular damage and reduction in antioxidants status of the tissues (Kukavica and Jovanovic, 2004).

Results in the present investigation demonstrated that salinity initiate lipid peroxidation which requires active O_2 uptake and/or induce oxidative stress in plant tissues (Bor *et al*., 2003). This initiation was accompanied with an increase in membrane leakage and H_2O_2 which minimized by gamma irradiation (Table 5). In addition, TSP and TS were counteracted by irradiation. Our results were supported by Bartels and Sunkar (2005). The protection might by result by significantly highest continuance and induced activities of antioxidative enzymes. Hence, constitutive and/or induced activity of SOD and other antioxidative enzymes such as POX, APOX, CAT and GR is essential. It is proposed that these enzymes may play an important role in the rapid defense responses of plant cells to oxidative stress due to their functions. Similar results were reported by Zabalza *et al*. (2006). Moreover, it may be suggested that SOD and APOX are working more efficiently in concern to decompose oxidants such as singlet oxygen (O_2^-) and H_2O_2 which might be produced during stress conditions. According to Ribeiro and Machado (2007), Citrus has several natural factors as cause of variability, therefore, the result of the present study strongly suggests that irradiated regenerated shoots of *Citrus limon* have a stronger potential to eliminate ROS through higher enzyme activities that involvement of the antioxidant system due to the increase in phenol availability. In addition, gamma rays can create variability from white useful variation can be selected. The embryogenesis regeneration under salinity was highest at 10 krad as shown in the preset investigation (Table 1-3). The natured cells, if survived as embryogenic calli can be useful for studying solid maturation. Therefore, it could be suggested that irradiation exhibit a better protection mechanism against oxidative damage by maintaining a higher inherited and induced activity of antioxidant enzymes than the non-irradiated protoplasts of *Citrus* (Table 6). Mittler (2002) supported our results and reported that, plant cells posses a variety of defense strategies against oxidative injury caused by salinity and/or irradiation stress. Such strategies involve induction in antioxidant status of the tissues (Kukavica and Jovanovic, 2004), nutritional relocation (Helaly *et al*., 2010), several stress proteid and proline (Afrasiab, 2006), specific detoxifying enzymes such as SOD, CAT and POX (Table 6) which decomposes superoxide radicals and hydrogen peroxide respectively (Bor *et al*., 2003) as well as various antioxidants quenchers including α-tocopherol and ascorbic acids as well as phenols (Gadalla, 2009). Reinforcement of plant defense mechanisms against oxidative damage may be successful achieved by using low does of

gamma rays. The beneficial effects of gamma rays at low does to salt-affected *Citrus* shoots are associated with the genetic action on certain cell membrane integrity (Bor *et al.*, 2003) and physiological components related to minerals constituents.

The effect of gamma rays on increasing K^+, Ca^{2+} and Mg^{2+} ions as shown in the present investigation (Table 8) led to an increase of osmotolerance and/or regulate physiological various processes including absorption of nutrients. The antagonistic relations between Na^+ and K^+ (Helaly *et al.*, 2010) may be taken as an indication of the role of gamma rays on modifying K^+/Na^+ selectivity under salt stress (Azooz, 2004). This promotion effect may be due to its role in improving content which protects the membrane and membrane bound enzymes. The positive effects of gamma irradiation on the nutritional status of the salt stressed plants may be ascribed to overcoming the substitution occurred between Na and K as well as their roles as an osmoregulators and as an activators of several enzymes required for normal plant metabolism and endogenous hormones synthesis (Ling *et al.*, 2008).

CONCLUSION

In conclusion, gamma irradiation induced *Citrus limon* protoplast to proliferation and continue their growth even at the highest level of salinity (8000 mg NaCl/L). Gamma irradiation may be produced *Citrus limon* line has a higher capacity to decompose H_2O_2 more rapidly and protect shoots against oxidative stresses caused by salinity. Irradiated shoots has a higher hereditary and induced capability under saline condition which provide to it a better protection from oxidative damage. This protection strategy might be resulted by significant higher constituents of photosynthetic pigments and compatible osmolytes (TSP, proline, TS, TSPh, Gly Bet and K). Decreases values of membrane leakage, lipid peroxidations, H_2O_2, Na and Cl may be considered. Higher activities of the most effective antioxidative enzymes (SOD, POX, APOX, CAT and GR) in preventing cellular damage may lead the irradiated plantlets to resist the potential oxidative damage. These results suggested a relationship between salt stress tolerance and osmoregulators as well as antioxidant defense system in *Citrus limon*. Most of these modifications appear to be a part of metabolic changes in response to salt tolerance throughout the developmental stages of *Citrus limon*. The regeneration of irradiative protoplast of lemon to plantlets in tissue culture system under NaCl stress up to 8000 mg L^{-1} is a clear indicator that this plant can be grown in adverse soil conditions such as salt and water stresses affected area. Irradiated shoots at 10 krad gamma rays and grown under 6000 mg NaCl/L salinity have high content of abiotic stress tolerant markers. This treatment can be straight forward included in the breeding abiotic stress tolerance lemon plants. Therefore, additional studies are needed to better understand the genetic stability of the regenerated plantlets and its relations with the physiological status of *Citrus limon*.

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