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Full Length Research Paper

The effects of NaCl pre-treatment on salt tolerance of tomato (*Lycopersicon esculentum* Mill.) callus grown under elevated saline conditions

El Fatih M Mahdi[1*], Tagelsir Ibrahim M Idris[2]

[1]Department of Science and Arts-Khulais, King Abdulaziz University, P.O. Box 355 Khulais, 21921 Saudi Arabia
[2]Department of Horticulture, Faculty of Agricultural Sciences, University of Sudan for Science and Technology, Khartoum North, 13314, Sudan

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The study was conducted to investigate the influence of NaCl pre-treatment on performance of callus cultures of salt sensitive *Lycopersicon esculentum* under salt stress conditions. Soluble proteins, proline, the activity of SOD and CAT antioxidative enzymes were used as indicators for salt tolerance.Increase in salinity level reduced survival rate and callus growth even in salt tolerant genotype at higher NaCl concentrations.The growth of untreatedand salt pre-treated *L. esculentum* calli was stopped at 70 and 105 mM NaCl respectively. The changes in soluble proteins, proline, and SOD and CAT enzymes activity in pre-treated callus of cultivated tomato due to the increased salinity level exhibited similar pattern of change of the wild salt tolerant tomato. The latter observation point to acquirement of salt pre-treated tomato callus tissues to a sort of salinity tolerance via a mechanism needs to be apprehended.

Keywords: *Lycopersicon esculentum*, *Lycopersicon pimpinellifolium*, salinity salt pre-treatment.

INTRODUCTION

The cultivated tomato, *Lycopersicon esculentum* Mill., of the family Solanaceae, is extensively distributed an annual vegetable crop throughout the world. It has high nutritive value and rich in vitamin C and phenolic compounds. Tomato is grown in a wide range of climates; however, the commercial production of tomatois concentrated in warm dry areas (Foolad, 2004). The overall world production exceeded 150 million tons in 2010 (FAOSTAT, Crop statistics, 2011).

Like most crop plants, tomato is moderately sensitive to salt stress which is an abiotic stress limits plant development and affects almost all the physiological and biochemical aspects of the plant and reduces yield (Bolarin *et al*. 1993; Sengupta and Majumder, 2009). For this reason, the production of tomato has been restricted by a high level of salinity of soils and irrigation water, though the demand for the fruit is increasing.

As drought and salinity are surged around the world, countless efforts have been devoted to developing and selecting salinity-tolerant crop plant genotypes. Therefore, changing salt tolerance of crops is expected to be an important aspect of crop improvement to maintain the global food production. The conventional selection and breeding techniques has achieved significant improvements in salinity tolerance of important agricultural crop plants (Ashraf and Harris, 2004). However, the techniques are exhaustive and expensive, and the character has a genetical and physiological basis, developmentally regulated and influenced by

*Corresponding Author's E-mail: emmahdi@hotmail.com, lmsarhan@kau.edu.sa

abiotic stressesthat add to the complexity of the trait (Foolad, 2004; Flowers, 2004).

Breeding for salt tolerance between *Lycopersicon esculentumLycopersicon pimpinellifolium* have been faced with difficulties of visualizing which of the main inheritable factors determine salt tolerance in the wild species,and the evidence of heterosis, dominance and additive gene effects (Shanon, 1985).

A number of cultural pracFtices were reported to contribute to endure the harmful effects of salts; such as, increasing salt tolerance by salt pre-treatment (Binzel *et al*., 1985), seed priming (Sivritepe *et al*., 2003) salt pre-treatment at the seedling stage (Cayuela*et al*., 2001) and drought pre-treatment applied at the seedling stage (Ryu *et al*., 1995).Cell Salt pre-treatments were presumed to result in effective adaptation of cell lines to salinity and Cuartero *et al*., (2006) suggested existence of genetic potency that tolerate high salt concentrations in the cells which triggered by exposure to salts. Although *in vitro* culture has emerged as a useful technique for the study of salt stress and being used for the selection of tomato and *Vigna radiata* tolerant to salinity (Hassan *et al*., 2008), yet the foregoing techniques are not employed.

The objective of this study was to explore variation in salt tolerance among cultivated and wild species of tomatoes via morphological, physiological and biochemical parameters. The research was also aimed towards induction of salt tolerance in cultivated tomatoes using *in vitro* salt pre-treatment.

MATERIALS AND METHODS

The cultivate species of tomato (*Lycopersicon esculentum*) and the wild species (*Lycopersicon pimpinellifolium*) were germinated *in vitro* according to Mahdi and Murashige (1995). After 3-5 days at 27^0C under complete darkness the uninfected germinating seeds were allowed to continue germination. Two weeks later, nodal segments were excised and transferred to nutrient gel callus induction medium in 25-X150 mm glass culture tubes. The medium contained Murashige and Skoog (1962) salts, 3% sucrose, 0.7% TC agar and in µM, 555*i*-inositol, 3 thiamineHCl, 2.4 pyridoxineHCl, 4.2 nicotinic acid, 26.6 glycine, 49.2 indole-3-butyric acid, and 1.32 N^6 benzyl adenine.25 ml of medium was dispensed in each tube and capped with natural polypropylene caps. The calli formed was subcultured three times on the same medium at monthly intervals.

Callus salt pre-treatment

Callus tissues from *Lycopersicon esculentum*was subjected to salt pre-treatment. 100 mg of callus tissue were placed on agar solidified distilled autoclaved water containing 100 M of NaCl for 36 hours. The pre treated calli were then transferred to the test media which consisted of Murashige and Skoog basal salts with minimal organics (M 6899- Sigma Chemical Co.) supplemented with 0,35, 70, 105and 140 mM of NaCl. The cultures were placed vertically and maintained at 27 ± 2^0C and under 16 h light exposure to 22.5 µmol $m^{-2}s^{-1}$ Gro Lux light. The number of culture tubes per treatment was 20 and they were transferred every four weeks to fresh medium of the same composition.

Data recorded were callus survival and weight. were also recorded. Statistical significance determined by comparing standard errors of means and obtaining 95% confidence limits from tables of binomials (Snedecor, 1957; Lentner, 1982).

Proline determination

Half gram of callus tissues was homogenized in 3% sulphosalicylic acid and filtered through Whatman No. 1 paper. Thefiltrate was used to proline quantification according a modification in Bates *et al*. (1973). Equal volumes (2mL) of filtrate, ninhidrin acid and glacial acetic acidwere thoroughly mixed and heated in awater bath set to 100^0C for 1h, an ice bath was used to stop thereaction to be cooled to room temperature. Toluene (4 mL) was added to the mixture and the extinction of toluene was measured spectrophotometrically at 520nm. One calibration curve was made using four replicates per dose, and those range from 0 to 80µg mL^{-1} spaced at 5µg mL^{-1} intervals. Values, higher than 80µg mL^{-1}, were diluted in glacial acetic acid and measured, after that data was converted to the original sample volume.

Enzyme assay and protein determination

The antioxidative enzyme activity was determined by homogenizing one gram oftomato callus tissues in 20 mL of cold100 mM potassium phosphate buffer (pH 6.8) containing 0.1 g Polyvinylpolypyrolidone(PVPP)for 2 min. The homogenate was filtered through nylon cloth lined in four layers. Filtrate samples were centrifuged for 20 min at 35.000 X G.All steps were performed at +4°C. Thesupernatant was collected in a tube and kept in an ice bath for enzyme assays.

The extract protein content was determined by Bradford (1976) method utilizing bovine serum albumin as the standard. SOD activity was assayed according to Giannopolitis and Ries (1977) method by monitoring

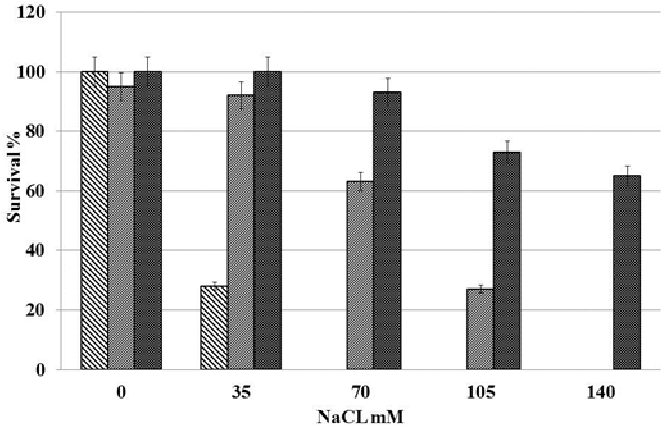

Figure1.Effect of NaCl concentration on callus tissue survival rate of untreated ( ), salt pre-treated) L. esculentum and L. pimpinellifolium( ). Bars on column indicate 95% confidence limits of binomial distribution

inhibition of photochemical reduction of 50% nitro blue tetrazolium (NBT). 3 ml of the reaction mixture containing;10 mM potassium phosphate buffer (pH 6.8), 13 mM methionine, 2 µM riboflavin, 75µM NBT, 100 nM EDTA and 200 µl enzyme extract was added to a 3mL disposable cuvets. The cuvets were incubated at 25 ℃ under 15 W fluorescent lamp illumination. The light was switched on to start the reaction and after 10 minutes it was switched off to stop the reaction. The absorbance of the reaction mixture was read at 560 nm. Each measurement was repeated three times for each enzyme extract. The values of SOD activity are expressed in units per g of protein.

CAT activity was determined according to Cakmak and Marschner (1992). The reaction mixture consisted of enzyme extract in combination with 10 mM potassium phosphate buffer (pH 6.8). 1.0 mM of Hydrogen peroxide (H_2O_2) was added to iniate the reaction and its decomposition was monitored at for 6 minutes 240 nm. Measurements were repeated three times for each enzyme extract. One unit of CAT activity was expressed as the amount of H_2O_2consumed (µmol per minute).A unit of catalase activity was expressed as the change in absorbance per minute and specific activity as enzyme units per gram soluble protein.

RESULTS AND DISCUSSION

Salinity is the major abiotic factor limiting plant growth and productivity through physiological and biochemical changes within the plant. The harmful effects of high salinity on plants can be observed at almost all stages of plant development including seed germination, vegetative growth and reproduction and occasionally associated with decreased productivity and eventual death of plants (Flowers, 2004).Innumerable approaches were used to achieve salt tolerance in plants. Some of the *in vitro* techniques were used only for selection, evaluation and/or characterization of salinity tolerant variants. In this research, callus salt pre-treatmentcultivated tomato callus tissues were used to in an attempt confer salinity tolerance.

Analysis of the callus growth revealed variability in salt response among the three types of calli used according to salinity level. The increase in salinity reduced callus survival rate and callus growth (Figure 1 and 2 respectively). Pre-treated callus of *L. esculentum*was able to survive higher salt concentrations (70 and 105 mM) compared to untreated one. Salt pre-treatment had been used in seed priming and was found to increase salt tolerance in seed germination and fruiting of tomato (Sivritepe *et al*., 2003). The positive effect of callus pre-treatment on callus growth wasnot recorded at 140 mM since the negative effect of high salinity culminates its growth. Similar results were observed in seed priming by Cano *et al.* (1991) who noticed the domination of the negative effect of salinity over the positive effect of seed priming. Inhibition of growth by salt stress has been observed even in tolerant plant species (Malik *et al*., 2010).

The effect of increasing salt concentration are also detected within the plant cells and manifested in changes in the proteins, oxidative enzymes and prolines. A higher content of soluble proteins has been observed in salt tolerant than in salt sensitive (pretreated and untreated) species of tomatoes (Figure 3). Ashraf and Harris (2004) reported similar results in barley, sunflower, finger millet, and rice. It was evident that soluble protein content decreased significantly with increasing salinity in the

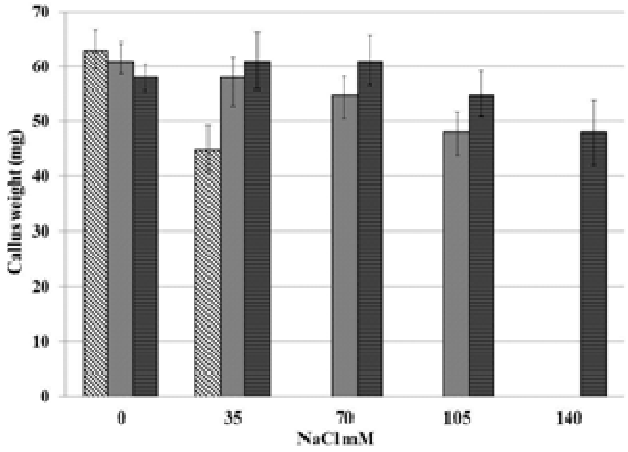

Figure 2. Effect of NaCl concentration on weightof callus of untreated (▨), salt pre-treated (▨) L. esculentum and L. pimpinellifolium(▩). Bars on column indicate S.E.

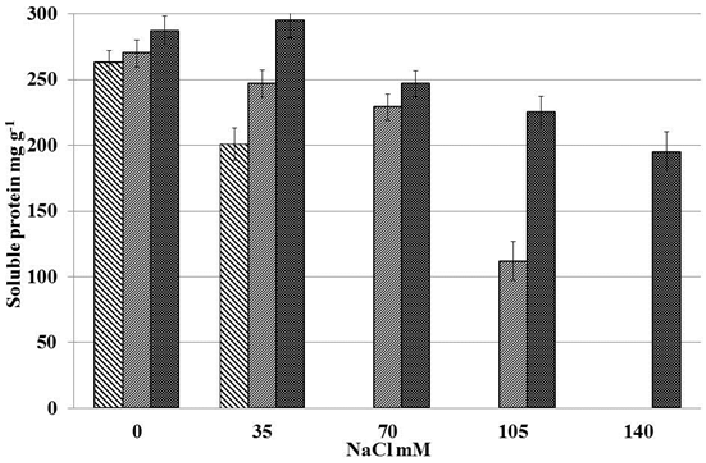

Figure 3. Soluble protein content in the callus tissues of untreated (▨), salt pre-treated (▨) L. esculentum and L. pimpinellifolium(▩) grown in different concentration of NaCl. Bars on column indicate SE

medium. The differences in soluble proteins between the sensitive and pre-treated sensitive and tolerant tomatoes were significant. A considerable variations in soluble proteins due to salt stress were observed in different plants, however, soluble proteins in leaves of all lentil lines was decreased upon salt stress (Ashraf and Waheed, 1993) which confirmed the findings. The endurance of salt pre-treated callus to higher salt concentrations, suggest acquiring of tissues to an adaptive response to salinity. Successful adaptation to salinity had been reported previously (Binzel *et al*., (1985) which advocates the persistence of the genetic potential for salt tolerance in the cells, and the expression is triggered by exposing them to salts. In such case, the response of pre-treated callus tissues to salinity resembled seed priming and salt pre-treatment applied at the seedling stage reported by Cuartero *et al*. (2006).

The concentration of proline increased significantly in the three types of callias the salt concentration increased to 35 mM (Figure 4). The tolerant genotype *L. pimpinellifolium* accumulated more than 6 folds proline in 105 mM NaCl, while the salt pre-treated *L. esculentum* accumulated about 5 folds proline compared to the untreated cultivated tomato that was perished at this particular concentration. A positive correlation between proline accumulation and salt tolerance in plants and they concluded that has been reported (Malik *et al*., 2010). This was probably ascribed to an adaptive role of proline in reconciliation of osmotic adjustment and protection of cellular structures in stressed plants. However, the

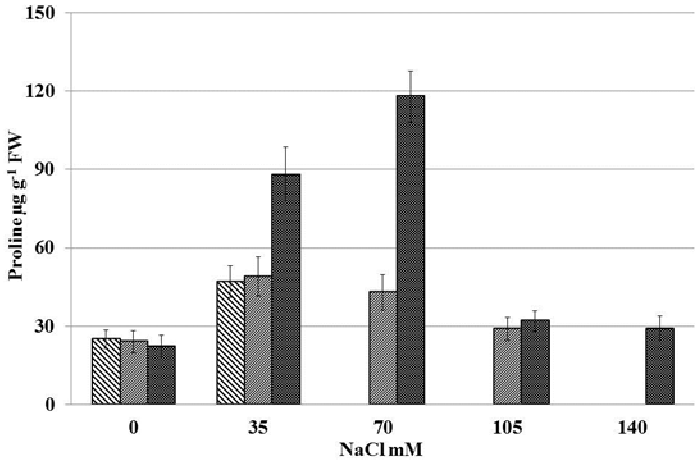

Figure 4. Proline content in the callus tissues of untreated ( ), salt pre-treated ( ) L. esculentum and L. pimpinellifolium(Bars on column indicate SE

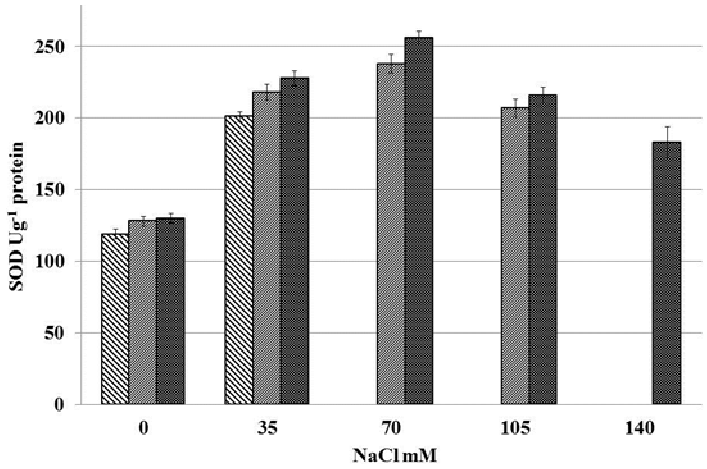

Figure 5. SOD enzyme activity in untreated ( ), salt pre-treated ( ) L. esculentumand L. pimpinellifolium( ) callus tissue as influenced by increased NaCl concentrations. Bars on column indicate SE.

probable adaptive components of salt tolerance are not fully understood (Malik *et al*., 2010).

Figure 5 illustrates a significant change of SOD antioxidant enzyme activity in response to the salinity. The activity in the three types of calli increased with increasing salinity up to 70 mM of NaCl and then decreased slightly at higher salinity level (105 mM for pre-treated cultivated tomato and the wild species and 105 and 140 for the wild species). Yet, the activity was higher than the control. The SOD activity in salt pre-treated callus showed similar trend of change to that of salt tolerant tissues of *L. pimpinellifolium*.CAT activity, on the other hand, showed a considerable decrease in activity in response to salt treatments in all calli tested, irrespective of the callus source. The decrease in the activity of the enzyme was more pronounced in untreated callus than salt pre-treated and callus of the salt tolerant genotype (Figure 6). The important aspect of salinity stress is the oxidative stress that leads to the formation of reactive oxygen species as a result of anaerobic metabolism. SOD and CAT are the main enzymes involved in the detoxification of the deleterious oxygen species (Mittova *et al*., 2003). Therefore, the significant change in SOD and CAT activity found in the salt tolerant genotype and the salt pre-treated cultivated tomato under increasing salinity stress denotes its relative tolerance to salinity, signifying the role of antioxidant enzymes in renovating tolerance against salt stress.

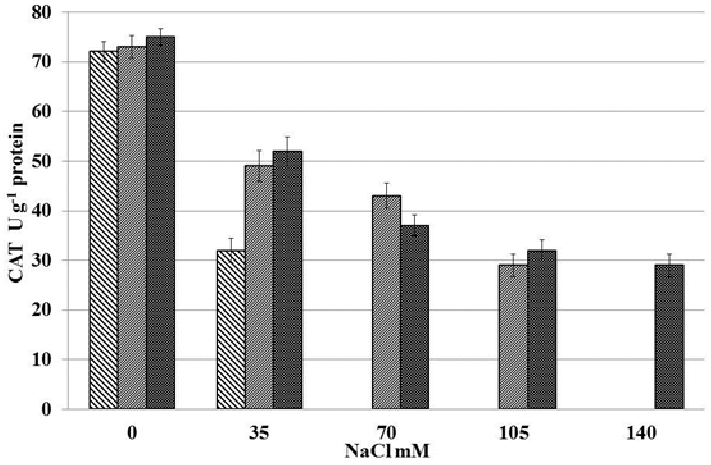

Figure 6. CAT enzyme activity in untreated ( ), salt pre-treated ( ) L. esculentumand L. pimpinellifolium( ) callus tissue as influenced by increased NaCl concentrations. Bars on column indicate SE.

In conclusion it was apparent that the difference in soluble protein and proline content and antioxidant enzyme activity could be ascribed to the difference in mechanisms underlying the harmful oxidative stress and consequent tolerance to salinity. It was also evident that salt pre-treatment rendered the salt sensitive *L. esculentum* to acquire the moderate salt tolerance qualities. In view of changes apparent on sensitive callus due to salt pre-treatment and the manipulability of callus cultures this model would be acceptable for future investigations of salt tolerance induction.

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