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Investigation of the effect of salt stress on the antioxidant enzyme activities on leaves of Date Palm (*Phoenix dactylifera*) seedling

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Abstract. The production of significant amount of reactive oxygen species (ROS) in salt stress condition causes damage to proteins, lipids, nucleic acids and other sites of cells, this process is a lethal factor for salt sensitive plants. Tolerant plants involved an antioxidant defense system which protects them against oxidative damage. Date palm is a salt-tolerant plant, to understand the regulation role of the antioxidant system provides protection against NaCl-induced oxidative damage in plants, the leaves used for the analysis of enzyme activities under long-term salt stress (NaCl with 0, 40, 80, 120, 160 and 200 mM) interaction on some antioxidant enzyme activities (superoxide dismutase (SOD), peroxidase (POD), catalase (CAT)) ascorbate peroxidase (APX), free proline and soluble protein contents in Date palm Hillawi cultivar (*Phoenix dactylifera*) was investigated. The activity of antioxidant enzymes catalase, peroxidase, ascorbate peroxidase, superoxide dismutase and prolin in the salt-tolerant cultivar increased markedly during salinity stress, while the soluble protein was mostly decreased by salinity stress. These results suggest that the antioxidant enzymes was a scavenging system forms the primary defense line in protecting oxidative damage under salt stress in date palm plants.
Key Words: Salinity, salt stress, antioxidant enzymes, proline, protein date palm.

Introduction. Date palm (*Phoenix dactylifera*) is an important horticultural crop, often cultivated in arid and semi-arid regions of the world, where salinity threatens to become, or is already a problem. In general, date palm is known to be tolerant to salinity. It has been shown that salinity causes several types of damage such as growth inhibition (Franco et al 1997), metabolic disturbances (Mavrogianopoulos et al 1999), and yield and quality losses (Del Amor et al 1999).

Salt tolerance can be defined as the ability of plants to survive and maintain growth under saline conditions. Plants have three mechanisms to tolerate high salt concentrations: cellular homeostasis which includes ion homeostasis and osmotic adjustment; detoxification which includes neutralization of ROS; and growth regulation (Zhu 2001). Physiological and metabolic changes possibly occurring as response to salinity stress are the production of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH), ROS have potential to interact with many cellular components, causing significant damage to membrane and other cellular structures, and consequently growth inhibition (Agarwal & Shaheen 2007; Gao et al 2008). ROS can seriously disrupt normal metabolism through oxidative damage to lipids, protein, and nucleic acids.

Plants, however, possess an impressive array of defense mechanisms against oxidative stress including the enzymatic and nonenzymatic antioxidant systems. The antioxidant enzymes include superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), while the nonenzymatic antioxidants include water-soluble (ascorbate, glutathione, phenolic compounds and flavonoids) and lipid-soluble (a-tocopherol, b-carotene, lycopene) metabolites. The latter of antioxidant enzymes includes catalase (CAT), superoxide dismutases (SOD), peroxidase (POD) and glutathione reductase (GR) (Ashraf 2002), which are essentially generated during normal metabolism as by-products of inevitable

leakage of electrons to molecular oxygen from the electron transport activities in chloroplast, mitochondria and plasma membrane (Foyer 1997). Some of other biochemical and physiological changes in tissues in response to several kinds of stresses can be verified through alterations in proteins. Kogan et al (2000) found that the accumulation of compatible solutes is one of the strategies that plants have developed to tolerate salt stress. Compatible osmolytes and proteins can therefore be used as potential biochemical markers useful in the identification and genetic manipulation of salt-resistant plants and plant cells (Shonjani 2002).

In the present study, was a part study of a mechanism salt tolerance of date palm, and it was aim to study the comparative effects of different concentrations of salinity on compatible osmolytes (prolin) and soluble proteins content and antioxidant enzyme activities (e.g., CAT, POD, APX and SOD) of date palm seedling to analyze the significance of these parameters in salinity stress tolerance. This study may be helpful in developing a better understanding and provide additional information on the mechanisms of salt tolerance.

Material and Method.

This study was conducted under controlled conditions in the Department of Horticulture and Landscape laboratory, College of Agriculture, Basrah University, Basrah, Iraq. The experiment was laid according to completely randomized Block design (CRBD) with four time replication. Date palm Hillawi cv. seeds were collected in July, of growth season at the private orchard, seeds were selected and stored in a gauze bag at 4 °C until to use. Seeds with uniform size were surface sterilized with 5% (w/v) calcium hypochlorite for 15 min, and rinsed four times thoroughly with distilled water. Date palm seeds were sown in plastic pots (15 cm in diameter and 20 cm in height) filled with sand culture. They were watered with a half-strength Hoagland's solution during the first 5 months following sowing, and then subjected to salt treatments. Salinized culture solutions were prepared by adding various concentrations of NaCl (0 mM, 40 mM, 80mM, 120 mM, 160 mM and 200 mM), and were added on the surface of the 1/2 Hogland. Controls were treated with 1/2 Hogland solutions. After 3 month of treatment leaf samples were collected, washed for 2 minutes by distilled water, and used immediately to examine, activities of SOD, POD, CAT, and POX accumulation of proline, and soluble protein, in leafs.

Assays of Antioxidant Enzymes.

Extraction of Enzymes. The plant samples consisting of fully expanded leaf were collected after 3 month from treated and untreated check pots. The samples weighing 0.3 g were directly dry frozen. The frozen leaf tissues were triturated in a cold mortar condition. A 6.0 mL solution containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 4% polyvinyll phrolidone (PVP) and 0.2 mM ascorbic acid was used to extract the enzymes. The extract material was centrifuged at 12,000 x g at 4 °C for 20 min for purification. The enzyme assays were performed in supernatant.

Determination of CAT activity. It was measured following the change in absorbance of the reaction mixture at 240 nm due to hydrogen peroxide reduction (Aebi 1984). Activity unit was calculated using the coefficient for H_2O_2 at 240 nm (40 mM^{-1} cm^{-1}). Enzyme activities were expressed as enzyme units per gram fresh weight (U/g fw).

Determination of POD activity. It was performed according to Kar & Choudhuri (1987) method with slight modifications. Reaction solution contained 2.85 ml 3 % guaiacol (water solution), 0.1 mL 2 % H_2O_2 and 50 µl enzyme extract. Activity unit was calculated using the coefficient of absorbance for tetraguaiacol at 470 nm (22.6 mM^{-1}). Enzyme activities were expressed as enzyme units per gram fresh weight (U/g fw).

Assay of APX activity. The activity of APX was assayed according to (Chen & Asada 1992). The reaction mixture (3 mL) contained 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.5 mM H_2O_2 and 0.1 mL enzyme extract.

The reaction was started by the addition of H_2O_2. The activity of enzyme was assayed by measuring the decrease in absorbance at 290 nm for 1 min of ascorbic as ascorbic acid oxidized.

Superoxide dismutase (SOD). The quantity of SOD activity was assayed following Giannopotitis & Ries (1977). The reaction solution was prepared by mixing of 150 mM potassium phosphate (pH 7.8), 13 mM methionine, 75 µM p-nitroblue tetrazolium chloride (NBT), 2 µM riboflavin, 0.1 mM EDTA. The 3 mL reaction solution was illuminated under light source to start reaction with the samples. After 20 minutes of initial reaction time, the lights were switched off. The absorbance readings were taken at wavelength of 560 nm. One unit of SOD activity was defined as the enzyme activity that reduced the photo reduction of nitroblue tetrazolium to blue formazan by 50 %. Enzyme activities were expressed as enzyme units per gram fresh weight (U/g fw).

Free proline and soluble protein content. Free proline content was quantified according to the method of Bates et al (1973). Protein content was measured according to Bradford (1976).

Statistical analysis. Analyses of variance (ANOVA) for all the variables were carried out using SPSS analysis program. Treatment means were compared using the protected least significant difference (LSD) test at $p<0.05$ levels following Snedecor & Cochran (1980).

Results and Discussion.

Antioxidant enzyme. Enzyme extract from the leaves of Hillawi Date Palm cultivar was assayed for CAT activity after exposure to different salinity concentrations (Figure 1). CAT enzyme activity increased gradually up to the level of 160 mM NaCl, while at the highest level (200 mM NaCl), the activity reduced but still higher than the absolute control.

Effects of NaCl on POD activity in the leaves were shown in Figure 2. POD activity increased markedly with salt levels increase. But there is no visible change in the activity of this enzyme at 160 and 200mM NaCl concentration treatment.

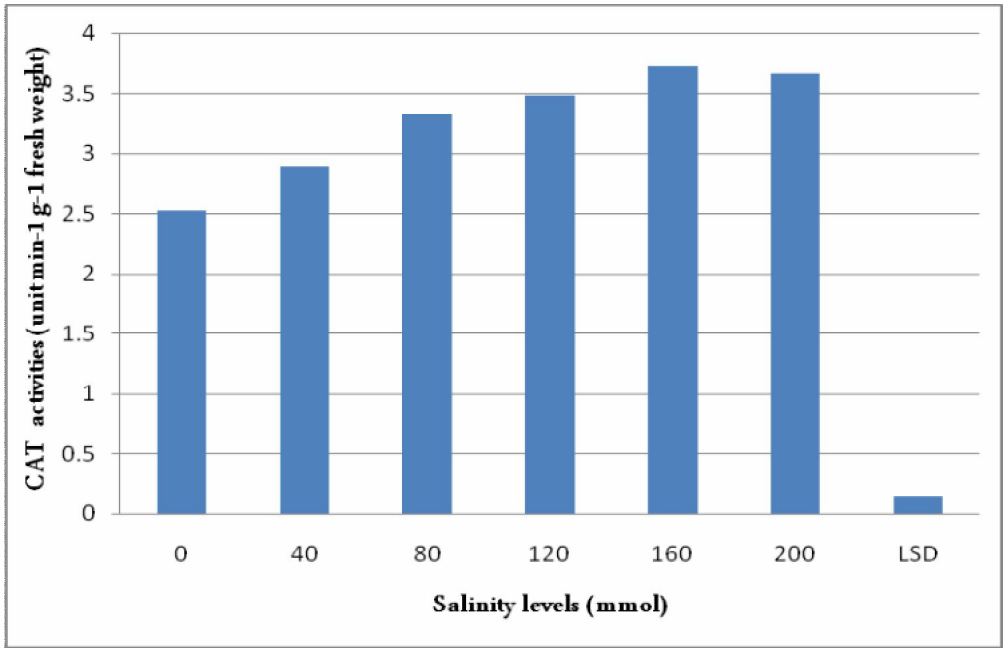

Figure 1. Effect of salinity on catalase (CTA) activities (unit min^{-1} g^{-1} fresh weight) in leaves of Date Palm cv. Hillawi.

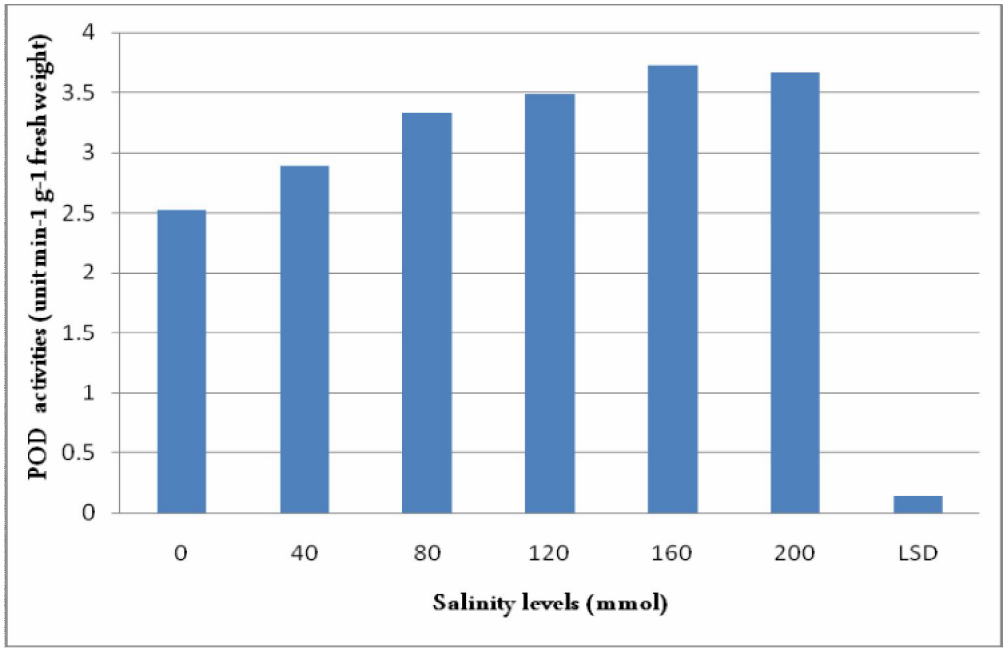

Figure 2. Effect of salinity on peroxidase (POD) activities (unit min^{-1} g^{-1} fresh weight) in leaves of Date Palm cv. Hillawi.

APX activity increased with increasing levels of NaCl up to 160 mM NaCl, but at the level of 200 mM NaCl, it reduced (about 0.03 %) in comparing with the 160 mM, the peak APX activity in the leaves was observed at 160 mM of NaCl concentration, increasing by 588.1 % compared to the control (Figure 3).
In Figure 3, the data indicated a highly significant increase in SOD activity in the salt-tolerant Hillawi date palm cultivar, but there was insignificant change in SOD content up to the level of 160 mM NaCl, the level of 200 mM NaCl, it reduced (about 0.14%) comparing with the 160 mM, but still higher than the absolute control.

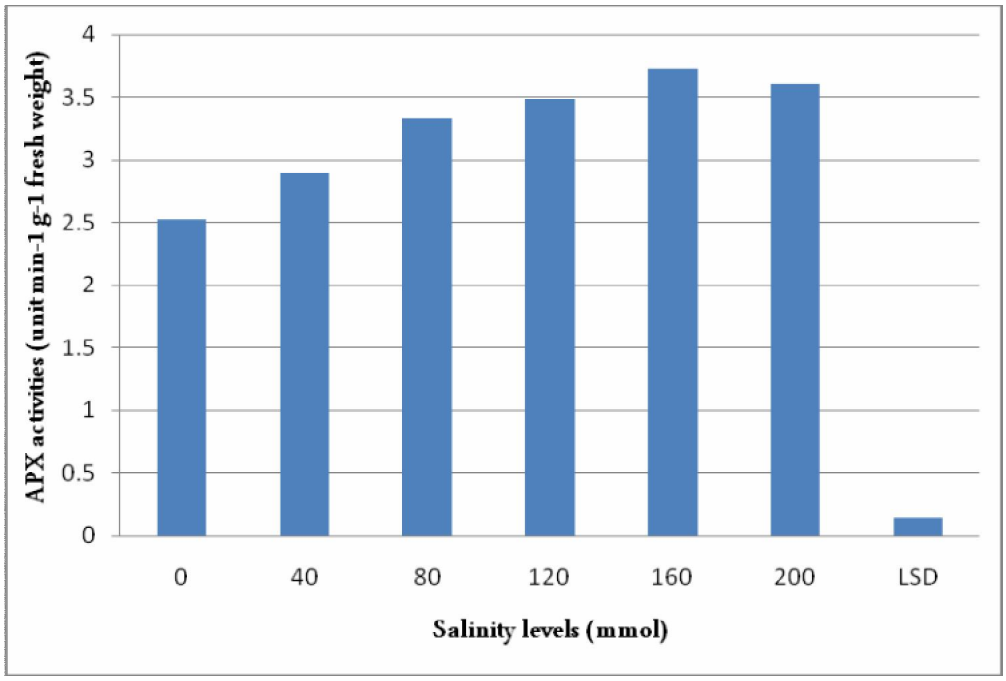

Figure 3. Effect of salinity on ascorbate peroxidase (APX) activities (unit min^{-1} g^{-1} fresh weight) in leaves of Date Palm cv. Hillawi.

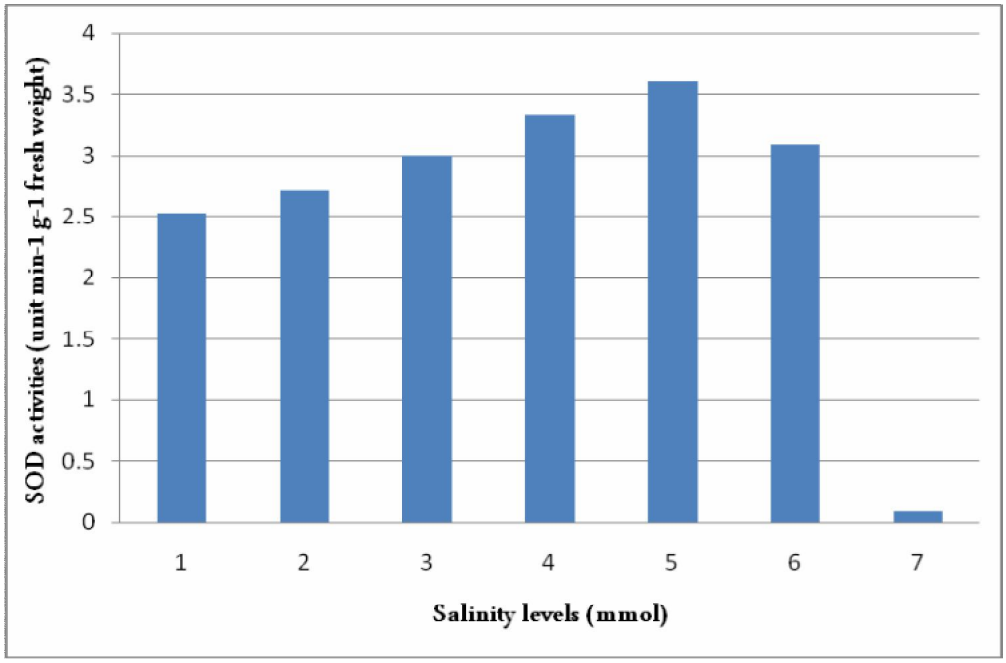

Figure 4. Effect of salinity on superoxide dismutase (SOD) activities (unit min^{-1} g^{-1} fresh weight) in leaves of Date Palm cv. Hillawi.

Free proline and soluble protein contents. It is clear from the present results that increasing concentrations of NaCl salinity increased leaf proline concentration of date palm cultivar (Figure 5). The magnitude of the increase was directly proportional to NaCl concentration. The maximam proline content was recorded at 250 mM NaCl treatment, but the quantitative analysis of the soluble protein contents during the present work indicated that soluble protein contents in the leaves of date palm Hillawi cultivar was significantly decreased in response to various NaCl treatments (Figure 6).

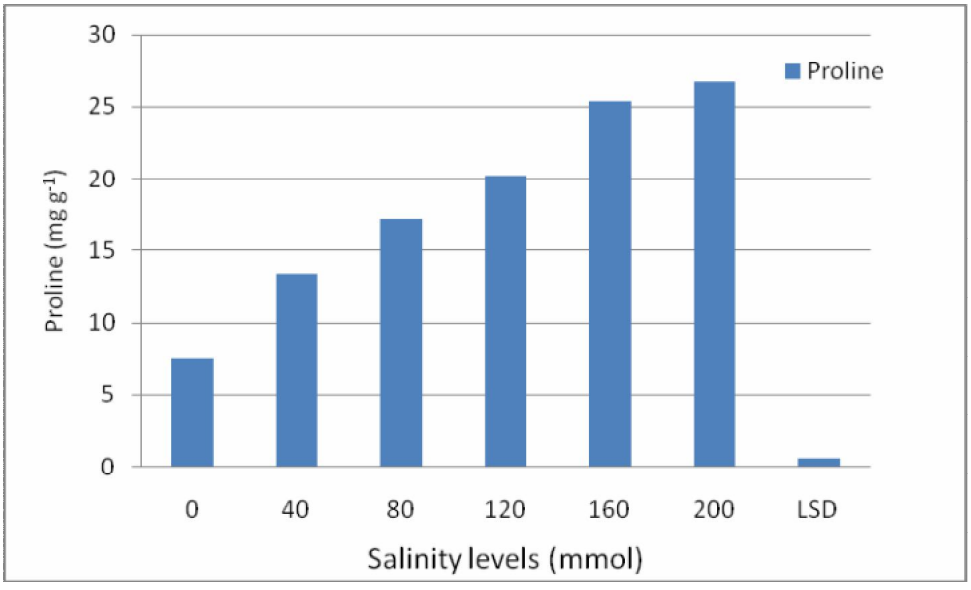

Figure 5. Effect of salinity on proline content (mg g^{-1}) in leaves of Date Palm cv. Hillawi.

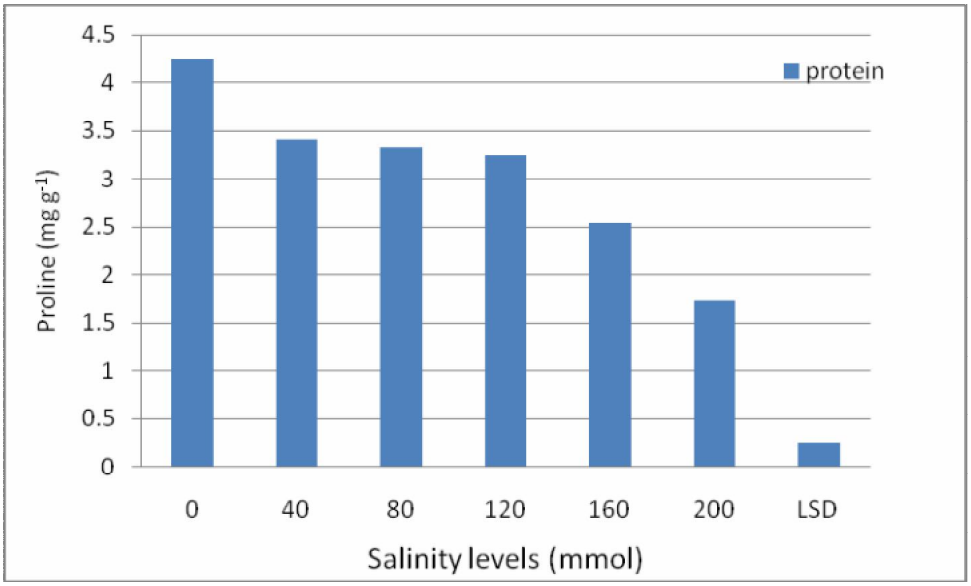

Figure 6. Effect of salinity on protein content (mg g^{-1}) in leaves of Date Palm cv. Hillawi.

One consequence of salt-stress in plants is the excessive generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals (Foyer et al 1994; Mittler 2002). Under normal growth conditions, the production of ROS in the cell is generally at low levels. However, under conditions of abiotic stress such as high salinity, cellular homeostasis is disrupted and leads to the production of relatively high levels of ROS (Mittler 2002). ROSs has potential to interact with many cellular components, causing significant damage to membranes and other cellular structures.

When ROS increases, chain reaction start, in which superoxide dismutase (SOD) catalyzes the dismutation of O_2^- radical to molecular O_2 and H_2O_2 (Meloni et al 2003). The H_2O_2 is then detoxified in the ascorbate-glutathione cycle (Mittler 2002), which involves the oxidation and re-reduction of ascorbate and glutathione through the ascorbate peroxidase (APX) and glutathion reductase (GR) (Noctor & Foyer 1998). If there is a serious imbalance in any cell compartment between the production of reactive oxygen species (ROS) and antioxidant defense, oxidative stress and damage occurs (Mittler 2002).

It is clear from the present results that increasing concentrations of NaCl salinity generates ROS and increase the activity of this antioxidant enzymes. CAT, which is involved in the degradation of hydrogen peroxide into water and oxygen, is the most effective antioxidant enzymes in preventing oxidative damage (Willekens et al 1997; Mittler 2002). The data indicated a highly significant increase in SOD activity which is thought to be one of the most important defense systems, which detoxifies superoxide anion free radicals, by the formation of H_2O_2 (Fridovich 1986), and the results suggest that the increased POD activity could contribute to the antioxidant mechanism of date palm seedlings against higher NaCl concentrations stress.

The increased activity of antioxidant enzymes during increased salt stress have been reported in wheat (Sairam et al 2002), tomato (Mittova et al 2002), rice (Vaidyanathan et al 2003), sugar beet (Bor et al 2003) and maize (Azevedo-Neto et al 2006). Bor et al (2003) reported a better protection from oxidative damage caused by salt treatment by increasing activities of SOD and CAT in beet leaves. According to Scandalios et al (1997), SOD and CAT are the most effective antioxidant enzymes in preventing cellular damage.

Salt stress caused a remarkable increase in free proline content. Proline has been considered as a carbon and nitrogen source for rapid recovery from stress and growth, a stabilizer for membranes and some macromolecules and also a free radical scavenger (Jain 2001).

One of the ways that plants develop ability to withstand against salinity by synthesizing organic compounds, is to accumulate proline and activating a battery of antioxidant enzyme system (Ashraf et al 2008). Aspinall & Paleg (1981) reported that high concentration of proline produced significant role in tolerance to drought and salinity. The production and accumulation of praline, protect the activity of intracellular macromolecules by making adjustment in osmotic potential (Tang 1989; Ashraf 1994), it is accumulated in greater quantity in cytosol in response to stress conditions (Ashraf 1994). Moreover, production of free radicals leads to increased cellular redox potential and destruction of membrane structures under oxidative stress, which are protected by accumulation of proline content (Mansour 1998; Gadallah 1999).

Different researchers (Athar et al 2009; Ashraf 1994; Ashraf & Harris 2004) reported high accumulation of proline in plants under stress conditions. The protein decrease with increase of NaCl concentration, may be is needed to synthesize hydroxyproline-containing protein (Arrigoni et al 1977), or the generation of reactive oxygen species that can cause oxidative damage to many cellular components including proteins (Halliwell & Gutteridge 1989), and salinity drastically inhibit the protein biosynthesis in plant tissues (Lutts et al 1999).

Conclusions. The date palm seedling may be possess defense mechanisms against oxidative stress which aspect under salt stress including the enzymatic antioxidant systems beside another defense mechanisms (Abdulwahid 2011, 2012) which interprets that date palm is salt tolerant plant.

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