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Seedling Growth and Biochemical Components of Lentil under Salinity Stress

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ABSTRACT

The present research was carried out in order to study the effect of salinity stress by NaCl on morphological traits and biochemical components of three lentil cultivars; Giza-9, Giza-51 and Giza-370. The experiment carried out was factorial in randomized complete block design (RCBD) with three replications. The results showed reduction of seedling growth of all lentil cultivars with increasing of NaCl concentration. Giza-9 had the highest germination percentage, shoot length, root length, seedling dry weight and seedling vigor index at high level of salinity. On the other hand, salinity level caused a marked reduction in total chlorophyll content. The activity of enzymes such as catalase (CAT), proline dehydrogenase (PDH) and ascorbate oxidase (AO) changed under salinity stress. In high level of salinity, Giza-9 cultivar had greater CAT and PDH activity but in Giza-51 cultivar ascorbate oxidase activity was higher. Moreover, salinity caused the appearance of new isozyme band and disappearance of others which may be indicator for salt tolerance in lentil. Finally, our results indicated that Giza-9 was more tolerant, Giza-51 was moderate and Giza-370 was the least salt tolerant.

Key words: *Lens culinaris* M., salinity stress, chlorophyll, enzyme activity, isozymes

Introduction

Lentil (*Lens culinaris* M.) is an important legume in the farming systems of the Mediterranean area, because it is a source of high quality protein in human diet and animal consumption (Thomson and Siddique, 1997; Katerji *et al*., 2001). The species are classified as salt sensitive (Ashraf and Waheed, 1990) like many other leguminous crops. Selection for salinity tolerance appears as a laborious and hazardous task and plant breeders are seeking for quick, cheap and reliable ways to assess the salt-tolerance of selected material. Salinity reduces the ability of plants to take up water, leading to growth reduction as well as metabolic changes similar to those caused by the water stress (Munns, 2002). High salt concentration in the roots affects the growth and yield of many important crops. Salinity may reduce crop yield by upsetting water and nutritional balance of the plant (Khan *et al*., 2007). Water availability and nutrient uptake by plant roots can be limited by high osmotic potential and toxicity of Na and Cl ions (Al-Karaki, 1997). It affects plant growth directly through its interaction with metabolic rates and pathways within the plants. That germination and early seedling growth of many crop plants are the most sensitive stages to environmental stresses (Cook, 1997). Puppala *et al*. (1999) demonstrated that elevated salinity slowed down water uptake by seeds, thereby inhibiting germination and root elongation. The responses of cultivated species to salinity in terms of growth and yield are the ultimate expression of several interacting physiological and biochemical parameters. NaCl salinity is known to decrease seed germination, root and shoot length, hydrolytic enzyme activity during germination (Mathew and Chandrasekhar, 1998; Promila and Kumar, 2000; Davenport *et al*., 2005) and also affects various metabolic processes such as photosynthesis, protein synthesis, respiration, nitrogen assimilation and phytohormone turnover (Arshi *et al*., 2002).

Sidari *et al*. (2007) reported that four lentil (*Lens culinaris* M.) genotypes were treated with salt stress (0, 50, 100, 150 or 200 mM NaCl), and it was reported that the increasing of NaCl concentration reduced the germination percentage, the growth parameters and the relative water content. Salt stress similar to many a biotic stress factors, is known to induce oxidative damage to plant cells from reactive oxygen species that affects the physiology and biochemistry of plants and

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that can lead to a reduction in plant yield (Azevedo-Neto *et al*., 2006). The reactive oxygen species can damage membranes and other essential macromolecules, such as photosynthetic pigments, proteins, DNA, and lipids (Fahmy *et al*., 1998). For stress protection, plants have developed enzymatic and non-enzymatic scavenging mechanisms for the reactive oxygen species (Demiral and Turkan, 2005). These scavenging mechanisms, such as the production of catalase to reduce hydrogen peroxide (Hernandez *et al*., 2000), enable the plant to maintain growth under stress conditions. Kennedy and De Fillippis (1999) reported that chlorophylls and carotenoids are significantly reduced under NaCl stress, but the rate of decline of protochlorophyll and chlorophyll is greater than that of Chl-a and carotenoids. The magnitude of the decreases was increased with increasing salinity level. The activities of the antioxidative enzymes such as catalase (CAT), increase under salt stress in plants and a correlation of these enzyme levels and salt tolerance exists (Lee *et al*., 2001; Mittova *et al*., 2003 and Parida and Das, 2005).

In the present study, the effects of salt stress on seed germination, seedling development and metabolic activity such as chlorophyll content, enzymes activity and isozymes profile were investigated during the seedling stage in lentil.

Materials and Methods

Plant material:

The present study was done in the lab of Seed Technology Research Dept., Field Crops Research Institute, Agricultural Research Center (ARC) and Biochemistry department, faculty of Agriculture, Al-Azhar University. Three Lentil (*Lens culinaris* M.) cultivars Giza-9, Giza-51 and Giza-370 were used in this study during germination stage. The seeds obtained from the Legume Crops Research Department of the Agricultural Research Center, Giza, Egypt. The seeds were surface sterilized by immersing in 0.5% sodium hypochlorite (NaOCl) solution for 5 min to prevent fungal infections and then washed three times with sterile, distilled water to remove any NaOCl residue.

Experimental:

To estimate the germination percentage, seedling growth and biochemical component effects of salinity levels on lentil, 50 randomly selected seeds from each cultivar were transferred into a sterile Petri dish (150 mm in diameter x 15 mm deep) containing two sheets of sterile Whatman No. 1. Each dish was moistened with 10 ml of NaCl solutions (0, 1000, 2000 and 3000 ppm) and were moved to a controlled environment chamber at 20 ± 2 ºC for germination under a 18 h light-6 h dark cycle. All solutions were made with distilled water.

Measurements of seedling vigor and metabolic activity were made at 14 days after transfer of the seeds to the Petri dishes. Seedling vigor was determined using the percent seed germination, shoot length, root length and seedling dry weight of ten randomly selected seedlings, and the seedling vigor index (seedling length in cm x germination percentage) outlined by (ISTA, 1999). Dry weight was determined after drying the plant tissue to a constant weight in a hot air oven at 85ºC for 12 h (Krishnasamy and Seshu, 1990).

Biochemical components within the seedlings were determined by measuring the chlorophyll content, enzyme activity of catalase, ascorbate oxidase and proline dehydrogenase and isozymes profile of catalase, peroxidase and esterase.

The chlorophyll content of the seedlings was measured using the spectrophotometric method described by Hipkins and Baker (1986). Total chlorophyll was calculated using the formula: Chlorophyll (mg/ml) $=25.8 \times A_{650} + 4.0 \times A_{665}$ and then converted into mg chlorophyll/g plant tissue.

Enzymes were extracted from 0.5 g leaf samples homogenized in a pre-chilled pestle and mortar containing ice cold 0.1 M phosphate buffer (pH 7.5) and 0.5 mM EDTA. Each homogenate was transferred to centrifuge tubes and centrifuged at 4°C in refrigerated centrifuge for 15 min at 15, 000 x g. The supernatant was decanted and used for measuring enzyme activity assays (Esfandiari *et al*., 2007).

Catalase activity was determined according to the method used by Aebi (1984) in which the disappearance of H_2O_2 in a reaction mixture containing 0.3 mL 3% H_2O_2, 2.5mL of 0.05 M phosphate buffer (pH 7), and 2.5 mL of plant extract was monitored by the decrease in absorbance at 240 nm.

Ascorbate oxidase activity was assayed at 25°C by following the decreasing in absorbance of the reaction mixture at 265 nm using previously described spectrophotometer. The reaction mixture consisted of 0.05 M potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 0.002% metaphosphoricacid, 0.15 mM L-ascorbic acid, and enzyme solution in a final volume of 3.0 mL according to the method of Oberbacher and Vines (1963).

Proline dehydrogenase was assayed by following $NADP^+$ reduction at 340 nm in a 0.15 M Na_2CO_3–HCl buffer (pH 10.3) containing 15 mM L-proline and 1.5 mM $NADP^+$ (Ruiz *et al*., 2002).

Isozymes electrophoresis, Native polyacrylamide gel electrophoresis (Native-PAGE) technique was used to separate the isozymes profile of lentil such as catalase, peroxidase and esterase. Isozymes fractionation was performed on vertical slab (19.8cm x26.8cm x.02cm) using the gel laconic electrophoresis apparatus according to Jonathan and Wendel (1990). The ingredients of compounds used are shown in Table 1.

Table 1: The ingredients of staining solutions

Enzyme	Compounds	Amount
Catalase	A-Na-thiosulphate 60 mM H_2O_2 3% B- KI 90 mM Acetic acid	30 ml 30 ml 100 ml 0.5 ml
Peroxidase	A-sodium acetate (1M, Ph 4.7) Acetic acid 3,3,5,5tetramethylbenzidine (TMBZ) B-0.30 % H_2O_2	50 ml 20 ml 125 mg 2 ml
Esterase	Sodium phosphate (100 mM, pH6.0) α -naphthyl acetate Fast blue RR salt	50 mg 25 mg 50 mg

Statistical analysis:-

Analysis for all data obtained was carried out using randomized complete block design with three replication and differences among means were calculated using L.S.D test according to Steel and Torrie (1980).

Results and Discussion

Effect of salinity on seedling growth:

Germination percentage, shoot length, root length, seedling dry weight and seedling vigor index of three lentil cultivars subjected to 14 days under four levels of salinity by NaCl (0, 1000, 2000 and 3000 ppm) are shown in Table (2). The results showed that germination percentage of lentil cultivars significantly decreased when salt stress level was increased. Under normal conditions (without stress), the highest germination percentage was recorded in cultivar Giza-51 (100%) followed by Giza-9 (98%), while the lowest germination percentage was recorded by Giza-370 (92%). Maximum reduction in germination percentage was observed in Giza-370 (70%) at 3000 ppm NaCl, while Giza-9 was found as salt tolerant (83%) at 3000 ppm NaCl. This reduction in seed germination caused by decreasing water content, a decreasing in the internal osmotic potential of germinating structures, which was limiting the hydration of seeds (Bliss *et al*., 1986). Reduction in seed germination by increasing salinity levels has been reported by numerous studies (Tsegay and Gebreslassie, 2014; Sharma and Vimala, 2016).

On the other hand, data presented in Table (2) showed that salinity significantly decreased shoot length and root length of the three lentil cultivars. Giza-9 had the tallest height in shoot length (3.7 cm) under normal condition and (1.7 cm) at 3000 ppm, while Giza-370 cultivar had the shortest height (1.2 cm) at 3000 ppm NaCl. Root length in Giza-9 insignificantly increased from 3.6 cm under

normal condition to 3.9 cm at 1000 ppm then significantly decreased to 1.7 cm at 3000 ppm NaCl. The maximum reduction in root length was observed in Giza-51 (1.3 cm) and Giza-370 (1.2 cm) at 3000 ppm NaCl. Salinity caused decrease of shoot and root growth by reducing turgor in expanding tissues resulting from lowered water potential in root growth medium. Furthermore, the toxic effect of the salt on the embryo and cell membranes of endosperm is one of the most important causes of decreased growth of shoot and root (Rehman *et al*., 1996).

Table 2: Effect of salinity on germination percentage and growth parameters of three lentil cultivars

Salinity NaCl (ppm)	Cultivar	Germination %	Shoot length (cm)	Root length (cm)	Seedling length (cm)	Seedling dry weight (mg)	Seedling vigor index
Control	Giza-9	98	3.7	3.6	7.3	156	715.4
	Giza-51	100	3.1	3.9	7.0	146	700.0
	Giza-370	92	3.3	2.9	6.2	143	570.4
1000	Giza-9	95	3.4	3.9	7.3	147	693.5
	Giza-51	88	2.7	2.0	4.7	120	413.6
	Giza-370	90	3.1	3.3	6.4	141	576.0
2000	Giza-9	93	3.3	2.2	5.5	140	511.5
	Giza-51	85	2.1	1.5	3.6	111	306.0
	Giza-370	82	2.5	2.7	5.2	126	426.4
3000	Giza-9	83	1.7	1.7	3.4	106	282.2
	Giza-51	76	1.5	1.3	2.8	98	212.8
	Giza-370	70	1.2	1.2	2.4	80	168.0
L.S.D $_{0.05}$		1.94	0.22	0.39	0.54	8.2	50.30

Results presented in Table (2) showed that increasing salinity stress led to decrease in seedling dry weight and seedling vigor index in all cultivars. The highest value of seedling dry weight was recorded by Giza-9 (156 mg) under normal condition, while the lowest value was observed in Giza-370 (80 mg) at 3000 ppm NaCl. Under normal condition, seedling vigor index in Giza-9 (715.4) and Giza-51 (700) was higher than Giza-370 (570). Under salinity stress, the highest value of seedling vigor index was (693.5) occurred at 1000 ppm NaCl in Giza-9, whereas the lowest value was (168) in Giza-370 followed by (212.3) in Giza-51 at 3000 ppm NaCl. Salinity reduced photosynthesis, which in turn limited the supply of carbohydrates needed for growth (Da Silva *et al*., 2011). Our results for reduction of seedlings growth of all lentil cultivars with increase in NaCl concentration are in conformity with those obtained by Abd El-Monem (2008). Azene *et al*. (2014) who found that salt stress by NaCl delays germination rate and decreases germination percentage, shoot and root length, seedling shoot and root weight of lentil cultivars.

Effect of salinity on total chlorophyll content:

The effect of salt stress by NaCl on total chlorophyll content in leaves of three lentil cultivars is shown in Figure (1). Total chlorophyll content was significantly decreased with increasing salinity levels. Giza-9 has the highest content of total chlorophyll at 2000 ppm NaCl compared to other cultivars. Also, at the highest level of salinity, total chlorophyll content in Giza-9 (0.352 mg/g F.W) was higher than Giza-51 (0.302 mg/g F.W) and Giza-370 (0.248 mg/g F.W). Salinity stress by NaCl caused increasing of free radicals in chloroplasts and destruction of chlorophyll molecules by Reactive Oxygen Species (ROS), which results in reduction of photosynthesis and growth (Zhang *et al*., 2003). Also, the reduction in chlorophyll content may be related to increase the enzyme activity of chlorophyll (Mishra and Sharma, 1994). Moreover, ion accumulation in leaves, especially chloride and sodium, affect chlorophyll biosynthesis due to its effect on the activity of iron-containing enzymes (Munne-Bosch *et al*., 1999).These results are in line with those obtained by Abd El-Monem (2008) and El-Hamamsy and Behairy (2015).

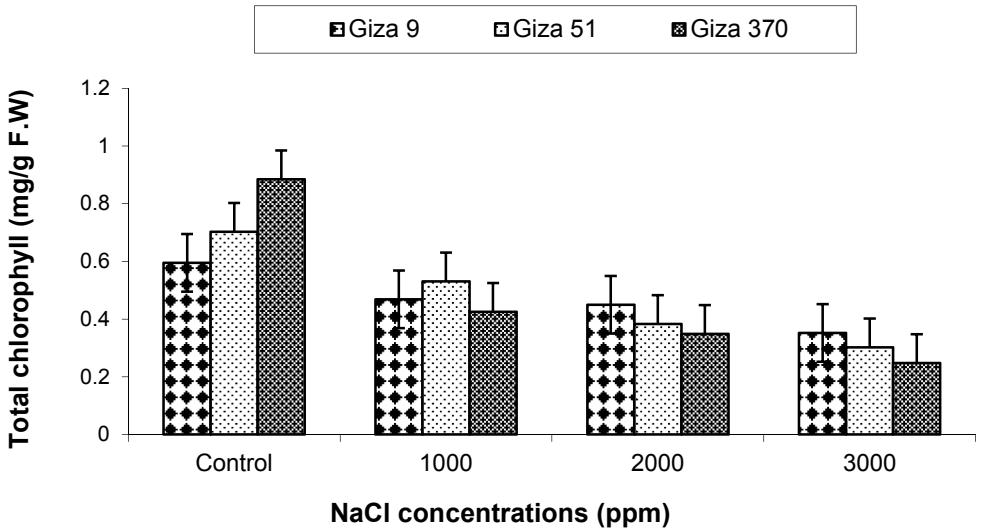

Fig. 1: Effect of salinity by NaCl on total chlorophyll content of three lentil cultivars

Effect of salinity on enzyme activity:

Figures 2, 3 and 4 showed the effect of increasing level of NaCl salinity on catalase (CAT), ascorbate oxidase (AO) and proline dehydrogenase (PDH) activities respectively, in all cultivars of lentil. In Giza-9, CAT activity was significantly increased with increasing of salinity level, while in Giza-51 increased up to 2000 ppm NaCl then decreased at 3000 ppm NaCl. CAT activity in Giza-370 insignificantly increased at 1000 ppm then decreased with increasing of NaCl level. The highest value of CAT activity (35 U/mg F.W) was observed in Giza-9 at 3000 ppm (Fig. 2). Seeds have various protective mechanisms one of which is the enzymatic antioxidative system including catalase (Pour *et al*., 2013). CAT, which is involved in the conversion of hydrogen peroxide into water and oxygen, is the most effective antioxidant enzymes in preventing oxidative damage (Mittler, 2002). Correlation of CAT activity and salt tolerance exists in plants under salt stress (Parida and Das, 2005).

On the other hand, Giza-370 has the highest value of AO activity (131 U/mg F.W) at 2000 ppm then significantly decreased to (83 U/mg F.W) at 3000 ppm NaCl. AO activity in Giza-9 significantly decreased to (23 U/mg F.W) at 1000 ppm then increased to (71 U/mg F.W) at 3000 ppm as compared to control (Fig. 3). Ascorbic acid (AsA) is oxidized to monodehydroascorbate (MDHA) and then to dehydroascorbate (DHA) in the process of enzymatic and non-enzymatic ROS scavenging reactions (Chen *et al*., 2003). Kwon *et al*. (2003) reported that the decreasing of DHA/AsA ratio lead to enhanced tolerance to NaCl. De Tullio *et al*. (2004) suggested that the function of ascorbic oxidase is likely to be related to oxygen management, rather than ascorbic acid consumption. The highest values of PDH activity (63 U/mg F.W) was recorded in cultivar Giza-370 under normal condition. At salinity of 1000 ppm, PDH activity of Giza-370 was higher than Giza-51 and Giza- 9, while at 3000 ppm , PDH activity in Giza-9 was higher than Giza-51 and Giza-370 (Fig. 4). Proline catabolism is catalyzed by proline dehydrogenase and pyroline-5-carboxylate dehydrogenase (Peng *et al*., 1996). A cycle of proline synthesis and its degradation is essential for buffering cellular redox potential in the cytosol as well as in plastids. Redox cycling is also important in plant antioxidant defense mechanisms under stress conditions (Hare *et al*., 1998 and Alia *et al*., 1991). The present results indicated that increasing of salinity level by NaCl on lentil cultivars caused the increase of enzymes activity. Also, Giza-9 has the highest value of CAT and PDH activity at the highest level of salinity. High enzyme activity under salinity stress can prevent damage and correlate with the plant resistance to that stress (Bernardi *et al*., 2004). Shalata *et al*. (2001) demonstrated that salt-tolerant species increase their antioxidant enzyme activities and antioxidant contents in response to salt stress, while salt-sensitive species failed to do so. These are consistent with the results reported by Behairy *et al*. (2012) and Aflaki-Manjili *et al*. (2012).

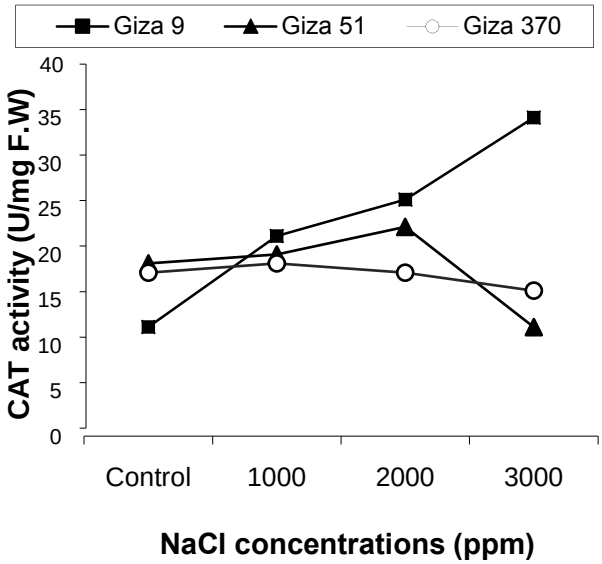

Fig. 2: Effect of salinity on CAT activity of three lentil cultivars

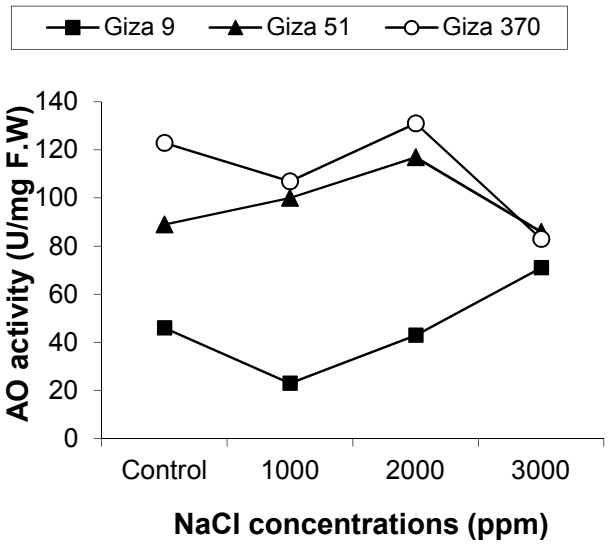

Fig. 3: Effect of salinity on AO activity of three lentil cultivars

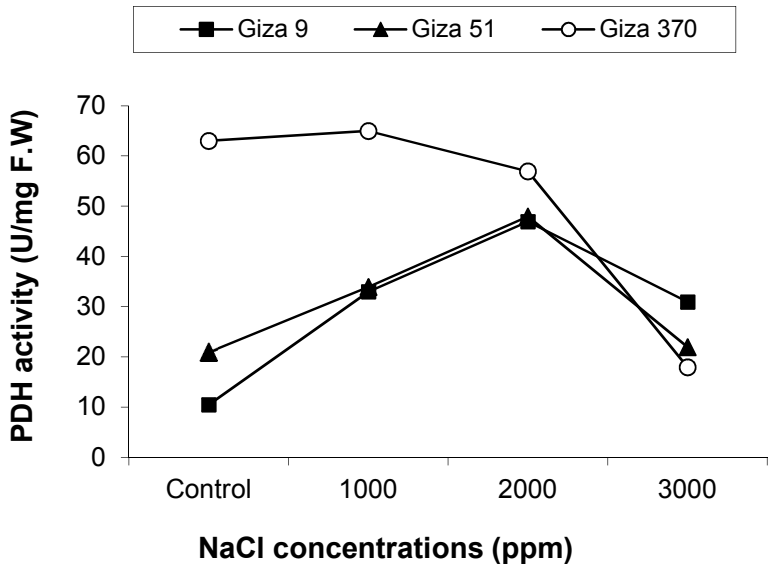

Fig. 4: Effect of salinity on PDH activity of three lentil cultivars

Effect of salinity stress on isozymes:

The electrophoretic profiles of Catalase (CAT), Peroxidase (POX) and Esterase (EST) isozymes were detected in leaves of lentil cultivars under salinity stress (Tables 3, 4 and 5). CAT isozyme bands at Rf 0.841 and 0.906 were present in the three lentil cultivars under control and salinity stress. At 1000 ppm NaCl, band at Rf 0.366 disappeared in Giza-9 and Giza-51, while appeared in Giza-370. Salinity treatments at 2000 ppm NaCl induced the appearance of new isozyme band at Rf 0.513 in Giza-370 as compared with the control. CAT isozyme band at Rf 0.513 was present in Giza-9 under control and 3000 ppm, while it was missing in Giza-51 and Giza-370 at the same level of salt stress (Table 3). In a similar way, POX isozyme band at Rf 0.671 appeared in shoot of the three lentil cultivars under control and all levels of NaCl. Salinity at 2000 ppm induced the appearance of new isozyme band at Rf 0.812 in Giza-9 and Giza-51, while it was missing under control, 1000 and 3000 ppm NaCl. POX isozyme band at Rf 0.355 was not present in Giza-51under control and the different

NaCl levels. At 3000 ppm NaCl, band at Rf 0.218 appeared in Giza-9 whereas disappeared in Giza-51 and Giza-370 (Table 4).

Table 3: Catalase isozymes

Rf of visible bands	Control			1000 ppm NaCl			2000 ppm NaCl			3000 ppm NaCl		
	Giza- 9	Giza-51	Giza-370	Giza- 9	Giza-51	Giza-370	Giza- 9	Giza-51	Giza-370	Giza- 9	Giza-51	Giza-370
0.366	+	+	+	-	-	+	+	+	+	+	+	+
0.513	+	-	-	-	-	-	-	-	+	+	-	-
0.841	+	+	+	+	+	+	+	+	+	+	+	+
0.906	+	+	+	+	+	+	+	+	+	+	+	+

Table 4: Peroxidase isozymes

Rf of visible bands	Control			1000 ppm NaCl			2000 ppm NaCl			3000 ppm NaCl		
	Giza- 9	Giza-51	Giza- 370	Giza- 9	Giza-51	Giza- 370	Giza- 9	Giza-51	Giza- 370	Giza- 9	Giza-51	Giza- 370
0.218	-	-	-	+	+	-	+	+	+	+	-	-
0.355	+	-	-	+	-	-	-	-	+	-	-	-
0.671	+	+	+	+	+	+	+	+	+	+	+	+
0.812	-	-	-	-	-	-	+	+	-	-	-	-

Table 5: Esterase isozymes

Rf of visible bands	Control			1000 ppm NaCl			2000 ppm NaCl			3000 ppm NaCl		
	Giza- 9	Giza-51	Giza- 370	Giza- 9	Giza-51	Giza- 370	Giza- 9	Giza-51	Giza- 370	Giza- 9	Giza-51	Giza- 370
0.172	+	+	+	+	-	+	+	+	+	+	+	+
0.315	+	+	+	+	+	+	+	-	+	+	+	+
0.553	+	+	+	-	-	-	-	-	-	-	-	-
0.821	+	+	+	+	+	+	-	+	-	-	-	-
0.882	+	+	+	+	+	+	+	+	+	+	+	+

Rf = retention factor band present = + band absent = -

On the other hand, EST isozyme band at Rf 0.882 was found in all the treatments of lentil cultivars. EST isozyme band at Rf 0.821 disappeared at 3000 ppm NaCl in all lentil cultivars as compared with control. Also isozyme band at Rf 0.553 disappeared under the different NaCl levels as compared with control. EST isozyme bands at Rf 0.172 and 0.315 were missing in Giza-51 at 1000 and 2000 ppm NaCl, respectively while, appeared in Giza-9 and Giza-370 in all treatments (Table 5). These results indicated that Giza-9 had the highest CAT and POX isozyme bands as compared with other cultivars. The induction of new isozymes bands in shoots of lentil cultivars under salinity stress may be considered as an indicator for salt tolerance in lentil. Isozymes and enzyme activities that provide a certain degree of tolerance to salt stress can be useful in understanding salt tolerance (Mhadhbi *et al*., 2011).These results are in harmony with those obtained by Vardhini and Rao (2003) who found that CAT activity decreased in maize varieties but enhanced in resistant varieties as compared to unstressed plants. Gao *et al*. (2008) found that high peroxidase isozyme activity was detected in salt-tolerant cultivars compared to salt-susceptible cultivar of Jatropha curcas seedlings.

Conclusion

In summary, exposure of lentil cultivars to salinity stress by different levels of NaCl caused reduction in seed germination, seedling growth and changed in metabolic activity such as chlorophyll content, enzyme activity and isozymes profile of all lentil cultivars. The present study indicates that Giza-9 was more tolerant, Giza-51 was moderate and Giza-370 was the least salt tolerant.

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