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Glutathione Induced Antioxidant Protection Against Salinity Stress in Chickpea (*Cicer arietinum* L.) Plant

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A POT experiment was conducted to evaluate the possible role of foliar treatment of glutathione in enhancing the antioxidant defense system of chickpea plant. Different concentrations (0, 50, 100 and 150 mg/L) of glutathione were used; namely GHS0, GHS1, GHS2 & GHS3, respectively under different levels of seawater salinity (0.23, 3.13 and 6.25 dS/m namely S0, S1 & S2, respectively). Seawater levels, S1& S2 caused obvious increases in proline, free amino acids and total soluble sugar contents. Moreover, foliar application of glutathione caused more significant increases in the above mentioned osmoprotectant contents of chickpea plants, as compared with those of corresponding controls. In addition, irrigation of chickpea plant with different levels of diluted seawater significantly increased hydrogen peroxide and lipid peroxidation, as compared with the tap water irrigated plants. Meanwhile, treating chickpea plants with different concentrations of glutathione resulted in significant decreases in hydrogen peroxide contents and lipid peroxidation levels in the control and salinity stressed plants. The applied concentrations of glutathione also resulted in marked increases of the antioxidant enzymes ascorbate peroxidase, glutathione reductase, peroxidase and superoxide dismutase. Maximum increases in the activities of antioxidant enzymes were observed on treating plants with glutathione at 100 mg/L either under normal irrigation or salinity stressed conditions. It could be concluded that foliar spray of glutathione was effective in improving chickpea performance by reducing hydrogen peroxide free radical and by enhancing compatible osmolytes, membrane stability and antioxidant enzyme activities.

Keywords: Chickpea, Glutathione, Salinity, Osmolytes, Mineral ions, Antioxidant enzymes,

Introduction

Chickpea plant (*Cicer arietinum* L.) is a popular crop in arid and semiarid areas and one of the important legumes cultivated under salinity stress conditions (Rao, et al., 2002 and Ahmed et al., 2016). Chickpea plant is particularly sensitive to salinity (Flowers et al., 2010). Chickpea plant is one of the largest legume crops in the world as being with highly nutritional value because it is rich in protein so, it is an important source of protein for human (Zaccardelli et al., 2013).

All over the world, abiotic stresses are considered as main causes of crop loss. Abiotic stresses such as salinity, drought or high temperature adversely affect plant growth and yield. Salinity is one of the serious abiotic stresses causing serious decline in the production of different plants (Sadak & Dawood, 2014). Saline water as seawater was considered unsuitable for plant irrigation but recently, it could be used for irrigation under certain conditions (Zeid, 2011). Salinity stress causes many changes in different metabolic and biochemical processes in plant cells, depending on the severity and the duration of this stress, thus finally results in decline of different crop production. Osmotic stress is the first effect that represses plant growth followed by ion toxicity (James et al., 2011). Reactive oxygen species (ROS) increased under different abiotic stresses thus could alter normal cellular metabolic activities via oxidative damage to nucleic acids, proteins and lipids (Imalay, 2003). The degree of damage resulted from the increased levels of ROS depends on the balance among the production of ROS and its removal via antioxidant scavenging

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mechanisms. So, plants have developed a complex defensive antioxidant system for mitigating the oxidative damages caused by increased levels of ROS. This system includes low molecular mass antioxidants and antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR) and the non-enzymatic antioxidants as ascorbate, glutathione and phenolic compounds (Hossain et al., 2013).

The production of different types of osmolytes is another mechanism developed to increase plant tolerance under stressed conditions. These osmolytes or osmoprotectants have an important function in lowering the osmotic potential to maintain cell turgid by increasing absorption of more water molecules. Soluble sugars, proline, glycinebetaine and trehalose are among different osmoprotectant compounds in plant cells. These compounds are highly soluble in water, low molecular weight and nontoxic compounds. These compatible solutes increase plant tolerance at salt stress via many ways as, protecting chloroplast and cytoplasm from sodium injury and as ROS scavengers (Smirnoff & Cumbes, 1989), stabilizing and protecting proteins (Bohnert & Jensen, 1996), adjustment of the osmotic balance and general improving stability of different physiological processes of plants under stressful conditions (Dawood & Sadak, 2014).

To increase plant tolerance to different abiotic stresses, different treatments with naturally occurring compounds in plant cells are used as foliar applications. Glutathione is a powerful antioxidant responsible for balances between oxidation and antioxidation, regulates many of the cell functions such as repair and synthesis of DNA and proteins, and in addition regulates plant enzymes. Glutathione also protects cells from free radicals and peroxides (Pompella et al., 2003) and is important for stress management. It is the main component of the glutathione–ascorbate cycle that decreases hydrogen peroxide (Noctor & Foyer, 1998).

So, this study is an attempt to improve the tolerance of chickpea (*Cicer arietinum* L.) plant under the conditions of salt stress, through improving some physiological and biochemical processes by using the naturally occurring substance glutathione.

Materials and Methods

Experimental procedure

Pot experiments were done at the greenhouse of National Research Centre, Dokki, Giza, Egypt, at the winter seasons of 2014/ 2015 and 2015/ 2016 to study the effect of foliar spray of glutathione on chickpea plant grown under saline conditions.

Chickpea (*Cicer arietinum* L.) cv. Giza 1 seeds were obtained from Agricultural Research Centre, Ministry of Agriculture and Land Reclamation, Egypt. Seeds were washed with distilled water, sterilized with 1% sodium hypochlorite for about 2 min, then washed again with distilled water. Ten chickpea seeds were sown along a center row per plastic pot containing 7 kg soil. Clay soil was mixed with yellow sand in a proportion of 3:1 in order to improve drainage. Ten days after sowing, the seedlings were thinned to three seedlings per pot. Experimental design was complete randomized blocks with three levels of glutathione (0, 50, 100 & 150 mg/L) and named as GSH0, GSH1, GSH2 and GSH3. Spraying of chickpea plants with glutathione was done at 45 and 60 days from sowing before the flowering stage. Tap water 0.23 dS/m and two diluted seawater levels 3.13 and 6.25 dS/m, were used and expressed as S0, S1 and S2, respectively. Levels of saline water were prepared by mixing tap water (0.23 dS/m) with seawater (51.2 dS/m) to obtain salinity levels of 3.13 and 6.25 dS/m. Analysis of the used water and soils are shown in Table1.

At 75 day plant age, some biochemical parameters such as lipid peroxidation (MDA), hydrogen peroxide content (H_2O_2), and the activities of some antioxidant enzymes were estimated in fresh plant leaves.

Biochemical analyses

Proline was extracted and calculated according to Bates et al. (1973). Free amino acids were extracted according to Vartainan et al. (1992) and determined with the ninhydrin reagent method (Yemm & Cocking, 1955). Total soluble sugars were extracted according to Homme et al. (1992) and assayed according to Yemm & Willis (1954). Hydrogen peroxide content was estimated using the method of Velikova et al. (2000). Malondialdhyde contents were measured according to Hodges et al. (1999).

TABLE 1: Analysis of irrigation water and soils.

Treatments	EC (dSm^{-1})	pH	Cationsmeq L^{-1}				Anions meq L^{-1}		
			Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	HCO_3^-	SO_4^{2-}	Cl^-
Tap water	0.23	7.39	1.0	0.57	2.48	0.24	0.11	1.31	2.7
Seawater	51.2	7.79	43.26	15.11	454.57	1.53	6.07	76.36	432
Sandy soil	0.13	8.16	2.67	2.39	1.31	0.24	1.12	4.21	0.7
Clay soil	1.48	7.67	5.75	1.98	5.97	0.37	1.51	6.76	5.51

Enzyme extracts were prepared according to Chen & Wang (2006). GR (EC 1.6. 4.2) activity was measured according to Lee & Lee (2000). APX (EC 1.11.1.11) activity was estimated as described by Nakano & Asada (1987). POX (EC 1.11.1.7) activity was evaluated according to Kumar & Khan (1982). SOD (EC 1.12.1.1) activity was calculated as described by Chen & Wang (2006). Macro element contents of chickpea plant were determined according to Chapman & Pratt (1978). N and P were determined using Spekol Spectrocololourimeter VEB Carl Zeiss. Ca, K and Na contents were estimated by using of flame photometer. Mg contents were calculated by atomic absorption spectrophotometer.

Statistical analysis

Data of the two seasons were statistically analyzed on complete randomized design system according to Snedecor & Cochran (1990). Means ± standard error (n = 3) were compared at P = 0.05 (Duncan, 1955).

Results

Proline and amino acid contents

The results presented in Fig. 1 clearly show the effect of chickpea plant treatments with different glutathione concentrations (GSH0, GSH1, GSH2 & GSH3) under tap water (S0) and different saline water levels. Irrigation with seawater levels S1& S2 caused marked increases in proline as well as free amino acids of chickpea leaves compared to the tap water irrigated plants S0 (control plant). Moreover, foliar treatment with glutathione (50, 100 & 150 mg/L); caused obvious increases in proline and free amino acids of treated chickpea plants, compared with the untreated plants irrigated with different seawater concentrations. The application of glutathione at the concentration of 100 mg/L resulted in maximum elevation of free amino acids as well as proline contents of chickpea plant under the studied salinity levels.

Total soluble sugar (TSS) contents

TSS contents of chickpea leaves of the control and foliar glutathione treated plants under salinity stress are presented in Fig. 1. The data showed that irrigation of chickpea with seawater (S1& S2) led to elevation of TSS of chickpea leaves, as compared to those irrigated with tap water S0 (control plants). Glutathione treatment markedly increased TSS accumulation either in the tap water or seawater irrigated chickpea plants.

Hydrogen peroxide (H_2O_2)

Variations in the hydrogen peroxide (H_2O_2) levels under salinity stress are presented in Fig. 2. H_2O_2 sharply increased in chickpea leaves by increasing the salinity level used. The H_2O_2 level increased by 38% in S1 and 92% under S2 salinity level, compared with that in the tap water irrigated plants (S0). Meanwhile, glutathione foliar treatment caused marked reductions in the levels of H_2O_2 in the chickpea plants grown under saline or tap water conditions, compared to corresponding controls. Glutathione at 100 mg/L was the most efficient concentration in decreasing H_2O_2 levels under normal and salinity conditions.

Lipid peroxidation

Foliar treatment with glutathione affected lipid peroxidation of chickpea plants grown under salinity stress as presented in Fig. 2. Salinity induced oxidative damage could be assessed by gradual elevation in lipid peroxidation contents (MDA contents) in the saline stressed plants, as compared with the control plants. Glutathione foliar treatment caused marked decrease in lipid peroxidation, especially at the GSH2 followed by GSH3, then GSH1 treatment was approximately comparable to the so untreated plants under different salinity levels (S0, S1& S2).

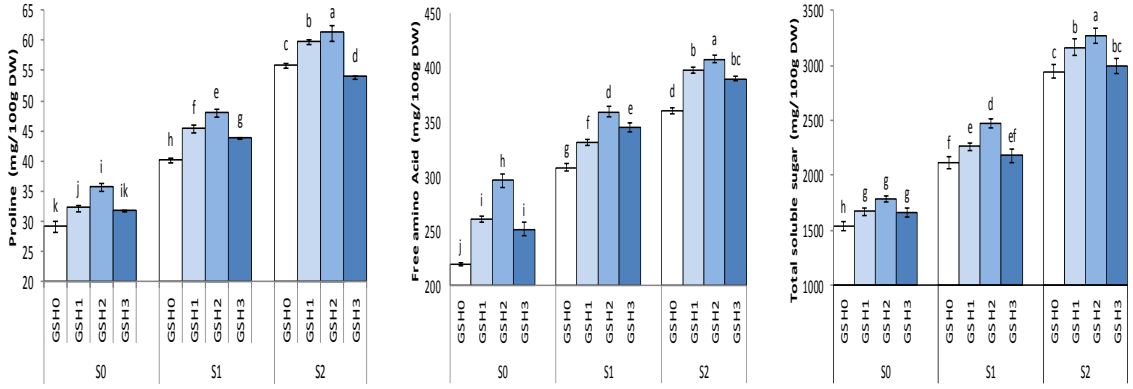

Fig. 1. Effect of glutathione (0, 50, 100 & 150 mg/ L) on proline, free amino acids and total soluble sugars (mg/100g DW) in leaves of chickpea plants (75 day-old) under salinity levels S0, S1& S2 (0.23, 3.13 & 6.25 dS/m, respectively). Each value is a mean of 3 replicates± SE. Bars with different letters indicate significantly different values at P 0.05.

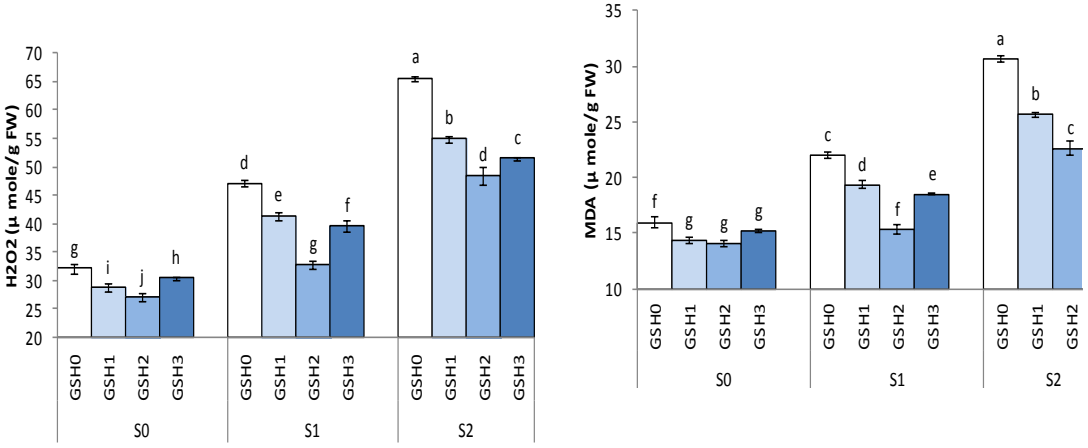

Fig. 2. Effect of glutathione (0, 50, 100 & 150 mg/l) on hydrogen peroxide (H_2O_2) and MDA (µ mole/g FW) in leaves of chickpea plants (75 day-old) under salinity stress levels S0, S1& S2 (0.23, 3.13 & 6.25 dS/m, respectively). Bars with different letters indicate significantly different values at P 0.05.

Antioxidant enzymes

The activities of various antioxidant enzymes in the seawater stressed plants and glutathione treated chickpea plants are presented in Fig. 3. Diluted seawater caused significant increases in the activities of GR, APX, POX and SOD. Glutathione foliar treatment with different concentrations increased the plant tolerance to stress via enhancing the studied antioxidant enzyme activities among the studied groups (S0, S1& S2). Application of 100 mg/L glutathione showed the highest stimulation in activities of chickpea plants. Moreover, this concentration gave higher stimulations in the four antioxidant enzymes under study under tap water irrigation (S0).

Mineral contents

Table 2 shows the changes in mineral ion contents of chickpea plants in response to glutathione treatment under different levels of diluted seawater. Irrigation of chickpea plant with different salinity levels caused a gradual significant decrease in the contents of potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{++}), phosphorus (P^{3+}), nitrogen (N) as well as the K^+/Na^+ ratio. In the same time, sodium contents increased significantly under salinity. The different applied concentrations of glutathione could improve the deleterious effect of salinity through increasing both the mineral ion (K, Ca, Mg, P, N) contents and the K^+/Na^+ ratios while, Na contents were decreased.

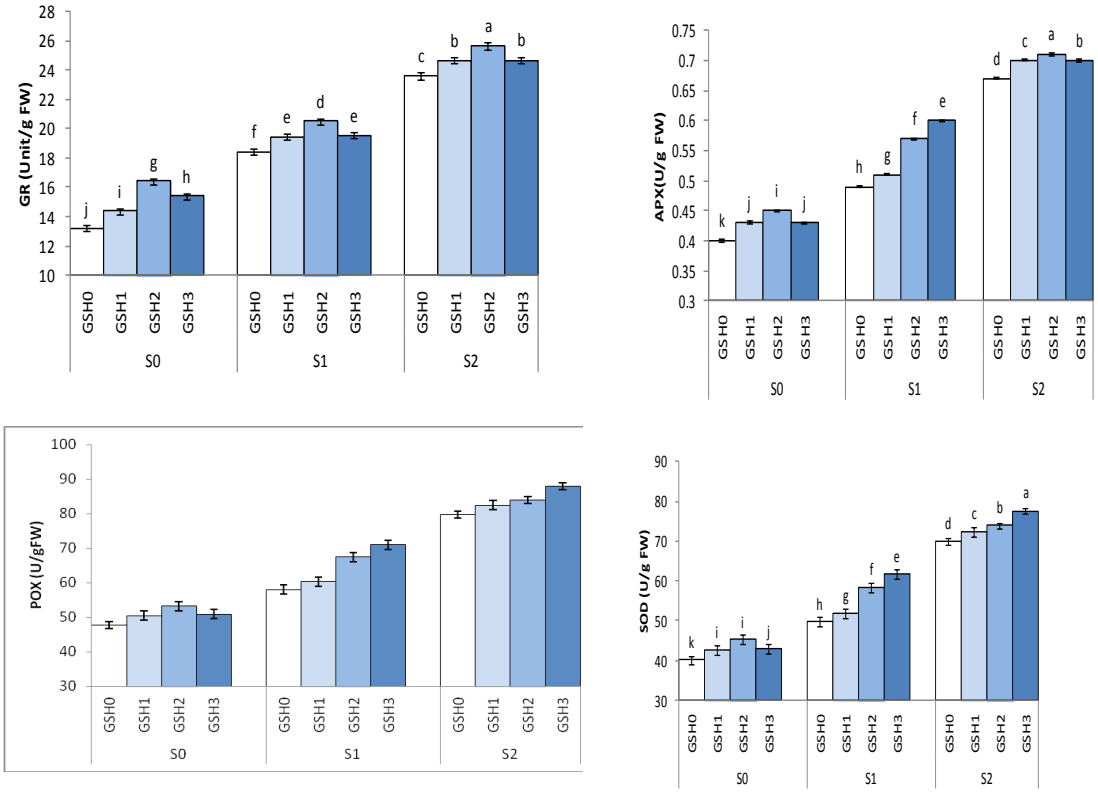

Fig. 3. Effect of glutathione (0, 50, 100 & 150 mg/l) on the activities of the antioxidant enzymes glutathione reductase (GR), ascorbate peroxidase (APX), peroxidase (POD) and superoxide dismutase (SOD) in leaves of chickpea plants (75 day-old) under salinity stress levels S0, S1& S2 (0.23, 3.13 & 6.25 dS/m, respectively). Each value is a mean of 3 replicates± SE. Bars with different letters indicate significantly different values at P 0.05.

TABLE 2. Effect of foliar spraying of glutathione (GSH) at different concentrations (0, 50, 100 & 150 mg/L) on mineral ion contents (K, Ca, Mg, P, N, Na and K/Na ratio) (mg/100g DW) of leaves of chickpea plants (75 day old) under three salinity stress levels (0.23, 3.13 & 6.25 dS/m) abbreviated as S0, S1& S2, respectively.

Salinity	GHS Conc.	Na^+	K^+	Ca^{2+}	Mg^{2+}	P^{3+}	N	K/Na
S0	0	184.287^{d}	1114.21^{d}	1979.00^{c}	173.66^{bc}	230.03^{de}	3723.83^{bc}	6.05^{bc}
	1	179.15^{d}	1181.52^{c}	2190.33^{b}	182.33^{b}	226.85^{e}	3881.01^{ab}	6.60^{b}
	2	174.02^{d}	1364.82^{a}	2307.33^{a}	199.33^{a}	220.35^{ef}	3991.30^{a}	7.84^{a}
	3	182.23^{d}	1221.31^{b}	2199.00^{ab}	191.00^{a}	230.75^{de}	3823.38^{b}	6.70^{b}
S1	0	216.11^{b}	1055.37^{f}	1660.66^{d}	152.66^{d}	273.65^{b}	3559.03^{d}	4.88^{e}
	1	196.60^{c}	1097.70^{e}	1879.66^{c}	171.66^{bc}	248.95^{cd}	3638.85^{c}	5.58^{cd}
	2	188.90^{cd}	1125.22^{d}	1977.66^{c}	169.66^{c}	239.20^{d}	3692.77^{bc}	5.96^{c}
	3	200.54^{c}	1092.76^{e}	1872.66^{dc}	164.66^{c}	253.93^{c}	3653.88^{c}	5.45^{cd}
S2	0	248.45^{a}	930.48^{h}	1439.00^{f}	131.00^{e}	314.60^{a}	3377.00^{e}	3.75^{f}
	1	199.17^{c}	1007.11^{g}	1550.33^{ef}	142.33^{de}	252.20^{c}	3476.20^{d}	5.06^{d}
	2	176.07^{d}	1054.10^{f}	1767.33^{d}	159.33^{cd}	222.95^{e}	3553.32^{d}	5.99^{c}
	3	199.17^{c}	1018.96^{g}	1658.66^{e}	150.66^{d}	252.20^{c}	3575.87^{cd}	5.12^{d}

Within the same column means with different superscripts are significantly differ (at $P \leq 0.05$).

Discussion

To avoid the harmful effect of osmotic stress resulted by salinity stress, plants evolved several techniques; one of these techniques is to increase the osmoprotectants (Abdel Latef & Miransari, 2014). In this study, an increased levels of proline, free amino acids and total soluble sugars were recorded in chickpea leaves of the plants subjected to salinity. Regarding to proline and free amino acids, it is well known that, accumulation of compatible osmolytes at high concentrations causes osmotic adjustment of plants under salinity stress. These increases due to salinity stress might be due to modulated amino acid metabolism. Proline has also a vital role in osmotic adjustment, stabilization and protection of enzymes, proteins and membranes from the harmful effects of salinity-osmotic stress (Ashraf & Foolad, 2007). This is in alliance with our results where glutathione treatment caused more accumulation of proline and free amino acids levels in chickpea plant. The increased level of proline in chickpea plant might be due to enhanced biosynthesis in a way or another via increasing key enzymes of proline biosynthesis (Amini & Ehasanpour, 2005).

In the present work, the enhancement of total soluble sugars under salinity treatments agreed with the previous results of Liu et al. (2016) in Nitraria tangutorum and (Bakhoum & Sadak, 2016) in *Helianthus annuus* L. The enhancement effect of salinity on total soluble sugars could be due to the improving effect of TSS on adjusting turgidity of cells and alleviating the resistance to water uptake by root (Bartels & Sunkar, 2005). Moreover, (Marschner, 1995) concluded that, the main solutes used in osmotic adjustment in many crops subjected to salinity stress are organic acids such as soluble sugars. The accumulation of total soluble sugars as a result of foliar treatment with glutathione might be due to a protective role on the photosynthetic system. However, our obtained results are consent with those of Kattab (2007) in two cultivars of canola plant. In addition, a protective role of glutathione on *Calendula officinalis* under salinity conditions was achieved via adjusting of redox system (Chaparzadeh et al., 2004).

Chickpea plants responded to salinity stress by inducing marked increase in H_2O_2 and MDA contents and this may be due to inadequate induction of antioxidant system (Hossain et al., 2013). MDA is considered as the potential biomarker of membrane lipid peroxidation in the cellular environment (Faried et al., 2016). Salinity stress caused changes in physical membrane organization and modification in the lipid matrix of the plasma membrane (Weisany et al., 2012). However, foliar application of glutathione lowered H_2O_2 production, and reduced MDA content. This might be achieved via glutathione-mediated direct ROS scavenging, antioxidative mechanism involved in eliminating ROS or stabilizing of membrane via its effect on antioxidant enzymes and/or increased contents of endogenous glutathione (Salama & Al-Mutawa, 2009). In addition, the effect of glutathione on stabilization of membrane permeability was concomitant with a decreased passive Na^+ influx (Foyer & Noctor, 2005).

In this investigation, our obtained data showed significantly increased activities of the studied antioxidant enzymes as GR, APX, POX and SOD of chickpea plant, as compared with those of corresponding control plants. These enzymes are responsible for ROS-scavenging (Sadak & Abdelhamid, 2015). The charged $\hat{O}_2$ molecule is impermeable through phospholipid membranes of the cell, thus SOD have the ability to remove $\hat{O}_2$ free radicals from different components of the cell. In our study, glutathione treatments enhanced SOD activity, thus stimulated the conversion of superoxide radical to hydrogen peroxide; a step important in protecting the cell. Similar results were obtained by Li et al. (2008) and Akladious & Abbas (2013). In the present work, POX was assumed to scavenge H_2O_2 so its activity was increased in response to salinity stress and glutathione treatment increased POX activity as well. The increased activities of the different studied enzymes (SOD, POX, APX and GR) in this work were concomitant with the increase in proline contents under salinity stress. Glutathione treatment enhanced the activities of these enzymes, as compared with those of the corresponding controls. The increased activities of enzymes in chickpea plant under irrigation with diluted seawater might be mainly attributed to its resistance.

The contents of potassium, calcium, magnesium, phosphorus, nitrogen as well as the K^+/Na^+ ratio was decreased with increased sodium contents in chickpea plants irrigated with diluted seawater levels, as compared with those irrigated with tap water. The mineral ion contents decreased but Na^+ content increased with salinity. The enhancement of Na^+ content under salinity stress in addition to the reduction

of other mineral contents was confirmed by Sadak & Abd Elhamid (2013) in flax plant and Rady et al. (2015) on soybean. Munns (2002) concluded that, salinity stress could affect plant by three means, namely water deficiency stress through decreasing water potential in the root, phyto-toxicity of Na^+ and Cl^{-1} and nutrient imbalances resulted from their improper uptake. In addition, Na^+ ions compete with K^+ ions for the binding sites necessary for biochemical activities. The increased levels of Na^+ with the reductions in K^+ contents, in response to salinity stress, results in marked reductions in K^+/Na^+. These could lead to disturbances in the accumulation of Na^+ in plant organs (Rady et al., 2015). This enhancement effect on Na^+ ion contents due to salinity stress was accompanied with decreases in phosphorus and potassium ions contents and this result might be due to the antagonism of phosphorus and potassium ions versus sodium ions (Alam, 1994). Foliar application of glutathione might alleviate the decreasing effects of salinity stress on ion concentrations through increases in K^+, Ca^{2+}, Mg^{2+}, P^{3+}, N as well as K^+/Na^+ ratio, as compared with untreated controls. Thus, the positive effects of glutathione treatments might have been arisen via improving the osmotic tolerance and/or regulation of the biochemical processes under study. The positive role of GSH might be also due to its effect in increasing membrane permeability (Kattab, 2007).

Conclusions

Foliar application of glutathione at different concentrations (50, 100 & 150 mg/L) could alleviate the harmful oxidative stress produced by irrigation of chickpea plant with two diluted seawater levels (3.13 and 6.25 dS/m). This appeared likely to take place via enhancing osmoprotectant compounds as proline, free amino acids and TSS. Meanwhile, glutathione treatments decreased H_2O_2 and MDA contents accompanied with increasing activities of antioxidant enzymes; (GR, APX, POX and SOD) and mineral ion contents. Finally, we recommend the use of 100 mg L^{-1} glutathione as foliar treatment to improve chickpea performance under diluted seawater irrigation conditions (EC < 6.25 dS m^{-1}).

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الجلوتاثيون يستحث الحماية بمضادات الأكسدة ضد الملوحة في نبات الحمص

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أجريت هذه التجربة لتقييم الدور المحتمل للجلوتاثيون في استحثاث نظام الدفاع المضاد للأكسدة في نبات الحمص. وقد استخدمت تركيزات مختلفة من الجلوتاثيون (50, 100, 150 ملجم/لتر) تحت تاثير مستويات مختلفة من ملوحة مياه البحر هي 0.23 و 3.13 و 6.25 دي اس/ ام وسميت اس0, اس1 واس2. وقد تسببت المستويات المختلفه من ملوحه مياه البحر في زيادة ملحوظه في البرولين والأحماض الأمينية الحرة والسكريات الكلية الذائبة. وعلاوة على ذلك، تسبب الرش الورقي للجلوتاثيون في زياده كبيره في المعايير موضع الدراسه والمسئوله عن الحمايه الأسموزيه في نبات الحمص مقارنه بنباتات الكنترول المناظره. وبالإضافة إلى ذلك، تسبب ري نبات الحمص بمستويات مختلفة من مياه البحر المخففة في ارتفاعات ملحوظة فى فوق أكسيد الإيدروجين وفوق أكاسيد اللبيدات مقارنة بالنباتات التي تم ريها بمياه الصنبور. وفي الوقت ذاته أدت معالجة نبات الحمص بتركيزات مختلفه من الجلوتاثيون إلي انخفاض كبير في فوق أكسيد الإيدروجين وفوق أكاسيد اللبيدات في كل من النباتات التي تم ريها بمياه مالحه او مياه الصنبور. وقد نتج عن استخدام تركيزات مختلفة من الجلوتاثيون زيادة ملحوظة في أنشطة الإنزيمات المضادة للأكسدة (أسكوربات بيروكسيديز ,خازل الجلوتاثيون، البيروكسيديز و سوبراكسيد ديسميوتيز). وقد لوحظت زيادة كبيره في أنشطة الإنزيمات المضادة للأكسدة في النباتات المعالجه بمركب الجلوتاثيون بتركيز 100 ملغ / لتر سواء تحت الري العادي أو الملوحة. ويمكن أن نخلص إلى أن رش الأوراق بالجلوتاثيون كان فعالا في تحسين أداء الحمص عن طريق الحد من شوارد فوق أكسيد الإيدروجين الحره وتحفيز الأيضيات المنظمه للاسموزيه وثبات الغشاء الخلوي وزيادة أنشطة الإنزيمات المضادة للأكسده.