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ORIGINAL ARTICLE

Osmotic adjustment in wheat flag leaf in relation to flag leaf area and grain yield per plant

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Background

Salinity stress causes ion toxicity and osmotic imbalances, leading to oxidative stress in plants. Antioxidants are considered ameliorators of saline stress and could develop salinity tolerance in crop plants. To ascertain the role of antioxidants in inducing osmotic adjustment in salt stressed wheat flag leaf in terms of compatible solutes accumulation, water relations parameters and osmotic adjustment as well as flag leaf area and grain yield per plant, in addition, flag leaf anatomy were examined.

Results

Salt stress up to 11.5 dSm^{-1} causes a significant reduction in water potential, osmotic potential, as well as relative water content, and water content. On the other hand, turgor potential and osmotic adjustment were significantly increased due to inducing increasing the higher accumulation of compatible osmolytes which leads to decreasing flag leaf area and grain yield per plant.

Application of both antioxidants, in particular, ascorbic acid increased significantly flag leaf area, and grain yield per plant due to osmotic adjustment and maintaining leaf turgor potential as a consequence of increasing leaf water potential, water content and relative water content as compared to control plants. On the other hand, application of both antioxidants under all salinity levels, nullify the harmful effects of salinity on flag leaf area and grain yield per plant due to increasing osmolyte accumulation, maintaining turgor potential and osmotic adjustment.

Anatomically, increasing salinity levels decreased thickness of leaf blade at midrib region, thickness of mesophyll tissue, tangential dimension of midrib vascular bundle, thickness of upper and lowerepidermis, thickness of big motor cell, and tangential dimension of big xylem vessel. Treatment with either ascorbic acid or tocopherol at 100 mg/L and their interactions with salinity increased all the above mentioned parameters in both nonsalinized and salinized plants. Ascorbic acid is the most effective in this concern.

Conclusion

In conclusion, wheat plants responded to an increased ion influx in their cells by increasing the osmolytes synthesis and accumulation under salt stress, which further increased with antioxidants treatment and helped in maintaining the osmotic balance.

Key words: Ascorbic, tocopherol, soil salinity, yield, wheat

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Soil salinization is one of the major factors of soil degradation. It has reached 19.5% of the irrigated land and 2.1% of the dry-land agriculture existing on the globe. Salinity problem considered a

significant factor affecting plant production and agricultural sustainability in many regions of the world as it reduces the value and productivity of the affected land. In most saline soils Na^+ and Cl^- are the dominant ions, and usually they exceed by far the plant demand/necessity. The excess of soluble salts in the root environment alter the aqueous and ionic thermodynamic equilibrium, which results in hyper-osmotic stress, ionic imbalance and toxicity (Munns 2002). As a result of these changes, the activities of various enzymes and the plant metabolism are affected (Munns 2002, Lacerda et al 2003). The maintenance of turgor has been reported to be essential for keeping a normal cell activity and contribute to growth under low water availability. Osmotic adjustment (OA) has been reported to contribute to maintain the turgor pressure (Martınez et al 2004) and has drawn much attention during the last years. It has been hypothesized that these compounds benefit stressed cells in two ways: by acting as cytoplasmic osmolytes, thereby facilitating water uptake and retention, and by protecting and stabilizing macromolecules and structure (i.e. proteins, membranes, chloroplast, and liposomes) from damage induced by stress conditions (Martınez et al 2004).

Osmotic adjustment (OA) is usually achieved by uptake of inorganic ions i.e. K^+, Na^+ and Cl^- from the soil solution or synthesizing and accumulation of organic compounds as sugars, and amino acids, mainly proline (Farouk 2005). Energy is needed for the synthesis or transport of solutes for osmotic adjustment (Munns 2002). Taking into consideration energy efficiency, it is predicted that the accumulation of ions, which is not needed in the metabolism and is of low molecular weight, is efficient for the OA, and that the ions can be accumulated quickly in response to osmotic stress (Raven 1985). However, the excessive accumulation of ions may disrupt the balance of the absorption and the function of other ions in the cell.

In mature leaf, OA plays an important role for plant cell survival, facilitative higher stomatal conductance and leaf expansion (Westgate and Boyer 1985) to sustain photosynthesis under stress conditions. It is accepted that during osmotic adjustment the cells tend to compartmentalize most of the absorbed ions in vacuoles at the same time that they synthesize and accumulate compatible organic solutes in the cytoplasm in order to maintain the osmotic equilibrium between these two compartments (Hasegawa et al 2000). As a consequence of solute accumulation, the osmotic potential of the cell is lowered, this, in turn, attracts water into the cell and, thereby, tends to maintain its turgor. In fact, OA is an effective component of salt tolerance, which has a positive direct or indirect effect on plant productivity, because it contributes to the maintenance of turgor and cell volume (Ludlow and Mu-Chow 1990). The reaction of different wheat cultivars to salt stress with respect to accumulation organic and inorganic solutes is different. In addition, there are some constricting reports regarding to the pattern of these osmotica and their contribution to osmotic adjustment.

Natural osmoprotectant concentrations in cytoplasmic compartments are osmotically significant because they have pivotal roles in maintaining cell turgor and the driving gradient for water uptake under stress (Rontein et al 2002), allowing physiological processes, such as stomatal opening, photosynthesis and cell expansion (Serraj and Sinclair 2002). In addition to their role in cell water relations, organic solutes accumulation may also help towards the maintenance of ionic homeostasis and of the C/N ratio, removal of free

radicals, and stabilization of macromolecules and organelles, such as proteins, protein complexes and membranes (Bray et al 2000). In plant the major compatible osmoprotectant solutes are glycinebetaine and proline (Misra and Gupta 2005) are thought to function as osmoprotectants for protein (Bohnert and Jenson 1996) these solutes also provide a protective environment for enzymes and macromolecular structure and function. The contributory role of osmoprotectants i.e. glycinebetaine and proline to osmotic adjustment under salt stress was confirmed by several investigations (Yeo 1998, Meloni et al 2001), but the significance of its osmotic adjustment is still in debate and varies according to the species. Hence, improvement of crop performance by increasing osmotic potential-adjusting ability might be more significant in increasing plant growth and yield. Osmotic adjustment may be achieved by application of some osmoprotectants, ions, plant growth substances and finally by antioxidants, but there are few report in this respect. OA leads to better extraction of water from the soil, maintain the volume of protoplast and turgor pressure, stimulates root growth (Leport et al 1999) and facilitates a better translocation of pre-anthesis carbohydrates reserves to the grain during the grain filling periods (Subbarao et al 2000b). Additionally, there is a positive relationship between OA and grain yield in water-deficit environments (Blum et al 1999). Many reports regard OA to be a causal mechanism favoring crop productivity under salinity stress. However, there are also conflicting reports indicating a negative relationship between OA and seed yield under stress condition (Subbarao et al 2000a). Other reports indicate no relationship between OA and growth and/or seed yield under stress condition (Tangpremsri et al 1995). Thus, OA as an adaptation mechanism for salinity resistance is somewhat debatable and requires further analysis.

Recently, application of antioxidants has been reported to successfully mitigate the adverse effects of salinity on plants (Beltagi 2008). Of these, application of antioxidants has recently gained a ground as a very promising means of mitigating the adverse effects of salt on plant growth and metabolism (Shalata and Neumann 2001). Although the role of antioxidants in improving plant water relations and osmotic adjustment is very rare. Ascorbic acid (AsA) is exceptional antioxidants that react with oxidizing agents much more readily than anything else and mops them up before they have a chance to damage anything (Foyer 1993). It is a strong reducatant that services in cells as electron donor, reducing and thus, running the risk of many different compounds (Smirnoff 1996). Much evidence has suggested that AsA affects biosynthesis, levels and signaling of many phytohormones including ethylene, gibberellic acid and abciscic acid. Therefore, AsA has proposed roles in regulating many physiological and developmental processes including photosynthesis, cell division and growth, flowering and senescence (Barth et al 2006). Many studies indicate that foliar application of AsA exerted positive effects on leaves content of photosynthetic pigments, growth and yield of many plants (Singh et al 2001, Talaat 2003).

Tocopherols are believed to protect chloroplast membranes from photo-oxidation and help to provide an optimal environment for the photosynthetic machinery. Most of proposed α-tocopherols functions are related to their antioxidant properties, the most prominent of which is protection of polyunsaturated fatty acids from lipid peroxidation by quenching and scavenging various reactive oxygen radicals. Also, in plants, tocopherol

levels vary in different tissues and fluctuate during development and in response to a biotic stress. Tocopherols and its effect on growth and metabolism of plants and its role in amelioration of plants against stresses were studied by many authors i.e. Jahnke and White (2003) and Hussein et al (2007).

In spite of these controversies, osmotic adjustment is receiving increasing recognition as a major plant acclimatization mechanism to salt stress (Misra and Gupta 2005). Several ions, amino acids, quaternary amines, organic acids, and sugars were found among the solutes that accumulate during osmotic adjustment of salt stressed plants (Meloni et al 2001). Antioxidants can play an important role in the development of salt tolerance in crops. However, there is little information about the role of antioxidants on regulation of osmotic adjustment processes in plants under normal or salinized condition. Keeping in view the above reports on the role of exogenous antioxidants on wheat cultivar there is a need for better understanding of antioxidants mechanism of action and the magnitude of its effects in wheat plant to improve crop stress tolerance. Thus, the main objective of the present study was to test the hypothesis that are the application of antioxidants improving wheat grain yield under salinity is due to improvement plant water relations and osmotic adjustment processes.

MATERIALS AND METHODS

Fifteen uniform wheat grains (Giza 168 cultivar) were sown on 10th November, 2006 and 15th November, 2007 (First and second season, respectively), in closed-bottom plastic pots containing 15 kg clay loam soil, (containing 0.786 meq/100g soil sulphate, 0.27 meq/100g bicarbonate, 0.51 meq/100g chloride, 0.38 meq/100g calcium, 0.60 meq/100g magnesium, 0.006 meq/100g potassium and 0.45 meq/100g sodium) with or without additional salinity. Soils were salinized prior to sowing by adding sodium chloride (NaCl) solution to adjust salt concentrations to 0.12, 0.35 and 0.70 % NaCl of oven dry soil. Actual salinity levels expressed as ECe (dSm^{-1}) were determined at three times before and during cultivation. The means of salinity levels in soil were 0.8, 7.5 and 11.5 dSm^{-1}.

Two weeks after sowing, the seedlings were thinned to 10 uniform seedlings per pot. Phosphorous and potassium fertilizers were added to the soil before sowing at the rate of 5 g P_2O_5 per pot in the form of calcium super phosphate (15.5% P_2O_5) and 2 g K_2O per pot in the form of potassium sulphate (48%). Ammonium nitrate (33.5%) was added at the rate of 4 g N/pot in two equal portions; the first during the seedling stage and the second at the appearance of the flag leaf. At 40 days from sowing, the pots at each salinity levels were divided into three groups. The first group was sprayed twice with water (control), while the other two groups were sprayed twice (i.e. after 40 and 50 DFS) with aqueous solutions of either ascorbic or α-tocopherol at the rate of 100 mg/l until run-off, with Tween 20 as a wetting agent. At heading (65 DFS), three randomly selected plants were harvested per pot and then removed for determination of flag leaf area and biochemical constituents. Flag Leaf Area (cm^2) which was calculated by the following formula; a = L x W x 0.75 (Gardner et al 1985).

Flag leaf Water relations parameters and osmotic adjustment: Quantification of flag leaf water status was made by measuring the leaf water relations parameters; water content (WC), relative water content (RWC), water potential (WP), osmotic potential (OP), turgor potential, and osmotic

adjustment (OA) during the crop productive phase at early flowering (65 DAS). Water content was determined according to Fernandez-Ballester et al (1998). Meanwhile the relative water content (RWC) was determined, briefly, flag leaf discs were weighted to obtain fresh weight (FW). The plant materials were floated in distilled water inside a closed Petri dish and determined the turgid weight (TW), and then the plant materials were placed in a pre-heated oven at 80 ^{o}C for determination dry weight (DW). RWC (%) = {(FW-DW)/ (TW-DW)} x100. Leaf WP (Ψw) was determined according to the method of Taiz and Zeiger (1998). A proportion of the same leaves used for water potential, was divided into two portion, the first portion composed have of lamina (without the midrib vine) that used for determination of osmotic pressure, meanwhile the second portion having lamina with midrib vein that used for measuring osmotic pressure at turgidity. Osmotic potential (Ψ0) was determined using total soluble solids percentage (TSS) in leaf sap using hand refracto-meter and the corresponding values of water potential were then obtained from tables given by Gossev (1960). The leaves were directly taken from different treatments, immediately frozen for 2 weeks, after which time plant material was thawed and the frozen sap was extracted in the laboratory by crushing the material with pestle. After filtration, the sap was directly used for osmotic pressure determination through determination TSS values then converted to OP from Gossev table. The remaining half from different treatments was immediately placed in a suitable container, with distilled water for 12h. The sap was then extracted in the laboratory. The TSS at full turgor converted to OP from Gossev table. The osmotic adjustment (OA) is determined using the following equation according to Kiani et al (2007) OA=ΨFT (ww)- ΨFT (ws), where ΨFT (ww) is osmotic pressure at full turgor of unstressed plants and ΨFT (ws) is osmotic pressure at full turgor of stressed plants or antioxidant treated plants. Turgor potential (Ψp) was calculated as the difference between leaf water potential and osmotic potential values.

Total free amino acids were extracted and determined according to the modified method of Dubey and Rani (1989a, b). Proline was determined by the modified ninhydrine methods of Magne and Larher (1992). Plant materials were placed into test tube containing distilled water. The tubes were kept for 30 min in a boiling water bath then cooled at room temperature. To 150 µL of the corresponding water extract, 1 ml of ninhydrine reagent was added and maintained in a boiling water bath for 20 min. the mixture was cooled and the product formed was extracted with toluene. Absorbance was measured at 520 nm on a spectrophotometer. Glycinebetaine content (GlyBet) content was estimated by the method of Grieve and Grattan (1983). Oven dried leaves were finally ground with deionized water at 100 0C for 60 min. GlyBet concentration was determined spectrophotometrically (Spekol-11) at 365 nm. Total water soluble organic acids (TWSOA) extraction was performed according the methods of Huang and Redmann (1995) using water: methanol/chlorophorm (2:1): water, and Chloroform in the ratio of 1.1:3.5:1.2:1.2. Following extraction for 12 h, the extract was separated by filtration. The organic acids in the supernatant were aspirated into covered vials and determined by titration with 0.005 N NaOH using 0.04% aqueous bromothymol blue as an indicator which became green at pH 7. Total soluble sugars extracted by Ethanol and then determined by phenol-sulphoric acid methods as described by Sadasivam and Manickam (1996). Flag

leaf sample from the 2nd season were taken 65 DAS. The samples were killed and fixed in formalin-acetic-alcohol for 48 h, then dehydrated in a series of ethanol and embedded in paraffin wax (52-54oC melting points). Sections were made at 15-17 µm thick using rotary microtome, stained with erythrosin/crystal violet and mounted in Canada balsam, then examined microscopically for determining the anatomical changes in leaves.

Statistical analysis: The data were analyzed following Analysis of Variance (ANOVA) technique and mean separations were adjusted by the Multiple Comparison test (Norman and Streiner 2003) using the statistical computer programme MSTAT-C v.1.2. Means were compared by using LSD test at 5% level of significance.

Table 1a. Total free amino acids (mg/g FW), proline (mg/g FW), glycinebetaine (mg/g FW), total soluble sugars (mg/g FW) and total water soluble organic acids (mg/g FW) in wheat flag leaf as affected by soil salinity.

Salinity (dSm^{-1})	Total Free Amino Acids		Proline		Glycinebetaine		Total Soluble Sugars		Total Water Soluble Organic Acids	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
0.8	85.578	85.567	13.541	13.309	1.353	1.556	19.778	19.778	39.622	39.722
7.5	94.282	93.532	17.197	17.020	3.482	3.256	26.116	26.437	53.211	51.656
11.5	106.47	105.66	20.463	20.146	5.341	5.236	31.017	31.176	62.833	62.044
LSD (0.05)	2.347	1.546	0.715	0.562	0.310	0.177	0.855	0.526	2.971	1.965

Table 1b. Total free amino acids (mg/g FW), proline (mg/g FW), glycinebetaine (mg/g FW), total soluble sugars (mg/g FW) and total water soluble organic acids (mg/g FW) in wheat flag leaf as affected by antioxidants application.

Antioxidants	Total Free Amino Acids		Proline		Glycinebetaine		Total Soluble Sugars		Total Water Soluble Organic Acids	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
Water	91.820	91.640	15.226	15.240	2.806	2.701	22.484	22.696	47.178	45.778
AsA	100.80	98.881	18.490	17.917	3.984	3.838	28.911	28.672	56.378	54.711
Toc	93.707	94.236	17.486	17.390	3.387	3.508	26.114	26.022	52.011	52.933
LSD (0.05)	2.345	1.544	0.712	0.564	0.310	0.176	0.856	0.527	2.974	1.966

RESULTS

Organic solutes accumulation:

Significant differences were observed among the salt treatments for total free amino acids (TAA), proline (Pro), and glycinebetaine (GlyBet), total soluble sugars (TSC), and Total water soluble organic acids (TWSOA) accumulation under salinity levels up to 11.5 dSm^{-1} in the soil. Data presented in Table (1a, b) indicate that, wheat plants under salt-stressed conditions responded to an increased ion influx in their cells by increasing the synthesis and accumulation of flag leaf organic solutes i.e TAA, Pro, GlyBet, TSC and TWSOA in comparison with the control, which further increased with applications of either AsA or Toc under normal or salinity conditions and helped in maintaining the osmotic balance and thus helped in enhanced salt

tolerance. The maximum concentration of organic solutes was recorded with the application of AsA and Toc combined with high salinity levels in comparison with antioxidants alone. AsA was more effective than Toc in this concern (Figure, 1).

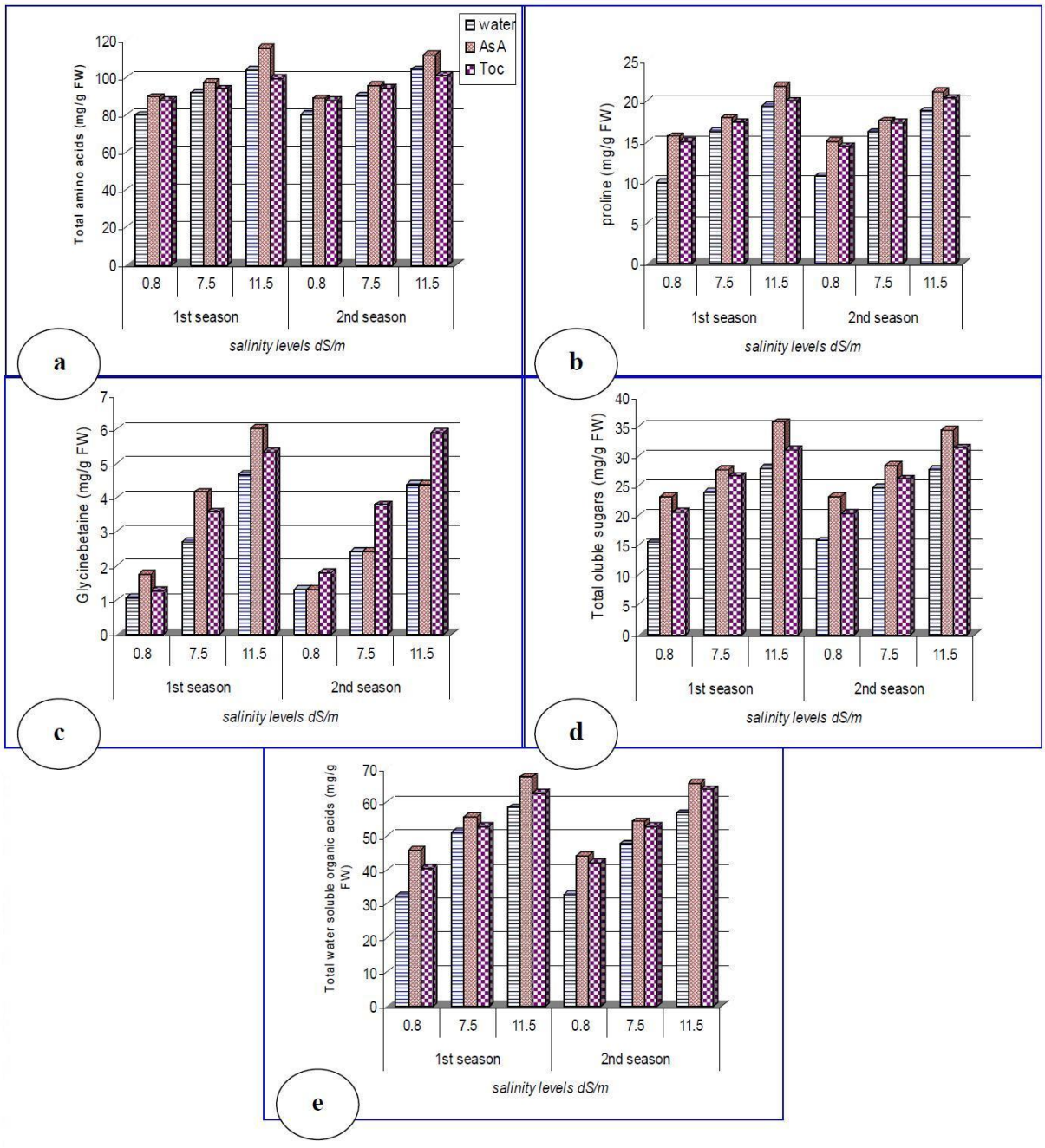

Figure 1. Total free amino acids (a), proline (b), glycinebetaine (c), total soluble sugars (d) and total water soluble organic acids (e) in wheat flag leaf as affected by the interactions between soil salinity and antioxidants in the two growing seasons

Table 2a. Leaf water potential (-MPa), leaf osmotic potential (-MPa), leaf turgor potential (MPa), osmotic adjustment (MPa), relative water content(%) and water content (%) in wheat flag leaf as affected by soil salinity.

Salinity (dSm-1)	Leaf Water Potential		Leaf Osmotic Potential		Leaf Turgor Potential		Osmotic Adjustment		Relative Water Content		Water content	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
0.8	-0.231	-0.228	-0.657	-0.649	0.426	0.426	0.074	0.092	87.03	83.48	87.53	88.69
7.5	-0.310	-0.316	-0.777	-0.759	0.449	0.428	0.192	0.187	78.17	77.84	85.30	85.23
11.5	-0.346	-0.350	-0.889	-0.923	0.544	-0.573	0.256	0.257	70.08	68.70	79.96	76.36
LSD (0.05)	0.0162	0.010	0.0162	0.0054	0.043	0.0354	0.0073	0.024	0.74	1.90	2.228	0.95

Table 2b. Leaf water potential (-MPa), leaf osmotic potential (-MPa), leaf turgor potential (MPa), osmotic adjustment (MPa), relative water content(%) and water content (%) in wheat flag leaf as affected by antioxidants application.

Antioxidants	Leaf Water Potential		Leaf Osmotic Potential		Leaf Turgor Potential		Osmotic Adjustment		Relative Water Content		Water content	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
Water	-0.318	0.315	-0.721	-0.741	0.403	0.426	0.122	0.132	69.59	71.70	80.97	78.36
AsA	-0.279	-0.286	-0.830	-0.817	0.533	0.518	0.218	0.213	84.65	80.90	86.38	87.51
Toc	-0.290	-0.293	-0.772	-0.772	0.482	0.479	0.182	0.192	81.04	77.43	85.45	84.41
LSD (0.05)	0.0101	0.010	0.0195	0.0093	0.0407	0.0333	0.0069	0.0231	0.713	1.87	2.16	0.92

Leaf water relations parameters:

Water status is highly sensitive to salinity and is, therefore, dominant in determining the plant responses to stress. Progressively increasing salt stress up to 11.5 dSm^{-1}, affected all water relations parameters significantly (Table 2). Both water potential, (Ψ_w) and osmotic potential, (Ψ_s), decreased significantly (became more negative) progressively with increasing salt stress, therefore, the values were the lowest at high salinity level. Likewise, water content, (WC), and relative water content, (RWC), decreased with increasing salinity levels. The decrease was more pronounced in high salinity level. Osmotic adjustment (OA) capacity and leaf turgor potential, (Ψ_p) of wheat flag leaf increased significantly with decreasing Ψ_w regardless of stress levels up to 11.5 dSm^{-1} (Tables 2).

Either AsA or Toc foliar spray increased (less negative values) leaf water potential, water content and relative water content in flag leaf area as compared with unsprayed plants, meanwhile decreased osmotic potential in flag leaf. Osmotic adjustment increased significantly in flag leaf with application of both antioxidants due to maintaining turgor potential of leaf. AsA was more effective than Toc in increasing leaf turgor under normal and saline conditions. However, the magnitude of OA increased as the water deficit intensified as a result of decreasing Ψ_w (Tables 2).

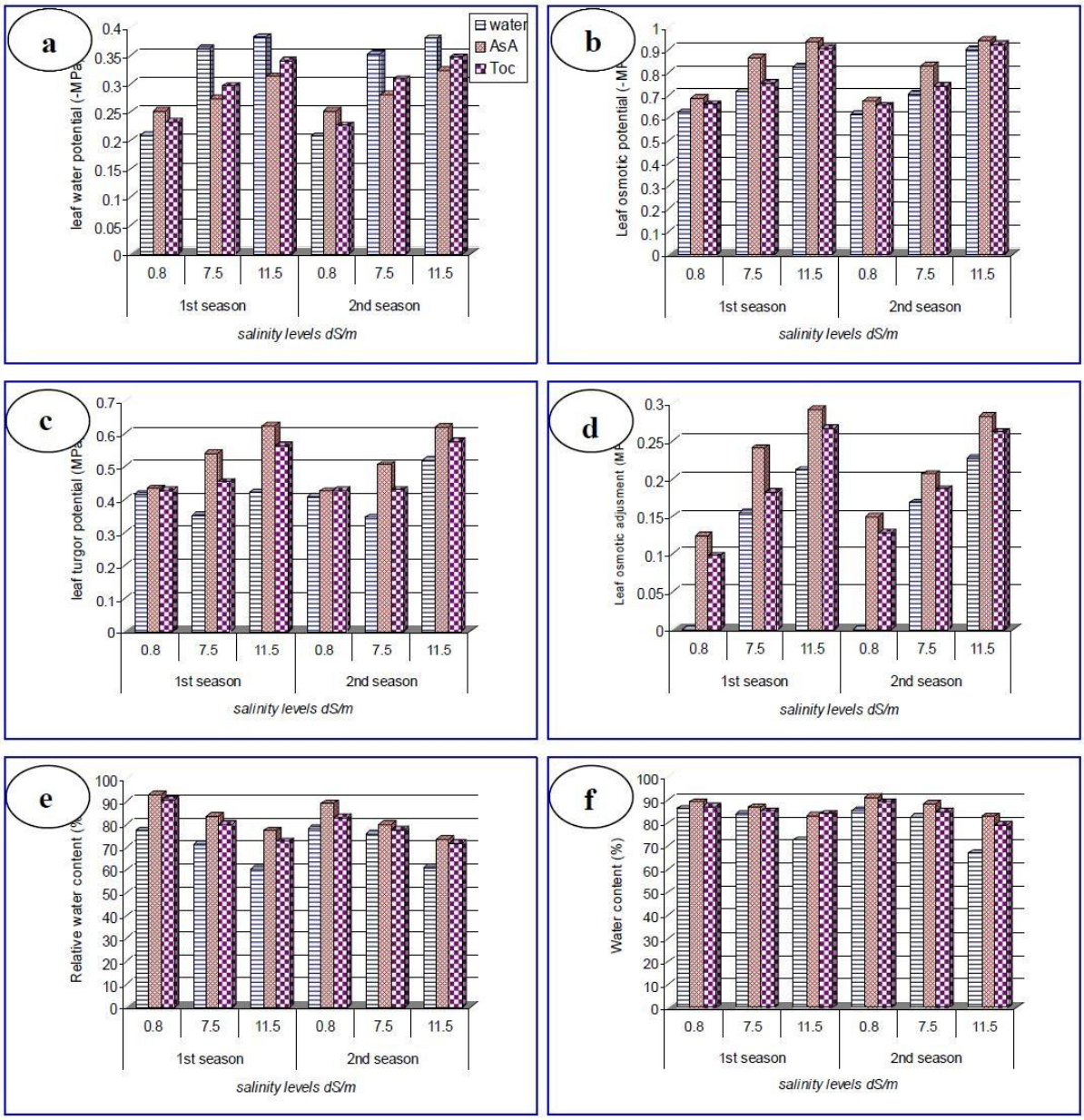

Figure 2. Leaf water potential (-MPa), leaf osmotic potential (-MPa), leaf turgor potential (MPa), osmotic adjustment (MPa), relative water content(%) and water content (%) in wheat flag leaf affected by the interactions between soil salinity and antioxidants in the two growing seasons

Table 3. leaf anatomical characteristics of flag leaf of wheat plants 65 DAS as affected as affected by salinity or antioxidants as well as their combinations

Treatment		Thickness of upper epidermis (UE)		Thickness of lower epidermis (LE)		Thickness of big motor cell (BMC)		Thickness of leaf through midrib (TL)		Tangential dimension of midrib vascular bundle (TDMVB)		Tangential dimension of big xylem vessel (TDBXV)		Thickness of mesophyll tissue (MT)	
Salinity (dSm^{-1})	Antioxidants mg/L	µ	%	µ	100%	µ	100%	µ	100%	µ	100%	µ	100%	µ	100%
Control (0.8)	Water	5.00	100	4.33	100	9.66	100	185.3	100	65.0	100	8.66	100	93.6	100
	AsA	5.66	113.2	5.66	130.7	11	113.87	250.6	135.24	82.6	127.07	10	115.47	111.6	116.12
	Toc	5.66	113	5	115.4	10.66	110.35	234	126.28	73.0	112.30	9.66	111.54	105.6	112.82
7.5	Water	4.33	86.6	3.66	84.52	7.66	79.29	122.3	66.00	54.6	84	7.66	88.45	79.0	84.40
	AsA	5.33	106.6	4.66	107.6	10	103.51	214.6	115.81	65.3	100.46	9.00	103.92	101.3	108.22
	Toc	4.66	93.2	4	92.37	9	93.16	151.6	81.81	62.3	95.84	8.00	92.37	86.3	92.20
11.5	Water	2.33	46.6	2	46.18	5	51.75	54.6	29.46	43.0	66.15	6.66	76.90	49.00	52.35
	AsA	3.66	73.2	3.33	76.90	6.66	68.94	118.6	64	53.3	82	7.66	88.45	75.00	80.12
	Toc	3.33	66.6	3	69.28	6	62.11	109.3	58.98	45.3	69.69	7.66	88.45	69.6	74.35

As regard to the interaction between antioxidants and salinity levels, the date in Figure 2 proved that application of antioxidants, in particular, AsA under normal or salinized condition improved flag leaf water status due to decreasing leaf water potential, osmotic potential, and improving osmotic adjustment, and maintaining turgor potential. it is noted that application of both antioxidants, in particular, AsA increased significantly relative water content under control or low salinity level, then decreased under high salinity level. On the other hand water, application of both antioxidants, nullifies the harmful effect of salinity on water content.

Flag leaf structure:

Leaf anatomical characters, such as thickness of upper epidermis (UE), thickness of lower epidermis (LE), thickness of big motor cell (BMC), thickness of leaf through midrib (TL), tangential dimension of midrib vascular bundle (TDMVB), tangential dimension of big xylem vessel (TDBXV), and thickness of mesophyll tissue (MT) of flag leaf were studied. Cross section of wheat flag leaves showed that there were significant changes in leaf anatomical characteristics induced by both antioxidants application. Application of either AsA or Toc increased the thickness of wheat leaf blade respectively, due to the increase in the thickness of mesophyll tissue as well as thickness of both lower and upper epidermis cells. In addition, the thickness of leaf blade through midrib region was also increased respectively, due to the increase in the midrib vascular bundle, as well as tangential and radial dimensions of big metaxylem vessel. Moreover, antioxidants increased the thickness of big motor cells. Antioxidants resulted in increasing the area of xylem and phloem tissues, due to the stimulation of pro-cambium activity in the midrib

bundle during their differentiation. Ascorbic acid was more effective in increasing the thickness of the blade, dimension of xylem and phloem as well as the thickness of the mesophyll tissue (Table 3 and Figure 3).

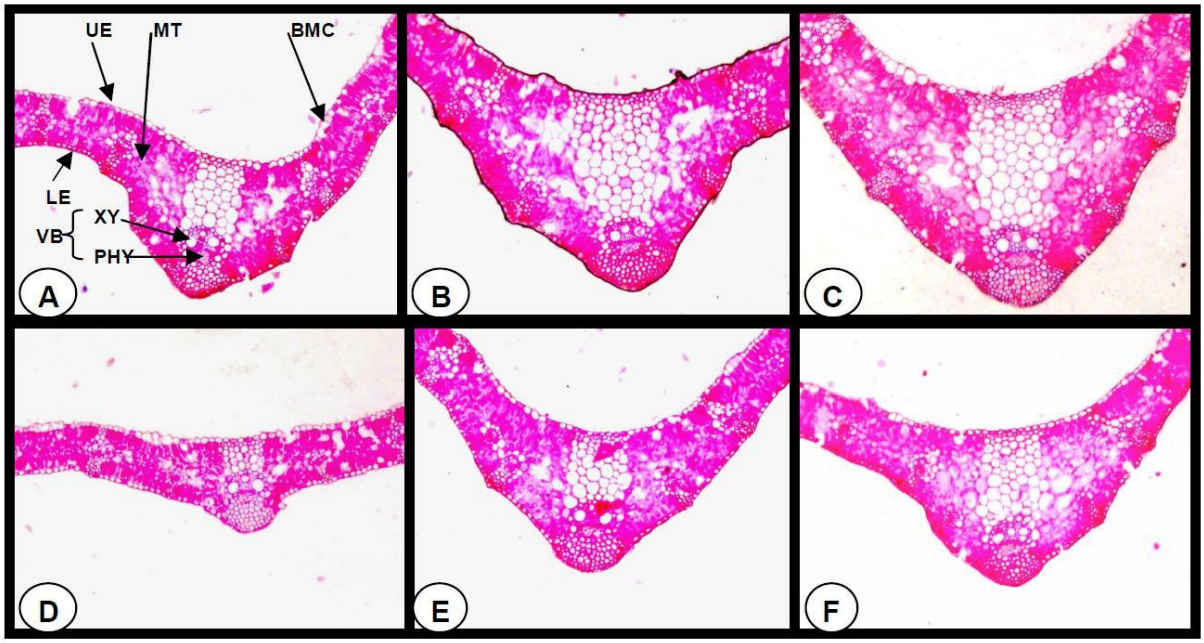

Figure 3. Leaf anatomical characteristics of flag leaf of wheat plants 65 DAS as affected by salinity or antioxidants as well as their combinations

Table 4a. Flag leaf area (cm^2) and grain yield per plant (g) of wheat plant as affected by soil salinity.

Salinity (dSm^{-1})	Flag leaf area (cm^2)		Grain yield per plant (g)	
	1st season	2nd season	1st season	2nd season
0.8	24.910	26.423	8.398	8.220
7.5	20.068	22.222	6.919	6.783
11.5	15.691	15.386	4.768	4.528
LSD at 0.05	0.591	0.930	0.358	0.321

Table 4b. Flag leaf area (cm^2) and grain yield per plant (g) of wheat plant as affected by antioxidants application.

Antioxidant	Flag leaf area (cm^2)		Grain yield per plant (g)	
	1st season	2nd season	1st season	2nd season
Water	16.656	17.773	3.647	5.417
AsA	23.871	25.188	7.572	7.442
Toc	20.117	21.070	6.866	6.672
LSD at 0.05	0.570	0.924	0.360	0.322

Regarding the effect of salinity on flag leaf structure, the thicknesses of wheat flag leaf blade through the midrib region as well as the mesophyll tissue thickness were decreased under salinity levels. In addition, the tangential and dimension of midrib vascular bundle and big metaxylem vessels, and

thickness of xylem and phloem were also decreased. The decrease in mesophyll tissue, xylem and phloem leads to a slow rate on the translocation of photoassimilates towards the developing grains through the peduncle and spike rachilla. Furthermore, the decrease in the diameter of metaxylem vessels in the leaf blade results in lowering the accumulation of necessary water required for photosynthesis (Table, 3 and Figure, 3).

Concerning the interaction between salinity and antioxidants, the interactions increased the wheat flag leaf blade thickness grown under high salinity level. On the other hand, antioxidants used partially overcome the depression effect of high salinity levels on the thickness of the midrib region and mesophyll tissue. Ascorbic was more effective than tocopherol in this concern.

Flag Leaf area and grain yield per plant:

Restriction of leaf growth is among the earliest visible effects if many stress conditions, including salinity. Because leaves determine radiation interception and are the main photosynthetic organs, salinity stress effects on leaf expansion and functions are directly related to yield constraints under saline conditions. It is evident from results presented in Table 4, that increasing NaCl in rooting medium up to 11.5 ds/m had a significant adverse effect on flag leaf area and grain yield per plants. The great reduction in these parameters was observed under high salinity level.

It was noted that application of either AsA or Toc under normal or salinized conditions distinctly increase flag leaf area and grain yield per plant as compared with control plants or untreated plants under such salinity levels (Table 4 and Figure 4). The highest values of both parameters were recorded due to application of ASA. In general, AsA was more effective than Toc in this concern.

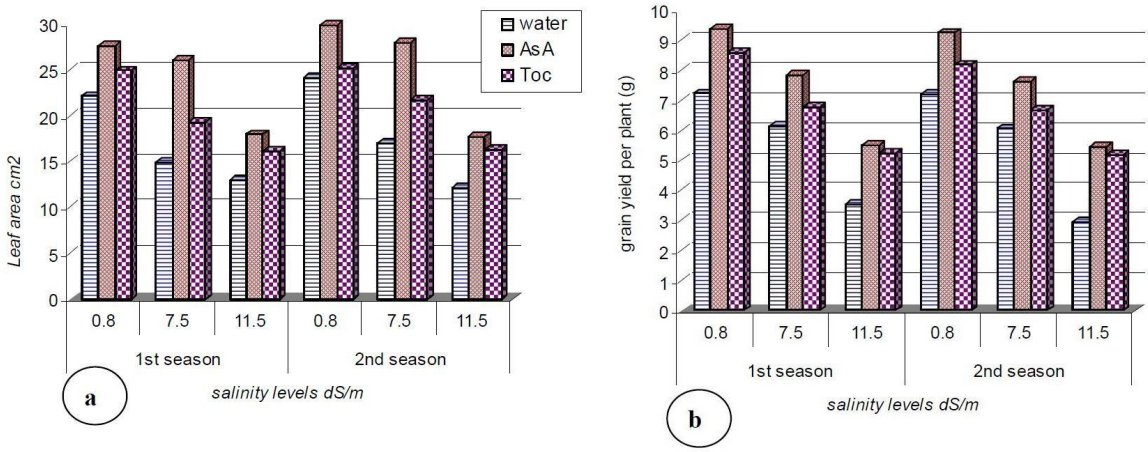

Figure 4. Flag leaf area (a) and grain yield per plant (b) of wheat plant as affected by the interactions between soil salinity and antioxidants in the two growing seasons

DISCUSSION

Salinity stress is the major factor limiting plant growth and productivity (Farouk 2005). The nature of salinity stress was of great importance in the water relations of the wheat plant treated with antioxidants compared with the untreated plant under normal or salinized conditions. These different responses could be due to the fact that

wheat treated with antioxidants has some tolerance-avoiding mechanisms, such as osmotic adjustment (OA), decrease in leaf water potential and decrease in leaf osmotic potential, to maintain their water status at values similar to those of the control plant (Table 2 and Figure 2). When OA has occurred, rigidity of the cell wall is necessary to maintain the cell/tissue integrity. Thus, these two processes allow an increase in the water potential gradient between the soil and plant and improving the water absorption under soil water deficit, so the tissues do not suffer water stress.

OA involves the net accumulation of organic or inorganic solutes/osmolytes; total soluble sugars, total free amino acids, proline, glycinebetaine, sodium, chloride and potassium (Munns 2005, Bandeh-hagh et al 2008) in cells in response to a fall in the water potential of their environment. As a consequence of this net accumulation, the cell osmotic potential is lowered, and turgor pressure tends to be maintained (Blum et al 1996). In the present investigation, salinity treatments markedly reduced the leaf water potential and this change was not compensated for by a reduction in leaf osmotic potential (Table 2). In glycophytes, the concentrations of compatible solutes that accumulate are not so high, on order of 10 mM, but if partitioned exclusively to the cytoplasm, they could generate a significant osmotic pressure and functions as an osmolytes. At low concentrations, these solutes presumably have another role, perhaps in stabilizing the tertiary structure of proteins, and function as osmoprotectants (Chen and Murata 2002). Compatible solutes synthesis comes with energy cost and hence involved a potential growth penalty. In leaf cell, approximately seven moles of ATP are needed to accumulate one mole of NaCl as an osmoticum, whereas the amount of ATP required to synthesis one mole of an organic compatible solute is an order of magnitude higher i.e. 34 for mannitol, 41 for proline, 50 for GlyBet, and approximately 52 for sucrose (Raven 1985). But, the mechanism of leaf turgor maintenance inorganic solute like Cl and Na accumulation could also have negative effects in plants as leaf death. Upon exposure to salinity stress, many plants accumulate organic compatible solutes that are non-toxic at high concentrations; compatible solutes are defined as water-soluble organic compounds of a low molecular weight (termed also as osmoprotectants) (Chen and Murata 2002). It is generally accepted that the increase in cellular osmolarity which results from the accumulation compatible solutes is accompanied by the influx of water into, or at least a reduced efflux from, cells, thus providing the turgor necessary for cell expansion. Water potential, solute potential and turgor potential are inter-related in plant cells and are markedly affected when plants are exposed to salt stress. Although accumulation of organic solutes increased in both non-stressed and stressed plants due to foliar applied antioxidants (Table 2), leaf osmotic potential was not greatly changed due to accumulation of organic solutes. From these finding, it is plausible to propose that changes in organic solutes accumulation caused slight change in leaf osmotic potential which resulted in improved leaf turgor potential and thus contribute in osmoregulatory processes. In the present investigation, foliar application of antioxidants, in particular, AsA, improved leaf water potential and leaf turgor potential, whereas leaf osmotic potential slightly decreased in the stressed plants due to its role in increasing compatible organic solutes and potassium (Table 1).

It is well known from the present investigation that the organic osmolytes were enhanced in

response to NaCl and/or antioxidant treatments, where their interactions had an additive effect. Moreover, the toxic effects generated by sodium chloride were completely overcome by the application of AsA or Toc. Higher osmolytes accumulation, especially proline seems to be related to salt tolerance in wheat not to be a consequence of tissue reaction to salt stress damage. Antioxidants especially AsA appears to confer greater osmoprotectant by the additive role with NaCl in osmolyte accumulation.

It has been reported that free amino acids contribute to osmotic adjustment, but experimental results are inconsistent (Ford 1984). Free amino acids increased due to salt stress in wheat flag leaf up to 11.5 dsm^{-1}. These results were confirmed by Farouk (2005) and Younis et al (2009). The accumulation of amino acids in stressed plant could be caused by 1) protein degradation (Yadav et al 1999) for providing amino acids needed for synthesis of new proteins suited for growth or survival under the modified conditions, and 2) inhibition of protein synthesis. In contrast, the results of present investigation proved that application of antioxidants increased significantly total free amino acids.

Proline "Pro" concentrations, of all the organic solutes analyzed, showed the highest relative increase in response to salt stress (Table 1). Proline accumulation may contribute to osmotic adjustment at the cellular level (Tripathi et al 2007); hence, these solutes play an important role in osmoregulation. The significance of proline accumulation in osmotic adjustment is still debated and varies according to the species. However, convincing evidence is still lacking as to whether accumulation of proline can provide any biochemical adaptation for plants during salt stress. Antioxidants are directly involved in the changes taking place in the plant under salt stress. Pro has multiple functions, such as osmotic pressure regulation, protection of membrane integrity, stabilization of enzymes/proteins, maintain appropriate NADP+/NADPH ratios and scavenger of free radicals (Tripathi et al 2007, Kaymakanova and Stoeva 2008, Misra and Saxena 2009), a major source of energy and nitrogen during immediate post-stress metabolism, thereby inducing salinity tolerance (Jain et al 2001). Over-accumulation of proline under either salt stress or antioxidants application or their interactions, in plants, has been attributed to the strategies adapted by plants to cope up with stress conditions (Alqurainy 2007). The increased or decreased regulation of enzymes of proline metabolism in response to salt stress or antioxidants has been demonstrated in many species (Sudhakar et al 1993, Misra and Saxena 2009). Such increase in proline content under salt stress or antioxidant application may be correlated with the increased synthesis of Δ1pyrroline carboxylate synthetase (P5CS) and P5CS mRNA levels (Hare and Cress 1996) and Pyrroline 5 carboxylate reductase (P5CR) (Misra and Gupta 2006), and γ-glutamyl kinase activity (Misra and Saxena 2009) or the low activity of degrading enzyme, proline oxidase (EC 1.5.99.8), localized in the inner mitochondrial membrane (Misra and Saxena 2009) and cytoplasmic proline dehydrogenase (EC 1.5.1.2) (Delauny and Verma 1993) to negligible rate. But till date, proline metabolism in presence of antioxidants is not known and need more studies. Many authors indicate that, the importance of soluble carbohydrates in stimulating of proline accumulation through an inhibition of the degradation enzymes of proline (Heineke et al 1992), and stimulated the synthesis enzymes of proline. In this concern Hare and Cress (1996) find

that mRNA transcript encoding P5CR was increased in phloem tissue in response to water deprivation.

Glycinebetaine (GlyBet), a quaternary ammonium compound, is regarded as one of the most effective osmoprotectants owing to its many advantages besides its efficacy as a compatible solute. The molecular features of GlyBet enable its interaction with both the hydrophobic and hydrophilic domains of macromolecules without perturbing the cellular functions (Sakamoto and Murata 2002). It has been reported that GlyBet protects the cells from stresses by maintaining an osmotic balance between the intracellular and extracellular environments and by stabilizing the quaternary structures of complex proteins like antioxidants enzymes and biomembranes and other functional units like oxygen-evolving photosystem II complex (Rhodes and Hanson 1993). In the present study, it has been noticed that GlyBet over-accumulated contributes to the maintenance of OA in antioxidants treated plants under normal or salinized conditions. Some researchers have also reported that GlyBet induced the accumulation of osmolytes, such as soluble sugars, and free proline (Ma et al 2004). Treatment of wheat plants with AsA or Toc increased significantly GlyBet level in flag leaf. Such an increase may be attributed to the fact that the addition of AsA promotes betaine formation by stimulating its biosynthesis. In this concern, Alqurainy (2007) revealed that application of AsA acid increased significantly GlyBet content in bean and pea seedlings grown under salinity stress.

Among the organic solutes, soluble carbohydrates contributed the most to the leaf osmotic potential, and they also seemed to be important in the leaf osmotic adjustment under salt stress conditions, as suggested by Tajdoost et al (2007) and Kholova et al (2009). The increment in soluble carbohydrates due to salinity or antioxidants application may in turn play an important role in increasing the osmotic pressure of the cytoplasm. This conclusion is in accordance with the results obtained by Greenway and Munns (1980) who stated that these organic molecules act as osmotica and play an important role in osmotic adjustment in non-halophytes, moreover, sugars as osmolytes enable plants to keep better water relation under salt stress conditions. The current hypothesis is that sugars act as osmotica and/or protect specific macromolecules and contribute to the stabilization of membrane structure. The accumulation of sugars was the result of an enhanced efficiency in the use of carbon coupled to a reduction in cellular metabolism, which could favor the accumulation of respiratory substrate to support the osmotic adjustment required to survive in saline media (Schnapp et al 1990).

The measurement of increases in organic acids produced by mitochondria and cytosol can give information about respiratory activities and the equilibration of any cation excess, since catalytic mechanisms of hydrolysis of insoluble reserves are the first reactive to be activated by the passive absorption of water. Very little has been reported about the role of organic acids in plants due to salinity stress or antioxidant application. Former studies revealed increase in organic acid levels (citrate, malat… all linked to oxdation) in response to salinity addition (Bourgeais-Chaillou and Guerrier 1992). Organic acid have a role as osmotica as was previously described for *Phaseolus vulgaris* after exposure to sodium chloride shock under short-term experimental conditions (Ortiz et al 1994). The role of antioxidants on increasing the accumulation of organic acid under normal or

salinized conditions needs further work to explain the actual role of antioxidants on organic acid metabolism and accumulation in plant cell.

It is well known from the result of the present investigation that flag leaf area was progressively decreased with the increase of salinity (Table, 4). The decreased rate of leaf growth after an increase in soil salinity is primary due to reduction in water potential in the root zone which transmitted via the xylem to the leaves, causing leaf cells to loss water and reduced its elongation rates (Fricke and Peters 2002). Over days, reduction in cell elongation and also cell division lead to smaller final size. It is well known that cell expansion is dependent on water uptake, which relies on water potential gradients between the expanding cells and the water source. The relationship between solute uptake and leaf elongation under salinity has been examined in various systems. Soluble sugars (Kholova et al 2009) and other organic solutes such as proline (Misra and Saxena 2009) accumulate in the leaves under saline conditions and contribute to osmotic adjustment. They also help to sustain cell wall synthesis. The actual relationship between turgor and leaf growth is complex. In agreement with more recent ideas about the mechanism of cell wall extensibility, cell enlargement beings with a reduction or relaxation of wall stress. As a consequence, turgor pressure and water potential are reduced, and water is drawn into the cell. The result is that the cell enlarges by uptake of water, initiated by a yielding of the wall. Synthesis and deposition of new wall materials is needed during or after cell enlargement to prevent wall rupture in subsequent growth. This was evident in our results, where the thickness of the mesophyll tissue, epidermis cell and vascular bundles decreased in the stressed plants indicating a reduction in cell size (Table 3, Figure 3) due to inhibition of the pro-cambial activity from, primary vascular tissues as well as with a decrease in the number and size of mesophyll tissue. The reduction in cell size under salt stress conditions may be considered as salinity adaptation mechanisms (Steudle 1997). On contrast, application of both antioxidants, in particular, AsA, increased significantly flag leaf area under control or salinized conditions (Table 4 and Figure 4) due to hyper-accumulation of compatible solutes (Tables 1 and Figure 1) and potassium, and/or decreasing both sodium and chloride in flag leaf (unpublished results). Such accumulation provides the turgor necessary for cell expansion resulting in increasing leaf area (Munns and Termaat 1986). This conclusion was supported by our results which indicate that application of both antioxidants increased leaf water potential and leaf osmotic potential as well as leaf turgor potential which resulted in increasing water uptake to cells and increasing relative water contents, resulted in increasing leaf cell elongation and finally flag leaf area. In addition, application of antioxidants increased total soluble sugars which serve as a substrate for increasing initiation of leaf primordial and decreasing plastochron duration (Munns et al 1979) which leads to increasing leaf area. This result was supported by several studies which confirmed that application of antioxidants increased significantly leaf area (Abd EL-Aziz et al 2007). This was evident in our results, where the thickness of the mesophyll increased in plant treated with antioxidants under normal or salinized conditions indicating an increase in cell size (Table 3, Figure 3).

Osmotic adjustment has received increasing interest during recent years. Associations between OA and grain yield under water deficit in wheat

(Moustafa et al 1996), and sorghum (Santamaria et al 1990) have been reported. However, the utility of OA as a mechanism of salinity tolerance is open to debate. Such a favorable effect of OA on yield and its components could presumably by attributed to the well-established role of OA in maintaining turgor and plant growth under water deficit as observed in various crops (Grammatikopoulos 1999). Recently, Subbarao et al (2000b) have recorded a significant positive relationship between OA and RWC under water deficit that lead to a significantly positive association between OA and leaf area, indicating maintenance of crop growth by OA under stress condition. That is, genotypes that adjusted osmotically, could maintain high photosynthetic rate because of more favorable leaf water status, which could, in turn, lead to higher crop growth rate and dry matter production, maintaining, ultimately, a higher productivity under salt stress. Thus, it could be inferred that maintenance of higher RWC at high salinity level in this study (Table 2, Figure 2) could maintain growth and metabolic activities in plants, including, photosynthesis and other physiological processes (Subbarao et al 2000b). Additionally, antioxidants treated plants could, presumably, translocate the pre-anathesis carbohydrates reserves to developing grains more efficiently than untreated plants. Moreover, OA could play a role in maintenance of turgor and better water content of leaves, which might help the plant, under salinity stress, to survive and maintain growth and metabolic activities so as to result, ultimately, in improved crop productivity.

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