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Effect of Salt Treatment on the Expression of Phenolics and Peroxidase Activity Assessed in Two Barley Cultivars Acsad 1230 and Arig 8

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Abstract: Phenolic compounds and peroxidase activity in both leaves and roots were used to assess salt tolerance in Acsad 1230 and Arig 8 barley cultivars originated from Lybia and Morocco, respectively. This assessment was carried out at Z2.1 and Z4.5 of Zadock's scale using 70 and 140 mM NaCl treatments. Rates of soluble phenolic compounds and its various constituents were carried out using HPLC. Results indicated that Acsad 1230 is less sensitive to salt stress than Arig 8. Increased accumulation of soluble phenolic compounds and higher peroxidase activity were observed in the Acsad 1230 treated with 140 mM of NaCl. Phenolic compounds increased by 30% while peroxidase activity increase ranged from 50 and 239% at Z2.1 and Z4.5, respectively in Acsad 1230. Phenolic compounds were not affected by greater salt treatment, whereas an increase of 159% of peroxidase activity was observed in Arig 8 at Z4.5 growth stage only. Results of HPLC showed significant accumulation and modification of the composition of derived benzoic acid, several ferulic, apigenin and luteolin derivatives in extracts from leaves for both cultivars under salt stress. Extracts from roots of both cultivars showed that the profile of phenolics is characterized by an accumulation of benzoic acid, p-coumaric acid derivatives and other non identified chemical compounds. These results suggest that genetic variability for salt tolerance could be associated with phenolic compounds and peroxidase activity. The magnitude of expression of these compounds may be considered as valuable tool to select for salt stress.

Key words: Barley, phenolic compounds, peroxidases, salt stress

INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the major cereal crops in Tunisia and covers the second largest area after durum wheat. However, barley is cultivated mostly in harsh growing conditions where drought and irregular rainfall are frequently encountered. Thus, barley has been widely adapted as an alternative cereal crop in semi-arid areas of the central and southern part of Tunsia and particularly cultivated for double end use purposes: grain production and forage crop. In these areas, soil salinity is considered as main limiting factor and causes a significant yield reduction of most cereal crops[1]. Increased salinity level is attributed to rain shortage, frequent drought and particularly to an increased use of poor quality water with a greater salt concentration. Previous investigations[2] indicated that elevated temperature increased salt stress in the semi-arid areas. Several investigations indicated that salinity affects metabolic process and induces irreversible physiological disorders[3]. However, differential plant responses to salt stress were also reported. Identifying potential mechanisms of salt tolerance within plant species is becoming an increasing research priority in several countries in order to efficiently select for tolerant cultivars that can withstand increased concentration of NaCl in the nutrient medium. Along with morphological selection criteria, the use of physiological related traits such as peroxidases which oxydate several substrates may provide not only an indication of salt tolerance mechanism but also as a tool to improve selection efficiency for salt tolerant cultivar[4,5]. The large distribution pattern of these enzymes within vegetative

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parts would suggest that these peroxidases are involved in several physiological processes such as lignin synthesis[6] and other abiotic and biotic stresses[7]. The high catalytic activity of peroxydases was found to regulate several plant growth processes[4,8]. Peroxidase and phenolic compounds were both considered as potential indicators of plant related stress reaction. However, limited results suggested the implication of phenolic compounds in salt tolerance. Several authors reported that these organic compounds are involved in the expression of plant tolerance and resistance to several biotic and abiotic stresses[6,9-11].

Limited investigations were carried out on barley cultivars using phenolics and peroxidase activity as selection criteria for salt tolerance. Therefore, the aim of this study was to evaluate two selected barley cultivars: Acsad 1230 and Arig 8 for salt tolerance on the basis of the magnitude of expression of phenolic compounds and peroxidase activity during the vegetative growth.

MATERIALS AND METHODS

Plant material: This investigation was conducted during October, 2002. Two six row barley cultivars (Arig 8 and Acsad 1230) were selected on the basis of their relative salt tolerance among six cultivars investigated that includes: Souihli (landrace from Tunisia); Rihane, Faiez (new selections from ICARDA Barley Breeding Program that are widely grown in CEWANA regions), Arig 8 (landrace from Morocco), Tichtdret (from Algeria) and Acsad 1230 (local barley population from Libya). The cultivar Arig 8 is originated from Morocco and was found the most susceptible to increased level of NaCl concentration. Acsad 1230 is originated from Lybia and was found to be the most tolerant to salt stress.

These cultivars were cultivated using pot and treated with 0.70 and 140 mM of NaCl. Pots were irrigated every 5 to 6 days with tap water alone or supplemented with respective NaCl concentrations. Alternate washing irrigation is used after every salt treatment application. At Z2.1 and Z4.5 Zadock's growth scale corresponding to four weeks and twenty weeks of post sowing growth periods, third leaves and flag leaves as well as root are sampled and lyophilised.

Analysis of phenolic compounds lyophilised plant material of 50 mg is grinded in cool temperature using 1.5 mL of MeOH (80%)[12]. The supernatant was agitated during 5 min at 4°C. The mixture is centrifugated at 7000 g x 3 min and the supernatant is recovered and conserved at -20°C.

The residue is supplemented with 2 mL of NaOH and placed at 100°C x 2 h to extract non soluble phenolic compounds. After incubation the extracts were acidified to pH 2 with HCl 2 N. Ethyl acetate is then used to extract phenolic compounds and evaporated under dry. The residues were redissolved in MeOH and stored at -20°C for HPLC analysis.

Soluble phenolic compounds evaluation was determined using Folin Ciocalteu's reactif[12] Optic density recorded at 760 nm and phenolic compounds rates are expressed as mg of chlorogenic acid per g DM.

HPLC measurements were carried out using HPLC Waters 600 E type equipped with a photodiodes array detector Waters 990 associated with an unit of data logger to analyse recordings. Separation is carried out with acetonitrile and bi-distilled acidified water at pH 2.6 with *o*-phosphoric acid then filtrated on millipore of (0.45 µ) along an analytical column with inversed phase Interchrom C18, 5 µm. Phenolic compounds were detected using the following wave lengths 280, 320 and 350 nm. Results are expressed in mg equivalent acid chlorogenic per g of DM. Phenols are identified using retention time span of UV in comparison with standards.

Extraction and analyses of peroxidase activity: Peroxydase extraction was carried out using Tris-maleate buffer (0.1 M, pH 6.5) enriched with Triton X-100 (0.1 g L^{-1}). Peroxidasic activity was measured at 470 nm using gaïacol as substrate[13]. 100 to 200 µL of the enzymatic extract (200 mg MS/2 mL) is added to 2 mL reaction mixture of 0.1 M Tris-maleate and 25 mM gaïacol solution. Reaction is initiated with 10 µL of H_2O_2 (10%) and data recorded during 3 min. Results are expressed as δ DO min^{-1} mg^{-1} DM.

RESULTS

Effect of salt stress on the phenolic compounds: Screening for salt tolerance was carried out using six barley cultivars originated from North Africa (Souihli and Rihane from Tunisia, Arig 8 and Faiz from Morocco, Acsad 1230 from Lybia and Tichdrett from Algeria). Arig 8 was found to be the most susceptible cultivar to increased salt treatment, whereas Acsad 1230 has shown a greater level of salt tolerance. This tolerance was associated with a normal growth and physiological parameters as sodium content and water retention that were comparable to check treatments (Table 1).

The accumulated sodium in the Acsad 1230 leaves was found to be linked to a greater level of hydratation. Lower water retention was noted in leaves from extracted from Arig 8 cultivar in presence of a greater salt application. These results suggest that Na^+ compartimentation is more efficient in Acsad 1230 than in

Table 1: Relationship between rate of sodium of leaves and the relative water content during juvenile and mature growth stages

	Acsad 1230		Arig 80	
	Rate of sodium (μeq/g DM)	Relative water content (%)	Rate of sodium (μeq/g DM)	Relative water content (%)
Growth stage Z2.1				
Control	240.4	79.9	365.7	88.1
70 mM	1038.2	85.4	1082.5	87.6
140 mM	1496.2	83.0	1093.4	75.9
Growth stage Z2.1				
Control	450.3	69.2	578.6	72.0
70 mM	1212.9	69.7	1038.6	68.4
140 mM	1304.9	75.5	1140.2	66.7

Table 2: Comparison of NaCl effects on rates of soluble phenolic extract from leaves and roots at Z2.1 growth stage and from the flag leaf using two barley cultivars Acsad 1230 and Arig 8

	3-4 Leaves		Roots	
	Acsad 1230	Arig 8	Acsad 1230	Arig 8
Growth stage Z2.1				
Control	14.34	11.10	2.94	3.67
70 mM	12.11	13.46	2.89	3.59
140 mM	12.04	12.28	4.10	4.79
Growth stage Z4.5	Flag leaf		Roots	
Control	14.34	11.10	2.94	3.67
70 mM	12.11	13.46	2.89	3.59
140 mM	12.04	12.28	4.10	4.79

Table 3: Comparison of NaCl effects on bound rates of phenolic rates extracted leaves and roots at Z2.1 growth stage and from the flag leaf using two barley cultivars Acsad 1230 and Arig 8

	3-4 Leaves		Roots	
	Acsad 1230	Arig 8	Acsad 1230	Arig 8
Growth stage Z2.1				
Control	8.35	1.12	4.43	4.29
70 mM	9.74	1.05	5.25	2.42
140 mM	9.54	5.78	4.60	5.44
Growth stage Z4.5	Flag leaf		Roots	
Control	9.89	11.05	2.61	14.65
70 mM	3.99	10.45	3.12	16.62
140 mM	10.18	11.36	6.30	11.91

Arig 8. Using lower salt application (70 mM NaCl), both cultivars Acsad 1230 and Arig 8 exhibited comparable level of phenolic compounds as observed for the check salt treatment. At 140 mM of NaCl, phenolic compounds accumulation rates discriminate between both cultivars (Table 2). Stable rates of soluble phenolics compounds were noted in Acsad 1230 and reduced rates were observed in Arig 8 (-22%). At 70 mM of NaCl, the phenolics compounds into roots were not affected. However, the treatment with 140 mM NaCl at 3-4 leaves growth stage increased phenolic compounds in both cultivars but with a more pronounced effect observed in Acsad 1230 (+39,%) than in Arig 8 (+31%) (Table 2). Moreover, superior accumulation rates of soluble phenolic compounds in leaves and roots from check plants and those treated with NaCl of both cultivars were found at Z4.5 than at Z2.1 growth stage.

Slight increase of bound phenolic compounds in the leaves have been found in Acsad 1230 at Z2.1 growth stage when 70 mM NaCl was applied, whereas these compounds were not affected in Arig 8 cultivar as compared to check treatment. At Z4.5 growth stage, reduced phenolic compounds was detected in Acsad 1230, while the level of these compounds remained unchanged in Arig 8. Using 140 mM, bound phenolic compounds did not vary in Acsad 1230 whereas a pronounced reduction of 50% of these compounds was obtained in Arig 8 at Z2.1 growth stage. At Z4.5 growth stage, results indicated no significant effect of NaCl treatment on the rate of bound phenolic compounds for both cultivars: Arig 8 and Acsad 1230 (Table 3).Under 70 mM of NaCl, bound phenolics fraction into roots increased by 20% in Acasd 1230 and reduced by 44% in Arig 8 at Z2.1 growth stage. At Z4.5 growth stage, a slight increase of bound phenolic compounds in the roots of both cultivars, but this pattern was more pronounced in Acsad 1230. When greater NaCl treatment concentration is applied (140 mM), important accumulation of bound phenolic compounds (+141.88%) was obtained in Acsad 1230 and reduced accumulation rate was found in Arig 8 at Z4.5 (Table 3).

Analysis of HPLC data of soluble phenolic compounds extracts from leaves of both cultivars indicated that they are rich in flavonoids as well as phenolic acids at Z2.1 growth stage.

Significant accumulation of derived benzoic acid, several ferulic, apigenin and luteolin derivatives were all also noted in extracts of leaves from both cultivars (Fig. 1). The response to salt treatment appeared to be in relation with a major modification in levels of each of these compounds. Among these modifications, it has been found that compounds 1 to 9 (benzoic acid and luteolin derivatives) increased by 35 and 58%, respectively in Acsad 1238 treated with 140 mM of NaCl as compared to zero salt treatment. Similarly, compound 8 (luteolin derivative) increased by 35%. A significant increase of 72, 204, 689 and 205% of the specific compounds 1, 3, 7 and 8, respectively was also observed in cultivar Arig 8 (Table 4).

Extracts from roots showed that the profile of phenolics is characterized by a higher accumulation of

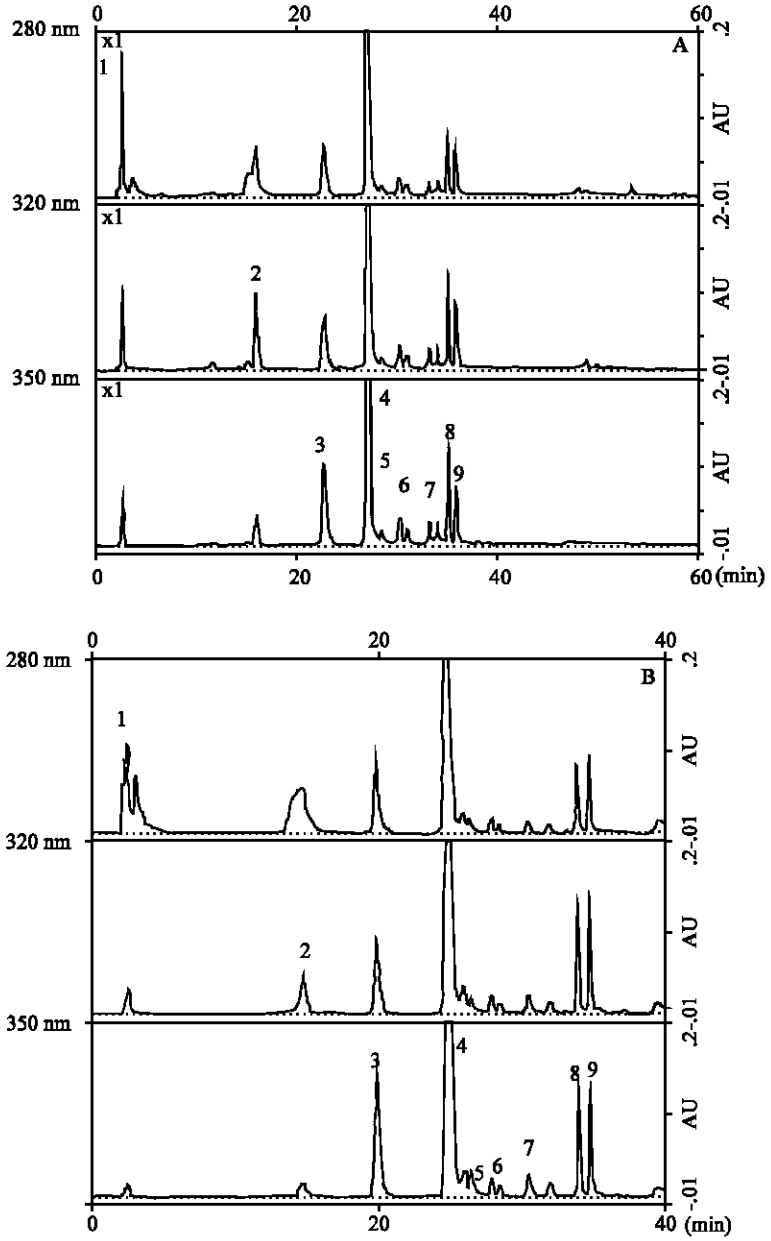

Fig. 1: HPLC chromatogram of phenolics extract from leaves of Acsad 1230 barley cultivar grown in absence (A) and in presence of NaCl 140 mM (B). 1: Benzoic acid derivative; 2: Ferulic acid derivative; 3, 4,5 and 6: Apigenin derivative; 7,8 and 9: Luteolin derivative

benzoic acid, p-coumaric acid derivatives and other non identified chemical compounds (Fig. 2). Under 70mM NaCl treatment, the cultivar Acsad 1230 expressed a significant increase of 273 of the non identified compound 2 (Table 5). When 140 mM was used, both cultivars expressed a higher rates of the compounds 3 and 4 (p-coumaric acid derivatives) with a pronounced expression rates in Acsad 1230 (177 and 156%, respectively) (Table 5).

Effect of salt stress on peroxidase activity: Unchanged peroxidase activity in both leaves and roots were noted at Z2.1 growth stage for either salt 70 and 140 mM application in cultivar Arig 8. Inversely, in Acsad 1230 treated with 140 mM, peroxidase activity increased by 50 and 60% for both 3rd leaf and roots, respectively (Table 6). Peroxidase activity appeared to be positively associated with increased levels of salt treatment in Z4.5 growth stage for both cultivars. However, higher rates

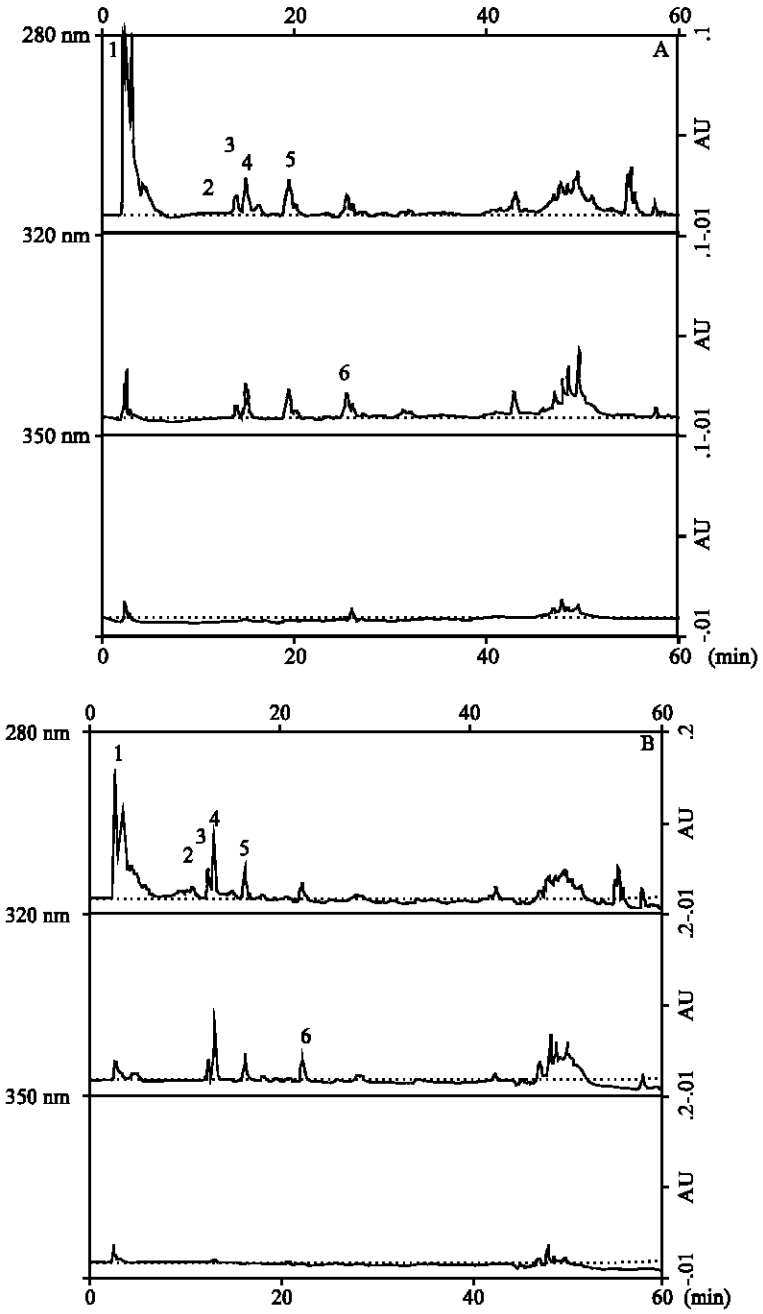

Fig. 2: HPLC chromatogram of phenolics extract from roots of Acsad 1230 barley cultivar grown in absence (A) and in presence of NaCl 140 mM (B) 1: Benzoic acid derivative; 2: not identified; 3, 4 and 6: p-coumaric acid derivative; 5: not identified

of peroxidase activity were depicted in Acsad 1230 and reached 88 and 239% for 70 and 140 mM, respectively. Lower peroxidase activity rates of 68 and 160% were observed in Arig 8 for both salt treatments, respectively. Induced peroxidase activity of 163 and 90% in the root system in Acsad 1230 was noted for 70 and 140 mM, respectively. This pattern was not found in Arig 8 (Table 6).

Table 4: Effect of salinity stress on different rates of phenolic compounds in leaves during Z2.1 growth stage in two barley cultivars

		Acsad 1230 μg g^{-1} DM			Arig 8 μg g^{-1} DM		
		Control	70 mM	140 mM	Control	70 mM	140 mM
Benzoic acid derivative	Pic 1	68.00	49.76	92.01	58.00	100.00	52.58
Ferulic acid derivative	Pic 2	73.29	73.55	46.21	55.80	54.11	38.01
Apigenin derivative	Pic 3	104.6	68.03	113.80	33.60	102.13	25.31
	Pic 4	565.6	699.10	540.54	549.2	426.54	393.84
	Pic 5	22.53	-	8.92	-	-	-
	Pic 6	12.34	-	5.75	-	-	-
Luteolin derivative	Pic 7	12.73	15.08	14.40	9.48	-	7.88
	Pic 8	48.19	65.02	60.84	32.60	-	79.30
	Pic 9	33.31	40.00	52.58	25.00	21.11	29.40

Table 5: Effect of salinity stress on different rates of phenolic compounds in roots during Z2.1 growth stage in two barley cultivars

		Acsad 1230 μg g^{-1} DM			Arig 8 μg g^{-1} DM		
		Control	70 mM	140 mM	Control	70 mM	140 mM
Benzoïc acid derivative	Pic 1	152.61	80.77	52.38	40.39	59.26	35.38
p-coumaric acid derivative	Pic 3	11.31	13.65	15.68	13.32	6.3	26.77
	Pic 4	23.99	18.85	30.68	42.19	20.19	97.05
	Pic 6	20.12	25.52	15.6	12.5	12.28	18.64
Not identified	Pic 2	2.26	8.43		13.92	9.91	9.58
	Pic 5	24.83	11.87	20.48	22.51	1.93	17.18

Table 6: Comparison of peroxidase activity in leaves and in roots at Z2.1 growth stage and in the flag leaf using two barley cultivars Acsad 1230 and Arig 8

	3-4 Leaves		Roots	
	Acsad 1230	Arig 8	Acsad 1230	Arig 8
Growth stage Z2.1				
Control	1088.75	1745.63	370.00	779.38
70 mM	1105.63	1746.25	456.88	781.25
140 mM	1615.00	1762.50	590.63	798.13
Growth stage Z4.5	Flag leaf		Roots	
Control	1072.50	641.25	795.00	1183.75
70 mM	2015.00	1077.50	2094.38	1224.38
140 mM	3637.50	1665.00	1506.88	1465.63

DISCUSSION

The two barley cultivars investigated showed significant differences for growth and yielding ability. Peroxidase activity was not similarly affected by salt treatments in both cultivars. Positive peroxidase activity response to salt treatment was noted in Acsad 1230 while no significative response was noted for Arig 8. This could be in relation with the higher sensitivity of this cultivar to salt leading to an inhibition of peroxidase activity. Comparable results were reported in transgenic tobacco using UV treatment[11]. However, Gosset *et al.*[14] found significant increase of peroxidase activity and greater decomposition of H_2O_2 in rice cultivars grown under salt treatment. They concluded that salt susceptible cultivars are characterized by a significant reduction of peroxidase activity. Using sensible cultivars of cotton Martinez and Lauchll[15] argued that salinity enhances the peroxidation of membrane lipids which are found to be associated with reduced ascorbate peroxidase activity, catalase and peroxidases. However, anti-oxidant enzymes activity appears to induce the protection of membrane structures in salt tolerant cultivars[15]. Increased peroxidase activity was found to be associated to salt tolerance in cereals[16] and in two Zucchini cultivars[17]. It has been recognized that peroxidases are involved in H_2O_2 decomposition that initiates toxic oxygen production. These radicals are implicated in lipid per oxidation and in the denaturation of proteins and DNA[18,19] as a result of the cellular decompartimentation.

Several investigations demonstrated the implication of these enzymes in the lignification, defense mechanism against invading pathogens, salt tolerance and senescence[6,20]. Peroxidases were found to be involved in salt tolerance in Acsad 1230 barley cultivar and contribute to strengthening cell walls of the leaves and roots. This process could regulate the absorption of toxic ions and the detoxification as well as the protection of cells from toxic oxygen derivatives that are induced by salt stress. Increased peroxidase activity under salt stress could result from the synthesis of isoperoxidases and/or the activation of existing peroxidases[14,21]. Peroxidases are often used as molecular markers of biotic and abiotic stresses and could be used as tool for selection and as prediction model for greater yielding ability[22].

Results of bound and soluble phenolics indicated an increase of these compounds in Acsad 1230 treated with greater salt application. However, in Arig 8 these compounds decreased or remained stable under salt stress. Comparable results were found in transgenic tobacco cultivars[11]. Hence, phenolic compounds represent useful indicators to select for salt tolerant cultivars.

In conclusion, the barley Arig 8 was determined as a susceptible cultivar more than Acsad 1230 to salt stress. This susceptibility and tolerance is being in relation with the metabolic response of tissue to salt in terms of phenolics and peroxidases activities.

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