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Antioxidative Responses of Shoots and Roots of Wheat to Increasing NaCl Concentrations

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Summary

The effect of NaCl was studied in seedlings of two cvs. of *Triticum durum* Desf., differently sensitive to drought and to heavy metals (cv. Ofanto more tolerant than cv. Adamello). The seedlings were grown for 9 days in Hoagland's 2 solution, added with increasing NaCl concentrations (0, 50 and 100 mmol/L). Comparisons of control and salt-stressed plants included ascorbate and glutathione contents, their reduction states and the activities of ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase. The results indicated an involvement of activated oxygen species in the mechanism of cellular toxicity of NaCl and pointed out differences in the induction of antioxidant defences among the two cvs. Indeed, notwithstanding a higher constitutional content of ascorbate in cv. Adamello, cv. Ofanto was able to induce ascorbate synthesis when subjected to salt stress. Higher levels of NaCl resulted in increasing glutathione contents in the roots of both cvs., likely for an increased requirement of antioxidants in the organs that firstly suffer stress. Only the roots of cv. Ofanto showed glutathione oxidation following treatments. The general trend for the antioxidative enzyme activities was an increase in the shoots and a decrease in the roots.

The experimental evidence suggested a more marked effect of NaCl stress on cv. Adamello when compared with cv. Ofanto.

Key words: Ascorbate, Ascorbate peroxidase, Dehydroascorbate reductase, Glutathione, Glutathione reductase, Monodehydroascorbate reductase, NaCl, Salt stress, Triticum durum, Wheat.

Abbreviations: APX = Ascorbate peroxidase; AsA = Ascorbate; DHA = Dehydroascorbate; DHAR = Dehydroascorbate reductase; EDTA = Ethylendiaminetetraacetic acid; GR = Glutathione reductase; GSH = Reduced glutathione; GSSG = Oxidized glutathione; MDHA = Monodehydroascorbate; MDHAR = Monodehydroascorbate reductase; PVP = Polyvinylpolypyrrolidone.

Introduction

Salt interaction with plant physiological processes is complex, depending on salt type and concentration, on plant genotype, growth stage and environmental conditions (Shannon et al., 1990). The alterations in plant growth in saline environment may be due either to unspecific osmotic effects (Cramer et al., 1994), and/or to specific ion toxicity (Cheeseman, 1988).

Plants may react to the salt damage by ion compartmentation, ion exclusion and osmotic adjustment (Gorham et al., 1985). There are some indications that salt excess can induce conditions of oxidative stress. Singha and Choudhuri (1990) reported that $O_2^{\cdot -}$ and H_2O_2 could play an important role in the mechanism of salt injury. In addition, many other authors correlate changes in antioxidative molecule contents and redox state, and in antioxidative enzyme activities under salt stress in plant tolerance to salinity (Gossett et al., 1994, 1996; Hernández et al., 1993 a, b, 1995; Olmos et al., 1994; Streb and Feierabend, 1996).

Unspecific osmotic effects may determine partial stomatal closure, while specific ion toxicity may alter membrane struc-

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ture and functionality (Navari-Izzo et al., 1988 a, b), so allowing electron leakage from the electron transport chain. As a consequence, an improved production of activated oxygen species may derive. When the balance between the production of active oxygen species and the quenching ability of antioxidants is upset, oxidative damage may occur.

To defend themselves against oxidants plants have evolved specific protective mechanisms, involving antioxidant molecules and enzymes. Among the specific protective mechanisms evolved by plants, the importance of ascorbate (AsA) and glutathione (GSH) is now well known. In biological systems, AsA is a scavenger of oxygen radicals with a relatively high rate constant for the most damaging form of activated oxygen, i.e. $OH^{\cdot}$. AsA also scavenges $O_2^{\cdot -}$ and $^1O_2$, reduces thiyl radicals and dismutes H_2O_2 through the action of ascorbate peroxidase (APX, EC 1.11.1.11) (Smirnoff and Pallanca, 1996). GSH can metabolize free radicals and can act in the protection of the thiol status of proteins, representing the major cytoplasmic thiol-disulfide redox buffer in most plant cells (Gilbert et al., 1990). This compound is also required to activate the transcription of defensive genes (Wingate et al., 1988).

APX utilizes AsA as an electron donor in the neutralization of H_2O_2, both in cytosol and in different cellular compartments (Foyer, 1996; Smirnoff and Pallanca, 1996; Jiménez et al., 1997). In a previous study on wheat chloroplasts we found an induction of these components following NaCl treatments (Meneguzzo et al., 1998). Since AsA is continuously required by APX, a regenerating cycle involving GSH and specific enzymes is also present (Smirnoff and Pallanca, 1996).

Two cvs. of wheat with different sensitivity to drought (Quartacci et al., 1994, 1995; Loggini et al., 1997) and to heavy metal excess (Ciscato et al., 1997) showed different antioxidative responses in chloroplasts when subjected to salt stress (Meneguzzo et al., 1998). Given this fact, we address questions concerning the effects of salinity on shoots and roots of the two cvs. and the possible induction of antioxidative mechanisms.

Materials and Methods

Plant material

Seeds of two cvs. of wheat (*Triticum durum* Desf.), differently sensitive to drought and to heavy metal excess, were provided by the Istituto Sperimentale per la Cerealicoltura (Foggia, Italy). Seedlings of the two cvs. (cv. Ofanto more tolerant than cv. Adamello) were grown for 9 days in a half-strength Hoagland's 2 solution, added with increasing concentrations of NaCl (0, 50, 100 mmol/L) and renewed every 3 days. Perlite was utilized as an inert substrate. Plant growth was carried out in a growth chamber at a constant day/night temperature of 17 °C, a 16-h photoperiod, 80–90 % relative humidity and a photon flux density of 220 $\mu mol\ m^{-2}\ s^{-1}$ supplied by fluorescent OSRAM L36 lamps. At harvest, shoots and roots were immediately separated and washed quickly with distilled water to remove any possible salt surface contamination and immediately air-dried on absorbing paper for Cl^- and enzyme analyses.

Na and Cl determination

Aliquots of shoots and roots were dried and used for Na^+ analysis by spectrophotometrical absorption (Perkin-Elmer model 373), after sample digestion with concentrated HNO_3. Chloride ions were extracted according to Binzel et al. (1987) and determined utilizing a selective ion electrode (Metrohom AG CH-9101 Herisau).

AsA and DHA determination

Aliquots of fresh shoot and root tissues were homogenized in ice-cold 5 % (w/v) trichloroacetic acid containing 2 % insoluble polyvinylpolypyrrolidone (PVP), using a cold mortar and pestle. AsA and total ascorbate (AsA + DHA) were determined in the supernatant after centrifugation at 12,100 g_n for 20 min, using a method based on the reduction of ferric ion to ferrous ion with AsA in acid solution followed by formation of the red chelate between ferrous ion and 4, 7-diphenyl-1, 10-phenanthroline (bathophenanthroline), which absorbs at 534 nm, as described by Wang et al. (1991). Total ascorbate was determined through a reduction of DHA to AsA by 0.97 mmol/L dithiothreitol. The concentrations of DHA were estimated on the basis of the difference between total ascorbate and AsA values. A standard curve covering the range of 0–25 nmol AsA was used, as described in Sgherri et al. (1994).

GSH and GSSG determination

Aliquots of fresh shoot and root tissues were homogenized in ice-cold 5 % 5-sulfosalicylic acid using a cold mortar and pestle, and centrifuged at 12,100 g_n for 20 min; the supernatants were used for total (GSH + GSSG) and oxidized glutathione determinations by the DTNB-GSSG reductase recycling procedure (Anderson et al., 1992). Changes in absorbance of the reaction mixtures were measured at 412 nm at 25 °C and the total glutathione content was calculated from a standard curve in which the GSH equivalents present (1–10 nmol) were plotted against the rate of change in absorbance at 412 nm. GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. A specific standard curve with GSSG (0.1–2 nmol) containing 1.8 % 2-vinylpyridine and 3.6 % triethanolammine was used. GSH was determined by subtraction of GSSG (as GSH equivalents) from the total glutathione content.

Enzyme extractions and assays

All operations were carried out at 0–4 °C. Aliquots of shoot and root tissues were ground in a cold mortar, using a specific buffer and pH for each enzyme extraction. The homogenates were squeezed through two layers of muslin, centrifuged at 12,100 g_n for 20 min and assayed as described below. Conditions for all assays were chosen so that the rate of reaction was constant for the whole experimental period and proportional to the amount of enzyme added. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as a standard.

The extraction of APX was performed in 50 mmol/L Tris-HCl, pH 7.2, containing 2 % (w/v) insoluble PVP and 1 mmol/L Na_2EDTA; 2 mmol/L AsA was added to the medium to avoid inactivation during extraction and assay. APX activity was assayed by measuring the oxidation of AsA, operated by H_2O_2, at 290 nm and 25 °C according to Wang et al. (1991). Briefly, the reaction mixture contained 0.25 mmol/L AsA, 0.1 mmol/L H_2O_2 and 50 mmol/L potassium phosphate, pH 6.6, in a final volume of 1 mL. The reaction was initiated by H_2O_2 addition. Corrections were made for the low, non-enzymatic oxidation of ascorbate by H_2O_2 and for the oxidation of ascorbate in the absence of H_2O_2.

The homogenizing medium for MDHAR (EC 1.6.5.4) was 50 mmol/L Tris-HCl, pH 7.2, containing 1 mmol/L Na_2EDTA, 0.05 % (w/v) cysteine and 2 % insoluble PVP. MDHAR activity was assayed by measuring the oxidation rate of NADH at 340 nm and 25 °C according to Arrigoni et al. (1992). The reaction mixture was composed of 0.2 mmol/L NADH, 2 mmol/L AsA, 0.1 mmol/L Tris-

HCl, pH 7.2, in a final volume of 1 mL. The reaction was initiated by the addition of 1.1 U ascorbate oxidase to generate saturating concentrations of MDHA.

The extraction buffer for DHAR (EC 1.8.5.1) was the same as used for MDHAR. The activity of DHAR was determined according to Gillham and Dodge (1986), monitoring AsA formation via DHA reduction at 265 nm. Briefly, the assay mixture, maintained at 25 °C, contained 0.2 mmol/L DHA, 2.5 mmol/L GSH, 0.1 mmol/L Na_2EDTA and 50 mmol/L potassium phosphate, pH 6.6. The reaction was started by adding the enzyme extract. Controls without enzyme and without GSH were carried out to correct for non-enzymic reduction of DHA and for AsA present in the fraction being assayed, respectively.

Glutathione reductase (GR, EC 1.6.4.2) was extracted with 100 mmol/L potassium phosphate, pH 7.0, containing 1 mmol/L Na_2EDTA and 2 % (w/v) insoluble PVP. According to Sgherri et al. (1994), the GSSG-dependent oxidation of NADPH was monitored by the decrease in absorbance at 340 nm and 30 °C. Briefly, the assay mixture contained 200 mmol/L potassium phosphate, pH 7.5, 0.2 mmol/L Na_2EDTA, 1.5 mmol/L $MgCl_2$, 0.25 mmol/L GSSG and 25 µmol/L NADPH in a final volume of 1 mL. The reaction was initiated by NADPH addition. Corrections were made for the background absorbance at 340 nm, without NADPH.

Results

Different than the cv. influence, which determined a significantly higher content only for Na in shoots and for Cl in roots of cv. Adamello, the effect of NaCl was always significant, inducing an increasing accumulation of the two ions (Table 1). The interaction of cv. and NaCl, apart for Na in shoots, did not exert any significant effect on the ions in shoots and roots.

The effect of cv. on shoots was significant on DHA, but not on AsA (Fig. 1). Indeed, DHA content was higher in the shoots of cv. Adamello than in cv. Ofanto. Moreover, no differences were observed between the two ascorbate forms in the roots of the two cvs. On the other hand, the effect of NaCl was significant on both AsA and DHA of shoots and roots. In particular, in the shoots of 50 mmol/L NaCl-treated plants, AsA content decreased by about 16 % and DHA content decreased by 30 %. On the contrary, at 100 mmol/L NaCl, 22 and 64 % increases of AsA and DHA of shoots, respectively, were shown. In the roots, the highest NaCl concentration induced a 22 % decrease in AsA content and a 32 % increase in DHA content. The interaction of cv. and NaCl exerted significant effects on AsA content of both shoots and roots. Indeed, AsA contents of shoots and roots were higher in the control plants of cv. Adamello in comparison with cv. Ofanto. At 50 mmol/L NaCl, in the shoots of cv. Adamello the content of AsA decreased, whereas in cv. Ofanto, at 100 mmol/L NaCl, it showed a sensible increase. Moreover, in the roots of cv. Adamello AsA content progressively decreased with the increase in NaCl concentration, whereas in the roots of cv. Ofanto it increased by 46 % in 50 mmol/L NaCl-treated plants. On the other hand, the

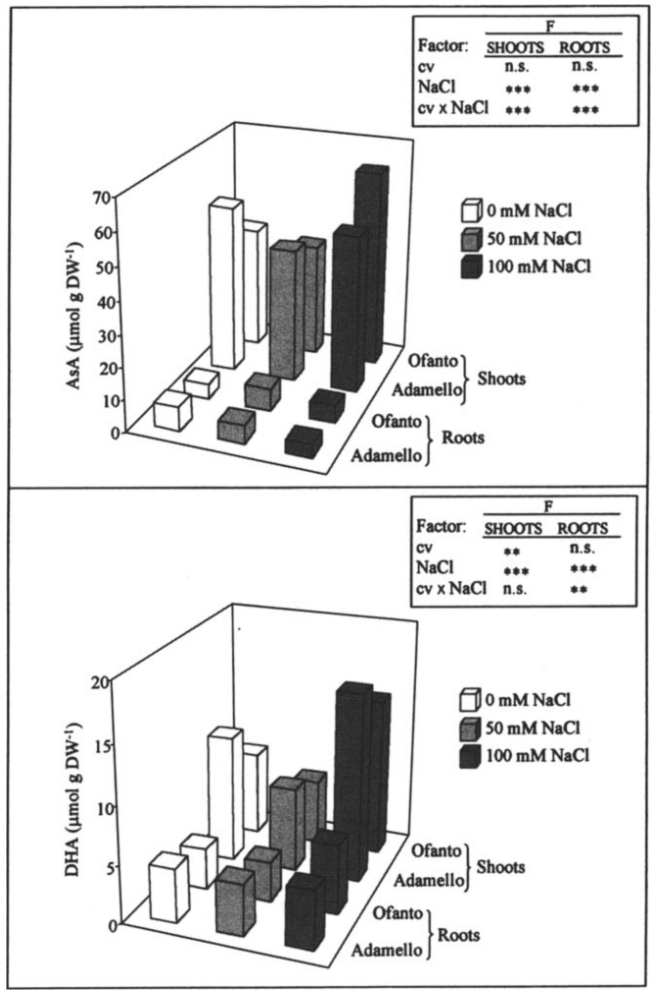

Fig. 1: Effect of cv. (Adamello and Ofanto) and NaCl (0, 50 and 100 mmol/L) upon AsA and DHA contents of shoots and roots of wheat (*Triticum durum* Desf.). A two-way analysis of variance was used to evaluate cv. effect (cv.), NaCl effect (NaCl) and interaction between cv. and NaCl (cv. × NaCl). Significance of the F ratio was as follows: n.s. = not significant; *, ** and *** = significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

Table 1: Effect of cv. (Adamello and Ofanto) and NaCl (0, 50 and 100 mmol/L) upon Na and Cl contents of shoots and roots of wheat (*Triticum durum* Desf.).

(mg g DW^{-1})	NaCl (mmol/L)	Shoots		Roots		Factor	F	
		Adamello	Ofanto	Adamello	Ofanto		Shoots	Roots
Na	0	0.52	0.46	0.60	0.61	cv.	**	n.s.
	50	26.14	22.08	30.68	27.27	NaCl	***	***
	100	29.78	29.20	34.63	30.36	cv.×		
						NaCl	*	n.s.
Cl	0	1.06	0.98	0.61	0.67	cv.	n.s.	*
	50	9.95	9.77	11.01	9.55	NaCl	***	***
	100	12.37	11.93	14.30	13.82	cv.×		
						NaCl	n.s.	n.s.

A two-way analysis of variance was used to evaluate cv. effect (cv.), NaCl effect (NaCl) and interaction between cv. and NaCl (cv. × NaCl). Significance of the F ratio was as follows: n.s. = not significant, *, ** and *** = significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

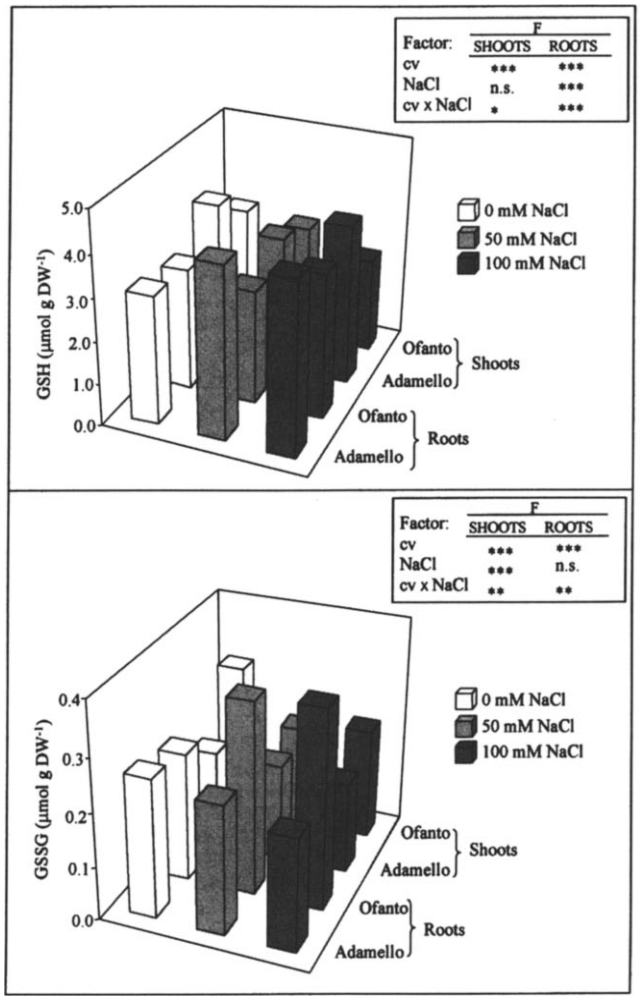

Fig. 2: Effect of cv. (Adamello and Ofanto) and NaCl (0, 50 and 100 mmol/L) upon GSH and GSSG contents of shoots and roots of wheat (*Triticum durum* Desf.). Otherwise as for Fig. 1.

interaction between cv. and NaCl resulted in changes in the content of DHA of roots, but not of shoots.

The cv. had a significant effect on both forms of glutathione of shoots and roots (Fig. 2). In particular, GSH content was slightly higher in the shoots and the roots of cv. Adamello than in cv. Ofanto, whereas the opposite was shown by GSSG. Regardless of the cv., in the shoots no change was observed in GSH content due to the increase in NaCl concentration, whereas both concentrations of NaCl induced a decrease in GSSG content. The effect of salt on roots was quite different from that on shoots. Indeed, 50 and 100 mmol/L NaCl caused a 24 % increase in GSH, while no change was observed in GSSG. The interaction of cv. and NaCl exerted a significant effect on GSH and GSSG contents of both shoots and roots. In particular, in the shoots of cv. Ofanto GSSG content decreased by 34 and 28 %, following the increase in NaCl concentration, whereas cv. Adamello was not influenced. In the roots, at both NaCl concentrations, we observed an increase in GSH in cv. Adamello and in GSSG in cv. Ofanto.

The effect of cv. was significant on all of the enzyme activities of roots and on those of MDHAR and DHAR of shoots (Figs. 3 and 4). Indeed, cv. Adamello showed higher activities of MDHAR and DHAR of shoots in comparison with cv. Ofanto, whereas in the roots of cv. Ofanto, MDHAR, DHAR and GR activities were higher than in the other cv. With regard to the effect of NaCl on enzyme activities, it was significant both in shoots and in roots. In particular, in the shoots the specific activities of APX and DHAR showed a similar trend, although at different levels: at the highest salt concentration they increased by 24 and 90 %, respectively. At both NaCl concentrations, an increase in the activity was also shown by MDHAR and GR of shoots, with a more marked effect on the latter enzyme (40 and 50 % at 50 and 100 mmol/L NaCl, respectively). In the roots of treated wheat, a 20–30 % decrease in the specific activities of APX, DHAR and GR was observed, while the activity of MDHAR

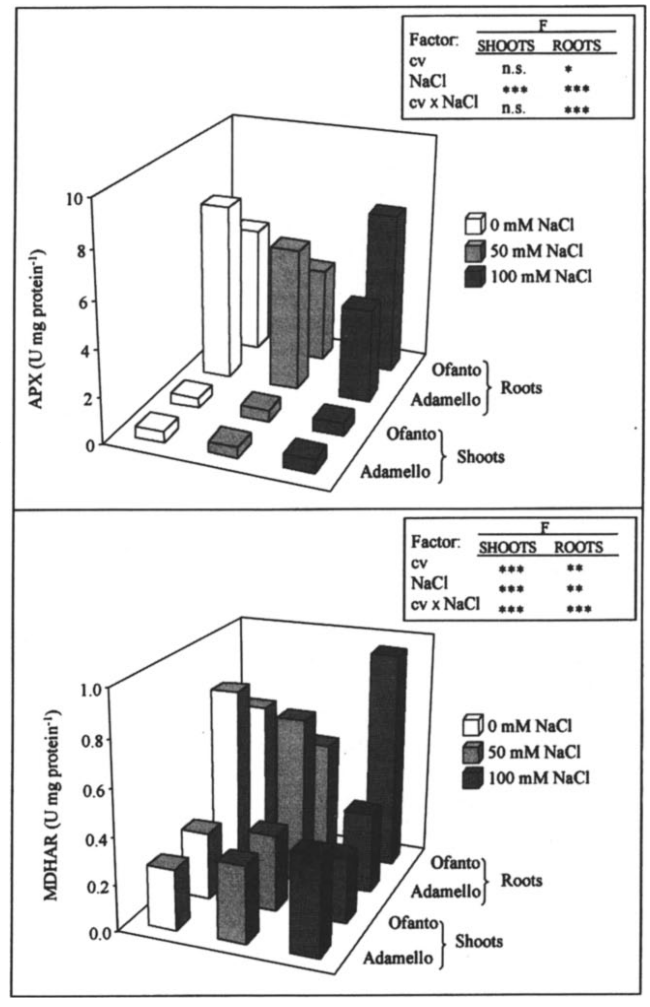

Fig. 3: Effect of cv. (Adamello and Ofanto) and NaCl (0, 50 and 100 mmol/L) upon APX and MDHAR activities of shoots and roots of wheat (*Triticum durum* Desf.). Otherwise as for Fig. 1.

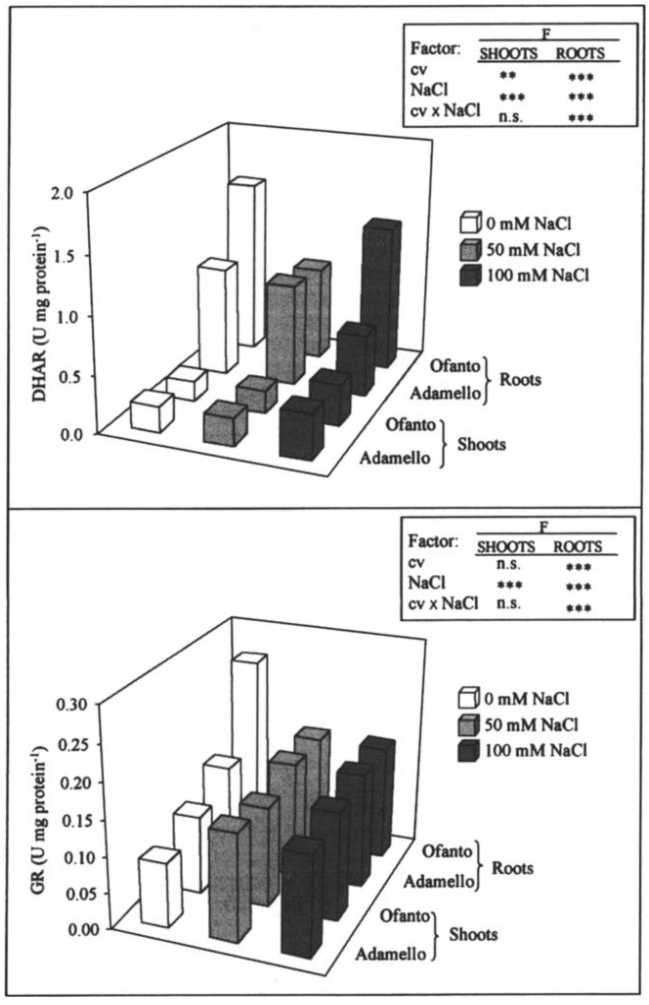

Fig. 4: Effect of cv. (Adamello and Ofanto) and NaCl (0, 50 and 100 mmol/L) upon DHAR and GR activities of shoots and roots of wheat (*Triticum durum* Desf.). Otherwise as for Fig. 1.

decreased by 16 % only at 50 mmol/L NaCl. Different as in shoots, where the interaction of the two factors exerted a significant effect only on the activity of MDHAR, in the roots the interaction was significant on the activities of all the enzymes studied. In particular, in the roots of cv. Ofanto, 100 mmol/L NaCl caused an increase in APX and MDHAR and no change in DHAR, whereas in the roots of cv. Adamello the activities of the same enzymes decreased. Moreover, in the roots of cv. Ofanto, at 50 and 100 mmol/L NaCl, the activity of GR decreased by 38 %, while in the roots of cv. Adamello no change was observed.

Discussion

Salt tolerance of plants primarily requires the maintenance of appropriate ionic and osmotic conditions in the cytoplasm and in the vacuole, which allow cellular functions to proceed in an unaffected way and simultaneously ensure sufficient cell turgor in the presence of salt (Shannon et al., 1990). The different concentrations of Na and Cl observed in roots and shoots of the two cvs. (Table 1) suggest a different pattern of their uptake and translocation. The effect of the interaction cv. × NaCl on Na content of shoots shows a more marked effect of salt stress on cv. Adamello, which is less tolerant to environmental stress (Ciscato et al., 1997; Loggini et al., 1997; Quartacci et al., 1994). When adaptive mechanisms are insufficient, salt tolerance may be improved to some extent when the accompanying activated oxygen damage is scavenged by enhanced antioxidative defence systems.

The reduced pool of ascorbate represents an antioxidant reserve, so that it may assume great importance in adaptive responses of plants to stress conditions. The different pattern of the forms of ascorbate showed by the two cvs. (Fig. 1) may contribute to explain their different responses to salt stress. In cv. Adamello the decrease in the AsA to DHA ratio at 100 mmol/L NaCl may lead to a depletion of the ascorbate pool, because DHA is unstable at physiological pH and may be non-enzymatically degraded (Smirnoff and Pallanca, 1996). In the shoots of both cvs., the induction of MDHAR at 50 mmol/L NaCl (Fig. 3) could have contributed to maintain the AsA to DHA ratio at control level, in spite of the possible decrease in AsA content. However, at 100 mmol/L NaCl, in the shoots of cv. Adamello the rate of AsA oxidation exceeded the capacity of the regenerative systems to reduce MDHA and DHA, so that in the absence of a *de novo* synthesis, it resulted in a decrease in AsA as compared with DHA. The individual factors did not induce any effect on total ascorbate of the salt-treated wheat roots, nevertheless the interaction of cv. and salt affected all of the ascorbate forms, resulting in a quite different behaviour of the two cvs. (Fig. 1), so that in cv. Ofanto a reserve of antioxidant power was also present in the roots to cope with a further stress. This evidence suggests that the constitutive quantity of the antioxidant may not be so important as the capacity to induce its synthesis, owing to an oxidative stress. Moreover, cv. Ofanto, having higher ascorbate turnover rates under salt-stress conditions, seemed to be able to acclimate to the stress better than cv. Adamello.

On the other hand, the glutathione pool behaved differently from ascorbate (Fig. 2). Indeed, in cv. Adamello we found an increase in GR activity in shoots and higher content and reduced state of glutathione in shoots and roots when compared with cv. Ofanto. When the same cvs. were subjected to water deficit, Loggini et al. (1997) found that only the more sensitive cv. Adamello required the induction of GR during stress. Generally, higher GSH to GSSG ratios and GR activities in plants subjected to oxidative stress, such as high salt conditions, are correlated to acclimation or even tolerance to the stress (Gossett et al., 1994, 1996). Likely, the two antioxidant molecules play a different role in the responses of the two wheat cvs. to NaCl. Differences in the redox state of ascorbate and glutathione were also found by Streb and Feierabend (1996) in rye leaves treated with 0.2–0.6 mol/L NaCl. Differences in the subcellular localization of these antioxidants might exist. A majority of glutathione is present outside the chloroplasts, so that a decline in the GSH to GSSG ratio may reflect the predominant oxidation of the cytosolic antioxidant, while a higher proportion of ascorbate seemed to be

localized in the chloroplasts (Meneguzzo et al., 1998), where it increased in NaCl-treated seedlings (Hernández et al., 1995). The ascorbate was presumably maintained in the reduced state through the photosynthetic electron transport (Streb and Feierabend, 1996).

The increase in total glutathione content observed in the roots of both cvs. following NaCl imposition (Fig. 2) may be due both to a GSH transport from shoots, as suggested by our results, and/or to a direct synthesis of GSH in the roots, as suggested by Rüegsegger et al. (1990). The transport could satisfy an increased requirement of antioxidants in the organs firstly suffering stress (Rennenberg, 1982). Moreover, GSH represents the main form of reduced S transport from shoots towards roots (Lappartient and Touraine, 1996) and seems to be an important signal molecule in defence responses of plants (Rennenberg and Brunold, 1994), as suggested by its role in genic expression modulation (Wingate et al., 1988). The existence of a GSH transport would be sustained by a GSH synthesis in the shoots (Fig. 2). In the roots of cv. Ofanto treated-plants, the GSH content decreased with respect to that of GSSG, likely for a reduction in GR activity. However, GSH seemed not to be utilized longer by DHAR. At 100 mmol/L NaCl, in the roots of cv. Adamello the enhanced reduction state of GSH could be due to the decrease in DHAR activity (Fig. 4).

The opposite patterns in enzyme activities shown by shoots and roots at 50 and 100 mmol/L can lead one to hypothesize that the salinity level may modulate the response of plants.

It is not clear how salt stress elicits the antioxidant responses described. It might be possible that Cl^- toxicity somehow disrupts normal electron flow to photosystem II (Gossett et al., 1994). Such a disruption could also result in excess electron leakage, which in turn could increase the generation of activated oxygen species and induce the antioxidant responses. Hence, it is also possible that a change in membrane integrity caused by a high Na^+ to Ca^{2+} ratio (Izzo et al., 1993) could result in an increase in electron leakage and in activated oxygen species, stimulating the general antioxidant responses.

Both cvs. appeared to react to the stress, even if the different behaviour shown by the two cvs. indicates that cv. Adamello underwent a greater stress when compared with cv. Ofanto. Moreover, the different pattern in ion accumulations (Table 1) as well as in antioxidative molecules and enzymes suggests a different kind of reaction to the stress.

It remains to be investigated whether the behaviour of cv. Ofanto is sufficient to make it more resistant to NaCl treatment, as already shown in water deficit conditions (Quartacci et al., 1994, 1995; Loggini et al., 1997) and upon treatment with heavy metals (Ciscato et al., 1997), so thar it could be considered cross-tolerant to different stress conditions.

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