Antioxidative protection in wheat varieties under severe recoverable drought at seedling stage

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ABSTRACT

The antioxidative protection in leaves of four winter wheat (*Triticum aestivum* L.) varieties with different field drought resistance was studied under severe recoverable soil drought at seedling stage by withholding irrigation for 7 days (57–59% leaf water deficit) followed by rewatering. A 3-fold raise in electrolyte leakage and a sharp increase in proline accumulation corresponded to drought severity. Hydrogen peroxide content and catalase (CAT) activity were maintained low under stress. Peroxidase (GPX) activity increased, whereas superoxide dismutase (SOD) activity only slightly changed. The content of ascorbate and low-molecular thiols diminished under severe drought and was restored in recovery. Malondialdehyde level was not changed significantly in drought-treated plants but raised after re-watering. In recovery CAT activity became significantly higher whereas GPX activity diminished. Three isoforms of SOD, one of catalase and three of GPX were revealed. Proline accumulation had a predominant role in drought response. As for varieties, drought sensitivity or tolerance was not necessarily correlated with differences in the antioxidative response at early vegetative stage.

Keywords: antioxidative protection; drought; proline; recovery; seedlings; wheat (*Triticum aestivum* L.)

Drought stress is an important environmental factor constraining crop productivity. Wheat is essential nourishment for more than 1/3 of the world population and crop yield will be considerably influenced in the perspective of global climate change and limitation of water resources in the environment (Chaves and Oliveira 2004). Much of the injury on plants under abiotic stress is linked to oxidative damage at the cellular level (Smirnoff 1993). Development of oxidative stress is a result of the imbalance between the formation of reactive oxygen species (ROS) and their detoxification (Mittler 2002). Electron transport chains in chloroplasts and mitochondria and excited chlorophyll are the most active intracellular producers of ROS such as superoxide anion radical and singlet oxygen. Other important sites of ROS production, especially of hydrogen peroxide, are peroxisomes (sources – photorespiration and fatty acid β-oxidation), plasmalemma and cell walls (Mittler 2002). Oxidative stress under drought is a consequence of the inhibition of photosynthetic activity and the resulting exposure of chloroplasts to excess excitation energy and increased activated oxygen formation via the Mehler reaction (photoreduction of O_2 yielding superoxide radical) along with decrease in photorespiratory H_2O_2 production in peroxisomes (Smirnoff 1993). The mitochondrial respiration is also activated under stress (Mittler 2002) Greater oxidative load on chloroplasts and mitochondria under drought stress was previously reported (Munné-Bosch and Lalueza 2007). If not quenched, the above mentioned ROS can be converted to the highly toxic hydroxyl radical that can randomly damage cell membranes, proteins, and nucleic acids.

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Due to the short living period of ROS, the damage effects are usually restricted at the sites of their production (Mittler 2002). In this respect, the antioxidant protection in plant cells is complex and highly compartmentalized, comprising enzymic and non-enzymic components. The enzymes superoxide dismutase (SOD, EC 1.15.1.1), a family of enzymes catalysing the dismutation of superoxide anion radical to hydrogen peroxide in organelles and in the cytosol; catalases in peroxisomes (CAT, EC 1.11.1.6) which remove the bulk of hydrogen peroxide generated in photorespiration, and peroxidases with broad specificities (GPX, EC 1.11.1.7) located in vacuoles, cell walls and the cytosol which use hydrogen peroxide for substrate oxidation play central role in the defense against ROS. The low molecular antioxidant compounds, such as ascorbate and glutathione, are present in the tissues in millimolar concentrations (Noctor and Foyer 1998). Ascorbate is the major primary antioxidant that reacts directly with hydroxyl radical, superoxide anion radical and singlet oxygen, as well as secondary antioxidant in the ascorbate-glutathione pathway. Glutathione is the predominant non-protein thiol, redox buffer, and substrate for keeping the ascorbate in reduced form in the ascorbate-glutathione pathway (Noctor and Foyer 1998).

Usually enhanced anti-oxidative protection is related to better drought resistance (Sairam and Srivastava 2001). Four Bulgarian varieties of wheat were compared under field water limitation in terms of yield and oxidative stress response and it was established that a loss of membrane integrity and oxidative damage to lipids were more pronounced in the sensitive varieties under field drought (unpublished data). The effect of the drought on given plant species depends on variety, intensity and duration of the stress as well as on the developmental stage. It was interesting to observe the antioxidative protection of the same varieties under drought at early seedling stage in order to evaluate the usefulness of oxidative stress parameters as an additional screening criterion for detecting water stress tolerance or sensitivity in plants. Different models were used to study water stress in plants – water or soil cultures, shock treatment or gradual imposition, which influence the antioxidative response differently and the obtained results may not be quite comparable (Srivalli et al. 2003). It was considered important to use soil cultures and to induce water deficit stress gradually by withholding irrigation because these conditions are closer to the natural ones than a rapid artificial decrease in water potential of the root environment. Gradual stress imposition gives the possibility for plants to adapt cell processes to the adverse conditions (Simova-Stoilova et al. 2006). Relatively few data describe recovery from drought, especially antioxidative protection (Sgherri et al. 2000, Srivalli et al. 2003).

The aim of this work was to evaluate the oxidative stress and the response of the protective systems to soil drought and subsequent re-watering in an early growth stage of wheat varieties differing in their field drought resistance.

MATERIAL AND METHODS

Plant material and growth conditions. Four varieties of winter wheat (*Triticum aestivum* L.) with different field drought resistance (based on grain yield reduction under field drought during a three-year period) were studied – two drought-tolerant (Yantar and Zlatitsa) and two drought-sensitive (Dobrudjanka and Miziya). Plants were grown in pots with 400 g of leached meadow cinnamonic soil, pH 6.2, optimally fertilized with N, P and K, 16 plants per pot, under 150 μE/m^2/s PAR, 21–25°C and 14 h photoperiod. Relative soil humidity 70% of the maximal soil capacity was maintained by daily watering. Drought stress was imposed on 8-day old seedlings with fully expanded first leaf and developing second one by withholding irrigation for seven days, followed by three days recovery of treated plants by resuming optimal water supply. Controls were watered daily. All biochemical analyses were performed on the first leaf. Leaf samples were quickly frozen and stored in liquid nitrogen until analyses.

Leaf water status, membrane stability, protein and dry weight measurements. Relative water deficit (wd %) of the first leaves was determined according to the formula:

$$[(tw - fw)/tw] \times 100$$

where: tw is leaf weight at full turgidity and fw is the actual leaf fresh weight

Samples were taken at a fixed hour in the morning. The cell membrane integrity was evaluated as relative electrolyte leakage from 2 cm leaf segments floating on distilled water for 16 h at 8°C and expressed in percentage of total leaf electrolyte content released after boiling the segments for 10 min in the same effusate.

Protein quantity was determined spectrophotometrically according to Bradford (1976) with bovine serum albumin as a standard. Plants were dried at 105°C to constant weight for the determination of dry weight.

Proline, hydrogen peroxide and malondialdehyde determination. Leaves (0.5 g fw) were homogenized in 5 ml of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 10 000 g for 30 min. Proline was determined by the method of Bates et al. (1973). Hydrogen peroxide content was assayed with the redox active indicator xylenol orange according to Wolff (1994). Values were calculated using standard curve with known amount of H_2O_2. Lipid peroxidation was estimated using the thiobarbituric acid reactive substances assay. The optical density was read at 440, 532 and 600 nm and malondialdehyde (MDA) content was calculated according to Hodges et al. (1999) with correction for sugar interference in the test.

Antioxidant enzyme activities and isoforms. Leaf material (0.5 g fw), frozen in liquid nitrogen prior to the extraction, was homogenized in ice-cold 50 mmol/l Tris-HCl buffer pH 7.5 containing 2 mmol/l $MgCl_2$, 2 mmol/l $CaCl_2$, 10 mmol/l β-mercaptoethanol, 2 mmol/l phenylmethanesulphonylfluoride, 0.005% Triton X 100, 50 mg Polyclar AT and centrifuged at 15 000 g for 30 min at 4°C. Enzyme activities and isoenzyme staining were analysed as described previously (Demirevska-Kepova et al. 2004). SOD activity was measured spectrophotometrically at 560 nm based on inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50%. Isoenzymes of SOD were resolved on 10% nondenaturing polyacrylamide gel at 4°C. After electrophoresis, SOD activity in gels was visualized with NBT. SOD isoforms were differentiated by pre-stain incubation for 30 min in 50 mmol/l potassium phosphate buffer, pH 7.8 containing 5 mmol/l H_2O_2. The Mn SOD is resistant to such treatment, whereas Cu-Zn SODs are inhibited. Catalase (CAT) activity was assayed following H_2O_2 decomposition at 240 nm ($\varepsilon = 0.0394 mM^{-1}.cm^{-1}$). CAT isoenzymes were separated on 7.5% nondenaturing polyacrylamide gel at 4°C. The gels were stained for CAT activity

Table 1. Effect of drought and re-watering on leaf water status, membrane stability and protein content

Variety	Treatment	Leaf water deficit (%)	Electrolyte leakage (%)	dw (g/g fw)	Leaf protein (mg/g dw)
Yantar	C	5.96 ± 1.79^{a}	7.13 ± 2.82^{a}	0.0796	170.26 ± 11.68^{cde}
	D	59.60 ± 2.32^{e}	21.99 ± 2.72^{d}	0.2165	189.37 ± 22.21^{de}
	R	8.17 ± 1.65^{ab}	5.11 ± 0.37^{a}	0.1016	137.23 ± 25.16^{abc}
	RC	10.73 ± 1.68^{bc}	4.87 ± 0.29^{a}	0.0911	140.85 ± 6.81^{abc}
Zlatitsa	C	5.45 ± 1.44^{a}	5.36 ± 0.63^{a}	0.0803	154.05 ± 38.22^{abcd}
	D	59.65 ± 4.39^{e}	17.10 ± 3.53^{c}	0.2323	155.72 ± 14.82^{abcd}
	R	6.95 ± 1.69^{ab}	8.18 ± 2.25^{ab}	0.0979	154.21 ± 8.41^{abcd}
	RC	8.88 ± 5.53^{ab}	5.03 ± 0.33^{a}	0.0926	144.09 ± 4.47^{abc}
Miziya	C	5.49 ± 0.85^{a}	5.21 ± 0.91^{a}	0.0849	162.20 ± 30.47^{bcde}
	D	57.14 ± 3.81^{de}	18.32 ± 6.44^{cd}	0.2563	144.09 ± 1.38^{abc}
	R	8.03 ± 0.56^{ab}	11.84 ± 1.28^{b}	0.1017	148.11 ± 31.32^{abc}
	RC	10.05 ± 1.69^{bc}	3.61 ± 1.20^{a}	0.0907	138.45 ± 4.01^{abc}
Dobrudjanka	C	6.14 ± 0.88^{a}	4.67 ± 0.70^{a}	0.0817	197.86 ± 4.59^{e}
	D	55.54 ± 1.99^{d}	19.61 ± 5.28^{cd}	0.2331	152.36 ± 4.76^{abcd}
	R	10.08 ± 0.61^{bc}	7.62 ± 1.06^{ab}	0.1024	127.30 ± 8.09^{ab}
	RC	13.3 ± 5.71^{c}	7.70 ± 0.80^{ab}	0.1016	123.47 ± 7.03^{a}

C – control plants; D – drought-treated plants; R – recovery; RC – age control of recovery
Values are mean ± standard deviation of three replicates. Statistically significant difference among values ($P < 0.05$) is indicated with different letters following the figures

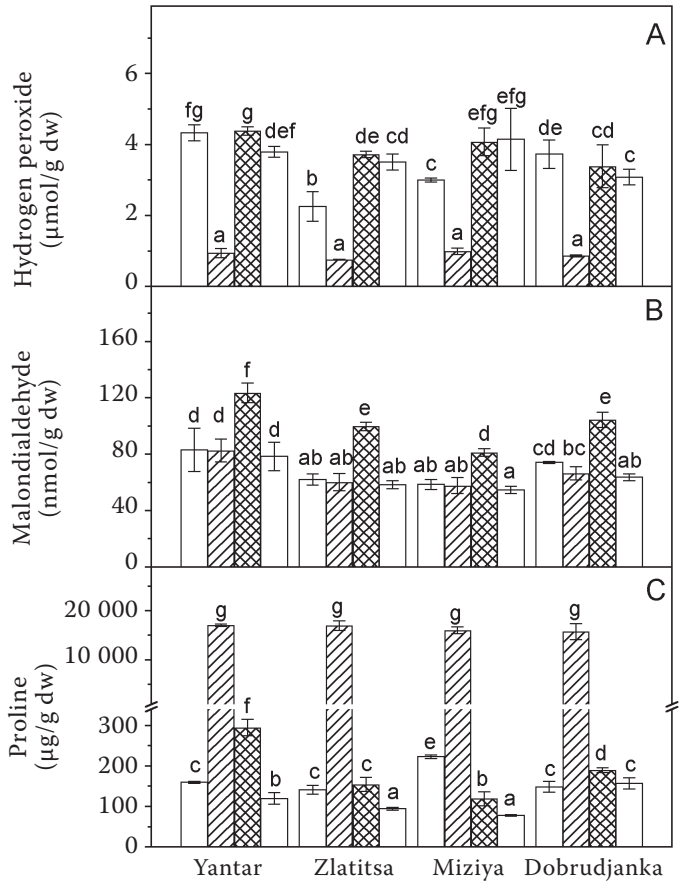

Figure 1. Hydrogen peroxide (A), malondialdehyde (B) and proline (C) content in control, drought-treated and recovered plants on a dw basis. White columns – age controls (adjacent to the treatments), stripped columns – drought treatment, checked columns – recovery. The columns represent means ± SD of three replicates from two independent experiments. Statistically different values ($P < 0.05$) are indicated with different letters above the columns

with ferricyanide. GPX activity was assayed with guaiacol as a substrate following the formation of the reaction product ($\varepsilon = 26 mM^{-1}.cm^{-1}$) at 420 nm. GPX isoenzymes were analysed on 7.5% nondenaturing polyacrylamide gel at 4°C. The gels for isoenzyme staining were loaded with 20 μg protein per lane and stained for GPX activity with substrate guaiacol.

Antioxidant compounds. Nonprotein SH groups were determined as described by Edreva and Hadjiiska (1984) with Ellman's reagent and were assumed to represent mainly GSH. Optical density at 412 nm was registered and the level of nonprotein SH groups was calculated using $\varepsilon = 13600 M^{-1}.cm^{-1}$ for the reaction product 2-nitro-5-benzoic acid. Ascorbate pool (total and reduced) was assayed according to the protocol of Hodges et al. (1996) on the basis of reduction of Fe^{3+} to Fe^{2+} by ascorbate in acid solution and complexation of Fe^{2+} with α,α'-dipyridyl leading to a pink colour. The absorbance at 525 nm was measured and ascorbate content was quantified using a standard curve. Oxidised ascorbate was estimated from the difference between total and reduced ascorbate.

Statistical analysis. Results were based on at least three replicates from two independent experiments. The values were analysed by multifactor ANOVA (Statgraphics plus, version 2.1) at the level of significance $P < 0.05$. Values followed by different letters are significantly different for a given parameter.

RESULTS AND DISCUSSION

The establishment of the experimental design and physiological response of wheat plants to drought treatment was described in details in Simova-Stoilova et al. (2006). To achieve uniform development of soil water deficit, which was important for correct comparison among varieties, seeds of the four varieties in this study were sown together

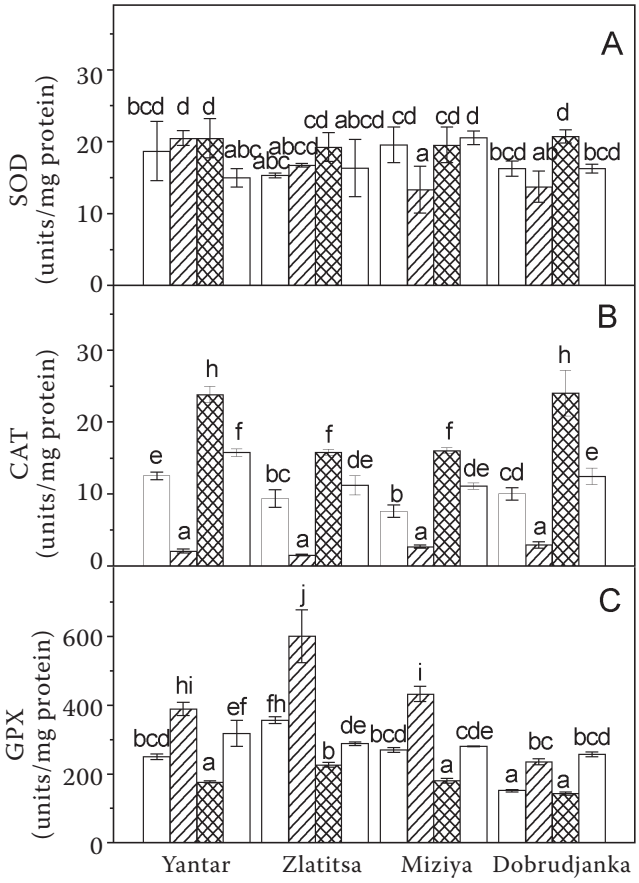

Figure 2. Activities of SOD (A), CAT (B) and GPX (C) in control, drought treated and recovered plants on a protein basis. White columns – age controls (adjacent to the treatments), stripped columns – drought treatment, checked columns – recovery. Units were defined as: the quantity of enzyme required to inhibit the reduction of NBT by 50% per min (SOD), nmol H_2O_2 decomposed per min (CAT), μmol tetraguaiacohinone produced per min (GPX). The columns represent means ± SD of three replicates from two independent experiments. Statistically different values ($P < 0.05$) are indicated with different letters above the columns

in the same pots. Water deprivation of 8-day old seedlings for 7 days resulted in an increase in leaf wd to 56–59% (Table 1). Water loss from the first leaf was also seen by the increase in dw per g fw ratio and corresponds to severe stress conditions (Smirnoff 1993). As a result of the treatment, the electrolyte leakage from cell membranes, reflecting membrane intactness, increased about 3-fold. This damage was recoverable after 3 days of optimal water supply for all varieties under study except the most drought-sensitive one (Miziya). Leaf total soluble protein levels did not clearly indicate senescence symptoms driven by severe drought at seedling stage. This is in contrast to field drought experiments on the same varieties, where accelerated senescence was observed at reproductive stage of development (unpublished data). The physiological response to drought was similar among the varieties. Leaf water status and membrane intactness recovered three days after re-watering.

Data concerning first leaf hydrogen peroxide level, oxidative damage to membranes as malondialdehyde content as well as proline accumulation are presented in Figure 1. Hydrogen peroxide content was low under drought compared to 7-day old control plants (Figure 1A). Values of the recovered plants were comparable to the respective controls. Malondialdehyde level (Figure 1B) was not changed significantly in the leaves of drought-treated plants but raised during recovery. These findings indicate increased oxidative strain on membranes in recovery from severe drought stress but rather strict control on ROS formation in the cells. The varieties responded similarly to drought and re-watering. Proline content (Figure 1C) in drought-treated plants reflected to a great extent the stress severity without significant differences

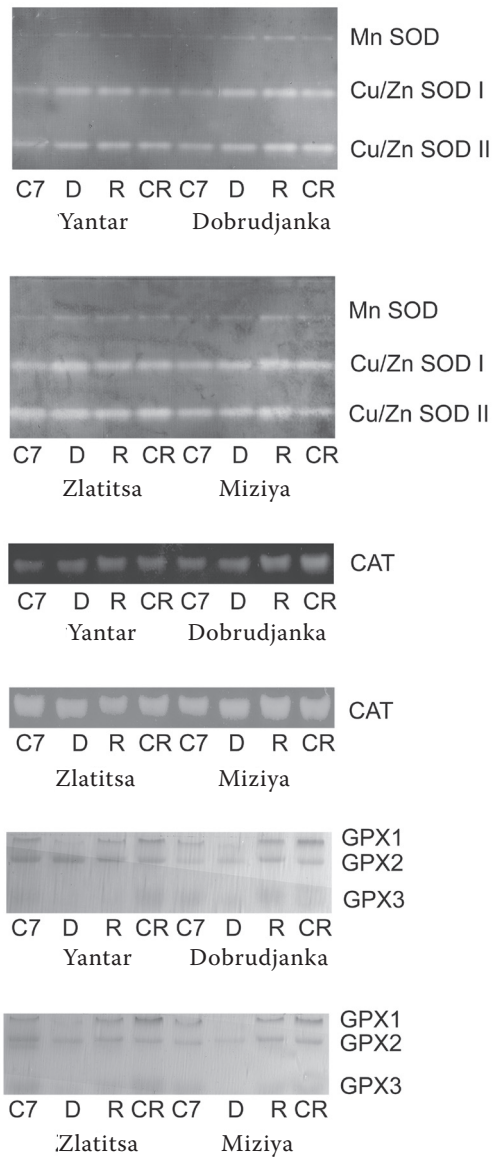

Figure 3. Isoenzyme profiles of SOD, CAT and GPX. Abbreviations below the images: C7, CR – controls, D – drought treatment, R – recovery. Different isoforms are indicated on the right. Gels are loaded with 20 µg protein per lane

among varieties. After 3 days of recovery it diminished without reaching the values of the respective control plants. Accumulation of compatible solutes like proline helps maintaining cell water status and protecting membranes and proteins from the denaturing effects of the osmotic stress. The ability of proline to scavenge free radicals is another point to be considered in this context (Ashraf and Foolad 2007). Genotype-dependent differences in proline accumulation were observed in wheat seedlings subjected to drought, which corresponded to the stress intensity (Yadav et al. 2004). In our study, proline accumulation under severe drought was most probably linked to membrane protection and also to antioxidative defense and did not reveal differences among varieties.

The activities of the main antioxidative enzymes SOD (A), CAT (B) and GPX (C) are presented in Figure 2. Data on leaf protein basis reflect the relative proportion of the enzyme in the total protein content. SOD activity was little changed as a consequence of drought stress. CAT activity was very low in drought-treated plants and after recovery it was significantly higher than that of controls. It corresponded to the low hydrogen peroxide level under drought stress and most probably was a consequence of the inhibition of photosynthesis and photorespiration under drought, which is well documented (Chaves and Oliveira 2004). GPX activity was significantly higher under severe drought and diminished again during recovery. It paralleled the changes in electrolyte leakage and was in accordance with the membrane-stabilizing function of this enzyme. Different trends of changes in antioxidative enzyme activities were described depending on the mode of imposition, duration and severity of the drought stress (Sgherri et al. 2000, Sairam and Srivastava 2001). In general, increased SOD and CAT activities were reported for a mild water deficit (Feng et al. 2004), whereas severe or prolonged drought stress caused a decline in activities of this enzyme (Guo et al. 2006).

The total enzyme activity may not reflect opposite changes in the activities in enzyme isoforms. The results of in-gel staining for SOD, CAT and GPX activity after separating the proteins in non-denaturing PAGE (PAGE – polyacrylamide gel electrophoresis) are presented in Figure 3. The bands of the SOD isoforms were established preliminarily using selective inhibitor treatments. Three isoforms of SOD (the mitochondrial Mn SOD, the cytosolic CuZn SOD I and the chloroplastic CuZn SOD II), one of CAT and three of GPX were clearly visible. Under the treatment, no major changes were detected in the isoenzyme bands of SOD except a slight enhancement of CuZn SOD I. The CAT band was slightly reduced under drought and was enhanced again during the recovery phase. Conserved isoenzyme profile of GPX in the controls but dynamic changes in drought treated and recovered plants were detected. GPX1 and GPX3 were reduced in activity under drought and restored after recovery whereas GPX2 was persistent. The opposite results obtained for GPX activity spectrophotometrically in whole extract and after electrophoretic separation were surpris-

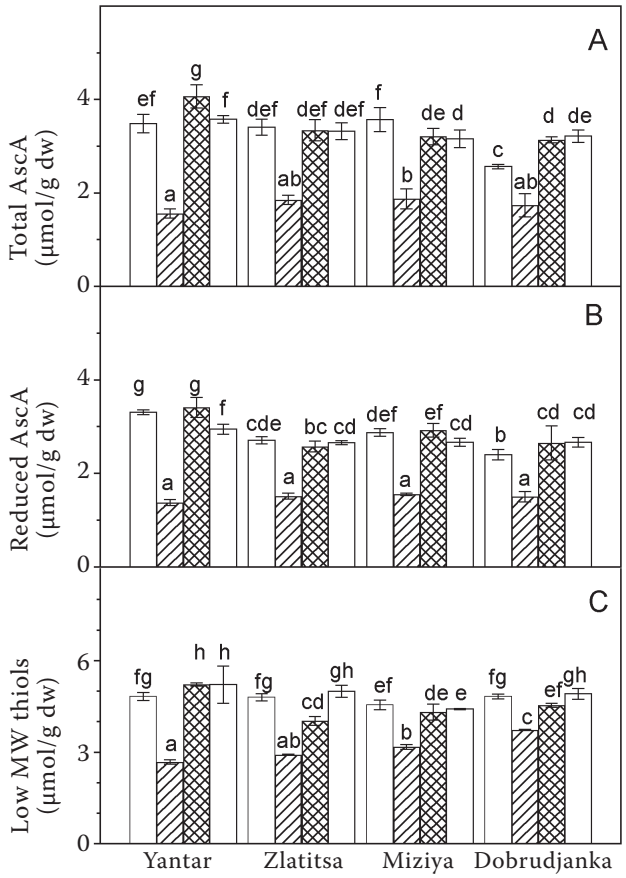

Figure 4. Total ascorbate (A), reduced ascorbate (B) and low molecular thiol (C) content in control, drought treated and recovered plants on a dw basis. White columns – age controls (adjacent to the treatments), stripped columns – drought treatment, checked columns – recovery. The columns represent means ± SD of three replicates from two independent experiments. Statistically different values ($P < 0.05$) are indicated with different letters above the columns

ing but might be explained by different conditions of analysis. Electrophoresis might lead to separation of the proteins from low-molecular activators or inhibitors or cause some conformational changes in the enzyme protein thus affecting its activity. Moreover, in the izoenzyme staining for GPX, combined guaiacol and benzidine substrates were used. No clear differences in the isoenzyme response were noticed among varieties.

Changes in the contents of low-molecular antioxidant compounds ascorbate (A, B) and thiols (representing mainly glutathione, C) after severe drought and subsequent recovery are presented in Figure 4. The levels of both compounds strongly decreased during drought and were restored during recovery from water deficit stress. The ratio between reduced and oxidised ascorbate was more or less conserved. These results strongly suggest participation of the low-molecular antioxidative compounds in the defense against ROS under severe drought and rather good functionality of the ascorbate/glutathione cycle, which allowed wheat plants to maintain a low hydrogen peroxide level. An involvement of antioxidant metabolites in ROS detoxification under drought with increased pools of ascorbate and glutathione at the beginning of the water stress and diminution when the stress becomes more severe was observed (Dalmia and Savhney 2004). No variety-specific differences in the response of low molecular antioxidants to drought were found.

Drought resistance is defined as a higher relative yield of a certain genotype compared with another at the same stress intensity. However, many factors could influence yield under drought at the whole plant level: morphology, net photosynthesis, assimilate mobilization and redistribution, accelerated senescence. It appears likely that a better antioxidative protection plays a role in drought resistance at the reproductive stage in the field. However, it is questionable to consider such plant properties to distinguish between drought-sensitive and drought-tolerant varieties at early seedling stage under artificial growth conditions. The results

reported here lead to the conclusion that stress protection in these plants was achieved mainly by proline accumulation and by a high plasticity of the metabolic processes.

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