Plant Growth Regul (2009) 58:107–117
DOI 10.1007/s10725-008-9356-6

ORIGINAL PAPER

Antioxidative protection and proteolytic activity in tolerant and sensitive wheat (*Triticum aestivum* L.) varieties subjected to long-term field drought

Lyudmila Simova-Stoilova · Klimentina Demirevska · Tatyana Petrova · Nikolay Tsenov · Urs Feller

Received: 22 July 2008 / Accepted: 12 December 2008 / Published online: 10 January 2009

Abstract Field drought studies were performed in order to assess oxidative stress, proteolytic activity and yield loss under natural stress conditions. Flag leaves of two drought-tolerant (Yantar and Zlatitsa) and two drought-sensitive (Miziya and Dobrudjanka) winter wheat varieties were analyzed. Stress intensity was assessed by relative electrolyte leakage and proline accumulation. Senescence progression was followed by loss of chlorophyll and protein. Lipid peroxidation, H_2O_2 content, activities of superoxide dismutase (SOD), catalase (CAT), and non-specific peroxidase (GPX) isoforms, as well as proteolytic activities were analyzed from heading throughout grain filling. Weakening of membrane integrity and oxidative damage to lipids were more pronounced in the sensitive varieties under field drought. The activities of Fe- and Cu/Zn SOD isoforms decreased in the controls, but remained high in drought-treated plants. The activities of MnSOD isoforms and CAT were enhanced towards grain filling, especially in the sensitive varieties under drought. GPX activities were rised under drought but progressively diminished. Accelerated senescence under field drought was linked to higher proteolytic activity with variety specific differences in the protease response, but without a clear correlation to drought resistance or sensitivity. Field drought led to higher oxidative stress more pronounced for drought sensitive varieties, especially during the grain filling period.

Keywords Anti-oxidative enzymes · Field water deficit · Oxidative stress · Proteolysis · Wheat · Yield

Abbreviations

DAS	Days after sowing
DI	Drought intensity
FW	Leaf fresh weight
GPX	Non-specific peroxidase
CAT	Catalase
ROS	Reactive oxygen species
WD	Water deficit
SOD	Superoxide dismutase
S	Drought susceptibility index
TW	Leaf weight at full turgidity

Introduction

Drought is one of the most significant factors that reduce crop productivity and scenarios for global

L. Simova-Stoilova (✉) · K. Demirevska
Department of Plant Stress Molecular Biology, Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bldg. 21, 1113 Sofia, Bulgaria
e-mail: lsimova@mail.bg

T. Petrova · N. Tsenov
Dobrudja Agricultural Institute, General Toshevo, Bulgaria

U. Feller
Institute of Plant Sciences, University of Bern, Altenbergrain 21, 3013 Bern, Switzerland

environmental change suggest an increase in aridity in many agricultural areas (Chaves and Oliveira 2004). At a whole plant level crops have developed different strategies to counteract the negative effects of water limitation: increased water use efficiency, drought escape by shortening life cycle, dehydration avoidance by maintaining lower water deficit (WD) in the cells, dehydration tolerance at higher WD (Lascano et al. 2001). Drought tolerance of crops is regarded as higher yield of a certain genotype compared to others at the same level of stress intensity (Fisher and Maurer 1978).

In wheat, the most critical stage for yield reduction under stress is the spike-growing period around anthesis (Fisher 1985; Gupta et al. 2001). Water stress usually shortens the life cycle and the grain-filling period of crops, reducing photosynthesis and accelerating senescence (Yang et al. 2000; Chaves and Oliveira 2004). At the same time, better and faster remobilization of leaf constituents has been observed under mild field drought, which could minimize the effect of the shorter grain-filling period (Yang and Zhang 2006). Flag leaves in wheat are strongly involved in source–sink relations and are important source of nutrients during grain filling trough photo assimilate partitioning and nutrient remobilization. Amino acid remobilisation in senescence is closely linked to protein degradation and occurs relatively early in senescence, when the cell integrity is still well maintained, whereas strong oxidative events occur at later senescence stage (Hortensteiner and Feller 2002; Prochazkova and Wilhelmova 2007). Increased endopeptidase activity is reported in wheat flag leaves during grain filling (Srivalli and Khanna-Chopra 1998). The activity of intracellular proteases considerably increases under drought in comparison to well-watered plants (Zagdańska and Wiśniewski 1996; Martinez et al. 2007). Although an induction of cysteine proteinases takes place in senescence, which is more enhanced by drought, it is not clear how genotype specific differences in the protease response are related to yield. Certain data suggest that higher proteolytic activity is rather negatively correlated to drought resistance of wheat (Grudkowska and Zagdańska 2004).

Development of oxidative stress is a result of the imbalance between the formation of reactive oxygen species (ROS) and their detoxification (Mittler 2002, Zimmermann and Zentgraf 2005). Electron transport chains in chloroplasts and mitochondria membranes 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). Photosynthesis inhibition under drought leads to 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). Proliferation of mitochondria and peroxisomes has been reported in senescing tissues, which become the main source of activated oxygen at this stage of development (Del Rio et al. 1998). If not quenched, the above mentioned ROS can be converted to the highly toxic hydroxyl radical which can damage randomly cell membranes, proteins, and nucleic acids. All ROS except H_2O_2 are short living (2–4 μs) so their damage effect is mainly at the sites of their production. Only H_2O_2 (half-time 1 ms) can diffuse apart and penetrate membranes (Mittler 2002). It has a double role as ROS and as signalling molecule—a cellular indicator of stress and secondary messenger involved in the stress-response signal transduction pathway and activation of the defense systems.

The ROS detoxifying enzymes play a central role in the defence against ROS, besides the non-enzymic components (α-tocopherol, carotenoids, redox-couples of ascorbate and glutathione). Superoxide dismutases (SODs, EC 1.15.1.1, a family of enzymes catalyzing the dismutation of superoxide to H_2O_2) located in chloroplasts, cytosol, mitochondria, peroxisomes, and apoplast, acts in close cooperation with the enzymes and metabolites of the ascorbate-glutathione cycle. Catalases in peroxisomes (CAT, EC 1.11.1.6) remove the bulk of H_2O_2 generated in photorespiration. Peroxidases with broad specificity (GPX EC 1.11.1.7) located in vacuoles, cell walls, and the cytosol use H_2O_2 in substrate oxidation (Mittler 2002). Better resistance and acclimation to drought is experimentally correlated with enhanced anti-oxidative protection (Sairam et al. 1998; Sairam and Srivastava 2001; Chen et al. 2004; Khanna-Chopra

and Selote 2007). However, weakening of the enzymic antioxidative protection is reported in senescing tissues, which can partly be explained by the protein degradation characteristic for this developmental stage (Casano et al. 1994). Generally, higher antioxidative protection has been linked to delayed senescence (Zimmermann and Zentgraf 2005).

The link oxidative stress–senescence–proteolytic activity is only partially elucidated. Most of the reports concerning drought tolerance and oxidative stress under drought are undertaken in controlled laboratory conditions on hydroponic cultures with PEG treatment or on pot soil experiments with some constraints for the development of the root system. They used shorter experimental duration and imposition of more severe drought than that under natural field conditions. Limited data exist on the basis of field trials, where WD usually co-occurs with high light and high temperature stress and superimposition of stresses could modify the response. For example, WD may have a protective role against heat stress but may exacerbate photoinhibition (Chaves et al. 2002). Comparative field and in vitro studies on different wheat varieties established no clear correlation of water stress tolerance with some components of the antioxidant system activity under field conditions, but the existence of such a link was found under osmotic stress in vitro (Lascano et al. 2001). Moreover, source-sink relations are a predominant factor at the reproductive stage on plant growth.

In this study, the response to field water stress was compared in four winter wheat (*Triticum aestivum* L.) varieties differing in yield under field drought in order to understand the response to oxidative stress, alterations in proteolytic activities, and effect on yield under agronomically relevant drought conditions in the field.

Materials and methods

Field experiments

Four winter wheat varieties—two relatively drought-tolerant (Yantar and Zlatitsa) and two relatively drought-sensitive (Miziya and Dobrudjanka)—were compared. Field studies with terminal drought were carried out in 2006/2007 on haplic chernozem soil (Fao-Unesco-Isric international classification of soil resources) at the Experimental Station of Dobrudja Agricultural Institute, General Toshevo, Bulgaria (43°42′N, 28°2′E). Plants were grown in a "dessication" greenhouse covered with a transparent polyethylene rainout shelter. The penetration of water from control plots into those of drought treatments was prevented by drainage. Seeds were sown at the end of October in 1 m rows, 70 germinating seeds per row in four random replicates for each variety and treatment. In the autumn all the plants received water (about 120 l m^{-2}). Thereafter, drought-treated plants were left on soil humidity reserves and developed gradual water stress, whereas control plants were regularly watered, receiving about 440 l m^{-2} of water in the spring. First signs of drought suffering of the treated plants were observed at the end of April, during the stem extension phase. Flag leaves were harvested at regular time intervals in May, at 170, 184, and 197 days after sowing (DAS, around heading, anthesis, and grain filling stages). Leaf material was quick frozen and stored in liquid nitrogen until analysed.

Growth and yield parameters

The heading date, stem height and spike length, spike number per row, grain number per spike, grain weight per row, grain weight of one main spike, and weight of 1,000 grains were registered in four plots (rows) per treatment (measurements on ten plants per plot). Drought susceptibility index (S) was calculated according to Fisher and Maurer (1978): $S = (1 - Y_d/Y_c)/DI$ where Y_d and Y_c are the yields of the drought-treated and control plants of one particular variety and DI is the drought intensity for the conditions of the given year. DI is defined as $DI = 1 - X_d/X_c$ where X_d and X_c are the average yields of all compared varieties under drought and control conditions, respectively. Thus, $S = (1 - Y_d/Y_c)/(1 - X_d/X_c)$ and if $S < 1$ the variety has high drought tolerance, if $S > 1$ the variety has low drought tolerance compared to the mean yield of the group of varieties under study.

Chlorophyll and protein content

Total chlorophylls were extracted with 80% acetone, estimated according to Arnon (1949) and calculated using the formula of McKinney (1941). Protein

quantity was determined spectrophotometrically according to Bradford (1976) using bovine serum albumin as a standard.

Water status and membrane stability

Relative WD (%) of the flag leaves was determined according to the formula: (TW − FW)/TW × 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 flag leaf membrane integrity was evaluated as relative electrolyte leakage from 2 cm leaf segments floating on distilled water for 24 h at 8°C and expressed in percentage of total leaf electrolyte content released after boiling the segments for 10 min in the effusate. Soil water content was measured every sampling day at 10-cm depth intervals up to 100 cm deep using a soil drill between plots subjected to the same watering regime.

Proline, hydrogen peroxide and malondialdehyde determination

Leaves (0.5 g FW) were homogenized in 5 ml of 0.1% trichloroacetic acid. The homogenate was centrifuged at 10,000 g for 15 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 a standard curve with known amount of H_2O_2. Lipid peroxidation was estimated using the assay for thiobarbituric acid-reactive substances. 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 isoforms

Leaf material (0.5 g FW) was homogenized in ice-cold 50 mM Tris–HCl buffer pH 7.5 containing 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol, 0.005% Triton×100, and 50 mg Polyclar AT. The extracts were centrifuged at 14,000 g for 40 min at 4°C. Extracts for proteolytic activity were prepared without protease inhibitors. Phenylmethanesulphonylfluoride was added to the extracts for SOD, CAT, and GPX in a final concentration 2 mM. Isoenzymes of SOD were resolved on 10% non-denaturing polyacrylamide gel at 4°C. The gels were loaded with extract volume equivalent to 2 mg leaf FW. After electrophoresis SOD activity in gels were visualized according to González et al. (1998). SOD types were differentiated by pre-stain incubation for 30 min in 50 mM potassium phosphate buffer, pH 7.8 containing 5 mM H_2O_2 or 3 mM KCN. The MnSOD is resistant to both treatments; FeSOD is inhibited by H_2O_2 but not by KCN. Cu–Zn SODs are inhibited by both reagents. Catalase (CAT) isoenzymes were separated on 7.5% non-denaturing polyacrylamide gel at 4°C. The gels were loaded with protein equivalent to the same FW and were stained for CAT activity following Woodbury et al. (1971). GPX isoenzymes were analysed on 7.5% non-denaturing polyacrylamide gel at 4°C. The gels were loaded with protein equivalent of equal FW and were stained for GPX activity according to Hart et al. (1971).

Proteolytic activity

Endopeptidase activity was assayed spectrophotometrically using azocasein as a substrate according to Fischer and Feller (1993) with some modifications. The pH-optima were determined previously.

Statistics

Results were based on at least three replicates. The values were statistically analyzed by multifactor ANOVA (Statgraphics plus version 2.1.) Values are presented as a means ± standard deviation ($n = 3$ or more). Statistically significant differences among values at the significance level $P < 0.05$ are indicated by different letters.

Results

Plant development and yield parameters

Dobrudja region has temperate continental climate with severe winters and hot summers. Under our experimental conditions, soil water content was not a limiting factor for plant growth and development during the autumn. First drought symptoms were observed only from stem extension stage onwards. Considerable differences were detected between soil

Table 1 Soil water content (moisture percentage related to weight of absolutely dry soil)

Soil depth (cm)	Soil water content (%)					
	Drought (DAS)			Controls (DAS)		
	170	184	197	170	184	197
0–10	12.8	10.0	9.5	22.3	22.2	22.7
10–20	13.0	10.8	11.0	23.4	23.2	23.8
20–30	14.9	12.2	12.1	24.4	23.9	24.7
30–40	15.2	13.5	13.6	25.1	24.6	25.2
40–60	15.2	14.2	14.4	25.2	24.8	24.3
60–80	14.5	14.3	13.6	24.5	23.9	23.8
80–100	13.8	14.3	13.3	24.3	23.2	23.5

Means of three replicates are shown

water content of drought and watered plots in the depth of the greatest part of the wheat root system (Table 1). Significantly faster heading was observed for the stressed plants of all varieties, indicating shortened vegetation cycle under drought (Table 2). Comparing the controls, the heading of the variety Miziya was earlier than that of the other three varieties. In terms of total yield, the varieties Yantar and Zlatitza behaved as relatively drought-tolerant and the varieties Miziya and Dobrudjanka as drought-sensitive (Table 2, drought sensitivity index). Drought-tolerant varieties had shorter stems than the sensitive ones. The stem height, the weight, and the number of grains per spike were not affected by drought in the most tolerant variety Zlatitsa.

Flag leaf water status, membrane damage, and accumulation of proline and hydroperoxides

Flag leaf WD (Table 3) had a tendency of slow increase in control plants towards leaf ageing. In drought stressed plants, a pronounced rise in WD was detected at the last sampling for Yantar, Miziya, and Dobrudjanka. For the variety Miziya, drought caused a significant increase of WD compared to control plants of the same age during the whole sampling period. The WD in the flag leaf of stressed Miziya plants was the highest at the last sampling compared to that of the other three varieties. This finding corresponded to faster increase in membrane instability for the same variety compared to the other varieties (Fig. 1a). The electrolyte leakage from cell membranes rose sharply in drought treated tolerant plants at second sampling but remained thereafter relatively constant. In sensitive varieties (Miziya and Dobrudjanka), membrane instability was highest at last sampling. Changes in the time course (increase followed by a decline) were observed for the content of lipid hydroperoxides (Fig. 1b). Highest MDA levels were detected in sensitive varieties at the second sampling. Proline content (Fig. 1c) was increased 1.6- to 3-fold at first sampling, 5.5- to 12-fold at second sampling, and 3.6- to 13-fold at last sampling in drought treated plants compared to the controls of the same age. The highest proline content was observed in drought treated varieties Yantar and Miziya, second sampling. The variety Dobrudjanka had the highest level of proline in drought treated plants at first sampling, compared to the other varieties. Hydrogen peroxide contents (Fig. 1d) were relatively constant in tolerant varieties. Drought treated plants of the sensitive varieties Dobrudjanka and Miziya contained significantly higher levels of H_2O_2 at 184 DAS compared to controls, followed by a considerable decrease at 197 DAS.

Isoenzyme profiles of SOD, CAT, and GPX activities

Activity changes of SOD, CAT, and GPX isoforms in the flag leaf of control and drought stressed plants are shown in Figs. 2 and 3. The bands of the respective SOD types (Fig. 2) were established using inhibitory analysis (data not shown). Two MnSOD, one FeSOD, and two Cu/Zn SOD isoforms were revealed. The activity of the second MnSOD was enhanced under drought at anthesis and during the grain filling period, especially in the sensitive varieties Miziya and Dobrudjanka. The activities of FeSOD progressively diminished in the controls but persisted in drought treated plants, without visible differences between varieties. Highest activities possessed Cu/Zn SOD I (cytosolic) and Cu/Zn SOD II (chloroplastic). Cu/Zn SOD I activity decreased more rapidly in the controls than in the drought-treated plants. Cu/Zn SOD II activity was lower only in the last phase in the controls of drought-tolerant varieties (Yantar and Zlatitza) whereas it was at a constant level in the controls and in stressed sensitive varieties (Miziya and Dobrudjanka).

One isoform of CAT was revealed (Fig. 3). Its intensity increased progressively during grain filling,

Table 2 Growth and yield parameters

Variety and treatment		Heading (DAS)	Stem height (cm)	Spike length (cm)	Number of spikes per row	Number of grains per spike	Weight of 1,000 grains (g)	Grains weight per main spike (g)	Yield per row (g)	Drought sensitivity index
Yantar	D	174.8 ± 1.0^{a}	91 ± 2.9^{ab}	9.3 ± 0.3^{bc}	97 ± 2.9^{a}	38 ± 1.6^{a}	35.4 ± 3.1^{bc}	$1.35 \pm 0.\ 2^{b}$	130.9 ± 1.8^{bc}	0.821
	C	179.8 ± 0.5^{c}	98 ± 3.2^{cd}	9.9 ± 0.5^{de}	116 ± 14^{bcde}	44 ± 5^{bc}	45.3 ± 5.0^{e}	2.01 ± 0.4^{c}	172.9 ± 21.5^{e}	
	K	Δ 5.0	92.6%	94.7%	83.6%	86.5%	78.2%	67.16%	75.7%	
Zlatitsa	D	174.8 ± 1.0^{a}	88 ± 1.7^{a}	8.9 ± 0.2^{ab}	127 ± 7.8^{e}	38 ± 3^{a}	37.0 ± 0.9^{bcd}	1.41 ± 0.1^{b}	137.2 ± 15.7^{cd}	0.657
	C	179.5 ± 0.6^{c}	91 ± 2.6^{ab}	9.6 ± 0.4^{cd}	126 ± 4.8^{de}	42 ± 1.6^{ab}	40.8 ± 1.1^{de}	1.72 ± 0.1^{c}	170.3 ± 27.5^{e}	
	K	Δ 4.8	96.2%	92.4%	100.8%	90.7%	90.6%	81.97%	80.5%	
Miziya	D	172.8 ± 1.6^{b}	100 ± 0.8^{d}	9.7 ± 0.2^{cde}	109 ± 4.2^{b}	41 ± 3^{ab}	32.0 ± 1.1^{b}	1.31 ± 0.1^{ab}	104.8 ± 10.1^{ab}	1.282
	C	176.5 ± 1.3^{d}	108 ± 3.4^{e}	10.1 ± 0.4^{e}	115 ± 5.6^{bcd}	50 ± 2.1^{d}	39.7 ± 5.0^{cd}	2.01 ± 0.3^{c}	168.9 ± 30.8^{e}	
	K	Δ 3.8	93.0%	95.7%	94.8%	81.3%	80.7%	65.17%	62.0%	
Dobrudjanka	D	174.3 ± 0.8^{ab}	95 ± 6.1^{bc}	8.5 ± 0.4^{a}	110 ± 9.8^{bc}	39 ± 2.6^{a}	26.4 ± 1.7^{a}	1.04 ± 0.1^{a}	99.0 ± 4.1^{a}	1.264
	C	180.0 ± 0.8^{c}	105 ± 0.8^{e}	9.1 ± 0.1^{b}	122 ± 10.8^{cde}	47 ± 2^{cd}	37.3 ± 5.3^{cd}	1.72 ± 0.1^{c}	158.2 ± 7.1^{de}	
	K	Δ 5.8	90.2%	92.9%	90.2%	83.5%	70.7%	58.14%	62.6%	

Means ± SD from four plots (measurements on ten plants per plot) are shown

Numbers followed by the same letter are not significantly different at $P < 0.05$ level (multifactor ANOVA test)

D drought, *C* controls, $K = C - D$ (Δ) for the time of heading and $K = D/C$ (%) for the other parameters, *DAS* days after sowing

Table 3 Flag leaf WD (%)

Variety	Water deficit (%)					
	Drought (DAS)			Controls (DAS)		
	170	184	197	170	184	197
Yantar	12.50 ± 2.89^{cd}	12.92 ± 2.31^{cde}	17.55 ± 2.80^{fg}	8.36 ± 3.59^{ab}	11.32 ± 1.79^{d}	14.00 ± 0.37^{f}
Zlatitza	11.04 ± 2.08^{bcd}	13.96 ± 1.84^{def}	16.43 ± 2.27^{efg}	8.49 ± 1.63^{ab}	11.04 ± 2.29^{bcd}	11.62 ± 2.86^{bcd}
Miziya	12.33 ± 3.33^{cd}	17.46 ± 1.51^{fg}	28.36 ± 4.82^{h}	6.99 ± 1.81^{a}	11.99 ± 2.07^{bcd}	14.35 ± 0.85^{def}
Dobrudjanka	12.75 ± 4.16^{cde}	14.28 ± 1.95^{def}	18.84 ± 4.29^{g}	9.97 ± 3.55^{abc}	12.64 ± 2.01^{cd}	13.62 ± 1.19^{cde}

Means ± SD of four replicates are shown

Numbers followed by the same letters are not statistically different at $P < 0.05$ (multifactor ANOVA test)

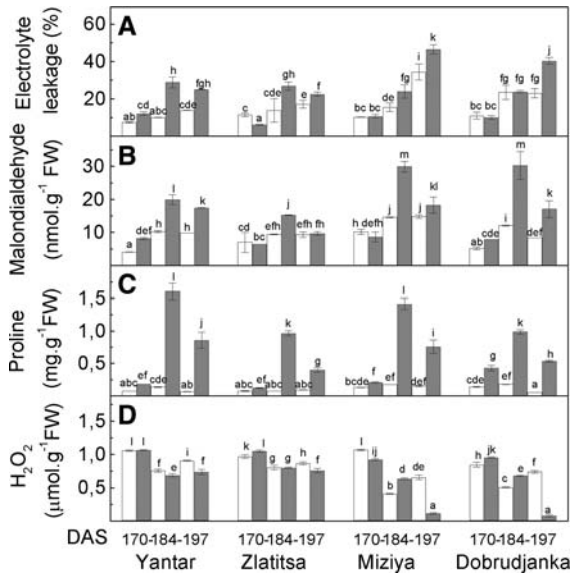

Fig. 1 Relative electrolyte leakage in %, contents of proline, MDA, and H_2O_2 in the flag leaves. Abscissa, DAS (*days after sowing*) and varieties. *White columns* controls, *gray columns* drought treatment. Values are means of three replicates. Standard deviations are presented as *vertical bars*. Statistically significant differences ($P < 0.05$) are indicated by *different letters*

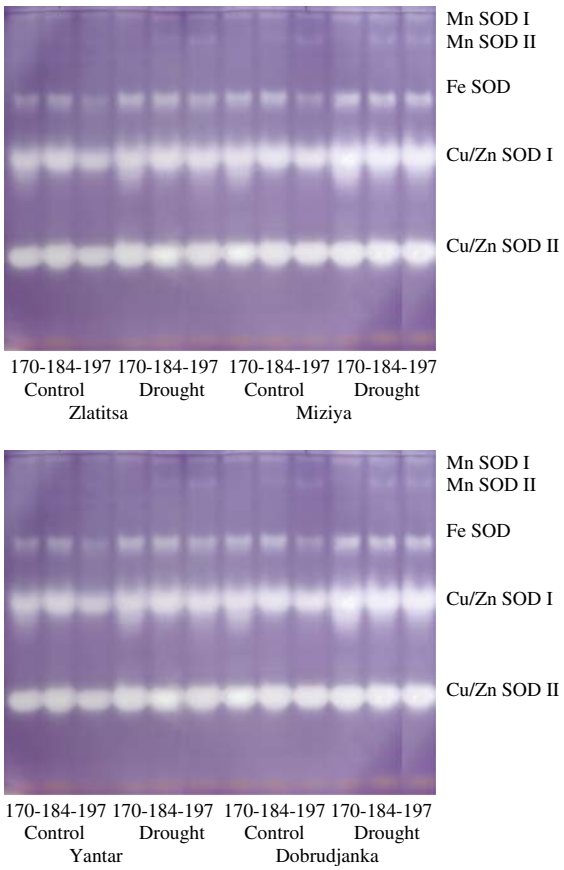

Fig. 2 Profiles of the activities of SOD isoforms. Below the images: 170, 184, and 197 days after sowing. The different isoforms are indicated on the right. Gels were loaded with an extract volume equivalent to 2 mg leaf fresh weight

especially in the drought-sensitive varieties under stress, in accordance with the diminished level of H_2O_2 in drought treated variants at last sampling. GPX activity (Fig. 3) was higher in the drought-stressed plants than in controls at the first sampling but progressively declined thereafter. An additional isoform was clearly visible in the controls of Dobrudjanka at the last sampling, which was absent in the drought treated plants.

Senescence progression and endoprotease activity

Time courses for chlorophyll and soluble protein contents in the flag leaves are presented in Fig. 4.

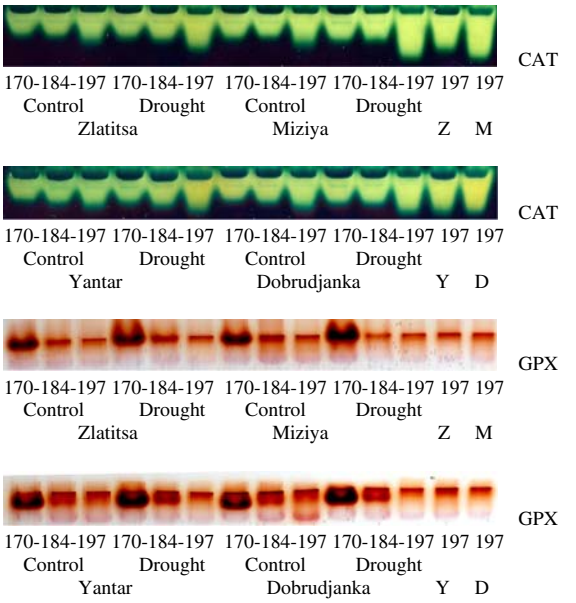

Fig. 3 Profiles of the activities of CAT and GPX isoforms. Below the images: 170, 184, and 197 days after sowing; Z, Zlatitsa; M, Misiya; Y, Yantar; D, Dobrudjanka. The different isoforms are indicated on the right. Gels were loaded with an extract volume equivalent to 2 mg leaf fresh weight

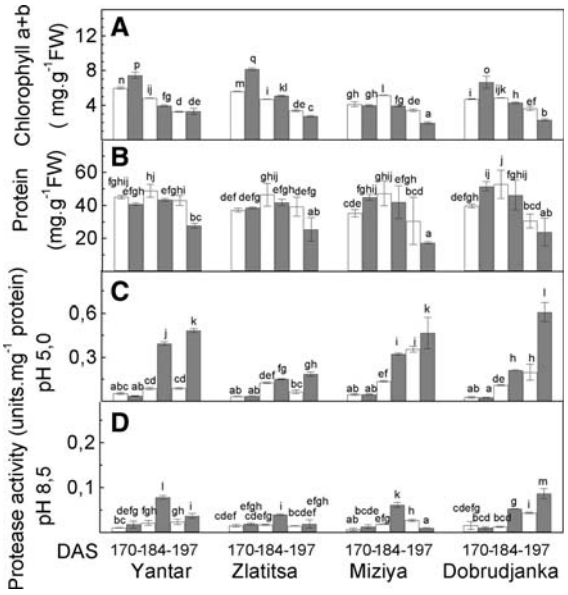

Fig. 4 Contents of chlorophyll a + b, soluble protein, as well as of azocaseinolytic activity at pH 5.0 and 8.5 in flag leaf extracts. Units are defined as increase in optical density at 450 nm after incubation for 1 h at 37°C. Abscissa, DAS (*days after sowing*) and varieties. *White columns* controls, *gray columns* drought treatment. Values are means of three replicates. Standard deviations are presented as vertical bars. Statistically significant differences ($P > 0.05$) are indicated by *different letters*

Tolerant varieties Yantar and Zlatitsa had highest chlorophyll a + b content in drought treated plants at first sampling followed by very sharp decline. The most sensitive variety Miziya had under stress the lowest chlorophyll content compared to the others which remained unchanged at the second sampling (Fig. 4a). Time course changes of variety Dobrudjanka were intermediate. At last sampling, lowest pigment level was detected in the flag leaves of the sensitive varieties under drought. Total soluble protein (Fig. 4b) diminished more in the controls of the resistant varieties compared to those of the sensitive ones at 197 DAS. At the last sampling, protein remobilization was enhanced by unfavourable conditions in all varieties. Increases in proteolytic activities were more pronounced in the controls of the sensitive than of the tolerant varieties (Fig. 4c, d). Drought increased the activity of proteases with pH optimum around 5 for all varieties. The tolerant variety Zlatitsa showed the lowest increase in activity. However, in the other tolerant variety (Yantar) the rise in activity at pH 5 was comparable to that of the sensitive Miziya. Proteolysis in the alkaline range increased at second sampling under drought and then decreased, except for the variety Dobrudjanka, in which it remained strongly enhanced.

Discussion

The response to water shortage depends on the plant species, the intensity and the duration of the stress, the developmental stage as well as the concurrence of other stresses such as high temperature and high irradiance, especially in the field (Sharma and Singhal 1993). Under field conditions, the gradual imposition of WD enables plants to accommodate to stress and to develop efficient defense mechanisms to resist to intense drought. Our results showed that under field conditions these wheat plants developed no strong WD (only 10–28% at the last sampling). The faster phenological development and maintenance of a quite good water status in the flag leaf suggests rather drought avoidance than tolerance mechanisms. Only the most sensitive variety Miziya developed significantly higher WD during the whole

sampling period and correspondingly presented the highest drought suffering.

Clear differences between control and stressed plants were observed in the time-courses for most parameters considered and might be explained by the intensity of metabolic processes at a given developmental stage. Badiani et al. (1996) found seasonal fluctuations of cellular antioxidant capacity in field grown wheat, which was closely linked to the photosynthesis and the transient oxidative strain associated with certain developmental stages. Thus, the highest values of proline and MDA in flag leaves established at 184 DAS could be linked to higher metabolic activity around anthesis, whereas later lower values of these parameters were at hand probably due to a shift toward senescence (evidenced by chlorophyll and protein loss).

In many plants, free proline accumulates in response to the imposition of a wide range of biotic and abiotic stresses (Hare and Cress 1997). The ability of proline to mediate osmotic adjustment, stabilise subcellular structures, and scavenge free radicals are well known. However, a controversy exists on the extent to which proline accumulation is a consequence of stress or benefits plants under adverse environmental conditions (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 studies, terminal drought in winter wheat resulted in up to 12 times more proline in the flag leaves of drought-treated plants compared to the controls. No clear differences in proline accumulation were found comparing sensitive and tolerant varieties. Highest proline accumulation under drought presented the tolerant variety Yantar at 184 DAS; however, the sensitive variety Miziya had also such a high level at the same time. This finding might reflect the complex character of the field drought tolerance in which proline accumulation may not be the main determinant.

In this study, the drought-sensitive varieties Dobrudjanka and Mizyia suffered more from oxidative stress under field drought than the drought-tolerant genotypes. The membrane instability was higher in drought-sensitive than in tolerant varieties and did not diminish during grain filling. Higher oxidative injuries to membranes and higher level of H_2O_2 were observed in these varieties in the middle of May. It was followed by a sharp decrease in the H_2O_2 content along with increased CAT activity at the third sampling. It is interesting to note that this drop in H_2O_2 content at 197 DAS was paralleled with relatively high MDA level. The bulk of H_2O_2 production in cells is linked to peroxisomes and photorespiration, whereas the steady-state level of H_2O_2 in organelles such as chloroplasts and mitochondria is maintained relatively low (Mittler 2002). With drought stress progression, when carbon dioxide fixation becomes limited by WD, the rate of active oxygen production in chloroplasts increases but mainly in the forms of superoxide and singlet oxygen which could damage membrane lipids to form MDA (a breakdown product of lipid peroxidation), whereas photorespiratory H_2O_2 production in peroxisomes decreases (Smirnoff 1993). Tolerant varieties had less fluctuation on the level of H_2O_2 in the flag leaves and less induced CAT activities compared to the sensitive ones. CAT is confined to peroxisomes and related organelles and is not typically induced by drought (Smirnoff 1993). Its higher activity at 197 DAS could rather be linked to its function probably to detoxify H_2O_2 derived from fatty acid oxidation (important source of energy in senescing tissues). Proliferation of peroxisomes in senescence has been reported (Pastori and del Rio 1997).

The isoforms of SOD revealed in our study corresponded to that reported by Feng et al. (2004). Decreased activities of SOD and CAT were reported for wheat undergoing natural senescence (Srivalli and Khanna-Chopra 2001) and under PEG-induced WD in wheat (Nair and Ramaswamy 2006). In this study, control plants were characterized by a clear diminution in the activities of FeSOD and of both chloroplastic and cytosolic CuZnSODs, whereas drought-treated plants tended to a very slow decrease in the activity of cytosolic CuZnSOD I and to maintained activities of FeSOD and chloroplastic CuZnSOD II. MnSODs (most probably of mitochondrial and peroxisomal origin) were up-regulated in sensitive varieties under drought. At a subcellular level, greater oxidative load on chloroplasts and mitochondria than on other compartments were found under drought (Munné-Bosch and Lalueza 2007). The results support the observation that SOD and CAT activities are enhanced under drought in wheat (Feng et al. 2004), and that the degree of oxidative stress and antioxidant activity seems to be associated with

the tolerance/susceptibility of a genotype to water stress (Sairam and Srivastava 2001).

The drought-sensitive varieties Dobrudjanka and Mizyia presented higher senescence rates in the controls than the resistant ones. Field water stress had additional impact on chlorophyll and protein loss from flag leaves. However, no clear correlation was observed between enhanced proteolytic activity and drought sensitivity. The tolerant variety Yantar had proteolytic response similar to that of sensitive varieties. The variety Dobrudjanka had higher protease activity at pH 8.5 compared to the other varieties both under field drought and during drought at the seedling stage (Simova-Stoilova et al. 2006). The response may depend on the variety. Proteolysis was enhanced under field drought in all varieties. The source–sink relations during the reproductive stage have a high impact on the flag leaves. The increase in protease activity could contribute to amino acid remobilization during grain filling but is most likely not connected directly with drought tolerance or sensitivity. The methods used for assessment of proteolytic activity in this study detect predominantly that of vacuolar origin, while the most proteinaceous cell compartment is the chloroplastic one. It is quite possible that chloroplasts are the main place for amino acid remobilization during grain filling. The different response of SOD isoforms reported here indirectly suggests well-conserved subcellular structures in wheat flag leaves. Additional studies including compartmentalization of proteolysis could explain these results.

In conclusion, field drought led to higher oxidative stress as indicated by increased lipid peroxidation and enhanced activities of CAT and some SOD isoforms. The membrane damage and the increases in CAT and MnSOD activities were more pronounced for drought sensitive varieties, especially during the grain filling period. Accelerated senescence under field drought was paralleled by higher azocaseinolytic activities, without a clear correlation to drought resistance or sensitivity.

Acknowledgments This study was supported by grants from Ministry of Education and Science of Republic Bulgaria (projects CC 1503 and PISA) and from Swiss National Science Foundation, SCOPES (project DILPA). The authors are grateful to A. Kostadinova and B. Juperlieva-Mateeva for their skilled technical assistance.

References

Arnon DI (1949) Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24:1–15

Ashraf M, Foolad MR (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59:206–216. doi:10.1016/j.envexpbot.2005.12.006

Badiani M, Paolacci AR, Miglietta F, Kimball BA, Pinter PL Jr, Garcia RL, Hunsaker DL, La Morte RL, Wall GW (1996) Seasonal variations of antioxidants in wheat (*Triticum aestivum*) leaves grown under field conditions. Aust J Plant Physiol 23:687–698

Bates LS, Waldren RO, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207. doi:10.1007/BF00018060

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Casano LM, Martin M, Sabater B (1994) Sensitivity of superoxide dismutase transcript levels and activities to oxidative stress is lower in mature-senescent than in young barley leaves. Plant Physiol 106:1033–1039

Chaves MM, Oliveira MM (2004) Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. J Exp Bot 55:2365–2384. doi:10.1093/jxb/erh269

Chaves MM, Pereira JS, Maroco J, Rodrigues ML, Ricardo CPP, Osorio ML, Carvalho I, Faria T, Pinheiro C (2002) How plants cope with water stress in the field. Photosynthesis and growth. Ann Bot (Lond) 89:907–916. doi:10.1093/aob/mcf105

Chen KM, Gong HJ, Chen GC, Wang SM, Zhang CL (2004) Gradual drought under field conditions influences the glutathione metabolism, redox balance and energy supply in spring wheat. J Plant Growth Regul 23:20–28. doi:10.1007/s00344-003-0053-4

Del Rio LA, Pastori GM, Palma JM, Sandalio LM, Sevilla F, Corpas FJ, Jimenez A, Lopez-Huertas E, Hernandez JA (1998) The activated oxygen role of peroxisomes in senescence. Plant Physiol 116:1195–1200. doi:10.1104/pp.116.4.1195

Feng Z, Jin-Kui G, Ying Y, Wen-Liang H, Li-Xin Z (2004) Changes in the pattern of antioxidant enzymes in wheat exposed to water deficit and rewatering. Acta Physiol Plant 26:345–352. doi:10.1007/s11738-004-0024-9

Fischer A, Feller U (1993) The pattern of peptide hydrolase activities in shoots of field-grown winter wheat during the cold season. Agronomie 13:293–299. doi:10.1051/agro:19930406

Fisher RA (1985) Number of kernels in wheat crop and the influence of solar irradiation and temperature. J Agric Sci 105:447–461

Fisher RA, Maurer R (1978) Drought resistance in spring wheat cultivars. I. Grain yield response. Aust J Agric Res 29:897–912. doi:10.1071/AR9780897

González A, Steffen KL, Lynch J (1998) Light and excess manganese. Implications for oxidative stress in common bean. Plant Physiol 118:493–504. doi:10.1104/pp.118.2.493

Grudkowska M, Zagdańska B (2004) Multifunctional role of plant cysteine proteinases. Acta Biochim Pol 51:609–624

Gupta NK, Gupta S, Kumar A (2001) Effect of water stress on physiological attributes and their relationship with growth and yield of wheat cultivars at different stages. J Agron Crop Sci 186:55–62. doi:10.1046/j.1439-037x.2001.00457.x

Hare PD, Cress WA (1997) Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regul 21:79–102. doi:10.1023/A:1005703923347

Hart MA, Tyson H, Bloomberg B (1971) Measurement of activity of peroxidase isoenzymes in flax. Can J Bot 49:2129–2137. doi:10.1139/b71-301

Hodges DM, de Long JM, Forney CF, Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207:604–611. doi:10.1007/s004250050524

Hortensteiner S, Feller U (2002) Nitrogen metabolism and remobilization during senescence. J Exp Bot 53:927–937. doi:10.1093/jexbot/53.370.927

Khanna-Chopra R, Selote DS (2007) Acclimation to drought stress generates oxidative stress tolerance in drought-resistant than—susceptible wheat cultivar under field conditions. Environ Exp Bot 60:276–283. doi:10.1016/j.envexpbot.2006.11.004

Lascano HR, Antonicelli GE, Luna CM, Melchiorre MN, Gomez LD, Racca RW, Trippi VS, Casano LM (2001) Antioxidant system response of different wheat cultivars under drought: field and in vitro studies. Aust J Plant Physiol 28:1095–1102

Martinez DE, Bartoli CG, Grbic V, Guiamet JJ (2007) Vacuolar cysteine proteases of wheat (*Triticum aestivum* L.) are common to leaf senescence induced by different factors. J Exp Bot 58:1099–1107. doi:10.1093/jxb/erl270

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410. doi:10.1016/S1360-1385(02)02312-9

Munné-Bosch S, Lalueza P (2007) Age-related changes in oxidative stress markers and abscisic acid levels in a drought-tolerant shrub, *Cistus clusii* grown under Mediterranean field conditions. Planta 225:1039–1049

Nair JS, Ramaswamy NK (2006) Differential response of antioxidant enzymes in wheat (*Triticum aestivum* L.) under PEG-induced water stress. Physiol Mol Biol Plants 12:95–99

Pastori GM, del Rio LA (1997) Natural senescence in pea leaves: an activated oxygen-mediated function for peroxisomes. Plant Physiol 113:411–418

Prochazkova D, Wilhelmova N (2007) Leaf senescence and activities of the antioxidant enzymes. Biol Plant 51:401–406

Sairam RK, Srivastava GS (2001) Water stress tolerance of wheat (*Triticum aestivum* L.): variations in hydrogen peroxide accumulation and antioxidant activity in tolerant and susceptible genotypes. J Agron Crop Sci 186:63–70

Sairam RK, Deshmukh PS, Saxena DC (1998) Role of antioxidant systems in wheat genotypes tolerance to water stress. Biol Plant 41:387–394

Sharma PK, Singhal GS (1993) Effect of water stress on primary photosynthetic processes: interaction with light and temperature. Indian J Biochem Biophys 30:10–14

Simova-Stoilova L, Vassileva V, Petrova T, Tsenov N, Demirevska K, Feller U (2006) Proteolytic activity in wheat leaves during drought stress and recovery. Gen Appl Plant Physiol Spec Issue 32:91–100

Smirnoff N (1993) The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol 125:27–58

Srivalli B, Khanna-Chopra R (1998) Drought-induced enhancement of protease activity during monocarpic senescence in wheat. Curr Sci 75:1174–1176

Srivalli B, Khanna-Chopra R (2001) Induction of new isoforms of superoxide dismutase and catalase enzymes in the flag leaf of wheat during monocarpic senescence. Biochem Biophys Res Commun 288:1037–1042

Wolff SP (1994) Ferrous ion oxidation in presence of ferric ion indicator xylenolorange for measurement of hydroperoxides. In: Packer L (ed) Methods in Enzymology, vol 233. Academic Press, New York, pp 182–189

Woodbury WA, Spencer K, Stahmann MA (1971) An improved procedure using ferricyanide for detecting catalase isozymes. Anal Biochem 44:301–305

Yadav RS, Sharma RK, Yadav RM (2004) Effect of simulated drought on free proline accumulation in some wheat (*Triticum aestivum* L.) genotypes. Indian J Agric Biochem 17:43–44

Yang J, Zhang J (2006) Grain filling of cereals under soil drying. New Phytol 169:223–236

Yang J, Zhang J, Huang Z, Zhu Q, Wang L (2000) Remobilization of carbon reserves is improved by controlled soil-drying during grain filling of wheat. Crop Sci 40:1645–1655

Zagdańska B, Wiśniewski K (1996) Endoproteinase activities in wheat leaves upon water deficit. Acta Biochim Pol 43:515–520

Zimmermann P, Zentgraf U (2005) The correlation between oxidative stress and leaf senescence during plant development. Cell Mol Biol Lett 10:515–534