PROTEOLYTIC ACTIVITY IN WHEAT LEAVES DURING DROUGHT STRESS AND RECOVERY

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Summary. Intracellular proteases could be involved in stress induced protein degradation and metabolism reorganization. Data on this topic are still quite limited. Eight varieties of winter wheat (*Triticum aestivum* L.) with different field drought resistance were examined in this study. Plants were grown as soil cultures in a growth chamber and watered daily to maintain 70% relative soil humidity. Progressive water stress was induced in plants with a fully developed first leaf by withholding irrigation for seven days, followed by three days recovery. Water deficit in the treated plants reached approximately 60% and differences in membrane stability among varieties were observed. After the recovery period, leaf water status and membrane stability were close to the values of the controls. Depending on the variety, leaf protein content was unchanged or declined after drought treatment and was restored upon recovery. Decreased protein content was in agreement with the higher azocaseinolytic activity at pH 5.0 and pH 8.5 under water deprivation. Upon recovery, the level of proteolytic activity diminished. Gel activity staining of one variety, showing good drought resistance ("Zlatitsa") and one with high drought sensitivity ("Miziya") revealed two protease bands with different response upon drought and recovery and towards protease inhibitors. The changes in SDS-PAGE protein profiles were more markedly expressed in the sensitive variety compared to the resistant one. The high

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total endopeptidase activity was related to drought sensitivity rather than resistance.

Keywords: drought, electrolyte leakage, proteolysis, recovery, wheat (*Triticum aestivum* L.).

Abbreviations: EDTA – Ethylendiaminetetracetic acid, FW – Leaf fresh weight, PMSF – Phenylmethylsulfonyl fluorid, PHMB – p-Chloromercurybenzoate, SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis, TCA - Trichloroacetic acid, TW - Leaf weight at full turgidity, WD – Water deficit.

INTRODUCTION

Drought is one of the most significant factors among abiotic stresses that limit plant performance, growth and productivity (Chaves and Oliveira, 2004). Nowadays many physiological, biochemical and molecular biology studies on the mechanisms of drought tolerance of agriculturally important crops have been performed (Yamaguchi-Shinozaki et al., 2002). It is established that water deficit stress induces the expression of many genes among which are some genes coding proteases (Bray, 2002, Cruz de Carvalho et al., 2001). Intracellular proteases have an important role in the degradation of damaged or unnecessary proteins, metabolism reorganisation and nutrient remobilization under stress (Feller, 2004, Grudkowska and Zagdańska, 2004). Contribution of cysteine proteases to total proteolytic activity increases drastically in response to water deficit in wheat (Zagdańska and Wiśnievski, 1996). It is important for the agricultural practice to understand the relation between proteolysis and plant performance in drought conditions and recovery from stress (Chaves and Oliveira, 2004). It is not clear whether high proteolytic activity under stress conditions is advantageous for the plant allowing reorganization of protein pattern or it leads to cell disintegration (Zagdańska and Wiśnievski, 1996). Some experimental evidence suggests that drought sensitive species and varieties have higher proteolytic activity compared to resistant ones (Roy-Macauley et al, 1992, Zagdańska and Wiśnievski, 1996, Hieng et al., 2004), however, data on relation of proteolytic activity to drought sensitivity or resistance are still quite limited.

The aim of this study was to analyze changes in proteolytic activities, protein pattern and membrane intactness during drought stress and subsequent recovery in order to compare varieties differing in their drought resistance.

MATERIALS AND METHODS

Plant material

Eight varieties of winter wheat (*Triticum aestivum* L.) with different field drought resistance were studied. Two of them are recognized in Dobrudja Agricultural Institute, General Toshevo, North Bulgaria as drought tolerant ("Yantar" and "Zlatitsa") and two – as drought sensitive ("Dobrudjanka" and "Miziya"). Four other varieties were chosen on the basis of their resistance to different abiotic stresses at the Institute of Plant Genetic Resources, Sadovo, South Bulgaria, assuming that they will have some differences in drought resistance, too. "Katya" was recognized as the most drought resistant variety in Bulgaria, "Pobeda" – as cold resistant, "Sadovo 1" – as disease resistant, and "Yunak" – as an N-deficiency tolerant variety.

Growth and treatment

Plants were grown in pots (400 g of leached meadow cinnamonic soil with pH 6.2, obtained from the experimental field near Gorni Lozen, optimally fertilized with N, P and K), 12 plants per pot, under 150 μE m^{-2} s^{-1} PAR irradiance, 21-25 °C and 16 h photoperiod. Relative soil humidity of 70% was maintained by daily watering. Drought stress was imposed on 8 day old plaing by withholding irrigation for seven days, followed by three days recovery. Controls were watered daily. Soil humidity, growth parameters, water deficit and electrolyte leakage were monitored during the whole drought period.

Methods

The water deficit (WD) was determined following the formula (TW-FW)/TW in percentages, where TW is leaf weight at full turgidity, FW – the actual leaf fresh weight. Membrane integrity of first leaf was evaluated by relative electrolyte leakage from 2 cm leaf segments floating on distilled water and expressed in percentage of the total leaf electrolyte content released after boiling the segments in effusate.

Biochemical analyses were performed on the first leaf which was fully expanded at the beginning of the treatment. Leaf material (0.5 g FW), frozen in liquid nitrogen prior to extraction, was homogenized in 2.5 ml (for controls and recovered) or 3 ml (for drought-treated) 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 X 100, 50 mg Polyclar AT and centrifuged at 14000 g for 40 min at 4°C. Total soluble leaf protein content was measured by the method of Bradford (1976). Proteolytic activity was assayed spectrophotometrically using azocasein as a substrate according to Fisher and Feller (1993) with some modifications. The reaction mixture contained 300 μl extract with equal protein contents (2 mg/ml), 300 μl of 200 mM phosphate-citrate buffer pH 5.0 or

borate buffer pH 8.5, 100 µl freshly prepared 2% w/v azocasein. Following incubation for 3h at 30°C, the proteolysis was stopped by addition of 150 µl 50 % trichloroacetic acid. Optical density of the supernatant was registered at 450 nm after mixing 1:1 v/v with 1N NaOH. TCA was added immediately after azocasein in blank samples. The pH-optima were determined preliminarily.

The 12.5 % SDS-PAGE of leaf soluble proteins was performed according to Laemmli (1970). Gel activity staining for cysteine proteases at pH 6.0 was made following the protocol of Beyene et al. (2006) using gelatine as a substrate. For inhibitory analysis, aliquots of extracts were pre-incubated for 30 min at room temperature with the following protease inhibitors: PMSF - for serine proteases, PHMB - for cysteine proteases, DL-Norleucine - for aspartic proteases, EDTA - for metalloproteases.

RESULTS AND DISCUSSION

Withholding irrigation resulted in gradual diminution in soil humidity by 2% per day reaching 56-58% on the 7th day of drought. Leaf water deficit (WD) remained unchanged during the first four days of water deprivation, afterwards it sharply increased to reach an average of 35-40% on the 5th day of the drought period (mild water stress) and 55-60% at day 7 (severe water stress). Drought resistant varieties developed WD more slowly, however at day 7 the differences among varieties tended to efface. After recovery, leaf water status was similar to the controls (Fig. 1 - left). Growth inhibition was observed at day 5 of drought treatment (data not shown) without differences among varieties. Pronounced differences were found in electrolyte leakage on the 7th day of drought (Fig. 1 – right). The increased electrolyte leakage indicates mechanical strain on membranes under severe drought (Chaves and Oliveira, 2004). The membrane injury seemed to be partially reversible as after recovery from 7 days water deprivation, membrane stability tended to be restored without completely reaching the level of the control plants (Fig. 1 - right). The observed differences in membrane stability among varieties were not completely consistent with their yield reduction under field drought conditions. Probably, field drought resistance is a much more complex phenomenon, which includes concurrent stresses such as high temperature and irradiance (Chaves and Oliveira, 2004). Moreover, plant response to drought at the whole plant level is more complex than at cellular level and is related to morphology, cell division, cell expansion, net photosynthesis, assimilate partitioning and many other factors (Blum, 1996).

Leaf protein content was expressed per leaf area because the leaf FW and DW underwent considerable changes during the treatment. The changes in leaf soluble protein in control, stressed (drought for 7 days) and recovered plants of the eight varieties under study are presented on Fig. 2. Some differences among varieties

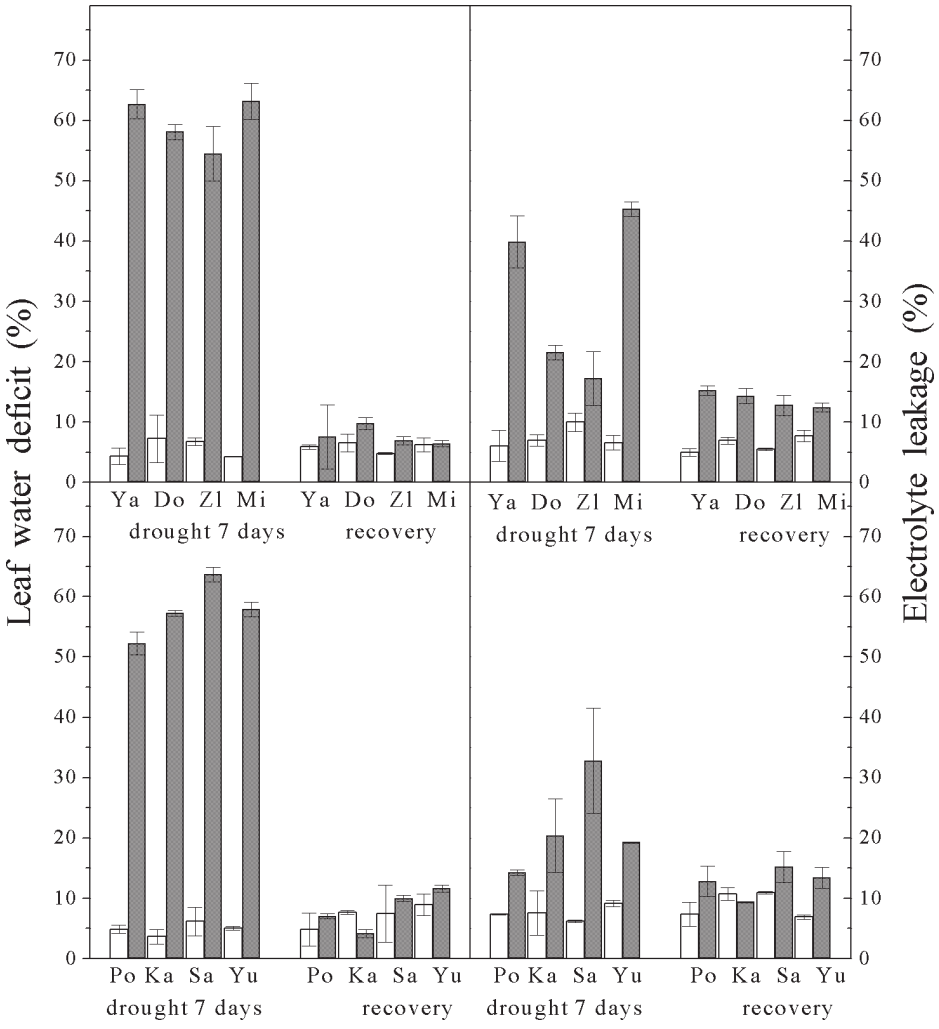

Figure 1. Leaf water deficit (%) and electrolyte leakage (%) after 7 days drought and subsequent recovery. Varieties: Ya - "Yantar", Do - "Dobrudjanka", Zl - "Zlatitsa", Mi - "Miziya", Po - "Pobeda", Ka - "Katya", Sa - "Sadovo 1" and Yu - "Yunak". White columns – control plants, grey columns – stressed plants. Values are means of three replicates. Vertical bars – standard deviations.

were observed which could be due to other factors besides proteolysis, such as inhibition of protein synthesis under unfavourable conditions.

In the varieties with dynamic protein changes ("Miziya", "Pobeda", "Sadovo 1"), decreased protein content was in agreement with higher azocaseinolytic activity at pH 5 and pH 8.5 on the 7th day of drought (Fig.3). Vacuolar proteases had major contribution to proteolytic activity at pH 5. Azocaseinolytic activity was not significantly increased under mild drought (day 5, data no shown) and diminished after

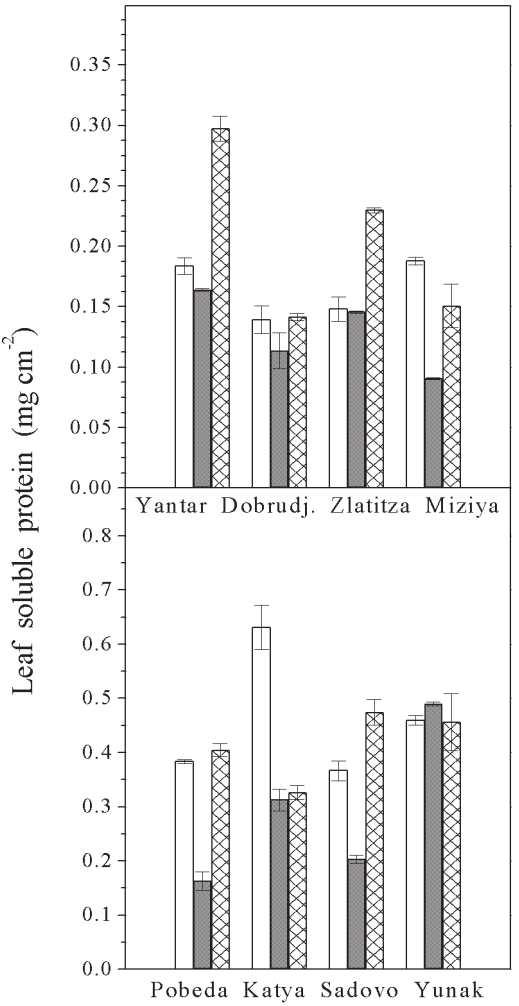

Figure 2. Soluble protein content on leaf area basis in the eight varieties considered. White columns – control plants, grey columns – plants after 7 days drought, hatched columns – recovered plants. Values are means of three replicates. Vertical bars – standard deviations.

recovery. It seems that vacuolar proteases are involved in drought sensitivity rather than drought resistance mechanisms and are not linked with membrane repair mechanisms.

For further analyses, one drought sensitive ("Miziya") and one drought resistant ("Zlatitsa") variety were chosen. Some changes in SDS-PAGE protein profiles (declining intensity of the band representing the intact large subunit of Rubisco, appearance of not yet identified new bands) of the sensitive variety were more ex-

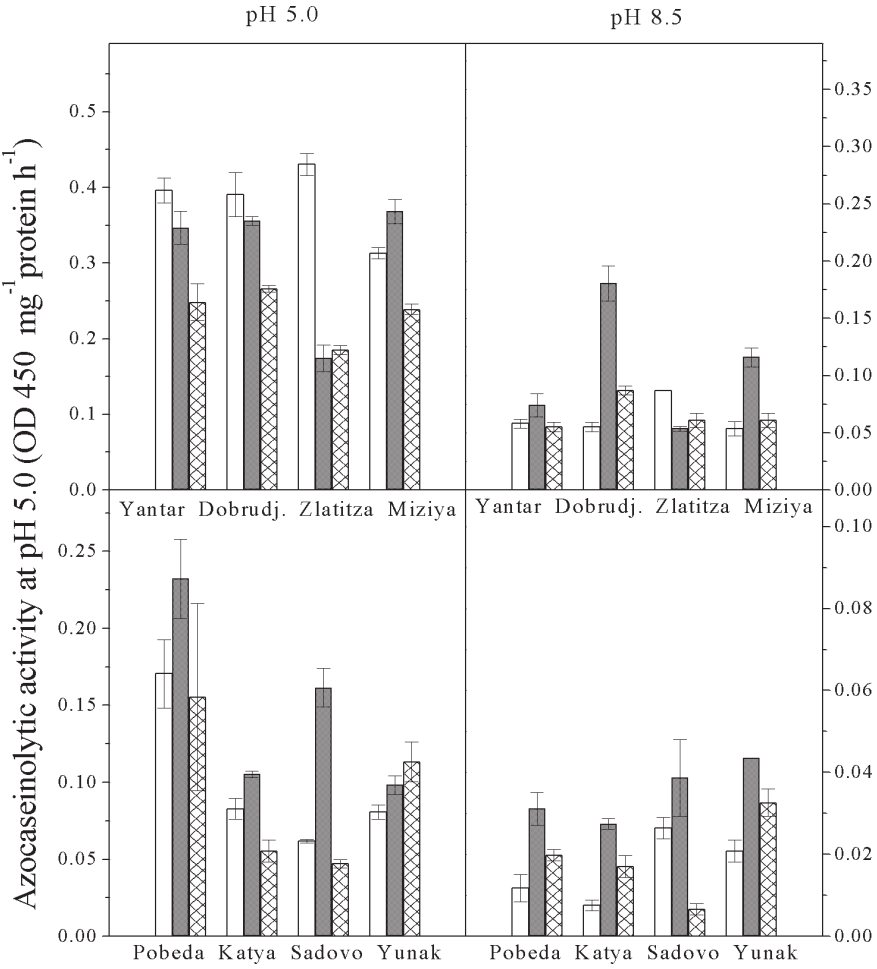

Figure 3. Proteolytic activity at pH 5.0 and pH 8.5 with substrate azocasein. White columns – control plants, grey columns – plants after 7 days drought, hatched columns – recovered plants. Values are means of three replicates. Vertical bars – standard deviations.

pressed compared to the resistant one (Fig. 4). The results are in concert with the diminution of protein content and the enhancement of proteolytic activity in drought sensitive variety "Miziya".

Activity staining for cysteine proteases after SDS-PAGE on 10% gel containing gelatin (Fig. 5) revealed two protease bands with different behavior during drought and recovery. In the drought resistant variety ("Zlatitsa") the controls exhibited the highest activity, which diminished following drought. It could be supposed that endogenous protease inhibitors could participate in drought resistance (Pernas et al, 2000) and interfere with proteolytic activity measured. Another possibility is that the ATP-dependent proteolytic response will be more relevant in drought resistance

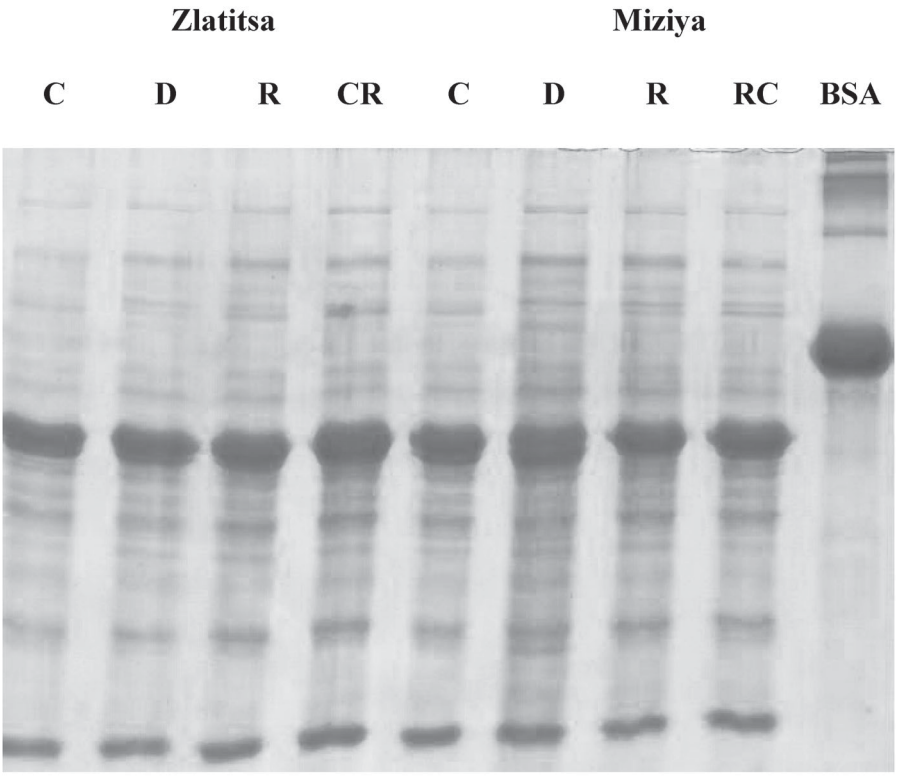

Figure 4. SDS-PAGE leaf protein profiles in 12.5 % gel of the varieties "Zlatitsa" – drought resistant and "Miziya" – drought sensitive. Equal protein quantity (30 μg) is loaded on the starts. C – controls, D – drought treated for 7 days, R – recovered for 3 days, RC – age controls of the recovery.

mechanisms than the ATP–independent one (Wisniewski and Zagdańska, 2001) and our results with azocasein reflect rather the ATP independent proteolysis. Water deprivation enhanced mainly one of the bands with proteolytic activity in the drought sensitive variety ("Miziya"). The inhibitory analysis (data not shown) revealed that one of the bands was inhibited by PHMB (cysteine protease inhibitor), the other one by DL-norleucine (aspartic protease inhibitor). Probably, the two bands with proteolytic activity belonged to different types of proteases. Enhancement of the more slowly moving band in variety "Miziya" after the drought period could be implicated in some stress-induced protein degradation in this sensitive cultivar.

In conclusion, after severe drought total proteolytic activity increased in the sensitive varieties and declined upon recovery. The obtained results suggest involvement of the main endogenous proteolytic activities in the mechanisms of drought sensitivity rather than in drought resistance mechanisms.

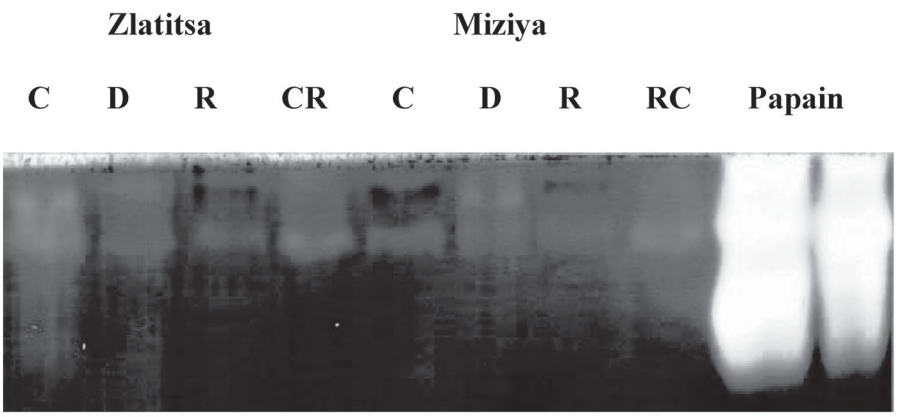

Figure 5. In gel activity staining for cysteine proteases in gelatine containing gel of the varieties "Zlatitsa" – drought resistant and "Miziya" – drought sensitive. Equal protein quantity (30 g) is loaded on the starts. **C** – controls, **D** – drought treated for 7 days, **R** – recovered for 3 days, **RC** – age controls of the recovery.

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