A COMPARISON OF THE EFFECTS
OF DROUGHT ON PROLINE ACCUMULATION AND PEROXIDASES ACTIVITY
IN LEAVES OF *FESTUCA RUBRA* L. AND *LOLIUM PERENNE* L.

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ABSTRACT

The effect of soil drought on leaf water content, proline content, and the activity of guaiacol (GuPX) and ascorbate (APX) peroxidases as well as the level of lipid peroxidation were investigated in leaves of drought resistant red fescue (*Festuca rubra*) and drought sensitive perennial ryegrass (*Lolium perenne*). Plants were grown under glasshouse conditions in soil pot culture. 26 day-old grasses were exposed to drought by withholding irrigation for 18 days. Water content in leaves of perennial ryegrass decreased more than in red fescue throughout the experimental period. On the other hand, proline content (PC) was higher in red fescue. The activity of APX and GuPX increased in leaves of red fescue, while it did not change in perennial ryegrass. Our data demonstrate that both red fescue and perennial ryegrass were able to survive applied drought, as shown by a lack of stress-induced lipid peroxidation and hence no evidence of oxidative damage. We speculate, that the observed drought stress tolerance at cellular level was associated with the ability to accumulate proline, and to maintain high activity of APX and GuPX, resulting in protection against oxidative damage and lipid peroxidation. It seems that this mechanism works better in red fescue.

KEY WORDS: red fescue, grasses, lipid peroxidation, peroxidases, proline, ryegrass.

INTRODUCTION

Drought is one of the most important environmental factors limiting productivity of many crops in numerous regions of the world (Bray 1997; Tubiello et al. 2007). Red fescue (*Festuca rubra*) and perennial ryegrass (*Lolium perenne*) are two major cool-season forage and turf grasses in temperate regions of the world (Stadelman et al. 1998). These species constitute valuable components of universal grass mixtures used for turf on sports fields, golf courses, roadsides and pasture. They also play a considerable role in the restoration of natural value of degraded areas and areas requiring reclamation (Wang and Ge 2005; Martinello and D'Andrea 2006). Cool-season forage and turf grasses species often suffer from drought, which deteriorates sward density as well limits its survival and persistence (Huang et al. 1998; Huang and Gao 1999; Jiang and Huang 2000; Martiniello and d'Andrea 2006). Due to extensive, deep rooting, fescue is able to avoid leaf water deficit and is considered to be drought resistant grass (Sheffer et al. 1987; Karsten and MacAdam 2001). Perennial ryegrass is characterized by a high seed production and a high persi-

stence capability, but is rather susceptible to drought (Volaire et al. 1998; Lucero et al. 1999).

The capacity of plant to survive in water-limiting conditions depends on induction of mechanisms involved in avoidance of dehydration and tolerance to dehydration in cells (Farooq et al. 2009). Water deficit enhances the production of reactive oxygen species (ROS) such as superoxide radical ($O_2^{.-}$) and hydrogen peroxide (H_2O_2), which may cause lipid peroxidation and consequently membrane damages (Smirnoff 1993; Van Breusegem et al. 2001; Mittler 2002; Apel and Hirt 2004). The maintenance or increase in the activity of enzymes involved in removing these toxic ROS, to avoid cellular damages, is regarded as an important factor in tolerance to dehydration (Sairam et al. 1997; Fu and Huang 2001; Terzi and Kadioglu 2006). The enzymatic antioxidant systems include, among others, peroxidases (EC 1.11.1.7, etc.), which are important hydrogen peroxide scavenging enzymes (Hiraga et al. 2001). Guaiacol peroxidase (GuPX) is characterized by broad specificity with respect to substrate, and both guaiacol and pyrogallol have been used as electron donor in assays their activity. Many isoenzymes of GuPX occur in vacuole, cell

wall and cytosol (Amako et al. 1994; Mika and Lüthje 2003). Ascorbate peroxidase (APX) is distributed in at least four cell compartments: stroma, tylakoid membrane, microbody and cytosol, and is characterized by high degree of specificity for ascorbic acid and high affinity to H_2O_2 (Yoshimura et al. 2000). Its essential role is the scavenging of H_2O_2 in the chloroplast (Asada 1992).

One of the most common metabolic responses to drought is the accumulation of free proline, which is considered to be involved both in dehydration avoidance and in tolerance mechanisms (Serraj and Sinclair 2002; Chaves et al. 2003; Nayyar 2004; Ashraf and Foolad 2007; Trovato et al. 2008). Proline as a non-toxic compatible compound protects the cell membranes against detrimental effects of dehydration and lowers generation of ROS (Smirnoff and Cumbes 1989; Bandurska 2000; Kocsy et al. 2005). Likewise, accumulation of proline permits osmotic adjustment, which results in water retention and avoidance of cell dehydration (Blum 2005; Kavi Kishor et al. 2005).

The objective of the present study was to compare the effect of soil drought on metabolic changes associated with the maintenance of stable leaf water content as well with leaf dehydration tolerance in drought resistant red fescue (*Festuca rubra*) and drought sensitive perennial ryegrass (*Lolium perenne*). Plant responses were assessed by evaluating leaf water content, proline content, level of lipid peroxidation as well the activity of guaiacol and ascorbate peroxidases.

MATERIALS AND METHODS

Plant material and growth conditions

Red fescue (*Festuca rubra)* cv. 'Areta' and perennial ryegrass (*Lolium perenne)* cv. 'Tivoli' were used in this study. 'Areta' is a Polish cultivar classified as slender creeping red fescue, which runs strongly by rhizomes and is able to cover a large area as well as increase sward density (Golińska and Goliński 2005; Goliński and Domański 2005). These properties make it the principal component in the universal lawn mixture. 'Tivoli' is a Danish forage cultivar of ryegrass, which is also added to the universal lawn mixture to ensure rapid greening.

Seeds obtained from the Antoniny Plant Breeding Station were sown in two plastic containers filled with soil and allowed to germinate under glasshouse conditions. Each container was divided in two parts, one for perennial ryegrass and the other for red fescue. Plants were watered regularly to maintain soil moisture at about 60% of water capacity. 26 day-old plants grown in one container were exposed to drought by withholding irrigation for 18 days. Control plants grown in the second container were watered regularly. The samples of leaves at similar developmental stage from control and drought treated plants were collected 3 times during the stress period and used for the estimation of water content (WC), lipid peroxidation (MDA), proline content (PC), as well as ascorbate peroxidase (APX) and guaiacol peroxidase (GuPX) activities.

Measurements

Water content (WC) in leaves was estimated by measuring leaf fresh weight and dry weight following oven drying of fresh leaf samples at 70°C. WC (%) was calculated using the following formula:

$$WC = \frac{\text{fresh matter} - \text{dry matter}}{\text{fresh matter}} \times 100\ (\%)$$

For proline determination, samples (300 mg) of fresh leaf tissues were freeze-dried and stored at -20°C until analysis. PC was determined according to Bates et al. (1973) by measuring the quantity of the coloured reaction product of proline with ninhydric acid. The absorbance was read at 520 nm. The amount of proline was calculated from a previously plotted standard curve for L-proline and expressed in $\mu g \cdot g^{-1}$ of leaf dry matter (DM).

For the assays of APX, GuPX and lipid peroxidation, samples of fresh leaf tissue (300 mg) were homogenized in a chilled mortar with 3 cm^3 0.1 M potassium phosphate buffer (pH 7.0) with 30 mg Polyclar AT added and centrifuged at 16 000 × *g* for 30 min at 4°C. The supernatant was collected for assays of soluble protein content, enzyme activities and lipid peroxidation.

The activity of APX was determined according to Nakano and Asada (1987). The reaction mixture contained 2.3 cm^3 0.1 M potassium phosphate buffer (pH 7.0), 0.2 cm^3 5 mM L-ascorbate, 0.3 cm^3 1 mM H_2O_2 and 0.2 cm^3 enzyme extract. The hydrogen peroxide dependent oxidation of ascorbate was followed by a decrease in absorbance at 290 nm (absorption coefficient 2.8 $mM^{-1} \cdot cm^{-1}$). A unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 mol of substrate per second. APX activity was expressed as nkat $\cdot mg^{-1}$ protein.

The activity of GuPX was estimated according to Hammerschmidt et al. (1982). The reaction mixture contained 0.5 cm^3 enzyme extract, 0.5 cm^3 3.4 mM guaiacol and 0.5 cm^3 0.9 mM H_2O_2. The oxidation of guaiacol to tetraguaiacol in the presence of H_2O_2 was measured as an increase in absorbance recorded at 470 nm. The enzyme activity was calculated using absorption coefficient for tetraguaiacol (26.6 $mM^{-1} \cdot cm^{-1}$) and was expressed as nkat $\cdot mg^{-1}$ protein.

The level of lipid peroxidation was measured by determination of malondialdehyde (MDA) content according to the method described by Dhindsa and Matowe (1981). The volume of 2 cm^3 of 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA) was added to 500 µl of the supernatant. The mixture was incubated at 95°C for 30 min, quickly cooled in an ice bath to room temperature and then centrifuged at 10 000 × *g* for 10 min. Absorbance of the supernatant was determined at 520 nm and 600 nm. The value for nonspecific absorption at 600 nm was subtracted from the value at 532 nm. The concentration of MDA was calculated using extraction coefficient of 155 $mM^{-1} \cdot cm^{-1}$ (Heath and Packer 1968).

Protein was determined according to the method applied by Bradford (1976), using bovine serum albumin as a standard.

Statistical analysis

The experiment was done twice and in each case similar trends were observed. Analyses for each investigated parameter were made in five biological replicates. Values shown in Figures represent means of five replicates from one representative experiment. Significance of differences was performed using Tukey's multiple range tests. Pearson correlation coefficients were determined for the relationships between *WC* and *PC* in leaves. Statistical analyses were performed using Statistica 8.0 software.

RESULTS

The restriction of water supply caused various decline in leaf WC of examined grasses. During 18 days of stress leaf WC in red fescue decreased gradually to 78.8%. However, in perennial ryegrass WC decreased to 76.3% after 5 days of stress already. Then, after 13-day stress, it slightly increased to 84.6% and at the end of stress decreased to as little as 67.2% (Fig. 1).

Proline content in control plants remained stable during the experimental period and no significant differences were detected between species (Fig. 2). Free proline level changed differently throughout the drought period in the examined grasses. After 18 days of stress red fescue was found to exhibit about a 2-fold higher PC than perennial ryegrass. However, in the former, a pronounced increase in proline level was observed already after 5 days of stress. An enhanced proline accumulation in leaves accompanied the stress-induced reduction of WC. The analysis of correlations between WC and proline level in leaves of both red fescue and perennial ryegrass revealed a negative and statistically significant relationship (Fig. 3).

The examined grasses responded differently to drought in respect to the activity of GuPX and APX (Figs 4 and 5). In leaves of red fescue the activity of GuPX did not change after 5 and 13 days of drought stress, but then at day 18 it increased to about 38% above the control. APX activity decreased after 13 days of drought and then it increased to about 12% above the control at day 18. In the case of perennial ryegrass the activity of both enzymes practically did not change in relation to non-stressed plants.

The protein contents in both grasses were rather stable throughout experiment. Only at 18 day of stress slight but statistically significant higher protein content was observed in leaves of stressed plant as compared to control (data not shown).

Stress-induced changes in the level of lipid peroxidation (MDA) are shown on Fig. 6. In leaves of red fescue subjected to drought for 5 days lipid peroxidation level was about 25% lower than in control, but no difference was observed in MDA content between control and stressed plants after 13 and 18 days of stress. However, during the entire experimental period the MDA content in leaves of the stressed perennial ryegrass was lower by about 18% to 46% than that in the control.
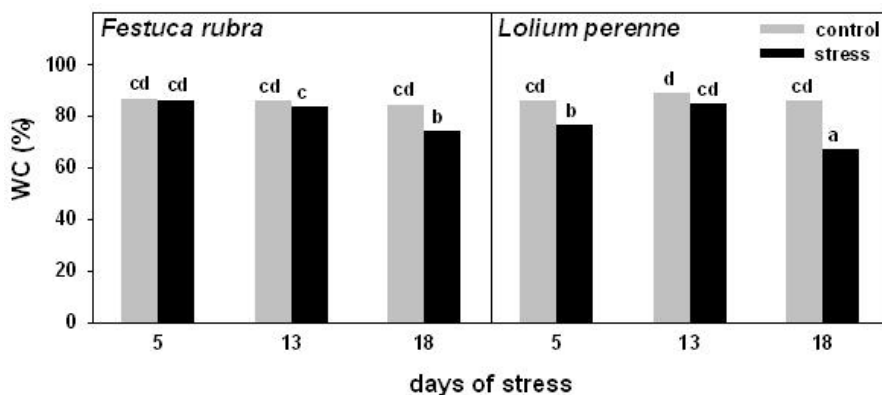

Fig. 1. Water content in leaves of *Festuca rubra* L. 'Areta' and *Lolium perenne* L. 'Tivoli' in response to drought. Different letters indicate significant differences between means of five replicates (p<0.05).
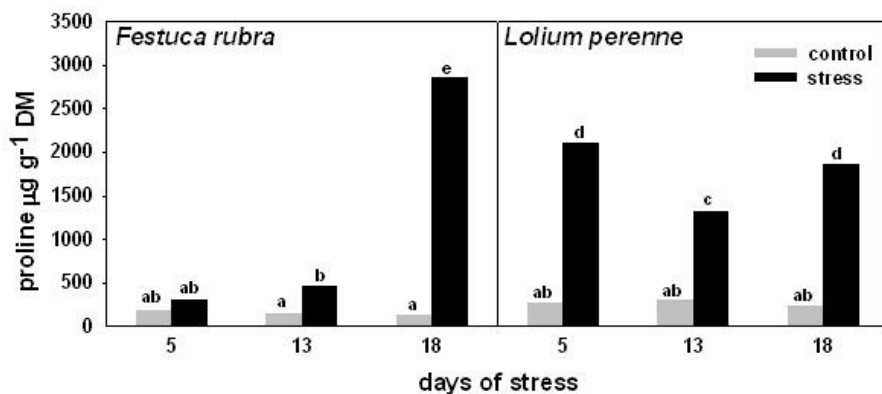

Fig. 2. Proline content in leaves of *Festuca rubra* L. 'Areta' and *Lolium perenne* L. 'Tivoli' in response to drought. Different letters indicate significant differences between means of five replicates (p<0.05).
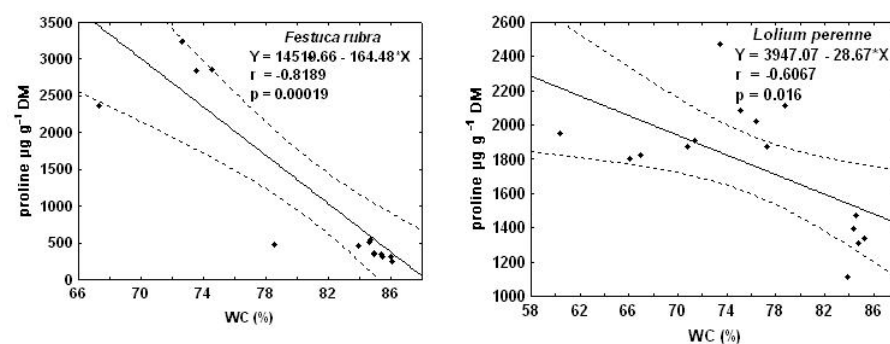

Fig. 3. The relationship between proline accumulation and water content in leaves of *Festuca rubra* L. 'Areta' and *Lolium perenne* L. 'Tivoli'.
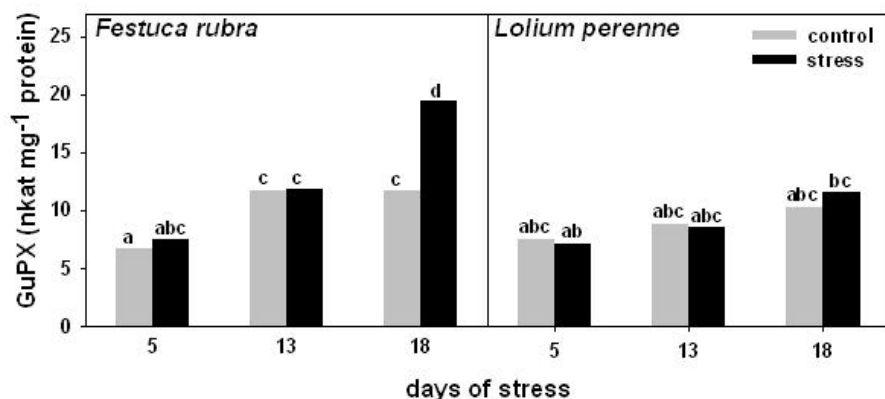
Fig. 4. Guaiacol peroxidase activity in leaves of *Festuca rubra* L. 'Areta' and *Lolium perenne* L. 'Tivoli' in response to drought. Different letters indicate significant differences between means of five replicates (p<0.05).
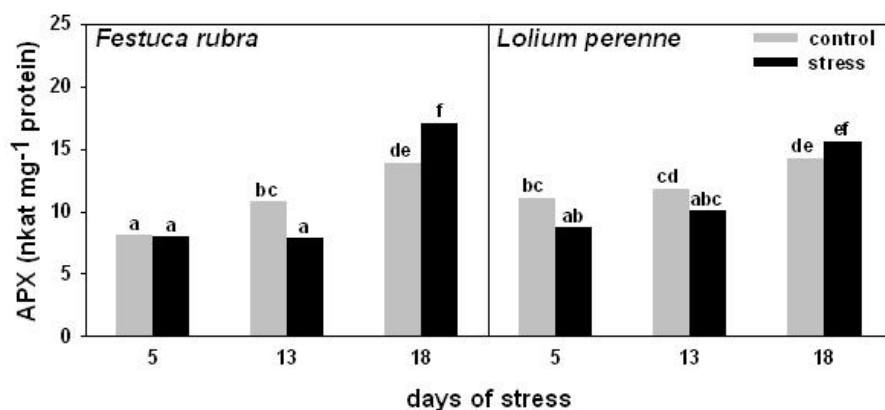

Fig. 5. Ascorbate peroxidase activity in leaves of *Festuca rubra* L. 'Areta' and *Lolium perenne* L. 'Tivoli' in response to drought. Different letters indicate significant differences between means of five replicates (p<0.05).
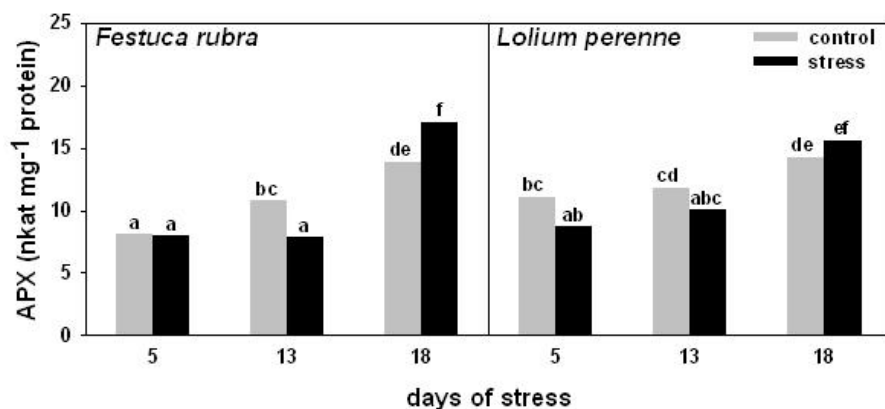

Fig. 6. Malondialdehyde (MDA) content in leaves of *Festuca rubra* L. 'Areta' and *Lolium perenne* L. 'Tivoli' in response to drought. Different letters indicate significant differences between means of five replicates (p<0.05).

DISCUSSION

The maintenance of stable water content in perennial herbaceous swards is a trait well correlates with drought survival (Volaire 2008). In our study, the limitation of water supply had a different effect on WC in leaves of the investigated grass species. In drought sensitive perennial ryegrass a significant decrease in leaf WC was shown at the 5th day of stress and at the end of stress duration decreased more than in drought resistant red fescue. The ability of Festuca species to thrive with limited soil moisture and delayed appearance of stress symptoms have been reported earlier (Aronson et al. 1987). Studies of White et al. (1992) revealed that fescue survival during drought was associated with osmotic adjustment and turgor maintenance. This process involves the accumulation of compounds, which decrease cellular osmotic potential, thus helping the movement of water into the cell resulting in increase of leaf turgor. These compounds include proline, glycine-betaine,

mannitol, sorbitol as well water-soluble carbohydrates (Kavi Kishor et al. 2005; Mahajan and Tuteja 2005; Trovato et al. 2008). The increase in the concentration of osmotic active carbohydrates in response to drought was observed in tall fescue and perennial ryegrass (Karsten and MacAdam 2001). Our objective was to test the effect of water deficit on proline content. The presented results revealed negative and statistically significant correlation between PC and WC in leaves of both grasses. However, the pattern of stress induced proline accumulation was different in the examined grasses. At the 13th day of stress, different PC (higher in red fescue) in leaves of both grasses was accompanied by similar WC. Lower WC at day 18 of stress and lower PC were shown in leaves of perennial ryegrass than red fescue. Thus, it may be assumed that threshold level of WC decisive for proline accumulation is different in examined grass species. Our results indicate that red fescue has better ability to maintain turgor under drought conditions than perennial ryegrass. Bringing in mind higher pro-

line accumulation in leaves of red fescue is possible to suppose that this amino acid could be responsible for maintenance of water status. Likewise, higher PC and higher water retaining capacity was shown in drought-tolerant *Phaseolus acutifolius* than drought-sensitive *Phaseolus vulgaris* (Türkan et al. 2005).

The high activity of antioxidant enzymes under water deficit conditions is an important factor in dehydration tolerance, since it prevents the accumulation of active oxygen species and oxidative damage (Mittler 2002; Farooq et al. 2009; Sairam et al. 1997; Gupta et al. 2005; Terzi and Kadiolglu 2006). A study by Fu and Huang (2001) showed that red fescue cv. 'Falcon II' was characterized by high resistance to moderate drought due to maintenance of a stable or enhanced activity of antioxidant enzymes, including APX. In this study the activity of APX and GuPX increased under stress conditions in red fescue, whereas it did not change in perennial ryegrass. We suppose that these differences in response could be one of the causes responsible for higher drought resistance of red fescue in comparison to perennial ryegrass.

Increasing evidence show on possible role of proline in effective protection of antioxidant enzymes from detrimental effect of different stresses *in vivo* and *in vitro* (Jain et al. 2001; Öztürk and Demir 2002). Sharma and Dubey (2004) revealed that proline alleviated APX inactivation under PEG induced water deficit in rice and assumed that this amino acid might be involved in conferring a direct protection of APX under osmotic stress. More efficient activity of APX and other peroxidases in drought-tolerant *Phaseolus acutifolius* was related to higher PC than in drought-sensitive *Phaseolus vulgaris* (Türkan et al. 2005). Recent report revealed that drought tolerant maize genotype exhibited a higher proline accumulation, higher peroxidase activity and lower accumulation of malondialdehyde (Moussa and Abdel-Azis 2008). In view of the cited data it may be supposed that the maintenance of a high level of APX and GuPX activity in leaves of examined grasses under drought conditions may be associated with the accumulation of proline.

Our results indicate that both red fescue and perennial ryegrass were capable of surviving the applied drought because of a lack of stress-induced lipid peroxidation and hence no evidence of occurrence of oxidative damage. The level of lipid peroxidation measured as MDA content in leaves is used as an indicator of oxidative damage, which takes place under various stress conditions (Smirnoff 1993). Drought stress results in an increase in membrane lipid peroxidation due to a decrease of antioxidant enzyme activities was shown under the conditions of severe and prolonged drought in red fescue and other grass species (Fu and Huang 2001; Jiang and Huang 2001). Drought stressed dwarf mutant lines of bermudagrass, which maintained higher levels of antioxidant enzymes, were characterized by lower malondialdehyde content, and improved stress resistance than the wild type (Lu et al. 2008). However, in a drought-tolerant *Coffea canephora* clone the level of lipid peroxidation increased under drought conditions, but the increase was lower in a drought-sensitive one, due to the greater activation of the antioxidant system (Lima et al. 2002). The lower MDA increase in drought-tolerant *Phaseolus acutifolius* was related to higher activity of APX and other peroxidases, while a significant MDA increase in drought-sensitive *Phaseolus vulgaris* was derived from the decreased activities of these enzymes (Türkan et al. 2005).

In conclusion, based on the data obtained it seems that the examined grass species differ in the reaction to applied soil drought. Red fescue had better ability to control water loss from leaves and was characterized by higher proline accumulation. In spite of leaf dehydration the applied stress did not cause cell membrane damages. It was probably due to maintain high activity of APX and GuPX, protecting against oxidative damage and lipid peroxidation. We suppose that this mechanism of stress tolerance works better in drought resistant red fescue than drought sensitive perennial ryegrass.

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