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ORIGINAL PAPER

# Antioxidant response to drought in red and white clover

**Irina Vaseva · Yasar Akiscan · Lyudmila Simova-Stoilova · Anelia Kostadinova · Rosa Nenkova · Iwona Anders · Urs Feller · Klimentina Demirevska**

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**Abstract** Antioxidant response to drought in red (*Trifolium pratense* L., cv. "Start") and white clover (*Trifolium repens* L, cv. "Haifa" and cv. "Debut") grown as soil cultures was evaluated in water-deprived and recovered plants. Drought provoked oxidative stress in leaves confirmed by the considerable changes in electrolyte leakage, malondialdehyde, hydrogen peroxides and proline contents. Immunoblot of Δ-1-pyrroline-5-carboxylate synthetase (P5CS), which catalyzes the first two steps in proline biosynthesis, revealed strong induction of the enzyme in red clover plants submitted to drought. Water-deprived white clover plants exhibited distinct P5CS profiles. This was related to different drought tolerance of the studied *T. repens* cultivars. Isoenzyme analyses of superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) demonstrated certain differences in antioxidant defence among the tested varieties. It was confirmed that MnSOD (in both *T. repens* and *T pratense*) and FeSOD (in *T. repens*) isoforms were the most affected by drought. The red clover cultivar "Start" exhibited the lowest FeSOD and POX activities which could contribute to its poor performance under water deprivation.

**Keywords** Drought stress · Isoenzyme analysis · ROS detoxifying enzymes · Δ1-pyrroline-5-carboxylate synthetase · Red clover (*Trifolium pratense* L.) · White (*Trifolium repens* L.) clover

**Abbreviations**

| | |
|---|---|
| CAT | Catalase |
| EL | Electrolyte leakage |
| FW | Fresh weight |
| GS | Glutamine synthetase |
| MDA | Malondialdehyde |
| PAGE | Polyacrylamide gel electrophoresis |
| P5CS | Δ1-pyrroline-5-carboxylate synthetase |
| PGP | Phosphoglycolate phosphatase |
| POX | Peroxidase |
| Pro | Proline |
| R | Recovery |
| ROS | Reactive oxygen species |
| SOD | Superoxide dismutase |
| WD | Water deficit |

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I. Vaseva (✉) · L. Simova-Stoilova · A. Kostadinova · R. Nenkova · K. Demirevska
Plant Stress Molecular Biology Department, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria
e-mail: irina.vaseva@abv.bg

Y. Akiscan
Department of Field Crops, Faculty of Agriculture, Mustafa Kemal University, Antakya 31120, Hatay, Turkey

I. Anders · U. Feller
Institute of Plant Sciences and Oeschger Centre for Climate Change Research (OCCR), University of Bern, Altenbergrain 21, 3013 Bern, Switzerland

## Introduction

Water scarcity resulting from global climate change is accompanied by more frequent and more severe summer droughts in many regions (Seneviratne et al. 2006; Koleva and Alexandrov 2008; Jaeger and Seneviratne 2011). This causes drought stress in plants and limits crop yield worldwide (Hamdy et al. 2003). The agricultural practice

for drought-tolerant crops turns into topical demand (Cázares et al. 2010). Water loss can induce production of highly reactive molecules such as hydrogen peroxide, superoxide anion and hydroxyl radicals, singlet oxygen which all are named reactive oxygen species (ROS). The role of ROS as stressors and factors of protection and signaling was discussed widely in the literature (Chaves et al. 2003; Apel and Hirt 2004; Asada 2006).

Plant response to drought is often accompanied by oxidative damage (Chaves et al. 2003; Noctor et al. 2002; Reddy et al. 2004; Ślesak et al. 2007; Foyer and Noctor 2009). Antioxidant protection in plant cells is a complex and highly compartmentalized phenomenon and includes both enzymatic and non-enzymatic components (Mittler 2002). ROS detoxifying enzymes are induced during different kinds of biotic and abiotic stresses for maintenance of normal growth (Blokhina et al. 2003). The key role of antioxidant enzymes is to reduce or scavenge ROS which are normally produced in different cell organelles and the cytosol. Their activities increase considerably under stress conditions. Superoxide dismutase (SOD, EC 1.15.1.11), peroxidase (POX, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6) are the important ROS scavenging enzymes. They participate in removal of superoxide radical and hydrogen peroxide ($H_2O_2$), produced directly or indirectly by Mehler reaction and photorespiration in plants, preventing the formation of the highly toxic hydroxyl radical via Haber–Weiss or Fenton reactions (Mittler 2002). SODs are localized in chloroplasts, mitochondria, peroxisomes and the cytosol. POX activities are distributed in vacuoles, the cell walls and the cytosol, whereas CAT enzymes are presented only in peroxisomes. Hydrogen peroxide was considered also as a signaling molecule involved in plant response to wide range of biotic and abiotic stresses (Laloi et al. 2004). It was demonstrated that elevated levels of reactive oxygen species, such as hydroxyl radicals, under drought are capable to induce oxidative stress, causing lipid peroxidation and consequently membrane injury (Mittler 2002).

Lipid peroxidation is linked to the activity of antioxidant enzymes, e.g., with the increase of SOD, APX, GPX and CAT (Esfandiari et al. 2007; Mittler 2002). Malondialdehyde is a product of lipid peroxidation and is regarded as a biomarker for evaluation of the damages in plasmalemma and organelle membranes caused by oxidative stress. MDA content in plants increases under environmental stresses. Usually, the better oxidative stress tolerance is accompanied with lower MDA levels. Lipid peroxidation can be estimated as the amount of malondialdehyde present in plants as an effect of oxidative damage (Bailly et al. 1996).

Electrolyte leakage measured in leaf fragments has been extensively used for assessing stress intensity in plants. Most often cellular membranes represent the first target of various abiotic stresses and this parameter characterizes their integrity (Bajji et al. 2002). According to Bandurska (2000), the maintenance of the physical–chemical integrity of membranes under drought stress can be considered as one of the best physiological indicators of protoplasmic tolerance in plants.

Proline (Pro) is accumulated as an osmoprotectant of cellular structures in response to osmotic stress, including drought (Hanson and Hitz 1982). Smirnoff and Cumbes (1989) have suggested that Pro could scavenge ROS. Results of Alia and Matysik (2001) show that Pro is a very effective agent in reducing the production of singlet oxygen; therefore, in its free form it is an important singlet oxygen quencher. Pro accumulation has been frequently correlated with tolerance to drought and high salinity (Kishor et al. 1995; Hmida-Sayari et al. 2005).

The first two steps of proline biosynthesis in plants are catalyzed by the bifunctional enzyme Δ-1-pyrroline-5-carboxylate synthetase (P5CS, EC not assigned) that encompasses both $\gamma$-glutamyl kinase and glutamic-$\gamma$-semialdehyde dehydrogenase activities. P5CS plays a key role in plant intracellular accumulation of proline (Kishor et al. 1995; Pérez-Arellano et al. 2010) and is subjected to feedback inhibition by Pro, controlling the level of the free imino acid under both normal and stress conditions (Hong et al. 2000). Usually, Δ-1-pyrroline-5-carboxylate synthetase is encoded by two differentially regulated genes (Turchetto-Zolet et al. 2009) and this was confirmed for many different plant species such as *Arabidopsis thaliana* (Strizhov et al. 1997), *Lycopersicon esculentum* (Fujita et al. 1998), *Medicago sativa* (Ginzberg et al. 1998), *Medicago truncatula* (Hur et al. 2004), etc. In most of the studied plant species, one of the P5CS isoforms is usually osmo-regulated and the other is associated with developmentally regulated dehydration processes (Hur et al. 2004; Verdoy et al. 2006; Szèkely et al. 2008; Pérez-Arellano et al. 2010).

Increased frequency of extreme temperatures and drought cause oxidative stress which negatively influences crop plant productivity including perennial clovers. Clovers are important legumes with high forage quality and they are cultivated in diverse ecological conditions as a pure stand or mixed with other grassland species. Drought adaptation strategies of plants, including clovers, involve different mechanisms which contribute to avoidance of water scarcity, stabilization of damaged proteins and accumulation of antioxidant molecules and enzymes. There is only a limited amount of published data on the antioxidant response of *Trifolium spp.* to drought (Bermejo et al. 2006; Lee et al. 2007; Wang and Chang 2008; Wang et al. 2008).

Clovers belong to genus comprising approximately 300 different species, which are characterized with big genetic diversity (Yu et al. 2001; Malaviya et al. 2008). The most

widely cultivated clovers are white (*Trifolium repens*) and red clover (*Trifolium pratense*). Genotypic variation observed in clover plants is a factor that contributes to their wide range of adaptation capacity to limited water regimes.

The main objective of this study was to characterize the symptoms of oxidative stress in red (cv. "Start") and white clover cultivars (cv. "Haifa" and cv. "Debut") subjected to drought by measuring electrolyte leakage, malondialdehyde, hydrogen peroxides and free proline content and to compare their antioxidant defense capacities represented by SOD, CAT and POX enzymatic activities. In addition, we expected that the evaluation of changes in ROS detoxifying enzymes will contribute to selection of reliable isozyme markers for drought tolerance or drought susceptibility.

## Materials and methods

### Plant material, growth and drought stress application

Plant material used for the analyses was the same as the one described and used in our recent publication (Vaseva et al. 2011). Red (*Trifolium pratense* L., cv. "Start") and white clover (*Trifolium repens* L, medium—cv. "Haifa" and small leafed cv. "Debut") plants were sown in pots (9.5 cm diameter, 12 cm deep) containing 321 g Florabella® soil (electrical conductivity 40 mS/m ± 25%, pH 5.5–6.5, Klasmann-Deilmann GmbH, 49744 Geeste, Germany) and grown in a growth chamber with 14-h photoperiod and 250 μmol $m^{-2}$ $s^{-1}$ light intensity at 25/21°C day/night temperature. The three clover varieties were grown together in one and the same pot (with replicates) to ensure equal water potential in the root environment. Soil moisture was controlled daily by pot gravimetric measurements. Water loss was compensated to maintain relative soil humidity at 65% of the maximum field capacity. Plants received optimal water supply for the first 21 days of their growth until the first and the second leaves were fully developed. Drought was imposed for 14 days followed by 21-day period of recovery by optimal watering. The control plants were watered daily during the whole experimental period. Leaf samples were taken from control and stressed plants submitted to 14 days drought (35 day olds plants) as well as from age controls and individuals recovered from stress (56-day-old plants). The applied water deprivation increased water deficit in leaves of cv. "Start" and cv. "Haifa" with more than 50%, while cv. "Debut" developed approximately 40% as it was already published earlier (Vaseva et al. 2011). All analyses were performed with samples derived from fully developed second and third leaves. Leaf material was frozen in liquid nitrogen and preserved at −80°C until analyses.

### Determination of electrolyte leakage, MDA, $H_2O_2$ and proline

Membrane integrity was evaluated by the relative electrolyte leakage (EL) from 2-cm leaf segments floating on distilled water for 24 h at 4°C according to the method of Nunes and Smith (2003). The initial conductivity (IC) of the effusate and the conductivity after boiling the segments for 10 min in the same solution and cooling (final conductivity—FC) were measured using a conductivity meter. The relative electrolyte leakage was calculated as a percentage of the initial to the final conductivity: EL = IC/FC × 100.

The level of lipid peroxidation in clover leaf samples was determined in terms of malondialdehyde (MDA) content according to the method of Hodges et al. (1999). Leaves (150 mg FW) were homogenized in 3 ml of 0.1% trichloroacetic acid (TCA) (4°C) and centrifuged at 10,000*g* for 15 min. The supernatant was used in the subsequent determination. The supernatant (0.5 ml) was mixed with 0.5 ml of 0.1 M Tris/HCl buffer (pH 7.6) and 1 ml of TCA–TBA–HCl reagent (containing 15% w/v trichloroacetic acid—0.375% w/v thiobarbituric acid (TBA)—0.25 N hydrochloric acid). This mixture was boiled for 15 min in water bath and centrifuged at 2,000*g* for 5 min. The absorbance of the samples was read at 440, 532 and 600 nm (Specol 10, Germany) to determine malondialdehyde content (expressed as nmol MDA $g^{-1}$ FW).

Leaves (0.5 g FW) were homogenized in 5 ml of 0.1% trichloroacetic acid. Polyclar AT (50 mg) was added at the time of grinding. The homogenate was centrifuged at 10,000*g* for 30 min. 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 hydrogen peroxide, expressed as nmol $H_2O_2$ $g^{-1}$ FW.

Proline content was determined by the method of Bates et al. (1973). Leaf samples (0.5 g FW) from each variety were homogenized in 10 ml of 3% aqueous sulphosalicylic acid at room temperature and the homogenate was centrifuged at 2,000*g* for 5 min. Two milliliters of the extract reacted with 2 ml ninhydrine and 2 ml glacial acetic acid for 1 h at 100°C in a water bath. The reaction was stopped using an ice bath. The reaction mixture was extracted with 4-ml toluene. The chromophore containing toluene was separated and the absorbance read at 520 nm (Specol 11 Jena, Germany). Proline content was determined using calibration curve and expressed as mg Pro $g^{-1}$ FW.

### Protein content

Total soluble protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

### Isoenzyme staining of SOD, CAT and POX

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 $\beta$-mercaptoethanol, 2 mM phenylmethanesulphonylfluoride, 0.005% Triton X 100, 50 mg Polyclar AT, and centrifuged at 15,000*g* for 30 min at 4°C. Activities of the antioxidative enzyme isoforms were estimated using in-gel staining methods. Thirty micrograms of total protein per lane was separated at 4°C on a native 7.5% PAGE (CAT and POX) or 10% native PAGE (SOD). SOD activity was visualized and SOD types differentiated according to González et al. (1998). Gels were soaked for 25 min in 50 mM potassium phosphate buffer pH 7.8, containing 28 mM TEMED and 28 μM riboflavin. After the incubation, gels were placed in the same buffer supplemented with 2 mM NBT for 30 min in darkness with shaking. Subsequently, the gels were exposed to a light source until white bands appeared (15 min), then washed extensively in 50 mM potassium phosphate buffer with pH 7.8. SOD isoforms were differentiated by pre-stain incubation for 30 min in 50 mM potassium phosphate buffer, pH 7.8 containing 2 mM KCN or 5 mM $H_2O_2$. Cu/ZnSODs were inhibited by cyanide, FeSODs were resistant to cyanide, but were inactivated by hydrogen peroxide and MnSODs were resistant to both inhibitors. CAT isoenzymes were stained following Woodbury et al. (1971). POX isoenzymes were separated and revealed according to Hart et al. (1971). Representative images obtained from three individual experiments with in-gel SOD, POX, CAT activity assays are shown.

### Protein extraction and immunoblotting

Soluble leaf proteins for SDS-PAGE were extracted as described previously (Vaseva et al. 2011). For immunoblot analysis, the extracted proteins were separated by 12% SDS-PAGE and then transferred to nitrocellulose membrane (BioRad). The level of Δ-1-pyrroline-5-carboxylate synthetase was analyzed on immunoblots developed with primary antibodies raised in a rabbit against the synthetic peptide EITFGDKSRVGRGGM representing the highly conserved amino acids 225 to 239 of this enzyme from soybean. Equal loading was confirmed by two additional immunoblots with antibodies against phosphoglycolate phosphatase (PGP) performed with the same protein samples. The bands were visualized as described previously by Mitsuhashi and Feller (1992).

### Statistical analysis

The experiment was laid out in two-factor completely randomized design being genotypes and applications (namely, control, drought, control-recovery and drought-recovery). Data of electrolyte leakage, proline, malondialdehyde, hydrogen peroxides (Fig. 1) were obtained from three independent experiments and submitted to multifactor ANOVA analysis (MSTAT-C). Means were separated as a group by Duncan's Multiple Range Test at significant difference $P \leq 0.05$. Different letters above columns indicate significant differences between the groups, whereas the same letters show no significant difference.

## Results

Earlier published study using the same experimental material characterized the applied water stress as a moderate one, since plants developed up to 50% water deficit (Vaseva et al. 2011). Membrane electrolyte leakage and lipid peroxidation in terms of malondialdehyde (MDA) content were determined to assess the membrane damage after drought stress. Electrolyte leakage (EL) increased approximately 3–6 times under drought, depending on the cultivar (Fig. 1a), showing considerable membrane damage. Under recovery, EL diminished exhibiting only 1.2–1.7 times higher levels than the respective age controls (Fig. 1a), which characterized the observed membrane changes provoked by water deprivation as reversible ones. EL levels measured in drought stressed and recovered white clover cv. "Debut" showed the lowest difference (only 3 times), characterizing this cultivar as the less susceptible to membrane damage provoked by drought (Fig. 1a).

The oxidative damage of lipids was evaluated by measuring the changes in malonedialdehyde (MDA) content (Fig. 1b). MDA level in drought-stressed plants increased significantly and it diminished considerably during recovery.

Hydrogen peroxide content increased 5–6 times under drought in all clover cultivars (Fig. 1c). After recovery, $H_2O_2$ levels in previously stressed plants (DR) generally diminished but they reached the control values only in the recovered red clover cv. "Start" (Fig. 1c).

Proline content in leaves (Pro) increased significantly in drought-stressed plants—160-fold in white clover cv. "Haifa", 98-fold in red clover cv. "Start" and only 22-fold in white cv. "Debut" (Fig. 1d). Pro levels decreased and reached the control values during recovery (Fig. 1d).

The antioxidant capacity of leaf soluble proteins from well-watered and drought-stressed red and white clover plants was characterized via SOD, CAT and POX isoenzyme analyses using native PAGE (Figs. 2, 3).

Five forms of SOD were detected (Fig. 2a). According to the performed inhibitory analysis, two SOD bands disappeared completely after incubation with KCN and were

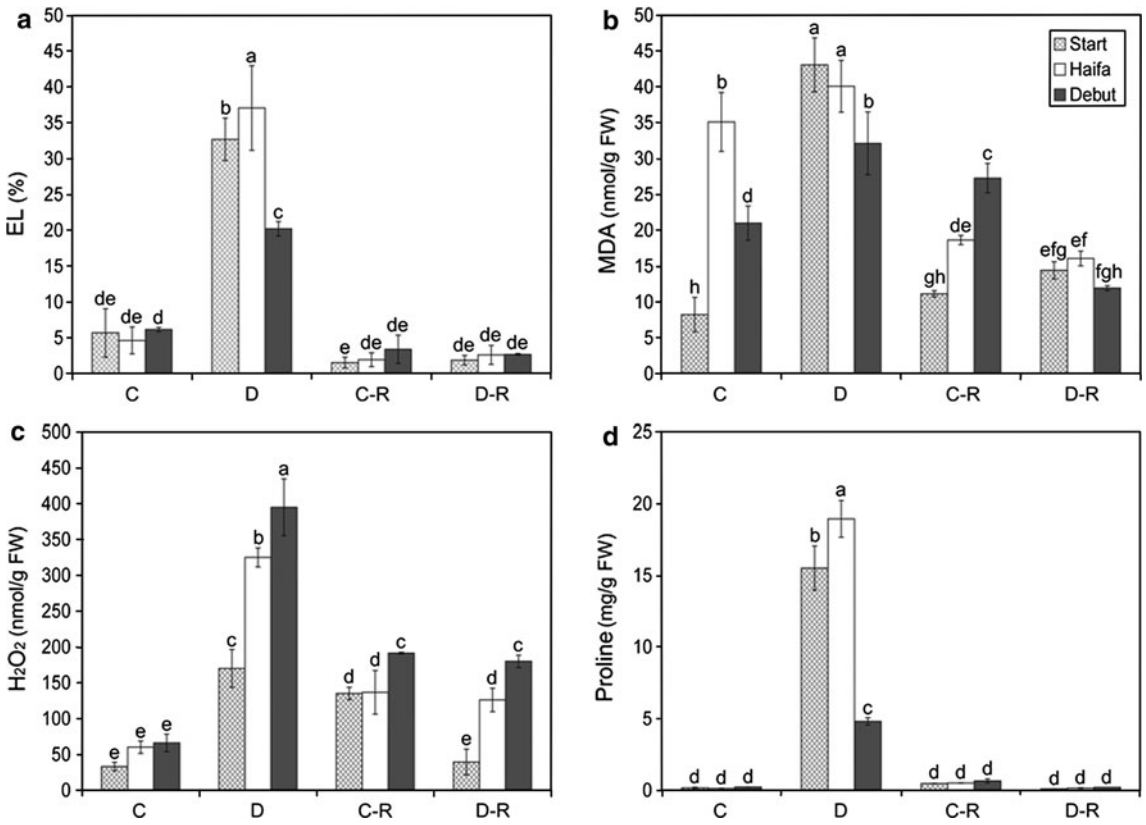


**Fig. 1** Electrolyte leakage (**a**), malondialdehyde (**b**), hydrogen peroxides (**c**), proline (**d**) content in leaves of well watered (C), drought stressed (D), age controls (CR) and recovered (R) red (cv. "Start") and white clovers (cv. "Haifa" and cv. "Debut"). Significant differences ($P \leq 0.05$) are indicated by *different letters*

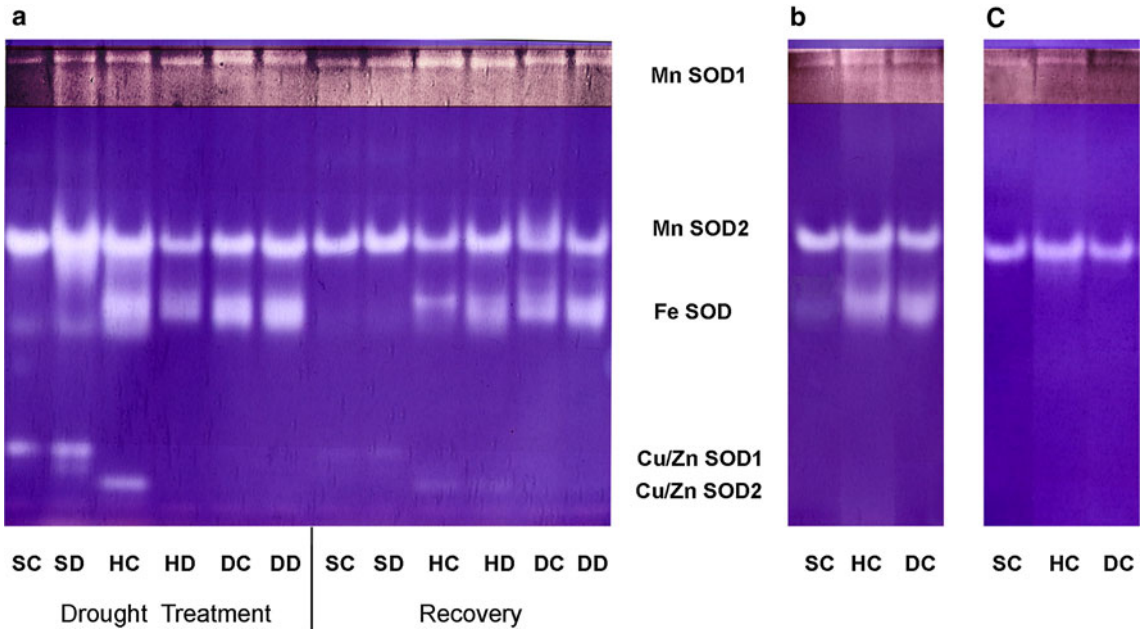


**Fig. 2** **a** Effect of drought on isoenzyme pattern of SOD from leaf extracts of control (C) and drought stressed (D) red (cv. "Start"—S), and white clovers (cv. "Haifa"—H and cv. "Debut"—D). **b** Inhibition of SOD activity with KCN. **c** Inhibition of SOD activity with $H_2O_2$. The *upper* field of the image was contrasted in order to visualize the weaker signals on the gel. Samples of 30 µg protein were loaded per lane

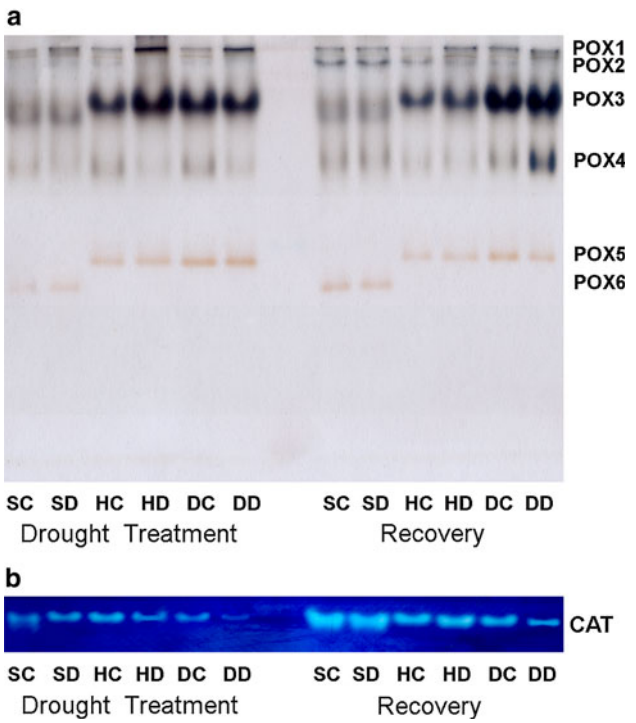


**Fig. 3** Effect of drought on isoenzyme pattern of POX (**a**) and CAT (**b**) from leaf extracts of control (C) and drought stressed (D) red (cv. "Start"—S) and white clovers (cv. "Haifa"—H and cv. "Debut"—D). Samples of 30 μg protein were loaded per lane

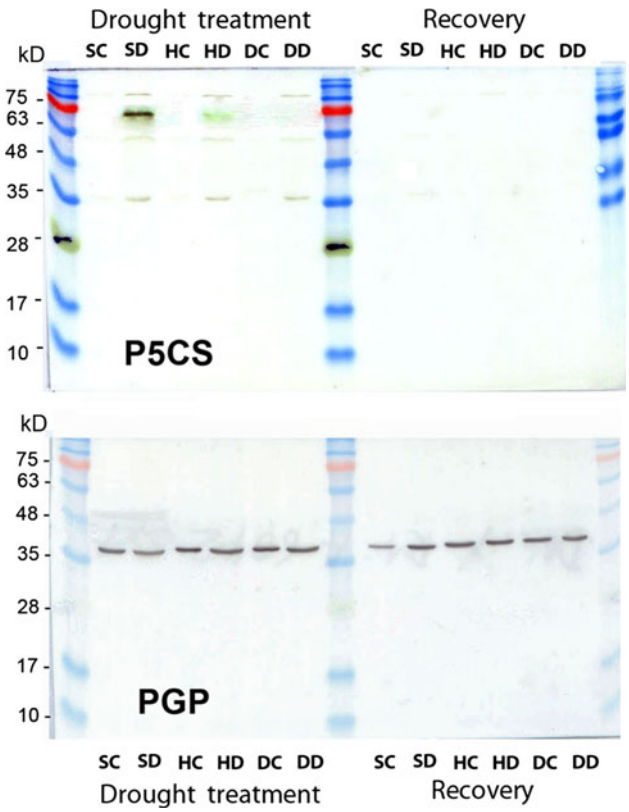


**Fig. 4** Immunoblot analysis of extracts from leaves of control (C) and drought stressed (D) red (cv. "Start"—S), and white clovers (cv. "Haifa"—H and cv. "Debut"—D) with polyclonal antibodies against Δ-1-pyrroline-5-carboxylate synthetase (P5CS). Phosphoglycolate phosphatase (PGP) immunoblot was used as a loading control. SDS-PAGE gels were loaded with equal amounts of soluble protein (10 μg/slot for P5CS and 5 μg/slot for PGP)

absent after $H_2O_2$ treatment, which means that they are most probably Cu/ZnSOD isoenzymes (Cu/Zn SOD1 and Cu/ZnSOD2). Three isoforms were stable upon KCN treatment but one of them turned out to be sensitive towards $H_2O_2$, which defined it as FeSOD. Two MnSOD isoforms were visualized after KCN (Fig. 2b) and $H_2O_2$ (Fig. 2c) treatments—MnSOD1, displaying a very weak signal, and MnSOD2.

Red clover cv. "Start" exhibited high intensity of MnSOD2 signal under drought, while the opposite trend was detected in water-stressed white clover "Haifa". White clover cultivar "Debut" displayed comparatively stable MnSOD2 levels both under drought and after recovery.

FeSOD isoform was with a very low intensity in red clover cv. "Start" in both well-watered and drought-stressed plants. Water deprivation tended to decrease the level of the FeSOD isoform in white clover plants cv. "Haifa", while it remained stable upon drought treatment in cv. "Debut".

Cu/Zn SOD1 band was present only in red clover cv. "Start" and its intensity increased slightly in water-deprived plants. Cu/ZnSOD2 band was detectable only in white clover, particularly in cv. "Haifa" control. The band was very faint in the other samples.

Six peroxidase (POX) isoenzymes were revealed after native electrophoresis of red and white clover leaf extracts (Fig. 3a). One additional POX isoform with a very weak and thin band migrating between POX1 and POX2 was detected as well. Most bands exhibited low peroxidase activity, except POX3 signal in samples derived from white clover plants. POX3 activity was enhanced in drought-stressed cv. "Haifa" and apparently it was not influenced in water-deprived cv. "Debut" plants. Red clover cv. "Start" showed lower POX activities than the two white clover varieties without any clear differences between stressed, control and recovered plants.

Clover leaf extracts revealed only one isoform with CAT activity with slightly decreasing intensity under drought in white clover samples (Fig. 3b). Stimulated CAT activity was revealed after recovery from drought in all leaf extracts but the white clover cultivar "Debut" still exhibited lower CAT activity compared to its age control.

Immunoblot of Δ-1-pyrroline-5-carboxylate synthetase (P5CS) (Fig. 4) visualized the expected signal (around 75–80 kDa) only in drought-stressed red (cv. "Start") and white (cv. "Haifa") clovers, although the later one was comparatively weak. P5CS bands were not detected in

control and recovered samples. Drought did not affect the levels of phosphoglycolate phosphatase (Fig. 4, PGP) which is involved in photorespiratory carbon metabolism. Therefore, this protein was chosen for loading control of P5CS immunoblot (Fig. 4, P5CS).

## Discussion

Summer in South-Eastern Europe is often characterized by long periods of drought which influence negatively growth and productivity of perennial clovers. One of the approaches to improve crop yield under water scarcity is the selection of tolerant cultivars which are capable to withstand unfavorable environmental conditions. The sensitivity or tolerance to water stress could be related to different genetically determined capacity of plants to cope with oxidative stress events which usually accompany drought (Apel and Hirt 2004). Identification of physiological and biochemical components of antioxidative defense system, which have a potential to confer drought tolerance, could be essential for the characterization of tolerant clover cultivars. The generation rate of reactive oxygen species (ROS) increases under drought conditions enhancing leakage of electrons to molecular oxygen. ROS comprise non-radical forms such as $H_2O_2$ (hydrogen peroxide) and $^1O_2$ (singlet oxygen), as well as $O_2^{\cdot-}$ (superoxide) and OH· (hydroxyl) free radicals. Peroxidation of lipid membranes further ensures a steady supply of ROS (Jain et al. 2001). Increased EL, MDA and $H_2O_2$ are among the biochemical changes provoked by water shortage and their accumulation has an effect on oxidative damage and ROS production in stressed plants (Alscher et al. 2002; Mittler 2002; Blokhina et al. 2003).

According to Erdei et al. (2002), the degree of plant drought tolerance differs among various species but also among different varieties of the same species. Earlier studies have shown that white clovers were more adaptable to a wide range of soil and environmental conditions then red clovers (Edmeades et al. 1991; Voigt and Mosjidis 2002; Vaseva et al. 2011; Simova-Stoilova et al. 2012). In our previous study with the same white clover varieties, an attempt has been made to explain the differences in potential to sustain unfavorable environmental conditions by analyses of some important proteins (Rubisco, Rubisco activase, Rubisco binding protein, calpains, heat shock proteins and dehydrins) associated with drought tolerance (Vaseva et al. 2011). The analyses showed that the small leafed white clover cv. "Debut" developed the lowest leaf water deficit (WD) and coped most efficiently with drought stress among the tested varieties. Detailed gene expression analysis revealed that the abundance of transcripts of the drought inducible dehydrin type $Y_2SK$ was higher in water-deprived cv. "Debut" plants. Another study with different and red clover cultivars confirmed that *Trifolium repens* was characterized with higher relative protein stability under waterlogging and it tended to express higher ascorbate activity involved in the antioxidative protection (Simova-Stoilova et al. 2012).

Hydrogen peroxide synthesis increases in response to environmental stresses such as excess excitation (light) energy, drought, and cold (Dat et al. 2000). $H_2O_2$ is a cytotoxic agent which can destroy normal metabolism through oxidative damage to lipids, proteins and nucleic acids (Halliwell and Gutteridge 1989) but is also considered to be a signal molecule (Neill et al. 2002). It has been confirmed that hydrogen peroxide directly regulates the expression of numerous genes involved in plant defense such as antioxidant enzymes, defense proteins and transcription factors (Hung et al. 2005). In addition, exogenously applied hydrogen peroxide is capable also to alleviate water stress in different crops (He et al. 2009; Ishibashi et al. 2011; Abass and Mohamed 2011) by serving as a second messenger in signal transduction pathways. All these previously published data regarding hydrogen peroxide content in relation to drought tolerance suggest that the high hydrogen peroxide content measured in white clover cultivars "Haifa" and "Debut" subjected to drought stress (Fig. 1c) could be one of the factors that contribute to the higher acclimation potential of *Trifolium repens* compared to *Trifolium pratense* towards water deprivation.

According to Ślesak et al. (2007), the mechanisms of $H_2O_2$ scavenging are regulated by both non-enzymatic and enzymatic antioxidants. Upon re-watering, recovered plants displayed similar but not equal enzymatic activities to the ones measured under pre-stressed control conditions (Fig. 3a, b). Subsequently $H_2O_2$, being a substrate of catalase and peroxidases, generally diminished after recovery, reaching the control values in *T. pretense* cultivar but still remaining above the control levels in white clover plants. This could be explained with the differences in the respective enzymatic antioxidant activities in the recovered plants from the two species.

The accumulation of low-molecular weight metabolites that act as osmoprotectants, such as proline, is part of plant adaptation towards water deficiency (Hoekstra et al. 2001). Drought provoked significant changes in free proline content in all of the tested clover cultivars (Fig. 1d). These result confirmed the well-known role of Pro as a molecule playing part in stabilization of proteins, membranes and subcellular structures, as well as a protector of cellular functions by scavenging reactive oxygen species (Kishor et al. 2005). According to Cushman et al. (1990) Pro accumulation in plant tissues could confer some adaptive advantages under osmotic stress but it could be also considered as a stress-induced marker for changes in

metabolism under unfavorable conditions. Hence, Pro content in leaves would be closely related to the degree of water stress experienced by the plant. The lowest Pro content measured in drought-treated white clover cv. "Debut" leaves could be explained by its comparatively good tolerance towards the imposed water stress (Fig. 1a, b). Previously published data showed that cv. "Debut" developed the lowest leaf water deficit during the 14 day water deprivation compared to the other two tested cultivars—red clover cv. "Start" and white clover cv. "Haifa" (Vaseva et al. 2011).

The measurement of specific antioxidant enzyme activities and expression analysis during water stress treatments has been generally accepted as an approach to assess the involvement of the scavenging system during drought stress. Often, the results describing the effect of water stress on antioxidant enzymes are contradictory which could be related to differences in the experimental set-up, the plant age or tolerance towards water deprivation (for review see Cruz de Carvalho 2008). Nevertheless, in some studies, a direct correlation between the induction of the antioxidant system and drought tolerance has been established (Loggini et al. 1999; Edjolo et al. 2001; Lascano et al. 2001; Türkan et al. 2005; Guo et al. 2006).

SOD isoforms are active in different subcellular compartments (Scandalios 1993) and an earlier study on antioxidant response of *Trifolium repens* subjected to water stress has shown that mitochondrial Mn-SOD, cytosolic and chloroplastic Cu/Zn-SOD, and chloroplastic Fe-SOD were among the major scavenger enzymes (Wang et al. 2008). Data reported in the present study demonstrated considerable impact of drought on FeSOD and MnSOD isoforms. CuZnSOD isoforms exhibited divergent profiles in red and white clovers in response to drought. This activity was up-regulated in *T. pretense* and inhibited in *T. repens* cultivars. Wang and Chang (2008) have also revealed three kinds of electrophoretically different SOD isozymes in the leaves of white clover *Trifolium repens.* They found that the chloroplast FeSOD isoform was the one mainly contributing to scavenging superoxide radicals produced during water deficiency. In the present study, we also found that FeSOD was the most abundant in the white clover cultivars "Haifa" and "Debut" and it was hardly detectable in *T. pretense* cultivar "Start". We confirmed that MnSOD2 and FeSOD forms were the most affected by water deprivation. In the medium-leafed white clover "Haifa" (the more susceptible to drought stress cultivar), these SOD activities were inhibited, while they remained unchanged in the stressed samples derived from the small-leafed drought-tolerant variety "Debut". Martinez et al. (2001) have studied differential responses of superoxide dismutase in freezing resistant *Solanum curtilobum* and freezing sensitive *Solanum tuberosum* subjected to oxidative and water stress. They observed that the frost resistant species *Solanum curtilobum* had higher SOD activity, with the FeSOD isozyme showing the greatest increase. The authors considered that the increase in SOD activity and the decrease in oxidative damage were closely related. The FeSOD abundance in white clovers especially the maintenance of SOD activity in cv. "Debut" could be related to its higher potential to alleviate the oxidative damage caused by drought stress. These observations suggest that MnSOD2 and FeSOD isoforms are candidates for drought-tolerance markers, particularly for white clover cultivars, which should be further elucidated.

CuZnSOD isoforms also exhibited divergent profiles in red and white clovers in response to drought. This activity was up-regulated in *Trifolium pratense* and inhibited in *Trifolium repens* cultivars. We assume that to maintain a certain level of total SOD, subcellular re-distribution of SOD activities is necessary. One may speculate that red clovers are characterized by predominant Cu/ZnSOD over FeSOD isoforms which maintain the required level of total SOD under particular growth conditions at this developmental stage. After recovery, the activity of SOD isoforms, especially these of Cu/ZnSOD and FeSOD, decreased which was most probably related to developmental processes.

Usually, high POX activity is related to detoxification of elevated hydrogen peroxide, restricting lipid peroxidation in membrane regions. Lee et al. (2007) have suggested that peroxidase enzymes were in relation with the intensity of water deficit in white clover (*Trifolium repens*). In agreement with this earlier publication, the highest POX activity, especially the one of isoforms POX1 and POX3, documented in the present study was detected in samples from white clover cultivars "Haifa" and "Debut". This coincided with the highest level of hydrogen peroxide measured under drought conditions. Previously published study on *Hordeum vulgare* cultivars with different tolerance towards drought (Acar et al. 2001) has shown that the most sensitive variety exhibited the lowest POX activity under water deprivation. In the present study, we observed similar trend—the red clover cultivar "Start" which was the most affected by drought exhibited the lowest POX activity. We assume that low POX activity under water deprivation could be regarded as a precondition for drought sensitivity.

Wang et al. (2008) have suggested that POX activity plays a major role in the scavenging $H_2O_2$, whereas cellular CAT occupies a subordinate place in scavenging water stress-induced $O_2^{\cdot-}$ and $H_2O_2$ in *Trifolium repens.* As it was previously shown by other studies with different plants (Zhang and Kirkham 1996; Fu and Huang 2001; Türkan et al. 2005), mild drought imposed on the red clover cultivar "Start" did not have any significant influence over CAT, although an inhibition of this activity occurred in

white clovers under water stress. Catalase is localized in peroxisomes and it eliminates $H_2O_2$ by breaking it down directly to form water and oxygen. The observation that its levels remained relatively stable in red clover subjected to drought compared to white clover, suggested more effective $H_2O_2$ dismutation capacity outside the chloroplasts for *T. pretense* (Türkan et al. 2005). After recovery, all clover varieties exhibited relatively higher CAT levels, which could be assigned to the need for more intense scavenging of $H_2O_2$ generated under water scarcity.

The obtained results suggest that SOD–CAT–POX system protects clover plants from oxidative damage during drought stress, but the individual enzymatic activities differ substantially between red and white clovers, as well as between the two tested *T. repens* cultivars.

Molecular phylogeny has shown that most of the legume P5CS genes belong to one and the same genetically preserved group (Falaknaz et al. 2011). PvP5CS from common bean (*Phaseolus vulgaris*) contains an open reading frame encoding a 716 amino acid polypeptide (Chen et al. 2009). Sequence analysis showed that bean PvP5CS shares 95.1% identity in nucleotide sequence and 93.2% identity in amino acid sequence with the mothbean (*Vigna aconitifolia*) P5CS. Another closely related *Trifolium* species *Medicago sativa* has two genes responsible for synthesis of P5CS (Ginzberg et al. 1998), and *MsP5CS-1* (2.6 kb) has an open reading frame of 717 amino acids. The two *M. sativa* P5CS clones share 65% identity in nucleotide sequences, 74% identity in deduced amino acid sequences. They both have high degree of resemblance with *Vigna aconitifolia* and *Arabidopsis thaliana P5CS* cDNA clones. The difference in the size of the two *M. sativa* P5CS isoforms is only three amino acids, so their approximate molecular weight should be very similar (75–80 kDa). Several studies have confirmed that abiotic stresses caused significant up-regulation of the expression of P5CS in leaves (Ginzberg et al. 1998; Chen et al. 2009) confirming that this enzyme is a stress-inducible one and it regulates the accumulation of proline in plants subjected to stress. The immunoblot of clover P5CS revealed a single band migrating at 75 kDa position (Fig. 4) in red clover cv. "Start" and white clover cv. "Haifa". This corresponded to the high proline content accumulated in the leaves of these two cultivars under drought. The more drought-tolerant white clover "Debut" had the lowest free proline amounts during water deprivation and did not express any signal in P5CS immunoblot which makes this enzyme a potential marker for monitoring of the physiological status during water deprivation.

In conclusion, the drought stress imposed on red and white clovers was accompanied by oxidative stress which was responded to differently by the tested cultivars. Red clover cv. "Start" seemed to be more susceptible to oxidative damage provoked by water deprivation than white clovers. *T. pratense* cv. "Start" exhibited the lowest FeSOD and POX activities which contributed to its poor performance under water deprivation, earlier demonstrated by profiling of Rubisco binding protein, low-molecular weight heat shock proteins, calpains and dehydrins (Vaseva et al. 2011). The extremely high accumulation of induced P5CS activity and the resulting high free proline content were indicators for the early occurred changes in metabolism of *T. pretense* under the imposed drought, which further supported its vulnerability towards unfavorable water supply. MnSOD2 and FeSOD isoforms remain to be elucidated as potential candidates for drought-tolerance markers, which are able to identify white clover cultivars resistant to water shortage.

**Authors Contribution** Klimentina Demirevska—study design, data collection, writing, literature search, figures, SOD, POX and CAT activities; Irina Vaseva—writing, literature search, figures; Urs Feller—data interpretation, immunoblot analyses; Lyudmila Simova-Stoilova—SOD, POX and CAT activities; Yasar Akiscan, Anelia Kostadinova and Rosa Nenkova—plant material, $H_2O_2$, MDA, proline, EL; Iwona Anders—immunoblot analyses.

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