RESEARCH PAPER

Peroxidases and lignification in relation to the intensity of water-deficit stress in white clover (Trifolium repens L.)

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Abstract

To investigate the lignification process and its physiological significance under drought-stressed conditions, the changes in enzymes responsible for lignification and the related physiological parameters were determined in white clover (Trifolium repens L.) leaves during 28 d of water deficit treatment. Water deficit gradually decreased leaf water potential ($\Psi_w$) to $-2.33$ MPa at day 28. For the first 14 d of water deficit, ascorbate peroxidase and phenylalanine ammonia-lyase were highly activated. Neither a change in the parameters symptomatic of oxidative stress nor growth inhibition was observed. The reduction of leaf biomass occurred from 21 d of water deficit treatment when $\Psi_w$ was $-2.27$ MPa or less, and was concomitant with the increase of lipid peroxidation and lignin content. As $\Psi_w$ decreased below $-1.67$ MPa from 14 d of water deficit, the enhanced activation of guaiacol peroxidase, coniferyl alcohol peroxidase, syringaldazine peroxidase, and benzidine peroxidase was involved in lignification rather than in protection of plant tissues against the oxidative damage. The data indicate that a high activation of lignifying enzymes during terminal stress may be a drought stress-induced injurious symptom, which leads to reduced forage growth and digestibility.

Key words: Drought, lignification, peroxidases, phenylalanine ammonia-lyase, polyphenol oxidase, Trifolium repens.

Introduction

The incorporation of lignin into cell walls results in structural rigidity and durability of plant tissues. Lignin, therefore, is responsible for inhibition of expansion growth in crop plants and for providing barriers to pathogen infection. Peroxidases (EC 1.11.1.7) are the enzymes most directly involved in lignin biosynthesis. Monomeric precursors of lignin are enzymatically dehydrogenated in the cell wall to phenoxy radicals. These radicals polymerize spontaneously, yielding a complex net of cross-linking among monolignols, proteins, and polysaccarides (Iiyama et al., 1994). Peroxidases have been implicated in these cross-linking reactions (Lewis and Yamamoto, 1990; Polle et al., 1994). According to the isoelectric point (pI) values of peroxidases, they are classified as cationic and anionic isozymes. Some articles suggested that anionic peroxidases were involved in lignin polymerization (Christensen et al., 1998; EL Chen et al., 2002), but Quiroga et al. (2000) reported that both cationic and anionic peroxidases showed the highest catalytic efficiency when the substrate used was syringaldazine, an analogue of lignin monomer.

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Abbreviations: APOX, ascorbate peroxidase; BPOX, benzidine peroxidase; CPOX, coniferyl alcohol peroxidase; FW, fresh weight; GPOX, guaiacol peroxidase; MDA, malondialdehyde; PAL, phenylalanine ammonia-lyase; PPO, polyphenol oxidase; SPOX, syringaldazine peroxidase; $\Psi_w$, leaf water potential.

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In addition, it has been postulated that the increase in cationic and anionic peroxidases is in part responsible for lignin synthesis and for the production and consumption of H₂O₂ required for the formation of phenoxy radicals (Mäder and Amberg-Fischer, 1982; EL Chen et al., 2002).

Moreover, phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) is considered to be responsible for the conversion of L-phenylalanine to trans-cinnamic acid, a key intermediate in the pathway of phenolics and lignin (Boudet, 2000; Rivero et al., 2001). Polyphenol oxidase (PPO; EC 1.14.18.1) catalyses the oxidation of polyphenols and the hydroxylation of monophenols (Rivero et al., 2001) and lignification of plant cells. Recent studies have indicated that phenol-oxidizing enzymes may participate in response to the defence reaction and hypersensitivity in inducing resistance of plants to biotic and abiotic stress (Jouili and El Ferjani, 2003; Jung, 2004).

It has been well documented that biotic and abiotic stresses are responsible for the increase in cell wall lignification (Chazen and Neumann, 1994; Polle et al., 1994; Katerji et al., 1997) which would be associated with decreased plant growth, nutrient content, and digestibility (Guenni et al., 2002). However, little has been known about the changes in peroxidases, PPO, and PAL activity as related to growth response and lignification under a water-deficit stressed condition. Moreover, peroxidase activities were not determined with natural substrates, and it was not possible to attribute specific roles to different isoforms (Polle et al., 1994).

In this study, it was hypothesized that the lignification-related enzymes, alone or in combination, which are responsible for the stress tolerance mechanism would be activated with different kinetics in relation to the intensity of drought stress (e.g. the decrease in leaf water potential, \( \Psi_w \)). To test this hypothesis, the physiological relationships between drought-induced activation of lignification-related enzymes, growth, and some stress symptomatic parameters in response to the change in leaf water status were investigated. Moreover, the activities of peroxidases were determined with important substrates used for \( \text{in vivo} \) lignification, such as coniferyl alcohol, benzidine, guaiacol, or syringaldazine.

**Materials and methods**

**Plant culture and sampling**

Sods of fully vegetative white clover were transplanted into 3.0 l pots containing a mixture of sand and fritted clay. Plants were regularly watered to field capacity during 2 weeks of adaptation. Water-deficit stress was imposed by decreasing the volume of water supply per day. Daily irrigation of 50 ml or 5 ml of water per pot was applied to the well-watered (control) and water deficit treatment, respectively. Half the volume of the daily irrigation for each treatment was applied at 10.00 h and the remaining half at 16.00 h to minimize the considerable differences between \( \Psi_{soil} \) predawn and \( \Psi_w \) midday that arise with once per day irrigation (Kim et al., 2004). Each treatment lasted for 28 d, and leaf tissues were sampled at intervals of 7 d just before the irrigation at 10.00 h. Tissue samples were immediately frozen in liquid nitrogen. Freeze-dried samples were finely ground and stored under vacuum for further analysis.

**Measurements of leaf water parameters**

\( \Psi_w \) was evaluated immediately as the petiole xylem pressure potential using a pressure chamber (PMS Instrument Co., Corvallis, OR, USA). Relative water content was determined gravimetrically as described previously (Kim et al., 2004). The measurements of leaf water status were carried out before dawn on the first or second fully expanded green leaf proximal to a stolon apex. The results of leaf water parameters are given as a pooled mean of three separate pots with three replicates in each.

**Determination of H₂O₂, lipid peroxidation, lignin, and total phenol**

H₂O₂ concentration was measured colorimetrically as described by Lin and Kao (2001a) using titanium sulphate. H₂O₂ concentration was calculated using the extinction coefficient 0.28 mM⁻¹ cm⁻¹ and was expressed as \( \mu \text{mol g}^{-1} \) tissue dry weight. The lipid peroxidation level was determined by measuring the concentration of malondialdehyde (MDA), as described previously (Heath and Parker, 1968). For lignin determination, 3 g of fresh leaves were homogenized with 95% ethanol. The homogenate was centrifuged at 10 000 g for 5 min. The pellet was washed three times with 95% ethanol and twice with a mixture of ethanol and hexane (1:2, v/v). The material was allowed to air dry and its lignin level measured. The dried sample was washed once with 2 ml of acetyl bromide in acetic acid (1:3, v/v). Then 1 ml of acetyl bromide in acetic acid (1:3, v/v) was added to the pellet and incubated at 70°C for 30 min. After cooling the mixture to room temperature, 0.9 ml of 2 M NaOH and 0.1 ml of 7.5 M hydroxylamine hydrochloride were added, and the volume was made up to 10 ml with acetic acid. After centrifugation at 1000 g for 5 min, the absorbance of the supernatant was measured against an NaOH blank at 280 nm (Lin and Kao, 2001b). The amount of lignin was calculated from a linear calibration curve (0–40 \( \mu \text{g} \)) built with commercial alkali lignin (Aldrich, Steinheim, Germany). Total phenol content was determined by the Folin–Ciocalteau method (Singleton and Rossi, 1965). The phenolic contents were estimated using a standard curve using gallic acid. Total phenolic content was expressed as gallic acid equivalents in mg g⁻¹ fresh weight (FW).

**Enzyme assays**

For extraction of enzymes, fresh samples (0.5 g) were homogenized with 1.5 ml of 100 mM K-PO₄ buffer solution (pH 7.0) containing 2 mM phenylmethylsulphonyl fluoride, and centrifuged at 14 000 g at 4°C for 20 min. The activities of peroxidases were measured using different substrates: ascorbate, guaiacol, coniferyl alcohol, and syringaldazine. Ascorbate peroxidase (APOX) activity was determined by measuring the decrease in absorbance at 290 nm (extinction coefficient of 2.8 mM⁻¹ cm⁻¹ for ascorbate) according to Chen and Asada (1989). The 1 ml reaction mixture contained 100 mM K-PO₄ (pH 7.5), 0.5 mM ascorbate, 0.2 mM H₂O₂, and 20 µl of extraction solution. APOX activity was expressed as \( \mu \)mol ascorbate oxidized min⁻¹ g⁻¹ FW. For guaiacol peroxidase (GPOX) activity, the oxidation of guaiacol was estimated by measuring the increase in absorbance at 470 nm for 1 min and the activity was calculated using an absorption for tetraguaiacol (26.6 mM⁻¹ cm⁻¹) (Lee and Lin, 1995). The reaction mixture
PPO activity was expressed as changes in absorbance at 420 nm of catechol (Sigma) as the substrate at a final concentration of 20 mM. Sodium phosphate buffer (pH 7.0) containing 10 mM DL-1,3-dihydroxy phenylalanine was used for incubation, dark bands indicative of PPO isozymes appeared in the gels. Reaction mixture containing 20 μl of enzyme extract and 10 mM phosphate buffer (pH 7.0) was used for different treatments. Regression analysis was used to determine the significance of relationships among the measured variables. Unless otherwise stated, conclusions are based on differences between means significant at $P < 0.05$.

Results

Leaf water potential and some physiological parameters

$\Psi_w$ reached a minimum value of $-2.33$ MPa after 28 d of water deficit treatment, while no significant changes occurred in the control (Table 1). As compared with the control plant, a significant ($P < 0.05$) increase (+22.7%) in H$_2$O$_2$ concentration occurred from day 21 of the water deficit treatment. The MDA concentration was not significantly affected over the first 14 d but then continuously increased until day 28 (+44.5%). A significant increase (+17.3%) in total phenol concentration was apparent within 7 d of water deficit treatment. The lignin concentration increased significantly from day 21 of water deficit treatment. A significant decline in dry mass (−20.9%) was observed from day 21.

Activities of lignification-related enzymes and their activity staining

GPOX activity was not significantly different between watering treatments for the first 7 d. However, GPOX activity in drought-stressed leaves subsequently increased to a 1.9-fold higher level compared with the control at day 28 (Fig. 1A). In water deficit plants, a rapid increase (+73%) in APOX occurred within the first 7 d, and then gradually decreased until day 28 (Fig. 1B). CPOX was significantly increased from 14 d of water deficit treatment (Fig. 1C). SPOX activity was induced 8.7-fold by water deficit treatment during 28 d (Fig. 1D). PAL activity in water deficit leaves rapidly increased for the first 7 d (+62%) and then decreased at day 14, but was maintained at a higher level than the control (Fig. 1E). A significant increase (+13%) in PPO in water deficit leaves was apparent at day 14, and it was maintained at the same level (Fig. 1F).

After PAGE, active staining of major enzymes involved in lignification was used to identify the expression of isozymes in leaves of white clover. The GPOX isozyme of 64.7 kDa was distinctly induced by drought stress (Fig. 2A). The SPOX isozyme of 51.4 kDa was commonly detected in both drought-stressed and control leaves, but the expression of this isozyme was much stronger in drought-stressed leaves (Fig. 2B). The BPOX isozyme of 48.9 kDa was active only in water deficit leaves, and the 30.3 kDa isozyme was strongly expressed in drought-stressed leaves (Fig. 2C). Active staining of PPO revealed that four isozymes (23.0, 25.5, 43.5, and 49.1 kDa) were actively induced by drought treatment (Fig. 2D).
Table 1. Changes in the leaf water potential and some physiological parameters of white clover under well-watered (control) or drought-stressed conditions for 28 days

Values are means of three replicates with three plants each. Different letters in a vertical column or a horizontal row are significantly different at P ≤ 0.05 by Duncan’s multiple range test.

<table>
<thead>
<tr>
<th>Leaf water parameters/treatment</th>
<th>Days after treatment</th>
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<tr>
<td></td>
<td>0</td>
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<tr>
<td>Water potential (Ψw, MPa)</td>
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<tr>
<td>Control</td>
<td>−0.43 a</td>
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<tr>
<td>Water-stressed</td>
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<td>Biomass (DM, g plant⁻¹)</td>
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<td>Control</td>
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<tr>
<td>Water-stressed</td>
<td>3.24 e</td>
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<tr>
<td>H₂O₂ (μmol g⁻¹ DW)</td>
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<tr>
<td>Control</td>
<td>40.90 d,e</td>
</tr>
<tr>
<td>Water-stressed</td>
<td>41.04 d,e</td>
</tr>
<tr>
<td>Lipid peroxidation (MDA, nmol g⁻¹ DW)</td>
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</tr>
<tr>
<td>Control</td>
<td>36.25 e</td>
</tr>
<tr>
<td>Water-stressed</td>
<td>36.59 e</td>
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<tr>
<td>Total phenol (mg gallic acid g⁻¹ FW)</td>
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</tr>
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<td>Control</td>
<td>4.26 c</td>
</tr>
<tr>
<td>Water-stressed</td>
<td>4.27 c</td>
</tr>
<tr>
<td>Lignin (mg g⁻¹ DW)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.25 c</td>
</tr>
<tr>
<td>Water-stressed</td>
<td>11.61 c</td>
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</tbody>
</table>

Activation of lignification-related enzymes in relation to the intensity of drought stress

To examine the response of peroxidases, PAL, and PPO to the intensity of drought stress, the relationships between Ψw and the activity of antioxidative enzymes were assessed. The activities of enzymes were normalized to the difference (Δ) between the values measured in drought-stressed and well-watered leaves (Fig. 3). The Δ GPOX activity was relatively low when Ψw was −1.50 MPa or more, but higher as the Ψw decreased below −1.67 MPa (Fig. 3A). The Δ APOX activity distinctly increased for the first 7 d (Ψw −1.44 MPa or more) and then declined with decreasing Ψw (Fig. 3B). As the Ψw decreased with developing water-deficit stress, the Δ CPOX and Δ SPOX increased (Fig. 3C, D). The Δ PAL activity rapidly increased when Ψw was −1.44 MPa or more (for the first 7 d in this study), but the relationship was less consistent as the Ψw decreased below this level (Fig. 3E). In response to water stress, the Δ PPO activity linearly increased until the Ψw was −1.67 MPa or more (during the first 14 d in this study) and it then remained at a similar level (Fig. 3F).

Relationships among physiological parameters and the activity of related enzymes

Linear correlations between the activity of antioxidant enzymes and H₂O₂, MDA, total phenol, lignin, and dry matter were assessed using the values measured in water deficit leaves (Table 2). For the early period of water deficit treatment (day 0 to day 14), all peroxidases examined in this study were significantly correlated with lipid peroxidation (MDA, except with APOX) and lignin. H₂O₂ concentration was significantly associated with CPOX and SPOX activities for day 0 to day 14, and with GPOX and CPOX for day 14 to day 28. The PAL activity was positively related to total phenol (P ≤ 0.05) and lignin (P ≤ 0.001) only for the early stress period.

Discussion

Drought stress was successfully induced in white clover leaves during this experiment, as the Ψw in drought-stressed leaves fell to a minimum value of −2.33 MPa at day 28 (Table 1). Biomass, H₂O₂, and MDA were not significantly affected by water deficit treatment for the first 7 d (Table 1). This indicates that the injurious symptoms of water deficit were not apparent until this period despite the significant decrease of Ψw. During the first 7 d when the Ψw was above −1.44 MPa, high activation of APOX and PAL by water deficit was clearly seen (Fig. 2B, E). This suggests that the ascorbate-glutathione pathway and phenylpropanoid metabolism were very highly induced during the early period of drought stress. It is well established that APOX has an active function in scavenging H₂O₂. These results are consistent with those of Foyer and Noctor (2003) who reported high activation of APOX and its role of scavenging H₂O₂ particularly during the early period of stress. On the other hand, phenylpropanoid metabolism was very active with high activation of PAL (Fig. 1), and total phenol content consequently increased after 7 d of water deficit treatment (Table 1). Rivero et al. (2001)
showed a highly positive relationship between PAL activity and the concentration of soluble phenolics in tomato and watermelon plants, and suggested that an accumulation of phenolic compounds in response to heat and cold stress would be attributed to the activation of the enzyme PAL. This would be beneficial to achieve acclimation and tolerance to water-deficit stress, since many kinds of plant phenolics have been considered to be the main lines of cell acclimation against stress in plants (Dixon et al., 1992; Rivero et al., 2001). Therefore, the data obtained from the mild water-stressed condition ($\Psi_w - 1.67$ MPa or more in this study) suggest that the rise of APOX and PAL activation is one of the important facets of drought tolerance which permits preservation of membrane integrity and leaf growth (Table 1).

The observed increase in H$_2$O$_2$ and MDA concentration in drought-stressed plants from 14 d of water deficit treatment (Table 1) might indicate extensive lipid peroxidation of cell membrane components caused by active oxygen species produced by the oxidative stress (Sairam et al., 2002). Significant decreases in dry mass occurred following 21 d of drought treatment ($\Psi_w - 2.27$ MPa or

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**Fig. 1.** Changes in the activities of (A) guaiacol peroxidase, GPOX; (B) ascorbate peroxidase, APOX; (C) coniferyl alcohol peroxidase, CPOX; (D) syringaldazine peroxidase, SPOX; (E) phenylalanine ammonia-lyase, PAL; and (F) polyphenol oxidase, PPO, in water-deficit stressed (filled circles) and well-watered control (open circles) leaves of white clover. Vertical bars indicate ±SE of the mean for $n=3$. 
less), which coincided with the increase in lignin concentrations (Table 1). As far as the relationship between plant growth and particular isoforms of peroxidases is concerned, the inhibition of cell growth was accompanied by the enhancement of cell wall peroxidase isoforms (Bruyant et al., 1996; YA Chen et al., 2002). Cell wall stiffening can also control cell growth through the reduction of plasticity. This may be done by the activity of ionically or covalently bound cell wall peroxidases involved in the polymerization of phenolic monomers of suberin (Quiroga et al., 2000) and lignification (Boudet, 2000). In this study, after 14 d of water deficit when the \( \Psi_w \) was below \(-1.67 \) MPa, the observed increase in GPOX, CPOX, and SPOX (Fig. 1) was significantly correlated with the increase of lignin content (Table 2). Additionally, Fig. 2C shows that BPOX is also induced after 14 d of water deficit. Lignification is catalysed by the oxidative polymerization of monolignols such as coumaryl, coniferyl, and sinapyl alcohol (Boudet, 2000) in the presence of \( \text{H}_2\text{O}_2 \). As expected, at day 28, when the lignin content increased by 16% in water deficit leaves (Table 1), some isozymes of GPOX and SPOX were much more strongly expressed (Fig. 2). Polle et al. (1994) reported that guaiacol and coniferyl alcohol POX activities were spatially correlated with lignin production in spruce needles, and suggested a co-regulation of enzymes involved in production and polymerization of monolignols. In this study, under the water-deficit stressed condition, the increased GPOX, CPOX, and SPOX activities were correlated with the lignin content (Table 2). Moreover, the negative relationship between lignin content and dry mass \((r=-0.805, P \leq 0.01; \text{data not shown})\) in water deficit leaves indicates that lignin accumulation is possibly responsible for the growth restriction caused by water-deficit stress. The isozymes of peroxidase participate in the modulation of cell wall properties during plant growth partly through catalysing the formation of covalent cross-links after oxidation of ester- and ether-bound phenolic acids and partly through the oxidative coupling of cinnamoyl alcohol moieties to generate lignin (Iiyama et al., 1994). The observed increases in peroxidase activities could reflect the modifications of mechanical properties of the cell wall, which, in turn, can be correlated with drought adaptation. A limitation of leaf growth observed from day 21 of water deficit treatment \((\Psi_w \leq 2.27 \text{ MPa or less})\) could be associated with lignin production which would be attributed to a high degree of GPOX, CPOX, and SPOX activities. The production of reactive oxygen species (ROS) in the apoplast can drive the oxidative cross-linking of cell wall components, such as hydroxyproline-rich glycoproteins. The cross-linking of structural proteins in the wall has been proposed as

![Fig. 2. Activity staining of (A) guaiacol peroxidase, GPOX; (B) syringaldazine peroxidase, SPOX; (C) benzidine peroxidase, BPOX; and (D) polyphenol oxidase, PPO, in white clover leaves. Samples were taken on the day of treatment (C, control), and 14 d and 28 d after well-watered (W) or water deficit (S) treatment.](image-url)
a mechanism to restrict cell growth (Iiyama et al., 1994) and to limit cell elongation (De Cnodder et al., 2005). The activities of lignifying peroxidase, assayed using coniferyl alcohol and syringaldazine, were stimulated in response to copper-induced oxidative stress (Jouili and El Ferjani, 2003). In the same way, the growth reduction caused by copper (EL Chen et al., 2002) and by cadmium excess (Chaoui and El Ferjani, 2005) was closely associated with the increased activity of lignifying peroxidase. Lignification decreases the cell wall plasticity and consequently reduces cell expansion. Concerning the mechanism of drought-induced lignification, it has been reported that a loss in antioxidant capacities (e.g. a decrease in APOX activation from day 14; Fig. 1B) results in an intrinsic accumulation of H₂O₂ (Table 1), which would then act as a signalling molecule triggering secondary reactions: mechanical strengthening of cell walls including lignification. The major phase of lignification, which occurred
after 21 d of water deficit treatment when the $\Psi_w$ was $\sim$2.27 MPa or less, was correlated with an increase in GPOX, CPOX, and SPOX, which is thought to sustain oxidative enzymes closely related to total phenol content, respectively, for the period day 14 to day 28 ($P \leq 0.001$). The metabolism of phenolic compounds also includes the action of oxidative enzymes such as PAL and peroxidases, which catalyse the oxidation of phenols to quinones. Some studies have reported that the activity of these enzymes increases in response to different types of stress. More specifically, both enzymes have been related to the appearance of physiological injuries (Ruiu et al., 1998; Rivero et al., 2001; Jouili and El Ferjani, 2003).

Taken together, the data obtained from this study indicate that physiological parameters and the lignification-related enzymes closely respond to the decrease in $\Psi_w$ as water-deficit stress develops. The kinetic analyses performed distinguished two distinct phases of drought stress development: (i) mild — endurance period (day 0 to day 14, $\Psi_w = -1.65$ MPa or more), characterized by high activation of APOX and PAL which have a potential role in providing antioxidative defence, as evidenced by no significant changes in $H_2O_2$ and lipid peroxidation between water deficit and control leaves; and (ii) severe — injury period ($\Psi_w = -1.67$ MPa or less), characterized by the enhanced activation of lignifying peroxidases (GPOX, CPOX, and SPOX) with a coincidental increase in lignin and lipid peroxidation leading to growth inhibition. In future investigations, it will be of interest to detect ROS accumulation using a different staining method, as recently described by De Cnodder et al. (2005). Such observations suggest that increased water deficit could induce lignification which is consequently responsible for reduction of forage biomass and digestibility in white clover leaves, where the antioxidative defence system seemingly fails to combat the oxidative damage.

Acknowledgement

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References


Table 2. Linear correlations among the descriptive parameters of physiological state and the activity of lignification-related enzymes

The values measured in the water-stressed plants were used for correlation analysis. The correlation coefficient ($r$) and significant differences are given; $n=15$.

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Period of stress</th>
<th>Dry matter</th>
<th>$H_2O_2$</th>
<th>MDA</th>
<th>Phenol</th>
<th>Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPOX d0–d14</td>
<td>$r=0.638^*$</td>
<td>$r=0.619$</td>
<td>$r=0.851^{**}$</td>
<td>$r=0.713^*$</td>
<td>$r=0.810^{**}$</td>
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<tr>
<td>d14–d28</td>
<td>$r=0.558$</td>
<td>$r=0.683^{**}$</td>
<td>$r=0.879^{***}$</td>
<td>$r=0.212$</td>
<td>$r=0.869^{**}$</td>
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<tr>
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<tr>
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<tr>
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<td>$r=0.687^*$</td>
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<td>$r=0.772^{**}$</td>
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<td>$r=0.753^*$</td>
<td>$r=0.798^{**}$</td>
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<tr>
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<td>d14–d28</td>
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</tbody>
</table>

$^*$ $P \leq 0.05$; $^{**}$ $P \leq 0.01$; $^{***}$ $P \leq 0.001$. 

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