Water stress generates an oxidative stress through the induction of a specific Cu/Zn superoxide dismutase in *Lotus corniculatus* leaves

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**Abstract**

Drought brings about different biochemical responses in plants in order to minimize its deleterious effects. Drought induces an oxidative stress in *Lotus corniculatus* leaves, measured as an increment in lipid membrane peroxidation and in situ detected superoxide. As a result, total superoxide dismutase (SOD: E.C. 1.15.1.1) activity increases after a 4-h drought, when the hydric potential has decreased to $-0.77$ MPa. Assays with specific inhibitors suggest the presence of MnSOD, Cu/ZnSOD and FeSOD activity in *L. corniculatus* plants, contribute 60, 30 and 10% of total SOD activity, respectively. The Cu/ZnSOD isoform proved to be the most responsive to drought showing a remarkable increase in its activity as that corresponds to the induction of three different isoenzymes. Expression analysis of SOD isoforms revealed an increase in Cu/ZnSOD transcripts with a maximum accumulation 4 h after drought imposition. The possible role of SOD enzymes as an antioxidant protector system under water stress conditions in *L. corniculatus* is discussed. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords**: Drought; *Lotus corniculatus*; Oxidative stress; Superoxide dismutase

1. Introduction

Although atmospheric oxygen is unreactive, it can be transformed by metabolic systems into highly reactive molecules such as singlet oxygen (‘$O_2$’), hydrogen peroxide (H$_2$O$_2$), superoxide radical (O$_2^-$) and hydroxyl radical (‘OH’). These forms are described as reactive oxygen species (ROS). Oxidative stress is used to define a cellular environment characterized by the increase of ROS [1].

Several environmental factors can generate ROS that causes oxidative injury to the plant cells. These factors include air pollutants [2], post-anoxic injury [3], freezing [4], extreme temperatures [5], high light [6] and drought [7].

Drought-induced physiological changes such as diminution of the cell hydric potential and stomatal closing result in a low CO$_2$ availability [8]. In this condition, the Mehler reaction is favored, and reduction equivalents from photosystem I are cannibalized from ferrodoxin to O$_2$ instead of NADP$^+$, resulting in the generation of O$_2^-$ [9]. Moreover, glycolate oxidase activity is induced during drought, generating H$_2$O$_2$ [10]. Anion superoxide can also be generated by ubiquinone autoxidation in the presence of oxygen, electron transport in the photosystems, and reactions with NAD(P)H in gloxisomal and peroxisomal membranes [7].

Stressful conditions such as drought associated with high light intensity is able to promote ROS production and oxidative stress [11], accelerating the senescence process [12].

The increased activity of antioxidant enzymes during stress has been reported in several organisms. In plants,
this enzymatic response could constitute an adaptive advantage in the protection from oxidative stress.

The O$_2^•−$ radical is the main source of oxidative injury in plants and several works have been carried out to establish the role of its terminator systems in relation to hydric stress tolerance [5].

The dismutation reaction catalyzed by the superoxide dismutase (SOD, E.C 1.15.1.1) maintains intracellular O$_2^•−$ within normal levels and various authors have implicated this enzyme in the protection of cells from hydric-oxidative stress [7].

Although SOD induction by different environmental stresses has been reported [13], its role as part of a putative protection mechanism in stress situations is still being discussed [5]. Functional approaches using transgenic plants have shown that plants overexpressing SOD are more tolerant to different stressful conditions [14].

The existence of three classes of SOD enzymes, each encoded by a small gene family, has probably complicated the elucidation of SOD roles in plants [15].

Studies of SOD have produced variable results due to the fact that isoforms are differentially affected by hydric stress [16]. In plants, the Cu/ZnSOD isoform—localized in the cytosol, chloroplast stroma and peroxisome—was recently found to be associated to tilacoidal membranes of spinach chloroplasts as well [5]. Although the cytosolic Cu/ZnSOD exhibits the strongest expression in plants, little is known about the cellular processes in which this enzyme is involved and its regulation [17].

Currently, the model forage legume Lotus corniculatus is widely sown in Uruguay not only as a monoculture but also in pastures with suitable companion grasses, particularly in infertile and acidic soils. While it is not as productive as clover and alfalfa, it has higher feeding values and an important advantage, it does not cause bloating in ruminants, a problem with other grasses, particularly in infertile and acidic soils. While it is not as productive as clover and alfalfa, it has higher feeding values and an important advantage, it does not cause bloating in ruminants, a problem with other legume pastures. However, *L. corniculatus* exhibits a progressive plant death that contributes to the loss of dry matter [18].

The aim of this study is to determine the incidence of fast–short drought treatment as the oxidative stress generator and the antioxidant responses mediated by SOD in *L. corniculatus* plants.

2. Materials and methods

2.1. Growth conditions, stress treatment and hydric status measurements

*L. corniculatus* cv. La Estanzuela San Gabriel (AGROSAN S. A. Montevideo, Uruguay) seeds were germinated at 28 °C for 2 days. The plants were grown in an hydroponic system [19] under controlled conditions, 16-h light:8-h dark cycle with photosynthetic photon flux density of 500 µmol/m²s, and 24/18 °C. After the plants had grown for 35 days, the flasks were randomly separated into a control group and three experimental groups receiving drought treatments for 2, 4 and 8 h. Drought was induced by withholding the nutrient solution.

Relative water content (RWC) was determined as described earlier [20]. Plant hydric potential (Ψ) was measured using a pressure chamber [21]. Hydric potential, RWC, analytical and biochemical determinations were done in pooled samples of leaves harvested after the stated time span.

2.2. Oxidative damage: lipid peroxidation and in situ superoxide detection

Lipid peroxidation was estimated using the method proposed by Iturbe-Ormaetxe et al. [22] with modifications. Lipid peroxides were extracted by grinding leaves with liquid nitrogen to a fine powder, adding 2.5 ml of 0.2 M sodium phosphate buffer, pH 7.6, 1% (v/v) TRITON X-100, 1% (w/v) butyl hydroxytoluene (in ethanol) and vortexing. The homogenate was centrifuged at 15 000 × g for 20 min at 4 °C and 0.150 ml of supernatant was mixed with 0.3 ml of 10% (w/v) trichloroacetic acid and boiled for 20 min. The mixture was cooled at room temperature and centrifuged at 12 000 × g for 2 min. The supernatant was mixed with 0.15 ml of 3% (w/v) SDS, 0.25 ml of 3% (w/v) thiobarbituric acid (in 50 mM NaOH) and 0.25 ml of 25% (v/v) HCl. The mixture was vortexed after addition of each solution, incubated at 80 °C in a water bath for 20 min and cooled on ice. The cromogen was extracted with 0.6 ml of isobutanol. Absorbance was read at 532 nm and non-specific 600 nm absorbance disregarded. Lipid peroxides were expressed as thiobarbituric acid reactive substances (TBARS) using a ε$_{532} = 156 \times 10^3$ per M cm [23]. Six to eight replicates of each sample were assayed.

Anion superoxide in situ localization was performed by treating leaves with nitroblue tetrazolium (NBT) following earlier described methods [24].

2.3. SOD activity and isoform identification

SOD enzymes were extracted as described earlier [25]. Tissues were ground using a pre-chilled mortar and pestle. The extraction buffer consisted of 50 mM potassium phosphate pH 7.8, 0.5 mM EDTA, 7.2 mM β-mercaptoethanol, 0.2% (v/v) TRITON X-100, 0.1% (w/v) bovine serum albumin and 0.1% (w/v) ascorbic acid. The ratio of plant tissue:extraction buffer was 1:1 (w/v). The homogenate was centrifuged at 10 000 × g
for 30 min at 4 °C and the supernatant was used for enzymatic assays and gel activity visualization. SOD activity was determined by the NBT reduction method [26].

In order to identify the different isoforms, either 10 μl of 100 mM KCN (inhibitor of the Cu/ZnSOD) or 25 μl of 80 mM H₂O₂ (inhibitor of the Cu/ZnSOD and FeSOD) were added to the incubation buffer [27].

One enzymatic unit (U) was defined as the volume of extract yielding a 50% inhibition and activity was expressed as U per mg of protein.

Detection of the different SOD isoforms was performed in 12% non-denaturing polyacrylamide gels with a 5% stacking gel. Forty micrograms of protein from original extracts used for SOD activity determination were loaded per well. Activity was detected in the gels following an earlier described method [28].

Protein in crude extracts was quantified by the Bradford method [29].

2.4. RNA isolation and analysis

Total RNA was extracted according to Botella et al. [30] with the following modifications, addition of 50 mM aurinic acid (freshly added) and an increase of the SDS concentration to 1% (w/v) in the extraction buffer. Nucleic acids were recovered by incubating at −20 °C 1 h in isopropanol. The pellet was resuspended in 0.5 ml of buffer containing 10 mM Tris pH 8.0, 1 mM EDTA and 1% (w/v) N-lauroyl sarcosine. Polysaccharides were removed from the RNA preparation according to a earlier described protocol [31].

Total RNA was fractionated on a 1.5% agarose gel containing 2.2 M formaldehyde. After electrophoresis, RNA was transferred to a nylon membrane (Hybond-N from Amersham) by capillary blotting with 20 X SSC overnight [32]. RNA was fixed by heating 2 h at 80 °C.

Filters were prehybridized at 42 °C in a solution containing 50 mM phosphate buffer pH 7.0, 50% (v/v) deionized formamide, 0.8 M NaCl, 5 mM EDTA, 10 X Denhardt’s solution, 0.1% (w/v) SDS, 100 μg/ml denatured fragmented salmon sperm DNA and 5% (w/v) dextran. Hybridization was performed overnight at 42 °C using 7 × 10⁶ cpm/ml of a SOD probe labeled with 32P-dCTP by the random priming method following the kit manufacturer instructions (BIORAD).

The probe consisted of a DNA fragment of the cytosolic Cu/ZnSOD (SOD3) obtained from N. plumangifolia and cloned in pUC18 [33]. Hybridized filters were washed at 42 °C in 2 X SSC, 0.1% SDS for 20 min, with a second incubation with 1 X SSC, 0.1% SDS during 30 min at 50 °C. Dried filters were exposed using Kodak X-OmatAR X-ray film for 2–5 days at −80 °C.

3. Results

3.1. Water stress induces oxidative damage in L. corniculatus

Drought treatment induced by withholding the nutrient solution from plants which had been grown hydroponically for 35 days, caused a drop in water content from 83.3 to 74.6% after 4 h and to 68.9% after 8 h, corresponding to a 10 and 17% reduction, respectively (Table 1). Water loss resulted in a reduction of Ψ by −0.40 MPa after 4 h and −0.26 MPa after 8 h of stress (Table 1). Moreover, a −0.26 MPa reduction was registered in Ψ after the initial 2 h of stress even though symptoms of water stress were not visible at this stage.

A distinct oxidative stress, as measured by lipid peroxidation, was detected at 4 h and was even more marked by 8 h after the onset of drought (Table 1). TBARS quantified in extracts from leaves subjected to a 2 h stress treatment amounted to 32 nmol/g DW (Table 1). Although the TBARS increased by another 57 nmol/g DW over the next 2 h of stress, the subsequent rise during the last 4 h of the 8-h time span slowed to only 42 nmol/g DW, indicating that the main impact of water stress occurred between 2 and 4 h after the treatment began. The loss of water after 8 h of hydric stress generated the most evident oxidative damage registered during the experiment, as measured by TBARS (Table 1).

These results could be correlated to the rise in superoxide radical content indirectly detected in leaves as water stress progressed. Although nitroformazan salt was observed in isolated areas of control leaves, it was detected in larger areas of leaves with stress progression. Furthermore, cell morphology was altered and the precipitate took up the entire foliole after an 8-h treatment (data not show).
Table 2

<table>
<thead>
<tr>
<th>Duration of dehydration (h)</th>
<th>Total SOD (U per mg protein)</th>
<th>MnSOD (%)</th>
<th>FeSOD (%)</th>
<th>Cu/ZnSOD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.98 ± 0.11</td>
<td>65</td>
<td>9</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>2.21 ± 0.18</td>
<td>53</td>
<td>9</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>2.32 ± 0.20</td>
<td>55</td>
<td>9</td>
<td>37</td>
</tr>
<tr>
<td>8</td>
<td>2.01 ± 0.15</td>
<td>58</td>
<td>7</td>
<td>35</td>
</tr>
</tbody>
</table>

3.2. SOD activity and hydric stress

The implemented drought treatments did not alter significantly total SOD activity in *L. corniculatus* leaves. However, the use of specific inhibitors allowed analysis of the activity of different SOD isoforms separately (Table 2).

Analysis of SOD activity of the different SOD isoforms shows that MnSOD was responsible for about 60% of total SOD activity, with a slight drop observed with the progression of drought. FeSOD contribution to total SOD activity never exceeded 10% and was not affected by the implemented drought treatments. On the other hand, the Cu/ZnSOD activity, which accounted for 30% of total SOD activity, increased when plants were subjected to a 4 h drought (Table 2).

Samples from the same crude extract used to determine SOD activity were subjected to native gel electrophoresis incubated with specific inhibitors (Fig. 1). A band of low electrophoretic mobility band was observed, which corresponded to MnSOD, due to its lack of inhibition by either H₂O₂ or KCN. Although this was the band with the highest intensity, it was not possible to detect any drought-induced intensity variation by gel visualization (Fig. 1).

A band of lower electrophoretic mobility was consistently observed during drought and disappeared when H₂O₂ was used as a specific inhibitor, suggesting FeSOD. The low intensity of this band was in accordance with the minor contribution of this isoform to total SOD activity (Table 2, Fig. 1).

Gel incubation in the absence of specific inhibitors revealed the three additional bands of higher electrophoretic mobility. The fact that these bands were not detected when H₂O₂ or KCN were used as inhibitors was indicative of the Cu/ZnSOD isoform (Fig. 1).

The Cu/ZnSOD band of less intensity and lowest mobility was detected after the 4 h hydric stress treatment and was named Cu/ZnSOD1 (Fig. 1). The highest mobility band in this subset was always present, although its intensity was lower in the control experiment (Fig. 1). This isoenzyme was named Cu/ZnSOD3.

The isoenzyme designated Cu/ZnSOD2, with an electrophoretic mobility between that of Cu/ZnSOD1 and Cu/ZnSOD3 but closer to the latter, was evident in plants subjected to a 2 h drought (Fig. 1). According to the isoform patterns observed for each treatment, the increase in Cu/ZnSOD activity in stressed plants (Table 1) could be attributed to isoforms of Cu/ZnSOD not present in control plants (Fig. 1).

3.3. Cu/ZnSOD mRNA analysis

In order to determine if the up-regulation of Cu/ZnSOD mRNAs level was involved in the drought-induced accumulation of Cu/ZnSOD proteins, a heterologous probe obtained from *N. plumbaginifolia* was used. Northern blot analysis of Cu/ZnSOD mRNA produced a hybridization signal in the plant subjected to a 2 h drought. Transcript accumulation reached its maximum level 4 h after the drought treatment began. The inductive response was transient as the Cu/ZnSOD mRNA levels declined 8 h after the onset of treatment (Fig. 2).
The signal from Northern blot analysis using FeSOD and MnSOD cDNA probes after low stringency washes was undetectable (data not shown).

4. Discussion

Low cellular hydric potential induces ROS, with \( \text{O}_2^{•−} \) and \( \text{H}_2\text{O}_2 \) radicals being the most abundant in plants under conditions of stress [5]. During drought, ROS are reportedly involved in lipid peroxidation, which results in membrane injury [7]. Despite the existence of cellular antioxidant systems, which account for the reduction of \( \text{O}_2^{•−} \) and \( \text{H}_2\text{O}_2 \) levels, membrane lipids are nonetheless damaged, probably due to 'OH generation [34]. The 8-h drought treatment applied to \( L. \ corniculatus \) plants resulted in a −0.46 MPa decrease in hydric potential. This water loss was enough to induce membrane lipid peroxidation in leaves. The TBARS were evident after 4 h of drought and was significantly different after 8 h of drought treatment (Table 1).

The results presented in this study clearly show that a diminution in leaf hydric potential during a 4 h time span generates an oxidative injury, likely due to 'OH reaction with biological membranes [35]. However, the \( \text{O}_2^{•−} \) radical, which is less reactive than 'OH, has been implicated as the most important factor in the generation of oxidative stress in plants [9]. We have indirectly detected an increase in the superoxide anion content, most probably related to drought progression. These results are similar to those reported for other plant species, using different techniques to detect the superoxide radical ([7] and references therein). Several works have been carried out in order to establish the role of the antioxidant system for this radical and its connection to hydraulic stress tolerance [5]. Thus, SOD enzymes play a crucial role in maintaining \( \text{O}_2^{•−} \) at sublethal levels. Like most plants, \( L. \ corniculatus \) possesses three SOD isoforms, the MnSOD, Cu/ZnSOD and FeSOD. Scandalios [5] proposes that the FeSOD isoform could be restricted to some plant species. This report demonstrates the existence of the FeSOD isoform in \( L. \ corniculatus \).

The absence of signal hybridization in the Northern analysis despite the presence of protein activity could be explained because FeSOD is not highly homologous among different plant species and so may not hybridize to the FeSOD message in \( L. \ corniculatus \) [16].

The chloroplastic location of FeSOD is related to the protection from oxidative stress provided by this organelle [28]. Studies of FeSOD activity under stress conditions have obtained variable results. Some authors have detected an induction of this isoform [36], while other studies have reported either an inhibition of its activity [33] or no change in activity [22]. The fact that \( L. \ corniculatus \) FeSOD activity remained unchanged following an 8-h drought treatment could be ascribed to a rapid stress establishment inducing a quick and dramatic injury of the chloroplasts. Alternatively, additional enzymatic antioxidant systems such as the chloroplastic Cu/ZnSOD described by other authors [37] or non enzymatic systems such as ascorbic acid or tocopherol, could contribute to \( \text{O}_2^{•−} \) elimination in the chloroplast [38].

The MnSOD activity—also detected in plant tissues—is, in most plant species, not superior to the combined activities of FeSOD and Cu/ZnSOD [16]. Similar results to those obtained in \( L. \ corniculatus \) plants have been found in pea [22], wherein MnSOD activity amounted to 50% of total SOD activity. High activities of the MnSOD isoforms could be related to \( \text{O}_2^{•−} \) generation by the mitochondrial electron transport chain. The induction of this isoform by water stress has only been reported for some plants [5]. Similarly to what has been reported for other plants with a high MnSOD activity, no drought-induced rise in \( L. \ corniculatus \) MnSOD was observed. Instead, a slight decrease in activity was observed after an 8-h drought [22].

Zhu and Scandalios [39] report the existence of MnSOD isoenzymes with a differential response to a wide range of stressful stimuli. Some of the isoenzymes responded to osmotic stress others did not, so the fact that MnSOD is not induced by hydric stress in \( L. \ corniculatus \) does not exclude the possibility of it being induced by different conditions of stress.

In plants, the isoform with the highest induction level in response to different stressful stimuli is the Cu/ZnSOD [10,11,32,40]. This isoform—found both in the cytosol and the chloroplast—is likely to be induced by metabolic alterations not directly connected with a hydric deficit [5].

An induction of Cu/ZnSOD activity in \( L. \ corniculatus \) plants subjected to a 2 h drought was observed. Maximum activity was reached between 4 and 8 h of nutrient solution deprivation. Northern-blot analysis of Cu/ZnSOD mRNA revealed transcript accumulation after 4 h of stress, possibly corresponding to the increment in Cu/ZnSOD activity registered at this time point.

Results presented in Figs. 1 and 2 suggest that the Cu/ZnSOD mRNA detected by Northern blot could be related to the accumulation of the Cu/ZnSOD2 protein, which contributes to the increase in total Cu/ZnSOD activity in \( L. \ corniculatus \). Rubber trees [42] and tomato plants [41] display a similar behavior regarding Cu/ZnSOD protein and mRNA accumulation. However, further studies must be carried out to confirm the relationship between both phenomena.

Studies with pea plants subjected to different water stress treatments have shown that the response of an-
tioxidants to water deficit depends on the severity of stress, the species and age of plants ([22] and references therein). This is the first report indicative of the involvement of Cu/ZnSOD activity in *L. corniculatus* response to water stress.

However, as the subcellular localization of Cu/ZnSOD was not addressed by the present work, it was not possible to draw conclusions about the cellular compartments which are more affected by the induced water stress in this legume species.

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