Antioxidant activities in mycorrhizal soybean plants under drought stress and their possible relationship to the process of nodule senescence

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Summary

• The mechanisms by which the mycorrhizal symbiosis protects soybean (*Glycine max*) plants against premature nodule senescence induced by drought stress is investigated here by evaluating the activity of a set of antioxidant enzymes in relation to nodule senescence.

• Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activity was determined in well watered or drought-stressed soybean plants inoculated with *Bradyrrhizobium japonicum* alone or in combination with *Glomus mosseae*.

• In roots, only GR activity was higher in mycorrhizal than in non-mycorrhizal plants. The other antioxidant activities were similar, or lower (APX), in droughted, mycorrhizal plants than in the corresponding nonmycorrhizal ones. Similarly, in nodules, SOD, CAT and APX activities were lower in droughted, mycorrhizal plants than in nonmycorrhizal plants whereas, again, GR activity was higher in nodules from mycorrhizal plants.

• We propose that the consistently higher GR activity in roots and nodules of mycorrhizal plants might have contributed to decreased oxidative damage to biomolecules, which are involved in premature nodule senescence. Additional drought-avoidance mechanisms induced by the AM symbiosis might also contribute to the lower oxidative stress in mycorrhizal plants.

Key words: antioxidant, arbuscular mycorrhizal symbiosis, drought, nodule senescence.

Introduction

The role of the arbuscular mycorrhizal (AM) symbiosis in the alleviation of drought-induced nodule senescence in soybean plants has been recently investigated (Ruiz-Lozano *et al.*, 2001). We observed that, under drought conditions, inoculation of soybean plants with the AM fungus *Glomus mosseae* enhanced nodule d. wt and increased its leghemoglobin and protein contents as well as the nodule activity (measured as acetylene reductase activity, ARA). Thus, we demonstrated that AM symbiosis alleviates drought-induced nodule senescence in legume plants.

The process of nodule senescence has been correlated with a marked decline in the major activities involved in removal of reactive oxygen species (ROS) (Evans *et al.*, 1999; Becana *et al.*, 2000). This is important because oxidative damage to biomolecules has been proposed as one of the most important mechanisms triggering nodule senescence in stressed nodules (Gogorcena *et al.*, 1995, 1997; Escuredo *et al.*, 1996; Becana *et al.*, 2000). Legume nodules have a high capacity to produce ROS, even though the concentration of free O_2 in the central zone is only 5–60 nM (Hunt & Layzell, 1993). The high concentration of oxygen-labile proteins, leghemoglobin and catalytic Fe in the nodules and the tendency of the oxygenated form of leghemoglobin to autoxidize are conductive to the production of ROS in the nodule cytosol (Dalton, 1995). This, in turn, can damage biomolecules such as lipids and proteins, thereby contributing to nodule senescence (Escuredo *et al.*, 1996).

The most remarkable result found in our previous study concerned the measurement of oxidative damage to biomolecules. Drought considerably enhanced oxidative damage to lipids and proteins in nodules of nonmycorrhizal plants whereas mycorrhizal treatments were protected against oxidative damage. Therefore, we concluded that the alleviation of oxidative damage in nodules of AM plants could be an important mechanism involved in the protective effects of the AM symbiosis against premature nodule senescence (Ruiz-Lozano *et al.*, 2001).

At this stage of the research two possibilities can be envisaged to explain the low oxidative damage found in nodules of mycorrhizal plants. Either mycorrhizal plants suffered less drought stress due to a primary drought-avoidance effect by the symbiosis (e.g. by direct water uptake by fungal hyphae from sources inaccessible to nonmycorrhizal plants and transfer to the host plant) and that kept plants protected against the generation of ROS, or mycorrhizal plants increased the activities of a set of enzymes involved in the elimination of active oxygen species. Plant cells contain an array of protective and repair systems that minimize the occurrence of oxidative damage. According to Smirnoff (1993), these can be divided into two categories: systems that react with active forms of oxygen and keep them at a low level (i.e. superoxide dismutases (SODs), catalase (CAT), or peroxidases), and systems that regenerate oxidized antioxidants (glutathione (GSH), glutathione reductase (GR), ascorbate and mono- and dehydroascorbate reductases). The first group of enzymes are involved in the detoxification of O_2^- radicals and H_2O_2, thereby preventing the formation of OH radicals. The GR, as well as the GSH, are important components of the ascorbate-glutathione pathway responsible for the removal of H_2O_2 in different cellular compartments (Dalton, 1995; Jiménez *et al.*, 1997).

In this study we analyzed the activity of representatives of the two enzymatic categories proposed by Smirnoff (1993) in root and nodule tissues. The aim was to get some clues on the mechanisms by which the mycorrhizal symbiosis protects legume plants against the premature nodule senescence induced by drought stress.

Materials and Methods

Experimental design and statistical analysis

The experiment consisted of a randomized complete block design with two inoculant treatments: plants inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, strain USDA 110 (Br); plants inoculated with the mycorrhizal fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm + Br). Twelve replicates of each treatment were done totalling 24 pots (one plant per pot) so that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 10 d before harvest.

Data were subjected to ANOVA with microbial treatment, water supply and microbial treatment–water supply interaction as sources of variation, and followed by Duncan's multiple range test (Duncan, 1955). Percentage values were arcsin transformed before statistical analysis.

Soil and biological materials

Loamy soil was collected from the Zaidin Experimental Station (Granada, Spain), sieved (2 mm), diluted with quartz-sand (< 1 mm) (1 : 1, soil : sand, v/v) and sterilized by steaming (100°C for 1 h for 3 d). The soil had a pH of 8.1 (water); 1.81% organic matter, nutrient concentrations (mg kg^{-1}): N, 2.5; P, 6.2 ($NaHCO_3$-extractable P); K, 132.0. The soil texture was made up of 35.8% sand, 43.6% silt and 20.5% clay.

Soybean (*Glycine max* L. cv Williams) seeds were sterilized in a 15% H_2O_2 solution for 8 min, then washed several times with sterile water to remove any trace of chemical that could interfere in seed germination, and placed on sterile vermiculite at 25°C to germinate. Seedlings (3-d-old) were transferred to plastic pots containing 600 g of the sterilized soil/sand mixture. A suspension (2 ml $seed^{-1}$) of the diazotrophic bacterium *Bradyrhizobium japonicum*, strain USDA 110 (10^9 cell ml^{-1}), was sprinkled over the seedling at the time of planting.

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia and infected root fragments. The AM species was *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, isolate BEG 122. Ten grams of inoculum were added to Gm + Br pots at sowing time just below soybean seedlings.

Growth conditions

Plants were grown in a controlled environmental chamber with 70–80% RH, day/night temperatures of 25/15°C, and a photoperiod of 16 h at a photosynthetic photon flux density (PPFD) of 460–500 µmol m^{-2} s^{-1} (Li-Cor, Lincoln, NE, USA; model LI-188B).

Soil moisture was measured with a ML2 ThetaProbe (AT Delta-T Devices Ltd, Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil. Volumetric soil water content is the ratio between the volume of water present and the total volume of the soil sample. It is a dimensionless parameter, expressed either as a percentage (% vol) or as a ratio (m^3 m^{-3}). Water was supplied daily to maintain constant soil water content close to field capacity (17% volumetric soil moisture) during the first 5 wk of plant growth. At this time half of the plants were allowed to dry until soil water content reached 80% field capacity (12% volumetric soil moisture) and maintained under such conditions for 10 d. In order to

control the level of water stress, the pot water content was daily measured with the ThetaProbe ML2 (at the end of the afternoon) and the amount of water lost was added to each pot in order to maintain soil water content at the desired level.

Each week throughout the experiment, plants received 10 ml of Hewitt's nutrient solution lacking N and P (Hewitt, 1952). 3 wk after planting, Br plants received nutrient solution amended with 0.35 mM K_2HPO_4 (Goicoechea *et al.*, 1997). That P concentration was chosen in an attempt to obtain well-watered plants of similar size and P content in both microbial treatments.

Parameters measured

Biomass production At harvest (45 d after planting), the root system was separated from the soil and nodule f. wt determined. Shoot d. wt was measured after drying in a forced draught oven at 70°C for 2 d.

Symbiotic development The percentage of mycorrhizal root infection was estimated by visual observation of fungal colonization after clearing washed roots in 18% KOH and staining with 0.05% trypan blue in lactophenol (v/v), according to Phillips & Hayman (1970). Parameters of mycorrhizal colonization were determined according to Trouvelot *et al.* (1986). The colonization frequency (F%) is a ratio between colonized root fragments and total number of root fragments observed. It gives an estimation of the root length colonized by the fungus. The colonization intensity (M%) is an estimation of the amount of root cortex which became mycorrhiza. Finally, the arbuscule abundance a% gives an estimation of the arbuscule richness in the mycorrhizal root fraction. Four replicates per treatment were used.

Nodule activity Nitrogenase activity was estimated by the C_2H_2 reduction technique (Hardy *et al.*, 1973). Although ARA measured in closed vessels does not represent the true nitrogenase activity (Minchin *et al.*, 1983), it can be appropriate in assays for comparative purposes (Irigoyen *et al.*, 1992). Intact nodulated roots were enclosed in a 300-ml glass flask and 15 ml of C_2H_2 were added. The flask was incubated at room temperature for 15 min. Samples of 500 µl were withdrawn from the flask and the ethylene content was quantified with a Hewlett Packard model 5890 gas chromatograph equipped with a Poropak-R column and a hydrogen flame ionization detector.

Hydrogen peroxide concentration and oxidative damage to lipids For determination of hydrogen peroxide concentration in nodules, aliquots of nodules were homogeneized with an ice-cold potter in HCl 25 mM and filtered through four layers of nylon cloth. The supernatants were adjusted to pH 7.0 for subsequent H_2O_2 quantification, which was performed by the 4-aminoantipyrine method (Frew *et al.*, 1983).

Lipid peroxides from roots and nodules were extracted by grinding 0.5 g or 0.25 g, respectively, with and ice-cold potter and 6 ml of 100 mM potassium phosphate buffer (pH 7.4) as described previously (Ruiz-Lozano *et al.*, 2001). Lipid peroxidation was estimated as the content of 2-thiobarbituric acid-reactive substances (TBARS) and expressed as equivalents of malondialdehyde (MDA) according to Halliwell & Gutteridge (1989).

Preparation of root and nodule extracts Enzymes were extracted at 0–4°C from 1 g (f. wt) root tissues using a mortar and pestle with 50 mg polyvinylpolypyrrolidone (PVPP) and 10 ml of the following optimized medium: 50 mM K-phosphate buffer pH 7.8 containing 0.1 mM EDTA for SOD, CAT and ascorbate peroxidase (APX) (Gogorcena *et al.*, 1995). The same medium supplied with 10 mM β-mercaptoethanol was used for glutathione reductase (Moran *et al.*, 1994). For nodules, enzymes were extracted from 0.3 g (f. wt) aliquots in 5 ml of the above-mentioned media using an ice-cold potter. Extracts were filtered through four layers of nylon cloth and centrifuged at 20 000 g, 20 min, 0–4°C. The supernatants were kept at −70°C for subsequent enzymatic assays.

Soluble protein was determined by the dye binding microassay (Bio-Rad) using BSA as the standard.

Enzyme assays Total SOD activity (EC 1.15.1.1) was measured according to Beyer & Fridovich (1987) based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25°C. CAT activity (EC 1.16.1.6) was measured by the disappearance of H_2O_2 (Aebi, 1984). The reaction mixture (3 ml) contained 10.6 mM H_2O_2. The reaction was initiated by adding 25 µl of the extract and monitoring the change in absorbance at 240 nm and 25°C for 3 min APX activity (EC 1.11.1.11) was measured in a 1-mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM hydrogen peroxide and 0.5 mM ascorbate. Adding the H_2O_2 started the reaction and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate of ascorbate (Amako *et al.*, 1994). Finally, GR activity (EC 1.6.4.2) was determined by the procedure of Carlberg & Mannervik (1985). The reaction mixture (1 ml) contained 0.1 M HEPES pH 7.8, 1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM oxidized glutathione, 0.2 mM NADPH and 150 µl of the enzyme extract. The rate of NADPH oxidation was monitored by the decrease in absorbance at 340 nm for 2 min. Two blanks, one without the enzyme extract and the other without oxidized glutathione were used as controls.

Treatment	Shoot d. wt	Nodule f. wt	Mycorrhizal infection		
			F	M	a
Br WW	2.40b	0.35c	0b	0b	0b
Br DS	2.00c	0.18d	0b	0b	0b
Br + Gm WW	2.90a	0.75a	95a	75a	81a
Br + Gm DS	2.30b	0.50b	94a	68a	88a

Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test ($n = 5$).

Table 1 Shoot d. wt (mg plant^{-1}), nodule f. wt (g plant^{-1}) and mycorrhizal root infection (F%, M%, and a%) in nonmycorrhizal (Br) or mycorrhizal (Br + Gm) soybean plants grown under well-watered (WW) or drought stress (DS) conditions

Results

Plant biomass and symbiotic development

Shoot d. wt was higher in the mycorrhizal treatments (well-watered and drought stressed) than in the corresponding nonmycorrhizal controls (Table 1). Drought stress decreased shoot d. wt in all plants, but stressed mycorrhizal plants had 15% more shoot d. wt than stressed nonmycorrhizal treatment.

The nodule f. wt was always higher in mycorrhizal than in nonmycorrhizal plants, regardless of the water treatment (Table 1).

No mycorrhizal colonization was observed in plants not provided with AM inoculum. The values of F, M and a were similar in both mycorrhizal treatments (unaffected by drought stress).

Nodule functioning and protein content

Nitrogenase activity was measured by the ARA test in total roots. ARA activity was higher in Gm + Br than in Br plants (Fig. 1). This increase was considerably more evident under drought stress conditions (240% increase).

Nodules of mycorrhizal plants cultivated under well watered conditions had enhanced protein content as compared to the nonmycorrhizal plants (Fig. 1). Drought decreased the protein content in nodules from the mycorrhizal plants, but it was similar to that of nodules from nonmycorrhizal plants.

Hydrogen peroxide accumulation and oxidative damage to lipids

No significant differences among the different treatments were found in hydrogen peroxide concentration in nodules (Fig. 2).

In roots, the oxidative damage to lipids increased as consequence of drought (Fig. 3). The increase was higher in nonmycorrhizal plants (by 42%) than in the nonmycorrhizal ones (by 15%). The oxidative damage to lipids was also higher in nodules from nonmycorrhizal plants than in those from mycorrhizal plants and that happened both under well-watered and under drought stress conditions. However, the differences in lipid peroxidation between nodules of mycorrhizal and nonmycorrhizal plants were higher under drought

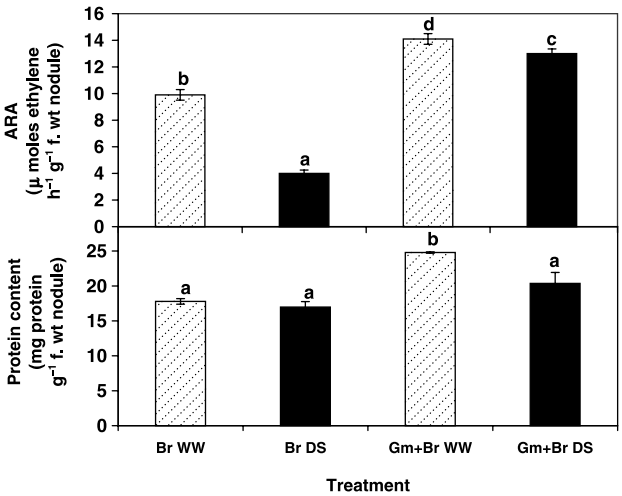

Fig. 1 Acetylene reductase activity (ARA) (μmol ethylene h^{-1} g^{-1} f. wt nodule) and protein content (mg g f. wt^{-1} nodule) in nodules of soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm + Br, *Glomus mosseae* plus *B. japonicum*.

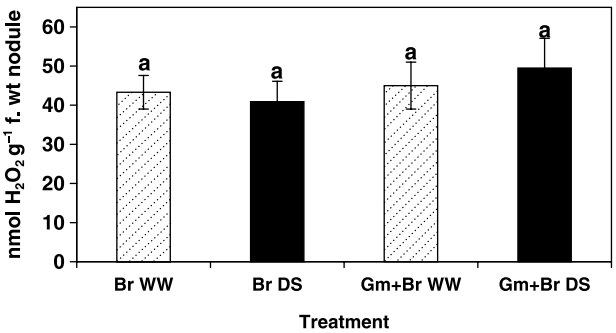

Fig. 2 Hydrogen peroxide concentration (nmol H_2O_2 g^{-1} f. wt nodule) in nodules of soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm + Br, *Glomus mosseae* plus *B. japonicum*.

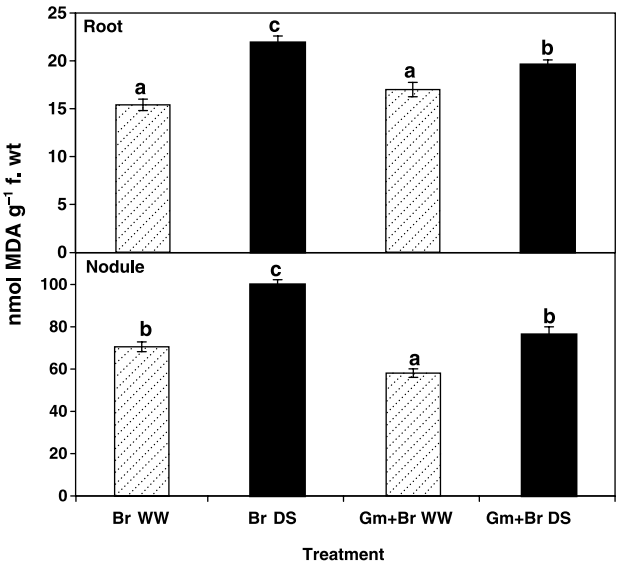

Fig. 3 Oxidative damage to lipids (nmol MDA g^{-1} f. wt) in roots and nodules of soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm + Br, *Glomus mosseae* plus *B. japonicum*.

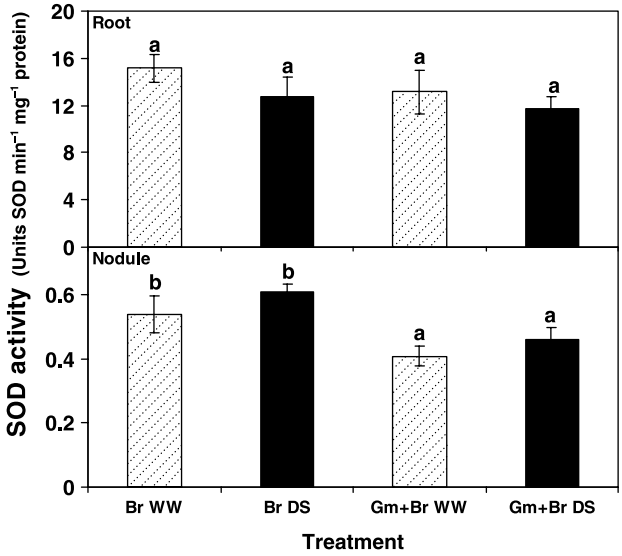

Fig. 4 Superoxide dismutase (SOD) activity (units min^{-1} mg^{-1} protein) in roots and nodules of soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm + Br, *Glomus mosseae* plus *B. japonicum*.

stress conditions (31% more lipid peroxidation in nonmycorrhizal plants) than under well-watered conditions (21% more lipid peroxidation in nonmycorrhizal plants).

Antioxidant activities

The specific SOD activity in roots was similar in all treatments. By contrast, in nodules, SOD activity was higher in non-mycorrhizal than in mycorrhizal plants (Fig. 4).

CAT activity in roots was 60% higher in mycorrhizal than in nonmycorrhizal plants under well-watered conditions (Fig. 5). Nonmycorrhizal plants subjected to drought showed the highest CAT activity in nodules.

APX in roots was enhanced by drought stress both in mycorrhizal and in non-mycorrhizal plants (Fig. 6). The highest APX activity was found in droughted nonmycorrhizal plants, which showed an increase of 80% relative to the droughted mycorrhizal plants. In nodules, however, the highest APX activity was found in well-watered nonmycorrhizal plants. Drought decreased APX activity by 23% in such treatment. The lowest APX activity was found in nodules from well-watered mycorrhizal plants although drought increased the APX activity in AM plants to a level similar to that of droughted nonmycorrhizal plants.

The most significant result was obtained regarding GR activity (Fig. 7). In roots, mycorrhizal plants had higher GR activity than nonmycorrhizal ones (over 350% increase). Drought had no significant effect on GR activity of roots of either treatment. In nodules, again the mycorrhizal treatment had more GR activity than the nonmycorrhizal ones. The

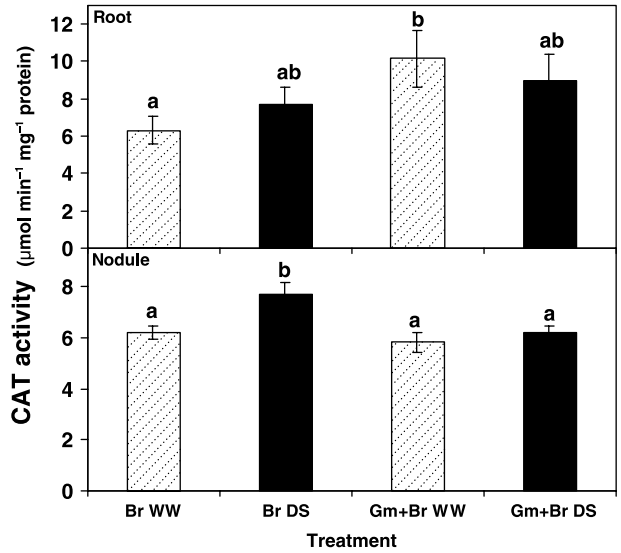

Fig. 5 Catalase (CAT) activity (μmol min^{-1} mg^{-1} protein) in roots and nodules of soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm + Br, *Glomus mosseae* plus *B. japonicum*.

difference was more evident under drought stress conditions, where the increase in GR activity reached 534%.

Water consumption

The daily water consumption was measured with the Thetaprobe ML2x by determining the volumetric soil

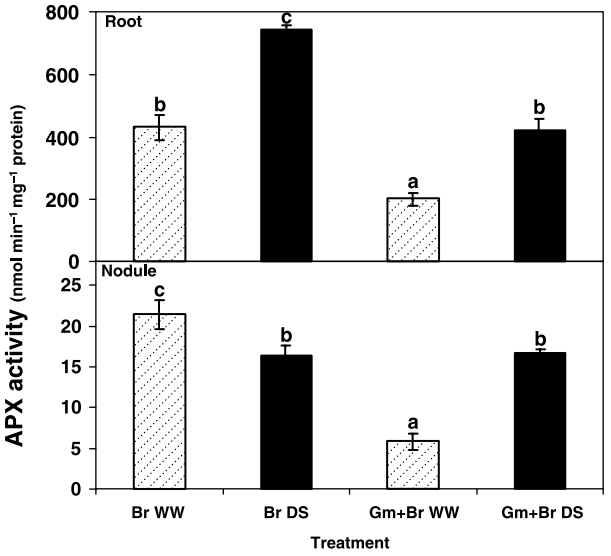

Fig. 6 Ascorbate peroxidase (APX) activity (nmol min^{-1} mg^{-1} protein) in roots and nodules of soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm + Br, *Glomus mosseae* plus *B. japonicum*.

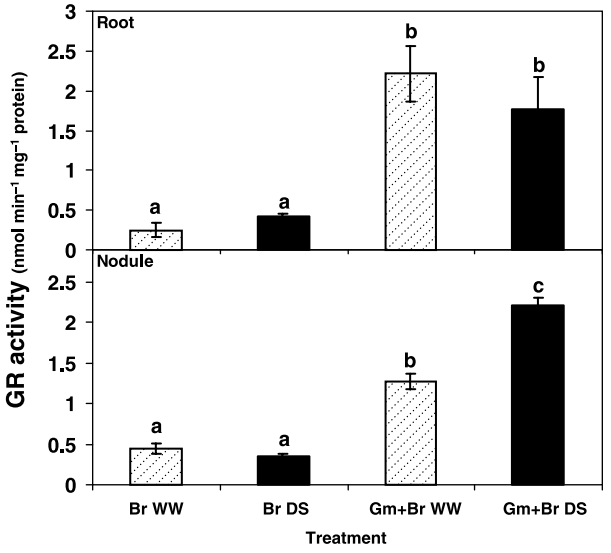

Fig. 7 Glutathione reductase (GR) activity (nmol min^{-1} mg^{-1} protein) in roots and nodules of soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm + Br, *Glomus mosseae* plus *B. japonicum*.

moisture in each pot. Since pots were daily watered to reach 17% of volumetric soil moisture (well-watered treatments) or 12% of volumetric soil moisture (drought stressed treatments), the reading of the ThetaProbe ML2x before watering was used to estimate water consumption per plant and per day. Under well-watered conditions, no differences in soil moisture were observed between mycorrhizal and

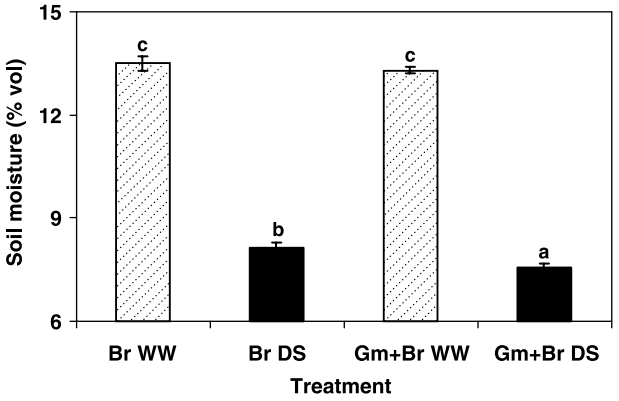

Fig. 8 Volumetric soil moisture (% vol) before watering in pots containing soybean plants cultivated under well-watered (WW) or drought-stress (DS) conditions. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm + Br, *Glomus mosseae* plus *B. japonicum*.

nonmycorrhizal plants (Fig. 8). By contrast, under drought stress conditions mycorrhizal plants depleted more the soil water content (average volumetric soil moisture of 7.55%) than nonmycorrhizal plants (average volumetric soil moisture of 8.15%). Such difference in volumetric soil moisture is equivalent to 3 ml pot^{-1} day^{-1} of extra water consumption in mycorrhizal plants.

Discussion

Many of the degenerative reactions associated with several biotic, abiotic and xenobiotic stresses are mediated by ROS. The term ROS is generic, embracing not only free radicals such as superoxide and hydroxyl radicals, but also H_2O_2 and singlet oxygen. While it is generally assumed that the hydroxyl radical and singlet oxygen are so reactive that their production must be minimized (Jakob & Heber, 1996), $O_2^{\cdot -}$ and H_2O_2 are synthesized at very high rates even under optimal conditions (Noctor & Foyer, 1998). The chief toxicity of $O_2^{\cdot -}$ and H_2O_2 is thought to reside in their ability to initiate cascade reactions that result in the production of the hydroxyl radicals. These radicals (and their derivatives) are among the most reactive species known to chemistry, capable of reacting indiscriminately to cause oxidative damage to biomolecules such as lipid peroxidation, denaturation of proteins and mutation of DNA (Halliwell & Gutteridge, 1989; Bowler *et al.*, 1992).

We concluded in our previous study that alleviation of oxidative damage to biomolecules in nodules might be involved in the protective effect of AM symbiosis against nodule senescence (Ruiz-Lozano *et al.*, 2001). In this study we have evidenced again a significantly lower oxidative damage to lipids in roots and nodules of droughted mycorrhizal plants than in nonmycorrhizal plants. Hence, in this investigation we analyzed in root and nodule tissues the activities of four enzymes well documented as involved in ROS scavenging.

The efficient destruction of $O_2^{\cdot -}$ and H_2O_2 requires the action of several antioxidant enzymes acting in synchrony. Superoxide is rapidly converted to H_2O_2 by the action of SOD (Bowler *et al.*, 1992). However, dismutation of $O_2^{\cdot -}$ simply serves to convert one destructive ROS to another. Since H_2O_2 is a strong oxidant that rapidly oxidizes thiol groups, it cannot be allowed to accumulate to excess (Noctor & Foyer, 1998). CATs convert H_2O_2 to water and molecular oxygen in peroxisomes. These enzymes have extremely high maximum catalytic rates but low substrate affinities (Willekens *et al.*, 1995). Furthermore, the absence of CAT in the chloroplast precludes a role in protection of the thiol-regulated enzymes of the Calvin cycle. An alternative mode of H_2O_2 destruction is via peroxidases, which are found throughout the cell and which have a much higher affinity for H_2O_2 than CAT (Jiménez *et al.*, 1997). Plants contain high activities for the enzymes of the ascorbate-glutathione cycle in which H_2O_2 is scavenged. In the first step of this pathway, APX, which is the most important peroxidase in H_2O_2 detoxification (Noctor & Foyer, 1998), catalyzes the reduction of H_2O_2 to water by ascorbate, and the resulting monodehydroascorbate and dehydroascorbate are reduced back to ascorbate by monodehydroascorbate reductase (MR) and by dehydroascorbate reductase (DR) plus GR, respectively (Iturbe-Ormaetxe *et al.*, 2001).

Curiously, the hydrogen peroxide concentration measured in nodules in this study was similar in all treatments. However, it should be considered that H_2O_2 is involved in virtually all major areas of aerobic biochemistry (e.g. respiratory and photosynthetic electron transport; oxidation of glycolate, xanthine and glucose) and is produced in copious quantities by several enzyme systems (Noctor & Foyer, 1998). Moreover, in some circumstances, the destructive power and signalling potential of ROS such as H_2O_2 are utilized as an effective means of defense (Levine *et al.*, 1994; Foyer *et al.*, 1997).

The results obtained in the present study in relation to four of these antioxidant activities showed that only the GR activity was higher in mycorrhizal roots than in the non-mycorrhizal ones. The other antioxidant activities were similar (SOD and CAT) or lower (APX) in the droughted mycorrhizal roots than in the corresponding nonmycorrhizal ones. Similarly, in nodules, the SOD, CAT and APX activities were lower in the droughted mycorrhizal plants than in the non-mycorrhizal plants while, again, the GR activity was higher in nodules from mycorrhizal plants. In previous works where several enzymes have been studied under the same stress condition differential responses have frequently been observed (Walker & McKersie, 1993; Willekens *et al.*, 1994; Conklin & Last, 1995). Moreover, determinations of total activities in crude homogenates may not adequately reflect the importance of compartment-specific changes (Noctor & Foyer, 1998) since it is not considering the activities at the sites where antioxidants are synthesized and required, that is, the various nodule and root cell organelles (Mittova *et al.*, 2000).

In all living organisms, glutathione (GSH) is the major low molecular weight thiol-containing compound. It is present in millimolar concentrations in different tissues, where it is a general reductant (Foyer *et al.*, 1991). In addition, it has several important functions, including the removal of toxic oxygen derivatives in the ascorbate-glutathione cycle, the induction of enzymes, and it participates in sulphur metabolisms and gene expression (Foyer *et al.*, 1995). Some legumes contain homogluthatione (hGSH) instead of or in addition to GSH. It is not known why hGSH is restricted to legumes and what specific role, if any, this thiol may play in nodule functioning. Matamoros *et al.* (1999) measured the thiol composition in 8 legumes of agronomic interest, including soybean. This study showed that hGSH predominated in soybean, bean and mungbean plants. In any case, both thiol compounds (GSH and hGSH) were present in nodules of these plants. The same study also showed that, under induced premature nodule senescence, the concentration of GSH in nodules of bean became higher than that of hGSH, while no measurement was done in soybean.

Although it has been suggested that enhanced GSH content is not in itself sufficient to improve resistance to high rates of ROS production (Noctor & Foyer, 1998), the increase of GR activity (the enzyme which regenerates the oxidized GSH into its reduced form) observed in mycorrhizal plants could be of importance regarding alleviation of damage caused by drought stress. In a recent study Kranner (2002) correlated the amount of reduced GSH with different degrees of desiccation tolerance in lichens. GR has been postulated to play an important role in plant protection against various forms of stress (Aono *et al.*, 1995). Enhanced GR activity has been associated with increases in ascorbate contents (Foyer *et al.*, 1995), better protection of ascorbate and glutathione pools against paraquat stress (Foyer *et al.*, 1991, 1995), decreased sensitivity to photoinhibition (Foyer *et al.*, 1995), and mitigated foliar damage during exposure to paraquat (Aono *et al.*, 1993). Moreover, the decrease in GR activity has been correlated with enhanced paraquat sensitivity in tobacco plants (Aono *et al.*, 1995).

In conclusion, the results obtained in this study suggest that the consistently higher GR activity in roots and nodules of mycorrhizal plants might have generated reduced antioxidants (GSH), which helped to decrease the oxidative damage to biomolecules that is involved in premature nodule senescence. In fact, the ASC-GSH cycle, of which the GR together with APX are important components, is one of the main antioxidant defenses in plants (Becana *et al.*, 2000) and is also present in the cytosol of nodule cells (Dalton, 1995). It has been proposed that the impairment of the ASC-GSH cycle in the nodules may be a common feature in the process of nodule senescence (Escuredo *et al.*, 1996). This idea agrees with recent findings by Burritt *et al.* (2002) who have postulated that the ability of seaweeds plants to prevent or reduce the production of ROS was due to increased activity of the enzymes required to regenerate ascorbate and glutathione, as

is the case of GR. However, we would also expect an increased APX activity in nodules of droughted mycorrhizal plants, while it was similar to that found in nodules of droughted nonmycorrhizal plants. Hence, it seems unlikely that only the increase of GR activity is responsible of the important protective effect observed (Ruiz-Lozano *et al.*, 2001). We propose that other mechanisms might have contributed to the protection of soybean plants against drought-induced nodule senescence. One possibility is that AM symbiosis leads to a lower drought-induced oxidative stress in mycorrhizal plants due to primary drought-avoidance mechanisms such as the higher water retention properties of a mycorrhizal soil as compared to the soil of nonmycorrhizal plants (Augé *et al.*, 2001a,b), as well as to the ability of AM hyphae to take up water from sources inaccessible to the nonmycorrhizal roots and transfer to the host plant (Hardie, 1985; Ruiz-Lozano & Azcón, 1995). Data on daily plant water consumption showed a higher ability of mycorrhizal plants to deplete soil water when water availability was limited and support the hypothesis that a primary drought-avoidance mechanisms, namely direct water uptake by hyphae, is also involved in the protection of mycorrhizal soybean plants against premature drought-induced nodule senescence.

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