Arbuscular mycorrhizal symbiosis can alleviate drought-induced nodule senescence in soybean plants

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Summary

• Drought stress causes premature senescence in legume root nodules, therefore decreasing their ability for nitrogen fixation. Many physiological plant processes affected by drought stress have also been proposed as inducers of nodule senescence. The objective of this research was to determine whether arbuscular mycorrhizal (AM) symbiosis, already proven to protect host plants against the detrimental effects of drought, can also help legume plants to cope with the premature nodule senescence induced by drought stress.

• Several parameters related to nodule senescence were evaluated in well watered or drought-stressed soybean plants singly or dually inoculated with *Bradyrhizobium japonicum* and/or the AM fungi *Glomus mosseae* or *Glomus intraradices*.

• Under drought conditions *G. mosseae* colonization stimulated nodule d. wt, increased acetylene reductase activity (ARA) by 112%, increased leghemoglobin content by 25% and increased protein content by 15%, relative to plants singly colonized by *Bradyrhizobium*. Drought considerably enhanced oxidative damage to lipids and proteins in nodules of nonmycorrhizal plants, whereas both mycorrhizal treatments were protected against oxidative damage.

• We propose that alleviation of oxidative damage is strongly involved in AM protection against nodule senescence. Differential influences of *G. mosseae* and *G. intraradices* are also discussed.

Key words: arbuscular mycorrhizal symbiosis, *Bradyrhizobium*, drought, nodule senescence, oxidative damage.

Introduction

Plants from the family *Leguminoseae* can benefit from symbiotic associations with N_2-fixing bacteria from the family *Rhizobiaceae*, as well as with AM fungi. Rhizobial symbiosis provides the plant with the N necessary for its growth. AM symbiosis enhances plant growth through the ability of extrarradical fungal hyphae to take up low-diffusing nutrients such as P from soil (Varma & Hock, 1998). The importance of AM symbiosis in legume plants has been traditionally attributed to the high P requirements of nodulation and N_2 fixation processes (Barea & Azcón-Aguilar, 1983). The nitrogenase activity is dependent on ATP for the reduction of atmospheric dinitrogen to ammonia. Approx. 21 mol of ATP are consumed per mol N_2 reduced (Salsac *et al.*, 1984). This explains why the scarcity of soluble P in soil is a critical limiting factor for the *Rhizobium*-legume symbiosis, which can be compensated by the AM symbiosis. However, the role of AM on the tripartite symbiosis *Rhizobium*-legume-AM fungus may not be limited only to this nutritional effect (Barea *et al.*, 1988).

Under natural conditions, legume plants are often exposed to various environmental stresses that decrease the efficiency of symbiotic system and plant production. In Mediterranean areas, drought stress is one of the most common abiotic factors affecting plant production. It has been shown that drought stress has a considerable negative impact on nodule functioning (Sprent, 1971). Among other detrimental effects, it inhibits plant photosynthesis and disturbs the delicate mechanisms of oxygen control in nodules that are essential for

active nitrogen fixation (Sprent, 1971; Becana *et al.*, 1986; Sprent & Zahran, 1988; Irigoyen *et al.*, 1992; Gogorcena *et al.*, 1995), causing premature root nodule senescence and thus decreasing its ability for nitrogen fixation. The physiological and biochemical factors which trigger nodule senescence are not yet well known. Kijne (1975) suggested that nodule senescence could be caused by a lack of nutrients, by hormonal changes or by accumulation of toxic metabolites. The possibility that hormonal imbalance (auxins and cytokinins) could be involved in the process of nodule senescence was then assessed by Noodén *et al.* (1979) and Sutton (1983). Another factor related to nodule senescence is a limitation in carbohydrate availability (Irigoyen *et al.*, 1992), which has also been shown by Gogorcena *et al.* (1997). They found complete correlation between carbohydrate depletion and a decrease of 95% in N_2ase activity. The inhibition of photosynthesis has also been considered as a factor which originates nodule senescence (Huang *et al.*, 1975; Swaraj *et al.*, 1988; Irigoyen *et al.*, 1992; Gordon *et al.*, 1993). Finally, one of the processes which seems to be highly involved in nodule senescence is oxidative damage to biomolecules (Gogorcena *et al.*, 1995, 1997; Escuredo *et al.*, 1996; Becana *et al.*, 2000). In fact, legume nodules are especially at risk from oxidative damage by activated oxygen species because they contain an abundance of oxygen-labile proteins – Lb and Fe potentially available for catalysing (catalytic Fe) free radical production through Fenton chemistry (Puppo & Halliwell, 1988; Becana & Klucas, 1992; Dalton, 1995). These processes, all of which originate nodule senescence, are in some way affected by drought stress (Hsiao, 1973; Turner, 1986).

In this context, it is well known that AM symbiosis can protect host plants against the detrimental effects caused by drought stress (Allen & Allen, 1986; Nelsen, 1987; Sánchez-Díaz & Honrubia, 1994; Ruiz-Lozano *et al.*, 1995a). Several nonnutritional mechanisms have been proposed to explain this protection by AM symbiosis: changes in plant hormones (Danneberg *et al.*, 1992; Goicoechea *et al.*, 1995, 1996); increased plant leaf gas exchange and photosynthetic rate (Ruiz-Lozano *et al.*, 1995b; Ruiz-Lozano *et al.*, 1996a); direct hyphal water uptake from soil and transfer to the host plant (Hardie, 1985; Ruiz-Lozano & Azcón, 1995); and enhanced activity of enzymes involved in antioxidant defence (Ruiz-Lozano *et al.*, 1996b) or nitrate assimilation (Ruiz-Lozano & Azcón, 1996). Hence, we wondered if AM symbiosis could help legume plants to cope with the premature nodule senescence induced by drought stress.

The objective of this research was to determine if drought-induced nodule senescence in legume plants could be ameliorated by AM symbiosis. For that purpose, a number of markers of nodule senescence (N_2ase activity and nodule contents in leghemoglobin, protein and lipids) were evaluated in well-watered or drought-stressed soybean plants singly inoculated with *Bradyrhizobium japonicum* or in combination with one of two AM fungus. We also determined plant growth and nutrition after colonization by each endophyte alone or by a combination of the endophytes in order to correlate the symbiotic efficiency of each endophyte alone with the AM protection against nodule senescence. We attempted to obtain information regarding those mechanisms involved in the amelioration of nodule senescence by AM. Thus, we determined parameters related with osmotic adjustment and oxidative damage in nodule tissues.

Materials and Methods

Experimental design and statistical analysis

The experiment consisted of a randomized complete block design with six 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); plants inoculated with the mycorrhizal fungus *Glomus intraradices* (Schenck and Smith) and *B. japonicum* (Gi + Br); plants inoculated with *G. mosseae* (Gm); plants inoculated with *G. intraradices* (Gi); and non-inoculated control plants (NI). The first three treatments were used for the study of parameters related to nodule senescence. The last three treatments were used for determination of plant growth and nutrition in order to compare the symbiotic efficiency of each endophyte alone with the AM protection against nodule senescence in terms of final plant yield and nutrition. Twelve replicates of each treatment were made totaling 72 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 sterilized soil/sand mixture (1 : 1, v/v). When appropriate, a suspension (2 ml seed) 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 for each endophyte was bulked in an open-pot culture of *Allium cepa* L. and consisted of soil, spores, mycelia and infected root fragments. The AM species were *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, isolate BEG 122 and *Glomus intraradices* (Schenck and Smith) isolate BEG 121. Ten grams of inoculum of the two *Glomus* isolates, possessing similar infective characteristics (about 115 infective propagules per gram, according to the MPN test), were added to appropriate pots at sowing time just below soybean seedlings.

Uninoculated control plants for each microbial treatment received the same amount of autoclaved rhizobial and/or mycorrhizal inoculum.

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 water potential was determined by a pressure plate apparatus (Soilmoisture Equipment Corp. 15 Bar Ceramic Plate Extractor, Cat. no. 1500), and soil water content was measured by weighing the soil before and after drying at 110°C for 24 h (Richards, 1954). A characteristic soil moisture curve was constructed and used to correlate soil water content and soil water potential (Ψ) by gravimetric measurement of soil water content in the pots (Richards, 1954). Water was supplied daily to maintain constant soil water potential close to −0.04 MPa (field capacity) during the first 5 wk of plant growth. At this time half of the plants were allowed to dry until soil water potential reached −0.17 MPa (2 d needed to ensure that all pots reached the level of water equivalent to this soil Ψ) and maintained under such conditions for another 8 d. In order to control the level of water stress, pots were weighed daily (at the end of the afternoon) and the amount of water lost was added to the pot in order to maintain soil Ψ close to −0.17 Mpa during the entire drought period.

Each week throughout the experiment, plants received 10 ml of Hewitt's nutrient solution lacking N and P (Hewitt, 1952). After planting (3 wk), plants received nutrient solution amended with N and/or P as follows (Goicoechea *et al.*, 1997): 0.18 mM K_2HPO_4 and 2 mM NH_4NO_3 (NI plants), 0.35 mM K_2HPO_4 (Br plants), 3 mM NH_4NO_3 (Gm and Gi plants). Nutrient concentrations were chosen in an attempt to obtain well-watered plants of similar size and nutrient contents in microbial treatments.

Parameters measured

Biomass production and nutrient content At harvest (45 d after planting), the root system was separated from the shoot and d. wt was measured after drying in a forced draught oven at 70°C for 2 d. Shoot contents (mg $plant^{-1}$) of N (micro-Kjeldahl) and P (Olsen & Dean, 1965) were also determined.

Symbiosis development The percentage of mycorrhizal root infection was estimated by visual observation of fungal colonization after clearing washed roots in 10% 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 parameters of arbuscule abundance a% and A% give an estimation of the arbuscule richness in the mycorrhizal root fraction and total root system, respectively. Four replicates per treatment were used. Nodule d. wt was measured after drying in a forced draught oven at 70°C for 48 h.

Nodule activity N_2ase activity was estimated by the C_2H_2 reduction technique (Hardy *et al.*, 1973). Although Acetilene reductase activity (ARA) measured in closed vessels does not represent the true nitrogenase activity (Minchin *et al.*, 1983; Chan *et al.*, 1994), it can be appropriate, however, in assays for comparative purposes (Becana *et al.*, 1986; 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 (Hewlett Packard Co., Barcelona, Spain).

Markers of nodule senescence Nodules to be used for all biochemical determinations were harvested, immediately frozen in liquid N_2 and stored at −80°C until used. Aliquots of nodules (0.3 g f. wt) were lyophilized and their d. wt measured, and then ground with 6 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 0.02% potassium ferricyanide and 0.1% potassium carbonate, using an ice-cold potter. The homogenate was centrifuged at 20 000 ***g*** for 20 min, and the supernatant was used for leghemoglobin (Lb) quantification. Free Lb was quantified by the fluorescence emitted by the tetrapyrrole group after removing the Fe with hot oxalic acid (LaRue & Child, 1979). The excitation was set at 405 nm and the emission at 600 nm. Unheated samples were used as blanks and haemoglobin from human plasma (Sigma, Alcobeudas, Spain) was used as standard.

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

Total lipids were extracted from 0.3 g nodules as described by Bligh & Dyer (1959). The organic phase (approx. 2.5 ml) was rinsed three times with 1% NaCl and evaporated to dryness with N_2. The residual solvent was removed in vacuo, and total lipids were determined gravimetrically.

Solute accumulation Free proline and total soluble sugars (TSS) were extracted from 0.3 g nodules as described previously for lipids (Bligh & Dyer, 1959). The methanolic phase was used for quantification of both substances. Proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates *et al.* (1973). TSS were analysed by 0.1 ml of the alcoholic extract reacting with 3 ml freshly prepared anthrone (200 mg anthrone + 100 ml 72% (w : w) H_2SO_4) and placed in a boiling water bath for 10 min according to Irigoyen *et al.* (1992). After cooling, the absorbance at 620 nm was determined in a Shimadzu UV-1603 spectrophotometer (Shimazdu Europe GmbH, Duisburg, Germany). The calibration curve was made using glucose in the range of 20–400 μg/ml.

Oxidative damage to lipids and proteins Lipid peroxides were extracted by grinding 0.3 g of nodules with an ice-cold potter and 6 ml of 100 mM potassium phosphate buffer (pH 7.4). Homogenates were filtered through one layer of Miracloth and centrifuged at 15 000 ***g*** for 20 min. The chromogen was formed by mixing 200 μl of supernatant with 1 ml of a reaction mixture containing 15% (w/v) Trichloro acetic acid (TCA), 0.375% (w/v) 2-thiobarbituric acid (TBA), 0.1% (w/v) butyl hydroxytoluene, 0.25 N HCl and by incubating the mixture at 100°C for 30 min (Minotti & Aust, 1987). After cooling at room temperature, tubes were centrifuged at 800 ***g*** for 5 min and the supernatant was used for spectrophotometric reading at 532 nm. 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). The calibration curve was made using MDA in the range of 0.1–10 nmol. A blank for all samples was prepared by replacing the sample with extraction medium, and controls for each sample were prepared by replacing TBA with 0.25 N HCl. In all cases, 0.1% (w/v) butyl hydroxytoluene was included in the reaction mixtures to prevent artifactual formation of 2-thiobarbituric acid-reactive substances (TBARS) during the acid-heating step of the assay.

Protein oxidation was measured as the total content of carbonyl groups by reaction with 2,4-dinitrophenylhydrazine (Levine *et al.*, 1990). 0.3 g of nodules were ground with an ice-cold potter and 2 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 2.5 μg of each one of the following protease inhibitors leupeptin, pepstatin A and aprotinin. The homogenate was clarified by centrifugation at 20 000 ***g*** for 15 min and the supernatant was used for carbonyl quantification after removal of possible contaminating nucleic acids with 1% (w/v) streptomycin sulphate (Levine *et al.*, 1990).

Results

Plant biomass production

All the microbial treatments increased plant shoot d. wt compared with the uninoculated control plants both under well-watered and under drought stress conditions (Fig. 1). However, under well-watered conditions there were few differences in shoot d. wt among the microbial treatments. Only *G. mosseae*-colonized plants increased their shoot d. wt when also inoculated with *Bradyrhizobium*. Drought decreased significantly shoot d. wt in all cases, but there were differences as a result of microbial effects. Plants dually colonized by *G. mosseae* and *Bradyrhizobium* showed the greatest shoot d. wt. This value was over 15% higher than that of plants single colonized by each microsymbionts alone or that of plants

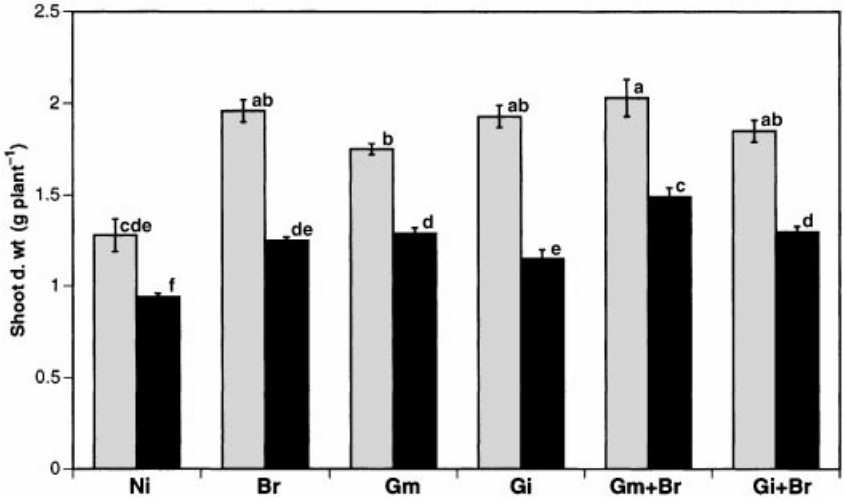

Fig. 1 Effect of drought stress and microbial treatment on plant shoot d. wt. Treatments are designed as Ni, noninoculated controls; Br, *Bradyrhizobium japonicum*; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*; Gm + Br, *G. mosseae* plus *Bradyrhizobium*; Gi + Br, *G. intraradices* plus *Bradyrhizobium*. Plants were either well-watered (light columns) or drought stressed for 10 d (dark columns). Data are means ± SE of six independent plants. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's Multiple Range test.

Table 1 Effect of drought stress and microbial treatment on mycorrhizal and rhizobial symbiosis development

Treatment	Water regime	F%	M%	a%	A%	Nodule d. wt
Ni	Well-watered	0b	0c	0c	0c	0d
	Droughted	0b	0c	0c	0c	0d
Br	Well-watered	0b	0c	0c	0c	71ab
	Droughted	0b	0c	0c	0c	48c
Gm	Well-watered	92.3a	46.5b	76.5b	37.1b	0d
	Droughted	89.0a	38.5b	91.7a	35.2b	0d
Gi	Well-watered	88.0a	41.5b	73.8b	31.0b	0d
	Droughted	87.0a	55.0ab	93.3a	52.0ab	0d
Gm + Br	Well-watered	96.0a	61.5ab	78.3b	48.0ab	80a
	Droughted	94.5a	48.5b	93.0a	46.0ab	65b
Gi + Br	Well-watered	96.0a	76.5a	89.3a	68.0a	63b
	Drougthed	86.0a	52.0ab	98.0a	52.0ab	36c

F (colonization frequency), M (colonization intensity), a (arbuscule richness in the mycorrhizal root fraction), A (arbuscule abundance in the whole root system). Nodule dry weight (mg plant^{-1}). Treatments are designed as Ni, noninoculated controls; Br, *Bradyrhizobium japonicum*; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*; Gm + Br, *G. mosseae* plus *Bradyrhizobium*; Gi + Br, *G. intraradices* plus *Bradyrhizobium*. Plants where either well-watered or drought stressed for 10 d. Within each parameter, means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's Multiple Range test ($n = 4$).

double colonized by *G. intraradices* and *Bradyrhizobium*. *G. intraradices* also benefited from dual colonization with *Bradyrhizobium*, which increased shoot d. wt compared with single colonization by this AM fungus.

Symbiotic development

Colonization frequency (F) was similar in all mycorrhizal treatments and resulted unaffected by the presence or absence of *Bradyrhizobium* as well as by drought stress (Table 1). Colonization intensity (M) and arbuscule abundance (A) were not significantly affected by drought stress. Only well-watered plants dually colonized by *G. intraradices* and *Bradyrhizobium* had higher M and A values than well-watered plants single colonized by either AM fungus. Curiously, another infection parameter evaluated, the arbuscule richness in the mycorrhizal root fraction (a), was significantly affected by drought stress. In fact, roots colonized by *G. mosseae*, either alone or in dual combination with *Bradyrhizobium*, enhanced their arbuscule richness as a consequence of drought. The same was observed in roots singly colonized by *G. intraradices*, while those dually colonized by *G. intraradices* and *Bradyrhizobium* did not significantly increase this parameter due to drought.

Nodule d. wt remained unaffected by mycorrhizal presence under well-watered conditions (Table 1). Only colonization by *G. intraradices* decreased nodule d. wt compared with *G. mosseae*-colonized plants, which produced the highest d. wt of nodule. Drought stress decreased nodule d. wt in all treatments. However, plants colonized by *G. mosseae* had higher nodule d. wt (35% increase) than plants single colonized by *Bradyrhizobium*. Inoculation with *G. intraradices* did not affect nodule d. wt compared with single *Bradyrhizobium*-inoculated plants.

Nodule functioning

Nitrogenase activity was measured by the ARA test in total roots. Colonization of plants by *G. mosseae* increased ARA activity compared with plants single inoculated with *Bradyrhizobium* (Fig. 2a). This increase was more evident under drought stress conditions (112% increase). By contrast, colonization by *G. intraradices* did not increase ARA activity compared with nonmycorrhizal plants either under well-watered (significant decrease) or under drought stress conditions. In all cases, drought decreased ARA activity compared with well-watered plants. However, this decrease was more evident in non-mycorrhizal- and *G. intraradices*-plants (80% decrease in both treatments) than in *G. mosseae*-colonized plants (64% decrease).

Other markers of nodule senescence

The leghemoglobin content in nodules (Fig. 2b) was enhanced in *G. mosseae*-colonized plants, both under well-watered (19%) and drought stress conditions (25%). Drought stress decreased this value in nodules from all treatments by over 44%. However, under the experimental conditions used here, nodules of plants colonized by *G. mosseae* showed the highest levels of leghemoglobin.

The protein content in nodules (Fig. 2c) decreased by 13% in plants colonized by *G. intraradices* under well-watered conditions. Nevertheless, drought stress decreased the protein content in nodules in all cases except in *G. intraradices*-colonized plants. This decrease was higher in plants single inoculated with *Bradyrhizobium* (28%) than in those inoculated with *G. mosseae* (12%).

Total lipid content of nodules (Fig. 2d) was decreased by dual inoculation with *G. intraradices* and *Bradyrhizobium*,

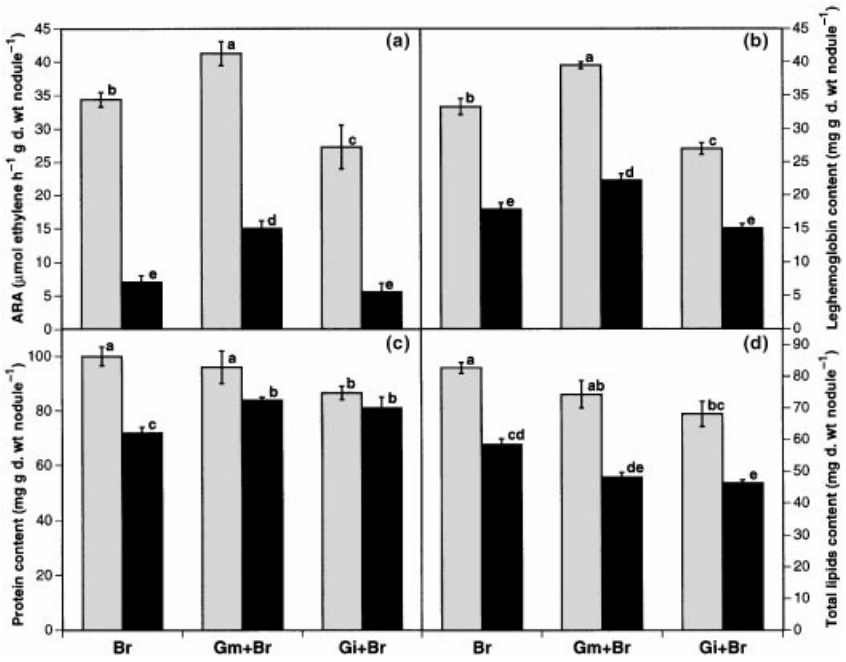

Fig. 2 Effect of drought stress and microbial treatment on nodule acetylene reductase activity (a), leghemoglobin content (b), protein content (c) or lipid content (d). Treatments are designed as Br, *Bradyrhizobium japonicum*; Gm + Br, *Glomus mosseae* plus *Bradyrhizobium*; Gi + Br, *Glomus intraradices* plus *Bradyrhizobium*. Plants were either well-watered (light columns) or drought stressed for 10 d (dark columns). Data are means ± SE of five independent nodule samples. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's Multiple Range test.

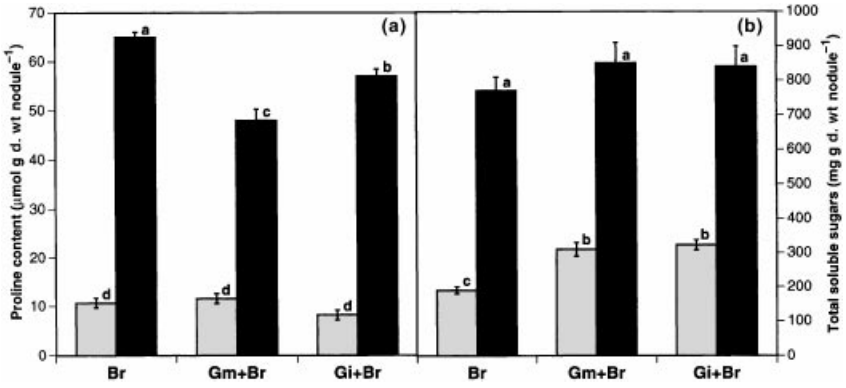

Fig. 3 Effect of drought stress and microbial treatment on nodule proline content (a) and total soluble sugars content (b). Treatments are designed as Br, *Bradyrhizobium japonicum*; Gm + Br, *Glomus mosseae* plus *Bradyrhizobium*; Gi + Br, *Glomus intraradices* plus *Bradyrhizobium*. Plants were either well-watered (light columns) or drought stressed for 10 d (dark columns). Data are means ± SE of five independent nodule samples. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's Multiple Range test.

both under well-watered (by 17%) and drought stress conditions (by 20%) compared with plants single colonized by *Bradyrhizobium*. By contrast, colonization by *G. mosseae* did not significantly change this value. Drought stress decreased total lipid content in nodules of all treatments by about 30%.

Solute accumulation

Well-watered plants accumulated little proline in nodules. Drought stress considerably enhanced proline accumulation (Fig. 3a). This enhancement was stronger in nonmycorrhizal plants (507% of increase) than in those colonized by either AM fungus. In fact, mycorrhizal plants exhibited the lowest nodule proline accumulation as a consequence of drought. This was more evident in the case of *G. mosseae*-plants which had 26% less proline in nodules than plants single colonized by *Bradyrhizobium*.

TSS in nodules (Fig. 3b) was affected by mycorrhizal colonization only under well-watered conditions since nodules from mycorrhizal plants had about 65% more TSS than nodules from nonmycorrhizal plants. Drought considerably increased the amount of sugars accumulated in nodule tissues. Under drought conditions, the total amount of sugar in nodules was similar in all treatments.

Oxidative damage to lipids and proteins

We measured oxidative damage to biomolecules such as lipids and proteins in nodules. Accordingly, the amount of lipid peroxides (thiobarbituric test) and oxidized proteins (total carbonyl groups) were quantified in the nodules of single or double colonized plants. Lipid peroxidation was not affected by mycorrhizal presence under well-watered conditions (Fig. 4a). Drought stress exposure enhanced accumulation of MDA in all treatments, but this increase was considerably

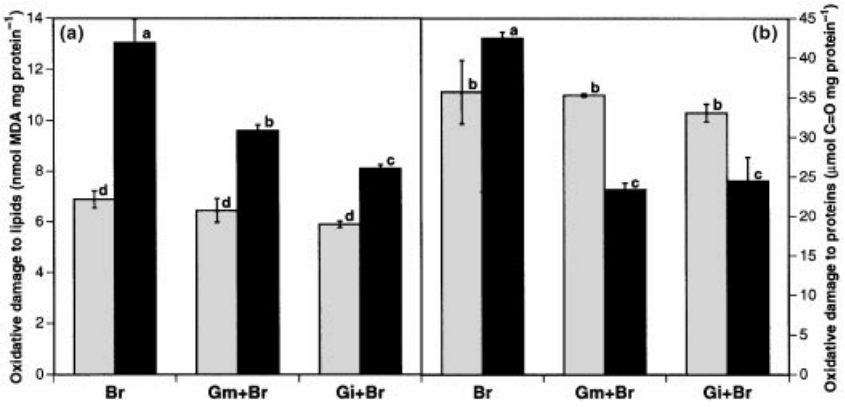

Fig. 4 Effect of drought stress and microbial treatment on oxidative damage to lipids (a) or proteins (b) in nodules. Treatments are designed as Br, *Bradyrhizobium japonicum*; Gm + Br, *Glomus mosseae* plus *Bradyrhizobium*; Gi + Br, *Glomus intraradices* plus *Bradyrhizobium*. Plants were either well-watered (light columns) or drought stressed for 10 d (dark columns). Data are means ± SE of five independent nodule samples. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's Multiple Range test.

higher in nonmycorrhizal plants than in mycorrhizal plants. *G. intraradices*-colonized plants showed the lowest value of lipid peroxidation. The amount of carbonyl groups (oxidative damage to proteins) was also similar in all treatments under well-watered conditions (Fig. 4B). Drought increased the content of carbonyl groups by 20% in nonmycorrhizal nodulated plants as compared with well-watered plants. By contrast, proteins were protected against oxidative damage in mycorrhizal plants, so that nodules from drought stressed mycorrhizal plants decreased their content in carbonyl groups by 34% (Gm + Br) or by 26% (Gi + Br) as compared with well-watered conditions.

Nutrient content

N content in well-watered plants was higher in nodulated plants than in nonnodulated plants (Table 2). *Bradyrhizobium* interaction with *G. intraradices* decreased N content (by 14%) compared with single colonization by *Bradyrhizobium*. By contrast, *Bradyrhizobium* interaction with *G. mosseae* did not negatively affect the plant N content. Drought stress decreased N content in all treatments, but this decrease was more evident in both uninoculated control plants and in those singly inoculated with *G. intraradices*.

Treatments did not change P content in plants cultivated under well-watered conditions. Drought decreased P content in plant tissues, and uninoculated controls and plants singly inoculated with *G. intraradices* were the most negatively affected. Plants double colonized by *G. mosseae* and *Bradyrhizobium* showed the highest P content.

Table 2 Effect of drought stress and microbial treatment on shoot N and P contents (mg plant^{-1})

Treatment	Water regime	N content	P content
Ni	Well-watered	23.1c	3.30a
	Droughted	13.8e	1.15e
Br	Well-watered	32.7a	3.20a
	Droughted	22.6c	1.88cd
Gm	Well-watered	22.2c	3.56a
	Droughted	19.4cd	2.24bc
Gi	Well-watered	22.2c	3.50a
	Droughted	16.3de	1.62de
Gm + Br	Well-watered	36.0a	3.50a
	Droughted	23.5c	2.53b
Gi + Br	Well-watered	28.0b	3.30a
	Drougthed	21.6c	1.90cd

Treatments are designed as Ni, noninoculated controls; Br, *Bradyrhizobium japonicum*; Gm, *G. mosseae*; Gi, *Glomus intraradices*; Gm + Br, *Glomus mosseae* plus *Bradyrhizobium*; Gi + Br, *G. intraradices* plus *Bradyrhizobium*. Plants where either well-watered or drought stressed for 10 d. Within each parameter, means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's Multiple Range test ($n = 6$).

Discussion

Senescence of legume root nodules is a complex process that implies the structural breakdown of both the bacteroids and the host cells (Becana *et al.*, 1986). Several aspects of N metabolism are negatively affected during senescence – nitrogenase activity being one of the most important (Sutton, 1983). Nodule senescence is commonly diagnosed by decreases in N_2 fixation, as well as in the content of leghemoglobin, total lipids and proteins (Sutton, 1983; Gogorcena *et al.*, 1997). In this study, drought stress exposition for 10 d induced nodule senescence in soybean plants as evidenced by the reduction in N_2ase activity and nodule contents of leghemoglobin, total proteins and lipids. However, most of the parameters measured to determine nodule senescence clearly showed a protective effect after root colonization by the AM fungus *G. mosseae* and, in some cases, also after colonization by *G. intraradices*. Under drought conditions *G. mosseae* colonization stimulated nodule d. wt, ARA activity was 112% higher than in plants singly colonized by *Bradyrhizobium* leghemoglobin content increased by 25% and protein content also increased by 15%. Total lipid content in nodules was the only index of nodule senescence which was not improved by AM symbiosis. This was more evident in the case of *G. intraradices*-colonized plants. This effect may be related to the fungal requirements for its own development. *G. intraradices* has been described as an aggressive AM fungus, producing high amounts of vesicles

and spores (Graham *et al.*, 1996). These structures have been estimated to contain 54–72% of lipids (Beilby & Kibdy, 1980; Jabaji-Hare *et al.*, 1984; Olsson & Johansen, 2000). Hence it is likely that the lower lipid content found in nodules of *G. intraradices*-inoculated plants may be due to the fungal use of lipids for the formation of vesicles and spores.

Accumulation of proline and TSS in nodules was determined as an index of dehydration in this tissue (Riazi *et al.*, 1985). Proline and sugars are important solutes that plants accumulate as osmoregulators when tissues are subjected to dehydration (Jones *et al.*, 1980; Meyer & Boyer, 1981; Morgan, 1984; Irigoyen *et al.*, 1992; Goicoechea *et al.*, 1998). Drought enhanced accumulation of both kinds of solutes in nodules. While all the treatments accumulated the same amount of TSS as a result of drought, mycorrhizal plants accumulated significantly less proline (26% in the case of *G. mosseae*) than plants single inoculated with *Bradyrhizobium*. The global balance between both solutes suggests that mycorrhizal plants suffered less dehydration than nonmycorrhizal ones, in agreement with other previous results on drought (Ruiz-Lozano & Azcón, 1997) and saline stress (Ruiz-Lozano *et al.*, 1996a).

Oxidative damage has been proposed by several authors as one of the most important mechanisms mediating nodule senescence in stressed nodules, due to the high content of nodules in oxygen-labile proteins, leghemoglobin and catalytic Fe (Gogorcena *et al.*, 1995, 1997; Escuredo *et al.*, 1996; Becana *et al.*, 2000). Leghemoglobin and catalytic Fe can catalyse free radical production through Fenton chemistry (Puppo & Halliwell, 1988; Becana & Klucas, 1992; Dalton, 1995), which in turn can damage biomolecules, such as lipids and proteins, thus contributing to nodule senescence (Escuredo *et al.*, 1996). Drought considerably enhanced oxidative damage to lipids in all treatments, but both mycorrhizal treatments showed less lipid peroxidation, mainly plants colonized by *G. intraradices*. More interestingly, drought increased oxidative damage to proteins in *Bradyrhizobium*-colonized plants, while both mycorrhizal treatments were protected against carbonyl group formation, showing even lower levels of carbonyl groups than under well-watered conditions. Nodule senescence has been correlated with a marked decline in the major activities involved in removal of reactive oxygen species (Evans *et al.*, 1999; Becana *et al.*, 2000). The mycorrhizal effect observed in this study can be explained if mycorrhizal plants enhanced their antioxidant machinery as a result of drought. This was reported in a previous study (Ruiz-Lozano *et al.*, 1996b) where we detected that mycorrhizal plants increased superoxide dismutase (SOD) activity both in shoot and root tissues as a consequence of drought, while P-fertilized uninoculated plants did not increase such activity under drought conditions. This observation could explain why mycorrhizal plants displayed less oxidative damage to lipids and proteins than nonmycorrhizal nodulated plants (Br), since SODs provide a powerful defence against reactive oxygen species formation (Tsang *et al.*, 1991; Bowler *et al.*, 1992; Foyer *et al.*, 1994).

All of these positive effects of AM symbiosis (mainly in the case of *G. mosseae*) ameliorating nodule senescence are expected to affect the final plant production. In this study the various microbial treatments produced plants of similar size and P content when cultivated under well-watered conditions. Only uninoculated control plants showed a reduced growth rate, indicating a positive symbiotic effect by each microsymbiont (combination of endophytes or each one separately). This was true both under well-watered and drought stress conditions. Drought decreased plant growth in all cases compared with well-watered conditions, but dual colonization by *G. mosseae* and *Bradyrhizobium* produced the largest plant size. This effect was directly correlated with the protection shown by this fungus against nodule senescence. In this study drought was imposed for only 10 d. We expect that if drought duration had been longer, this positive mycorrhizal effect on plant growth production would have also been amplified.

In relation to those parameters regarding nodule senescence, it is clear that the symbiotic efficiency of *G. intraradices* was lower than that of *G. mosseae*. Apparently, plants did not benefit from *G. intraradices* colonization to protect the nodules against premature senescence. In fact, the shoot d. wt of droughted plants colonized by *G. intraradices* was similar to that of plants single colonized by *Bradyrhizobium* and lower than that of *G. mosseae-Bradyrhizobium*-colonized ones. The diverse behaviours of *G. mosseae* and *G. intraradices* could be related to their varying developmental patterns. Normally, *G. intraradices* is a very aggressive AM fungus in terms of intensity of root colonization and production of fungal structures such as vesicles and chlamydospores (Graham *et al.*, 1996), and it may also require more photosynthetic products than *G. mosseae*. Graham *et al.* (1996) found that aggressive AM fungi such as *G. intraradices* used more nonstructural carbohydrates in roots than nonaggressive AM fungi. This could have lead to a competitive interaction between the AM fungus and *Bradyrhizobium* which resulted in a less effective combination of endophytes for protection against nodule senescence. Supporting this hypothesis, we found that soybean plants double-inoculated with *G. intraradices* and *Bradyrhizobium* displayed a reduction of nodule development in parallel with the lowest values of lipid accumulation in nodules. Antagonistic interactions between both endophytes have been reported. Ruiz-Lozano & Azcón (1993, 1994) described detrimental effects on chickpea plants after dual inoculation with an AM fungus and *Bradyrhizobium*. They found that a very infective strain of *Bradyrhizobium* reduced root colonization by *G. fasciculatum*, especially when photosynthesis was limited. Our hypothesis also agrees with results reported by Kucey & Paul (1982), who found that nodules utilized more photosynthates in a mycorrhizal host (12%) than in a nonmycorrhizal host (6%).

In conclusion, results from this study show that AM symbiosis can protect legume plants against the detrimental effects of premature nodule senescence induced by drought

stress. The exact mechanisms by which the AM symbiosis promote such an effect remain to be elucidated. It could be thought that mycorrhizal plants were simply better hydrated than nonmycorrhizal ones due to direct fungal water uptake and its transport to plant (Ruiz-Lozano & Azcón, 1995). However, it is unlikely that this was the only mechanism involved. Data from this study strongly suggest that alleviation of oxidative damage in nodules may be involved in the protective effect of AM symbiosis against nodule senescence. This will therefore be the first topic to be examined in future studies.

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