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The arbuscular mycorrhizal symbiosis enhances the photosynthetic efficiency and the antioxidative response of rice plants subjected to drought stress

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ABSTRACT

Rice (*Oryza sativa*) is the most important crop for human consumption, providing staple food for more than half of the world's population. Rice is conventionally grown under flooded conditions for most of its growing cycle. However, about half of the rice area in the world does not have sufficient water to maintain optimal growing conditions and yield is reduced by drought. One possible way to increase rice production in order to meet the rice demand is to improve its drought tolerance by means of the arbuscular mycorrhizal (AM) symbiosis. Thus, AM and non-AM rice plants were maintained under well-watered conditions or were subjected to moderate and severe drought stress for 15 d. After that, half of the plants from each treatment were harvested, while the other half were allowed to recover from drought for additional 25 d. The results showed that rice can benefit from the AM symbiosis and improve their long-term development after a drought stress period. In fact, at each watering level, AM plants showed about 50% enhanced shoot fresh weight as compared to non-AM plants. The AM symbiosis enhanced the plant photosynthetic efficiency under stress over 40%, induced the accumulation of the antioxidant molecule glutathione and reduced the accumulation of hydrogen peroxide and the oxidative damage to lipids in these plants. Thus, these combined effects enhanced the plant performance after a drought stress period.

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Introduction

Rice (*Oryza sativa*) is the most important crop for human consumption, providing staple food for more than half of the world's population. With around 600 Mt produced annually on 149 Mha in 2003, rice accounts for 23% of the world's caloric intake (Khush, 2003). There is evidence suggesting that rice was the first crop domesticated by humans, in the Yangtze River basin as early as 13,000 years ago (Lu et al., 2002).

Rice has the evolutionary peculiarity of being semi-aquatic and is conventionally grown under continuous submersion for most of its growing cycle. Thus, it has relatively few adaptations to water-limited conditions and is extremely sensitive to drought stress (Kamoshita et al., 2008). Rice needs a large amount of water, and water scarcity has a negative influence on plant growth, yield and quality determination (Vallino et al., 2009). However, about half of the rice area in the world does not have sufficient water to maintain flooded conditions and yield is reduced to some extent by drought. Even intermittent water stress at critical stages may result in considerable yield reduction and crop failure (Bernier et al., 2008). Indeed, drought is a major limitation for rice production in rain-fed ecosystems. Evenson et al. (1996) estimated global rice yield lost due to drought to be 18 million tons annually, or 4% of total rice production, which was valued conservatively at US $3.6 billion at that time. It is not simply the lack of water that lowers yield potential, but also the timing and duration of drought stress related to phenological processes (Jongdee et al., 2002).

Recurrent dry periods and scattered rainfall patterns have determined water shortage and consequent loss or damage in crop production in generally well-rain-fed areas (Vallino et al., 2009). Fortunately, it has been demonstrated that the arbuscular mycorrhizal (AM) symbiosis can protect host plants against detrimental effects of water deficit (for reviews see Augé, 2001, 2004; Ruiz-Lozano, 2003; Ruiz-Lozano et al., 2008). Studies carried out thus far have suggested several mechanisms by which the AM symbiosis can alleviate drought stress in host plants. The most important include: direct uptake and transfer of water through the fungal hyphae to the host plant (Hardie, 1985; Ruiz-Lozano and Azcón, 1995), changes in soil water retention properties (Augé et al., 2001), better osmotic adjustment of AM plants (Augé et al., 1992; Kubikova et al., 2001; Ruiz-Lozano et al.,

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1995a), enhancement of plant gas exchange and water use efficiency (Augé et al., 1992; Ruiz-Lozano et al., 1995a, 1995b) and protection against the oxidative damage generated by drought (Porcel and Ruiz-Lozano, 2004; Porcel et al., 2003; Ruiz-Lozano et al., 2001). This last mechanism has been recognized as crucial (Ruiz-Lozano, 2003), as many of the degenerative reactions associated with several environmental stresses, including water deficit, result in the production of reactive oxygen species (ROS) in plants, causing additional oxidative stress. The term ROS is generic, embracing not only free radicals such as superoxide (O_2^-) 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 and Heber, 1996), O_2^- and H_2O_2 are synthesized at very high rates even under optimal conditions (Noctor and Foyer, 1998). The main toxicity of O_2^- 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 and Gutteridge, 1989).

The improvement of stress tolerance is often related to enhancement of contents of antioxidant compounds in plants. Given the toxicity of ROS, plants need to have appropriate detoxification systems in place that allow rapid removal of these compounds. These systems include several antioxidant enzymes and also non-enzymatic compounds such as ascorbate, glutathione, flavonoids, carotenoids and tocopherols (Ma et al., 2008). Among these non-enzymatic compounds, glutathione and ascorbate are essential plant metabolites that regulate major cell functions and play a pivotal role in antioxidant defense (Noctor and Foyer, 1998). Ascorbate is a major substrate for reductive detoxification of H_2O_2 in plants. The H_2O_2 generated directly or after conversion by superoxide dismutases from other ROS species is initially degraded to H_2O by ascorbate peroxidases using ascorbate as electron donor. The resulting short-lived radical monodehydroascorbate disproportionates to ascorbate and dehydroascorbate, which in turn is reduced by dehydroascorbate reductase with glutathione as electron donor (Noctor and Foyer, 1998). Finally, the oxidized glutathione is reduced again to glutathione with electrons delivered from NADPH in a reaction catalyzed by glutathione reductase. Thus, the accumulation of antioxidant compounds is often used as an index of the capacity of plants to tolerate environmental conditions leading to oxidative stress (Noctor and Foyer, 1998).

Rice plants readily form mycorrhizal associations under upland conditions, but under submerged conditions infection is rare due to the anoxic environment (Ilag et al., 1987). However, some reports have revealed AMF colonization under flooded conditions (Secilia and Bagyaraj, 1994a, 1994b; Solaiman and Hirata, 1996). Rice can also be grown in a system of alternate irrigation to reduce water input needed for production. This system creates aerobic conditions in the soil that stimulate colonization of rice roots by arbuscular mycorrhizal (AM) fungi. However, there are only few studies providing an overview of the colonizing AMF in rice roots and there is still no c lear picture of how the association may be exploited to benefit crop yield under adverse environmental conditions (Vallino et al., 2009).

The demand for rice production is still rising because of the continuous increase in the global population. The world's population is predicted to reach approximately 8 billion by 2030, and there is therefore a need to further increase rice production by 40% in the next 20 years (Bernier et al., 2008). One possible way to increase rice production to meet the demand of population growth is to improve drought tolerance in rice by means of the AM symbiosis. Thus, the objective of this work was to investigate whether the AM symbiosis can help rice plants to overcome the negative effects of drought stress. The initial hypotheses are: (I) mycorrhizal inoculation would modify the responses of rice plant to water deficit, (II) the AM symbiosis would enhance the production of antioxidant compounds and decrease the accumulation of ROS in the host plant, and (III) these two effects would decrease the lipid peroxidation in AM plants. Plant physiological and biochemical parameters related to the oxidative status were determined in AM and non-AM rice plants just after a drought stress period and also after subsequent recovery from drought.

Materials and methods

Experimental design and statistical analysis

The experiment consisted of a randomized complete block design with two inoculation treatments: (1) plants inoculated with the mycorrhizal fungus *Glomus intraradices* and (2) uninoculated control plants. The watering treatments are described below in the "growth conditions" section.

At the two harvest times considered in this study [45 and 70 d after transplanting (DAT)], each watering and inoculation treatment was replicated four times. In addition, four AM plants and four non-AM plants were harvested 30 DAT, as controls just before starting the drought stress treatment, which totaled 56 pots (one plant per pot).

At each harvest time, data were subjected to analysis of variance (ANOVA) with AM inoculation, water supply and AM inoculation–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

Substratum consisted of a mixture of loamy soil (collected from the Zaidin Experimental Station, Granada, Spain), sieved (2 mm), quartz-sand (<1 mm) and vermiculite (1:2:6, soil: sand:vermiculite, v/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.

Rice (*Oryza sativa*, cv INCA LP-5) seeds were placed on sterile sand at 25 °C to germinate. Two-week-old seedlings were transferred to plastic pots containing 400 g of a sterilized substratum.

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 intraradices* (Schenck and Smith), isolate EEZ 01. AM inoculum was added to the seedbed in the germination container at sowing time and also to the appropriate pots at transplanting time (through banding 5 g of inoculum per pot), just below rice seedlings.

Growth conditions

Plants were grown in a controlled environmental chamber with 60–70% RH, day/night temperatures of 23/19 °C, and a photoperiod of 16 h at a photosynthetic photon flux density

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(PPFD) of 250 μmol m^{-2} s^{-1} (Licor, Lincoln, NE, USA, model LI-188B).

During the first 30 d after transplanting to the pots, each plant received 25 mL of aqueous nutrient solution containing complete formulation of nutrients (Hoagland and Arnon, 1950), except for P, which was reduced to 25% in order to avoid inhibition of AM colonization by excessive P. This volume of nutrient solution was applied three times per week on alternate days. At this stage, a group of plants was used for an initial measurement of plant biomass, root AM colonization, shoot water potential, photosynthetic efficiency and stomatal conductance before starting the water stress treatments (referred in the text as 30 DAT). Other measurements such as antioxidant compounds, hydrogen peroxide and proline contents, as well as of oxidative damage to lipids could not be carried out at this growing stage due to the low plant biomass obtained. The rest of the plants were subjected to drought, although a group of plants was kept as the control, receiving 25 mL of nutrient solution three times per week. For the moderate drought stress, the stock nutrient solution was diluted in only 10 mL of water (keeping the same total amount of nutrients than for the plants irrigated with 25 mL) and plants were irrigated with this amount of nutrient solution three times per week. For the severe drought stress, the stock nutrient solution was diluted in only 5 mL of water (keeping the same total amount of nutrients than for the plants irrigated with 25 mL) and plants were irrigated with this amount of nutrient solution three times per week. The drought stress treatment was maintained for 15 d. Then, half of plants from each treatment were harvested (which corresponded to 45 DAT), while the other half were allowed to recover from drought for an additional 25 d by irrigation with 25 mL of aqueous nutrient solution three times per week, and then harvested (which corresponded to 70 DAT).

Parameters measured

Biomass production

At 30, 45 and 70 DAT, the root system was separated from the shoot and their fresh weights (FW) determined. The shoot tissues were separated in 1 g aliquots and frozen in liquid nitrogen for future determination of antioxidant compounds, hydrogen peroxide and proline contents, as well as of oxidative damage to lipids. These determinations were carried out only in samples corresponding to 45 and 70 DAT, since the biomass of plant harvested at 30 DAT was too low to permit these determinations.

Symbiotic 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 lactic acid (v/v), according to Phillips and Hayman (1970). Quantification of the root colonization was performed according to the grid-line intersect method (Giovannetti and Mosse, 1980). Five replicates per treatment were used.

Photosynthetic efficiency

The efficiency of photosystem II was measured with FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant photosynthetic performance by measuring chlorophyll *a* fluorescence. FluorPen quantifies the quantum yield of photosystem II as the ratio between the actual fluorescence yield in the light-adapted state (F'_V) and the maximum fluorescence yield in the light-adapted state (F'_M), according to Oxborough and Baker (1997). Measurements were taken in the second youngest leaf of four different plants of each treatment.

Stomatal conductance

Stomatal conductance was measured two hours after the light turned on by using a porometer system (Porometer AP4, Delta-T Devices Ltd., Cambridge, UK) following the user manual instructions. Stomatal conductance measurements were taken in the second youngest leaf from four different plants from each treatment.

Proline content

Free proline was extracted from 250 mg of fresh leaves (Bligh and Dyer, 1959). The methanolic phase was used for quantification of proline content. Proline was estimated by spectrophotometric analysis at 530 nm of the ninhydrin reaction, according to Bates et al. (1973).

Glutathione and ascorbate contents

Glutathione content was measured as described by Smith (1985). Two hundred milligrams of the youngest fully developed leaves of each plant group were homogenized in a cold mortar with 5 mL 5% (w/v) sulfosalicylic acid, the homogenate was filtered and centrifuged at 1000*g* for 10 min. One milliliter of supernatant was neutralized by 1.5 mL 0.5 M K-phosphate buffer (pH 7.5). The standard incubation medium was a mixture of: 0.5 mL 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, 0.2 mL 6 mM 5,5′-dithiobis-(-2-nitrobenzoic acid), 0.1 mL 2 mM NADPH, and 0.1 mL (1 unit) glutathione reductase. The reaction was initiated by the addition of 0.1 mL glutathione standard or of extract. The change in absorbance at 412 nm was recorded for 9 min.

Ascorbate was assayed photometrically by the reduction of 2,6-dichlorophenolindophenol (DCPIP) as described by Leipner et al. (1997). Two hundred milligrams of the youngest fully developed leaves of each plant group were homogenized in 5 mL ice-cold 2% (w/v) metaphosphoric acid in the presence of 1 g NaCl. The homogenate was filtered through a filter paper. An aliquot of 300 μL was mixed with 200 μL 45% (w/v) K_2HPO_4. After 15 min incubation at 25 °C, 1 mL 2 M citrate-phosphate buffer (pH 2.3) and 1 mL 0.003% (w/v) DCPIP were added. The absorbance at 524 nm was measured immediately. The content of ascorbate was calculated by reference to a standard curve made of ascorbate.

Hydrogen peroxide content

Hydrogen peroxide content in leaves was determined by Patterson's method (Patterson et al., 1984), with slight modifications as described previously by Aroca et al. (2003). Two hundred and fifty milligrams of shoot fresh weight was homogenized in a cold mortar with 5 mL 5% (w/v) TCA containing 0.1 g of activated charcoal and 1% (w/v) PVPP. The homogenate was centrifuged at 18,000*g* for 10 min. The supernatant was filtered through a Millipore filter (0.22 μm). A volume of 1.2 mL of 100 mM potassium phosphate buffer (pH = 8.4) and 0.6 mL of the colorimetric reagent were added to 130 μL of the supernatant. The colorimetric reagent was freshly made by mixing 1:1 (v/v) 0.6 mM potassium titanium oxalate and 0.6 mM 4–2 (2-pyridylazo) resorcinol (disodium salt). The samples were incubated at 45 °C for 1 h and the absorbance at 508 nm was recorded. The blanks were made by replacing leaf extract by 5% TCA.

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Oxidative damage to lipids

Lipid peroxides were extracted by grinding 500 mg of leaves with and ice-cold mortar and 6 mL of 100 mM potassium phosphate buffer (pH 7). Homogenates were filtered through one Miracloth layer and centrifuged at 15,000*g* for 20 min. The chromogen was formed by mixing 200 μL of supernatants with 1 mL of a reaction mixture containing 15% (w/v) Trichloroacetic 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 and 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 and 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.

Results

Plant biomass and symbiotic development

The shoot fresh weight (SFW) and root fresh weight (RFW) before the stress treatment (30 DAT) were similar in AM and non-AM plants (Fig. 1A and B). After the stress period (45 DAT), both the SFW and the RFW decreased significantly in the treatments that received only 5 mL of aqueous nutrient solution as compared to the well-watered control treatment (25 mL aqueous solution), with this decrease being similar in AM and in non-AM plants. Finally, after the recovery from drought for an additional 25 d, the SFW showed a significant increase in AM plants as compared to non-AM plants. In fact, at each watering level, AM plants showed about 50% enhanced SFW as compared to non-AM plants. A similar trend was observed for RFW, but in that case, the differences were not significant for plants that had been irrigated with 10 mL of aqueous nutrient solution.

No mycorrhizal colonization was observed in plants not provided with AM inoculum. As expected, the values of root colonization increased with time (Fig. 2). Before the stress treatment (30 DAT), the percentage of root colonization was only 4%. After a drought stress period of 15 d (45 DAT), the root colonization increased to about 20%, with plants that had received 10 or 5 mL of aqueous nutrient solution having slightly higher root colonization than those that had remained under control conditions (25 mL of aqueous nutrient solution). After the recovery from drought for an additional 25 d (70 DAT), the root system reached over 45% AM colonization, with treatments that had received 10 mL of aqueous nutrient solution for 15 d showing the highest root colonization (53%).

Photosynthetic efficiency

The efficiency of photosystem II was assessed by measuring chlorophyll *a* fluorescence (Fig. 3A). Before the stress period (30 DAT), photosynthetic efficiency of photosystem II was similar in AM and non-AM plants. After the drought stress period (45 DAT), AM plants exhibited enhanced photosynthetic efficiency as compared to non-AM plants. The only exception

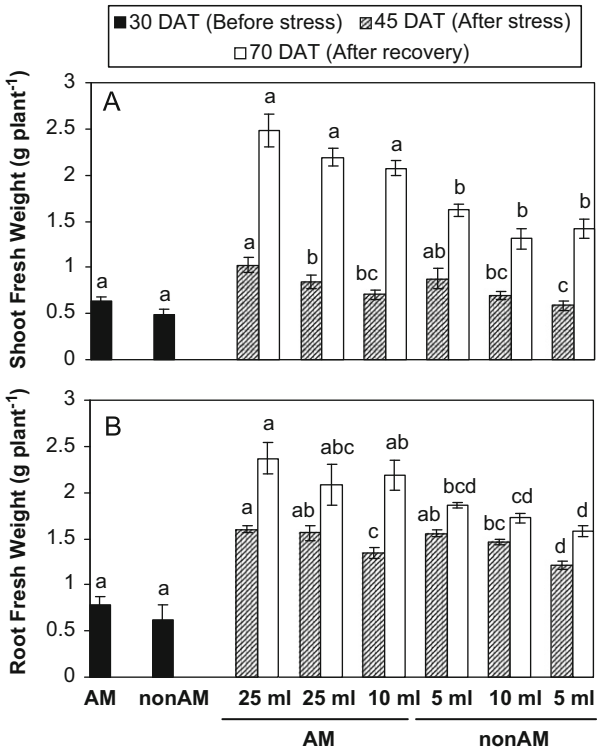

Fig. 1. Shoot (A) and root (B) fresh weights (g plant^{-1}) of rice plants inoculated or not inoculated with the AM fungus *G. intraradices*. A group of plants from both treatments were harvested 30 d after transplanting (30 DAT), just before starting the stress treatments. Plants were then subjected to a drought stress period of 15 d by irrigation with 10 or 5 mL of aqueous nutrient solution or remained as well-watered control receiving 25 mL of aqueous nutrient solution. A group of these plants were harvested after the 15 d drought stress period (45 DAT). Finally, a group of plants were allowed to recover from drought by growing for additional 25 d with 25 mL of aqueous nutrient solution. Then, the plants were harvested (70 DAT). For each harvest time, means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple range test ($n=4$).

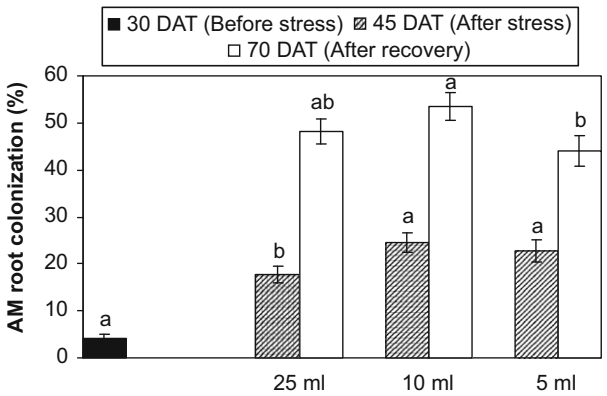

Fig. 2. Percentage of AM root colonization of rice plants inoculated with the AM fungus *G. intraradices*. A group of plants were harvested 30 d after transplanting (30 DAT), just before starting the stress treatments. Plants were then subjected to a drought stress period of 15 d by irrigation with 10 or 5 mL of aqueous nutrient solution or remained as well-watered control receiving 25 mL of aqueous nutrient solution. A group of these plants were harvested after the 15 d drought stress period (45 DAT). Finally, a group of plants were allowed to recover from drought by growing for an additional 25 d with 25 mL of aqueous nutrient solution. Then, the plants were harvested (70 DAT). For each harvest time, means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple range test ($n=4$).

was found in control non-AM plants that received 25 mL of aqueous nutrient solution, which showed no significant differences to AM plants. The AM symbiosis enhanced this

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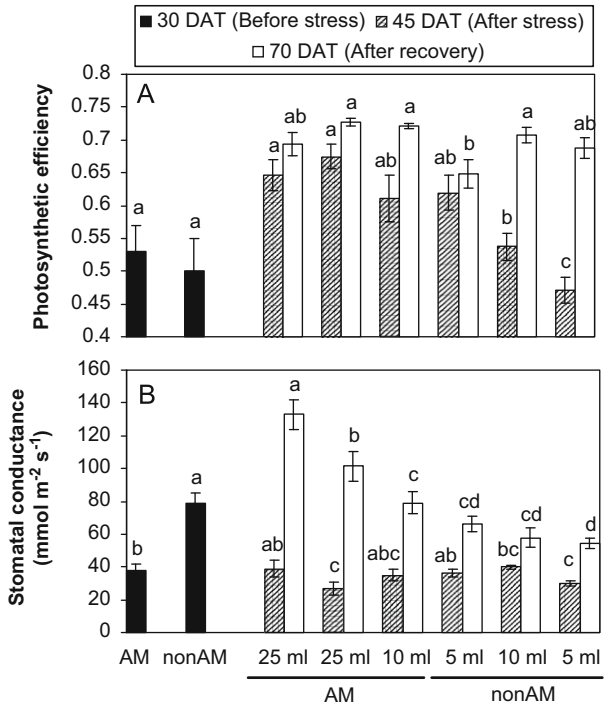

Fig. 3. Photosynthetic efficiency (A) and stomatal conductance (B) of rice plants inoculated or not with the AM fungus *G. intraradices*. See legend for Fig. 1.

parameter by 44% when the plants were irrigated with 10 mL of aqueous nutrient solution and by 40% in plants that were irrigated with 5 mL of aqueous nutrient solution. When plants were allowed to recover for 25 additional days (70 DAT), the photosynthetic efficiency still rose in all treatments with no significant differences between AM and non-AM plants.

Stomatal conductance

At the first measurement (30 DAT), the stomatal conductance of non-AM plants was almost two times higher than that of AM plants (Fig. 3B). After 15 d of drought stress (45 DAT), the stomatal conductance was reduced in the non-AM plants compared to the first measurement, showing similar values in all treatments. After recovery from drought (70 DAT), the AM plants considerably enhanced their stomatal conductance. However, the AM plants that had been previously irrigated with 10 or with 5 mL of aqueous nutrient solution had lower stomatal conductance than the AM plants kept as control with 25 mL of nutrient solution. At this stage, non-AM plants always exhibited lower stomatal conductance than their AM counterparts, regardless of water regime.

Proline content

At 45 DAT, the accumulation of proline in the shoots of AM and non-AM rice plants was induced by the drought stress imposed (Fig. 4). In fact, plants irrigated with 10 or 5 mL of aqueous nutrient solution showed enhanced proline content compared to their corresponding control plants irrigated with 25 mL of aqueous nutrient solution. In any case, the amount of proline accumulated at each water regime was slightly higher in non-AM than in AM plants. After recovery from drought (70 DAT), the proline content was still higher in the plants that had been previously subjected to drought (10 or 5 mL). However, at this

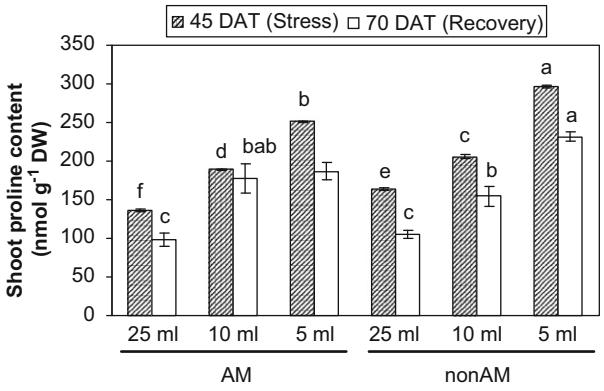

Fig. 4. Shoot proline content (nmol g^{-1} DW) of rice plants inoculated or not with the AM fungus *G. intraradices*. Plants were subjected to a drought stress period of 15 d by irrigation with 10 or 5 mL of aqueous nutrient solution or remained as well-watered control receiving 25 mL of aqueous nutrient solution. A group of these plants were harvested after the 15 d drought stress period (45 DAT). Finally, a group of plants were allowed to recover from drought by growing for additional 25 d with 25 mL of aqueous nutrient solution. Then, the plants were harvested (70 DAT). For each harvest time, means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple range test ($n = 4$).

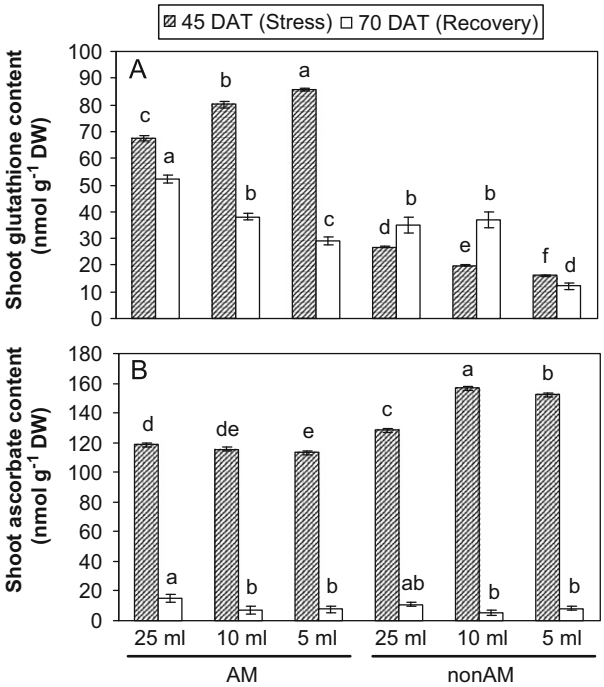

Fig. 5. Shoot glutathione (A) and ascorbate (B) contents (nmol g^{-1} DW) of rice plants inoculated or not with the AM fungus *G. intraradices*. See legend for Fig. 4.

stage, there were no significant differences between AM and non-AM plants for each water regime.

Accumulation of antioxidant compounds

When plants had been subjected to drought for 15 d (45 DAT), the amount of glutathione accumulated in plant shoots was considerably higher in AM than in non-AM plants (Fig. 5A). This effect was observed in all water regimes, including the non-stressed controls maintained with 25 mL of aqueous nutrient solution (152% of increase of glutathione content). However, the differences between AM and non-AM plants in the amount of

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glutathione accumulated increased as drought stress was more severe, reaching a 436% increase in AM plants fed with 5 mL of aqueous nutrient solution as compared to the corresponding non-AM plants.

When plants had recovered from drought (70 DAT), the glutathione content was still higher in AM than in non-AM plants, except for plants that had been previously irrigated with 10 mL of aqueous nutrient solution.

With respect to the ascorbate content (Fig. 5B), after the drought stress period (45 DAT), all the treatments showed a high level of ascorbate. However, non-AM plants accumulated more ascorbate than AM plants. The accumulation of ascorbate decreased considerably after recovery from drought (70 DAT) and, at this stage, no significant differences among treatments were found.

Hydrogen peroxide accumulation and oxidative damage to lipids

At 45 DAT, hydrogen peroxide accumulated in plants subjected to drought stress (Fig. 6A), especially in non-AM plants irrigated with 5 mL of aqueous nutrient solution (177% of increase as compared to well-watered non-AM plants). In contrast, in AM plants irrigated with 5 mL of aqueous nutrient solution, the accumulation of hydrogen peroxide increased by only 85% compared to well-watered AM plants. Under all water regimes, the amount of hydrogen peroxide accumulated was higher in the non-AM plants than in AM plants. When the plants were allowed to recover from drought for 25 additional days (70 DAT), the amount of hydrogen peroxide accumulated in plant shoots was low and did not show significant differences among treatments.

The oxidative damage to lipids was measured as the amount of lipid peroxides formed in the different treatments (Fig. 6B). The results clearly showed that AM plants did not enhance lipid peroxidation after the drought stress period (45 DAT). In contrast, non-AM plants subjected to drought accumulated more lipid peroxides than the corresponding AM plants. This effect was visible even in plants not subjected to drought (an increase of 97%), although it was more evident in the plants subjected to drought (an increase of 116% in plants irrigated with 10 mL of aqueous nutrient solution and of 155% in plants irrigated with 5 mL of aqueous nutrient solution). After recovery from drought (70 DAT), the level of lipid peroxides decreased in non-AM plants and equalized those of AM plants. No significant differences among treatments were found.

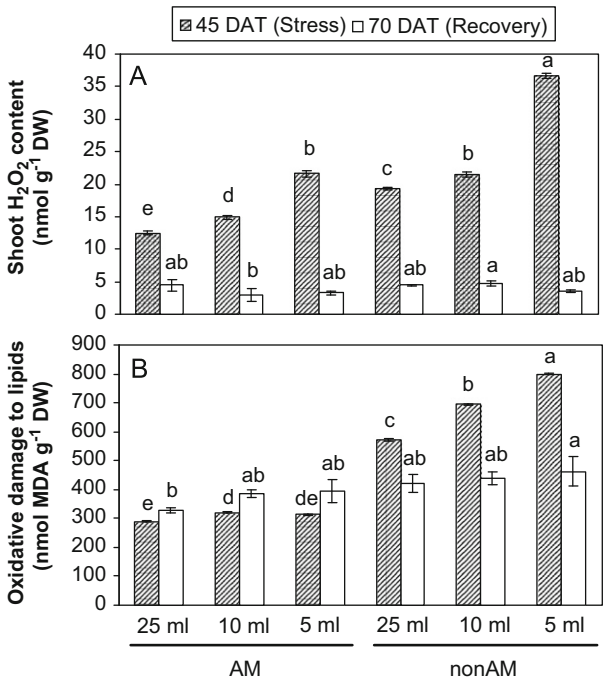

Fig. 6. Shoot hydrogen peroxide content (nmol g^{-1} DW) (A) and shoot oxidative damage to lipids (nmol MDA g^{-1} DW) (B) of rice plants inoculated or not with the AM fungus *G. intraradices*. See legend for Fig. 4.

Discussion

It is currently accepted that AM symbiosis protects host plants against the detrimental effects of water deficit and that the contribution of AM symbiosis to plant drought tolerance is due to a combination of nutritional, physical and cellular effects (Ruiz-Lozano, 2003). The AM symbiosis generally increases host plant growth due to improved plant nutrition (Smith and Read, 1997). The beneficial effects of different mycorrhizal fungi on plant growth under drought conditions have been demonstrated in various plant species (reviewed by Augé, 2001). In this study, the positive effects of AM symbiosis on plant growth were only visible 70 DAT. This indicates that, in rice plants and under the growing conditions assayed here, the colonization by *G. intraradices* effectively stimulated plant growth, although it took longer than for other host plants. This result may be related to the fact that it was only at 70 DAT that the rice plants reached a considerable level of AM colonization (ranging from 44% to 53%). In fact, at 30 DAT the level of root colonization was only 4% and at 45 DAT, the level of root colonization was about 20%. The stimulation of plant growth by AM symbiosis is not always linked to the degree of root colonization. The literature on AM symbiosis reflects that there is no threshold value of root colonization for plant growth enhancement. Rather, this depends on the plant and fungal species involved in the symbiosis and on the specific growth conditions. However, it is generally assumed that a higher rate of fungal root colonization will enhance AM effects on plant development (Smith and Read, 1997). Vallino et al. (2009) found comparable levels of AM root colonization (ranging from 14% to 51%) in 13 rice varieties cultivated also under aerobic conditions. In any case, to determine more precisely the cause of the plant growth enhancement, we evaluated the effects of mycorrhization on a variety of plant physiological and biochemical processes.

Many plants species decrease the osmotic potential of their cells by synthesizing and accumulating compatible osmolytes, such as proline, that participate in the osmotic adjustment. In this way, plants enhance their tolerance against drought stress (Yoshiba et al., 1997). In addition to acting as an osmoprotectant, proline also serves as a sink for energy to regulate redox potentials, as a hydroxyl radical scavenger, as a solute that protects macromolecules against denaturation and as a means of reducing acidity in the cell (Kishor et al., 1995; Smirnoff, 1993). In this study, the accumulation of proline was clearly visible in both AM and non-AM plants, which significantly enhanced their proline content when the water applied was reduced. In any case, the amount of proline accumulated was always lower in AM than in non-AM plants. This effect has been related to the protection of host plants from dehydration stress trough primary drought avoidance mechanisms. Thus, AM plants may need to accumulate less proline than their non-AM counterparts (Aroca et al., 2008; Porcel et al., 2004).

In this study, AM plants exhibited better performance of photosystem II when subjected to drought stress, as well as enhanced transpiration rates after recovery from drought. The higher values of photosynthetic efficiency in AM plants

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(closer to the theoretical maximum of 0.83) indicate that the photosynthetic apparatus of these plants was less damaged by the drought stress imposed (Germ et al., 2005). These two effects may also have accounted for the enhanced plant growth of AM plants, most likely by enhancing CO_2 fixation during and after drought stress. Indeed, several studies have shown a positive correlation between tolerance to drought stress and maintenance of efficiency of photosystem II, which also keep plant productivity (Loggini et al., 1999; Saccardi et al., 1998). In addition, the better performance of photosystem II and the enhanced transpiration in AM plants could have contributed to decreased photorespiration, leading to lower ROS production in these plants (Cadenas, 1989). Indeed, rice plants are very sensitive to oxidative stress (Maheshwari and Dubey, 2009). In this study, the accumulation of H_2O_2 increased in treatments subjected to drought, especially in non-AM plants irrigated with 5 mL of aqueous nutrient solution. The AM plants also enhanced their accumulation of H_2O_2, but to a lower extent than non-AM plants. The oxidation of membrane lipids is a reliable indication of uncontrolled free radical production, and hence, of oxidative stress (Noctor and Foyer, 1998). Accordingly, the amount of lipid peroxides was quantified in shoots of rice plants under the different treatments. Curiously, AM plants did not enhance lipid peroxidation after the drought stress period (45 DAT), while non-AM plants accumulated significant amounts of lipid peroxides.

In this study, we also measured the accumulation of glutathione and ascorbate in plant shoots after the different treatments. The most significant differences were found regarding the glutathione content. AM plants had a consistent enhancement of glutathione content as compared to non-AM plants. This was especially evident when plants were subjected to the most severe water deficit (irrigation with only 5 mL of aqueous nutrient solution), since AM plants showed 436% of glutathione increase as compared to their non-AM counterparts.

In all living organisms, glutathione (GSH) is the major low molecular weight thiol-containing compound. Glutathione exerts its antioxidant function by reaction with superoxide radicals, peroxy radicals and singlet oxygen followed by the formation of oxidized glutathione and other disulfides (Meyer, 2007). In addition, it has several important physiological functions, including the removal of toxic oxygen derivatives in the ascorbate–glutathione cycle, the induction of several enzyme activities, and it participates in sulphur metabolism and gene expression (Foyer et al., 1995).

The ascorbate is also an important non-enzymatic antioxidant compound because it is involved in the removal of H_2O_2 by ascorbate peroxidases, which use ascorbate as electron donor, and is closely related to glutathione in the ascorbate–glutathione cycle (Noctor and Foyer, 1998). However, in this study, the ascorbate content of AM plants was lower than in non-AM plants. We do not know why the accumulation of ascorbate and glutathione followed an opposite trend in AM plants, but it may be that rice plants have a preference for glutathione as an antioxidant compound compared to ascorbate. Indeed, in a recent study, Maheshwari and Dubey (2009) found that glutathione was also involved in the response of rice plants to the oxidative stress induced by nickel toxicity. The enhanced accumulation of glutathione found in AM plants in this study may also be related to the additional functions ascribed to glutathione, such as the induction of several enzyme activities or its participation in sulphur metabolism and regulation of gene expression (Foyer et al., 1995).

Kranner (2002) correlated the amount of glutathione with different degrees of desiccation tolerance in lichens. Thus, the consistently higher contents of glutathione in AM plants than in non-AM plants may have contributed to protecting rice plants against the oxidative stress generated by drought, preventing the oxidative damage to biomolecules such as lipids and protecting the host plants against the drought stress. In addition, previous studies carried out with AM and non-AM soybean plants demonstrated that the AM symbiosis consistently enhanced glutathione reductase activity in roots and nodules of AM plants. In these studies, it was suggested that the enhanced glutathione reductase activity contributed to generate reduced glutathione and to decrease the oxidative damage to biomolecules in soybean plants which, ultimately protected AM plants against drought stress (Porcel et al., 2003; Ruiz-Lozano et al., 2001). A similar reduction of oxidative damage to lipids by AM symbiosis has been observed in tomato plants subjected to salt stress (Zhong et al., 2007).

In conclusion, the results obtained in this study show that a crop plant such as *O. sativa* can reach a good level of AM root colonization when cultivated under aerobic conditions. Under these conditions, rice can also benefit from the AM symbiosis and improve their long-term development after a drought stress period. The AM symbiosis ameliorated the plant photosynthetic efficiency under stress, induced the accumulation of the antioxidant molecule glutathione and reduced the accumulation of hydrogen peroxide and the oxidative damage to lipids in these plants. Thus, these combined effects enhanced the plant performance after a drought stress period.

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