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Azospirillum and arbuscular mycorrhizal colonization enhance rice growth and physiological traits under well-watered and drought conditions

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ABSTRACT

The response of rice plants to inoculation with an arbuscular mycorrhizal (AM) fungus, *Azospirillum brasilense*, or combination of both microorganisms, was assayed under well-watered or drought stress conditions. Water deficit treatment was imposed by reducing the amount of water added, but AM plants, with a significantly higher biomass, received the same amount of water as non-AM plants, with a poor biomass. Thus, the water stress treatment was more severe for AM plants than for non-AM plants. The results showed that AM colonization significantly enhanced rice growth under both water conditions, although the greatest rice development was reached in plants dually inoculated under well-watered conditions. Water level did not affect the efficiency of photosystem II, but both AM and *A. brasilense* inoculations increased this value. AM colonization increased stomatal conductance, particularly when associated with *A. brasilense*, which enhanced this parameter by 80% under drought conditions and by 35% under well-watered conditions as compared to single AM plants. Exposure of AM rice to drought stress decreased the high levels of glutathione that AM plants exhibited under well-watered conditions, while drought had no effect on the ascorbate content. The decrease of glutathione content in AM plants under drought stress conditions led to enhance lipid peroxidation. On the other hand, inoculation with the AM fungus itself increased ascorbate and proline as protective compounds to cope with the harmful effects of water limitation. Inoculation with *A. brasilense* also enhanced ascorbate accumulation, reaching a similar level as in AM plants. These results showed that, in spite of the fact that drought stress imposed by AM treatments was considerably more severe than non-AM treatments, rice plants benefited not only from the AM symbiosis but also from *A. brasilense* root colonization, regardless of the watering level. However, the beneficial effects of *A. brasilense* on most of the physiological and biochemical traits of rice plants were only clearly visible when the plants were mycorrhized. This microbial consortium was effective for rice plants as an acceptable and ecofriendly technology to improve plant performance and development.

Introduction

Rice (*Oryza sativa* L.) is considered the most important crop for human consumption, providing staple food for more than half of the world's population. It accounts for 23% of the world's caloric intake (Khush, 2003). The demand for rice production is increasing as the global population increases (Bernier et al., 2008), with about two-thirds of the total rice production grown under irrigation (Maclean et al., 2002). Rice has the evolutionary particularity of being semi-aquatic. The conventional system for irrigating rice is to flood, which provides water and nutrient supply under anaerobic conditions and uses large amounts of water. However, about half of the rice area in the world does not have sufficient water to maintain flooded conditions, and yield is therefore 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. 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).

In addition to these environmental problems, a challenge for modern sustainable rice production is to decrease the amount of water used in rice production while maintaining or increasing the rice yield. However, rice itself has relatively few adaptations to

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water-limited conditions and is extremely sensitive to drought stress (Kamoshita et al., 2008). One possibility to increase plant water acquisition and/or drought tolerance is to use beneficial microorganisms as inoculants. Soil microorganisms such as AM fungi symbiotically associated with plant roots and interacting with specific microbial communities are able to develop a range of activities to increase plant growth and crop productivity under stressed conditions (Barea et al., 2005; Azcón and Barea, 2010). 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). Thus, to obtain benefits from the AM symbiosis, rice should be grown under non-flooded conditions, creating aerobic conditions in the soil that stimulate colonization of rice roots by AM fungi (Vallino et al., 2009; Ruíz-Sánchez et al., 2010).

It is accepted that microorganisms such as AM fungi and plant growth-promoting rhizobacteria (PGPR) such as *A. brasilense* are very effective in enhancing the ability of plants to become established and to cope with stress situations such as drought and nutrient limitation (Azcón and Barea, 2010). In a recent study, it was shown that the AM fungus *Glomus intraradices* enhanced rice growth, the photosynthetic efficiency and the antioxidative responses of rice plants to drought stress (Ruíz-Sánchez et al., 2010). The positive influence of inoculation with *A. brasilense* (the same strain used in this study) on biomass accumulation and grain productivity of rice plants grown under field conditions has also been shown (García de Salamone et al., 2010). Recently, the positive interactions developed under drought conditions between *Pseudomonas putida* or *Bacillus megaterium* and AM fungi in stimulating plant growth and drought tolerance have been reported (Marulanda et al., 2009). However, although there are studies using AM fungi and N_2-fixing bacteria to increase drought tolerance in legume plants (Barea et al., 1992; Vázquez et al., 2001), the available literature does not show information about the effects of dual inoculation AM fungi-bacteria on rice performance under drought conditions.

A. brasilense has been successfully inoculated under drought conditions in tomato (Creus et al., 2005), maize (Casanovas et al., 2002) and bean (German et al., 2000), but no information is available on rice under drought. In addition, there are no studies providing an overview of the activity and effect of dual *Azospirillum*–AM fungus inoculation of rice plants and the possible benefits under drought stress conditions. Thus, the present study must be considered as an attempt to increase drought tolerance of rice, one of the most important plants in the world by using a microbial consortium formed by two important endophytic microorganisms such as *A. brasilense* and an AM fungus, which were previously reported as being beneficial for rice development (Ruíz-Sánchez et al., 2010). This investigation can be considered as a next step from previous studies trying to increase the effectiveness of AM-colonization on rice by co-inoculation with the PGPR *A. brasilense*. It must, however, be taken into account that inoculation with AM fungi and with PGPRs often leads to plants with enhanced biomass production as compared to uninoculated plants. Under natural or field conditions, water deficits are produced because of a lowering in rainfall or water availability in the soil. This water limitation is of similar magnitude for large plants and for small plants. In contrast, larger plants have higher water requirements than smaller plants due to their higher evapotranspiration rates. Thus, the same water deficit will lead to a more pronounced drought stress in a plant with high biomass than in a plant with small biomass. In this study, we also assessed whether microbial treatments (AMF and *Azospirillum*), characterized by enhancing significantly plant biomass production, are still effective under a natural water deficit that affects larger plants more than smaller plants.

Materials and methods

Experimental design and statistical analysis

The experiment consisted of a randomized complete block design with four inoculation treatments: (1) plants inoculated with the AM fungus *Glomus intraradices*; (2) plants inoculated with *Azospirillum brasilense*; (3) plants dually inoculated with *Glomus intraradices* and with *Azospirillum brasilense*; and (4) uninoculated control plants. Two watering treatments were applied to these plants, as described in the "growth conditions" section. Each treatment was replicated six times, totaling 48 plants.

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

Soil and plant materials

The growth substratum consisted of a mixture of loamy soil (collected from Granada province, Spain), sieved (5 mm), diluted with quartz-sand (<1 mm) (1:1, v/v) and sterilized by steaming (100 °C for 1 h for 3 days). The soil had a pH of 8.2 (water); 1.5% organic matter, nutrient concentrations (g kg^{-1}): N, 1.9; P, 1; K, 6.9.

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 1 kg of sterilized substratum (one seedling per pot).

Inoculation treatments

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.

Azospirillum brasilense, strain AZ-39 (from University of Buenos Aires, Argentina) was used as bacterial inoculum. Liquid culture was prepared based on NFb medium (Döbereiner and Pedrosa, 1987). The medium was centrifuged (4500 × *g* for 5 min) and the pellet resuspended in sterile water. One milliliter of solution containing 10^8 cfu was added to the corresponding pots at transplanting time and 15 days later.

Growth conditions

The experiment was carried out under greenhouse conditions with temperatures ranging from 24 to 28 °C, 16/8 light/dark period, a relative humidity of 50–70% and a photosynthetic photon flux density of 800 μE m^{-2} s^{-1}, as measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B).

Plants were grown in pots containing 1 kg of sterilized substratum. During the 30 days after transplanting, plants were maintained under well-watered conditions (100% water holding capacity). At this growing stage, half of the pots were subjected to water stress for four weeks. To do that, the first two week, water application was reduced to 50% as compared to well-watered treatments and during the two last weeks of plant growth, the water application to drought stressed treatments was only 25% of that remaining for well-watered counterparts.

During the experiment, each pot received 10 mL a week of nutrient solution (Hoagland and Arnon, 1950) containing all the nutrients except for P, which was reduced to 25%. At the end of the experiment, plant biomass, AM colonization, stomatal con-

ductance, shoot water potential, photosynthetic efficiency, proline content, lipid peroxidation, and contents of ascorbate and glutathione were determined in rice plants.

Parameters measured

Biomass production

At harvest, the root system was separated from the shoot and their fresh weights (FWs) were determined. The shoot tissues were separated in 0.5 g aliquots and frozen in liquid nitrogen for future determination of antioxidant compounds, proline content and oxidative damage to lipids.

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 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 conducted in the second youngest leaf of four different plants of each treatment.

Shoot water potential

The mid-day leaf water potential (Ψ) was determined 1 day before harvest with a C-52 thermocouple psychrometer chamber and a HR-33T dew point microvoltmeter (Wescor Inc., Logan, UT, USA). Leaf discs were cut, placed inside the psychrometer chamber and allowed to reach temperature and water vapor equilibrium for 20 min before measurements were made by the dew point method.

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 performed in the second youngest leaf from four different plants from each treatment.

Shoot proline content

Free proline was extracted from 0.5 g 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).

Shoot glutathione and ascorbate contents

Glutathione content was measured as described by Smith (1985). Five 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 and 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). Five 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.

Shoot oxidative damage to lipids

Lipid peroxides were extracted by grinding 500 mg of leaves with an 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 then 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 (TBARSs) 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 TBARSs during the acid-heating step of the assay.

Results

Symbiotic development and plant biomass

AM colonization was not observed in non-inoculated plants. No significant differences in the percentage of root colonization were observed between well-watered and droughted rice plants. In contrast, coinoculation of *Azospirillum* considerably increased the percentage of mycorrhizal root colonization independent of the water level applied (Fig. 1).

As summarized in Fig. 2, the mycorrhizal colonization highly increased shoot and root growth, particularly under well-watered conditions. The greatest rice shoot development was reached in plants dually inoculated with AM fungus plus *Azospirillum* under well-watered conditions (560% of increase over uninoculated control plants). At the same time, drought stress had the greatest effect, reducing plant growth in AM-colonized rice plants, which where the largest ones. In any case, under drought stress conditions, AM plants (alone or in combination with *Azospirillum*) continued exhibiting higher shoot fresh weight than control or

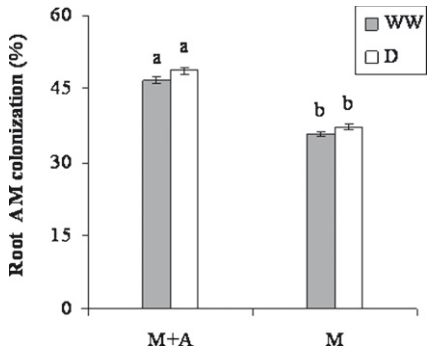

Fig. 1. Percentage of mycorrhizal root colonization in rice plants coinoculated or not with *Azospirillum brasilense* strain AZ-39 (A) and *Glomus intraradices* (M) under well-watered (WW) or drought stress (D) conditions.

Azospirillum-inoculated plants. Uninoculated control plants and *Azospirillum*-inoculated plants did not show any differences in plant growth under well-watered or under drought stress conditions (Fig. 2).

Plant height and number of leaves

Rice shoot height and number of leaves were also increased by AM-colonization under both water conditions and the greatest values were found in well-watered rice plants dually inoculated (Fig. 3). In non-mycorrhizal rice plants, no effects of *Azospirillum* or water level were found.

Photosynthetic efficiency and stomatal conductance

The efficiency of photosystem II was assessed by measuring chlorophyll fluorescence (Fig. 4). This parameter was enhanced by

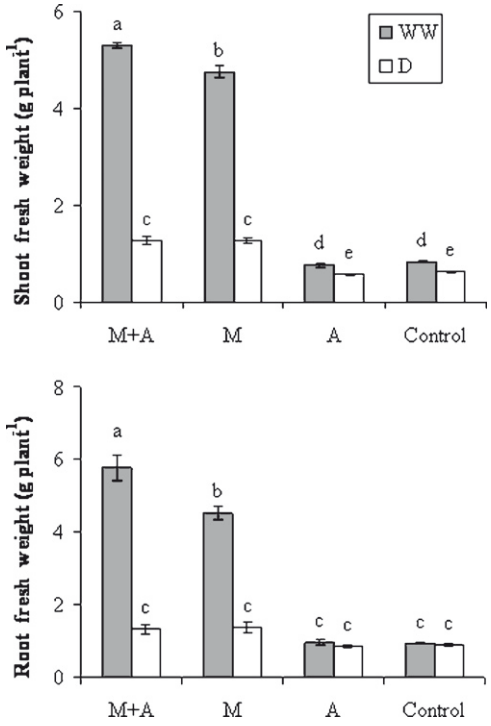

Fig. 2. Shoot and root fresh weights in rice plants inoculated with *Azospirillum brasilense* strain AZ-39 (A) and/or with the arbuscular mycorrhizal fungus *Glomus intraradices* or remaining as uninoculated controls. Plants were cultivated under well-watered conditions (WW) or subjected to drought (D).

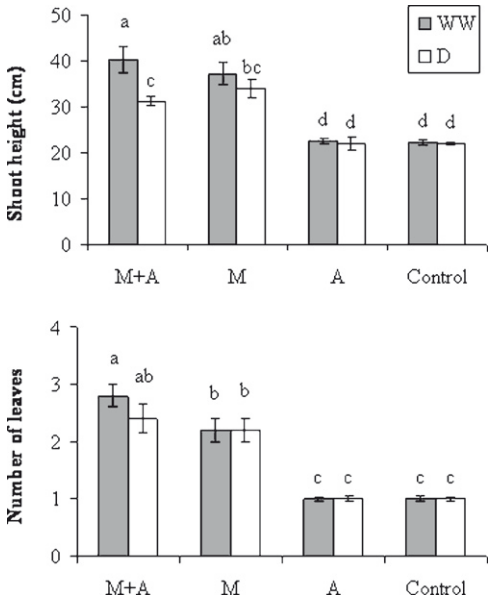

Fig. 3. Shoot height (cm) and number of leaves in rice plants. See legend for Fig. 2.

both AM colonization and *Azospirillum* inoculation, regardless of water regime applied. However, AM colonization increased this value to a greater extent than *Azospirillum* inoculation, with no additive effect after coinoculation of both microorganisms. Water conditions did not change photosynthetic efficiency.

Under well-watered conditions, the stomatal conductance of mycorrhizal plants was much higher than that of non-AM plants (Fig. 4). The inoculation of *Azospirillum* alone did not change this parameter. However, plants dually inoculated cultivated under well watered conditions reached the highest stomatal conductance. A similar trend was observed under drought stress conditions. Inoculation of *Azospirillum* increased this physiological value in AM-colonized plants by 80% under drought stress conditions and by 35% under well-watered conditions compared to plants singly inoculated with the AM fungus.

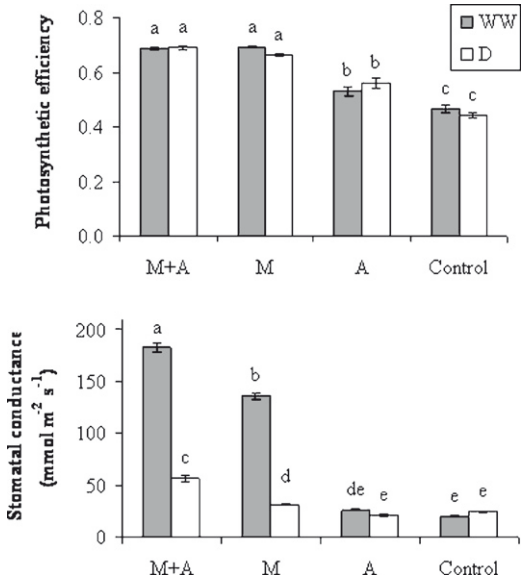

Fig. 4. Photosynthetic efficiency (photosystem II) and stomatal conductance in rice plants. See legend for Fig. 2.

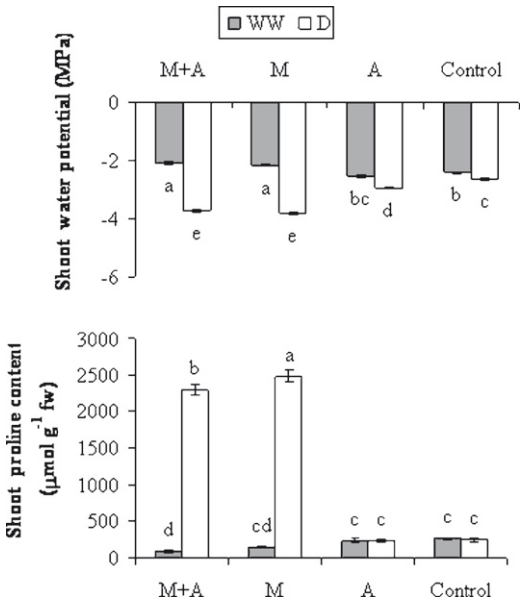

Fig. 5. Shoot water potential and shoot proline content in rice plants. See legend for Fig. 2.

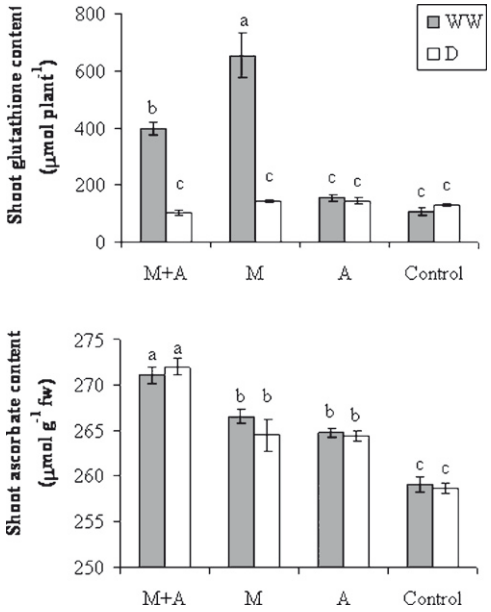

Fig. 6. Shoot glutathione and ascorbate content in rice plants. See legend for Fig. 2.

Shoot water potential and proline accumulation

Shoot water potential decreased in all treatments as a consequence of drought stress (Fig. 5). However, the decrease was more pronounced in AM plants, which showed a considerably higher biomass than non-AM plants when the stress began and were more affected by the stress imposed, with no effect of *Azospirillum* coinoculation. The decrease of shoot water potential in AM plants subjected to drought stress was parallel to the important accumulation of proline in shoot of these AM plants. In contrast, uninoculated control plants or plants singly inoculated with *Azospirillum* did not show significant differences in shoot proline content.

Accumulation of antioxidant compounds and oxidative damage to lipids

AM plants cultivated under well-watered conditions contained the highest levels of glutathione in their shoots, while control plants or those inoculated only with *Azospirillum* exhibited the lowest levels of glutathione (Fig. 6). In AM plants cultivated under well-watered conditions, the level of glutathione decreased by 39% when these plants were coinoculated with *Azospirillum*. When the plants were subjected to drought stress, the glutathione accumulated was similar in all treatments.

Regarding ascorbate content, both the single AM colonization and the inoculation with *Azospirillum* increased significantly this value related to the control treatments, with an additive effect after coinoculation of both microorganisms (Fig. 6). No effect of water regime was observed for any of the inoculation treatments.

The oxidative damage to lipids (measured as the amount of lipid peroxides formed) increased in plants singly inoculated with *Azospirillum* and in AM plants as compared to the uninoculated control ones, regardless of water regime (Fig. 7). Drought stress enhanced the amount of lipid peroxides only in AM plants, which had the bigger size and were more affected by the stress imposed. However, the coinoculation of AM plants with *Azospirillum* decreased significantly lipid peroxidation both under well-watered and under drought stress conditions as compared to single AM inoculated plants.

Discussion

The demand for rice production is still rising because of the continuous increase in world population. The world population is predicted to reach approximately 8 billion by 2030 and there is a need to further increase rice production by 40% in the next 20 years (Bernier et al., 2008). One possible way to enhance rice production is to improve yield and tolerance to stresses by means of rhizosphere microbial manipulation. Among the microbial groups, PGPR and AM fungi are able to promote activities which can improve agricultural development (Barea et al., 2005). In addition, the coinoculation with PGPR and AM fungi has been proposed as an efficient procedure to increase plant growth (Azcón and Barea, 2010). In fact, there are synergistic effects on plant growth when bacteria (PGPR) and AM fungi are coinoculated, particularly under growth limited conditions (Vivas et al., 2003). The reported results show that the growth responses of rice plants to AM inoculation and to the dual AM + *Azospirillum* inoculation have been really significant. Indeed, under well-watered conditions AM plants increased SFW by 500% as compared to uninoculated control plants and AM + *Azospirillum* plants which increased by 560%. Under drought stress conditions, both AM treatments increased SFW by 103% compared to the corresponding uninoculated control plants.

In the present study, performance of photosystem II was higher in AM-colonized plants (singly or in combination with *Azospirillum*) and was also improved, although to a lesser extent, in rice plants inoculated only with *Azospirillum*. This indicates better performance of the photosynthetic apparatus and is surely

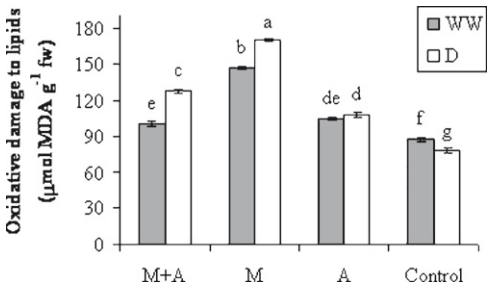

Fig. 7. Oxidative damage to lipids in rice plants. See legend for Fig. 2.

involved in the better growth observed in AM-colonized rice plants. Indeed, several studies have shown a positive correlation between tolerance to drought stress and maintenance of efficiency of photosystem II, which also keeps plant productivity (Loggini et al., 1999; Saccardi et al., 1998). Recently, a study carried out under very different growth conditions showed that AM colonization increased rice shoot biomass by 50%, and this effect was also attributed to enhancement of rice photosynthetic efficiency (Ruíz-Sánchez et al., 2010).

In this study, drought stress was imposed by reducing the amount of water applied to the plants, but all the droughted treatments (control, AM, *Azospirillum* or AM + *Azospirillum*) received the same amount of water daily. In contrast, AM and non-AM plants were remarkably different in plant size (see SFW, RFW, plant height and number of leaves). Thus, AM plants needed considerably more water than non-AM plants since their total evapotranspiration rate was significantly higher than non-AM plants. The low amount of water applied during the four weeks of stress treatment resulted in physiological drought stress for these plants. This was evidenced by the low water potential in their shoots tissues, by the important accumulation of proline or by the significant decrease of stomatal conductance. In contrast, the same amount of water applied to the non-AM plants, with a considerably lower plant development and total evapotranspiration rate, led to a less pronounced physiological drought in these plants, as evidenced by their higher shoot water potential or the low proline accumulated in their tissues. This fact must be considered when interpreting the different data obtained in this study. In spite of this, even after the drought stress period, the SFW of AM and AM + *Azospirillum* plants increased by 103% compared to the corresponding uninoculated control plants. These results demonstrated the importance of mycorrhization for rice development both under well-watered and under drought stress conditions. However, at this stage we cannot rule out the possibility that the negative effects of drought can appear at later stages of the rice life cycle, as suggested by Bernier et al. (2008), which also affects AM and non-AM plants differently. This should be examined in future studies by maintaining plants until tillering and grain-filling stages.

The results also showed that AM colonization was the most important factor for rice development, as mycorrhizal growth enhancement was more relevant than that produced by *Azospirillum.* Indeed, the single inoculation with *Azospirillum* did not improve rice plant development. Only when *Azospirillum* was coinoculated with the AM fungus did we observe a positive plant growth response in terms of SFW, SDW or number of leaves. The reasons for the lack of effect of *Azospirillum* when inoculated alone on rice plant growth are not known, and contrast with previous reports on this bacterium (García de Salamone et al., 2010). However, the rice cultivar, the soil used and the growing conditions in our study were very different than those in the former study, which was conducted under field conditions. Thus, interactions with many other soil microorganisms could occur in the study carried out by García de Salamone et al. (2010), while the present work was carried out on sterilized substratum where only the bacterium and/or the AM inoculum were applied. It is also interesting that *Azospirillum* increased the mycorrhizal colonization irrespective of the water level in the medium. This finding, together with the positive effect of coinoculation, suggests possible activity of *A. brasilense* as a mycorrhiza helper bacterium (Garbaye, 1994; Frey-Klett et al., 2007) that could involve promotion of fungal propagules germination, stimulation of mycelial growth or changes in the root architecture through the production of growth factors. The bacterium may also have contributed to nutrient mobilization from soil minerals and organic matter (Frey-Klett et al., 2007), which could contribute to the enhanced mycorrhizal performance observed in this study. These aspects were not addressed in this study, but merit future investigation. Although there is evidence that the degree of AM root colonization is not necessarily linked to the plant growth responses (Marulanda et al., 2003), in this study we found a positive correlation between the percentage of mycorrhizal root length achieved and plant growth stimulation. 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.

From the data obtained in this study, it is clear that only AM plants (inoculated alone or with *Azospirillum*) suffered the detrimental effects of drought stress. As noted above, this was due to the important differences in plant biomass between AM and non-AM plants. The amount of water added during the drought stress period to the two non-AM treatments was enough to maintain a normal physiological status in these plants. This is why non-AM plants did not accumulate proline in their shoots tissues or showed a lower oxidative damage to lipids than to AM plants. In contrast, AM or AM + *Azospirillum* plants subjected to drought experienced a severe drought stress and enhanced the accumulation of proline. This effect has been related to the protection of host plants from dehydration stress through primary drought avoidance mechanisms. Indeed, rice plants decreased the osmotic potential by synthesizing proline that participates in the osmotic adjustment in order to cope with drought stress (Yoshiba et al., 1997).

Environmental stresses, including water deficit, result in the production of reactive oxygen species (ROS) in plants, in that rice plants are very sensitive to ROS accumulation (Maheshwari and Dubey, 2009). 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). In the present study, under well-watered conditions, AM plants accumulated more glutathione than the rest of treatments, although when these plants were subjected to drought their content of glutathione decreased to equalize that of non-AM plants. On the contrary, plants dually inoculated with AM plus *Azospirillum* accumulated more ascorbate than uninoculated control plants and this did not change upon exposure to drought stress. However, when we measured the lipid peroxidation we observed that AM plants accumulated the highest amount of lipid peroxides. This effect must be linked to the notion that AM plants were more affected by the drought stress imposed than the rest of treatments due to their higher plant biomass. In any case, it is remarkable that *Azospirillum* coinoculation reduced significantly the synthesis and accumulation of glutathione, proline and lipid peroxides in AM plants. These decreasing effects on these compounds by *Azospirillum* inoculation seem to be related to the rice protection from dehydration through drought avoidance mechanisms and suggest that dually inoculated plants were better protected against the drought stress imposed.

In conclusion, the present results showed that, in spite of the fact that drought stress imposed to AM treatments was considerably more severe than to non-AM treatments, the biomass production in AM and AM + *Azospirillum* plants subjected to drought increased by 103% compared to the corresponding uninoculated control plants. The increase in biomass production under well-watered conditions was over 500%. Both results suggest that rice plants benefited not only from the AM symbiosis, but also from *A. brasilense* root colonization, regardless of the watering level. However, the beneficial effects of *A. brasilense* on most of the physiological and biochemical traits of rice plants were only clearly visible when

the plants were mycorrhized. In addition, the coinoculation of both microorganisms enhanced the mycorrhizal colonization of rice roots, suggesting also a possible mycorrhiza helper bacterium activity for *A. brasilense*. Thus, this microbial consortium was effective for rice plants as an acceptable and ecofriendly technology to improve plant performance and development.

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