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

Michel Ruíz-Sánchez[a], Elisabet Armada[b], Yaumara Muñoz[a], Inés E. García de Salamone[c], Ricardo Aroca[b], Juan Manuel Ruíz-Lozano[b,*], Rosario Azcón[b]

[a] Instituto Nacional de Ciencias Agrícolas, Estación Experimental del Arroz "Los Palacios", Gaveta Postal 1, San José de las Lajas, 37200 La Habana, Cuba
[b] Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín (CSIC), Profesor Albareda no. 1, E-18008 Granada, Spain
[c] Department of Crop Production, Faculty of Agronomy, University of Buenos Aires, Avda. San Martin 4453, Buenos Aires, Argentina

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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

* Corresponding author. Tel.: +34 958 121011; fax: +34 958 129600.
E-mail address: juanmanuel.ruiz@eez.csic.es (J.M. Ruíz-Lozano).

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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 ($4500 \times g$ 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 $\mu E\,m^{-2}\,s^{-1}$, 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-