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MYCORRHIZAE ARBUSCULAR SYMBIOSIS IN RICE PLANTS (*Oryza sativa* L.) UNDER WATER STRESS. PART II. BIOCHEMICAL RESPONSE

La simbiosis micorrízica arbuscular en plantas de arroz (*Oryza sativa* L.) sometidas a estrés hídrico. Parte II. Respuesta bioquímica

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ABSTRACT. It is estimated that the world population will continue to increase; however the water resources available to meet crop right now is not enough, it is working to find alternatives that save water and maintain or increase agricultural crop yields. The use of arbuscular mycorrhizal fungi (AMF) is certainly a way that contributes to such purposes. The research was conducted at the Experimental Station of Zaidín, Granada, Spain, in plastic pots with plants of mycorrhizal and non-mycorrhizal rice in semi-controlled conditions, with three water supplies, no stress (25 mL), moderate stress (10 mL) and severe stress (5 mL) for 15 days, with the aim of evaluating the effect of the inoculation of *Rhizoglomus intraradices* in rice plants under water stress and then retrieved on some biochemical parameters. The results showed that the symbiosis HMA reduces the accumulation of hydrogen peroxide and oxidative damage to lipids from an increased accumulation of the antioxidant glutathione. The combined effects of plant metabolism improved after a period of water stress and can be suggested as indicators under conditions of water deficit in plants mycorrhizal rice.

Key words: antioxidants, symbiosis, drought, stress, rice

RESUMEN. Se estima que la población mundial continúe en ascenso; sin embargo, el recurso hídrico disponible para enfrentar las cosechas en estos momentos no es suficiente, es por ello que se trabaja en buscar alternativas que ahorren agua y mantengan o incrementen los rendimientos en los cultivos agrícolas. El uso de los hongos micorrízicos arbusculares (HMA) es sin lugar a dudas, una vía que contribuye a tales propósitos. La investigación se realizó en la Estación Experimental del Zaidín, Granada, España, en macetas plásticas, con plantas de arroz micorrizadas y no micorrizadas, en condiciones semi-controladas, con tres suministros de agua, sin estrés (25 mL), estrés moderado (10 mL) y estrés intenso (5 mL), durante 15 días, con el objetivo de evaluar el efecto de la inoculación de *Rhizoglomus intraradices* en plantas de arroz sometidas a estrés hídrico y después de recuperadas, en algunas variables bioquímicas. Los resultados mostraron que la simbiosis HMA reduce la acumulación de peróxido de hidrógeno y el daño oxidativo a los lípidos a partir de un incremento en la acumulación del antioxidante glutatión. Estos efectos combinados mejoraron el metabolismo de plantas después de un periodo de estrés hídrico y se pueden sugerir como indicadores ante condiciones de déficit hídrico en plantas de arroz micorrizadas.

Palabras clave: antioxidantes, simbiosis, sequía, estrés, arroz

INTRODUCTION

Drought is the most important limiting factor for agricultural production and it is turning into an increasing problem in many world regions (1, 2). In the case of rice (*Oryza sativa* L.) it is an important restrictive factor in rain-fed ecosystems. It was estimated that 18 million tonnes (t) per year or 4 %

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of total rice production is lost because of drought (3), a quantity that has been conservatively valued in the United States of America in 3.6 thousand million dollars. Moreover, it is important to stand out that not only the lack of water reduces the yield potential, but also the time and duration of the drought in relation to phenological processes (4).

According to FAO reports, the United Nations estimated that world population will increase from 6,3 billion inhabitants in 2003 to 8 billion by 2025, so it is considered that rice production should rise over 40 % to meet world demand, at a time where there will be less water availability and less arable lands[A] (5).

In view to the inminent advance of desertification and drought, growers implement different strategies to mitigate the adverse effects of these phenomena on their crops. One of the strategies is the use of arbuscular mycorrhizal fungi (AMF) (6, 7). It has been proven that AMF can protect host plants against the deleterious effects of water deficit, a deficiency in nutrients uptake (phosphorus), protection against pathogens and other problems (8, 9, 10, 11, 12,). Studies done so far have suggested some mechanisms through which the symbiosis plants-AMF can relief drought effects in host plants. The most important ones are the direct uptake and water transfer by the fungus hyphae to the host plant (13, 14, 15), changes in the properties of water retention on the soil (9), better osmotic adjustment of MA plants (6, 7), improved gas exchange, efficient use of water (13), and protection against the oxidative damage generated by drought (16, 17, 18, 19).

This latter mechanism has been recognized as crucial (10, 18), since several degenerative reactions are associated to a series of environmental tensions, water deficit included, that bring about the production of reactive oxygen species (ROS) in plants causing an additional oxidant stress. In general ROS include not only free radical like the superoxide (O_2-) y and hydroxyl radicals (OH^-), but also hydrogen peroxide (H_2O_2); likewise, it is known that oxygen and radicals OH- are not reactive so its production should be minimal (20), while O_2- and H_2O_2 are synthesized at very high rates, even under optimum conditions (21, 22). These radicals and its byproducts are found among the most reactive species known in chemistry, able to indiscriminately react and cause oxidant damage to biomolecules. It favors the occurrence of phenomena like lipidic peroxidation and protein denaturalization (23).

Taking into account all the above, this research looked at evaluating the effect of inoculating rice plants with *Glomus intraradices* subjected to water stress after 30 days of being transplanted (DDT) and after recovery (DR) on some biochemical variables.

MATERIALS AND METHODS

Experimental design

The research was carried out at Zaidín Experimental Station, Granada, Spain, in rice (*Oryza sativa* L.) under semi-controlled conditions, for which the short-cycle cultivar INCA LP-5 was used. At the initial stage (seedbed) 50 % of the seeds were inoculated with *Glomus intraradices*, at the rate of 100 g per each kg of substrate. Plants were transplanted 15 days after germination (DDG) to pots of 500 g of substrate. Another inoculation was done at transplanting (5 g of inoculum per pot), just beneath rice roots.

Experimental conditions

Plants were grown between 60-70 % relative humidity, day and night temperatures were 23 and 19 ºC, respectively, with a photoperiod of 16 hours light and 8 hours darkness at a light intensity of 250 µE m^{-2} s^{-1}, measured with a Licor (Lincoln, NE, EE.UU. model LI-188B).

During the first 30 days after transplanting (DDT), each plant received 25 mL of nutritive solution (24), except phosphorus (P) that decreased to a 25 % in order to avoid the inhibition of the arbuscular mycorrhizae colony. This volume of nutritive solution was applied three times a week in alternate days. Control treatments (without water stress) received 25 mL of nutritive solution three times a week. This moderate water stress consisted in the application of the same quantity of nutrients dissolved in 10 mL of water. The intensive water stress consisted in the application of the same quantity of nutrients dissolved in 5 mL of water.

Treatments:

T1. plants MA+25 mL (Control)
T2. plants MA+10 mL
T3. plants MA+5 mL
T4. plants noMA+25 mL (Control)
T5. plants noMA+10 mL
T6. plants noMA+5 mL

[A] FAO. *Perspectivas de cosechas y situación alimentaria* [en línea], 2010, [Consultado: 4 abril 2015], Disponible en: <www.fao.org/docrep/013/al972s/al972s00.pdf>.

Treatments were distributed on a totally ramdomized design. Data from each sampling were subjected to a simple ANOVA, inoculated plants with arbuscular mycorrhizae (MA) and non-inoculated plants (noMA), followed by Duncan's multiple range test for ($p \leq 0,05$) (25).

Soil and biological material

The substrate used consisted in a mixture of soil from the Zaidín Experiment Station in Granada, Spain, screened (2 mm), with sand (<1 mm) and vermiculite, at the rate of 1:2:6, soil, sand and vermiculite (v/v/v). Sand and vermiculite were sterilized at 120 ºC, for 20 minutes and the soil were sterilized with vapor (a 100 ºC, for1 h, three days in a row). Soil had a pH of 8,1 (water); 1,81 % of organic matter, and at the following nutrient concentrations (mg kg^{-1}): nitrogen (N) 2,5; phosphorus (P) 6,2 (extracted $NaHCO_3$- P); potassium (K) 132. The arbuscular mycorrhizal fungus used was the isolate EEZ 01, pertinent to the collection of the Zaidín Experiment Station in Granada, Spain.

Evaluations were performed after the period of water stress, 45 days after treatment and plants recovery (25 days post-stress) at 70 DDT, where the foliar water potential (MPa), hydrogen peroxide content (H_2O_2) (nmol g^{-1} dry mass (MS)), and the oxidative damage of the membrane were evaluated, through the titration of the malondialdehyde (MDA) (nmol g^{-1} MS), the reduced glutathione content (nmol g^{-1} MS), and reduced ascorbate content (nmol g^{-1} MS).

Measured parameters

Water potential

A system made up of the microvoltimeter HR- 33T, connected to a psychometric chamber C52 (Wescor Inc, Logan, UT, USA), as described by Porcel and Ruiz-Lozano (16) was used. A disk from the central part of the leaf was taken, pertinent to the upper third of the plant (0.0005 m^2 of diameter) and it was placed on the chamber. The temperature and water vapor of the disk was stabilized for 15 minutes before reading the water potential with the microvoltimeter. It was expressed in (MPa).

Hydrogen peroxide content

The hydrogen peroxide content was determined in the leaves (26), with slight modifications described by another author (27) at 508 nm absorbance in the spectrophotometer (Hitachi, model U-1900, Japan).

Oxidative damage to lipids

The oxidative damage to lipids was determined by the reading of the absorbance at 532 nm and 600 nm (28) in the spectrophotometer (Hitachi, model U-1900, Japan), this parameter was estimated from the content of reactive substances of tiobarbituric acid and it was expressed as equivalents of MDA (23). The calibration curve was made with MDA, in the range of 0.1-10 nmol. The target for all samples was prepared by the substitution of the sample by the extraction medium.

Reduce gluthathione content

The glutathione content was determined at 412 nm of absorbance. A patter curve was made from a glutathione pattern solution (50 mM) at the following concentrations: 0, 10, 20, 30, 40, and 50 µM (29).

Reduced ascorbate content

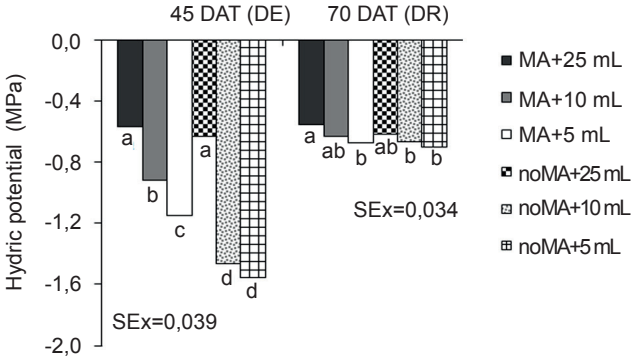
Ascorbate was quantified by photometry (30), upon the reduction of 2,6-dichlorophenolindophenol (DCPIP). Absorbance was immediately measured at 524 nm. Ascorbate content was estimated by reference to a patter curve with ascorbic acid at 2 mM con 0; 0,2; 0,3 and 0,4 mM.

RESULTS AND DISCUSSION

The first part of this article reviewed and discussed the response of rice plantas inoculated and non-inoculated subjected to different water stress intensities, from the physiological and photosynthetic efficiency point of vew, the percentage of symbiotic colonization of the roots, fresh mass of the aerial and radical part in addition to proline content (31).

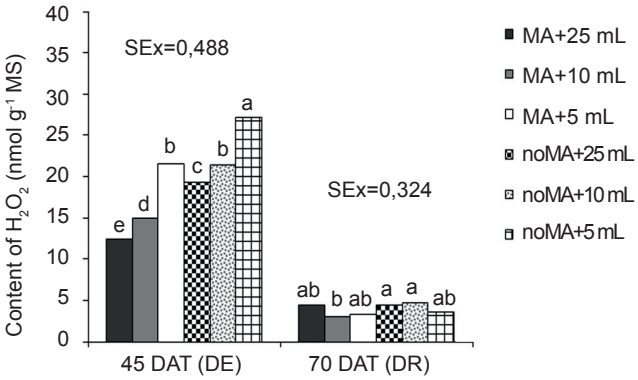
Figure 1, shows the water potential of the MA and noMA plants subjected to water stress for 15 days (evaluated at 45 days after treatment). The results confirmed the effect induced by a water deficit in plants with potential that became more negative regarding control treatments that were less negative (MA+25 mL and noMA+25 mL), without significant differentes between MA and noMA plants. On the other hand, it is important to stand out that water potentials of MA treatments (10 and 5 mL) were less negative than noMA (10 and 5 mL), a behavior reported by other authors in tomato (*Solanum lycopersicon* L.) and lettuce (*Lactuca sativa* L.) (16, 32) respectively.

After the recovery of the plants (70 days after treatment), the water potential decreased in those treatments subjected to water stress, to the degree of not finding differences between the MA plants MA with 10 mL and the control, and in turn, between irrigated plants with 5 mL and those irrigated with 10 mL. As to noMA plants, no significant differences were observed among treatments.



Means with equal letters do not differ significatively (p≤0,05) according to Duncan's multiple range test

Figure 1. Water potential (MPa) of rice plants inoculated or not with AMF *Glomus intraradices*, subjected to water stress and evaluated after the stress (DE) and recovery (DR)



Means with equal letters do not differ significatively (p ≤ 0.05) according to Duncan's multiple range test

Figure 2. Hydrogen peroxide content (nmol g^{-1} MS) in rice plants inoculated or not with AMF *Glomus intraradices* subjected to water stress and evaluated after the stress (DE) and recovery (DR)

The increase or reduction of the water potential in plants is very much related to the presence of mycorrhizal symbiosis and with the time in which water stress was applied, in addition to its severity. This behavior is explained because fungal hyphae improve this indicator as well as the hydraulic conductivity of the root, which reduces resistance to water flow (7, 8), this aspect has been proven in in corn plants (*Zea maiz*) inoculated with AMF and exposed to water stress (11) and in grape (*Vitis vinífera* L.) (33). The development of the extraradical mycelium allows roots having a greater access to soil and thus increase its hydration with the consequent improvement of plant metabolism even under environmental stress conditions (8, 9, 12).

Accumulation of hydrogen peroxide

45 after treatment, hydrogen peroxide (H_2O_2) accumulated in plants subjected to water stress (Figure 2), particularly in noMA plants irrigated with 5 mL of nutritive solution (177 % increase compared to noMA well-irrigated plants). On the contrary, in MA irrigated plants with 5 mL of nutritive solution, the accumulation of hydrogen peroxide increased compared to MA well-irrigated plants. In all water regimes, the quantity of accumulated hydrogen peroxide was higher in noMa plants than in MA plants. When plants recovered from the water stress during 25 additional days (70 days after treatment), the quantity of accumulated hydrogen peroxide in the aerial part of the plant was low and did not show significant differences among treatments.

The H_2O_2 accumulation was higher in the treatments subjected to drought, especially in noMA plants and irrigated with 5 mL of nutritive solution. MA plants also increased the accumulation of H_2O_2, but to a lesser degree than noMA plants. Similar behavior was observed in *Arabidopsis thaliana* plants, when subjected to drought stress, in addition to reduce transpiration and stomas opening (11, 19).

Oxidative damage to lipids

The oxidative damage to lipids was measured as the quantity of lipidic peroxides formed in the different treatments (Table I). Results clearly showed that MA plants did not increase lipids peroxidation after the drought period (45 days after treatment). On the contrary, noMA plants subjected to drought accumulated more lipidic peroxide than MA. This effect was visible even in plants subjected to drought (a rise of 97 %), though it was more evident in plants subjected to drought (a rise of 116 % in irrigated plants with 10 mL of nutritive solution and of 155 % in plants irrigated with 5 mL of nutritive solution). After the recovery from drought (70 days after treatment), the lipidic peroxide level decreased in noMA plants, but retained a higher peroxide content than MA plants. In this case, there were no significant differences among treatments subjected to water stress.

Table I. Oxidative damage to lipids (nmol MDA g^{-1} MS) in rice plants inoculated with *G. intraradices* subjected to water stress 30 days after transplanting (DAT), evaluated after water stress (DE) and recovery (DR)

Treatments	Quantity of peroxides lipidic (nmol MDA g^{-1} MS)	
	45 DDT (DE)	70 DDT (DR)
MA+25 mL	289,09 e	289,08 d
MA+10 mL	321,16 d	321,15 c
MA+5 mL	313,85 d	313,85 cd
noMA+25 mL	571,40 c	420,91 b
noMA+10 mL	694,18 b	437,97 ab
noMA+5 mL	799,82 a	462,09 a
ES$\bar{x}$	14,554	9,075

Means with equal letters do not significatively differ (p≤0,05) according to Duncan's multiple range test

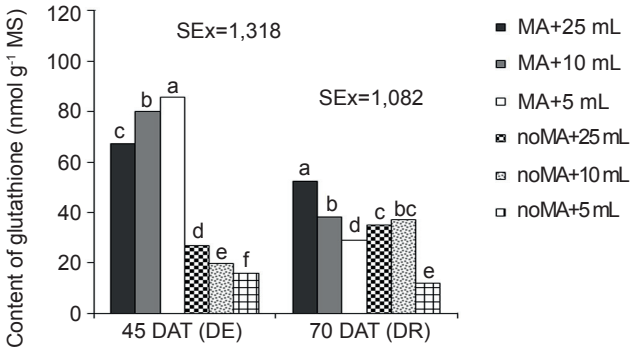
Rice plants are very sensitive to oxidative stress (34). Consequently, the quantity of lipidic peroxides was quantified in the aerial part of rice plants for the different treatments. Lipidic peroxidation was smaller in MA plants after the drought period (45 days after treatment), while in noMa plants important quantities of lipidic peroxides accumulated. Similar results were observed in tomato plants (*Solanum lycopersicon* L.) inoculated with MA, but under salt stress conditions (35).

Reduced glutathione and ascorbate contents

When plants were subjected to water stress for 15 days (45 days after treatment), the quantity of accumulated glutathione was considerably higher in Ma plants than in noMa plants (Figure 3).

This effect was observed under all water regimes, including the controls treated with 25 mL of nutritive solution (66,73 % increased glutathione content). However, the differences between MA plants and noMA plants, in the quantity of accumulated glutathione increased with drough was more severe, reaching 321,22 % in MA plants irrigated with 5 mL of nutritive solution compared to their corresponding noMa plants. When plants recovered from drought (70 days after treatment) glutathione content kept on being higher in MA plants than in noMa plants, except in those that had been previously irrigated with 10 mL of nutritive solution.

The glutathione exists in two different forms, the reduced form, and the oxidated one; however, glutathione in plants remains only in the reduced form (36). It has an antioxidant function by reacting with superoxide radicals and singlete oxygen for the formation of oxidated glutathione (18, 22, 23, 37).



Means with equal letters do not differ significatively (p≤0,05) according to Duncan's multiple range test

Figura 3. Glutathione content (nmol g^{-1} MS) in rice plants inoculated or not with the AMF *Glomus intraradices* subjected to water stress and evaluated after the stress (DE) and recovery (DR)

As to ascorbate content (Table II), after the drought period (45 DDT), all treatments showed a high level, however, noMa plants accumulated more ascorbate than MA plants. The ascorbate accumulation considerably decreased after the recovery from drought (70 DDT) and in this stage, no important differences among treatments were found.

Table II. Reduced ascorbate content (nmol g^{-1} MS) in rice plants inoculated with *G. intraradices* subjected to water stress 30 days after transplanting (DAT), evaluated after water stress (DE) and recovery (DR)

Treatments	Reduced ascorbate (nmol g^{-1} MS)	
	45 DAT (DE)	70 DAT (DR)
MA+25 mL	118,71 d	14,81 a
MA+10 mL	115,45 d	7,05 c
MA+5 mL	113,10 d	7,60 c
noMA+25 mL	128,24 c	11,01 b
noMA+10 mL	156,07 a	5,68 d
noMA+5 mL	152,60 b	8,22 c
ES$\bar{x}$	1,457	0,470

Means with equal letters do not differ significatively (p≤0,05) according to Duncan's multiple range test

These results permit to infer that MA plants show an improvement of tolerance to stress which is usually related to an improvement of antixidant compounds in plants (Figure 3; Table II). Due to ROS toxicity, plants need adequate desintoxication systems that allow a fast elimination of these compounds. These systems include some antioxidant enzymes of non-enzymatoc

compounds as ascorbate, glutathione, flavonoids, carotenoids, and tocopherols (21, 38). Among these non-enzymatic compounds, glutathione and ascorbate are essential metabolytes that regulate major cell functions and play a key role in the antioxidant defense (22, 23, 39).

Ascorbate is an important indicator for reducing the desintoxication of hydrogen peroxide (H_2O_2) in plants. The peroxide directly generated or after conversion of demutase superoxide is initially degraded to H_2O by ascorbate peroxidase using ascorbate as donor of electrons (20, 39, 40).

Under these conditions, the antixocidant response of the plant to water stress is activated by different mechanisms since in MA plants more glutathione is accumulated 45 days after treatment; however, in the case of ascorbate, it significantly accumulates in noMa plants.

From this research it can be generally concluded (part 1 and 2) that rice plants were mycorrhized with percentages of 20 and 50 % under aerobical conditions. Mycorrhization has a marked influence on the growth of rice plants in the long-term. The accumulation of proline considerably increased, both in MA plants as in noMa plants, after being subjected to water stress. In any case, the quantity of accumulated proline was always lower than in MA plants MA (31). Mycorrhized plants showed less oxidative damage favored by the accumulation of antioxidant glutathione and a tendency to accumulate less peroxide and oxidative damage to lipids after the stress.

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