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Arbuscular Mycorrhiza Interaction with *Medicago sativa* Plants: Study of Abiotic Stress Tolerance in Sustainable Agriculture

Micorrizas arbusculares en interacción con plantas de *Medicago sativa*: Estudio de la tolerancia al estrés abiótico en agricultura sustentable

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Resumen

Objetivo: Investigar los efectos de la inoculación con *Rhizophagus intraradices* sobre parámetros fisiológicos y defensa antioxidante en plantas de *Medicago sativa* bajo estrés salino, sequía y frio. Materiales y métodos: *Semillas de M. sativa,* var. Dekalb (DK166**)**, fueron desinfectadas y sembradas en una mezcla de tierra y arena esterilizada; 10 g de *R. intraradices* fue adicionado a la mitad de las macetas y todas ellas recibieron 5 ml de un cultivo de *Shinorhizobium meliloti*. Crecieron en un invernáculo a 25/20 °C (día/noche), con 16 h luz, 50-60% humedad relativa y riego normal durante 45 días. Después de ese tiempo se realizaron los tratamientos de control, sequía, salinidad y frio. La colonización de *R. intraradices* fue calculada en todos los tratamientos. La conductancia estomática fue medida usando un porómetro y la eficiencia del fotosistema II *(PE)* fue medida con Fluor Pen FP100. El peso seco de tallos y

Abstract

Objetive: This study investigated the effects of inoculation with *Rhizofagus intraradices* on physiological parameters and antioxidant defense in *Medicago sativa* plants under salt, drought and cold stress. Material y methods: *M. sativa* seeds, var. Dekalb (DK166**)**, were disinfected and seeded in sterilized mixture of soil/sand. 10 g of *R. intraradices* was added to half of the pots and all plants received 5 ml of a culture of *Shinorhizobium meliloti*. They were grown in a greenhouse at 25/20°C (day/night), with 16 light, 50-60% relative humidity and regular watered during 45 days. After this time, were place the treatment of control, drought, salinity and cold. *R. intraradices* colonization was calculated in all treatments. Stomatal conductance was measured using a porometer and the efficiency of photosystem II (PE) was measured with Fluor Pen FP100. The shoot and root dry weight (DW), proline content, the oxida-

raíces, el contenido de prolina, el daño oxidativo de lípidos y la actividad enzimática de SOD, CAT, APX fueron determinadas en todos los tratamientos después del estrés. Resultados: Todos los parámetros fisiológicos y enzimáticos medidos fueron significativamente (*t*-Student) mejores en plantas inoculadas, mostrando una interacción positiva entre micorrización-estándar fisiológico. La conductancia estomática, en plantas inoculadas, incrementó bajo sequía y salinidad, mostrando protección frente a estos estreses abióticos. La eficiencia fotosintética en plantas no micorrizadas disminuyó bajo todos los estreses, pero en plantas micorrizadas se mantuvo similar en todos los tratamientos de estrés, mostrando su mitigación. El peso seco aéreo y de raíces fue similar bajo diferentes estreses abióticos en plantas no micorrizadas, pero en plantas micorrizadas fueron mayores y mostraron diferencias entre ellos. La peroxidación lipídica en plantas no micorrizadas aumentó en condiciones de sequía y salinidad, mientras que en plantas inoculadas se mantuvo constante, mostrando mitigación. Las plantas inoculadas incrementaron las actividades de superóxido dismutasa (SOD) y ascorbato peroxidasa (APX) en tallos y raíces bajo todos los estreses, mostrando defensa antioxidante. Catalasa tuvo un comportamiento variable. Conclusión: La micorrización tuvo una interacción positiva con diferentes parámetros fisiológicos y defensa antioxidante en *M. sativa*, y promovió mitigación ante el estrés abiótico.

Palabras clave

Antioxidantes, fotosíntesis, frio, hongos, prolina, salinidad, sequía.

tive damage to lipids and the enzyme activities of SOD, CAT, APX, were determined in all treatments after stress. Results: All physiological and enzymatic measurements were significative (*t*-Student) better in inoculated plants, showing a positive interaction mycorrhization-physiological status. Stomatal conductance, in inoculated plants, increased under drought and salinity, showing protection to these abiotic stresses. Photosynthetic efficiency in non-mycorrhizal plants decreased under all stresses, but in mycorrhizal plants, it remained similar in all stress treatment, showing their mitigation. The aerial and roots dry weights were similar under different abiotic stress in non-mycorrhizal plants, but in mycorrhizal plants, were higher and showed different from each stress. Lipids peroxidation in non-inoculated plants increased under drought and salinity conditions, while in inoculated plants remained constant showing mitigation. Inoculated plants increased activities of superoxide dismutase and ascorbate peroxidase in shoots and roots under all stresses showing antioxidant defense. Catalase had a variable performance. Conclusion: The mycorrhization have a positive interaction with different physiological parameters and antioxidant defense in *M. sativa* and promoted mitigation under abiotic stresses.

Keywords

Antioxidants, cold, drought, fungi, photosynthesis, proline, salinity.

Introduction

Alfalfa (*Medicago sativa* L.) is a fundamental resource for agricultural production in temperate regions of the world, based on its high nutritional quality, forage production, growth habit, persistence, plasticity and capacity for symbiotic fixation of atmospheric nitrogen (Basigalup *et al.*, 2007).

Arbuscular mycorrhizal fungi (AMF) represent the most widespread symbiosis with land plants. The associated fungi colonize the plant roots and reside in the internal tissues of their host plant. This mutualistic association not only plays a key role enhancing plant growth by facilitating the uptake of water and essential nutrients but also protects the plant from adverse soil conditions. The application of mycorrhizal fungi is a promising alternative strategy for sustainable crop production under normal as well as biotic and abiotic stress conditions. The mycorrhizal plants have an improved ability for nutrient uptake and have ability to tolerate stress environments (Nadeem *et al.*, 2017)

The abiotic stresses such as drought, salinity, and extreme temperature regimes are linked by the fact that they all decrease the availability of water and nutrients to plant cells. The production of crops and their sustainability is mostly influenced by abiotic stress, such as drough (Barea *et al.*, 2011), salinity (Al-Khaliel, 2010), extreme temperature (Canci and Toker, 2009).

Arbuscular mycorrhizal fungi *(AMF)* can promote plant growth increasing plants production under stress due to their benefits: establishments of extensive hyphal networks and secretion of biomolecules like glomalin, which ameliorates soil structure and enhances water and nutrient uptake (Pagano, 2011). One of the most used species to alleviate the biotic stress in plants and increase their yields is *R. intraradices*.

R. intraradices inoculation improved the shoot and root dry weights, total root length, root surface area, root volume, and the number of root forks under heavy metal stress, in *Robinia pseudoacacia* (Zang *et al.*, 2020) and increasing abiotic stress tolerance in *Digitharia eriantha* (Pedranzani *et al.*, 2016) and increased the essential oil yield and dry biomass in aromatic plant (Colombo *et al.*, 2013).

Under stress conditions, AMF can modify plant physiology in order to cope with those environmental factors (Miransari, 2010). Several reports have shown that mycorrhizal symbiosis improves plant health through increased protection against environmental stresses, either biotic (*e.g.*, pathogen attack) or abiotic (*e.g.*, drought, salinity, heavy metals, organic pollutants) (Azcón and Barea, 2010).

Plant stress responses are dynamic and involve complex cross-links among different regulatory levels, including adjustment of metabolism and gene expression for physiological and morphological acclimation (Krasensky and Jonak, 2012). During abiotic stress, different metabolic pathways are uncoupled and electrons are transferred to molecular oxygen to form reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), or hydroxyl radicals (OH•) (Noctor *et al.*, 2014). The scavenging of ROS is achieved through the action of different enzymatic and non-enzymatic compounds, including superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), ascorbate peroxidise (APX), and the enzymes of the ascorbate-glutathione pathway. Non-enzymatic mechanisms include compounds, such as ascorbic acid, glutathione, and α-tocopherol, capable of directly scavenging several ROS (Scheibe and Beck, 2011). Abiotic stress symptoms include plant wilting, reductions in the rate of net photosynthesis, reductions in stomatal conductance, and the gradual decrease in total chlorophyll content and biomass.

The aim of this study was to determine the effects of the symbiotic association of *Medicago sativa* and the mycorrhiza *R. intraradices* in relation to the physiological parameters and antioxidant response under different abiotic stresses.

Materials and methods

Treatments and experimental design

Eight treatments were compared under a randomized complete block design with five replications. Treatments came from the combination of two factors: (1) inoculated and non-inoculated (control) plants with *Rhizophagus intraradices*, (2) four growing conditions non-stressed (control), and any of three stresses drought, salt, or cold. The experimental unit was a pot as seen in figure 1.

Figure 1

Experimental units (pots) lay-out

Soil and biological material

A loamy soil was collected from the ground at the Zaidin Experimental Station (Granada, Spain). The soil had a pH of 8.1 (measured in water, 1:5 w/v); 1.5% organic matter; nutrient concentrations (g/kg): N, 1.9; P, 1 ($NaHCO_3$-extractable P); K, 6.9. The soil was sieved (5 mm), diluted with quartz-sand (2 mm) (1:1, soil: sand, v/v), and sterilized by steaming (100 ºC for 1 h on 3 consecutive days).

M. sativa seeds, var. Dekalb (DK166), were disinfected with 70% ethanol for 3 min and rinsed three times with sterile water. One seed per pot was sown in 500 ml pots containing the sterilized mixture of soil/sand.

Mycorrhizal inoculum of *R. intraradices* (Schenck and Smith) strain EEZ 58 (Ri) was prepared as described by Porcel *et al.* (2006) and 10 g of the inoculum was added to half of the pots at the time at sowing. All plants received 5 ml of a culture of *Shinorhizobium meliloti,* 10^6 cell per ml.

Growing conditions

All plants were grown in a greenhouse at 25/20 °C (day/night), with 16 light,50-60% relative humidity, an average photosynthetic photon flux density of 800 μmol m^{-2} s^{-1}, (LI-COR, Lincoln, NE, USA, model LI-188B) and watered to field capacity for 45 days.

Stress conditions were applied after the 45-day mentioned above, protocol for each stress condition 1-25 °C and soil at field capacity; 2-25 ºC and 60% of field capacity for one week (drought); 3-25 ºC and soil irrigated with 200 mMNaCl for two weeks (salinity); 4-4 °C for 72 h (cold) continuously (Pedranzani *et al.*, 2016). Before harvest, stomata conductance and photosynthetic efficiency were taken. At the end of each stress, the plants were harvested and the weights were taken and the samples were placed in liquid nitrogen for enzymatic measurements.

Mycorrhizal development

R. intraradices colonization was estimated by visual inspecting of fungal structures after treatments with different stresses, clearing of roots in 10% KOH and staining with 0.05% (w/v) trypan blue in lactic acid according to Philips and Hayman (1970). The percentage of *R. intraradices* colonization was calculated according to the gridline intersects method (Giovannetti and Mosse, 1980).

Stomatal conductance, photosynthetic efficciency and aerial and root dry weight

Stomatal conductance was measured using a porometer (Porometer AP4, Delta-T Devices Ltd., Cambridge, UK). The efficiency of photosystem II (PE) was measured with Fluor Pen FP100 (Photon Systems Instruments, Brno, Czech Republic). Fluor Pen 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 (FM), according to Oxborough and Baker (1997) both were measured in five different plants per treatment. After finishing the treatments, aliquots of aerial and root tissue were extracted and place in an oven at 70 C° for 48 h for shoot and root dry weight (DW) measured.

Proline content

Free proline content was quantified according to the method of Bates *et al.* (1973) and was measured in five different plants per treatment.

Oxidative damage to lipids

500 mg of leaves were ground with mortar on ice with 6 mL of 100 mM of potassium phosphate buffer (pH7) and the homogenate was filtered with 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) butylhydroxytoluene and 0.25 N HCl, 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 measurement at 532 nm in the spectrometer. 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).

Determination of enzyme activities

Among the enzymes known to be involved in ROS detoxification, SOD, CAT, APX were selected and their activities were determined in shoots and roots of all treatments.

Oneg of leaves or roots, with 50 mg polyvinylpolypyrrolidone (PVPP) and 10 ml of 50 mM K-phosphate buffer (pH 7.8) at 0-4ºC were used for enzyme extraction, containing 0.1 ml EDTA for superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). Extracts were filtered through four layers of nylon cloth and centrifuged at 20 000 g for 20 min at 0-4 ºC. The supernatants were kept at -70 ºC for subsequent enzymatic assays. Total SOD activity (EC 1.15.1.1) was measured according to Beyer and Fridovich (1987), based on the ability of SOD to inhibit the reduction of nitrobluetetrafolium (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 ºC. CAT activity (EC 1.16.1.6) was measured by the disappearance of H_2O_2 (Aebi, 1984). The reaction mixture (3 ml) contained 10.6 mM H_2O_2. The reaction was initiated by adding 25 μl of the extract and monitoring the change in absorbance at 240 nm and 25 ºC for 3 min. APX activity (EC 1.11.1.11) was measured in a 1 ml reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2 and 0.5 mM ascorbate. Adding the H_2O_2started the reaction and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate of ascorbate (Amako *et al.*, 1994).

Statistical analysis

First, a *t*-test for independent samples (data were homoscedastic and normal) was performed to observe the correlation among measured variables and the two types of plants: inoculated and not inoculated. ANOVA's were carried out to identify the influence of the four growing conditions on measured variables, mean multiple comparisons analyses were done when a significant effect was found. Data manipulation and analysis were done by SPSS (Statistical Pack for Social Sciences) version 27.

Results

Mycorrhizal development

The application of different stresses did not affect root colonization. The mycorrhization percentage of *R. intraradices* to *M. sativa* plants was 60% in non-stress plants; 59.3% in drought stress plants; 66.6% in salinity and 63.8% in salinity and cold stress respectively.

Mycorrhizal and non-mycorrhizal plants interaction

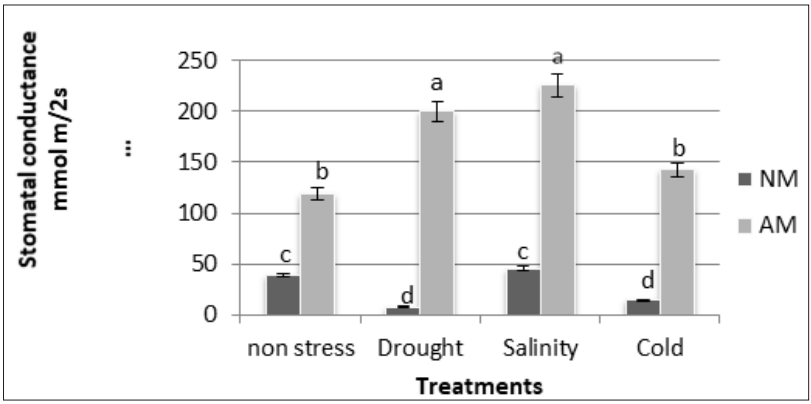
A *t*-test for independent samples (data were homoscedastic and normal) was performed to observe the correlation between the study variables and the two groups: mycorrhizal (AMA) and non-mycorrhizal (NM). For all the parameters (variables) studied, the difference between AM and NM plants was significant, being a positive interaction.

Stomatal conductance, photosynthetic efficiency and aerial and root dry weight

Stomatal conductance increased considerably with AM colonization in all conditions in comparison with NM plants. In AM plants, drought and salinity stress increased stomata conductance and cold stress remained similar to non-stressed plants. In NM plants decreased by drought and cold stress (figure 2).

Figure 2

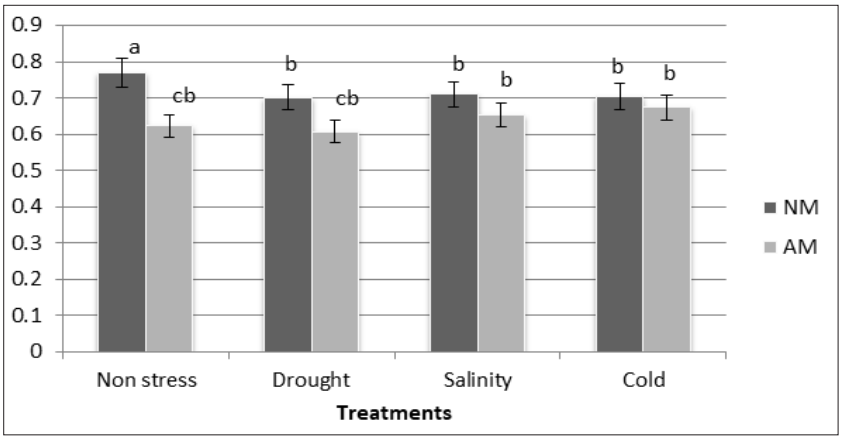
Stomatal conductance (mmol m/2s) in AM and NM plants of *M. sativa* under non-stress and drought, salinity and cold conditions. Different letters mean significant differences ($P \leq 0.05$) as determined by the ANOVA test (n=5)



In NM plants, the photosynthetic efficiency decreased in salinity, drought and cold stress compared with non-stressed plants. The photosynthetic efficiency was not affected by abiotic stress in AM plants and it remained similar in all treatment respect the control (figure 3).

Figure 3

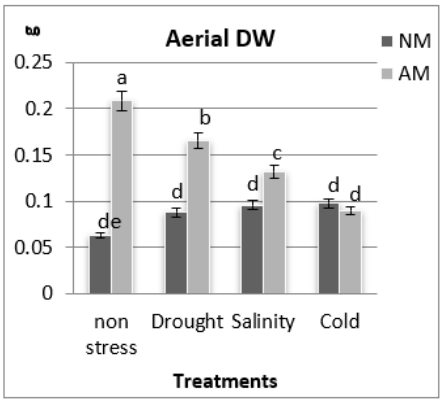
Photosynthetic efficiency in AM and NM plants of *M. sativa* under non-stress and drought, salinity and cold conditions. Different letters mean significant differences ($P \leq 0.05$) as determined by ANOVA test (n=5)

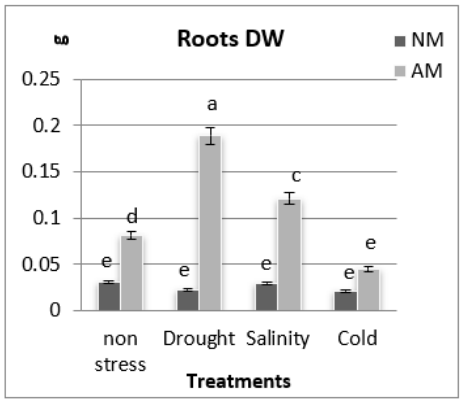


The DW of shoots and roots of *M. sativa* plants varied in relation to colonization, thus AM plants increased DW, in relation to those that were NM (figure 4a and b). Aerial and roots DW increased in non-stressed, drought and salinity condition. Cold showed similar behavior in NM and AM plants, both aerial and roots DW (figure 4a and b).

Figure 4

Photosyntetic efficiency aerial and roots DW in AM and NM plants of *M. sativa* under non-stress and drought, salinity and cold. Different letters significant mean differences ($P \leq 0.05$) as determined by ANOVA test (n=5)



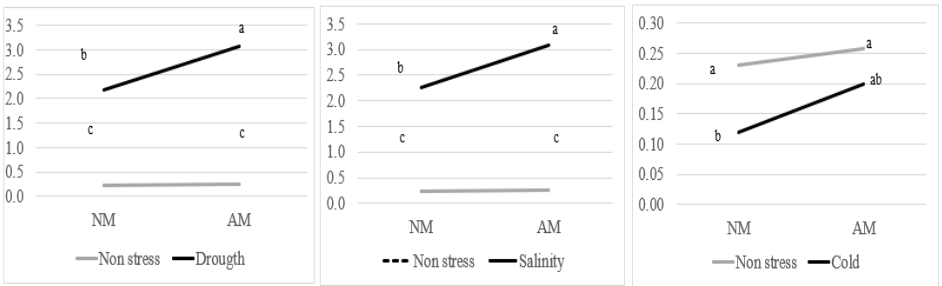


Proline

There is an interaction between mycorrhization and proline production under abiotic stress in *M.sativa* plants. Proline contents increased in NM and AM plants with drought and salinity stress in contrast with the control, and these changes were greater in AM plants compared with NM plants (figure 5a, b). Proline decreased under cold stress in NM and AM plants; in addition, there was mycorrhizal-proline interaction, in which it increased in AM plants concerning NM plants (figure 5c). This shows that proline was a protective osmolyte against drought and salinity being the protection greater, in AM plants.

Figure 5

Determination of proline content (μmol/g FW) in *M. sativa* NM and AM plants under non-stress and drought (a), salinity (b) and cold (c) conditions



Oxidative damage to lipids (MDA)

Under non-stress conditions, Malondialdehyde (MDA) was significantly higher in AM plants than in NM plants. The MDA content increased significantly in shoots of NM plants when subjected to drought, salinity and, cold. In contrast, in shoots of AM plants, there were no significant differences between non-stressed plants and those subjected to abiotic stress (figure 6), showing that lipid peroxidation, as an alarm element against abiotic stress, is not necessary for the presence of symbiosis with mycorrhizae.

Antioxidant enzymes

In roots and shoots, SOD activity was significantly higher in AM plants with respect to NM plants under non-stress, drought, salinity and cold conditions (table 1). In roots and shoots from AM plants, CAT activity was elevated compared to NM plants in control and cold condition. In shoots, CAT activity decreased under drought and salinity stress in AM plants as compared with NM plants (table 1). APX activity was always significantly higher in AM plants than in NM plants in both roots and shoots tissues under the entire conditions tested (table 1).

Figure 6

Oxidative damage to lipids (nmol MDA/g FW) in shoots (a) of non-mycorrhizal (NM) and mycorrhizal (AM) *M. sativa* plants under non-stress, drought, salt, or cold stress conditions. Different letters mean significant differences ($P \leq 0.05$) as determined by the ANOVA test (n=5)

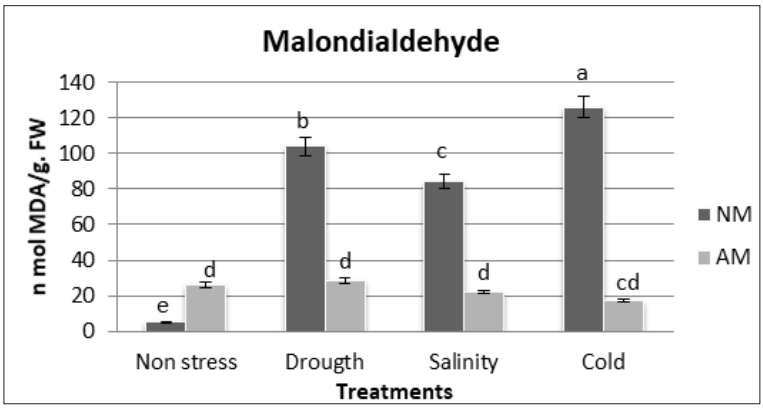


Table 1

Activities of antioxidant enzymes (SOD, CAT, APX) in roots and shoots of non-mycorrhizal (NM) and mycorrhizal (AM) *M. sativa* plants under non-stress, drought, salt, or cold stress

	SOD U min/gFW		CAT μmol H_2O_2/min/gFW		APX μmol ASC min/g FW	
	Root	Shoot	Root	Shoot	Root	Shoot
NM						
NS	14.58±0.19^{d}	15.83±0.14^{d}	0.3821±0.87^{c}	0.4621±0.96^{b}	13.22±3.46^{de}	15.13±3.82^{de}
D	20.71±0.10^{c}	18.67±0.03^{c}	0.3221±0.46^{c}	0.4524±0.26^{b}	19.88±3.56^{cd}	22.37±3.45^{cd}
S	22.78±0.25^{c}	23.76±0.07^{c}	0.3515±0.56^{c}	0.4172±0.38^{b}	17.86±3.11^{d}	16.57±3.33^{d}
C	17.89±0.45^{d}	13.99±0.09^{d}	0,3475±0,63^{c}	0.4065±0.56^{b}	10.19±3.42^{de}	13.16±2.45^{de}
AM						
NS	32.27±0.29^{a}	30.58±0.36^{b}	0.6826±0.82^{a}	0.6772±0.82^{a}	25.58±5.05^{c}	29.53±5.43^{bc}
D	27.55±0.85^{b}	41.43±0.41^{a}	0.3256±0.58^{c}	0.3256±0.57^{c}	35.97±5.90^{a}	45.80±5.46^{a}
S	27.70±0.18^{b}	33.41±0.07^{b}	0.2704±0.51^{c}	0.2704±0.52^{c}	31.10±5.57^{b}	27.51±5.25^{c}
C	32.80±0.1^{a}	32.71±0.05^{c}	0.7461±0.86^{a}	0.6919±0.83^{a}	28.61±5.34^{bc}	22.80±4.77^{cd}
P	($P \leq 0.05$)	($P \leq 0.05$)	($P \leq 0.05$)	($P \leq 0.05$)	($P \leq 0.05$)	($P \leq 0.05$)

NS= Non stress, D= Drought, S= Salinity, C=Cold.

Columns represent means ±Standard error. Different letters mean significant differences ($P \leq 0.05$) undetermined by the analysis of variance (ANOVA) test (n=5).

Discussion

The values for colonization observed in *M. sativa* plants were similar than in other species (Abdel Latef and Chaoxing, 2011; Porcel and Ruiz-Lozano, 2004; Ruiz-Sánchez *et al.*, 2010).

M. sativa plants inoculated with *R. intraradices* had higher DW than NM plants under control and all abiotic stress conditions and these results were consistent with those in maize (Feng *et al.*, 2002), fenugreek (Evelin and Kapor, 2014), and *Pigeon pea* (Gard and Manchanda, 2009) under salt stress. Wu and Xia (2006) found that *Citrus tangerine* plants colonized by *Glomus versiforme* increased the DW compared with NM plants under similar drought stress conditions.

Stomatal conductance is regulated primarily by the aperture of the stomata pore and stomata density (Yan *et al.*, 2012; Huang and Xu, 2015; Gamage *et al.*, 2018). In this study stomatal conductance increased in AM plants under control, drought, salt and cold stresses compared with NM plants. AM symbiosis often alters the stomata behavior of the host (Augé *et al.*, 2016; Chitarra *et al.*, 2016). Smith an Read (2008) explained that the ability of AM plants to maintain stomata opening longer than NM plants as soils dry is because hyphae penetrate pores that are inaccessible to roots, and they spread beyond the root zone, effectively increasing the available volume of the soil solution. There is evidence that AM root systems can better exploit bound water in drying soils, in some cases providing access to soil water below the permanent wilting Ψ of NM plants (Auge *et al.*, 2015). In NM plants, stomatal conductance decreased under drought and cold. Changes in stomatal conductance are always accompanied by changes in leaf water potential (Ψ) or osmotic adjustment. The influence of AM on stomatal conductance may also be associated with altered chemical signals and carbon dynamics of the plant leaves (Ruiz-Lozano and Aroca, 2010b).

The photosynthetic efficiency remained unchanged in AM plants under different conditions, however decreased significantly with the drought, salt, and cold stress in NM plants, in correlation with the stomatal closure. A positive correlation between tolerance to abiotic stresses in AM plants and maintenance of photosystem II efficiency has been demonstrated, which, in turn, maintains (Porcel and Ruiz-Lozano, 2004) or even increases the productivity of the plant (Ruiz-Sánchez *et al.*, 2010). In *M. sativa* AM plants, photosystem II efficiency under stress conditions remain similar to the unstressed control values because AM plants maintained higher water status and hence more opened stomata (Auge *et al.*, 2015).

In order to tolerate abiotic stress, plants accumulate a high concentrations of low molecular mass organic solutes, such as soluble sugars, proline and other amino acids, to regulate the osmotic potential of cells aiming at improving absorption of water under drought stress (Zang *et al.*, 2010). Our data indicate that the concentration of proline in leaves increased during drought and saline stress in both AM and NM-treated plants. The results are in agreement with previous reports of drought stress (Zang *et al.*, 2010; Ruiz Lozano and Aroca, 2010b).

Abiotic stress is accompanied by the formation of ROS, such as superoxide radicals (O2-) and H_2O_2, that damage membranes and macromolecules and the oxidation of membrane lipids is an indication of uncontrolled free-radical production and oxidative stress (Noctor *et al.*, 2014). Accordingly, the number of lipid peroxides was quantified in shoots of *M. sativa*, the lipid peroxidation in AM plants subjected to drought, salinity, and cold was 77, 81, and 85.6% lower than NM plants respectively, indicating a clear defense of the plant against stress by the AM symbiosis. These were observed in tomato plants subjected to salinity (Abdel Latef and Chaoxing, 2011) and in soybean and rice plants under drought stress conditions (Porcel and Ruíz-Lozano, 2004; Ruíz-Sánchez *et al.*, 2010).

As a defense mechanism, the activity of antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) is enhanced under drought (Sankar *et al.*, 2007; Manivannan *et al.*, 2008a), salinity (Jaleel *et al.*, 2008b), cold (Garbero *et al.*, 2011) and oxidative stress (Pérez Chaca *et al.*, 2014). In the present study, APX activities of *M. sativa* inoculated with *R. intraradices* increased significantly as compared to NM plants, whether host plants were under well-watered, and drought, salinity, or cold conditions. The behavior of SOD was identical to that of APX, increasing its activity in all treatments of mycorrhizal plants. The behavior of CAT was similar, but only for unstressed and cold treatment. Previous research has also reported increases in SOD, CAT, and APX activity in AM tomato plants subjected to saline stress (Abdel Latef and Chaoxing, 2011; Abbaspour *et al.*, 2012) and in *Digitaria eriantha*, were CAT, APX and SOD showed significant increases in AM plants as compared to non-AM plants under salinity, drought and cold stress (Pedranzani *et al.*, 2016). CAT and APX activities are both involved in the scavenging of hydrogen peroxide, although APX has a much higher affinity for H_2O_2 than CAT (Estrada *et al.*, 2013). CAT activity was lower in the roots and shoots of AM plants subjected to drought and salinity than AM plants without stress, which may indicate that in *M. sativa* in these conditions, hydrogen peroxide could be preferentially scavenged by APX. Indeed, in these plants, APX activity was significantly higher than in control plants not subjected to stress.

Conclusion

Alfalfa var. Dekalb 166, which was considered moderately tolerant to drought and salinity stresses, associated with mycorrhizae, presents many antioxidant defense mechanisms that strongly prevent possible damage from drought and salinity, and cold improved performance and physiological parameters.

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