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Jasmonates, Aquaporins and Nutritional Response in *Medicago Sativa* in Symbiosis with *Arbuscular* Mycorrhizae under Abiotic Stresses

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) can be considered a crucial tool in the development of modern agriculture that respects the environment. Among the plant species that can stablish symbiosis with AMF Medicago sativa is included. M. sativa produces high quality forage in various parts of the world and by partnering with AMF they improve plant growth and development. The present study investigated the effects of inoculation with the arbuscular mycorrhizal (AM) fungus Rhizophagus intraradices on physiological, proteomic and nutritional parameters in M. sativa plants during salt, drought or cold stress. The fresh weight of shoots and roots increased in AM plants under non-stressed, droughted and saline condition. Cold affected similarly non-mycorrhizal and AM plants. The precursor of the phytohormone jasmonic acid, OPDA showed a significant increase in non-mycorrhizal (NM) and mycorrhizal (AM) plants in the absence of stress and under drought and salinity stresses. JA showed a Cold stress did not affect this hormone. The proteomic study reported that the accumulation of PIP1 aquaporins varied in NM and AM plants with stress treatments. Micronutrients such as Fe, Mn and macronutrient such as Ca and Mg increased in AM plants. In contrast, the concentration of heavy metals, such as Cu, Pb, Ti, Hg, Bi, Cr, As and Be was lower in AM roots relative to NM controls. These results suggest that the salt and drought stress tolerance of M. sativa plants could be enhanced by R. intraradices, and this symbiosis could be used to restore and improve productivity in stressful lands in semiarid ecosystems.

Keywords: Aquaporin protein; arbuscular mycorrhiza; jasmonates; ion balance

Introduction

The associations between arbuscular mycorrhizal fungi (AMF) and various plant species is a close relationship that occurs in the soil and can be used to study the mitigation of stress factors such as drought, cold, salinity or the presence of heavy metals (Ahkami *et al.*, 2017) [1]. Mycorrhizal-plant relationship is beneficial for both, as they can contribute to the absorption of nitrogen and phosphorus; in addition to some micronutrients that are found in trace amounts such as some metals, whose limitation in plants causes poor plant growth (Ahkamiet *al.*, 2017) [1].

AMF constitute an important functional group in soil biodiversity since they can contribute enormously to crop productivity and ecosystem sustainability (Smith and Read, 2008) [46]. Through their nutritional and non-nutritional activities, they profoundly influence agroecosystem processes, providing ecosystem services of soil stabilization, bioprotection, biofertilization and bioregulation (Gianinazzi *et al.*, 2010) [21]. AMF play also an important role in biofortification and the development of a sustainable modern agriculture, due to ability of AMF to significantly improve soil structure, nutrient and water availability, plant tolerance to drought, high temperature, salinity, heavy metals pollution and pathogens, and the stimulation of secondary metabolites synthesis resulting in higher crop quality (Golubkina*et al.*, 2020) [24]. AMF becoming an important biological resource in the structure and functioning of soils and influencing the ecological behavior, productivity and composition of natural plant communities, as well as agricultural crops (Pérez *et al.*, 2011) [40]. AMF are vital endosymbionts playing an effective role in plant productivity and the functioning of the ecosystem. They are of key importance for sustainable crop improvement (Gianinazzi*et al.*, 2010) [21].

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) [11]. *Medicago sativa* in symbiosis with AMF can improve many mechanisms of tolerance to abiotic stress, such us increasing the aerial and roots dry weights, increasing stomatal conductance, produce osmo-compatible substances such as proline and increasing antioxidant activity through superoxide dismutase and ascorbate peroxidase (Pedranzani *et al.*, 2021) [39]

Plant hormones, including jasmonic acid (JA) and its derivatives, commonly called jasmonates, are among the substances used by plants to regulate their responses to abiotic and biotic stresses. The elevation of jasmonate levels is usually correlated with the activation of genes encoding for JA biosynthetic enzymes (Wasternack and Hause 2013). Hause *et al.* (2002) showed that the mycorrhization of barley roots with *Glomus intraradices* was accompanied by an elevation of the JA and JA-Ile levels, both suggesting a causal link to mycorrhization. In *Digitaria eriantha,* the levels of OPDA (JA precursor) and JA were significantly higher in AM plants under drought and salinity stress compared with NM plants (Pedranzani *et al.* 2016) [37].

In addition, altered levels of important proteins such as aquaporins have been reported in AM plants (Bárzana *et al.* 2014 [9]; Quiroga *et al.* 2019 [44]). As a consequence, the AMF symbiosis regulates the root hydraulic properties, including root hydraulic conductivity (Bárzana *et al.* 2014 [9]; Quiroga *et al.* 2019 [44]).

AM fungi provide their host plants an efficient supply of low mobility mineral nutrients, mainly phosphorus and some micronutrients such as Cu and Zn. Due to the hyphal network, they develop in the soil, AMF acquire nutrients and deliverer them to the host plant in exchange for carbon compounds produced by the plants (Finlay, 2008) [19].

The aim of this study was to determine the effects of the symbiotic association of *M. sativa* and the AM fungus *R. intraradices* in relation to the regulation of aquaporins, stress hormones and ionic balance under abiotic stresses.

Materials and Methods

Experimental design

The experiment consisted of a randomized complete block design with five replications per treatment. The experiment had two factors: (1) inoculation treatment, with non-inoculated control plants and plants inoculated with the AM fungus *Rhizophagus intraradices*, (2) abiotic stress application, in which plants were cultivated under optimal conditions throughout the entire experiment

and other groups of plants were subjected to either drought, salt or cold stress. Thus, there were a total of eight treatments with five replicates, giving a total of 40 pots. Table 1 – Experimental design for the evaluation of mycorrhization protective effect (antioxidant) of AM fungus *Rhizophagus intraradices* upon *Medicago sativa* development under abiotic stress conditions (Table 1)

Pot	Plant	Experiment
1-5	Non Mycorrhizal*M. sativa*	Non stress (control)
6-10	Non Mycorrhizal *M. sativa*	Cold (4º)
11-15	Non Mycorrhizal *M. sativa*	Drought
16-20	Non Mycorrhizal *M. sativa*	Salinity
21-25	Mycorrhizal *M. sativa*	Non stress (control)
26-30	Mycorrhizal *M. sativa*	Cold (4º)
31-35	Mycorrhizal *M. sativa*	Drought
36-40	Mycorrhizal *M. sativa*	Salinity

Table 1: Experimental design

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^{-1}): 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 (100ºC for 1 h on 3 consecutive days). Seeds of *M. sativa* were washed for 3 min in pure ethanol and rinsed three times with distilled water. Seeds were then 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) [42] and 10 g of the inoculum was added to half of the pots at the time at sowing. Non-inoculated pots received the same amount of autoclaved mycorrhizal inoculum together with 5 ml of a filtrate of the AM inoculum in order to provide a general microbial population free of AM propagules. All plants received 5 ml of a culture of *Shinorhizobium meliloti,* 10^6cell per mL.

Growing conditions

Inoculated (AM) and non-inoculated (NM) plants were cultivated in a greenhouse at 25°C:20°C (day:night), with 16:8 photoperiod, a relative humidity of 50-60% and an average photosynthetic photon flux density of 800 µmol m^{-2} s^{-1}, as measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B). Plants were watered to field capacity and maintained under optimal conditions for 45 days. After that period, AM and NM plants were divided in four groups and each of them was subjected to the following treatment: 1) 25°C and soil at field capacity (non-stress); 2) 25ºC and 60 % of field capacity for two weeks (drought); 3) 25ºC and soil irrigated with 200 mM NaCl for two weeks (salinity); 4) 4°C for 72 h (cold) (Pedranzani *et al.,* 2021) [39].

Mycorrhizal development

At the end of the experiment, the roots were separated from the leaves and the roots were carefully rinsed with tap water. Root fragments 1 cm long were cut from four plants, and placed in a tube. Mycorrhizal colonization was estimated by visual observation of fungal structures after 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 samples were stored in lactoglicerol: lactic acid, glycerol, water 1:1:1 (v/v/v). The percentage of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980) using 200 roots segments of each sample.

Plant fresh weigh

At the end of each stress, the plant system was extracted, and the fresh weights of the aerial part and root of 5 plants were taken on a precision scale.

Extraction, purification and determination of jasmonates.

JA was extracted and pre-purified according to Gidda *et al.* (2003).1 g FW shoot samples from five control and treated plants were homogenized with 10 ml methanol and 100 ng (D_6) JA, as an internal standard. The homogenate was filtered under vacuum on a column with cellulose filter. The extract was taken to dryness, dissolved with 10 ml methanol, and loaded on columns filled with 3ml DEAE-Sephadex A25 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) (Ac-form, methanol). The column was washed with 3 ml methanol, following elution with 3 ml 0.1 M acetic acid in methanol, elution with 3 ml 1 M acetic acid in methanol and elution 3 ml 1.5 M acetic acid in methanol, all fractions were combined, evaporated and separated on preparative HPLC, and analyzed by gas chromatography (GC-MS). Determinations were performed for triplicate.

Western blot analysis

For microsome isolation, roots were homogenized in grinding buffer (100 mM Tris-HCl pH = 7.5, 1 mM EDTA, 12% (w/v) sucrose, 0.2 mM aminoethyl-benzene-sulfonyl-fluoride, 2 g ml^{-1}aprotinin, 1 g ml^{-1}leupeptin, and the supernatant collected after centrifugation at 15,000 rpm / 10 min. The supernatant was filtered through a single layer of cheesecloth and centrifuged again at100,000 rpm for 2h. The pellet was resuspended in buffer plus 0.1% (w/v) SDS. Ten micrograms of protein were loaded in each line after incubating for 30 min at 37 °C in presence of denaturing buffer (20 mM Tris-HCl pH = 8.6, 1% (w/v) SDS, 0.3% (v/v) β-mercaptoethanol, 8% (v/v) glycerol, 0.2% (w/v) bromophenol-blue). Proteins were transferred to a PVDF membrane at 100 mA for 1 h. The membranes were blocked during 2 h at room temperature with 5% (w/v) nonfat milk in Tris-buffered-saline (TBS) with 0.05% Tween 20. Membranes were incubated overnight at 4ºC with 1:2,000 dilution of an antibody raised against a 42-amino acid N-terminal peptide of AtPIP1; 1, which recognizes most PIP1 proteins from several plant species. The antibody was kindly provided by Dr. Maurel (INRA, Montpelier, France). Goat anti-rabbit Ig coupled to horseradish peroxidase was used as secondary antibody at a 1:20,000 dilutions. The signal was developed using a chemiluminescent substrate (West-Pico, Super Signal, Pierce, and Rockford, IL, USA). Microsomes were isolated from two different root samples. Western blots were developed on both microsome samples of each treatment without significant differences between them. The equal loading of the proteins in the different lanes was confirmed by staining the gel with coomassie brilliant blue. To quantify the inmunoblot signal, the intensity of each band was measured using Adobe Photo Shop 8.0.1 (Adobe Systems, Mountain View, CA), corrected for the background and normalized against the intensity of the corresponding coomassie brilliant blue band.

Elemental analysis in control samples

Concentrated nitric acid (65% v/v) from Sigma-Aldrich (Germany) and hydrogen peroxide (30% v/v) from J. T. Baker (Mexico) were used for the digestion of the samples. For the determination of the total amount of the elements of interest, the samples (0.2 g dry mass) were digested by microwave-assisted mineralization with 5 mL of HNO_3 and 3 mL of H_2O_2. A microwave digestion system model START-D from Milestone (Sorisole, Italy), and Milestone hermetically sealed 100 ml internal volume, 1 cm wall thickness polytetrafluoroethylene (PTFE) reactors was used for total digestion of the samples. The homogenate was diluted to a final volume of 15 mL with Milli-Q water. The mineralization of samples was performed in triplicate. Multi-element calibration standard 3 from Perkin Elmer Pure Plus containing 10 mg L^{-1} of Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V and Zn in 5% HNO_3 was used. An inductively coupled plasma mass spectrometer, Perkin-Elmer SCIEX, ELAN DRC-e (Thornhill, Canada) was used. An HF-resistant and high performance perfluoracetate (PFA) nebulizer model PFA-ST, coupled to a quartz cyclonic spray chamber with internal baffle and drain line, cooled with the PC 3 system from ESI (Omaha- NE, USA) was used. Tygon black/black 0.76 mm i.d. and 40 cm length peristaltic pump tubing was used. External calibration was done with aqueous multielemental standards of 0, 2, 4, 8, 10, 16, 20, 40 and 80 µg/l. Samples were run in parallel with adequate reagents blanks.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 program (SPSS, Inc. Chicago IL, USA). All data were subjected to analysis of variance (ANOVA) with inoculation treatment (NM, M) and a biotic stress (well-watered (control), drought, salinity and cold)as sources of variation. Posthoc comparisons with Duncan's Multiple Range Test (Duncan 1955) were used to determine differences between the groups with the aid of the Statistical Analytical Software (SAS) program, version 3.5. Ion analysis was made for comparison of medianstrough Box Plot with Minitab Program.

Results

Mycorrhizal development and plant fresh weigh.

Plant colonization by *R. intraradices* ranged from 54.3 to 66.6% of root length under control and stress conditions (Figure 1). Figure 2 shows the roots of *M. sativa* colonized by *R. intraradices,* with vesicles and hyphae, in all treatment. Stress conditions such as drought, salinity and cold did not affect the percentage of mycorrhization

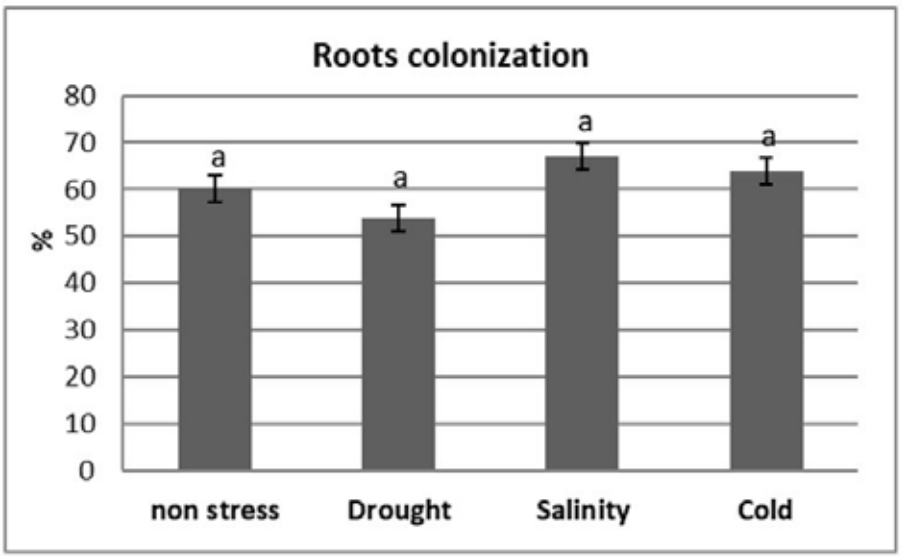


Figure 1: Percentage of roots AM colonization with the AM fungus *R. intraradices* in *M. sativa* plants cultivated either under optimal, drought, salt stress or cold stress conditions. Columns represent means ± Standard error and different letters mean significant differences ($P \leq 0.05$) as determined by the ANOVA test(n=5)

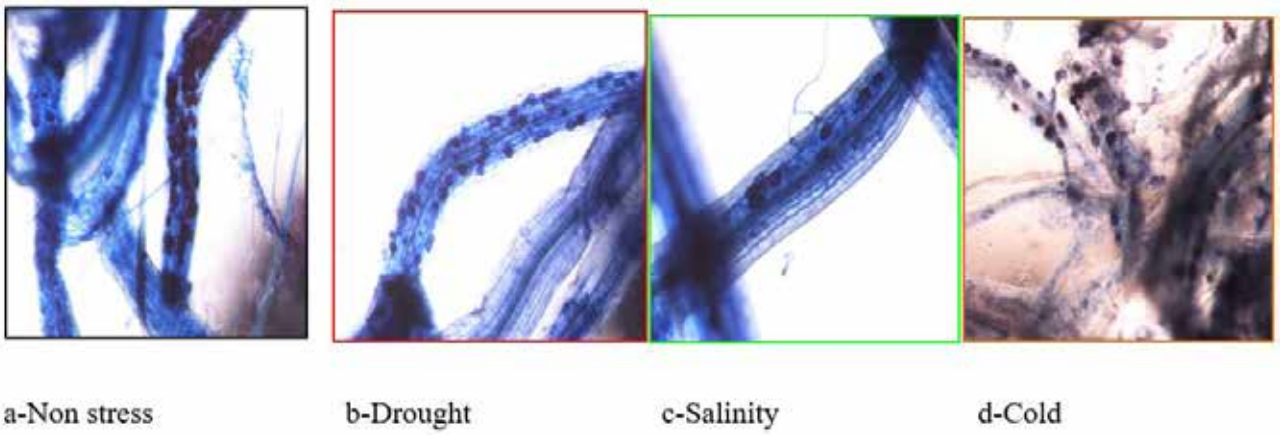


Figure 2: Images a, b, c and d show the colonization of *R intraradices* in the roots of *M. sativa* under conditions of no stress, drought, salinity and cold respectively

Fresh Weight

The FW of shoots and roots of *M. sativa* plants varied in relation to colonization, thus AM plants increased FW, in relation to non-mycorrhizal plants (Table 2). Aerial and roots FW increased in non-stressed, drought and salinity condition. Cold showed similar behavior in NM and AM plants, both aerial and roots FW (Table2)

	Fresh Weight (mg)			
	Shoots		Roots	
Treatment	NM	AM	NM	AM
Non-Stress	3257±2,50 c	4605± 2,40 b	1807±0,45c	3778 ±0,98a
Drought	1515±1,75 e	4602±1,55b	1969±1,99c	2919±0,67b
Salinity	2213±3,02 d	5515± 2,43ab	1774±1,65c	2575±1,78b
Cold	2311±2,40 d	2578±2,10d	2237±0,23b	2109±1,34b

Table 2: Columns: Treatment (NM = non-mycorrhizal plants; AM = Plants mycorrhized with *R. intraradices*), Non stress, Drought, Salinity, Cold. Columns represent means ± Standard error. Different letters mean significant differences (P ≤ 0.05) determined by Duncan's Multiple Range Test (n=5)

Jasmonate levels

Jasmonate levels were determined in the shoots of NM and AM plants. The levels of OPDA were 2 or 3 times higher in AM plants than in NM plants for all treatments, except under cold stress conditions, where the increase was not significant (Table 3). In NM plants, the levels of OPDA increased under salt and drought stress with respect to non-stress and cold stress conditions (Table 3). Similarly, in AM plants, OPDA contents were significantly higher under salinity and drought stress. In contrast, the accumulation of the JA precursor was inhibited in AM plants by cold stress. The level of JA in the shoots of AM plants was higher than in NM plants, under drought and salinity stress (Table 3). NM plants showed a similar JA content under drought, salinity and non-stress conditions.

	Jasmonates (pmol. g^{-1} FW)			
	OPDA		**JA**	
Treatment	NM	AM	NM	AM
Non-Stress	500 ± 2,50 d	2000 ± 5,10 b	1000 ± 1,45cd	1600 ± 2,56bc
Drought	1500 ± 4,05c	3100 ± 6,01 a	1300 ± 3,46 c	3400 ± 1,24a
Salinity	1600 ± 3,09c	3000 ± 7,04 a	1200 ± 3,67c	3150 ± 1,79 a
Cold	500 ± 5,80 d	1000 ± 8,70 cd	400 ± 0,23de	1000 ±1,34 cd

Table 3: Content of jasmonates (pmol g^{-1} FW) in *M. sativa* plants non-mycorrhized (NM) or mycorrhized (AM) with the AM fungus *R. intraradices* and cultivated either under optimal, drought, salt stress or cold stress conditions. OPDA and JA Columns represent means ± Standard error. Different letters mean significant differences (P ≤ 0.05) as determined by Duncan's Multiple Range Test (n=5)

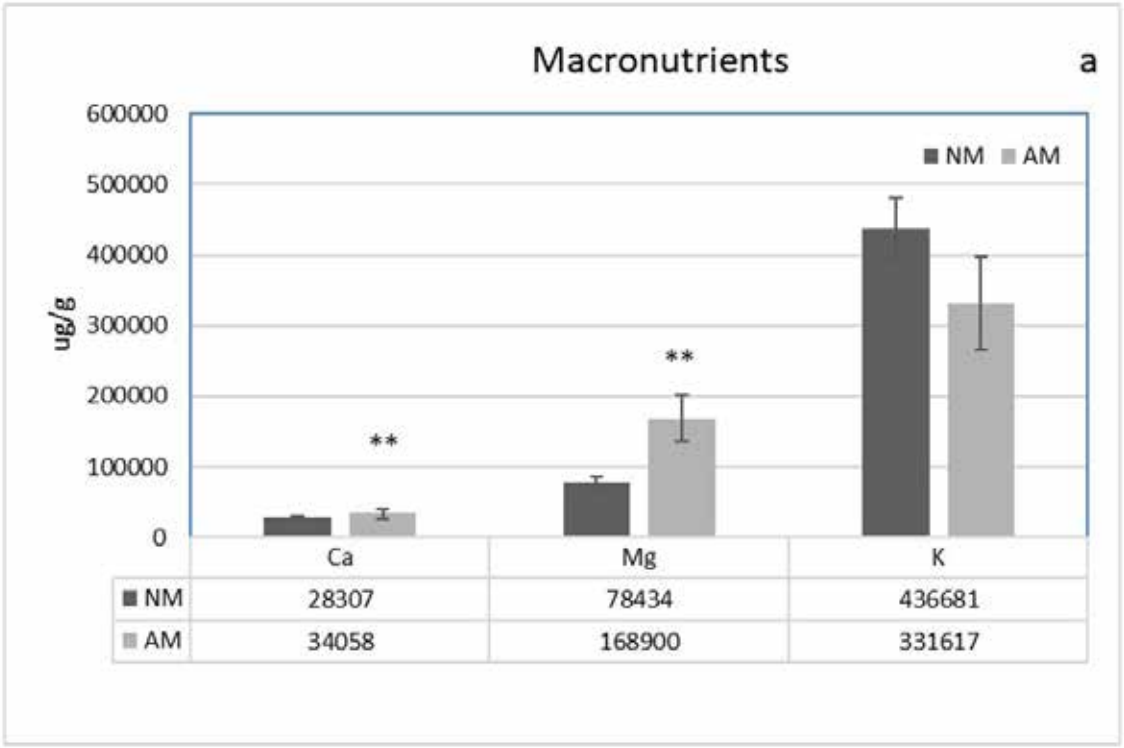
Aquaporin protein quantification

The accumulation pattern of PIP1 proteins was analyzed in roots of NM and AM plants under control and different stress conditions. An increase of accumulation of aquaporins PIP1 was observed in roots of AM plants in relation to NM plants under control conditions. In drought and saline conditions, the accumulation of aquaporins in NM plants was higher than in AM plants (Figure 3)

Figure 3: Western blot analysis of the accumulation of PIP1 aquaporins in roots of *M. sativa* plants non-mycorrhized (NM) or mycorrhized (AM) with the AM fungus *R. intraradices* and cultivated either under optimal, drought, salt or cold stress conditions. The values represent the mean value (n = 4) with two independent biological samples of the signal intensity, quantified by image analysis

Elemental analysis in control samples

Inoculated plants showed an AM-related increase in several mineral nutrients, including micronutrient such as Fe, Mn, (Figure 4a) and macronutrient as Ca and Mg (Figure 4b). In contrast, the concentration of many heavy metals (0 - 25 ug/g), such as Cd, Pb, Ti, Hg, and Bi (Figure 5a), were lower in mycorrhizal roots relative to NM controls. The heavy metals (0- 250 ug/g) such as Cr, As and Be (Figure 5b) were lower in mycorrhizal relative to NM controls too with significant differences ($P \leq 0.05$).



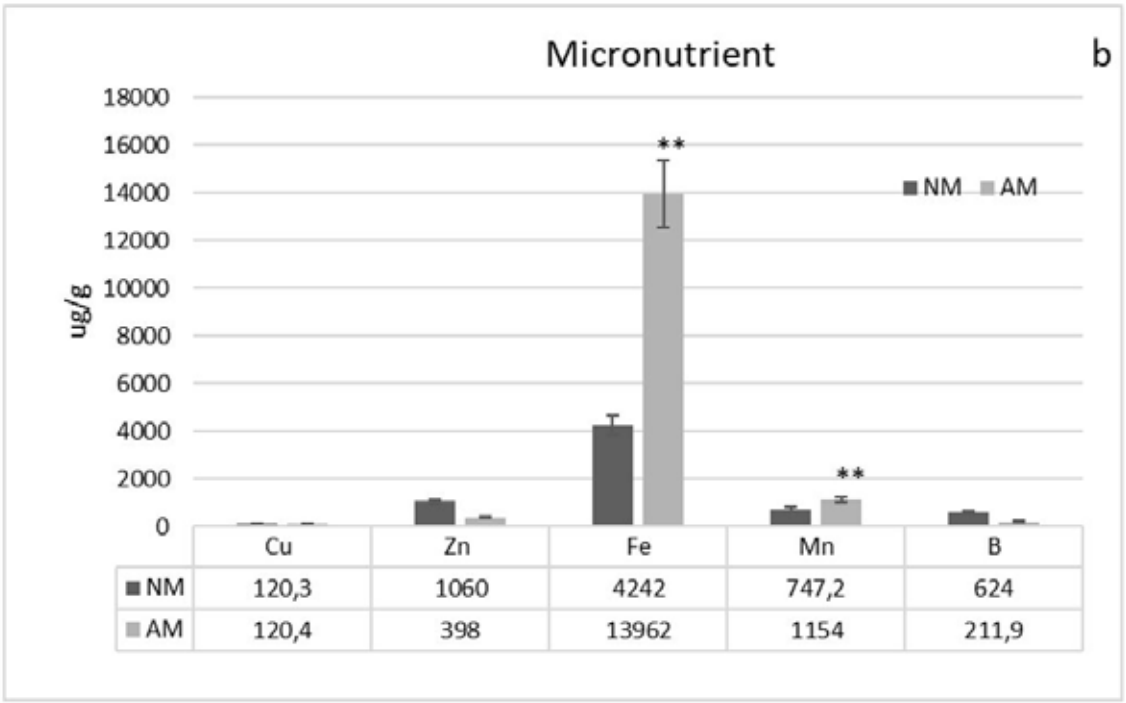
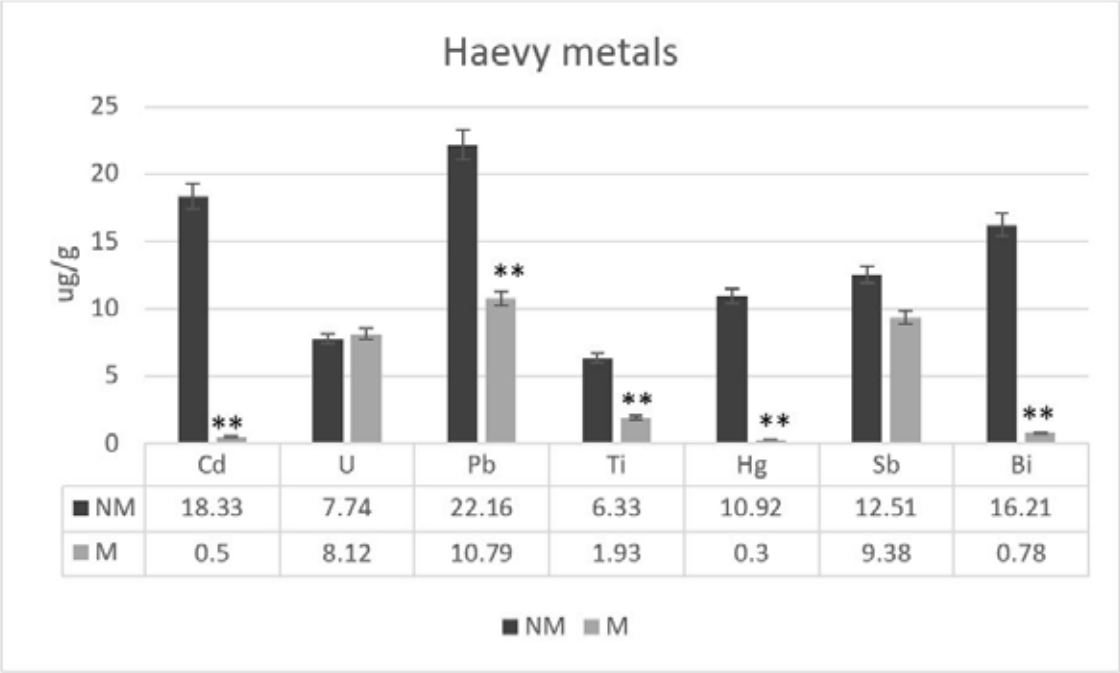


Figure 4: Micronutrients (a) and macronutrients (b) ions(µg/g) in *M. sativa* roots plants, non-mycorrhized (NM) or mycorrhized (AM) with the AM fungus *R.intraradices* ** significant differences ($P \leq 0.05$) (n=5)



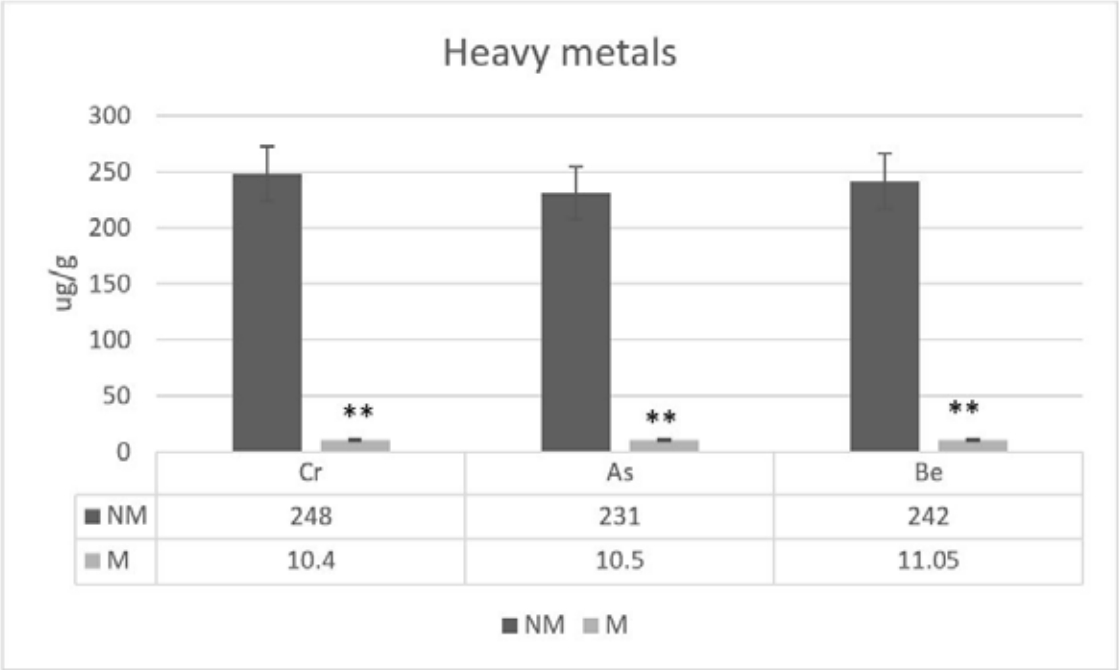


Figure 5: Heavy metals minor 25 (µg/g) (a) and more 25 (ug/g) (b) in *M. sativa* roots plants, non-mycorrhized (NM) or mycorrhized (AM) with the AM fungus *R. intraradices*. ** mean significant differences ($P \leq 0.05$) (n=5)

Discussion

AMFs form symbiotic associations with various plant species and have been shown to possess the potential to improve soil structure and plant growth under normal conditions and stressful environments ([47] Tang *et al.* 2009; Navarro *et al.* 2013 [35]; [26] Hashem *et al.* 2016).The values for colonization observed in *M. sativa* plants were similar than in other species and the application of abiotic stress did not affect root colonization and there were no significant differences between different stresses.

The results of this study show that the FW of stressed and non-stressed AM plants increased in relation to those that were NM. Various studies have reported that mycorrhizal colonization improves plant growth and vigor ([17] Evelin *et al.* 2014; Ahanger *et al.* 2014 [2]; [4] Alqaraui *et al.* 2014a, b; Hashem *et al.* 2016 [26]).

JA signaling can be induced by a range of abiotic stresses, including osmotic stress, salt, drought and cold stress (Pedranzani and Vigliocco, 2017) [38] and jasmonates are involvement in the formation and development of mycorrhizal symbiosis (Bucher *et al.* 2014) [12]. Increases in JA have been shown to correlate with the activation of genes for enzymes of the biosynthesis of this hormone (Hause *et al.*2007; Hause and Schaarschmidt, 2009) [28-29]. In our study, the levels of OPDA (JA precursor) were significantly higher in shoots of AM plants than in NM plants for all treatments except under cold stress conditions (Figure 2a). The level of JA in the shoots of AM plants was higher than in NM plants, showing significant difference under drought and salinity stress (Figure 2b). Apparently, the mycorrhizal plants of *M. sativa* activate defense mechanisms through the JA routes under salinity and drought conditions.

Aquaporins (AQPs) proteins have been shown to transport water and other small molecules through biological membranes, which is crucial for plants to combat abiotic stress, however, the precise role of *AQPs* in several stress response is not completely understood in plants (Zhou *et al.* 2012) [53]. Numerous studies have shown that AQPs play an important role in abiotic stress, such as salt ([45] Sade *et al.* 2010; Gao *et al.* 2010 [20]; Horie *et al.*2011 [30]), drought (Mahdieh *et al.* 2008) [34] and cold (Aroca *et al.* 2004 [6]; Chao *et al.*, 2014 [14]). The relationship between modulations by AM of plant AQPs and changes in root hydraulic conductance, plant water status and performance under stressful conditions is not well known (Bárzana *et al.* 2014 [9]; Johansson *et al.* 2000 [31]) The regulation of AQP subfamily depends on the watering conditions and the severity of stress imposed (Aroca *et al.* 2007) [7]. In this study we found induction of PIP1 *AQPs* in AM plants under control. In contrast, under drought and salinity, *AQP* accumulation was not raised or even is diminished, perhaps because other posttranscriptional regulatory mechanisms such as phosphorylation that could be altering their water channel activity (Calvo-Polanco *et al.* 2014 [13]; Quiroga *et al.* 2019 [44]).

The increment in PIP1 protein in AM roots under control conditions could be related to an increment of water needed to support the increased growth. Also, water and nutrients uptake by roots are directly related, as most of the nutrients are soluble and nutrient movement is water-dependent (Bárzana and Carvajal, 2020) [10]. In that way, the increment in Ca, Mg, Fe and Mn observed in AM plants are consistent with the observed increment in aquaporins contain.

By other side, the reduction in protein PIP1 root contain observed in AM plants under salinity and drought stress conditions could be related to a better control of water contain inside the cells as a defense mechanism to cope with de-hydration and to maintain the cell turgor needed to support growth rates. Another lecture could be done based in the requirement of stimulate the cation and anion balance needed to adequate response to the stress imposed. Should be taken into account that PIP1 aquaporins are related to different physiological functions as they have been proved to be related to water, H_2O_2, CO_2 but also to anions transport (Tyerman *et al.,* 2021) [49] and indirectly they are implicated in the regulation of water and cations channel activity of the PIP2 aquaporins. The heteromerization of PIP1 and PIP2 is determinant to regulation of membranes transport functions (Fetter *et al.,* 2004) [18] and also to the externalization of PIP1 from inner membranes to their final position in Plasmamembrane (Yepes-Molina *et al.*, 2020) [51]. Also, it has been observed that HvPIP2;8 water channel activity is stimulated by the PIP1 heteromerization with 5 different PIP1 isoforms, and at the same way, the cation Na+/K+ transport of the same PIP2 aquaporin was proportionally reduced (Tran *et al,* 2020) [48]. All these studies implies that PIP1 aquaporins regulation is essential to the osmotic and salinity stress resistance, and a deep regulation from AM plants was expected to have a role in the avoidance of the stressful conditions which lead to a better growth.

In both cases, the reduction in PIP1s under osmotic stress is related to the maintenance of a higher growth rates in AM plants at same conditions than NM plants.

Mycorrhizal plants have two routes of nutrient acquisition, the direct pathway through the root epidermis and roots hairs (representing the only uptake route of NM plants) and the mycorrhizal pathway through the fungal mycelium that delivers nutrients to the root cortex through the arbuscules (Nouri *et al.* 2014) [36]. In this study, we affirmed that the mycorrhization of *M. sativa* roots promoted the accumulation of beneficial ions. Mycorrhization improves *M. Sativa* nutrition by a better uptake of the elements such as calcium, magnesium, manganesium and iron. The most accepted hypothesis is that arbuscular mycorrhizal fungi allow the plant to explore a greater soil volume, far exceeding that of the rhizosphere, through the extracellular hyphae network. Thus, mycorrhizal plants can access an additional source of minerals (Li *et al.* 2014) [32]. Other studies show that AM symbioses increase the absorption of potassium, calcium, manganese, magnesium (Diaz *et al.*, 2010) [15] and iron (Aliasgharzad *et al.* 2009) [3]. The improvement in the acquisition of iron and manganese by AM plant roots is explained by the increase in the solubility of these

two elements by the associated AMF through the reduction of the ions Fe^{+++}into ions Fe^{++} (Diaz *et al.* 2010) [15]. Iron absorption improvement by mycorrhizal fungi is associated to siderophore and phytosiderophore production (Aliasgharzad *et al.* 2009) [3]. *Jacaranda* mycorrhization improves its growth by increasing of height, stems and leaves dry weight and the ability to acquire iron, magnesium and manganese (Zaouchi *et al.* 2015) [52]. In contrast, many heavy metals decreased in the roots of *M. sativa* plants inoculated with mycorrhizae. Indeed, Hammer *et al.* (2011) [25] proposed that AMF may act as a first barrier for ion uptake during the fungal transfer of nutrients from the soil to the host plant. They reported that *R. intraradices* can selectively take up elements such as K^+, Ca^{2+} and Mg^{2+}, while avoiding the uptake of Na^+ under salinity. Moreover, they observed that with increasing salinity levels the concentration of Na^+ increased in AM plants up to a certain level, but subsequently decreased at higher salinity. Similarly, in rice plants it has been shown that the AM symbiosis favours Na^+ extrusion from cytoplasm, its sequestration into the vacuole and its recirculation from photosynthetic organs to roots (Porcel *et al.*2016) [43]. Consequently, there is a decrease of Na^+ root-to-shoot distribution and an increase of Na^+ accumulation in rice roots which allows AM rice plants to maintain their growing processes under salt conditions, increasing it salt tolerance (Porcel *et al.* 2016) [43].

Evidence of accumulation of beneficial ions in AMF-inoculated plants, suggests that mycorrhizae are involved in improving the nutritional status of plants.

In summary, this study shows that AM symbiosis is able to alter the plant JA accumulation, the aquaporin protein profile and the accumulation of macro and micronutrients in order to improve the host plant tolerance to different abiotic stresses.

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