Effects of inoculation with Arbuscular Mycorrhizal Fungi on growth and water stress tolerance of Medicago sativa in arid region of Tunisia

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Abstract

Climate change, in particular drought and desertification were contributed to soil degradation. These phenomena constitute a limiting factor in crop yields of several plants characteristic arid ecosystem of Tunisia. Arbuscular Mycorrhizal Fungi can be contributed to solving this problem. The objective of this study was to evaluate the response of Medicago sativa to arbuscular mycorrhizal inoculation in a well-defined water stress regime of 25%, 50% and 75% of field capacity (CC). This plant was inoculated with native mycorrhizal strains from rhizospheric soils and by exogenous strains “Glomus spp”. The effects of different inoculum were evaluated by determining mycorrhizal colonization and growth parameters. The growth of seedlings was performed and the effects of AMF were evaluated after two months of culture. Mycorrhizal inoculation was showed a significant effect on the tolerance of the young seedlings to the water stress. A positive effect of AMF on the growth of Medicago sativa and biomass production was observed. The highest levels of mycorrhizal intensity were registered in roots of plants subjected to severe stress water. This resulted in an increase in studied morphological parameters and the efficiency of water use instant compared to uninoculated. In conclusion, natural mycorrhization would be as efficient as the mycorrhizal addition for growth stimulation and resistant to water stress.

Keywords: Arbuscular Mycorrhizal Fungi, Medicago sativa, water stress, inoculation

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Introduction

Several constraints prevent the increase of agricultural production. Drought was the main limiting factors affecting agricultural production in arid and semi-arid area [1]. It leads to low water availability whose was a crucial role, especially in the transport and accumulation of some nutrients necessary for plants growth and development. Plants have several adaptation strategies to water stress whose ability to associate with symbiotic micro-organism especially, Arbuscular Mycorrhizal Fungi (AMF). AMF are benefic micro-organisms in the soil and they are capable to associate with the roots of major plants. This symbiotic association improved the hydro-mineral nutrition [2,3]. They were significant drivers of nutrient cycling [4]. Mycorrhizal associations played also a significant role in soil structures and their stability [5]. AMF are important in ecosystem dynamics due to the adaptation of their symbiotic partners to different environmental
stresses [6]. Because of their importance on plant development, AMF played an important role in agriculture. Previous studies have shown that AMF may be benefic in agricultural ecosystems [7]. Until now, several studies have noted that the use of AMF as inoculum can increase plant biomass and minimized the different effects of drought and other environmental stresses [8,9]. Some AMF species improved significantly the aerial biomass of inoculated plants [10]. In the same context, several works showed that AMF allowed also a significant increase in root biomass [11]. All these works give evidence the importance of using AMF as inoculum in sustainable agriculture. In a situation of water stress, the inoculation with some AMF species could improve the tolerance of plants to water stress. However, the performance levels differ and depend on the first the ability of mycorrhizal strains to increase plant growth and health [12], second, the pedoclimatic conditions [13] and finally the mycorrhizal strain used [14].

The arid and semi-arid regions of the world and of Tunisia in particular, have been subjected to accelerated desertification mainly due to pressures associated with increased anthropogenic impacts (grazing) and climate changes which constituted a limiting factor in crop yields of several plants characteristic the arid region. Medicago sativa (M. sativa) was an important legume in the farming systems. It was an important forage legume. This plant was the most cultivated forage legume as animal consumption in the arid region of Tunisia [15]. There were large areas where M. sativa was cultivated. But, the culture and the productivity of this plant were constrained to environmental stresses, such as salinity and drought [16, 17]. Drought was one of the major stresses which severely limit crop production. However, the use of AMF as bio-fertilization technology, which would contribute effectively to improving the productivity and protection of plants, is still under-exploited and remains very limited and fragmented in the arid ecosystem of Tunisia. Indeed, to our knowledge, there was a little study that has been interested in the use of AMF as inoculum to improve the growth and the productivity of some cultured plants in arid region of Tunisia. Therefore, the present investigation was among first studies to evaluate the effect of AMF in the agriculture at Tunisia ecosystem. This work aimed to evaluate the response of M. sativa to different types of AMF inoculation in a well-defined water stress regime.

Materials and methods

Plant Material

To evaluate the efficacy of AMF inoculum, Fabaceae M. sativa was used in this study. This plant was among most cultivated forage legumes at arid region of Tunisia. The seeds of this variety are disinfected in diluted Sodium hypochlorite (90%) at 5 min, rinsed extensively and then imbibed for 30 min in sterile water. Then, they are germinated in nutrient agar (0.8%) and placed in the dark at 28 °C.
**Fungal inoculums**

For the composite native inoculum (indigenous strains), we selected four rhizospheric soils of “Medicago truncatula” at Bou-Hedma National Park with distinct proprieties. Three sites (Soil 1, soil 2 and Soil 3) were taken inside the Park and they were subjected to a light grazing (1 animal per 40 ha). The fourth site (Soil 4) outside the Park was subject to different management and more intensive grazing (80 animals per 40 ha). Table 1 shows the different properties of soils used as inoculum. Commercial inoculum of AMF (exogenous strains) containing some species of Glomus genus was obtained by the society “Inoculum plus” from France. Indeed, the genius of Glomus is considered the most abundant genera of AMF in arid and semi-arid regions [18,19]. For the production of the inoculums, Mycorrhizal strains have previously been multiplied separately in greenhouses using the maize variety (Zea mays) as a trap plant [20]. Zea Mays was grown under greenhouse in pots containing 250 g of soil (Rhizospheric of M. truncatula in different sites) and 1750 g a sterilized substrate. Each treatment was repeated three times. These pots were watered regularly with a solution of Long Ashton [21]. The inoculum was obtained after 3 months of cultivation and was consisted a mixture of spores and fragments of mycorrhizal roots.

<table>
<thead>
<tr>
<th>Sites</th>
<th>1*Soil texture</th>
<th>2*Spore density (spores/100 g of soil)</th>
<th>Grazing intensity</th>
<th>3*pH</th>
<th>3*Electrical conductivity (s.m⁻¹)</th>
<th>4*Organic matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sandy loam</td>
<td>517± 28</td>
<td>Light</td>
<td>8.0± 0.1</td>
<td>2.3± 0.3</td>
<td>1.9± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>Sandy</td>
<td>437± 12</td>
<td>Light</td>
<td>8.3± 0.1</td>
<td>2.0± 0.1</td>
<td>1.1± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>Sandy loam</td>
<td>464± 07</td>
<td>Light</td>
<td>8.0± 0.2</td>
<td>2.3± 0.1</td>
<td>1.4± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>Sandy loam</td>
<td>369± 14</td>
<td>Intensive</td>
<td>8.1± 0.1</td>
<td>1.7± 0.2</td>
<td>0.9± 0.1</td>
</tr>
</tbody>
</table>

1*Soil texture deetermined according to Naanaa and Susini [34].
2*AMF spores occurring in soil samples were extracted following the wet sieving method described by Gerdemann and Nicolson [35].
3*Soil pH and electrical conductivity were determined according to Miller et al [36].
4*Organic matter was determined according to the method of Tiessen and Moir [37].

**Experimental procedure**

Pots used in this experience have an appropriate volume for the root development of the young plant. They were filled with a sandy substrate sterilized by autoclaving at 120°C for 2 h two consecutive days. The inoculation was performed at an amount of 20 g (of mycorrhizal inoculums) by cultivating pot. The experiment included three treatments: uninoculated control,
inoculation with native AMF strains and inoculation with the commercial AMF (*Glomus* spp.). The uninoculated received only 2000 g of sterile sand. The different treatments were subjected to three water stress levels of 25%, 50% and 75% compared to the capacity of the floor field. The experiment lasted two months of culture. In this study, three factors were studied: inoculation types, water stress and sampling dates. Fig. 1 explains the experimental procedure used in this study.

Sampling of plants and dying of root samples

The samples were collected after 40 days, 50 days and 60 days of culture. For each treatment, aerial and root parts of three plants were separated to determine their fresh biomass and then dried in an incubator at 30°C for 48 hours to evaluate their dry biomass. The other roots of plants were carefully sampled in order to evaluate the parameters of mycorrhization. For the status of mycorrhization, a fine root sample was taken from each plant collected. Root segments of 1-2 cm in length were submerged in 10% potassium hydroxide (KOH) at 90°C for 45 min to clear them, bleached in Hydrogen peroxide (H$_2$O$_2$) for 3 min and acidified in 1% hydrogen chloride (HCL). After this, root segments were stained for 90 min with 0.05% Trypan Blue at 60°C. For each species, 30 root fragments were preserved in lactoglycerol to distain the plant material (while the fungal structures retained their blue color). All stained roots were viewed through a microscope at 400x magnification and the presence of hyphae, vesicles, and arbuscules inside the root bark was determined.

Parameters evaluated and Statistical analyses

The growth of plants in height was followed for each type of inoculation. The dry biomass of shoot and root of plant samples were determined. The mycorrhizal parameters (mycorrhizal frequency and intensity) were evaluated by observation of fungal colonization of 30 root fragments per plant, according to the technique of Trouvelot et al [22]. The collect of plant samples was done each time after 40, 50 and 60 days of growth for each type of inoculation. Statistical analyses were performed by using the SAS statistical package. The statistical effects of AMF inoculums were ascertained by ANOVA for repeated measures. The least significant difference values at 5% level of significance (P ≤ 0.05) were calculated to assess differences between parameters. Three ways-ANOVA was used to determine relationships between variables.
Fig. 1: Experimental procedure used in this study.

Results and discussion

Effect of Inoculation types, water stress and sampling date on the colonization roots of *M. sativa* by mycorrhizal fungi.

The identification of symbiotic structures of AMF inside the roots of *M. sativa* as a response to different types of inoculums could confirm the natural mycorrhization of this plant. The infection
rate of the root system by AMF appears to be affected by the type of inoculums (Fig. 2). The values of mycorrhizal frequency (F %) varied between the six types of inoculum. Mycorrhizal intensity (M %) is higher in the case of inoculation with Glomus spp. and the rhizospheric soil on Site 1 (soil 1). Results illustrated in Fig. 2 revealed an effect of the sampling date for the mycorrhizal colonization. The highest values of mycorrhization were apparent from the collection to 50 days of growth. As indicated in the Fig. 2, the uninoculated control was not containing any indication of mycorrhization in the three collection dates. The roots of this plant did not present any structures of the endomycorrhizal fungi.

At the six types of inoculation, the mycorrhization infection rate of the root system of M. sativa by the AMF appears to be affected by the imposed water deficit (Fig. 2). For the three water treatment, the mycorrhization frequency (F %) and intensity (M %) was higher in the stress water by 25% CC than 50 and 75% CC. Thus, the application of water stress of 75 CC reduces the progression of AMF in root cortex of this plant in the different inoculation types (Fig. 2). The higher value of mycorrhization infections of roots registered in the inoculation with Glomus spp. and Soil 1. Roots of M. sativa were present some structures of AMF (arbuscular and vesicular) in stress water by 25% CC even if the roots were collected after 40 days. It is a clear correlation between the water treatment imposed and the collection dates.

Effect of inoculation types, water stress and sampling date on the growth of M. sativa.

Our study revealed that, compared with non-inoculated plants, the inoculated plants had a significantly higher growth for all analysis variables (p<0.001). Regardless of the type of inoculation considered, all growth parameters were more pronounced for inoculated plants than non-inoculated plants (Figs. 3, 4, 5 and 7). After 40 days of growth, the effect of AMF has not yet been very significant. The inoculated plants had slightly higher growth than non-inoculated plants. The growth parameters were characterized by rapidly and highly value of inoculated plants after 50 days of growth.

On the other hand, the type of used inoculation had a significant effect on all growth parameters (p<0.001). Therefore, the responses of M. sativa to mycorrhizal symbiosis was dependent the type of AMF inoculation (Figs. 3, 4, 5 and 7). The highest value was registered in the inoculation with Soil 1 and Glomus spp. However, no difference was observed between those two type inoculations for the major studied variables. The uninoculated control was not mycorrhizal suggesting that differences in all measured parameters are mainly due to the effect of inoculated strains.
The results of the Figs 3, 4, 5 and 7 showed that water stress had a negative effect on the most studied morphological parameters of *M. sativa*. The application of water stress has a negative effect on the aerial part of this plant. When the water stress is accentuated, the elongation of the aerial part of this plant is reduced (Fig. 6). Root length is significantly higher with the applied stress by 50% and 25% CC and is without significant difference between the different types of the water regimes of 75% CC (Fig. 5). The length root of *M. sativa* achieved 15 cm at the water stress by 25%. Statistical analysis of aerial and root biomass data for different inoculation (Fig. 4 and 5) showed the variability effect of water stress. The negative effect was observed mainly for the regime of 25% CC. Indeed, the aerial biomass of all inoculated plants was significantly greater than non inoculated plants.

Fig. 6 is demonstrated that the imposed water stress favored the increase of the underground biomass compared to the aerial biomass. The importance of the root part is generally associated with the search of water, especially in the water regime of 25% CC.

**Correlation and Interaction between AMF Inoculation, sampling date and water stress**

Generally, mycorrhizal frequency (F %) and intensity (M %) increase at 50 and 60 days of growth. At the first sampling (40 days), mycorrhizal parameters appear only for the water stress of 25% CC. Indeed, this parameter is only observed for plants inoculated with *Glomus* spp. and those with Soil 1. Mycorrhizal frequency and intensity appear correlated with AMF inoculation, sampling date and water stress (Table 2). At the same context, all studied growth parameters was varied according to AMF inoculation, date of sampling and water stress.

This work was a permit to evaluate and compare the response of *M. sativa* to AMF inoculation: native mycorrhizal strains and exogenous strains “*Glomus* spp.”. In addition, the effect of typical inoculum factor and water stress in the culture was also studied. As first result, as observed in the root of *M. sativa*, all characteristic structures of root colonization by AMF (intracellular aseptate hyphae, vesicles and arbuscules) were observed. This plant responded significantly with AMF inoculation used in this study. The response of *M. sativa* to the different strains of AMF inoculation is due generally a preference of the host plant shown against some AMF strains. The highest rate colonization of root samples could be explained by the high mycotrophy of this Fabaceae, which is formed naturally a dense mycelium filament at the soil. The results indicated that AMF has a significant influence on the growth of this plant. However, the response degree to AMF inoculation depended on the variety and the types of fungal strain. Similar results were observed in other plant species under semi-controlled conditions. The positive effect of AMF inoculation in the growth of *M. sativa* is due in large part to a better improvement in water nutrition. Indeed, the inoculated plants were able to absorb the water...
beyond several centimeters to the absorption zone of roots through to better exploration of the soil by hyphae of AMF [23, 24].

Based on the literature [25, 26], Mycorrhizal inoculation can improve plant growth and their resistance to drought. The application of water stress had a negative effect on the growth of *M. sativa*, and when the water stress accentuated, the morphological parameters of these plants reduced. According to our finding, other studies showed that drought reduced the morphological parameters of plants [27]. AMF has a significant effect on the development of *M. sativa*, in the face of imposed water stress. To compare with the non-inoculated plant, the mycorrhizal plant will acquire a better resistance to water deficit [28], the major biotic and abiotic stresses [6]. However, the highest rate values of frequency and intensity of mycorrhization were registered in the application of 25% CC water stress compared to other applied water stresses. Our study demonstrated clearly the important role of AMF inoculation and their ability to improve the tolerance of *M. sativa* to water stress.

Recent research corroborated and emphasized the crucial role of AMF in improvement the plant nutrition. This nutrition improved in the aerial part of mycorrhizal plants compared those non-mycorrhizal [6, 29]. In agreement with our results, the biomass and length of aerial parts (Fig. 4) of mycorrhizal *M. sativa* are very important compared to those non mycorrhizal (control soil). AMF inoculation increased significantly also biomass root of studied plant (Fig. 5). These results are consistent with the work of Avio et al. [11] when studying the effect *Rhizophagus irregularis* in the growth of *M. sativa*. The study of Khan et al [30] and Fan et al. [31] was reported that AMF stimulates the biomass of root system. The increase of root biomass is generally associated with the water deficit condition. In addition, according to Yadav et al. [32], it is evident that an increase in the root biomass allows obtaining high yields under conditions of water stress. The extra-radical mycelium formed by AMF grows and explodes far beyond the zone around roots comparatively to non-AM plant roots. Further, Guissou et al. [26] observed that root density and root length were positively correlated with mycorrhizal types and their dependence AMF species. The mixed AMF species, especially at Soil 1, had a strong effect on the major growth proprieties of *M. sativa*. The native mixed inoculum appears to be more effective as *Glomus* spp. for height growth and biomass production of this plant.
**Fig. 2**: Mycorrhizal frequency (F %) and intensity (M %) of *M. sativa*. Mean of three replicates and ± standard error. Differences between groups were significant α= 0.05.
Fig. 3: Inoculated and uninoculated *M. sativa* after 40 days of growth.
Fig. 4: Weight and dry weight of Aerial part of *M. sativa*. Mean of three replicates and ± standard error. Differences between groups were significant $\alpha= 0.05$. 
Fig. 5: Weight and dry weight of root part of *M. sativa*. Mean of three replicates and ± standard error. Differences between groups were significant α= 0.05
Fig. 6: Weight of Aerial part/ root part of *M. sativa*. Mean of three replicates and ± standard error. Differences between groups were significant $\alpha= 0.05$. 

![Weight of Aerial part/ Root part 75 % CC](image_url)

![Weight of Aerial part/ Root part 50% CC](image_url)

![Weight of Aerial part/ Root part 25% CC](image_url)
Fig. 7: Length of Aerial part and root part of *M. sativa*. Mean of three replicates and ± standard error. Differences between groups were significant α= 0.05.
Table 2: three way- ANOVA for Inoculation types, water stress and sampling date and their interactions

<table>
<thead>
<tr>
<th>Inoculation types</th>
<th>Water Stress</th>
<th>Sampling date</th>
<th>Inoculation types * Water Stress</th>
<th>Inoculation types* Sampling date</th>
<th>Inoculation types * Water Stress* Sampling date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycorrhizal frequency F%</td>
<td>1766.07 *** 2968.27 *** 2097.32 *** 130.93 *** 93.43 *** 8.2 ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycorrhizal Intensity M%</td>
<td>10123.36 *** 45788.37 *** 12176.4 *** 2095.76 *** 677.76 *** 97.72 ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight of Aerial part</td>
<td>787.27 *** 1040.69 *** 7167.43 *** 8.62 *** 124.25 *** 1.89 ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry Weight of Aerial part</td>
<td>13.7 *** 51.5 *** 1238 *** 8.3 *** 6.8 *** 9.5 ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight of root part</td>
<td>1983.18 *** 505.8 *** 5129 *** 7.32 *** 63.7 *** 4.35 ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry Weight of root part</td>
<td>57.6 *** 28 *** 175 *** 4 *** 28.6 *** 8 ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight of Aerial part / root part</td>
<td>290.05 *** 1518.07 *** 194.73 *** 29.07 *** 33.94 *** 4.62 ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of Aerial part</td>
<td>1642.01 *** 417.51 *** 1672.57 *** 80.35 *** 10.94 *** 4.39 ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length of root part</td>
<td>684.03 *** 695.77 *** 6022.73 *** 6.51 *** 7.72 *** 2.2 ***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: ***Significant at P<0.001, **Significant at P<0.01, *Significant at P<0.05, ns: non-significant

The effects of deficit water in our study are varied according mycorrhizal inoculation and levels of imposed stress. This is an agreement with the report of Ruiz-Lozano et al. [25] and Guissou et al [26]. At first finding, the application of water stress significantly increases the mycorrhizal colonization of roots. The highest intensity of roots mycorrhizal colonization of *M. sativa* was registered in is accentuated water stress comparison with 50% and 75% CC. Radi et al. [27]. are reported the same finding in their study. However, as consequence of deficit water, AMF appear in a very advanced step even if at the sampling after 40 days of growth (Fig. 2). The ratio of aerial biomass to root biomass (Fig. 6) showed that *M. sativa* favored root growth, especially, in the stressful water regime. This is may be a resistance strategy to water stress. This can be explained by the extra-radical mycelium formed by AMF to explode soil and increase the absorption surface of root system [27]. Furthermore, the tolerance of mycorrhizal plants in water stress is expressed by the maintenance of the turgescence of the foliar cells due to the accumulation of solutes [26]. Studies on osmoregulation are indicated that some composed (sugar and protein) play a significant role in the tolerance of water stress and contributed to maintenance balance of the osmotic force to keeping the turgescence of foliar cells as possible [27]. All the more, mycorrhizal plants can increase the accumulation of photosynthesis products...
and improve their growth and resistance to water stresses [33]. Continued interest on the roles of AMF as an aid to plants faced with water stress appears well-justified.

**Conclusion**

In conclusion, the present study showed clearly the importance of the mycorrhizal inoculum on the growth and the development of *M. sativa* and their capacity to tolerate the stress drought imposed. Our result demonstrated that this plant presents various aptitudes to be developed and resist to their ecosystem. AMF inoculation improves growth plants and their response varied according to the different treatments. Generally, the inoculum effects were varied depending AMF species used. Indigenous inoculums especially of “Soil 1” are effective on the growth of plants. However, their effectiveness was remained comparable of the exogenous inoculum (*Glomus* spp.). This study also pointed that AMF effect was depended the water deficit imposed. For the three water treatment, the highest mycorrhizal colonization was registered in the water stress of 25% CC than 50, 75% CC. Water stress can involve a depressive effect on the growth of the uninoculated plants. The results of the present study suggested that indigenous AMF species improved the capacity of *M. sativa* to be developed and to tolerate the drought which is the main limited factor in agriculture at arid and semi-arid ecosystem.

**Acknowledgments**

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**Conflict of Interest**

We declare that we have no conflict of interest.

**References**


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