Original article

Inoculation with selected indigenous mycorrhizal complex improves *Ceratonia siliqua*'s growth and response to drought stress

Issam Jadrane [a], Mohamed Najib Al feddy [b], Hanane Dounas [a], Lamfeddal Kouisni [c], Faissal Aziz [d], Lahcen Ouahmane [a,*]

[a] Laboratory of Microbial Biotechnologies, Agrosciences and Environment, Faculty of Science Semlalia, Cadi Ayyad University, Marrakesh, Morocco
[b] Phytobacteriology Laboratory, Plant Protection Research Unit, CRRA Marrakesh, National Institute for Agronomical Research, Marrakesh, Morocco
[c] University Mohammed VI Polytechnic, Agrobiosciences Program, Benguerir, Morocco
[d] Laboratory of Water, Biodiversity and Climate Change Cadi Ayyad University, Marrakesh, Morocco

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ABSTRACT

In the current study, we investigated the impact of inoculation with a selected indigenous arbuscular mycorrhizal fungi (AMF) complex on the growth and physiology of carob plants at increasing levels of watering (25, 50, 75 and 100% field capacity). The following growth and stress parameters were monitored in carob seedlings after 6 months of growth and 2 months of applied drought stress: fresh and dry weight, root and shoot lengths, leaf surface area, relative water content, stomatal conductance and membrane stability. Chlorophyll a and b, total soluble sugars, proline and protein contents were also determined along with the activities of stress enzymes: Catalase, Peroxidase and Superoxide dismutase. The obtained results indicate that inoculation with the indigenous AMF complex has a positive impact on the plant's growth as all the assessed parameters were significantly improved in the mycorrhizal plants. Additionally, our results show that mycorrhization contributes to the minimization of the impact of drought stress on the carob plants and allows a better adaptation to dry conditions.

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1. Introduction

The Mediterranean region is one of the most vulnerable to global warming with a drought frequency that has been dangerously increasing for the last 30 to 40 years (Mariotti, 2010). Ten of the driest winter seasons in this region were recorded in the last twenty years (Hoerling, 2012) leading to an increased water short-

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age and a bigger threat on the local ecosystems and agriculture which utilizes the vast majority of the water supply in the region (Espere, 2006). This trend is expected to exacerbate in the next few decades (Giorgi, 2006) which, combined with the increasing prevalence of low fertility degraded soils in the region, is bound to have catastrophic consequences on the local plant cover (Séré et al., 2008). The carob (*Ceratonia siliqua*), a leguminous of the species Caesalpinaceae sub-family, is an important component of Mediterranean ecosystems and one of few plants able to thrive in the marginal calcareous soils that are prevalent in the region, it also tolerates the semi-arid to arid climate conditions (Battle and Tous, 1997). Whether it is pods, pulp or seeds, carob trees have a multitude of ancient and newly developed uses in the food and agricultural industries (Parrado et al., 2008) in addition to numerous promising pharmaceutical and cosmetic applications (Guardiola et al., 2018; Papakonstantinou et al., 2018). The carob has thus become an important candidate in agriculture as an additional candidate for diversification (Janick and Paull, 2008) but also for use in the restoration of soils and as a natural barrier to desertification (Manaut et al., 2015). The success of the carob in these harsh environments is in part due to its symbiotic relationship with arbuscular mycorrhizal (AM) fungi. This symbiotic association

is the most common type of symbiosis among vascular plants (Simon et al., 1993), 80% of them are able to form these types of relationships. By mobilizing minerals and nutrients in root systems (Smith and Read, 2008), improving the quality of the soil and minimizing the effects of various stresses on the plant host (Bompadre et al., 2014; Auge et al., 2014; Wu et al., 2017). AMF symbiosis greatly contribute to the maintenance and sustainability of soil-plants systems (Van der Hejden et al., 2006), in various climates and environmental conditions. This protective role of AMF symbiosis becomes vital in the environments most vulnerable to the effects of abiotic stresses (Bothe et al., 2010; Chitarra et al., 2016; Wu et al., 2017). As ecosystems worldwide are increasingly endangered by environmental change, new strategies that rely on AMF are being developed to alleviate the negative consequences associated with these changes (Barea et al., 2011). These strategies aim to improve the growth and stress tolerance of the plant host as well as re-establish the indigenous structural, chemical and biological properties of the surrounding soils through the use of native mycorrhizal potential (Manaut et al., 2015; Fakhech et al., 2019). The carob tree has been shown to withstand harsh climactic and geographic conditions (Correia et al., 2010; Ozturk et al., 2010) and its high dependence on its AM fungal partners has also been established (Ouahmane et al., 2012). In fact, inoculation with different commercial AM fungi has been shown to improve the carob's tolerance to drought stress by reinforcing its stress-coping mechanisms, particularly its water relations, photosynthetic ability and the management of oxidative stress (Essahibi et al., 2018). In this study, we investigated the effects of inoculation with an indigenous mycorrhizal complex on carob seedlings' response to various levels of drought stress (25%, 50%, 75% and 100% Field Capacity). Specifically, we analysed the fresh and dry biomass, root and shoot lengths, membrane stability, stomatal conductance, relative water content, chlorophyll a and b, total soluble sugars, protein and proline content and the activities of three major oxidative stress enzymes: Catalase, Peroxidase and Superoxide Dismutase.

2. Materials and methods

2.1. Experimental design, field sampling and inoculum production

2.1.1. Mycorrhizal spores origin and isolation
The source soil for the mycorrhizal fungi was collected in the region of Marrakesh, Morocco where the carob tree is indigenous. Sampling was conducted at depths varying between 10 and 40 cm.

2.1.2. Inoculum preparation
The endomycorrhizal inoculum was produced using Maize (*Zea mays L.*) as an endophytic plant. The Maize seeds were disinfected and germinated in pots containing the disinfected soil. Maize plantlet were then inoculated with a suspension of sterilized mycorrhizal spores mixture formerly extracted using the wet sieving method (Ouahmane, 2007) then allowed to grow for a period of 3 months. The roots colonized by the mycorrhizal fungi complex were used as fresh mycorrhizal inoculum; they were rinsed three times with sterile distilled water and cut into 1 cm fragments.

2.1.3. Carob seed origin and preparation
Carob pods were collected from a single carob tree in the region of Marrakesh, Morocco. The seeds were surface sterilized with 10% bleach solution then placed in 97% sulfuric acid solution for 20 min (Gunes et al., 2013). After being thoroughly rinsed they were soaked in water for 48 h, placed in Petri dishes containing wet cotton and allowed to germinate. The seeds were considered to be germinated after the radicle has exceeded a length of 5 mm.

2.1.4. Inoculation and planting
Germinated seeds were placed in 2 g of fresh mycorrhizal root fragments then planted in pots containing 2 kg of sterilized soil 50% soil/50% sand (v/v). The pots were then placed in a glasshouse at the national institute for agricultural research in Marrakesh, Morocco at temperatures varying between 22 and 38 °C, 50–60% humidity and a photoperiod of about 16 h light/8 h dark. They were watered daily for an initial period of growth of 24 weeks at 100% field capacity (FC). Drought stress was introduced over four weeks at 25%, 50%, 75% and 100% FC, then maintained for 8 weeks. The plants fell into 2 categories: Mycorrhizal (AMF) and non mycorrhizal (NM).

2.2. Physiological and mycorrhization parameters

At the end of the water stress cycle, the plants were harvested, the roots and shoots were weighed separately to determine their fresh and dry weights, the shoot and root dry weights were measured after placing the fresh plant materials in an oven for 48 h at 80 °C. Shoot heights and root lengths were measured. Leaf surface area was determined using image analysis software imagej (NIH). Mycorrhization parameters were determined through microscopic observation of colorized fresh carob root fragments using the method developed by Phillips and Hayman (1970). After washing and de-colorization in 10% KOH at 90 °C for 2 h, the roots were placed in a 7.5% hydrogen peroxide solution for 5 min then treated with 1% HCl for 5 min. A 0.5% trypan blue stain (1:1:1 water, glycerol and lactic acid) was used as a coloring agent at 90 °C for 15 min. Mycorrhization parameters were determined according to the method of Trouvelot et al., (1986).

2.3. Relative water content

Relative water content (RWC) was determined using the formula developed by Talaat and Shawky (2014). RWC = $100 \times [(FW-DW)/(TW-DW)]$ in which FW, DW and TW represent Fresh weight, dry weight and turgid weight respectively. The turgid weight (TW) was determined after placing the leaves, fully submerged, in water in the dark for 24 h at 4 °C.

2.4. Membrane stability

Membrane stability was determined according to the method developed by Shanahan et al., (1990), a conducto-metric technique which assesses membrane damage by measuring electrolyte leakage. 100 mm^2 leaf fragments were rinsed then placed in test tubes containing 10 mL of distilled water and placed in test tube shaker racks for 24 h, the initial conductivity C1 is then measured. Final conductivity C2 was measured after autoclaving the samples for 10 min at 0.1 MPa and cooling them down to room temperature (25 °C). The membrane stability index is then calculated based on the formula: MSI = $[1 - (C1/C2)] \times 100$.

2.5. Stomatal conductance

Stomatal conductance was measured at a temperature of 25 °C using a leaf porometer (Model SC-1, Decagon devices).

2.6. Total Chlorophyll

Fresh leaf material (50 mg) was ground in 3 mL of 90% acetone solution then centrifuged at 100 rpm for 10 min. After 2 h incubation in the dark, optical density (OD) was read at 663 and 645 nm and chlorophyll a, chlorophyll b and total chlorophyll contents were calculated according to Arnon (1949) and Raimbault et al., (2004).

2.7. Proline content

Leaf material (400 mg) were homogenized in 5 mL of 95% Ethanol and rinsed three times using 70% Ethanol. For each sample, 5 mL of the combined supernatant were recovered and 2 mL of chloroform were added along with 3 mL of water. The samples were then allowed to incubate for 12 h (Nguyen and Paquin, 1971). A 0.2 to 1 mL aliquot of the superior phase was then added to a ninhydrin solution and glacial acetic acid and placed in a 100 °C water bath for 45 min. After cooling, 2 mL of toluene were added and the samples were allowed to rest for 30 min. Optical density (OD) of the superior phase was then read at 520 nm and a standard curve was used to determine proline concentration (Singh et al., 1973, modified).

2.8. Total Soluble sugars (TSS)

Total soluble sugar content was determined according to Dubois et al. (1956, modified). 100 mg of fresh plant matter were ground in 4 mL of 80% ethanol then centrifuged at 4000 rpm for 10 min. 2.5 mL of 5% phenol and 2.5 mL of 97% sulfuric acid were added to 0.5 mL of the supernatant, the mixture was homogenized then allowed to rest for 5 min. Optical density was measured at 485 nm and a glucose standard curve was used to determine TSS content.

2.9. Protein content and oxidative enzyme activity

The protein extract was obtained by grinding and homogenizing 100 mg of leaves sample in 0.1 mL of 50 mM potassium phosphate buffer (pH 7.5), 1% pvpp (polyvinylpolypyrrolidone) and 0.1 mM EDTA. The resulting mixture was centrifuged for 20 min at 4 °C (12,500g) and the supernatant was used for protein content enzymatic activity determination.

Total proteins were determined using the method of Bradford (1976). 100 µl of diH$_2$O were added to 100 µl of the protein extract and 2 mL of Bradford's reagent. The samples were then incubated for 5 min and the optical density (OD) was read at 595 nm. Protein content was determined using a serum bovine albumin standard curve. Catalase (CAT) activity was determined in the protein extract by determining the rate of disappearance of the 15 mM hydrogen peroxide. The reaction mixture contained 940 µl of 50 mM phosphate buffer (7.0 pH), 40 µl of hydrogen peroxide and 40 µl of the protein extract. The change in OD was determined by spectrophotometry at 240 nm for 3 min (ε = 39.4 mM cm^{-1}) (Hwang et al., 1999). Guaiacol-peroxidase (G-POD) activity was also determined in the protein extract by following the change in OD for 5 min at 470 nm (ε = 26.6 mM cm^{-1}). The reaction mixture contained 2290 µl of 60 mM phosphate buffer (6.8pH), 100 µl of 18 mM guaiacol and 40 µl of the enzyme extract with the addition of 100 µl of 20 mM hydrogen peroxide to initiate the reaction (Hwang et al., 1999). Superoxide dismutase (SOD) activity was determined by measuring the reduction of Nitroblue tetrazolium according to the method of Beyer and Fridovich (1987). The reaction mixture contained 2550 µl of 100 mM phosphate buffer (pH 7.8), 75 µl 55 mM methionine, 300 µl 0.75 mM nitro blue tetrazolium (NBT) and 50 µl of the enzyme extract, 60 µl of 0.1 mM riboflavin were added and the mixture was subsequently incubated under 2 fluorescent lamps (20 W) for 15 min at 25 °C. The OD was read at 560 nm. An enzymatic unit was defined as the amount necessary to inhibit the reduction of the NBT by 50%.

2.10. Statistical analysis

Statistical analysis was conducted using two-way ANOVA on statistical analysis software SPSS 20 (IBM) with AMF inoculation (AMF) and field capacity (FC) as first and second factors respectively. The significance of the differences and among treatments and factor interactions was calculated at 5% whereas mean comparisons were determined using Tukey's post-hoc test (P $\leq$ 0.05). A minimum of three repetitions was used for all the analyzed parameters.

3. Results

3.1. Physiological and mycorrhization parameters

Both shoot and root fresh weights were significantly improved by the presence of the mycorrhizal complex particularly at high levels of water stress (25% FC). The shoot fresh weight of the mycorrhizal plants was 33% greater than the non-mycorrhizal plants at 25% FC and 51% greater at 50% FC. The beneficial effect of the mycorrhizal complex was also observed in the absence of drought stress at 75% and 100% FC. At 75% FC, the shoot and root fresh weight were improved by 61% and 30% respectively (Table 1). Overall, both the AMF treatment and the water regime had a significant impact on the fresh weight. The shoot and root dry weights were also significantly impacted by the presence of the mycorrhizal complex at all levels of stress. At 25% and 50% field capacity, shoot dry weight nearly doubled in the presence of the mycorrhizal complex indicating a positive effect, the root dry weight was also improved by the AMF inoculation at all stress levels (Table 1). However, the combined effect of the water regime and the mycorrhizal inoculation was less significant. Shoot heights and leaf surface areas were significantly negatively impacted by the imposition of drought stress; both these parameters were improved by the AMF inoculation (Tables 1 and 2). Drought stress caused a slight increase in root lengths which were also improved by the presence of the mycorrhizal complex (Table 1). Mycorrhization frequency determination indicated a successful colonization of the carob roots by the mycorrhizal complex (Fig. 1).

3.2. Relative water content, stomatal conductance and membrane stability

The relative water content (RWC) was heavily impacted by the presence of the mycorrhizal complex, although no significant differences were observed between non-mycorrhizal and inoculated plantlings in the absence of stress (75% and 100% FC), the relative water content was improved going from 88.21% to 94.41% at the highest level of water stress (25% FC) (Table 2). Stomatal conductance was significantly decreased in all water-stressed plants compared to the well-watered plants, this effect was alleviated in the mycorrhizal plants which had stomatal conductance values that are comparable with their non-stressed counterparts (Table 2). A similar effect was observed with membrane stability which maintained relatively high values in the non-stressed plants regardless of their mycorrhization status. In the stressed plants, mycorrhization allowed the maintenance of a higher membrane stability index in the AMF plants whereas significant decreases were noted in the non-mycorrhizal water stressed plants (Table 2).

3.3. Chlorophyll a and b content

Chlorophyll a and b and total chlorophyll contents were severely impacted by drought stress in absence of the mycorrhizal complex. Expectedly, no significant differences were observed between the non-mycorrhizal (NM) and inoculated (AMF) carob seedlings in the absence of drought stress (75% and 100% FC), however, drought stress did cause a significant decline in Chl a, Chl b and total chlorophyll in the non-mycorrhizal seedlings (25% and

Table 1
Root length (RL), Shoot height (SH), Shoot fresh weight (SFW), Root fresh weight (RFW), Shoot dry weight (SDW), Root dry weight (RDW) and Relative water content (RWC) in non-mycorrhizal (NM) and inoculated (AMF) carob seedlings. Mean values ± SE in the same column followed by the same lower case letters are not significantly different at $P \leq 0.05$ by Tukey test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Water regime (% FC)	AMF inoculation	RL (cm)	SH (cm)	SFW (g)	RFW (g)	SDW (g)	RDW (g)
100%	NM	44 ± 0.58 [d]	6 ± 0.10 [bc]	1.02 ± 0.01 [c]	0.76 ± 0.02 [bc]	0.62 ± 0.01 [de]	0.31 ± 0.01 [b]
	AMF	63.67 ± 0.67 [a]	9.57 ± 0.09 [a]	2.13 ± 0.26 [a]	1.17 ± 0.05 [a]	1.49 ± 0.13 [a]	0.69 ± 0.06 [a]
75%	NM	43.33 ± 0.67 [d]	5.7 ± 0.06 [cd]	1.04 ± 0.03 [c]	0.68 ± 0.02 [bcd]	0.64 ± 0.01 [de]	0.27 ± 0.01 [b]
	AMF	64.67 ± 0.33 [a]	9.1 ± 0.12 [a]	1.96 ± 0.15 [a]	0.92 ± 0.1 [ab]	1.26 ± 0.05 [ab]	0.64 ± 0.01 [a]
50%	NM	48.33 ± 0.88 [c]	5.2 ± 0.06 [de]	1.07 ± 0.09 [c]	0.54 ± 0.03 [cd]	0.58 ± 0.01 [de]	0.18 ± 0.01 [b]
	AMF	54.67 ± 0.88 [b]	6.43 ± 0.24 [b]	1.81 ± 0.02 [ab]	0.93 ± 0.04 [ab]	1.05 ± 0.03 [bc]	0.65 ± 0.06 [a]
25%	NM	50.33 ± 0.88 [c]	4.93 ± 0.09 [e]	0.9 ± 0.04 [c]	0.44 ± 0.02 [d]	0.48 ± 0.03 [e]	0.16 ± 0.01 [b]
	AMF	52.67 ± 0.88 [bc]	6.13 ± 0.12 [bc]	1.26 ± 0.06 [bc]	1.053 ± 0.02 [a]	0.86 ± 0.08 [cd]	0.58 ± 0.03 [a]
AMF inoculatin (AMF)		555.02***	750.23***	93.27**	109.97***	192.09***	310.61***
Field Capacity (FC)		7.03**	171.50***	7.18***	7.19**	15.62***	5.92**
Fc × AMF		81.23***	58.33***	3.81*	3.71*	6.11**	0.89 **ns**

Table 2
Relative water content (RWC), Stomatal conductance (SC), leaf surface area (LSA) and Membrane stability (MS) in non-mycorrhizal (NM) and inoculated (AMF) carob plantlings. Mean values ± SE in the same column followed by the same lower case letters are not significantly different at $P \leq 0.05$ by Tukey test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

| Water regime (% FC)	AMF Inoculation	RWC (%)	SC (mmol/m^{2}S)	LSA (cm^2)	MS (%)
100%	NM	94.73 ± 0.34 [b]	137.18 ± 0.91 [c]	6.13 ± 0.03 [d]	85.33 ± 0.07 [b]
	AMF	96.51 ± 0.09 [a]	182.63 ± 0.62 [a]	10.17 ± 0.03 [a]	85.83 ± 0.18 [b]
75%	NM	94.78 ± 0.06 [b]	133.04 ± 1.28 [cd]	5.83 ± 0.03 [d]	83.4 ± 0.12 [c]
	AMF	95.9 ± 0.18 [ab]	180.28 ± 0.99 [a]	10.0 ± 0.06 [a]	86.67 ± 0.09 [a]
50%	NM	90.06 ± 0.20 [c]	119.24 ± 0.99 [e]	5.17 ± 0.03 [e]	61.8 ± 0.23 [e]
	AMF	94.34 ± 0.43 [b]	153.44 ± 0.69 [b]	8.5 ± 0.12 [b]	83.03 ± 0.12 [c]
25%	NM	88.21 ± 0.63 [c]	99.72 ± 0.84 [f]	4.7 ± 0.06 [f]	54.1 ± 0.12 [f]
	AMF	94.41 ± 0.32 [b]	131.12 ± 0.34 [d]	7.6 ± 0.12 [c]	80.3 ± 0.12 [d]
AMF inoculation (AMF)		201.41***	4106.82***	5514.38***	18577.13***
Field Capacity (FC)		85.94***	1113.74***	372.34***	9600.30***
FCx AMF		24.714***	41.32***	37.75***	4656.90***
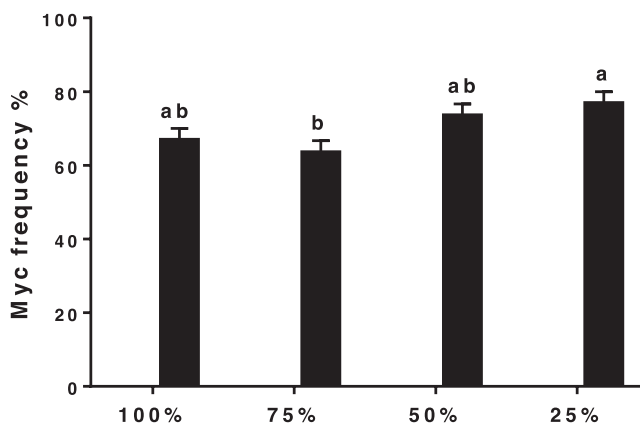
Fig. 1. Mycorrhization frequency of the inoculated seedlings at different water regimes: 100%, 75%, 50% and 25% field capacity (FC).

50% FC) (Table 3). On the other hand, with values of 0.243 mg/g FW, 0.807 mg/g FW and 1.05 mg/g FW at 25% FC for Chl a, Chl b and total chlorophyll respectively (Table 3), the AMF inoculated plants had chlorophyll levels comparable to those observed in the absence of stress. These values correspond to a 92%, 38% and 49% increase compared to the non-mycorrhizal plantlings. These results indicate that inoculation with the mycorrhizal complex greatly diminishes the negative impact of drought stress on photosynthetic activity and chlorophyll content specifically. In fact, in the presence of the complex at low field capacity, chlorophyll contents were similar to those observed in the well- watered seedlings at 75% and 100% field capacity. The specific and combined effects of both drought stress and mycorrhization were all highly significant (Table 3).

3.4. Proline, total soluble sugars and protein content

In the presence of drought stress, proline accumulation was significantly attenuated by the mycorrhizal complexe inoculation with a 23% and 19% decrease in proline content at 25% and 50% FC respectively compared to the NM plants (Fig. 2a). In the absence of drought stress, a slight difference in leaf proline content was observed between the inoculated and non-mycorrhizal carob plants at 75% FC, however, no significant differences were noted at 100% FC (Fig. 2a). Total soluble sugar (TSS) accumulation was also more significant in the stressed plants compared to the non-stressed plants (Fig. 2b), similarly to what was observed with proline contents, this effect was even more pronounced in the mycorrhizal plants. On the other hand protein content varied greatly between the inoculated and non-mycorrhizal plants in the presence and absence of drought stress. In the absence of mycorrhizal complex, protein content decreased as the level of drought stress imposed on the carob plantlings was increased with values going from 2.9 mg/g DM at 100% to 1.34 mg/g DM at the highest stress level (25% FC) (Fig. 2c). The mycorrhizal inoculation allowed the treated plants to maintain a significantly higher protein content at all levels of stress, 3.54 mg/g DM at 100% FC and 1.54 mg/g DM and 25% FC (Fig. 2c).

3.5. Antioxidant enzymes activity

Catalase (CAT) activity increased remarkably in the water stressed plants (25 and 50% FC) compared to the non-stressed (75 and 100% FC) plants, this activity was further increased in the presence of the AM complex indicating a stronger response to the ROS resulting from water stress. No significant differences in CAT activity were recorded between the mycorrhizal and non-

Table 3
Leaf chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (Chl a + b) content in non-mycorrhizal (NM) and inoculated (AMF) carob plantlings. Mean values ± SE in the same column followed by the same lower case letters are not significantly different at $P \leq 0.05$ by Tukey test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

| Water regime (% FC)	AMF Inoculation	Chl a (mg/g FW)	Chl b (mg/g FW)	Chl a + b (mg/g FW)
100%	NM	0.22 ± 0.00[c]	0.89 ± 0.000[b]	1.11 ± 0.00 [ab]
	AMF	0.25 ± 0.00 [a]	0.88 ± 0.00[b]	1.13 ± 0.00 [a]
75%	NM	0.19 ± 0.00 [d]	0.91 ± 0.00 [a]	1.10 ± 0.00[b]
	AMF	0.23 ± 0.00 [bc]	0.88 ± 0.00[b]	1.12 ± 0.00 [ab]
50%	NM	0.16 ± 0.00 [e]	0.66 ± 0.00 [e]	0.82 ± 0.00 [e]
	AMF	0.25 ± 0.00 [a]	0.83 ± 0.00[c]	1.08 ± 0.01[c]
25%	NM	0.09 ± 0.00[f]	0.55 ± 0.00[f]	0.64 ± 0.01[f]
	AMF	0.24 ± 0.00 [ab]	0.81 ± 0.00 [d]	1.05 ± 0.01 [d]
AMF Inoculation (AMF)		1504.17***	1682.00***	3024.60***
Field Capacity (FC)		203.28***	2080.33***	1739.80***
FCx AMF		201.94***	838.99***	917.93***
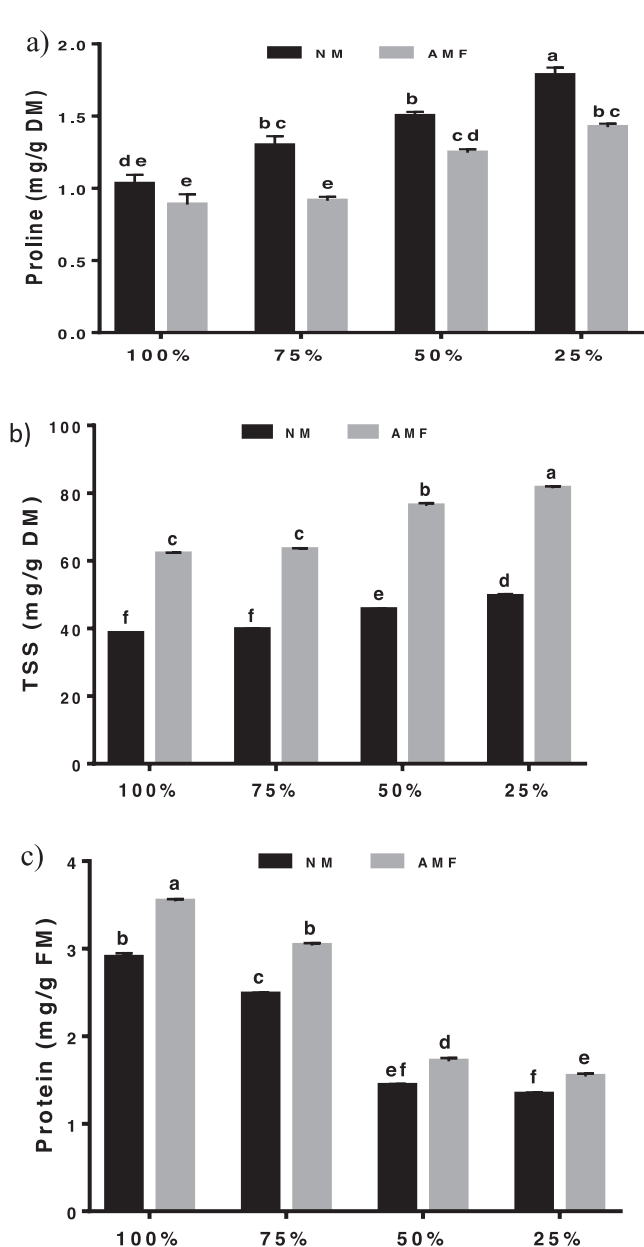
Fig. 2. Leaf proline (2a), total soluble sugars content (2b) and protein content (2c) in carob seedlings non-inoculated (NM) or inoculated with the native mycorrhizal complex (AMF) at 100%, 75%, 50% and 25% field capacity (FC) regimes.
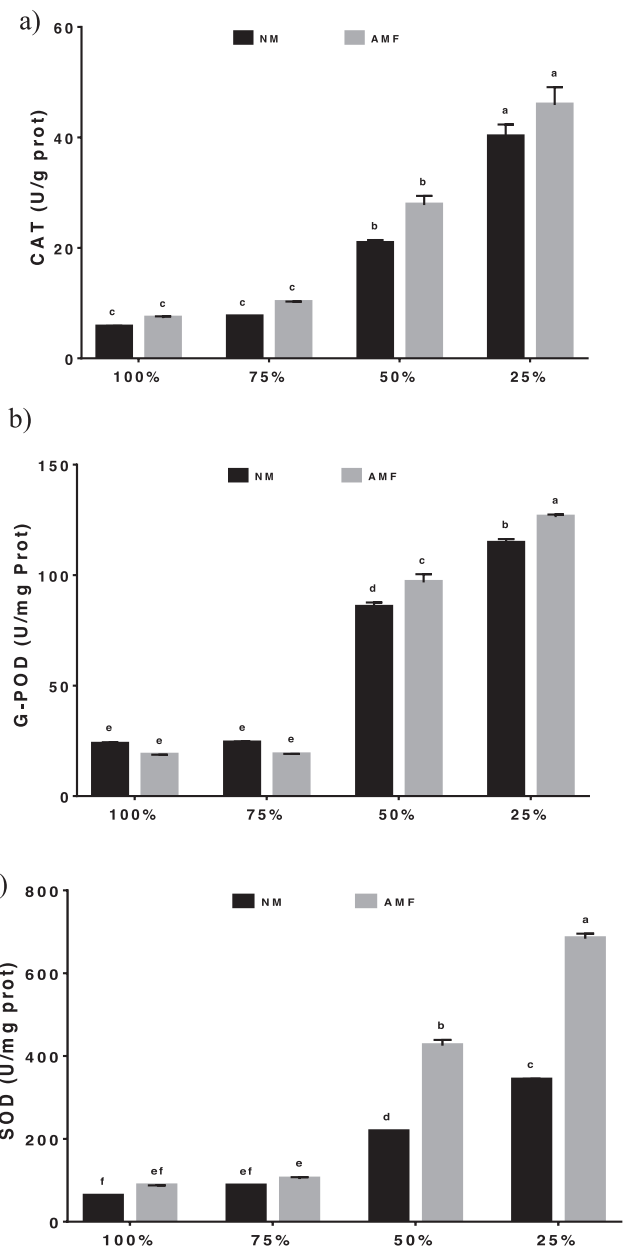

Fig. 3. Leaf catalase (3a), guaiacol peroxidase (3b) and superoxide dismutase (3c) activities in carob seedlings, non-inoculated (NM) and inoculated with the native mycorrhizal complex (AMF) at 100%, 75%, 50% and 25% field capacity regimes.

mycorrhizal carob plants when the water stress was not applied (75% and 100% FC) (Fig. 3a). Similarly, peroxidase (G-POD) activity drastically increased in the stressed plants, particularly at the highest stress level (25% FC) where AM inoculation led to a more pronounced enzymatic response compared to the NM plants. In the absence of drought stress (75% and 100% FC), no significant differences were recorded between control plants and those inoculated with the AM complex (Fig. 3b). The changes recorded in superoxide dismutase (SOD) activity in response to water stress were similar to those observed in catalase and guaiacol-peroxidase. SOD activity increased significantly in the stressed plants (25% and 50% FC) compared to the well-watered ones (75% and 100%) (Fig. 3c). In the absence of water stress (75% and 100% FC) AM plants had a slightly lower, though non-significantly, SOD activity than NM plants. However, at the highest stress level (25% FC), SOD activity nearly doubled in the AM plants compared to the NM plants, a similar but less marked effect was observed in the mildly stressed (50% FC) carob plants (Fig. 3c).

4. Discussion

Water deficit is one of the biggest challenges currently facing humankind, the effects of the resulting drought stress, even intermittent, can be very damaging ecologically and agriculturally. More than 50% of all crop production worldwide is subject to drought stress (Grant, 2012; Naeem et al., 2013) and drastic decreases in yield have been recorded at different growth stages in multiple plant species (Martínez et al., 2007). In legumes, the second important food source for human populations around the world after cereals (Kudapa et al., 2013), drought stress has been shown to negatively impact biomass, pod and seed numbers as well as their overall quality and yield (Hasanuzzaman et al., 2013; Pagano, 2014). Most plant species rely on three major mechanisms for drought tolerance, escape, avoidance or resistance (Rapparini & Penuelas, 2014). The carob (Ceratonia siliqua) which is an important member of the leguminous family and a staple of the Mediterranean flora, has been shown to adapt to drought stress through drought avoidance (Correia et al., 2001). Like many leguminous plants, the carob is able to form symbiotic associations with arbuscular mycorrhizal fungi which have been shown to support plant processes and alleviate many of the consequences of drought stress (Ouahmane et al., 2012; Chitarra et al., 2016; Ruiz-Lozano et al., 2016; Quiroga et al., 2017). In this study, inoculation with selected AMF complex greatly improved biomass production in the carob seedlings in both the presence and absence of drought stress. The expected decrease in plant wet and dry biomass as well as the shoot heights and leaf surface area caused by the introduction of drought stress, mild and severe, was significantly alleviated by AMF inoculation. A similar effect was recorded in a different study where AMF inoculation with single mycorrhizal species, *Rhizophagus fasciculatus*, *Funneliformis mosseae* and *Rhizophagus intraradices* led to an improved plant growth and biomass production (Essahibi et al., 2018) in the presence of drought stress. Loss of turgor pressure is an important consequence of drought stress and is responsible, along with the limited cell division, for the reduction in plant growth making the maintenance of high relative water content in the plant an important factor in the plant's ability to withstand drought stress. In this study, RWC was improved in the AMF plants as a response to mild and severe drought stress (50% and 25% FC) compared to the NM plants whereas no significant differences were recorded in the non-stressed plants indicating a direct implication of the mycorrhizal complex in maintaining a high RWC in the plant. The maintenance of cell turgor and water relations within the plant in response to

water deficit is also greatly dependent on the management of osmotically active molecules and ions within the plant cell (Farooq et al., 2009). Specifically, the accumulation of such compounds lowers the osmotic potential in the cell, causing water to move into the cell and increase cell turgor. Proline is an important osmo-protectant and its accumulation is known to occur as a result of drought stress in a variety of species contributing greatly to the induction of drought tolerance (Yamada et al., 2005). In this study, drought stress caused an increase of proline and total soluble sugars content in the carob plants consistent with that observed in other carob studies (Essahibi et al., 2018), AMF inoculation led to a significant decrease in proline and sugar accumulation in the treated plants compared to NM carob plants suggesting an increased tolerance to drought stress (Tang et al., 2009). A similar protective effect of AMF inoculation was observed at the cell membrane level with the maintenance of a relatively high membrane stability index. The production of reactive oxygen species (ROS) is another important consequence of drought stress, the accumulation of these ROS can be highly damaging to numerous essential molecules such as nucleic acids, proteins and photosynthetic pigments (Ruiz-Lozano, 2003; Kavas et al., 2013; Fouad et al., 2014). Numerous studies have linked ROS accumulation from oxidative stress to protein denaturation and an overall decrease in protein contents (Schwanz et al., 1996; Sgherri and Navari-Izzo, 1995; Yordanova, 2004). In this study, drought stress caused a significant decrease in protein content in the carob plants, however, inoculation with the mycorrhizal complex significantly improved protein content and attenuated the negative effects of the oxidative stress caused by water deficit. In fact, protein content was significantly improved in the mycorrhizal plants compared to the NM plants in both the absence and presence of drought stress. A drop in Chlorophyll a and b content as a result of drought stress has also been recorded in a number of species (Mafakheri et al., 2011). A similar decrease was recorded in our study when drought stress was applied to NM plants. On the other hand, in the presence of AMF complex, Chlorophyll a and b content was significantly increased indicating an improved maintenance of the plants photosynthetic ability. This improvement in chlorophyll content through mycorrhizal inoculation was reported in a number of studies in response to oxidative stress (Zuccarini, 2007; Beltrano and Ronco, 2008). In order to counteract the various negative effects of oxidative stress and the resulting reactive oxygen species damage, plants have evolved various complex defense processes involving the action of antioxidant molecules. These molecules can be enzymatic or non-enzymatic and participate in ROS scavenging pathways such as the water-water and the ascorbate glutothione cycles in chloroplasts, mitochondria and other cellular compartments (Zagorchev et al., 2013). Antioxidant enzymes such as CAT, G-POD and SOD play an important role in the defense processes against oxidative stress and their increased activity has been shown to improve tolerance to induced stress (Chen et al., 2010; Fikret et al., 2013). Superoxide dismutase catalyses the dismutation of ROS to hydrogen peroxide and molecular oxygen (Giannopolitis and Ries, 1977) whereas Catalase (CAT) and G-POD act by scavenging hydrogen peroxide and converting it to water and molecular oxygen (Shao et al., 2008). Inoculation with AMF was shown to improve the activity of SOD, CAT and G-POD in response to drought stress in carob (Essahibi et al., 2018) and other plant species (Benhiba et al., 2015; Tyagi et al., 2017). In this study, we recorded a similar significant increase in CAT, G-POD and SOD activities in response to drought stress; inoculation with the mycorrhizal complex further improved this activity and therefore allowed the plant to develop a stronger antioxidant response to the oxidative damage caused by water deficit.

5. Conclusion

The results of this study support the drought avoidance mechanisms observed in *Ceratonia siliqua* and other plant species in response to water deficit. The response is based on the maintenance of high water content and the improvement of antioxidant enzyme activities against damaging reactive oxygen species (ROS) in the plant cells. Our study also showed that inoculation with the AMF complex greatly contributes to the carob's ability to withstand the negative effects of oxidative stress resulting due to water deficit by the up-regulation of oxidative stress enzymes and the attenuation of the effects of the biochemical processes that result from drought stress. Inoculation with a native mycorrhizal complex thus constitutes a potentially efficient eco-engineering method for treatment of nursery-grown carob seedlings prior to their transplantation into areas affected by drought stress.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts, polyphenoxidase in *Beta vulgaris*. Plant Physiol. 24, 1–15.

Augé, R.M., Toler, H.D., Saxton, A.M., 2014. Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis Mycorrhiza

Barea, J.M., Palenzuela, J., Cornejo, P., Sánchez-Castro, I., Navarro-Fernández, C., López-García, A., Azcón-Aguilar, C., 2011. Ecological and functional roles of mycorrhizas in semi-arid ecosystems of Southeast Spain. J. Arid Environ. 75 (12), 1292–1301.

Battle, I., Tous, J., 1997. Carob tree *Ceratonia siliqua* L. International Plant Genetic Resources Institute, Rome.

Beltrano, J., Ronco, M.G., 2008. Improved tolerance of wheat plants (*Triticum aestivum* L.) to drought stress and rewatering by the arbuscular mycorrhizal fungus Glomus claroideum: effect on growth and cell membrane stability. Braz. J. Plant Physiol. 20, 29–37.

Benhiba, L., Fouad, M.O., Essahibi, A., Ghoulam, C., Qaddoury, A., 2015. Arbuscular mycorrhizal symbiosis enhanced growth and antioxidantmetabolism in date palm subjected to long-term drought. Trees 29, 1725–1733.

Beyer, W.F., Fridovich, I., 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal. Biochem. 161, 559–566.

Bompadre, M.J., Pérgola, M., Fernández Bidondo, L., Colombo, R.P., Silvani, V.A., Pardo, A.G., Godeas, A.M., 2014. Evaluation of arbuscular mycorrhizal fungi capacity to alleviate abiotic stress of olive *(Olea europaeaL.)* plants at different transplant conditions. Sci. World J. 2014, 1–12.

Bothe, H., Turnau, K., Regvar, M., 2010. The potential role of arbuscular mycorrhizal fungi in protecting endangered plants and habitats. Mycorrhiza 20 (7), 445–457.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chen, F., Wang, F., Sun, H., Cai, Y., Mao, W., Zhang, G., et al., 2010. Genotype-dependent effect of exogenous nitric oxide on Cd-induced changes in antioxidative metabolism, ultrastructure, and photosynthetic performance in barley seedlings (Hordeum vulgare). J. Plant Growth Regul. 29, 394–408.

Chitarra, W., Pagliarani, C., Maserti, B., Lumini, E., Siciliano, I., Cascone, P., Schubert, A., Gambino, G., Balestrini, R., Guerrieri, E., 2016. Insights on the impact of arbuscular mycorrhizal symbiosis on tomato tolerance to water stress. Plant Physiol.

Correia, M.J., Coelho, D., David, M.M., 2001. Response to seasonal drought in three cultivars of *Ceratonia siliqua*: leaf growth and water relations. Tree Physiol. 21, 645–653.

Correia, P.J., Gama, F., Pestana, M., Martins-Loução, M.A., 2010. Tolerance of young (*Ceratonia siliqua* L.) carob rootstock to NaCl. Agric. Water Manage. 97, 910–916.

DuBois M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Fred, 1956. Smith Analytical Chemistry 28 (3), 350–356 doi:10.1021/ac 60111a 017.

ESPERE, cited 2006: Climate encyclopedia-Food and climate [Available online at http://espere.mpch-mainz.mpg.de/documents/pdf/].

Essahibi, A., Benhiba, L., Babram, M., Ghoulam, C., Qaddoury, A., 2018. Influence of arbuscular mycorrhizal fungi on the functional mechanisms associated with drought tolerance in carob (*C.siliqua* L.). Trees 32, 87–97.

Fakhech, A., Manaut, N., Ouahmane, L., Hafidi, M., 2019. Contributions of indigenous arbuscular mycorrhizal fungi to growth of *Retama monosperma* and *acacia gummifera* under water stress (case study: Essaouira sand dunes forest). J. Sustainable For., 1–11

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., Basra, S.M.A., 2009. Plant drought stress: effects, mechanisms and management. Agron. Sustain. Dev. 29, 185–212.

Fikret, Y., Uzal, O., Taylan, O., Ozlem, Y., 2013. Investigation of the relationship between the tolerance to drought stress levels and antioxidant enzyme activities in green bean (*Phaseolus Vulgaris L.*) genotypes. Afr. J. Agric. Res. 8, 5759–5763.

Fouad, M.O., Essahibi, A., Benhiba, A., Qaddoury, A., 2014. Effectiveness of arbuscular mycorrhizal fungi in the protection of olive plants against oxidative stress induced by drought. Span J. Agric. Res. 12, 763–771.

Giannopolitis, C.N., Ries, S.K., 1977. Superoxide Dismutases: I. Occurrence in Higher Plants. Plant Physiol. 59 (2), 309–314.

Giorgi, F., 2006. Climate change hot spots. Geophys. Res. Lett. 33, L08707.

Grant, O.M., 2012. Understanding and exploiting the impact of drought stress on plant physiology. In: Ahmad, P., Prasad, M.N.V. (Eds.), Abiotic Stress Responses in Plants: Metabolism, Productivity and Sustainability. Springer Science Business Media, pp. 89–104.

Guardiola, F.A., Barroso, C., Enes, P., Couto, A., Díaz-Rosales, P., Afonso, A., Costas, B., 2018. Humoral and mucosal immune responses in meagre (*Argyrosomus regius*) juveniles fed diets with varying inclusion levels of carob seed germ meal. Fish Shellfish Immunol. 79, 209–217.

Gunes, E., Gubbuk, H., Ayala Silva, T., Gozlekci, S., Ercisli, S., 2013. Effects of various treatments on seed germination and growth of carob (*Ceratonia siliqua* L.). Pak. J. Bot. 45, 1173–1177.

Hasanuzzaman, M., Gill, S.S., Fujita, M., 2013. Physiological role of nitric oxide in plants grown under adverse environmental conditions. In: Tuteja, N., Gill, S.S. (Eds.), Plant Acclimation to Environmental Stress. Springer Science + Business Media, New York, pp. 269–322.

Hoerling, M. et al., 2012. On the increased frequency of mediterranean drought. J. Clim. 25, 2146–2161.

Hwang, S.Y., Lin, H.W., Chern, R.H., Lo, H.F., Li, L., 1999. Reduced susceptibility to water logging together with high light stress is related to increases in superoxide dismutase and catalase activity in sweet potato. Plant Growth Regul. 27, 167–172.

Janick, J., Paull, R.E., 2008. The Encyclopedia of Fruit and Nuts. CABI Publishing, p. 954.

Kavas, M., Baloğlu, M.C., Akça, O., Köse, F.S., Gökçay, D., 2013. Effect of drought stress on oxidative damage and antioxidant enzyme activity in melon seedlings. Turk. J. Biol. 37, 491–498.

Kudapa, H., Ramalingam, A., Nayakoti, S., et al., 2013. Functional genomics to study stress responses in crop legumes: progress and prospects. Funct. Plant Biol. 40, 1221–1233.

Mafakheri, A., Siosemardeh, A., Bahramnejad, B., Struik, P.C., Sohrabi, Y., 2011. Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. Aust. J. Crop Sci. 10, 1255–1260.

Manaut, N., Sanguin, H., Ouahmane, L., Bressan, M., Thioulouse, J., Baudoin, Ezékiel, Galiana, A., Hafidi, M., Prin, Y., Duponnois, Robin, 2015. Potentialities of ecological engineering strategy based on native arbuscular mycorrhizal community for improving afforestation programs with carob trees in degraded environments. Ecol. Eng. 79, 113–119. ISSN 0925-8574.

Mariotti, A., 2010. Recent changes in the Mediterranean water cycle: a pathway toward long-term regional hydroclimatic change? J. Climate 23, 1513–1525.

Martínez, J.P., Silva, H., Ledent, J.F., Pinto, M., 2007. Effect of drought stress on the osmotic adjustment, cell wall elasticity and cell volume of six cultivars of common beans (*Phaseolus vulgaris* L.). Eur. J. Agron. 26, 30–38.

Naeem, M., Nasir Khan, M., Masroor, M., Khan, M.M.A., Moinuddin, 2013. Adverse effects of abiotic stresses on medicinal and aromatic plants and their alleviation by calcium. In: Tuteja, N., Gill, S.S. (Eds.), Plant Acclimation to Environmental Stress. Springer Science + Business Media, New York, pp. 101–146.

Nguyen, S.T., Paquin, R., 1971. Méthodes d'extraction et de purification des acides aminés libres et des protéines de tissus végétaux. J. Chromatogr. A 61, 349–351.

Ouahmane, 2007. Rôles de la mycorhization et des plantes associées (Lavande et Thym) dans la croissance du cyprès de l'Atlas (Cupressus atlantica G.) : conséquences sur la biodiversité rhizosphérique et la réhabilitation des milieux dégradés.Thèse de Doctorat, Faculté des Sciences Semlalia, Université Cadi ayyad Marrakesh, Morocco.

Ouahmane, L., Ndoye, I., Morino, A., Ferradous, A., Sfairi, Y., Al Faddy, M., Abourouh, M., 2012. Inoculation of *Ceratonia siliqua* L. with native arbuscular mycorrhizal fungi mixture improves seedling establishment under greenhouse conditions. Afr. J. Biotechnol. 11, 16422–16426.

Ozturk, M., Dogan, Y., Sakcali, M.S., Doulis, A., Karam, F., 2010. Ecophysiological responses of some maquis (*Ceratonia siliqua* L., *Oleaoleaster Hoffm.* & *Link, Pistacia lentiscus* and *Quercus coccifera* L.) plant species to drought in the east Mediterranean ecosystem. J. Environ. Biol. 31, 233–245.

Pagano, M.C., 2014. Drought stress and mycorrhizal plants. In: Miransari, M. (Ed.), Use of Microbes for the Alleviation of Soil Stresses. Springer Science + Business Media, New York, pp. 97–110.

Papakonstantinou, E., Chaloulos, P., Papalexi, A., Mandala, I., 2018. Effects of bran size and carob seed flour of optimized bread formulas on glycemic responses in humans: a randomized clinical trial. J. Funct. Foods 46, 345–355.

Parrado, J., Bautista, J., Romero, E.J., García-Martínez, A.M., Friaza, V., Tejada, M., 2008. Production of a carob enzymatic extract: Potential use as a biofertilizer. Bioresour. Technol. 99 (7), 2312–2318.

Phillips, J.M., Hayman, D.S., 1970. Improve procedures for clearing roots and staining parasitic and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55, 158–161.

Quiroga, G., Erice, G., Aroca, R., Chaumont, F., Ruiz-Lozano, J.M., 2017. Enhanced drought stress tolerance by the arbuscular mycorrhizal symbiosis in a drought-sensitive maize cultivar is related to a broader and differential regulation of host plant aquaporins than in a drought-tolerant cultivar. Front. Plant Sci. 8, 1056.

Rapparini, F., Penuelas, J., 2014. Mycorrhizal fungi to alleviate drought stress on plant growth. In: Miransari, M. (Ed.), Use of Microbes for the Alleviation of Soil Stresses. Springer Science+Business Media, New York, pp. 139–159.

Raimbault, P., Lantoine, F., Neveux, J., 2004. Rapid measurement of chlorophyll a and of phaeopigments a by fluorimetry after extraction with methanol. Comparison with the elastic acetone extraction method. Oceanis 30, 189–205.

Ruiz-Lozano, J.M., 2003. Arbuscular mycorrhizal symbiosis and alleviation of osmotic stress. New perspectives for molecular studies. Mycorrhiza 13, 309–317.

Ruiz-Lozano, J.M., Aroca, R., Zamarreño, Á.M., Molina, S., Andreo-Jiménez, B., Porcel, R., et al., 2016. Arbuscular mycorrhizal symbiosis induces strigolactone biosynthesis under drought and improves drought tolerance in lettuce and tomato. Plant Cell Environ. 39, 441–452.

Schwanz, P., Picon, C., Vivin, P., Dreyer, E., Guehl, J.M., Polle, A., 1996. Responses of antioxidative systems to drought stress in pendunculate oak and maritime pine as modulated by elevated CO2. Plant Physiol. 110 (2), 393–402. https://doi.org/10.1104/pp.110.2.393.

Séré, G., Schwartz, C., Ouvrard, S., Sauvage, C., Renat, J.C., Morel, J.L., 2008. Soil construction: a step for ecological reclamation of derelict lands. J. Soils Sediments 8 (2), 130–136. https://doi.org/10.1065/jss2008.03.277.

Sgherri, C.I.M., Navari-Izzo, F., 1995. Sunflower seedlings subjected to increasing water deficit stress: oxidative stress and defence mechanisms. Physiol. Plant 93, 25–30.

Shanahan, J.F., Edwards, I.B., Quick, J.S., Fenwick, J.R., 1990. Membrane thermostability and heat tolerance of spring wheat. Crop Sci. 30, 247–251.

Shao, H.B., Chud, L.Y., Jaleel, C.A., Zhaoe, C.X., 2008. Water-deficit stress-induced anatomical changes in higher plants. C. R. Biol. 331, 215–225.

Simon, L., Bousquet, J., Lévesque, R.C., Lalonde, M., 1993. Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363 (6424), 67–69.

Singh, T.N., Paleg, L.G., Aspinall, D., 1973. Stress metabolism I. Nitrogen metabolism and growth in the barley plant during water stress. Aust. J. Biol. Sci. 26, 65–76.

Smith, S.E., Read, D.J., 2008. Mycorrhizal Symbiosis. Academic press, San Diego, CA, USA.

Talaat, N.B., Shawky, B.T., 2014. Protective effects of arbuscular mycorrhizal fungi on wheat (Triticum aestivum L.) plants exposed to salinity. Environ. Exp. Bot. 98, 20–31.

Tang, M., Chen, H., Huang, J.C., Tian, Z.Q., 2009. AM fungi effects on the growth and physiology of Zea mays seedlings under diesel stress. Soil Biol. Biochem. 41, 936–940.

Trouvelot, A., Kough, J.L., Gianinazzi-Pearson, V., 1986. Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de methodes d'estimation ayant une signification fonctionnelle. In: Gianinazzi-Pearson, V., Gianinazzi, S. (Eds.), Physiological and genetical aspects of mycorrhizae =Aspects physiologiques et genetiques des mycorhizes. Institut National de la Recherche Agronomique Press, Paris.

Tyagi, J., Varma, A., Pudake, R.N., 2017. Evaluation of comparative effects of arbuscular mycorrhiza (Rhizophagus intraradices) and endophyte (Piriformospora indica) association with finger millet (Eleusine coracana) under drought stress. Eur. J. Soil Biol. 81, 1–10.

Van der Heijden, M.G.A., Streitwolf-Engel, R., Riedl, R., Siegrist, S., Neudecker, A., Ineichen, K., Boller, T., Wiemken, A., Sanders, I.R., 2006. The mycorrhizal contribution to plant productivity, plant nutrition and soil structure in experimental grassland. New Phytol. 172, 739–752.

Wu, H.-H., Zou, Y.-N., Rahman, M.M., Ni, Q.-D., Wu, Q.-S., 2017. Mycorrhizas alter sucrose and proline metabolism in trifoliate orange exposed to drought stress. Sci. Rep. 7 (1).

Yamada, M., Morishita, H., Urano, K., Shiozaki, N., Yamaguchi-Shinozaki, K., Shinozaki, K., Yoshiba, Y., 2005. Effects of free proline accumulation in petunias under drought stress. J. Exp. Bot. 56, 1975–1981.

Yordanova, R., 2004. Antioxidative enzymes in barley plants subjected to soil flooding. Environ. Exp. Bot. 51 (2), 93–101.

Zagorchev, L., Seal, C.E., Kranner, I., Odjakova, M., 2013. A central role for thiols in plant tolerance to abiotic stress. Int. J. Mol. Sci. 14, 7405_7432.

Zuccarini, P., 2007. Mycorrhizal infection ameliorates chlorophyll content and nutrient uptake of lettuce exposed to saline irrigation. Plant Soil Environ.. 53, 281–287.