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ORIGINAL ARTICLE

Estimating the contribution of arbuscular mycorrhizal fungi to drought tolerance of potted olive trees (*Olea europaea*)

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Abstract

The aim of the present study is to investigate the contribution of mycorrhization to the resilience of olive trees to drought. One-year-old olive plants were inoculated (Myc^+) or not (Myc^-) with arbuscular mycorrhizal fungi (AMF), and subjected to a 40-day-drought period. At regular intervals of the watering-off period and after rehydration period, water relations and gas exchanges parameters were measured. Similarly, the total soluble sugars, proline, and mineral nutrients concentrations were determined. The results revealed that Myc^+ plants were less affected by drought than Myc^- plants proving the involvement of the AMF in the alleviation of drought impact on olive tree. In fact, the turgor potential (Ψ_p) in Myc^+ plants exhibited positive values during the whole treatment period, while Ψ_p in Myc^- plants was negative mainly under severe stress intensity. Moreover, the stomatal function of Myc^+ plants was less affected by drought compared to Myc^- plants. The maximum of mycorrhizas relative drought alleviation rate (RDAR) was estimated to be 40% for Ψ_{pd} and RWC, 36% for the osmotic potential (Ψ_S), 86% for Ψ_p, 16% for g_s, and 27% for E. The osmotic adjustment by proline was earlier in Myc^+ plants than in Myc^- ones. The inoculation with AMF also improved mineral uptake (K, N, Zn, and Fe). After 40 days of drought, Myc^+ plants survive but not Myc^- ones. In addition, the restoration of the irrigation permitted the Myc^+ plants to recuperate from severe drought stress. To sum up, inoculation of young olive trees with the AMF improved their resilience to drought.

Keywords Gas exchange · Mineral nutrition · Mycorrhization · Drought resilience · Water relations · Osmotic adjustment

Abbreviations

RDAR	Relative drought alleviation rate
DI_{Myc^-}	Drought impact in non-mycorrhizal plants
DI_{Myc^+}	Drought impact in mycorrhizal plants

Introduction

In their natural environment, plants are frequently subjected to various environmental stresses which negatively impact their growth and development and threaten their survival (Ruiz-Lozano 2003). Drought is the main abiotic constraint often blamed for limiting plant growth and yield in various regions of the world (Kramer and Boyer 1997). Around the Mediterranean and especially the southern rim, water scarcity is the main factor limiting agricultural development (Chartzoulakis 2005). In these areas, rainfall is highly variable in space and time. For instance, in Tunisia where average annual rainfall ranges from 1500 mm on the northwestern mountains to less than 50 mm in the southern tip of the country (Arnould and Hotyat 2003), agricultural activity is largely dictated by precipitation distribution.

The prominence of the olive oil sector in Tunisia is due to its economic, social, and environmental importance (Boudiche et al. 2003). Currently, Tunisia occupies the second position in the world after the European Union in terms of olive oil production and export (Jackson et al. 2015). Olive groves cover about 1.7 million hectares which represent 79%

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of the total arboreal area and 45% of the total arable lands. This is no surprise, since Olive is a drought tolerant species (Lo Gullo and Salleo 1988; Ennajeh et al. 2009) making it one of the most widely cultivated fruit trees in the arid and semi-arid regions despite high temperatures and water scarcity (Rejsková et al. 2007).

When experiencing sub-optimal water supply, plants manifest a combination of anatomical, physiological, and metabolic adaptations (Bray 2004; Rossi et al. 2013). Some plant species evolved to avoid drought whereas others adopted drought tolerance strategies. In this respect, root colonization by mycorrhizal fungi plays a crucial role in the response of plants to drought (Rapparini and Peñuelas 2014). It allows an integrative drought response combining avoidance and tolerance strategies (Augé 2001; Ruiz-Sánchez et al. 2010; Rapparini and Peñuelas 2014). The presence of microorganisms, as arbuscular mycorrhizal fungi (AMF), in the soil is a key factor in the response of olive trees to drought (Calvo-Polanco et al. 2016).

Most plants develop mutualistic mycorrhizal associations with fungi (Pozo and Azcón-Aguilar 2007). Under favorable conditions, these associations improve growth through enhanced water and nutrients uptake (Lovato et al. 1996; Azcon-Aguilar and Barea 1997; Manuela and Luciano 2002). Under constraining conditions, mycorrhizas improve resilience of plant against environmental stresses like nutrient deficiency, water scarcity, and soil-borne pathogens (Lovato et al. 1996; Azcon-Aguilar and Barea 1997; Barea et al. 2011). Mycorrhizas act as biofertilizers and bioprotectors for plants (Gianinazzi et al. 2010; Bücking et al. 2012). On low fertile soils, mycorrhizas improve assimilation of essential minerals (Berruti et al. 2016). This is especially true for P which absorption is highly improved in mycorrhized plants (Ruiz-Lozano et al. 1995; Smith and Smith 2011; Lone et al. 2015; Taylor et al. 2015). AMF also enhance the uptake of N (Subramanian and Charest 1999; Taylor et al. 2015), S (Allen and Shachar-Hill 2009), K (Garcia and Zimmermann 2014), Zn (Lehmann et al. 2014), Cu, Fe, and Mn (Lehmann and Rillig 2015).

The role of mycorrhizas in plants' response to drought has been well documented in maize, wheat, barley, soybean, onion, lettuce tomato, and olive trees (Augé 2001; Davies et al. 2002; Caravaca et al. 2003; Mena-Violante et al. 2006; Boomsma and Vyn 2008; Khalvati et al. 2010; Calvo-Polanco et al. 2016; Ruiz-Lozano et al. 2016; Yooyongwech et al. 2016). The contribution of AMF to plants' tolerance of drought is mainly due to the larger volume of soil explored by roots and the extra-radical hyphal (Gianinazzi et al. 2010; Orfanoudakis et al. 2010; Gutjahr and Paszkowski 2013; Zou et al. 2015; Zhang et al. 2016), the enhanced osmotic adjustment (Augé et al. 1992; Kubikova et al. 2001), and more efficient stomatal regulation by controlling abscisic acid metabolism (Duan et al. 1996). In olive seedlings, AMF increase growth, nutrients uptake, and root hydraulic conductivity (Porras-Soriano et al. 2009; Calvo-Polanco et al. 2016). They also help to reduce damage due to soil dryness by the activation of antioxidant defenses (Bompadre et al. 2013). Under field conditions prevalent in semi-arid olive-growing areas of the southern Mediterranean region where fairly long periods of drought are interrupted by the sporadic torrential rains of fall and winter. We can assume that inoculation with AMF can help trees withstand severe soil dryness and recuperate rapidly after the rains. This aspect has not been properly studied so far. Therefore, we carried out a study to investigate the contribution of AMF to the resilience of olive trees to drought. We tested the hypothesis that AMF inoculation enhanced the ability of olive plants to resist a severe soil dryness and to recuperate after this stress. We assessed the role of mycorrhizas in maintaining olive trees gas exchange and water-relation parameters, as well as their influence on the accumulation of common osmotica during the treatment period. The ability to regain pre-stress status after resuming watering was specifically examined in inoculated young-potted trees.

Materials and methods

Plant culture and treatment

One-year-old (*Olea europaea* L., cv Zarrazi) trees comparable in size were used in this study. They were provided by the nursery of the National Office of Oil (ONH, Tunisia). Sixty trees were individually transplanted into 17-l pots filled with sterile soil (1% silt, 39% coarse sand, 42% fine sand, and 8% very fine sand), having pH of 8.17 and known mineral composition (0.133% N, 2.07% Ca, 0.127% K, 0.26% Mg, 0.06% Fe, 0.066% Na, 0.000065% P, and 1.39% OM). The pots were covered with plastic film and aluminum foil to reduce evaporation from the soil surface and to minimize solar heating. The experiment was conducted outdoor on the campus of the Faculty of Science of Gabes (Southern Tunisia: 33°50′ N, 10°5′ E) during the dry season between June and August of 2015.

The pots were organized into six groups of ten following a Completely Randomized Design. Three groups (i.e., replicates) of plants were randomly chosen and were inoculated with AMF (Myc^+) by adding 80 g of the commercial product '*Symbivit*' (INOCULUMplus, Dijon, France) into the potting medium. The other three groups were not inoculated (Myc^-). '*Symbivit*' contains propagules of six different identified AMF: *Glomus etunicatum, Glomus microaggregatum, Glomus intraradices, Glomus claroideum, Glomus mosseae,* and *Glomus geosporum*. The plants were watered weekly with tap water.

After 4 months, AMF colonization of Myc^+ root plants was verified, whereas those of Myc^- plants did not. At this point, watering was stopped for 40 days (drought period). Thereafter, irrigation was resumed (recuperation period). A separate set of 30 trees were similarly transplanted in pots but were not inoculated and were regularly watered so to maintain the soil near field capacity. They were considered as control plants (CTR). They were kept on the same lot as the treated trees. The CTR plants were only used to calculate the relative drought alleviation rate (RDAR) but not included in the comparison between treatments (Myc^+ and Myc^-).

At regular intervals during the drought period (0, 7, 14, 23, 36, and 40 days) and after 1 month of recovery, water relations and gas exchange parameters were measured. On each date, three plants from each of the three groups were randomly selected and harvested; their leaves and roots were separated, dipped in liquid nitrogen then stored in a freezer (− 30 °C) for biochemical analysis.

Microscopic observations and estimation of mycorrhizal colonization

This symbiotic association was assessed on fresh root from three *Symbivit*-inoculated olive trees. The roots were stained with trypan blue (0.05%) (Philips and Hayman 1970), while the mycorrhization parameters were evaluated by the overall assessment of 30 root fragments with 1 cm length per plant (Trouvelot et al. 1986). The Mycorrhizal frequency (*F*%) and the intensity of colonization (*M*%) were determined with the Mycocalc program (http://www.dijon.inra.fr/mychintec/). For arbuscules, we have just verified their existence, the arbuscules score (*A*%) was not calculated, because they were difficult to detect. The microphotographs of AMF-colonized roots were taken with a digital camera (Cmex 5, Euromex, Holland) coupled with photonic microscope (OX Range, Euromex, Holland) interfaced to a computer using image manager Zeiss software.

Determination of soil moisture

Soil water status was characterized by measuring soil moisture content (SM) using the gravimetric method described by Davies et al. (2002). A soil sample was taken by coring through the whole vertical mass of the soil in the pot. First, the soil fresh mass (SFM) was determined; then, the soil sample was dried in an oven at 105 °C for 48 h, and the soil dry mass (SDM) was measured. The SM was calculated as follows:

$$\mathrm{SM}\ (\%) = (\mathrm{SFM} - \mathrm{SDM})/\mathrm{SDM} \times 100.$$

Plant–water relations

The plant–water status was characterized by taking predawn leaf water potential (Ψ_{pd}) and leaf relative water content (RWC) measurements. Ψ_{pd} was measured in the early mornings before sunrise on small terminal brindles using a Scholander pressure chamber (PMS Instrument Company, Albany, OR, USA) (Scholander et al. 1965).

The RWC was measured on mature fully expanded leaves according to Kramer and Brix (1965), as follows:

$$\mathrm{RWC}\ (\%) = (\mathrm{FW} - \mathrm{DW})/(\mathrm{SW} - \mathrm{DW}) \times 100,$$

where FW is fresh weight, SW is saturated weight, and DW is dry weight.

To obtain FW, the leaves were detached from the stem and immediately weighed individually. To determine the SW, the leaves were rehydrated by dipping their petioles in distilled water in a covered beaker and kept in the dark at 4 °C temperature for 24 h. DW was determined by drying the leaves in an oven at 80 °C for 48 h.

Gas exchange measurements

Stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) and transpiration rate (E, mmol H_2O m^{-2} s^{-1}) were measured on mature leaves using the LCi portable gas exchange system (ADC BioScientific Ltd, Hoddesdon, UK). Measurements were done between 09.30–10.30 h under saturating light conditions at temperatures between 20–30 °C. The measurements were repeated three times for each of three leaves per plant. A total of three plants per treatment were used.

Osmotic and turgor potentials

The osmotic potential (Ψ_s) was determined by the method of Nobel (1991). The same plant samples used for measuring the Ψ_{pd} were used for determining Ψ_s. To obtain cell contents, discs of 0.5 cm diameter obtained from fresh leaves were enclosed in an Eppendorf tube perforated at its base. The tube was immerged in liquid nitrogen for a few seconds, and then removed and left to thaw for 5 min; three freeze–thaw cycles were performed for each sample. The perforated Eppendorf was placed in another larger non-perforated tube which was centrifuged at 8000×*g* for 15 min at a temperature of 4 °C. The exudates from the discs were collected in the larger tube. Ψ_s of the exudates was read using an osmometer (WESCOR, VAPRO model 5600, UT, USA). To express Ψ_s in MPa, the following equation was used:

$$\Psi_s\ (\mathrm{MPa}) = (\Psi_s\ (\mathrm{mosmol/kg\ H_2O}) \times 2.577433)/1000.$$

Turgor potential (Ψ_p) was calculated as the difference between Ψ_{pd} and Ψ_s:

$$\Psi_p = \Psi_{pd} - \Psi_s.$$

Soluble sugars content

The soluble sugar content of the leaves was determined using the phenol–sulfuric acid method (Robyt and White 1987). For the extraction of soluble sugars, frozen leaves were grinded into a fine powder in liquid nitrogen. 0.2 g of the powder was mixed with 5 ml of 80% methanol. The homogenate was heated in a water bath at 70 °C for 30 min. 1 ml of the extract was added to 1 ml of 5% phenol (v/v) and 5 ml of concentrated sulfuric acid. After agitation and cooling, the absorbance (DO) was measured at 640 nm wavelength. The concentration of the soluble sugars was determined by reference to a standard curve prepared using glucose solutions ranging in concentration from 0.05 to 0.3 mg ml^{-1}.

Proline content

Proline content was determined using the ninhydrin method described by Troll and Lindsley (1955). 0.2 g of ground frozen leaves were homogenized in 5 ml of 40% methanol then placed in a water bath at 80 °C for 30 min. After cooling, 1 ml of the extract was mixed with 2 ml of concentrated glacial acetic acid, 1 ml of ninhydrin solution (25 mg ml^{-1}), and 2 ml of a mixture containing 24% distilled water, 60% glacial acetic acid and 16% orthophosphoric acid. The mixture was heated in a water bath at 100 °C for 30 min. After ice-cooling, 3 ml of toluene were added to this mixture. After agitation of the mixture, the upper phase was transferred into a new tube, dehydrated with anhydrous Na_2SO_4. The sample was kept in the dark for at least 2 h. After that, the absorbance was measured at a wavelength of 528 nm. Proline concentration in the samples was determined using a standard curve established with proline solutions ranging in concentration from 0.001 to 0.005 mg ml^{-1}.

Ion content

The contents of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), zinc (Zn), and iron (Fe) were determined on samples of dried olive leaves collected at the end of water treatment period. P was quantified by the colorimetric vanadate–molybdate–yellow method (Chapman and Pratt 1961). The absorbance was measured at 430 nm wavelength. The concentration of P was deduced from a standard curve obtained by plotting the concentration of known P_2O_5 solutions against their absorbencies. Total N content was determined using the Kajeldhal method (Kjeldhal 1883).

K, Mg, Zn, and Fe were quantified by atomic spectrometric absorption method. First, the samples were mineralized by mixing 20 mg of dry leaf material with 20 ml of 1% nitric acid. The mixture was subjected to slight stirring in the dark for 48 h. After that, the solution was filtrated on Whatman paper No. 1. Finally, the ion concentrations were measured using an atomic absorption spectrometer (Avanta GBC spectrometer, Australia), using an air-acetylene flame.

Calculated physiological parameters

For the evaluation of the role of AMF inoculation in the resilience of olive trees to drought, a specific physiological parameter was calculated. This parameter was adapted from the index used by Plenchette et al. (1983): relative field mycorrhizal dependency (RFMD) index which appreciates the role of mycorrhizal inoculation in the improvement of plant growth under field conditions. We used a "Relative Drought Alleviation Rate" (RDAR) which measures the contribution of AMF to the alleviation of drought impact for each measured physiological parameter. RDAR was calculated as follows:

$$\text{RDAR}\ (\%) = \left[\frac{(DI_{Myc^-}) - (DI_{Myc^+})}{(DI_{Myc^-})}\right] \times 100,$$

where DI_{Myc^-} is the drought impact on the measured physiological parameter in non-mycorrhizal plants (Myc^-). It is the maximum difference between CTR and Myc^- values for each measured parameter. DI_{Myc^+} is the drought impact on the measured physiological parameter in mycorrhizal plants (Myc^+), which is calculated as the maximal difference between CTR and Myc^+ values for each measured parameter. ($DI_{Myc^-} - DI_{Myc^+}$): appreciates the difference in drought impact on the measured physiological parameter between non-inoculated and inoculated plants; so it approximates the percentage of alleviation of drought impact accomplished by AMF inoculation. Thus, RDAR is the rate of alleviation of the impact of drought brought by AMF inoculation ($DI_{Myc^-} - DI_{Myc^+}$) as a percentage of total drought impact without AMF (DI_{Myc^-}). In the figures of each ecophysiological measured parameter, the RDAR is presented as the percentage of the alleviated part (grilled area) which was accomplished by AMF inoculation in Myc^+ plants versus the total impact of drought on non-inoculated (Myc^-) plants (hatched area + grilled area).

Statistical analysis

The experiment was arranged as a Completely Randomized Design with three replicates. All values of variables are the means of at least three replicates ± SE. The data were subjected to a statistical analysis of variance using GLM procedure of SAS software (SAS Institute 1999) followed by separation of means by Duncan post hoc test with a level of significance $P = 0.05$.

Results

Mycorrhizal colonization

The images result only from some pot checks on colonization and were not regularly done for all samples (Fig. 1). The microscopic observations showed that the roots of inoculated olive plants (Myc^+) were colonized by endomycorrhizal fungus. This colonization exhibited a frequency (*F*%) of 42.94% and an intensity (*M*%) of 5.39%.

Soil moisture

In control pots (CTR), soil moisture (SM) remained high throughout the trial period (Fig. 2). In contrast, SM in not-watered pots (treatments Myc^+ and Myc^-) decreased steadily from 16 to 0.5% during the watering-off period. Soil water status during this period was similar in all pots which were not watered. Therefore, any differences in plant response to drought between stressed Myc^+ and Myc^- plants were likely due to mycorrhization rather than to differences in soil hydration.

Plant–water relations

Changes in leaf RWC of Myc^+ and Myc^- plants during the stress period indicate that mycorrhization had a significant effect on plant water status (Fig. 3). Soil drying led to a decrease in leaf RWC in stressed plants. This decrease was more acute in Myc^- plants (68.6%) than Myc^+ ones (44.2%) especially towards the end of the water stress

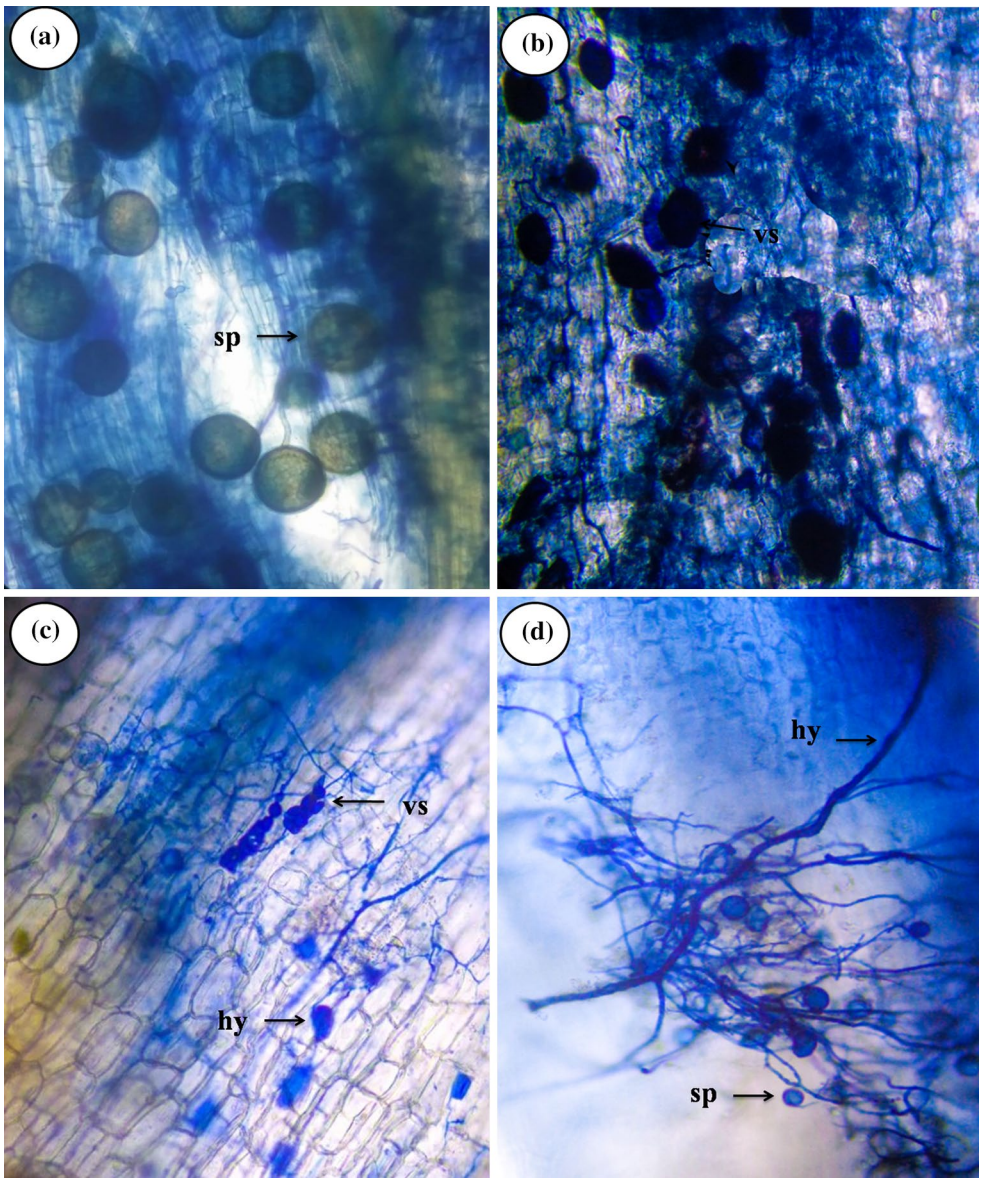

Fig. 1 Arbuscular mycorrhizal colonization in roots of olive trees: spore (sp) attached to extra- and intra-radicular hyphae (hy) (**a**, **d**, G: ×400); vesicles (vs) formed between cells in root cortex of olive trees (**b**, **c**, G: ×400)

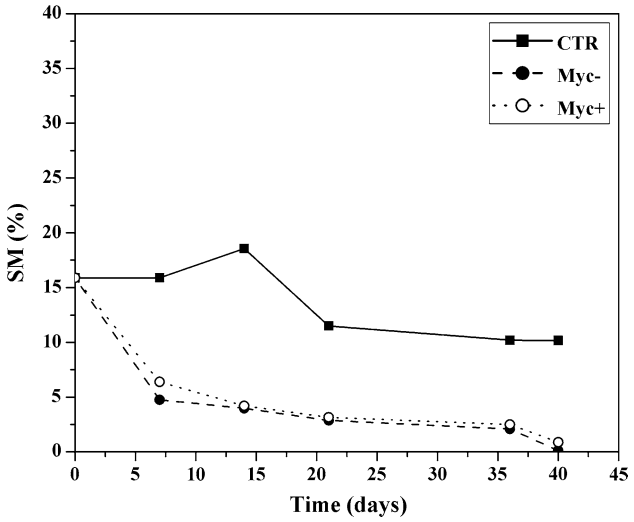

Fig. 2 Soil moisture (%) as a function of time (days) during 40 days of watering-off period. Three types of pots were assessed: pots containing well-watered plants (CTR) and pots containing not-watered plants inoculated (Myc^+) or not (Myc^-) with AMF

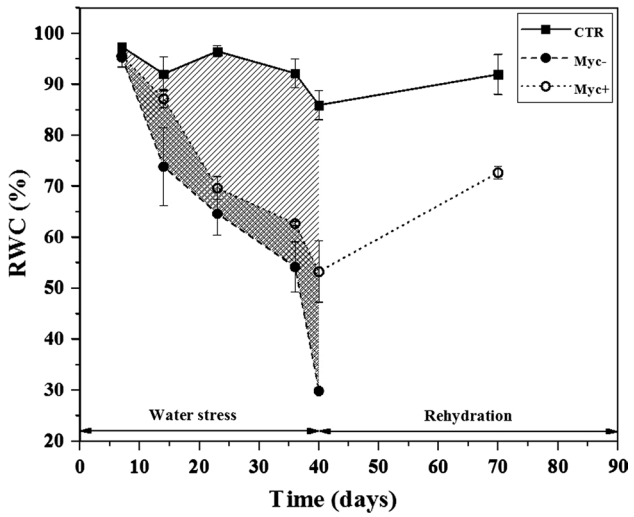

Fig. 3 Leaf relative water content (RWC) as a function of time (days) in well-watered (CTR) olive plants and in water-stressed olive plants inoculated (Myc^+) or not (Myc^-) with AMF. Water-stressed plants were subjected to 40 days of watering-off period followed by 30 days of rehydration. The oblique hatched area represents the no-alleviated part of the drought impact on the studied parameter, but the grilled area represents the alleviated part accomplished by AMF inoculation. Vertical bars indicate SE ($n=3$). Asterisk indicate that, at the same measurement date, differences between Myc^- and Myc^+ treatments are significant at $P \leq 0.05$

period. However, there were no significant differences between Myc^- and Myc^+ plants in leaf RWC ($P=0.93$). After 1 month of resuming irrigation (rehydration), Myc^+ plants recuperated their pre-stress RWC passing from 53% at the beginning of rehydration to 74% at the end. On the contrary, Myc^- plants did not recuperate and wilted. CTR plants maintained their RWC stable and high throughout the experimental period.

On the first day of the drought period, Ψ_{pd} was high (−0.45 MPa) in all trees (Fig. 4). However, as SM decreased, Ψ_{pd} diminished but with no significant difference between Myc^- and Myc^+ plants ($P>0.11$) until the 14th day of treatment. The differences between treatments increased and became significant from the 23rd day until the end of the drought treatment period ($P<0.006$). However, Myc^- plants were more affected than Myc^+ plants; Ψ_{pd} was −8.47 MPa in Myc^- plants versus −7.37 MPa in Myc^+ plants after 40 days of no irrigation. Rehydration allowed Myc^+ plants to recuperate their pre-stress Ψ_{pd} level while Myc^- plants did not.

Stomatal conductance and transpiration rate

Under favorable water conditions (until 7 days), stomatal conductance (g_s) in Myc^+ was significantly higher than that in CTR plants ($P=0.003$) (Fig. 5a). Under water stress conditions, changes in g_s reveal significant differences between Myc^- and Myc^+ plants ($P<0.05$). Myc^+ plants showed leaf g_s less affected, and they had higher values than Myc^- plants. Leaf g_s was 0.026 mol H_2O m^{-2} s^{-1} after 40 days of no irrigation in Myc^+ plants and was nil in Myc^- plants. In CTR plants, leaf g_s remained high during the experimental period.

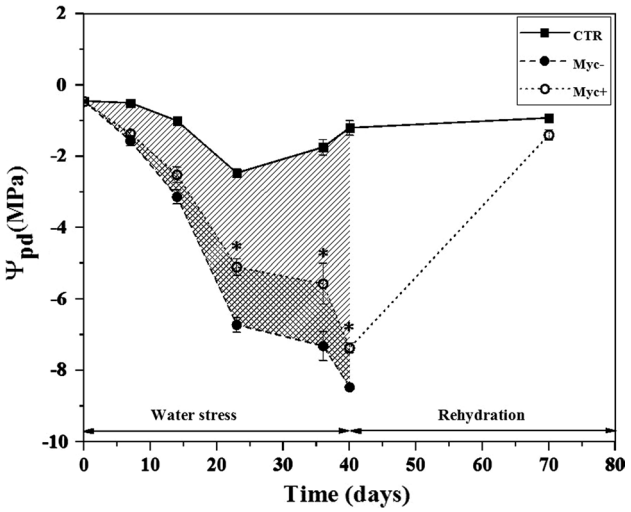

Fig. 4 Predawn leaf water potential (Ψ_{pd}) as a function of time (days) in well-watered (CTR) olive plants and in water-stressed olive plants inoculated (Myc^+) or not (Myc^-) with AMF. Water-stressed plants were subjected to 40 days of watering-off period followed by 30 days of rehydration. The oblique hatched area represents the no-alleviated part of the drought impact on the studied parameter, but the grilled area represents the alleviated part accomplished by AMF inoculation. Vertical bars indicate SE ($n=3$). Asterisk indicate that, at the same measurement date, differences between Myc^- and Myc^+ treatments are significant at $P \leq 0.05$

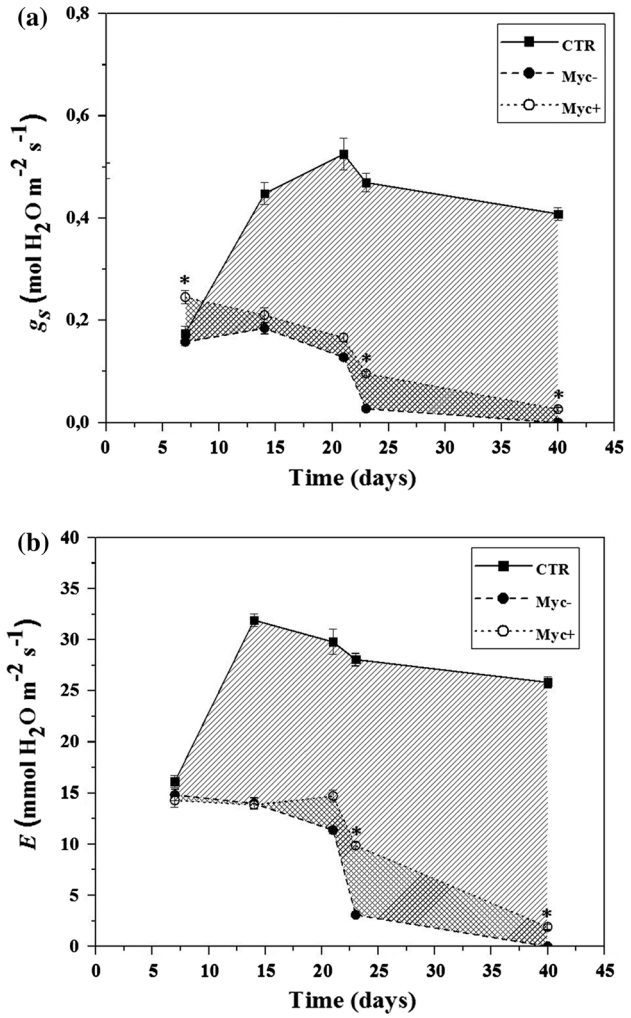

Fig. 5 Evolution of **a** stomatal conductance (g_s) and **b** transpiration rate (E) as a function of time (days) in well-watered (CTR) olive plants and in water-stressed olive plants inoculated (Myc^+) or not (Myc^-) with AMF. Water-stressed plants were subjected to 40 days of watering-off period. The oblique hatched area represents the no-alleviated part of the drought impact on the studied parameter, but the grilled area represents the alleviated part accomplished by AMF inoculation. Vertical bars indicate SE ($n=27$). Asterisk indicate that, at the same measurement date, differences between Myc^- and Myc^+ treatments are significant at $P \leq 0.05$

Leaf transpiration was also affected differently by drought depending on mycorrhization status (Fig. 5b). Leaf E of all plants was high and nearly constant until 21 days after stopping watering. After this period, the transpiration rate decreased but more acutely in Myc^- plants than Myc^+ plants ($P < 0.04$). After 40 days of stress, E of Myc^- plants was nil, while it was 1.91 mmol H_2O m^{-2} s^{-1} in Myc^+ plants. In CTR plants, E was high throughout the experimental period.

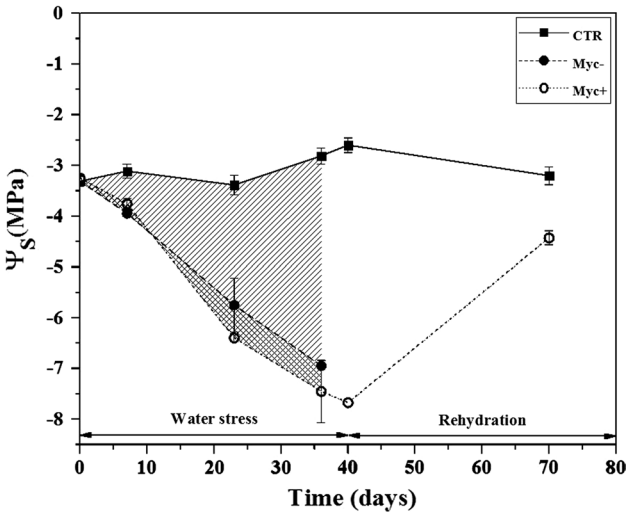

Fig. 6 Evolution of osmotic potential (Ψ_S) as a function of time (days) in well-watered (CTR) olive plants and in water-stressed olive plants inoculated (Myc^+) or not (Myc^-) with AMF. Water-stressed plants were subjected to 40 days of watering-off period followed by 30 days of rehydration. The oblique hatched area represents the no-alleviated part of the drought impact on the studied parameter, but the grilled area represents the alleviated part accomplished by AMF inoculation. Vertical bars indicate SE ($n=3$). Asterisk indicate that, at the same measurement date, differences between Myc^- and Myc^+ treatments are significant at $P \leq 0.05$

Osmotic and turgor potentials

The osmotic potential (Ψ_s) was affected in both Myc^+ and Myc^- trees during the no irrigation period (Fig. 6). The Ψ_s decreased more acutely in Myc^- plants than Myc^+ ones, but the differences between these two groups of plants were not significant ($P > 0.19$). After resuming irrigation, Ψ_s of Myc^+ plants increased but remained lower than its pre-stress level. Ψ_s was approximately -3 MPa in CTR plants during all the experimental period.

Figure 7 shows the changes in turgor potential (Ψ_p) as a function of time. Myc^+ plants maintained a positive Ψ_p during the experiment showing values near to those of CTR plants. In Myc^- plants, cell turgor was more clearly affected by water deficit. It decreased with time reaching zero after 18 days of stopping irrigation, and it registered negative values when the drought stress was severe. Despite this global distinct behavior between Myc^+ and Myc^- plants, the differences between the two treatments concerning the Ψ_p were not significant ($P > 0.10$). After 1 month of rehydration, Myc^+ plants recuperated their pre-stress cell turgor and their Ψ_p value became higher than that of control plants which remained positive throughout the experimental period.

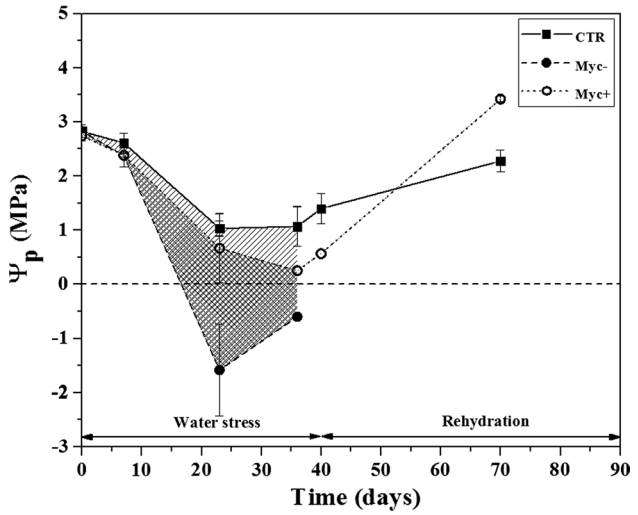

Fig. 7 Evolution of turgor potential (Ψ_P) as a function of time (days) in well-watered (CTR) olive plants and in water-stressed olive plants inoculated (Myc^+) or not (Myc^-) with AMF. Water-stressed plants were subjected to 40 days of watering-off period followed by 30 days of rehydration. The oblique hatched area represents the no-alleviated part of the drought impact on the studied parameter, but the grilled area represents the alleviated part accomplished by AMF inoculation. Vertical bars indicate SE ($n=3$). Asterisk indicate that, at the same measurement date, differences between Myc^- and Myc^+ treatments are significant at $P \leq 0.05$

Soluble sugars and proline contents

The evolution of soluble sugars concentration in leaves according to the decrease of Ψ_{pd} was influenced by AMF treatment (Fig. 8a). Indeed, before imposing water deficit and under its moderate level (-2 MPa $< \Psi_{pd}$), soluble sugar concentrations were not significantly different between Myc^+ and Myc^- plants ($F_1 = 1.63$, $P = 0.23$). However, when the Ψ_{pd} decreased, the accumulation of sugars was higher in Myc^- plants than in Myc^+ plants ($P = 0.019$).). Myc^- plants accumulate twice as much sugars than Myc^+ plants. It reached its maximum (185 mg g^{-1} FW) for Ψ_{pd} about -6.8 MPa. Soil rehydration reduced soluble sugars content in the leaves of Myc^+ plants to about the level of pre-stress or under moderate stress.

The evolution of leaf proline content as a function of Ψ_{pd}, was influenced by AMF treatment (Fig. 8b). The differences between Myc^+ and Myc^- plants were significant ($F_1 = 4.47$; $P = 0.04$). Under well-watered conditions, proline content was slightly higher in Myc^+ plants than Myc^- ones. Proline accumulated in the leaves of both Myc^+ and Myc^- plants as a result of water stress. However, it reached its maximum at $\Psi_{pd} = -5$ MPa for Myc^+ plants and -7.4 MPa for Myc^- plants. After that, proline content decreased at levels slightly lower than pre-stress values for each group of plants. Rehydration caused proline concentration drop in Myc^+ plant leaves lower than under well-watered conditions.

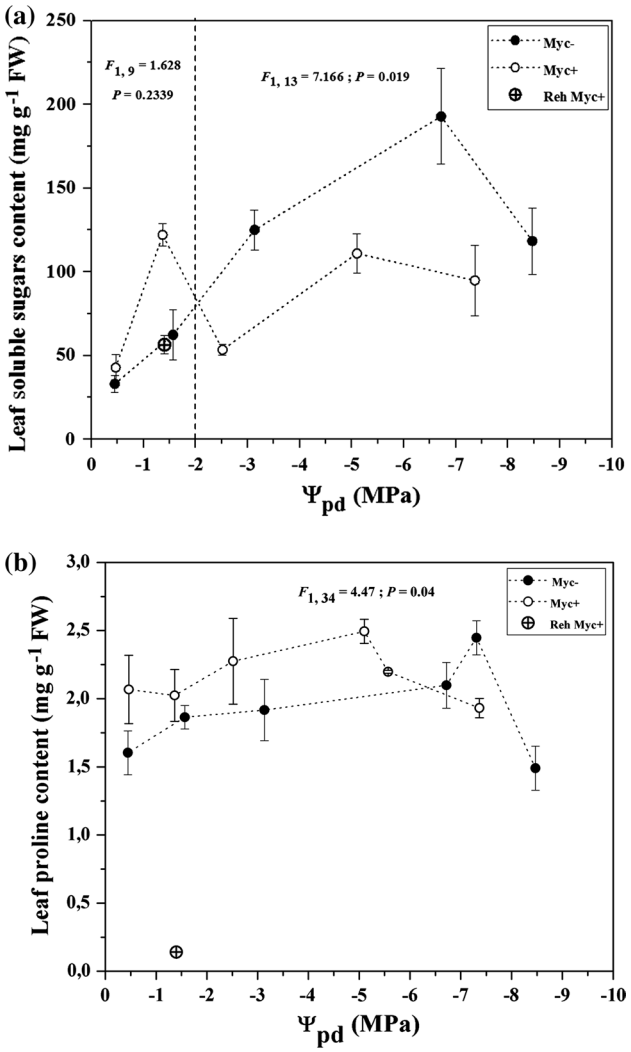

Fig. 8 Variation of soluble sugars content (**a**) and proline content (**b**) as a function of predawn leaf water potential (Ψ_{pd}) in water-stressed olive plants inoculated (Myc^+) or not (Myc^-) with AMF. Water-stressed plants were subjected to 40 days of watering-off period followed by 30 days of rehydration (Reh). Vertical bars indicate SE ($n=3$)

Ion content

Mineral analysis of olive leaves after 40 days of treatment revealed that water stress has affected the plants' mineral nutrition (Table 1). In leaves of non-inoculated plants, the reduction in mineral element concentration compared to the leaves of CTR plants was 22% for P, 33% for N, 70% for K, 6% for Mg, 82% for Fe, and 95% for Zn. However, mineral uptake in Myc^+ plants was less affected by drought. The reduction in mineral concentrations in the leaves of Myc^+ plants compared to the leaves of CTR plants was 7% for P, 47% for K, 16% for Mg, 57% for Fe and 87% for Zn. The exception was N concentration in leaves of Myc^+ plants which increased by 17% compared to CTR plants after

Table 1 Nutrient concentrations in dried leaves of well-watered (CTR) and water-stressed olive plants inoculated (Myc^+) or not (Myc^-) with AMF after 40 days of imposing the treatments

	% DW[a]				p.p.m	
	P	N	K	Mg	Zn	Fe
CTR	1.34 ± 0.19a	3.19 ± 0.19b	2.96 ± 0.9a	0.55 ± 0.06a	149 ± 12.4b	49.7 ± 3.9a
Myc^-	1.05 ± 0.03a	2.14 ± 0.20c	0.89 ± 0.1b	0.52 ± 0.04a	80.9 ± 3.6c	9.17 ± 0.88c
Myc^+	1.25 ± 0.2a	3.86 ± 0.16a	1.56 ± 0.2ab	0.46 ± 0.02a	190 ± 8.9a	21.21 ± 1.19b
F statistic	0.56	20.14	8.06	0.84	52.90	108.54
P	0.6096	0.0022	0.0395	0.4775	0.0013	< 0.0001
Significance level	NS	**	*	NS	**	**

The values are means of three replicates ± SE

The same letter within a column indicates no significant difference among treatments ($P < 0.05$)

p.p.m part per million parts of dry weight

[a]% DW percentage of dry weight

NS,*,**: indicate differences not significant or significant at $P \leq 0.05$ or at $P \leq 0.01$, respectively

Table 2 Maximum relative drought alleviation rate (RDAR) accomplished by AMF inoculation and the number of days to reach this maximum for five ecophysiological parameters measured in olive plants kept without watering for 40 days

	RWC	Ψ_{pd}	Ψ_s	Ψ_p	g_s	*E*
Maximum of RDAR (%)	41.7	38.0	36.2	86	15.5	27.1
Day of maximum	40	23	23	23	23	23

40 days of withholding watering. Under drought conditions, the uptake of four mineral elements (N, K, Zn, and Fe) was better maintained in AMF-inoculated plants than in non-inoculated ones. The differences between the two groups of plants were significant (Table 1).

Discussion

The establishment of the AMF symbiosis induces remarkable changes in the physiology of the host plant (Pozo et al. 2010; Azcón-Aguilar and Barea 2015). The association of AMF with plant roots could be a good strategy for enhancing the resistance of olive trees to severe drought. The addition of Symbivit product allowed the infection of olive plant roots through the AMF, this resulted their colonization by endomycorrhizal fungus after 4 months. In the present study, Myc^+ plants appeared more tolerant to drought stress than Myc^- plants. Under severe water stress conditions (40 days without watering), mycorrhizas appear to enhance water uptake, cell turgor, osmotic adjustment, and mineral uptake.

When the olive plants were fully irrigated, AMF had no effect on leaf RWC and Ψ_{pd}. However, under drought stress conditions, leaf RWC and Ψ_{pd} of Myc^- plants decreased more rapidly than Myc^+ plants. The differences between Myc^+ and Myc^- plants were not significant concerning their leaf RWC parameter. The Ψ_{pd} differed significantly between the two groups of plants mainly under severe drought stress ($P < 0.006$). This could be related to an improvement of water uptake by mycorrhizas external hyphae associated with Myc^+ plant roots. The hyphal water transport may be more important in dried soil than in hydrated one (Wu et al. 2013). Our results corroborate what was reported by the previous studies on citrus (Wu and Xia 2006) and soybean (Porcel and Ruiz-Lozano 2004). Under drought stress conditions, the significant differences between Myc^+ and Myc^- plants in their Ψ_{pd} could be detected also in the two principal components of Ψ_{pd}: Ψ_s and Ψ_p. Our results showed that water stress induced a decrease in Ψ_s. This decrease was slightly more important in Myc^+ plants than in Myc^- ones. This indicates that the former were more able to accumulate osmotic compounds. Augé et al. (1986) reported that Ψ_s of mycorrhizal rose plants decreased under water stress conditions indicating the occurrence of osmotic adjustment. While Myc^+ plants maintained their cells turgor under water stress conditions similar to well-watered plants (CTR). The Ψ_p of Myc^+ plants was positive during the drought treatment period. On the contrary, Ψ_p of Myc^- plants was positive at the beginning of the no-watering period and then became negative when water deficit increased reflecting the loss of cell turgor. Positive plant cell turgor is prerequisite for plant growth and important for survival (Jaleel et al. 2009). Maintaining cell turgor during a drought period enhances the tolerance to internal water deficit in the plant (Passioura 1996). Furthermore, inoculated trees maintained a high leaf RWC even under severe water deficit conditions. In our study, the alleviation of drought impact in olive tree by AMF inoculation was well detected in water-relation parameters

(Table 2). The Ψ_{pd}-RDAR due to AMF application indicates a large ability of AMF to alleviate drought effect on Ψ_{pd} especially when the stress is not too severe (Fig. 9). The determination of Ψ_p-RDAR showed a significant contribution of AMF to limiting the impact of drought on cell turgor (Fig. 9).

In addition to their role in improving drought tolerance, AMF appeared to help olive trees recuperate fast after watering restoration. Indeed, after irrigation was resumed, Myc^+ plants quickly regained their pre-stress levels of water status parameters, whereas Myc^- plants did not recover and wilted. Indeed, Ψ_{pd} reached pre-stress values after one month of resuming watering (Fig. 3). Ψ_s of Myc^+ plants remained more negative than that of CTR plants (Fig. 6). This may indicate that Myc^+ water-stressed plants acquired more drought resilience which may give the stronger defense against subsequent drought episodes. Furthermore, AMF inoculation may accomplish well cell turgor in olive tree. Thus, after 1 month, Myc^+-rehydrated plants showed Ψ_p higher than that of CTR plants (Fig. 7).

The stomata behavior is closely linked to the edaphoclimatic conditions of the plant specifically the water alimentation. The functionality of stomata is crucial for the modulation of leaf transpiration and photosynthesis. Stomatal conductance (g_s) and transpiration rate (E) are common parameters to gauge the plant's response to stress (Pinior et al. 2005). In the present study, under favorable water conditions, AMF inoculation allowed Myc^+ to get higher g_s than CTR and Myc^- plants. AMF inoculation significantly increased g_s in olive tree under well-watered

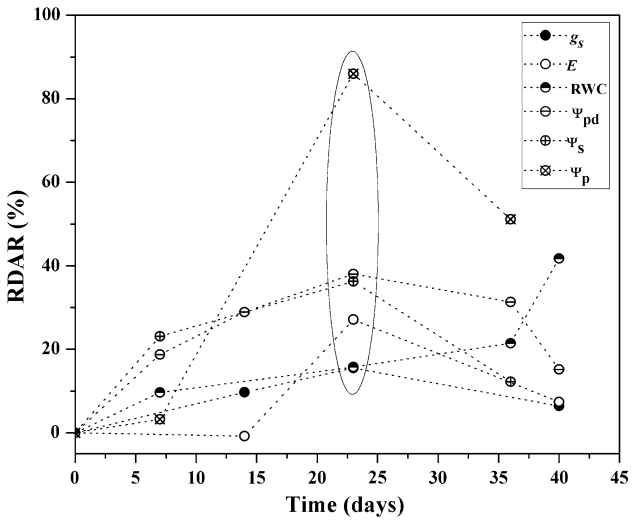

Fig. 9 Evolution of the relative drought alleviation rate (RDAR) of measured ecophysiological parameters as a function of water treatment time. For each measurement date, the RDAR estimates the contribution (%) of AMF inoculation to alleviate drought impact in olive trees

conditions (Calvo-Polanco et al. 2016). Water stress caused g_s and E decrease in both Myc^+ and Myc^- plants, but more so in the latter. After 40 days of watering-off, E and g_s were very low in Myc^+ plants and nil in Myc^- plants (at Ψ_{pd} of -8.47 MPa). Therefore, inoculation with AMF appeared to help Myc^+ plants to retain a level, albeit low, of gas exchange during water deficit treatments. For g_s and E, the RDAR achieved by inoculation with AMF were 15.5% at maximum and followed by 27.1%, respectively (Table 2). Therefore, the AMF appear to help plants to maintain their gas exchange only when water deficit is not severe (-5.1 MPa $< \Psi_{pd}$). Similar results were reported for other plant species subjected to similar treatments, such as date palm (Abohatem et al. 2011), other cultivars of olive tree (Caravaca et al. 2003), citrus (Wu and Xia 2006), and *Vigna unguiculata* (Duan et al. 1996).

One of the key components of drought tolerance mechanisms in higher plants is osmotic adjustment. The mechanism may be accomplished by the accumulation of numerous osmotic compounds including inorganic ions and organic solutes (Wu and Xia 2006). Our results show that, under drought conditions, Myc^- olive plants accumulated slightly more soluble sugars than Myc^+ plants for the same leaf water status (Ψ_{pd}). Myc^- plants accumulated soluble sugars until reached a peak at Ψ_{pd} of -6.8 MPa; thereafter, the concentration of these sugars decreased sharply to reach a level of 118.4 mg g^{-1} FW. In the whole water stress intensity studied range, Myc^+ olive plants accumulated less soluble sugars than Myc^- plants. The difference between Myc^+ and Myc^- plants appear to be related to AMF inoculation. In mycorrhized plants, the fungi use part of the carbohydrates for their nutrition. Similarly, shoots of water-stressed mycorrhized soybean plants, contained less sugars than those of non-mycorrhized plants (Porcel and Ruiz-Lozano 2004). On the contrary, other studies found that root colonization by AMF stimulates the accumulation of soluble sugars by the host plants, under drought conditions, like in maize (Subramanian and Charest 1995), in Citrus (Wu and Xia 2006), and in pistachio (Abbaspour et al. 2012). As well as the proline, another important organic compatible solute, accumulates in most plant species under water stress conditions and contributes to osmoregulation and osmoprotection (Rejsková et al. 2007; Hayat et al. 2012). The proline content increased in Myc^+ and Myc^- olive plants subjected to water deficit (Fig. 8b). However, the peak was reached at Ψ_{pd} values of -5 and -7.4 MPa in Myc^+ and Myc^-, respectively. The maximum of proline accumulation was reached in Myc^+ plants earlier than in Myc^- ones; while, in the reached levels, they were similar for both treatments. On the contrary, others found that proline content is higher in Myc^- plants than in Myc^+ plants subjected to water stress. This was reported in *Zingiber officinale* (Bhosale and Shinde 2011), citrus (Wu and Xia 2006), pistachio (Abbaspour

et al. 2012), and soybean (Porcel and Ruiz-Lozano 2004). In the present study, the early accumulation of proline in Myc^+ olive plants suggested that it is involved mainly in osmoregulation. Although, in Myc^- plants, the delay of proline accumulation suggested that it plays mainly the role of osmoprotector. After rehydration, leaf contents of soluble sugars and proline declined sharply to values equal or even lower than pre-stress level. This could suggest that, under favorable water conditions, carbohydrates and proline are principally diverted to the fungus partner in the symbiotic mycorrhizal association. In the contrary, this fungus partner alimented continuously the vegetal partner by water and mineral elements. In addition to their role in plant growth, ions may be involved in the osmoregulation process. Due to the low mobility of Cu, Zn, P, and Fe in the soil, mycorrhizas can improve the ability of host plants to absorb these nutrients via the extra-radical hyphae (Meddad-Hamza et al. 2010; Srivastava et al. 2002; Smith and Smith 2011). These hyphae allow the exploration of a larger volume of soil and concentrate ions near to roots thus reducing the diffusion distance (Krishnakumar et al. 2013). The inoculation of olive plants with AMF increased P and K contents of leaves (Calvo-Polanco et al. 2016). The extra-radical hyphae and the elevated acid phosphatase activity allow Myc^+ plants to absorb more phosphorus (Wu et al. 2011). In the current study, AMF-inoculated olive plants had considerably higher mineral nutrient concentrations (N, K, Zn, and Fe) than Myc^- plants. Nitrogen assimilation was improved in Myc^+ plants compared with Myc^- plants. This increase in N concentration may be due to higher activity of the main N-assimilating enzymes (Ruiz-Lozano and Azcón 1996). Caravaca et al. (2003) reported that P status in *O. europaea* was ameliorated by mycorrhizal inoculation under both watered and not-watered conditions. In addition, under similar conditions, K and Ca levels in citrus leaves were higher in Myc^+ seedlings than Myc^- ones (Wu and Xia 2006). In *Rhamnus lycioides* plants subjected to drought, mycorrhizas improve N and K uptake (Caravaca et al. 2003). Furthermore, Abbaspour et al. (2012) reported that AMF-colonized pistachio plants had considerably higher mineral nutriment contents (P, N, K, Ca, Zn, and Cu) than non-mycorrhizal plants under both irrigated and water-stressed conditions.

The evolution of the RDAR equivalent to all ecophysiological studied parameters was followed during the water deficit treatment period to determine the time of maximum AMF ability to alleviate drought stress (Fig. 9). Under favorable conditions or under moderate water stress, the AMF could be involved in the amelioration of growth and the development of the plant. However, when the stress is severe, the AMF is crucial for the survival of the plant (Meddad-Hamza et al. 2010). Our results show that the impact of drought on g_s, E, Ψ_{pd}, Ψ_s, and Ψ_p in olive tree is alleviated increasingly until the day 23 of water stress. At this time, the involvement of the mycorrhization is the most capable to alleviate the drought impact on gas exchanges and water-relation status. After that, the RDAR decreased according to the time of water stress. This behavior indicated that the AMF inoculation may highly alleviate drought impact on the stomatal function and the water status for more than 3 weeks of water stress.

In conclusion, our study showed that inoculation with AMF improves the resilience of olive trees to severe drought. The alleviation of drought impact on olive tree by AMF is high after 3 weeks and could be maintained until 40 days of severe water stress. Furthermore, AMF inoculation reinforces the capacity of drought-stressed olive tree to recuperate after severe water deficit period. Therefore, under arid and semi-arid climates characterized by severe drought stress, like that of the south Mediterranean region, the use of mycorrhizas could be a promising cultural practice to mitigate the effects of drought on olive trees.

To better understand the role of AMF in the resilience of plants to drought, further biochemical analyses are needed, e.g., the quantification of some plant growth regulators like abscisic acid (ABA) and strigolactone AMF-dependent is interesting. Furthermore, the inoculation of older olive trees with AMF to enhance their survival under severe drought conditions should be investigated.

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