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# Contribution of Native and Exotic Arbuscular Mycorrhizal Fungi in Improving the Physiological and Biochemical Response of Hulless Barley (*Hordeum vulgare* ssp. *nudum* L.) to Drought

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## Abstract

Water scarcity is considered as one of the most limiting factors of cereal productivity in Mediterranean agricultural systems. This study aims at investigating the contribution of arbuscular mycorrhizal fungi (AMF) inoculation in improving the tolerance of hulless barley (*Hordeum vulgare* ssp. *nudum* L.) to water stress. The experiment was carried out under semi-controlled conditions. Two AMF inoculants: a mixture of five native AMF species *Pacispora franciscana*, *Funneliformis mosseae*, *Funneliformis geosporum*, *Rhizophagus irregularis* and *Glomus tenebrosum* (NI) and a commercial inoculant containing six species of *Glomus* sp. (CI) were tested under two water regimes: well-watered regime (WW) and drought regime (D). Growth parameters such as plant height (PH) and shoot biomass; mineral nitrogen (N), phosphorus (P), potassium (K), sodium (Na), copper (Cu), iron (Fe) and zinc (Zn) contents; photosynthetic pigments chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and carotenoid (Car); chlorophyll fluorescence parameter (Fv/Fm); leaf relative water (RWC); and antioxidant enzyme catalase (CAT) and peroxidase (POX) activities were evaluated to investigate the effect of both factors. Water stress affected plant growth of hulless barley. However, application of AMF biofertilizers attenuated this negative effect. Both AMF inoculants NI and CI improved hulless barley growth (the PH by 11.3% and 19.8% and the shoot biomass by 26.1% and 41.3%, respectively) in comparison with their controls under a drought regime. Mineral nutrient N, P, K, Cu and Fe uptakes were significantly higher in AMF-inoculated plants compared with non-inoculated ones. Concerning the photosynthetic activity, a positive effect of AMF was observed as well under well-watered and drought conditions. Under drought regime, the activity of antioxidant enzymes increased in plants inoculated with AMF. These results were positively correlated with mycorrhizal root colonization, which was improved by AMF inoculation. In plants inoculated with NI, mycorrhizal root colonization was 2.3 times higher than in plants inoculated with CI under drought conditions. This finding was confirmed by the increase in AMF biomass assessed using specific phospholipid fatty acid (PLFA) and neutral fatty acid (NLFA) C16:1 $\omega$ 5 biomarkers in the rhizospheric soil of NI-treated plants in comparison with those treated with CI. In summary, the use of AMF could reduce drought damages by improving the physiological and biochemical responses of hulless barley. This study highlighted the potential role of AMF inoculation, in particular with native strains, as an innovative and eco-friendly technology for a sustainable crop growing system in arid and semi-arid areas.

**Keywords** Arbuscular mycorrhizal fungi · Biofertilization · Water stress · Hulless barley · Antioxidant activity · Nutrition

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## 1 Introduction

Abiotic stresses, such as drought, have become more common due to climate change, especially in arid and semi-arid areas. The Mediterranean area, and especially Tunisia, was identified as one of the regions most exposed to global climate change. Indeed, Tunisia has experienced 23 dry years from 1974 to 1997 (Giorgi 2006; USDA 2019). Drought is considered as one of the major factors decreasing crop

productivity (Lambers et al. 2008) and subsequently threatening food security (Dono et al. 2016). In fact, drought impairs various physiological processes in plant tissues as well as causes changes in both primary and secondary metabolites (Itam et al. 2020). It is well known that water stress induces oxidative damage in plant cells due to an overproduction of reactive oxygen species (ROS) such as: superoxide anion ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $\cdot OH$ ) (Sachdev et al. 2021; Sharma et al. 2012). Under normal conditions, the ROS are scavenged by different antioxidant mechanisms and therefore they are unable to cause any damage (Foyer and Noctor 2005; Silva et al. 2010). Oxidative stress occurs due to the imbalance between ROS generation and ROS scavenging, which reduces physiological and antioxidant defense systems (Dar et al. 2017). Moreover, the over-accumulation of ROS leads to a multitude of processes, such as lipid peroxidation and oxidative modification of proteins and nucleic acids, which extensively damage the normal cellular functioning of plants (Sallam et al. 2019).

Barley (*Hordeum vulgare* L.) is one of the oldest domesticated cereal crops (Zohary and Hopf 1993) and is widely cultivated all over the world (48 million hectares in 2019), ranking fourth in cereal production (FAO 2020). Barley is a versatile crop, used for animal feed, malting and human food (Elke and Emanuele 2013), yet is primarily used as grain or forage for livestock due to its higher fiber, protein and mineral nutrient contents (Rogers et al. 2017). It has plenty of health benefits for human nutrition due to its high content of beta-glucan, tocopherols (including tocopherols and tocotrienols) and phenolic compounds, which are known to reduce the risk of cardiovascular disease by lowering blood cholesterol and the glycemic index (Baik and Ullrich 2008). In Tunisia, barley is the second most cultivated cereal after durum wheat with more than 0.5 million hectares, representing 46.4% of the total area of cereals. It is frequently cultivated in semi-arid regions of the country, under rainfed conditions (DGPA 2020). Water availability is considered to be one of the main factors affecting wheat and barley production (Hossain et al. 2012). New strategies to overcome drought are needed, especially in the context of food sustainability and security.

Global climate models in semi-arid regions suggest that water scarcity is expected to intensify in the future (IPCC 2013). To cope with this environmental stress, soil microbiota may play a key role in improving plant growth and productivity (Berruti et al. 2016; Grover et al. 2011). Among these plant-growth promoting microorganisms, arbuscular mycorrhizal fungi (AMF) are the most widespread plant symbionts (Smith and Read 2008). Several studies have shown the beneficial effect of mycorrhizal symbiosis on plant growth by improving nutrient uptake efficiency under water deficit conditions (Al-Karaki et al. 2004; Begum et al.

2019a; Bowles et al. 2016; Gholamhoseini et al. 2013; Jayne and Quigley 2014). Moreover, it has been reported in several studies that AMF could improve the root surface area (Augé 2001) and thus increase plant water and nutrient uptake (Berruti et al. 2014; Marulanda et al. 2003). Many studies dealing with abiotic stresses have shown that mycorrhizal symbiosis had the ability to enhance plant growth properties as well as the photosynthesis and antioxidant enzymes activities of many species such as tomato, lettuce, citrus and maize (Mathur et al. 2019; Roupael et al. 2015; Xu et al. 2018a). Hence, few studies have been concerned with the physiological and the biochemical properties of barley subjected to drought stress.

Thus, the current research represents a first attempt to compare the potential of native and commercial AMF inoculations, under water stress conditions during tillering and grain filling development stages, in improving the growth of hullless barley (*Hordeum vulgare* ssp. *nudum* L.), one of the most important species for animal feed in semi-arid regions. To study the effect of AMF as biofertilizers, under water deficiency conditions, leaf water status, chlorophyll contents, antioxidant enzyme catalase and peroxidase activities, plant biomass and nutrient status were assessed.

## 2 Material and methods

### 2.1 Native AMF trapping and inoculant production

The soil sample used to trap and to multiply the native AMF was collected from the rhizospheric zone of durum wheat (*Triticum turgidum* L.) plants cultivated under rainfed conditions in Siliiana, in the northern region of Tunisia (36°23'47.24"N 10°11'8.01"E). Soil chemical properties were presented in Table S1. For the trap culture, the substrate was composed of one volume of soil mixed with one volume of autoclaved sand and perlite mixture (1:1 v/v). Common vetch (*Vicia sativa* L.) was the host plant. Pots were placed under a shelter during the period from January to August 2016. Plants were irrigated with Hoagland nutrient solution (Hoagland and Arnon 1950). The mycorrhizal inoculant produced contains a mixture of inert substrate, mycorrhizal roots, hyphae and spores of the different native AMF species. After the trapping period, spores were extracted from the soils using the method of wet-sieving (sieves of size 250, 125 and 40  $\mu m$ ) and decanting described by Gerdemann and Nicolson (1963), followed by the sucrose centrifugation method of Daniels and Skipper (1982). The spores were identified based on their morphological characteristics (Dalpé and Hamel 2007; <https://www.zor.zut.edu.pl/> *Glomeromycota*). Five AMF species were isolated from the prospected site: *Pacispora franciscana*, *Funneliformis*

*mosseae*, *Funneliformis geosporum*, *Rhizophagus irregularis* and *Glomus tenebrosum*.

### 2.2 Experimental design

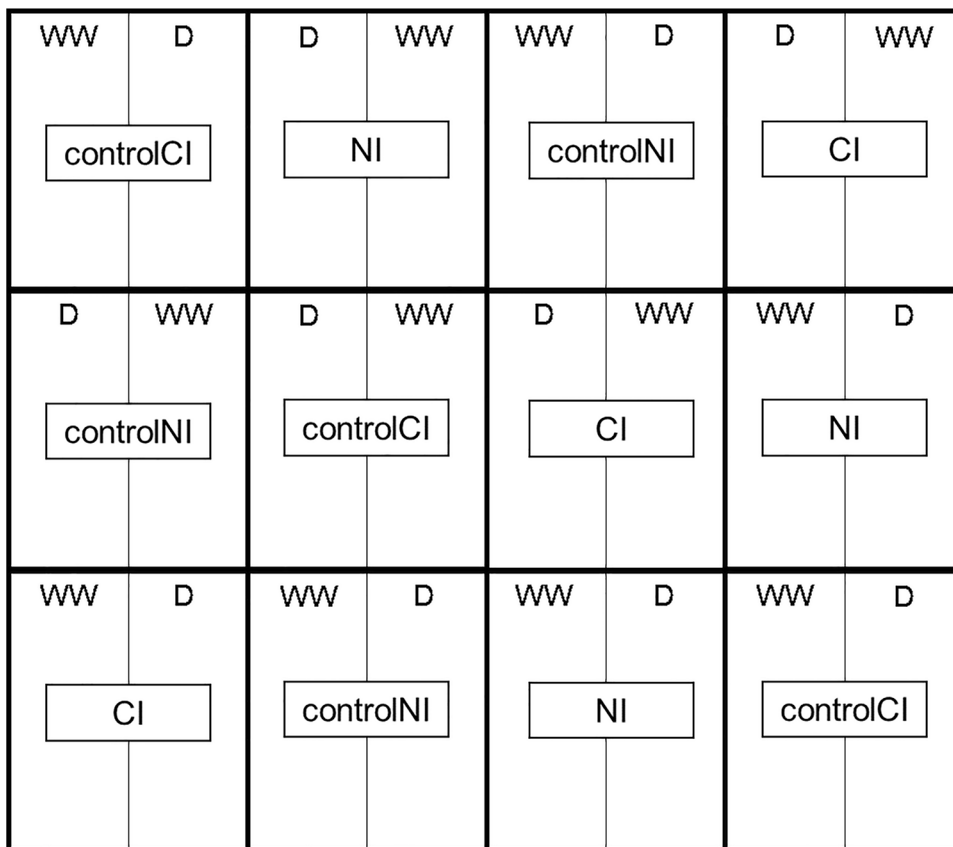
The experiment was conducted under shelter at the National Agronomic Institute of Tunisia (36°55'35.10"N 10°11'8.01"E). The plant species cultivated in this study was Hulless barley (*Hordeum vulgare* ssp. *nudum* L.). The experimental design was a split-plot arrangement in a completely randomized design (CRD) (Figure 1) with four mycorrhizal treatments: native inoculant containing a mixture of five AMF species as described previously (NI), commercial inoculant containing six species of *Glomus* sp. (CI), and two controls without inoculation (control NI and control CI) as main-plots and two water regimes: well-watered (WW) and drought (D) as sub-plots. Each treatment was replicated three times and, in each pot, four plants were maintained. For non-inoculated control plants, the same quantity of sterilized inoculant was added. Plastic pots (5 L) were surface-sterilized with sodium hypochlorite (NaClO) followed by ethanol and then filled with air-dried soil collected from the experiment station of the institute. Chemical and physical characteristics of the soil were presented in Table S1. The CI and the NI with an average of 500 propagules per pot were added homogeneously just below the seeds. Seeds of hulless

barley were surface-sterilized with 1% (v/v) NaClO for 5 min and washed several times with distilled water and were then sown on 13 January 2018. Gravimetric water contents were measured at the start of the experiment to determine the soil field capacity. The pots were arranged randomly with four plants per pot and were watered daily to maintain the soil at 70% of water holding capacity. Two water regimes were applied: well-watered, maintaining a holding capacity at 70%, and drought condition, maintaining the water holding capacity at 30-20%. The irrigation regimes were applied by weighing each pot and adding water to the weight calculated for the desired water regime. In fact, drought condition was induced twice by withholding the irrigation for 10 days at two critical growth stages: the tillering stage (68 days after sowing equivalent to the Zadoks stage Z20) and the grain filling stage (112 days after sowing equivalent to the Zadoks stage Z65). The drought period span was for 10 days (maintaining 30-20% of the holding capacity). Plants were then re-irrigated when the holding capacity of the pot reached 20%. The crop was harvested on 5 June 2018.

### 2.3 Plant biomass and mineral nutrient analysis

At harvest maturity, plants from each treatment (3 pots of four plants each) were hand-harvested and the shoot biomass was weighed. Then the shoot part was dried, crushed and

**Fig. 1** Experimental design layout of the shelter pot experiment using a split-plot arrangement in a completely randomized design (CRD); the main plot treatments consist of four arbuscular mycorrhizal fungi (AMF) inoculants: native inoculant containing a mixture of five AMF species *Pacispora franciscana*, *Funneliformis mosseae*, *Funneliformis geosporum*, *Rhizophagus irregularis* and *Glomus tenebrosum* (NI), commercial inoculant containing six species of *Glomus* sp. (CI), and two respective controls without inoculation (control NI and control CI); the subplot treatments consist of two water regimes: Well-watered (WW) and drought (D) with three replicates



then divided into two subsamples for mineral analysis. The first subsample (100 mg) was used to determine the total nitrogen (N) concentration using the Kjeldahl method (Jones 1987). The second one (100 mg) was ashed at 450 °C for 5 h in a muffle furnace. The ash was extracted in 20 mL of nitric acid (0.1 N) and then was digested for 10 min at 100 °C. After filtration, extracts were analyzed with a colorimetric method (Pauwels et al. 1992) to determine the total phosphorus (P) concentration at 840 nm. Total iron (Fe), copper (Cu) and zinc (Zn) concentrations were measured at 248.3, 324.8 and 213.5 nm, respectively, with an atomic absorption spectrophotometer and calcium (Ca), sodium (Na) and potassium (K) at 422.7, 589 and 769.9 nm, respectively, with a flame photometer according to the method described by Pauwels et al. (1992).

## 2.4 Mycorrhizal root colonization assessment

Fine roots of harvested barley plants were cleared in 10% (w/v) potassium hydroxide (KOH), acidified in 1 N hydrochloric acid (HCl) and stained with 0.05% (w/v) trypan blue as described by Phillips and Hayman (1970). The percentage of mycorrhizal root colonization was determined according to the method of McGonigle et al. (1990).

## 2.5 Soil AMF biomass quantification

After harvesting, the AMF biomass in the soil was determined through the evaluation of their specific fatty acid biomarker amounts according to the method of Frostegård et al. (1991) and Labidi et al. (2015). From each sample, lipids were extracted from 3 g of lyophilized soil with a mixture of chloroform, methanol and citrate buffer (1:2:0.8), then the lipids were fractionated into neutral lipids, glycolipids and phospholipids in a silica acid column. The phospholipid fatty acid (PLFA) and the neutral fatty acid (NLFA) were transformed into free fatty acid methyl esters by transesterification. The final extracts were analyzed using a PerkinElmer Autosystem gas chromatograph (GC) equipped with a flame-ionization detector (Norwalk, CT) with hydrogen as the carrier gas at a flow rate of 40 mL min<sup>-1</sup>. The peak areas of fatty acid were quantified by adding nonadecanoic acid methyl ester (C19:0) as an internal standard. Their identification relied on the retention times of fatty acids standard (Bacterial Acid Methyl Ester CP Mix in methyl coproate SUPELCO) and its mass analysis. The system was operated in the split mode (200 as split ratio) and helium was used as the carrier gas at a linear velocity of 37 cm s<sup>-1</sup>. Fatty acids were identified by comparing their mass spectra with the standard mass spectra in the NIST11s library. The amounts of the PLFA 16:1 $\omega$ 5 and NLFA 16:1 $\omega$ 5 representing membrane lipids and storage lipids of the AMF, respectively, were used as indicators of the AMF biomass (Frostegård

et al. 1991). Since the PLFA 16:1 $\omega$ 5 is not only specific for AMF but also for some Gram-negative bacteria, the ratio between NLFA and PLFA 16:1 $\omega$ 5 is always calculated. This ratio is high in AMF (1-200) and low in bacteria (< 1) (Olsson 1999).

## 2.6 Physiological and biochemical parameters measurements

During the two growth stages—tillering and grain filling—the plant height (PH) was measured from the stem base to the crossing point of the last leaf for the four barley plants per treatment. Then, the plant leaf samples were used to determine the effect of the water regime and mycorrhizal inoculation on the physiological and the biochemical response of barley plants.

### 2.6.1 Leaf relative water content

The relative water content (RWC) was determined as described by Turner (1986). From each treatment, leaf tissues were cut out from the most expanded leaf and weighed immediately to obtain the leaf fresh weight. The leaves were then placed in distilled water for 24 h at 10 °C in the dark and then the saturated weight, or turgid weight, was measured. After that, the leaves were dried in the oven at 80 °C for 48 h to obtain their dry weight. The RWC was calculated using the following equation:

$$\text{RWC} = \frac{[\text{fresh weight} - \text{dry weight}]/(\text{saturated weight} - \text{dry weight}) \times 100$$

### 2.6.2 Chlorophyll fluorescence analysis

Chlorophyll fluorescence (Fv/Fm) was measured using a portable multi-mode chlorophyll fluorometer (OS5p, Opti-Sciences, INC, Hudson, USA). The leaves were dark-adapted with a leaf clip holder for 15 min and were then illuminated with a saturating light. The measured Fv/Fm which corresponded to the potential quantum of photosystem II (PSII) was determined as follows: Fv/Fm = (Fm-F0) / Fm, where Fm and F0 are maximum and initial fluorescence yields of dark-adapted leaves, respectively (Maxwell and Johnson 2000). Six replications per leaf were taken for each treatment.

### 2.6.3 Chlorophyll *a*, chlorophyll *b* and carotenoid contents

The photosynthetic pigments chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and carotenoid (Car) were determined according to the method of Torrecillas et al. (1984). Chlorophyll was extracted from 100 mg fresh leaf tissues in 5 mL of 80% (v/v) acetone buffer for 72 h in darkness at 4 °C. The

extract absorbance was then measured at 645, 663 and 460 nm using a UV spectrophotometer (Cary 100/300 series, Agilent Technologies, France). The concentrations of chl *a*, chl *b* and carotenoid were calculated by the equations of Mackinney (1941) and Arnon (1949).

#### 2.6.4 Antioxidant enzymes activities

Peroxidase (POX) and Catalase (CAT) activities were assessed in mycorrhized and non-mycorrhized plants, 68 and 112 days after seeding. Leaf tissues (100 mg) were crushed and homogenized in 1 mL of 100 mM phosphate buffer (pH 7.0). After centrifugation, the supernatant was used to measure the absorbance of the protein content and the activity of CAT and POX enzymes at 750, 240 and 412 nm, respectively, with a UV/Visible spectrophotometer (Cary 100/300 series, Agilent Technologies, France). The POX assay was determined according to the Mitchell et al. (1994) method and the CAT assay was carried out using the method described by García-Limones et al. (2002) by following the decomposition of H<sub>2</sub>O<sub>2</sub>. Total proteins concentrations in the enzyme extracts were quantified according to the method of Lowry et al. (1951) using a commercial kit (BSA, Sigma, Saint-Quentin Fallavier, France).

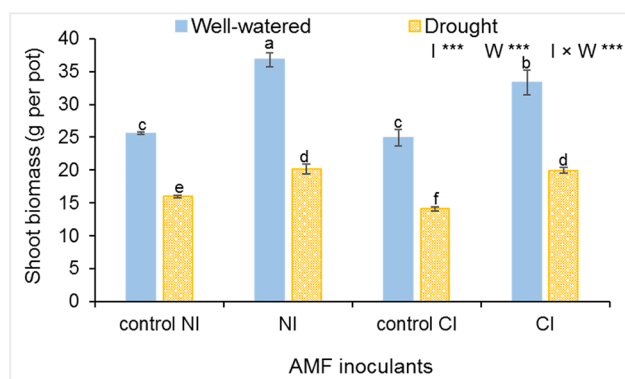
#### 2.7 Statistical analysis

The effect of AMF inoculation (4 levels), water regime (2 levels) and their interactions on measured parameters was performed using the two-way analysis of variance (ANOVA) followed by least significant difference (LSD) Fisher's test at  $P < 0.05$ . Before statistical analyses, data were checked for normality and homogeneity of variance using the Shapiro–Wilk test. The principal component analysis (PCA) was performed using the package 'factoextra' (Kassambara and Mundt 2020). All statistical analyses were performed using the RStudio software version 3.6.0 (R Core Team 2019).

### 3 Results

#### 3.1 Plant biomass and mineral nutrient contents

The analysis of variance showed that the AMF inoculation, as well as the water regime levels, significantly affected the shoot biomass of barley (Table S2). The results clearly indicated that the shoot biomass of AMF-inoculated plants by either NI or CI were higher compared to those of non-inoculated ones under both well-watered and drought regime (Fig. 2). Water stress significantly decreased the shoot biomass ( $P < 0.001$ , Table S2) by 41.7% compared with the well-watered regime. However, in AMF-inoculated plants, the barley shoot biomass significantly increased by 26.0%



**Fig. 2** Effect of arbuscular mycorrhizal fungi (AMF) inoculants and water regime on shoot biomass of hulless barley. NI, native inoculant containing a mixture of five AMF species (NI); CI, commercial inoculant; control NI and control CI, two controls without inoculation. Bars indicate standard error of the mean ( $n = 3$ ). Statistically significant effect at  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$

with NI and by 41.4% with CI in comparison with non-inoculated control plants, under drought regime (Fig. 2).

The nutrient concentrations of N, P, K, Cu, Fe and Zn in barley shoot were significantly and negatively affected by the water regime ( $P < 0.001$ , Table S2). In most cases, AMF-inoculated plants had a higher nutrient uptake as compared with non-inoculated ones. Under a drought regime, AMF inoculation with NI significantly improved N, P, K, Cu and Fe concentration by 1.0, 3.0, 3.5, 1.6 and 1.3 times, respectively, compared with their control plants. As for AMF-inoculated plants with CI, only N, P, Cu and Fe concentrations were increased by 1.2, 1.9, 1.7 and 1.4 times, respectively, in comparison with non-inoculated plants (Table 1). However, the K concentration showed no significant difference between AMF-inoculated plants with CI and their respective control plants. As for the Zn concentration, the interaction effect between the AMF inoculation and the water regime was not significant (Table S2). Unlike the other nutrients, the concentration of Na was significantly increased ( $P < 0.001$ , Table S2) by 38.8% with induced water stress. Under the drought regime, AMF-inoculated plants with CI showed a 31.8% lower Na concentration than non-inoculated plants. In contrast, the Na concentrations were similar between plants inoculated with NI and their respective controls (Table 1).

#### 3.2 Mycorrhizal root colonization and soil AMF biomass

The AMF root colonization was significantly affected by the application of AMF inoculants and the interaction between all the factors (AMF inoculants  $\times$  water regime) ( $P < 0.001$ , Table S3). Under well-watered condition, AMF inoculation significantly enhanced the mycorrhizal root colonization of barley 4.7 and 4.8 times for NI and CI, respectively, in

**Table 1** Effect of arbuscular mycorrhizal fungi (AMF) inoculation and water regime on nitrogen (N), phosphorus (P), potassium (K), sodium (Na), copper (Cu), iron (Fe) and zinc (Zn) contents of barley (*Hordeum vulgare* ssp. *nudum* L.) shoots

	N (mg g <sup>-1</sup> DW)	P (mg g <sup>-1</sup> DW)	K (mg g <sup>-1</sup> DW)	Na (mg g <sup>-1</sup> DW)	Cu (μg g <sup>-1</sup> DW)	Fe (μg g <sup>-1</sup> DW)	Zn (μg g <sup>-1</sup> DW)
<b>AMF inoculation</b>							
control NI	1.58 ± 0.15 <sup>c</sup>	0.21 ± 0.12 <sup>c</sup>	19.85 ± 9.53 <sup>b</sup>	82.01 ± 9.64 <sup>a</sup>	4.48 ± 2.15 <sup>b</sup>	22.83 ± 4.97 <sup>c</sup>	17.70 ± 4.98 <sup>b</sup>
control CI	1.70 ± 0.43 <sup>b</sup>	0.23 ± 0.06 <sup>c</sup>	21.17 ± 2.24 <sup>b</sup>	83.10 ± 16.67 <sup>a</sup>	4.23 ± 1.16 <sup>b</sup>	21.46 ± 4.80 <sup>c</sup>	18.68 ± 4.29 <sup>b</sup>
NI	1.92 ± 0.40 <sup>a</sup>	0.33 ± 0.06 <sup>b</sup>	43.44 ± 7.15 <sup>a</sup>	68.26 ± 22.64 <sup>b</sup>	7.16 ± 3.33 <sup>a</sup>	36.62 ± 13.29 <sup>a</sup>	29.20 ± 11.60 <sup>a</sup>
CI	1.87 ± 0.28 <sup>a</sup>	0.47 ± 0.11 <sup>a</sup>	37.70 ± 15.5 <sup>a</sup>	58.34 ± 13.82 <sup>b</sup>	6.90 ± 1.60 <sup>a</sup>	31.45 ± 6.35 <sup>b</sup>	37.74 ± 16.12 <sup>a</sup>
<b>Water regime</b>							
Well-watered	2.05 ± 0.22 <sup>a</sup>	0.36 ± 0.13 <sup>a</sup>	36.9 ± 13.70 <sup>a</sup>	61.08 ± 15.95 <sup>b</sup>	7.42 ± 2.13 <sup>a</sup>	34.00 ± 10.51 <sup>a</sup>	32.52 ± 13.83 <sup>a</sup>
Drought	1.48 ± 0.12 <sup>b</sup>	0.25 ± 0.12 <sup>b</sup>	24.21 ± 11.40 <sup>b</sup>	84.80 ± 12.98 <sup>a</sup>	3.97 ± 1.12 <sup>b</sup>	22.18 ± 4.32 <sup>b</sup>	19.14 ± 7.58 <sup>b</sup>
<b>AMF inoculation × Water regime</b>							
control NI × Well-watered	1.71 ± 0.06 <sup>c</sup>	0.31 ± 0.04 <sup>bc</sup>	28.35 ± 2.94 <sup>c</sup>	77.16 ± 12.29 <sup>bc</sup>	6.40 ± 0.46 <sup>c</sup>	27.03 ± 2.82 <sup>c</sup>	21.82 ± 2.64 <sup>a</sup>
control CI × Well-watered	2.09 ± 0.02 <sup>b</sup>	0.26 ± 0.03 <sup>cd</sup>	21.33 ± 1.94 <sup>c</sup>	68.83 ± 7.49 <sup>c</sup>	5.13 ± 0.70 <sup>cd</sup>	23.73 ± 4.98 <sup>cde</sup>	22.46 ± 1.22 <sup>a</sup>
NI × Well-watered	2.29 ± 0.03 <sup>a</sup>	0.33 ± 0.04 <sup>bc</sup>	46.65 ± 7.14 <sup>ab</sup>	48.11 ± 7.12 <sup>d</sup>	10.13 ± 1.00 <sup>a</sup>	48.53 ± 3.37 <sup>a</sup>	37.63 ± 10.61 <sup>a</sup>
CI × Well-watered	2.13 ± 0.02 <sup>b</sup>	0.56 ± 0.06 <sup>a</sup>	51.18 ± 6.89 <sup>a</sup>	50.24 ± 13.19 <sup>d</sup>	8.00 ± 1.51 <sup>b</sup>	36.70 ± 2.19 <sup>b</sup>	48.17 ± 14.18 <sup>a</sup>
control NI × Drought	1.45 ± 0.05 <sup>f</sup>	0.11 ± 0.06 <sup>e</sup>	11.37 ± 1.51 <sup>d</sup>	88.42 ± 3.54 <sup>ab</sup>	2.57 ± 0.65 <sup>f</sup>	18.63 ± 1.03 <sup>c</sup>	13.59 ± 2.08 <sup>a</sup>
control CI × Drought	1.31 ± 0.01 <sup>g</sup>	0.20 ± 0.07 <sup>de</sup>	21.02 ± 2.95 <sup>c</sup>	97.39 ± 5.25 <sup>a</sup>	3.33 ± 0.67 <sup>ef</sup>	19.20 ± 4.19 <sup>de</sup>	14.90 ± 1.34 <sup>a</sup>
NI × Drought	1.55 ± 0.04 <sup>e</sup>	0.33 ± 0.09 <sup>bc</sup>	40.22 ± 6.78 <sup>b</sup>	86.87 ± 3.29 <sup>ab</sup>	4.20 ± 0.62 <sup>de</sup>	24.70 ± 2.15 <sup>cd</sup>	20.75 ± 3.12 <sup>a</sup>
CI × Drought	1.61 ± 0.03 <sup>d</sup>	0.38 ± 0.05 <sup>b</sup>	24.23 ± 2.76 <sup>c</sup>	66.44 ± 10.35 <sup>c</sup>	5.80 ± 0.72 <sup>c</sup>	26.20 ± 3.67 <sup>c</sup>	27.31 ± 11.10 <sup>a</sup>
<b>Significance</b>							
AMF inoculation	***	***	***	***	***	***	***
Water regime	***	***	***	***	***	***	***
AMF inoculation × Water regime	***	*	***	*	**	***	ns

Plants were grown in agricultural soil (non-sterile) inoculated with native inoculant containing a mixture of five AMF species (NI); with commercial inoculant (CI); or non-inoculated with sterilized inoculants (control NI and control CI). Different letters in the same line indicate significant differences at 0.05 by Fisher's LSD test. Asterisks indicate significance at \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ , and ns indicates not significant ( $P > 0.05$ )

comparison with their respective non-inoculated control plants (Table 2). A drought regime significantly decreased the AMF root colonization of plants inoculated with CI by 32.7% and non-inoculated ones by 58.6% (Table 2). However, the root colonization rate reached a peak in AMF-inoculated plants with NI and was higher by 42.3% compared with a well-watered regime (Table 2). In order to evaluate the effect of mycorrhizal inoculation with NI and CI on the AMF biomass in the soil, the PLFA and the NLFA 16:1ω5 were determined in inoculated and non-inoculated treatments. It was found that the addition of NI and CI significantly increased the soil NLFA 16:1ω5 content, which represents mainly the vesicle storage structures, 12.3 and 8.0 times, respectively, compared with the non-inoculated treatments at both water conditions (Table 2). The highest amounts of NLFA 16:1ω5 were observed in soils inoculated with NI (6.8 and 6.03 μg g<sup>-1</sup> of soil under drought and well-watered regimes, respectively) (Table 2). Similarly, the soil PLFA 16:1ω5, used here to quantify the biomass of AMF in soils, showed a similar trend as the soil NLFA 16:1ω5 content (Table 2). On the other hand, the NLFA/PLFA 16:1ω5

ratio in the soil of inoculated barley plants ranging from 1.38 to 5.86 (> 1), which indicates the AMF predominance. However, low values (< 1) of the NLFA/PLFA 16:1ω5 ratio (range 0.20-0.35), were observed in the non-inoculated soil (Table 2). For AMF-inoculated plants with NI and CI, the NLFA 16:1ω5 was significantly and positively correlated with total root mycorrhizal colonization ( $R^2 = 0.81***$ ). However, there was a low and not significant correlation ( $R^2 = 0.16$ ) between the concentration of NLFA 16:1ω5 in the soil and total root colonization in non-inoculated plants (Fig. 3).

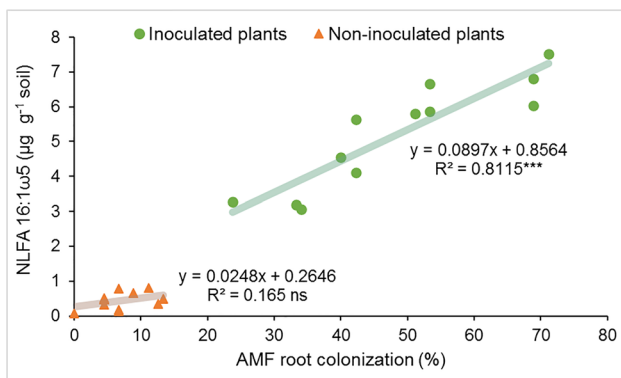
### 3.3 Plant height

The analysis of variance (Table 3a and b) showed that PH at both growth stages (tillering and grain filling) were significantly affected by the AMF inoculants, the water regime and their interactions ( $P < 0.001$ , Table S4). The drought regime reduced the PH of barley by 23.0% at both growth stages, compared with the well-watered regime (Table 3). Under the drought regime, the PH in

**Table 2** Effect of arbuscular mycorrhizal fungi (AMF) inoculation and water regime on total root colonization (Tot Myc), soil neutral lipid fatty acid (NLFA 16:1 $\omega$ 5), phospholipid fatty acid (PLFA 16:1 $\omega$ 5) amounts and ratio between NLFA and PLFA (NLFA/PLFA 16:1 $\omega$ 5) of hulless barley (*Hordeum vulgare* ssp. *nudum* L.)

	Tot Myc (%)	NLFA 16:1 $\omega$ 5 ( $\mu\text{g g}^{-1}$ dry soil)	PLFA 16:1 $\omega$ 5 ( $\mu\text{g g}^{-1}$ dry soil)	NLFA / PLFA 16:1 $\omega$ 5
<b>AMF inoculation</b>				
control NI	6.66 $\pm$ 4.86 <sup>c</sup>	0.39 $\pm$ 0.26 <sup>c</sup>	1.71 $\pm$ 0.70 <sup>bc</sup>	0.23 $\pm$ 0.13 <sup>c</sup>
control CI	7.27 $\pm$ 3.07 <sup>c</sup>	0.47 $\pm$ 0.23 <sup>c</sup>	1.58 $\pm$ 0.84 <sup>c</sup>	0.34 $\pm$ 0.18 <sup>c</sup>
NI	59.25 $\pm$ 11.97 <sup>a</sup>	6.41 $\pm$ 0.72 <sup>a</sup>	2.83 $\pm$ 1.89 <sup>a</sup>	3.61 $\pm$ 1.80 <sup>a</sup>
CI	37.77 $\pm$ 9.96 <sup>b</sup>	4.01 $\pm$ 1.08 <sup>b</sup>	2.26 $\pm$ 1.14 <sup>ab</sup>	2.01 $\pm$ 0.62 <sup>b</sup>
<b>Water regime</b>				
Well-watered	28.45 $\pm$ 19.95 <sup>a</sup>	2.98 $\pm$ 0.79 <sup>a</sup>	3.01 $\pm$ 1.10 <sup>a</sup>	0.86 $\pm$ 0.14 <sup>b</sup>
Drought	27.03 $\pm$ 18.17 <sup>a</sup>	2.66 $\pm$ 1.05 <sup>a</sup>	1.17 $\pm$ 0.21 <sup>b</sup>	2.23 $\pm$ 0.92 <sup>a</sup>
<b>AMF inoculation <math>\times</math> Water regime</b>				
control NI $\times$ Well-watered	10.4 $\pm$ 3.4 <sup>d</sup>	0.49 $\pm$ 0.31 <sup>d</sup>	2.31 $\pm$ 0.42 <sup>c</sup>	0.20 $\pm$ 0.10 <sup>c</sup>
control CI $\times$ Well-watered	9.4 $\pm$ 3.0 <sup>d</sup>	0.60 $\pm$ 0.23 <sup>d</sup>	2.12 $\pm$ 0.93 <sup>cd</sup>	0.35 $\pm$ 0.24 <sup>c</sup>
NI $\times$ Well-watered	48.9 $\pm$ 5.9 <sup>b</sup>	6.03 $\pm$ 0.56 <sup>a</sup>	4.42 $\pm$ 0.49 <sup>a</sup>	1.38 $\pm$ 0.20 <sup>bc</sup>
CI $\times$ Well-watered	45.2 $\pm$ 7.1 <sup>b</sup>	4.84 $\pm$ 0.91 <sup>b</sup>	3.23 $\pm$ 0.64 <sup>b</sup>	1.52 $\pm$ 0.32 <sup>bc</sup>
control NI $\times$ Drought	3.0 $\pm$ 2.6 <sup>d</sup>	0.30 $\pm$ 0.21 <sup>d</sup>	1.13 $\pm$ 0.14 <sup>e</sup>	0.26 $\pm$ 0.17 <sup>c</sup>
control CI $\times$ Drought	5.2 $\pm$ 1.3 <sup>d</sup>	0.36 $\pm$ 0.20 <sup>d</sup>	1.05 $\pm$ 0.22 <sup>e</sup>	0.33 $\pm$ 0.16 <sup>c</sup>
NI $\times$ Drought	69.6 $\pm$ 1.3 <sup>a</sup>	6.79 $\pm$ 0.74 <sup>a</sup>	1.23 $\pm$ 0.33 <sup>e</sup>	5.86 $\pm$ 2.13 <sup>a</sup>
CI $\times$ Drought	30.4 $\pm$ 5.7 <sup>c</sup>	3.18 $\pm$ 0.11 <sup>c</sup>	1.29 $\pm$ 0.18 <sup>de</sup>	2.50 $\pm$ 0.40 <sup>b</sup>
<b>Significance</b>				
AMF inoculation	***	***	**	***
Water regime	ns	ns	***	***
AMF inoculation $\times$ Water regime	***	**	**	***

Plants were grown in agricultural soil (non-sterile) inoculated with native inoculant containing a mixture of five AMF species (NI); with commercial inoculant (CI); or non-inoculated with sterilized inoculants (control NI and control CI). Different letters in the same line indicate significant differences at 0.05 by Fisher's LSD test. Asterisks indicate significance at \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ , and ns indicates not significant ( $P > 0.05$ )



**Fig. 3** The correlation between mycorrhizal root colonization and soil NLFA 16:1 $\omega$ 5 content of inoculated and non-inoculated plants with native inoculant (NI) and with commercial inoculant (CI) under the two different water regimes, ns = not significant, \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$

AMF-inoculated plants with NI and CI were increased by 11.2% and 19.8%, respectively, at the tillering stage (Table 3a) and by 10.1% and 27.3%, respectively, at the grain filling stage (Table 3b) compared with those in the non-inoculated plants.

### 3.4 Leaf relative water content

The leaf RWC was significantly influenced by the AMF inoculants, the water regime and by the interaction between the two factors ( $P < 0.001$ , Table S4) at both growth stages. The RWC in leaves was significantly diminished in response to drought stress (Table 3). In comparison with AMF-inoculated plants with NI and CI, the RWC in non-inoculated control plants were significantly reduced by 10.1% and 16.5%, respectively, at the tillering stage (Table 3a) and by 58.1% and 48.8%, respectively, at the grain filling stage (Table 3b) under a drought regime.



**Table 3** Effect of arbuscular mycorrhizal fungi (AMF) inoculation and water regime on plant height (PH), leaf relative water content (RWC), leaf chlorophyll fluorescence (Fv/Fm), leaf chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoid (Car) contents, catalase (CAT) and peroxidase (POX) activities of hulless barley (*Hordeum vulgare* ssp. *nudum* L.) at tillering (a) and grain filling (b) growth stages

a.		PH (cm)	Leaf RWC (%)	Fv/Fm	Chl <i>a</i> (mg g <sup>-1</sup> FW)	Chl <i>b</i> (mg g <sup>-1</sup> FW)	Car (mg g <sup>-1</sup> FW)	CAT (nkat g <sup>-1</sup> FW)	POX (nkat g <sup>-1</sup> FW)
<b>Tillering</b>									
<b>AMF inoculation</b>									
control NI		26.62 ± 3.38 <sup>c</sup>	59.36 ± 19.42 <sup>c</sup>	0.62 ± 0.07 <sup>c</sup>	1.52 ± 0.64 <sup>b</sup>	1.06 ± 0.27 <sup>c</sup>	0.78 ± 0.65 <sup>c</sup>	0.21 ± 0.18 <sup>b</sup>	7.62 ± 1.78 <sup>c</sup>
control CI		28.22 ± 5.05 <sup>b</sup>	49.80 ± 0.31 <sup>d</sup>	0.62 ± 0.1 <sup>c</sup>	1.26 ± 0.55 <sup>c</sup>	0.83 ± 0.36 <sup>c</sup>	0.66 ± 0.47 <sup>c</sup>	0.19 ± 0.15 <sup>b</sup>	7.24 ± 3.33 <sup>c</sup>
NI		31.21 ± 5.43 <sup>a</sup>	81.84 ± 7.27 <sup>a</sup>	0.72 ± 0.09 <sup>b</sup>	3.94 ± 0.94 <sup>a</sup>	2.24 ± 0.80 <sup>a</sup>	1.44 ± 0.95 <sup>b</sup>	1.50 ± 0.99 <sup>a</sup>	20.88 ± 8.71 <sup>a</sup>
CI		31.31 ± 3.43 <sup>a</sup>	72.88 ± 14.15 <sup>b</sup>	0.75 ± 0.05 <sup>a</sup>	3.78 ± 0.48 <sup>a</sup>	1.47 ± 0.14 <sup>b</sup>	1.67 ± 1.21 <sup>a</sup>	1.46 ± 1.02 <sup>a</sup>	18.14 ± 4.66 <sup>b</sup>
<b>Water regime</b>									
Well-watered		33.23 ± 2.55 <sup>a</sup>	77.01 ± 13.01 <sup>a</sup>	0.75 ± 0.05 <sup>a</sup>	3.21 ± 1.36 <sup>a</sup>	1.70 ± 0.74 <sup>a</sup>	1.88 ± 0.73 <sup>a</sup>	0.31 ± 0.19 <sup>b</sup>	11.59 ± 2.12 <sup>b</sup>
Drought		25.46 ± 2.21 <sup>b</sup>	54.93 ± 14.44 <sup>b</sup>	0.60 ± 0.07 <sup>b</sup>	2.04 ± 1.25 <sup>b</sup>	1.09 ± 0.53 <sup>b</sup>	0.40 ± 0.22 <sup>b</sup>	1.37 ± 1.07 <sup>a</sup>	15.35 ± 10.97 <sup>a</sup>
<b>AMF inoculation × Water regime</b>									
control NI × Well-watered		29.62 ± 0.66 <sup>d</sup>	77.04 ± 2.00 <sup>c</sup>	0.68 ± 0.01 <sup>b</sup>	2.11 ± 0.15 <sup>d</sup>	1.27 ± 0.22 <sup>bcd</sup>	1.37 ± 0.05 <sup>c</sup>	0.04 ± 0.01 <sup>c</sup>	9.23 ± 0.08 <sup>d</sup>
control CI × Well-watered		32.83 ± 0.44 <sup>c</sup>	56.83 ± 1.79 <sup>c</sup>	0.71 ± 0.01 <sup>b</sup>	1.75 ± 0.25 <sup>c</sup>	1.14 ± 0.07 <sup>cd</sup>	1.07 ± 0.19 <sup>d</sup>	0.041 ± 0.02 <sup>c</sup>	10.22 ± 1.09 <sup>d</sup>
NI × Well-watered		36.12 ± 0.44 <sup>a</sup>	88.43 ± 0.56 <sup>a</sup>	0.80 ± 0.00 <sup>a</sup>	4.77 ± 0.06 <sup>a</sup>	2.83 ± 0.47 <sup>a</sup>	2.30 ± 0.13 <sup>b</sup>	0.60 ± 0.14 <sup>b</sup>	13.01 ± 0.86 <sup>c</sup>
CI × Well-watered		34.33 ± 0.89 <sup>b</sup>	85.76 ± 1.74 <sup>b</sup>	0.79 ± 0.00 <sup>b</sup>	4.21 ± 0.10 <sup>b</sup>	1.57 ± 0.01 <sup>bc</sup>	2.78 ± 0.20 <sup>a</sup>	0.56 ± 0.03 <sup>b</sup>	13.92 ± 0.69 <sup>c</sup>
control NI × Drought		23.63 ± 1.07 <sup>f</sup>	41.68 ± 0.92 <sup>f</sup>	0.57 ± 0.06 <sup>d</sup>	0.93 ± 0.05 <sup>f</sup>	0.85 ± 0.08 <sup>de</sup>	0.19 ± 0.14 <sup>f</sup>	0.38 ± 0.13 <sup>b</sup>	6.01 ± 0.42 <sup>e</sup>
control CI × Drought		23.62 ± 0.33 <sup>f</sup>	42.78 ± 0.54 <sup>f</sup>	0.52 ± 0.01 <sup>e</sup>	0.78 ± 0.03 <sup>f</sup>	0.52 ± 0.15 <sup>e</sup>	0.26 ± 0.15 <sup>f</sup>	0.33 ± 0.04 <sup>bc</sup>	4.26 ± 0.18 <sup>f</sup>
NI × Drought		26.29 ± 1.02 <sup>e</sup>	75.25 ± 1.34 <sup>e</sup>	0.63 ± 0.00 <sup>c</sup>	3.11 ± 0.36 <sup>c</sup>	1.65 ± 0.58 <sup>b</sup>	0.58 ± 0.21 <sup>e</sup>	2.40 ± 0.14 <sup>a</sup>	28.76 ± 1.74 <sup>a</sup>
CI × Drought		28.29 ± 1.16 <sup>d</sup>	60.01 ± 0.86 <sup>d</sup>	0.69 ± 0.01 <sup>b</sup>	3.36 ± 0.16 <sup>c</sup>	1.37 ± 0.12 <sup>bc</sup>	0.57 ± 0.04 <sup>e</sup>	2.35 ± 0.46 <sup>a</sup>	22.37 ± 0.45 <sup>b</sup>
<b>Significance</b>									
AMF inoculation		***	***	***	***	***	***	***	***
Water regime		***	***	***	***	***	***	***	***
AMF inoculation × Water regime		***	***	**	*	*	***	***	***
<b>b.</b>									
<b>Grain filling</b>									
<b>AMF inoculation</b>									
control NI		49.54 ± 7.97 <sup>c</sup>	39.66 ± 18.72 <sup>c</sup>	0.63 ± 0.05 <sup>c</sup>	2.46 ± 1.21 <sup>c</sup>	0.99 ± 0.39 <sup>c</sup>	0.64 ± 0.30 <sup>c</sup>	0.17 ± 0.08 <sup>a</sup>	6.97 ± 2.3 <sup>c</sup>
control CI		49.75 ± 9.17 <sup>c</sup>	27.55 ± 6.37 <sup>d</sup>	0.62 ± 0.17 <sup>c</sup>	1.90 ± 0.78 <sup>d</sup>	0.83 ± 0.37 <sup>c</sup>	0.62 ± 0.38 <sup>c</sup>	0.14 ± 0.06 <sup>a</sup>	8.33 ± 2.6 <sup>c</sup>

Table 3 (continued)

NI	54.32 ± 8.45 <sup>b</sup>	67.15 ± 14.49 <sup>a</sup>	0.75 ± 0.05 <sup>a</sup>	3.75 ± 1.34 <sup>b</sup>	2.38 ± 0.83 <sup>b</sup>	1.90 ± 0.96 <sup>b</sup>	1.30 ± 0.58 <sup>a</sup>	26.04 ± 5.01 <sup>a</sup>
CI	56.87 ± 4.57 <sup>a</sup>	60.55 ± 19.53 <sup>b</sup>	0.71 ± 0.08 <sup>b</sup>	4.25 ± 0.59 <sup>a</sup>	3.20 ± 0.79 <sup>a</sup>	2.26 ± 0.98 <sup>a</sup>	1.08 ± 0.41 <sup>a</sup>	21.13 ± 6.24 <sup>b</sup>
<b>Water regime</b>								
Well-watered	59.46 ± 2.33 <sup>a</sup>	62.15 ± 19.98 <sup>a</sup>	0.76 ± 0.05 <sup>a</sup>	3.96 ± 1.06 <sup>a</sup>	2.36 ± 1.01 <sup>a</sup>	1.95 ± 0.09 <sup>a</sup>	0.42 ± 0.09 <sup>b</sup>	12.65 ± 2.76 <sup>b</sup>
Drought	45.78 ± 4.75 <sup>b</sup>	35.30 ± 14.30 <sup>b</sup>	0.59 ± 0.08 <sup>b</sup>	2.22 ± 1.04 <sup>b</sup>	1.34 ± 0.88 <sup>b</sup>	0.76 ± 0.49 <sup>b</sup>	0.93 ± 0.17 <sup>a</sup>	18.58 ± 4.63 <sup>a</sup>
<b>AMF inoculation × Water regime</b>								
control NI × Well-watered	56.76 ± 0.90 <sup>b</sup>	56.73 ± 1.16 <sup>b</sup>	0.67 ± 0.01 <sup>c</sup>	3.57 ± 0.09 <sup>b</sup>	1.44 ± 0.03 <sup>de</sup>	0.91 ± 0.10 <sup>d</sup>	0.09 ± 0.01 <sup>d</sup>	9.58 ± 0.52 <sup>d</sup>
control CI × Well-watered	58.08 ± 1.05 <sup>b</sup>	33.20 ± 1.45 <sup>c</sup>	0.78 ± 0.00 <sup>a</sup>	2.54 ± 0.57 <sup>c</sup>	1.00 ± 0.41 <sup>ef</sup>	0.96 ± 0.06 <sup>d</sup>	0.05 ± 0.01 <sup>d</sup>	10.66 ± 0.83 <sup>d</sup>
NI × Well-watered	62.00 ± 0.87 <sup>a</sup>	80.34 ± 0.91 <sup>a</sup>	0.80 ± 0.00 <sup>a</sup>	4.96 ± 0.22 <sup>a</sup>	3.13 ± 0.22 <sup>b</sup>	2.78 ± 0.06 <sup>b</sup>	0.76 ± 0.07 <sup>c</sup>	14.79 ± 0.86 <sup>c</sup>
CI × Well-watered	61.00 ± 0.50 <sup>a</sup>	78.35 ± 1.32 <sup>a</sup>	0.79 ± 0.00 <sup>a</sup>	4.78 ± 0.21 <sup>a</sup>	3.88 ± 0.12 <sup>a</sup>	3.14 ± 0.10 <sup>a</sup>	0.77 ± 0.07 <sup>c</sup>	15.58 ± 0.76 <sup>c</sup>
control NI × Drought	42.33 ± 1.33 <sup>c</sup>	22.60 ± 0.76 <sup>f</sup>	0.58 ± 0.01 <sup>c</sup>	1.37 ± 0.20 <sup>d</sup>	0.55 ± 0.09 <sup>f</sup>	0.37 ± 0.09 <sup>e</sup>	0.26 ± 0.13 <sup>d</sup>	4.36 ± 0.44 <sup>e</sup>
control CI × Drought	41.42 ± 0.99 <sup>c</sup>	21.89 ± 1.87 <sup>f</sup>	0.47 ± 0.02 <sup>f</sup>	1.27 ± 0.10 <sup>d</sup>	0.66 ± 0.54 <sup>f</sup>	0.28 ± 0.03 <sup>e</sup>	0.24 ± 0.03 <sup>d</sup>	6.01 ± 0.27 <sup>e</sup>
NI × Drought	46.63 ± 0.79 <sup>d</sup>	53.96 ± 1.59 <sup>c</sup>	0.70 ± 0.02 <sup>b</sup>	2.53 ± 0.02 <sup>c</sup>	1.64 ± 0.16 <sup>d</sup>	1.02 ± 0.12 <sup>d</sup>	1.83 ± 0.09 <sup>a</sup>	37.30 ± 2.89 <sup>a</sup>
CI × Drought	52.74 ± 0.89 <sup>c</sup>	42.75 ± 1.13 <sup>d</sup>	0.63 ± 0.01 <sup>d</sup>	3.72 ± 0.05 <sup>b</sup>	2.52 ± 0.39 <sup>c</sup>	1.38 ± 0.23 <sup>c</sup>	1.40 ± 0.36 <sup>a</sup>	26.68 ± 2.09 <sup>b</sup>
<b>Significance</b>								
AMF inoculation	***	***	***	***	***	***	***	***
Water regime	***	***	***	***	***	***	***	***
AMF inoculation × Water regime	***	***	***	***	*	***	***	***

Plants were grown in agricultural soil (non-sterile) inoculated with native inoculant containing a mixture of five AMF species (NI); with commercial inoculant (CI); or non-inoculated with sterilized inoculants (control NI and control CI). Different letters in the same line indicate significant differences at 0.05 by Fisher's LSD test. Asterisks indicate significance at \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ , and ns indicates not significant ( $P > 0.05$ )

### 3.5 Leaf pigments concentrations and chlorophyll fluorescence parameter (Fv/Fm)

The results revealed that Chl *a*, Chl *b*, Car concentrations and Fv/Fm were negatively and significantly ( $P < 0.001$ ) affected by the drought regime (Table 3). As for the non-inoculated plants, Chl *a*, Chl *b*, and Car contents and Fv/Fm were significantly ( $P < 0.001$ , Table S4) lower compared with the AMF-inoculated plants at both growth stages (Table 3). At the tillering stage and under a drought regime, the AMF-inoculated plants with NI and CI showed a 3.3 and 4.3 times increase, respectively, in Chl *a*, 1.9 and 2.6 times increase, respectively, in Chl *b*, 3.0 and 2.2 times increase, respectively, in Car, and 1.1 and 1.3 times increase, respectively, in Fv/Fm (Table 3a). At the grain filling stage, the same pattern was observed: AMF-inoculated plants presented the highest values under a drought regime (Table 3b).

### 3.6 Antioxidant Enzyme activities

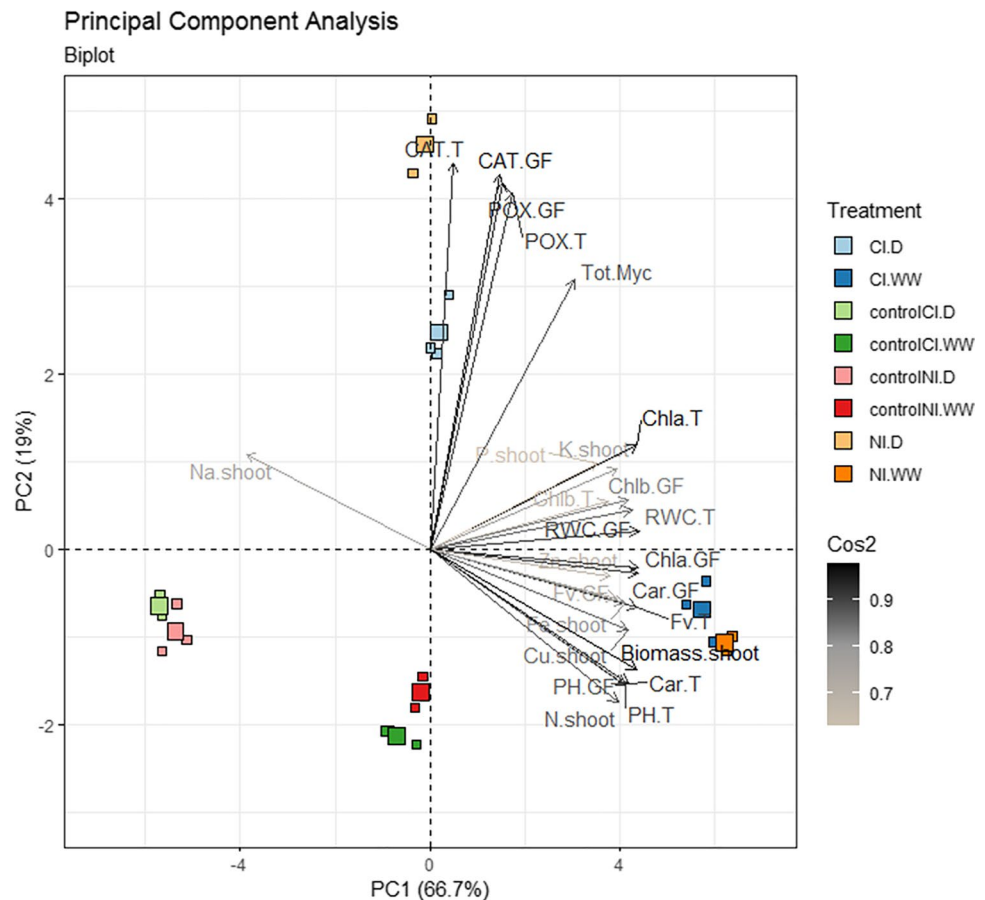
The CAT and the POX activities were significantly affected by the water regime, the AMF inoculants and the interaction of both factors ( $P < 0.001$ ), as shown by the two-way ANOVA results (Table S4). Under a drought regime, CAT and POX activities were significantly increased in

AMF-inoculated plants at both growth stages. However, a significant decrease in the activity of these antioxidant enzymes was observed in non-inoculated plants subjected to a drought regime (Table 3). Compared with the non-inoculated plants, AMF inoculation with NI and CI improved CAT activity by 6.3 and 7.1 times, respectively, at the tillering stage (Table 3a), and 7 and 5.8 times, respectively, at the grain filling stage (Table 3b) under a drought regime. Similarly, POX activity in AMF-inoculated plants with NI and CI significantly increased by 4.8 and 5.2 times, respectively, at the tillering stage, and by 8.5 and 4.4 times, respectively, at the grain filling stage compared with those in non-inoculated ones (Table 3a and b).

### 3.7 Relationships between the different AMF inoculants and the measured parameters under well-watered and drought regimes

A PCA was carried out to describe the relationships between the different AMF inoculants and the measured parameters under well-watered and drought regimes. The first two components (PC1 and PC2) explained 85.7% of the total variation (Fig. 4). In fact, the first component, PC1, explained the majority of the variation (66.7%), and was closely related to the relative water content, leaf photosynthetic pigments

**Fig. 4** Principal component analysis of the studied parameters of different arbuscular mycorrhizal fungi (AMF) inoculants under well-watered and drought regime. WW, well-watered, D, drought; NI, native inoculant; CI, commercial inoculant, control, non-inoculated; T, tillering growth stage; GF, grain filling growth stage; PH, plant height; RWC, relative water content in leaves; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; Car, carotenoid; Fv, the maximal quantum yield of PSII in dark-adapted state (Fv/Fm); POX, peroxidase activity; CAT, catalase activity; N. shoot, nitrogen uptake of shoots; P. shoot, phosphorus uptake of shoots; K. shoot, potassium uptake of shoots; Na. shoot, sodium uptake of shoots; Cu. shoot, copper uptake of shoots; Fe. shoot, iron uptake of shoots; Zn. shoot, zinc uptake of shoots; Tot. Myc, total root mycorrhizal colonization



Chl *a*, chl *b* and Car contents and chlorophyll fluorescence value at both the tillering and grain filling stages (Table S5). Also, most of the mineral nutrients were strongly and positively correlated with PC1 such as N. shoot ( $r^2 = 0.86$ ,  $P < 0.0001$ ), P. shoot ( $r^2 = 0.76$ ,  $P < 0.0001$ ) and K. shoot ( $r^2 = 0.85$ ,  $P < 0.0001$ ). However, the Na. shoot was negatively correlated with PC1 ( $r^2 = -0.84$ ,  $P < 0.0001$ ). The plant height and biomass were positively correlated with PC1 ( $r^2 = 0.94$ ,  $P < 0.0001$ ), as well as the total mycorrhizal root colonization ( $r^2 = 0.66$ ,  $P < 0.001$ ). The results showed that PC2, which explained 19% of the total variation, was predominantly influenced by the enzymatic activities such as POX ( $r^2 = 0.91$ ,  $P < 0.0001$ ) and CAT ( $r^2 = 0.93$ ,  $P < 0.0001$ ) at the grain filling growth stage, as well as the mycorrhizal root colonization ( $r^2 = 0.68$ ,  $P < 0.001$ ). Under a drought regime, the PCA indicated positive correlations with the AMF inoculation, plant height and biomass, nutrient concentrations of N, P, K, Cu, Fe and Zn, relative water content, photosynthetic pigments, chlorophyll fluorescence parameters and enzymatic activities. However, the concentration of Na increased during a drought regime, especially in non-inoculated plants. Plants inoculated with NI and CI tend to have higher values, especially for nutrient uptake, photosynthetic and enzymatic activities and biomass. The PCA results also confirmed the negative impact of water stress on these parameters; however, the inoculation with AMF mitigated these effects (Fig. 4, Table S5).

## 4 Discussion

Hulless barley (*Hordeum vulgare* ssp. *nudum* L.) is a very promising crop, as it can be used for food and feed, especially in semi-arid areas. However, there have been relatively few studies on it. This study is among the first ones analyzing in detail the effects of native AMF species on physiological and biochemical responses to water stress in hulless barley. In this regard, a pot experiment using native inoculant containing five AMF species of *Pacispora franciscana*, *Funneliformis mosseae*, *Funneliformis geosporum*, *Rhizophagus irregularis* and *Glomus tenebrosus* and commercial inoculant containing six species of *Glomus* sp. was carried out under well-watered and drought regimes. Total mycorrhizal colonization, plant growth parameters, mineral nutrient contents and antioxidant enzyme activities were investigated.

### 4.1 AMF inoculation increased mycorrhizal colonization of barley

In the present study, AMF inoculation significantly increased mycorrhizal root colonization of barley under both well-watered and drought conditions. We found that

root colonization with the CI was greater in barley plants grown under well-watered rather than under drought conditions. This result is in agreement with previous studies, which reported that AMF root colonization decreased under water stress condition in *Triticum aestivum* cultivars inoculated with *G. mosseae* and with *G. etunicatum* (Al-Karaki et al. 2004) and in *Cyclobalanopsis glauca* using *G. mosseae* (actually *F. mosseae* (Nicol. and Gerd.)) and *G. intraradices* (actually *R. intraradices* (Schenck and Smith) Walker and Schüßler comb. nov.) (Zhang et al. 2014). Furthermore, the NI showed the highest root colonization rate when barley plants were exposed to drought conditions. The results were in line with the amounts of the NLFA 16:1 $\omega$ 5 and the PLFA 16:1 $\omega$ 5 quantified in the rhizospheric soil. The same responses were obtained in *Zea mays* with *F. mosseae* (Ren et al. 2019) and in *Cucumis melo* with native consortium containing *Glomus* sp., *Sclerocystis* sp. and *Acaulospora* sp. (Meddich et al. 2021). Similarly, Chareesri et al. (2020) found that mycorrhizal colonization in rice roots was higher under dry conditions as compared with well-watered conditions due to the aerobic nature of AMF. In fact, the greater mycorrhizal root colonization with NI in comparison with CI, in addition to their high tolerance to water stress, may be related to the origin of the AMF. Similar results of the effectiveness of native AMF species in Mediterranean areas were reported in previous studies (Caravaca et al. 2005; Marulanda et al. 2007, 2009; Pellegrino et al. 2011). It has been suggested by Pellegrino and Bedini (2014) and Querejeta et al. (2006) that native AMF species had better adaptability to local climatic and edaphic conditions in semi-arid areas than non-native species. Thus, the mixture of native AMF species may be more beneficial to the plant growth due to the intra-specific synergism and the functional complementarity among locally adapted AMF species (Koch et al. 2011; Koide 2000).

### 4.2 AMF inoculation enhanced plant growth and mineral nutrition of barley under water stress through physiological and biochemical responses

In this study, the plant height and biomass production of barley were significantly higher in plants inoculated with NI as well as with CI as compared with non-inoculated ones. These results were in agreement with previous findings reported by Begum et al. (2019b) for maize, Bernardo et al. (2019) for wheat and Hashem et al. (2019) for chickpeas. In response to stress, abscisic acid (ABA) and strigolactones were induced which may actively promote AMF functioning and therefore plant performance (Pozo et al. 2015). In response to drought, the biosynthesis of strigolactones was increased in the presence of *R. irregularis* through the upregulation of gene *SICCD7* expression leading to better

development of lettuce and tomato (Ruiz-Lozano et al. 2016). The improvement in barley growth in inoculated plants under water stress conditions could be related to the well-developed root system and the increase in nutrient and water uptake (Hashem et al. 2018) via the establishment of an extensive extra-radical hyphae of AMF (Pagano 2014; Wu and Zou 2017). Moreover, AMF symbiosis can increase the biosynthesis of cellulose, hemicellulose, lipid molecules and carbohydrates, which lead to plant growth enhancement (Tarnabi et al. 2020). The boosting of plant growth observed with the AMF inoculation could be explained by the greater absorption of several nutrients from the soil by the plants, in particular nitrogen and phosphorus (Anli et al. 2020). Many studies also confirmed the correlation of growth enhancement with increased soil water level (Wu and Zou 2017). Our results revealed that plants inoculated with a combination of native AMF species and commercial AMF had considerably higher mineral nutrient N, P, K, Cu and Fe contents under drought conditions, compared with control plants. Similar results were reported by Al-Karaki et al. (2004) with *Glomus* sp., by Chen et al. (2017) with different AMF species from different genera such as *Claroideoglomus* sp., *Funneliformis* sp., *Diversispora* sp., *Glomus* sp., and *Rhizophagus* sp. and by Boutasknit et al. (2020) with a mixture of 25 species of *Glomus*, *Gigaspora*, *Acaulospora* and *Entrophospora*. The enhancement in mineral nutrition of inoculated plants could be linked to the ability of AMF to develop a hyphal network in order to access the unexplored region beyond the root depletion zone and thus to increase nutrient acquisition (Smith and Read 2008). Nitrogen and phosphorus are considered to be the most important elements for plant growth. Under water deficit condition, increased P and N in inoculated plants subsequently improved photosynthetic activities, which reflect the enhancement of plant growth by AMF (Begum et al. 2019a; Hameed et al. 2014; Zhu et al. 2014). Here, the increase in biomass and growth rates in AMF-inoculated plants can be directly linked to an increase of the P content in leaves, the major component in many metabolic processes, especially those related to nucleic acid synthesis, maintenance of membrane structures, cell division, energy metabolism and many other physiological processes during plant development (Malhotra et al. 2018). Also, the current work pointed out that barley plants inoculated with native AMF species had a higher K concentration but a lower Na concentration as compared with the non-inoculated ones. These results are in line with those of Al-Arjani et al. (2020) and Begum et al. (2019b). This might indicate the ability of AMF to pre-select elements for plants, such as K, which act as osmotic equivalents while they limit the uptake of toxic Na (Hammer et al. 2011) by retaining these ions in their intra-radical hyphae structures (Al-Karaki 2006). In addition, El-Mesbahi et al. (2012) demonstrated that the plant K concentration influences the regulation of root hydraulic

properties via AMF symbionts. Furthermore, an increased K concentration in barley can indirectly regulate osmotic adjustment and reduce the excessive accumulation of ROS in cell tissues (Begum et al. 2019b).

In the present study, mycorrhizal inoculation enhanced the leaf RWC of barley plants under well-watered and drought conditions, which is in agreement with the results of Aalipour et al. (2020a), Gholamhoseini et al. (2013), Mathur et al. (2019) and Zhu et al. (2012). This could be related to the ability of AMF to enhance the stomatal conductance capacity (Augé et al. 2008; Symanczik et al. 2018), osmotic adjustment (Augé 2004; Ruiz-lozano and Azcon 1995) and water retention (Augé et al. 2001) under such osmotic stress. Under drought conditions, mycorrhizal symbiosis regulates the level of ABA and cytokinin in roots in order to modulate better stomatal behavior by establishing a balance between root water uptake and leaf water transpiration (Ouledali et al. 2019). Xu et al. (2018b) demonstrated that *R. intraradices* upregulated the expression of 14-3-3 genes *TFT2* and *TFT3* through ABA signaling which resulted in enhanced stomatal behavior and water use efficiency in tomato plants subjected to drought stress. What was demonstrated is the implication of AMF in the regulation of plant aquaporin genes in order to increase the leaf water potential and reduce leaf transpiration (Aroca and Ruiz-Lozano 2009). Furthermore, Li et al. (2013) identified two aquaporin genes *GintAQPF1* and *GintAQPF2* in *R. irregularis* (formerly known as *G. intraradices*) which exhibit a higher capacity to transport water to plants via mycorrhizal hyphae under drought stress. Also, He et al. (2019) demonstrated that inoculation with *F. mosseae* upregulated the expression of aquaporin genes (such as *PtTIP1;2*, *PtTIP1;3* and *PtTIP4;1*) that led to a significant increase in leaf water potential of trifoliolate orange seedlings under drought stress. Both AMF inoculants improved the Fv/Fm in barley leaf, which is considered to be a good indicator of the potential of the photosynthetic system to respond to abiotic stresses (Araus et al. 1998; Krause and Weiss 1991). This result is consistent with *Ceratonia siliqua* under drought conditions (Meddich et al. 2021). The increase in chlorophyll fluorescence value could be linked to the stimulation of photosynthesis by AMF (Kaschuk et al. 2009). In fact, mycorrhizal symbiosis could mitigate the negative effect of water stress on PSII by inducing the activation of reaction centers, resulting in an increase of electron transport between PSII and PSI (Piniot et al. 2005). Mathur et al. (2019) revealed that inoculation with *R. intraradices*, *F. mosseae* and *F. geosporum* help *Triticum aestivum* plants to maintain the structure and the function of PSI and PSII under drought condition. Water stress induced significant declines in photosynthetic pigments Chl *a*, Chl *b* and Car. This result could be explained by the fact that thylakoid membranes, which contain protein photosynthetic

complexes that play a critical role in light absorption and conversion in chloroplast, are destroyed under water stress (Fang and Xiong 2015). Our findings showed that under water stress condition, barley plants inoculated with a mixture of native AMF species and a commercial AMF inoculant are able to maintain high chlorophyll contents compared with non-inoculated plants. This result is consistent with previous studies of Begum et al. (2019b) and Chen et al. (2017) carried out on other plant species such as cucumber (*Cucumis sativus* L. cv. Zhongnong No. 106) and maize (*Zea mays* L.), respectively. In fact, AMF enhance the rate of photosynthesis by stimulating rubisco activity, electron transport rates, adenosine tri-phosphate (ATP) production and even the ATP/ (adenosine diphosphate) ADP ratio in the chloroplast through increases in N and P mass fractions in leaves (Bechtaoui et al. 2021; Kaschuk et al. 2009). Thus, the results obtained were in agreement with other studies, suggesting that the higher water content in AMF mycorrhized plants led to a higher photosynthetic activity under stressful conditions (Boutasknit et al. 2020; Meddich et al. 2021). Interestingly, there was an increase in Car contents in the leaves of barley inoculated with AMF as compared with non-inoculated barley under both well-watered and drought conditions. It's well-known that carotenoid has an important photoprotective role against photoinhibition (Young 1991). The increase in carotenoid content in AMF-inoculated plants may also protect the plant system from being damaged by ROS (Krishna et al. 2005). Carotenoid represent a group of lipophilic antioxidants involved in detoxifying various forms of ROS. They are able to scavenge singlet molecular oxygen ( $^1O_2$ ) and triplet chlorophyll in order to protect the photosynthetic apparatus (Sharma et al. 2012). To cope with the oxidative damage, plant cells induced the production of antioxidant enzymes (Farooq et al. 2009) such as POX and CAT playing a critical role in quenching  $H_2O_2$  under water deficit stress (Fu and Huang 2001). In the present study, AMF inoculation, in particular with native AMF species, exhibited a more efficient antioxidant defense system as indicated by the increased POX and CAT concentrations under drought conditions, which may regulate the ROS level, thus reducing the oxidative damage. Similar results were observed by Aalipour et al. (2020b), Anli et al. (2020), Boutasknit et al. (2021) and Mo et al. (2016). It is well recorded that drought stress induces the accumulation of ROS within the cell, mainly  $O_2^{\cdot-}$  and  $H_2O_2$ , due to the disruption of cellular homeostasis (Sharma et al. 2012). In order to avoid the extensive oxidative damage of ROS, which can even cause cell death, plants use their antioxidant defense system using enzymatic and non-enzymatic components (Das and Roychoudhury 2014). Catalases and peroxidases represent the most important antioxidant enzymes, which are able to regulate the intracellular level of  $H_2O_2$ . Catalases are considered to be the most efficient

enzyme in  $H_2O_2$ -quenching since they convert two molecules of  $H_2O_2$  directly into water ( $H_2O$ ) and molecular oxygen and play a key role in dioxygen ( $O_2$ ) removal, while peroxidases reduce  $H_2O_2$  to  $H_2O$  and inhibit cell peroxidation of membrane lipids by decreasing the malondialdehyde (MDA) content (Ahmad et al. 2014; Dumanović et al. 2021). Under stress conditions, maintaining a high level of antioxidant enzymes, in particular catalase, may help plants to prevent cell damage through regulating the ROS level (Pedranzani et al. 2016; Ye et al. 2019). The contribution of AMF in regulating the ROS homeostasis through antioxidant enzyme activity has already been reviewed (Wu et al. 2014). Our results revealed that inoculation with either the native inoculant or the commercial one significantly enhanced the antioxidant defense system of hulless barley subjected to drought stress. These findings corroborate those of Gholin-zhad et al. (2020) who found that CAT and POX activity, as well as other antioxidant enzymes, were improved in sesame inoculated with *R. irregularis* and *F. mosseae* under water stress. The study of Zou et al. (2015) showed that inoculation with *R. irregularis* increased calcium ( $Ca^{2+}$ ) influxes resulting in less  $H_2O_2$  production in trifoliolate orange roots under drought stress. Langeroodi et al. (2020) demonstrated that *R. irregularis* increased the level of superoxide dismutase (SOD), peroxidase and other antioxidant enzymes in chicory, as well as reduced  $H_2O_2$  levels under drought stress. Similarly, Huang et al. (2014) suggested that inoculation with *F. mosseae* helps trifoliolate orange to maintain higher antioxidative enzyme activities, such as SOD isozymes and CAT, with lower  $H_2O_2$  accumulation. The improved tolerance of AMF-inoculated plants could be related to the up-regulated expression of antioxidants enzyme genes such as leaf *PtMn-SOD*, *PtPOD* and *PtCAT1* (He et al. 2020). Overall, AMF inoculation could enhance plant growth and photosynthesis through the up-regulation of the antioxidant plant system.

## 5 Conclusion

This study shows that arbuscular mycorrhizal fungi (AMF) inoculation, especially with the native inoculant, may be efficient in enhancing the growth and the biomass production of hulless barley under water stress condition during the tillering and grain filling growth stages. Our results demonstrate the beneficial role of AMF symbionts in mitigating the adverse effects of drought. The increase in plant growth was related to (i) an improvement of mycorrhizal root colonization, (ii) an increase in mineral nutrient nitrogen (N), phosphorus (P), potassium (K), copper (Cu) and iron (Fe) uptake and (iii) an enhancement of photosynthesis and antioxidant enzyme activities. In order to better understand the potential of AMF, field trials should be carried out in the future. The

use of native or exotic AMF strains as biofertilizer could represent a promising approach in assuring the sustainability of agricultural cropping systems in semi-arid Mediterranean regions, which are vulnerable to water deficit.

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## Declarations

**Conflict of Interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

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