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Native arbuscular mycorrhizal fungi enhance plant growth and productivity of hulless barley (*Hordeum vulgare* ssp *nudum* L.).

***M. JERBI*[1*], *S. LABIDI*[1], *A. LOUNES-HADJ SAHRAOUI*[2], *Y. DALPE*[3], *F. BEN JEDDI*[1]**

[1]*Laboratoire des Sciences Horticoles LR13AGR01, Université de Carthage, Institut National Agronomique de Tunisie, 43 Ave Charles Nicolle, 1082 Tunis, Mahrajène, Tunisia.*
[2] *Unité de Chimie Environnementale et Interactions sur le Vivant (UCEIV), Université du Littoral Côté d'Opale, SFR Condorcet FR CNRS 3417, 50 rue Ferdinand Buisson, 62228, Calais, France.*
[3] *Centre de recherche de l'est sur les céréales et oléagineux (CRECO), 960 Ave Carling, Ottawa K1A 0C6, Canada.*

***Corresponding author:** marouajerbi90@gmail.com

Abstract - A pot experiment was conducted to assess the growth response and grain yield of hulless barley (*Hordeum vulgare* ssp *nudum* L.) to arbuscular mycorrhizal fungal (AMF) inoculation. Three inoculants containing native AMF species, AI1 (*Funneliformis mosseae*, *Funneliformis geosporum* and *Scutellospora calospora*); AI2 (*Funneliformis mosseae* and *Funneliformis geosporum*); and AI3 (*Pacispora franciscana*, *Funneliformis mosseae*, *Funneliformis geosporum*, *Rhizophagus irregulare* and *Glomus tenebrosum*), isolated from agricultural soils in northern Tunisia were tested and compared to a commercial inoculant CI (*Glomus* sp.). Our findings showed that native AMF species, in particular AI3 mycorrhizal inoculum, performed better than commercial inoculant and presented the highest values for mycorrhizal root colonization (53.3%), followed by CI (43.7%), AI1 (38.5%) and AI2 (18.5%) and growth parameters. AMF inoculation also improved significantly macro and micro-nutrient contents such as N, P, K, Cu, Fe and Zn in hulless barley plants as compared to non-inoculated. This resulted in higher total biomass and grain yield of hulless barley. In fact, total biomass of hulless increased significantly about 1.6 and 1.5-fold in plants inoculated with AI3 and CI. Moreover, the highest grain yield was recorded in plants inoculated with AI3 (4.8 g/ pot), whereas the lowest (1.9 g/ pot) was observed with AI2. The obtained results highlight the efficiency of the native AMF species present in AI3 inoculum. This study underlines the potential of using efficient native AMF inoculants to enhance growth and yield of hulless barley.

Keywords: Mycorrhization, native inoculants, hulless barley, grain yield.

1. Introduction

Barley (*Hordeum vulgare* subsp. *vulgare*) is one on the oldest domesticated cereal crops all over the world. It takes the fourth place in world cereal production (USDA, 2019). In Tunisia, barley is the second cultivated cereal occupying 0.52 million hectares (FAOSTAT, 2018). More than 80% of barley is used for animal feed and the rest for human food and malt. In semi-arid regions, barley is mainly cultivated by sheep owners for winter grazing when forage and pasture are not available and it is also cultivated for grain production in other regions (El Felah and Medimagh 2005). There are two major categories of barley, hulled barley and naked (hulless) barley. The most used varieties are of the hulled form (Taketa et al. 2004). However, hulless is used for human food (Pourkheirandish and Komatsuda 2007). Hulless barley (*Hordeum vulgare* ssp *nudum* L.) attracted more interest as a food and feed because of its high content of soluble fibre β-glucan (Izydorczyk et al. 2005) and protein, essential amino acid lysine (Šterna et al. 2017). Despite its nutritional benefits, hulless barley has been rarely cultivated in the world (Narwal et al. 2017), particularly in Tunisia. Previous research has shown that hulless barley usually produce low yields as compared to the barley hulled forms (Bhatty et al. 1979). Because of its high nutritional value, it's necessary to improve its productivity by adopting sustainable crop practices.

Arbuscular mycorrhizal fungi (AMF) represent an important member of soil biota and play key roles in natural ecosystem (Smith and Read 2008). AMF are obligate symbionts, belonging to the phylum Glomeromycota, which form mutualistic symbioses with more than 80% of terrestrial plants (Smith and Read 2008). AMF are well known to promote plant growth by (i) improving mineral nutrition, in particular phosphorous and nitrogen (Clark and Zeto 2000; Smith and Smith 2011) and water uptake (Augé et al. 2001), (ii) increasing tolerance to abiotic stresses (Bencherif et al. 2019; Labidi et al. 2012; Lenoir et al. 2016) and enhancing plant protection against pathogens (Akhtar and Siddiqui 2008).
The current study aims to compare the effect of native mycorrhizal inoculants containing different AMF species isolated from agricultural Tunisian soils and a commercial inoculum on plant growth and grain yield of hulless barley as a promising cultivar for several application in food, feed and in agro-industries.

2. Material and methods

2.1. Collection of rhizospheric soil

Three different sites (S1, S2 and S3) from the regions of Nabeul, Zaghouan and Siliana, respectively, were chosen according to their bioclimatic stages (Table 1). The prospected sites represent the main cereal crops oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), and durum wheat (*Triticum turgidum* L.) cultivated under rainfed condition. From each site, plant roots with their rhizospheric soil were collected at a depth of 20 cm along a diagonal transect (three samples per site were taken). Table 2 represents the physico-chemical soil properties of the prospected sites.

Table 1. Description of prospected sites during the year (2015-2016).

	S1	S2	S3
Minimum temperature of the coldest month (°C)	6.5	8.4	11.5
Maximum temperature of the warmest month (°C)	26.6	27.5	29.0
Annual average rainfall (mm)	432.9	354	352.4
Bioclimatic stage	Subhumid	Superior semiarid	Middle semiarid

2.2. AMF inoculums

Three native AMF inoculums (AI1, AI2 and AI3) were isolated from the three prospected sites. The propagation of AMF inoculums was established in trap culture using the common vetch (*Vicia sativa* L.) as the host plant. After 12 months of culture, the native AMF inoculums produced containing a mixture of inert substrate, mycorrhizal roots, hyphae and spores of different native AMF species. The AMF spores were extracted through the wet-sieving and decanting method described by Gerdemann and Nicolson (1963). Spores were identified on the basis of their morphological characteristics, and on that available information in electronic resources such as Glomeromycota Phylogeny (http://www.amf-phylogeny.com/ and Janusz Blaszkowski web site (http://www.zor.zut.edu.pl/Glomeromycota/). Morphological identification revealed the presence of different AMF species in each inoculum with the most abundant as follows: AI1: *Funneliformis mosseae*, *Funneliformis geosporum* and *Scutellospora calospora*; AI2: *Funneliformis mosseae* and *Funneliformis geosporum*; and AI3: *Pacispora franciscana*, *Funneliformis mosseae*, *Funneliformis geosporum*, *Rhizophagus irregulare* and *Glomus tenebrosum*.

Table 2. Physico-chemical characteristics of the prospected sites.

	S1	S2	S3
pH	6.63 ± 0.07	8.23 ± 0.08	7.63 ± 0.03
EC (dS/ m)	0.09 ± 1.10	0.27 ± 1.74	0.19 ± 1.27
Total $CaCO_3$ (%)	1.27 ± 0.81	48.77 ± 0.45	40.42 ± 1.59
Active $CaCO_3$ (%)	0.77 ± 0.29	29.4 ± 0.53	23.01 ± 1.36
OM (%)	0.4 ± 0.20	0.13 ± 0.20	1.93 ± 0.15
N (%)	0.36 ± 0.08	1.25 ± 0.05	0.28 ± 0.05
P (ppm)	6.03 ± 0.92	15.27 ± 4.51	23.08 ± 2.08
Humidity (%)	4.3 ± 0.30	12.23 ± 0.38	15.08 ± 0.38
Clay (%)	33.2 ± 0.00	44.6 ± 0.00	42.7 ± 0.00

Silt (%)	12.7 ± 0.00	39.9 ± 0.00	29.4 ± 0.00
Sand (%)	54.1 ± 0.00	15.5 ± 0.00	27.9 ± 0.00

2.3. Pot experiment

The experiment was carried out under shelter at the National Institute of Agronomy of Tunisia (10° 11′ N, 36° 55′ E) in a completely randomized experimental design including eight treatments (with three replicates per treatment). Treatments consisted of three native AMF inoculants (AI1, AI2 and AI3), a commercial (*Glomus sp*.) inoculant (CI), and four controls (non-inoculated): control AI1, control AI2, control AI3 and control CI. A population-variety of hulless barley (*Hordeum vulgare* ssp *nudum* L.) named "Prophet barley" or "Moknine barley" was used in this experiment. Seeds were surface sterilized in 1% sodium hypochlorite and sown in sterile sand (twice autoclaved at 121°C for 30 minutes) in plastic cylinder pots (10×24 cm). For inoculated plants, plastic pots were added with the corresponding AMF inoculum (200 propagules/ pot). For non-inoculated plants (controls), the same quantity of sterilized inoculum was added. Plants were irrigated (at 75% pot capacity) twice a week with 20 ml Hoagland nutrient solution. Supplemental irrigation with distilled water was provided only on hot days. Plants were harvested after 5 months.

2.4. Determination of root mycorrhizal rates

Barley fresh roots were cleared in KOH (10%) and stained with trypan blue (0.05%) according to the method described by Phillips and Hayman (1970). The percentage of mycorrhizal colonization was determined with the method of McGonigle et al. (1990).

2.5. Measured parameters

Plant height was recorded and leaf chlorophyll content was measured using a portable SPAD meter (Minolta SPAD 502 m, Plainfield, IL, USA) starting from 20 days after sowing (DAS) (Markwell et al. 1995). After harvesting, plants were hand harvested and total biomass (g/pot) was determined. The total grain yield (g/pot) and thousand kernel weight (g) were also measured after threshing. The straw nitrogen concentration was obtained through the Kjeldahl method as described by Pauwels et al. (1992). P concentration was measured by the method of Olsen using spectrophotometer, K by the flame atomic absorption spectrometry, and Fe, Cu, Zn concentrations were determined with a flame photometer (Pauwels et al. 1992).

2.6. Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) and means comparison using LSD Fisher's test, at a significance level of $P < 0.05$. Statistical analyses were performed using RStudio v.3.6.0 software (R Core Team 2019).

3. Results

3.1. Mycorrhizal root colonization

AMF inoculation enhanced significantly mycorrhizal root colonization of inoculated hulless barley plants while non-inoculated were not colonized ($p < 0.001$, Table 3). Native inoculum AI3 showed significantly higher total root colonization rate than the other inoculants (Table 3). Moreover, plants inoculated with commercial *Glomus sp*. inoculum and with native AI1 recorded a greater percentages of total root colonization by 2.4 and 2.1-fold, respectively in comparison to those inoculated with native inoculum AI2. The same pattern was observed for arbuscular root colonization (Table 3). Compared to control, plants inoculated with AMF showed higher percentages of vesicles in their cell roots, whereas no significant differences were observed between the different mycorrhizal inoculants (Table 3).

Table 3. Effect of AMF inoculation on mycorrhizal root colonization of hulless barley

AMF treatment	Total root colonization (%)	Arbuscular colonization (%)	Vesicular colonization (%)
AI1	38.5^{b} ± 6.8	28.9^{c} ± 2.2	12.6^{a} ± 3.4
control AI1	0.0^{d} ± 0.0	0.0^{e} ± 0.0	0.0^{b} ± 0.0

AI2	18.5^{c} ± 3.4	14.1^{d} ± 3.4	9.6^{a}± 1.3
control AI2	0.0^{d} ± 0.0	0.0^{e} ± 0.0	0.0^{b} ± 0.0
AI3	53.3^{a} ± 2.2	43.7^{a} ± 3.4	14.1^{a} ± 5.6
control AI3	0.0^{d} ± 0.0	0.0^{e} ± 0.0	0.0^{b} ± 0.0
CI	43.7^{b} ± 3.4	37.0^{b} ± 1.3	14.1^{a} ± 5.6
control CI	0.0^{d} ± 0.0	0.0^{e} ± 0.0	0.0^{b} ± 0.0
ANOVA			
F values	167.3***	277.9***	14.96***

* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Different letters in the same row indicate significant differences according to the Fisher's LSD test ($p < 0.05$).

3.2. Plant height

Overall, plant height increased progressively during the growth stages to attain the highest level at maturity. Plant height of hulless barley was significantly affected by AMF inoculation ($p < 0.001$). The highest growth rates were observed in plants inoculated with AI3, CI and AI1 while plants without inoculation showed minor heights (Figure 1). Plants inoculated with AI3 were taller and reached a height of 63 cm at 137 DAS which is about 14.8% higher as compared to their respective control (control AI3). Also, AI1 and CI increased plant height of hulless barley about 11.6 and 12.4%, respectively, in comparison to their respective controls. However, AI2 inoculant recorded lower growth rate than other AMF treatments (Figure 1).

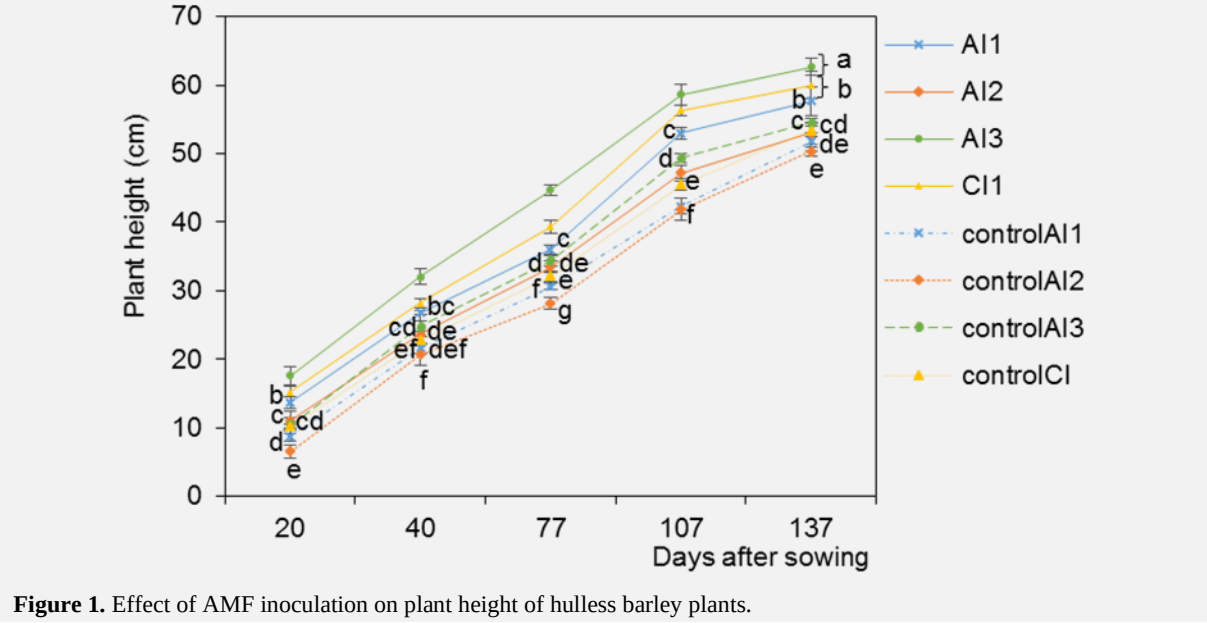

Figure 1. Effect of AMF inoculation on plant height of hulless barley plants.

3.3. Estimation of Leaf chlorophyll content

Leaf chlorophyll content estimated by SPAD increased linearly until reaching peak at 77 DAS and thereafter declined till maturity (Figure 2). Plant inoculated with AMF showed the highest leaf chlorophyll contents and maintained this trend until the end (107 DAS), compared to controls. Indeed, leaf chlorophyll contents were significantly greater in plants inoculated with AI3, CI, AI1 and AI2 (by approximately 42.4, 39.6, 28.9, and 23.4%, respectively) as compared to their respective controls (Figure 2). At 77 DAS, the greatest chlorophyll content was recorded at plants inoculated with AI3 and it was about 16.6, 31.3 and 4.6% higher than with AI1, AI2 and CI, respectively (Figure 2). The rate of decrease of leaf chlorophyll content after attaining maximum value was more rapid in control plants (between 6 and 6.7%) and also in those inoculated with AI2 (by 8.6%).

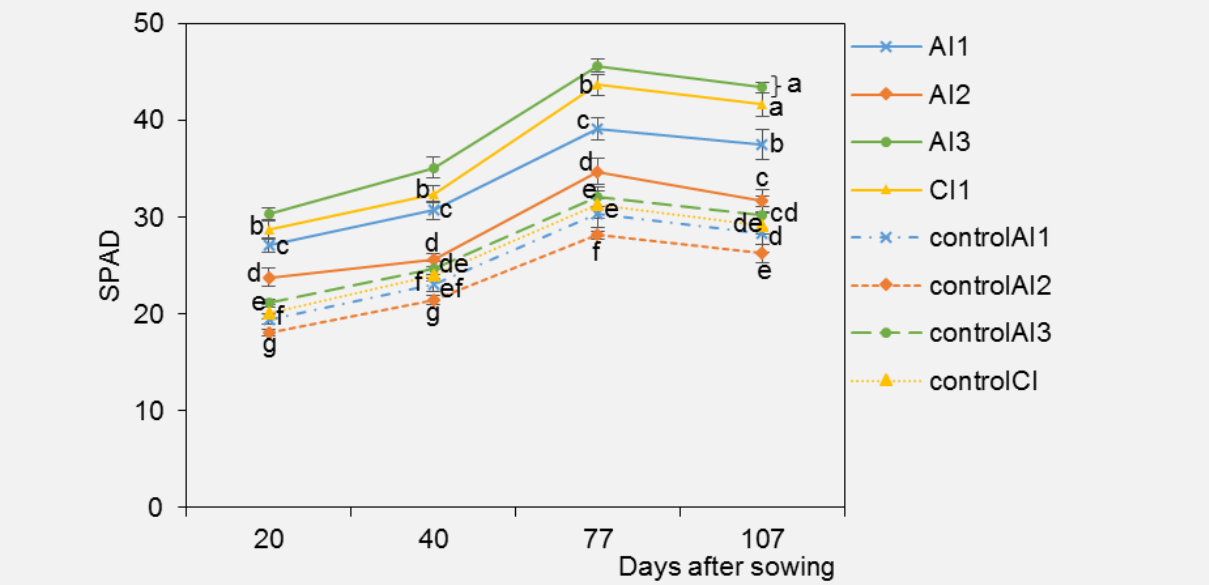

Figure 2. Effect of AMF inoculation on leaf chlorophyll content (SPAD) of hulless barley plants.

3.4. Total biomass, grain yield and thousand kernel of hulless barley

Regarding yield parameters, mycorrhizal inoculation, in particular with native inoculants (AI3 and AI1), as well as with commercial inoculant (CI), improved significantly total biomass, grain yield and thousand kernel weight of hulless barley (Figure. 3). In contrast, AI2 had the lowest values for all the parameters, and in some cases showed no significant differences compared to its respective control (Figure 3). Compared to controls, total biomass of barley was 1.6 and 1.5-fold greater in plants inoculated with AI3 and CI, respectively (Figure. 3a). In addition, the highest grain yield was recorded in plant inoculated with AI3 and it was about 1.8, 1.3, 2.3-fold higher as compared to AI1, CI and control, respectively (Figure. 3b). The same pattern was observed for thousand kernel weight (Figure. 3c). In fact, AI3 and CI recorded the greatest values of thousand kernel weight which were 29.9 and 26.8 g, respectively, followed by AI1 with 20.5g (Figure. 3c).

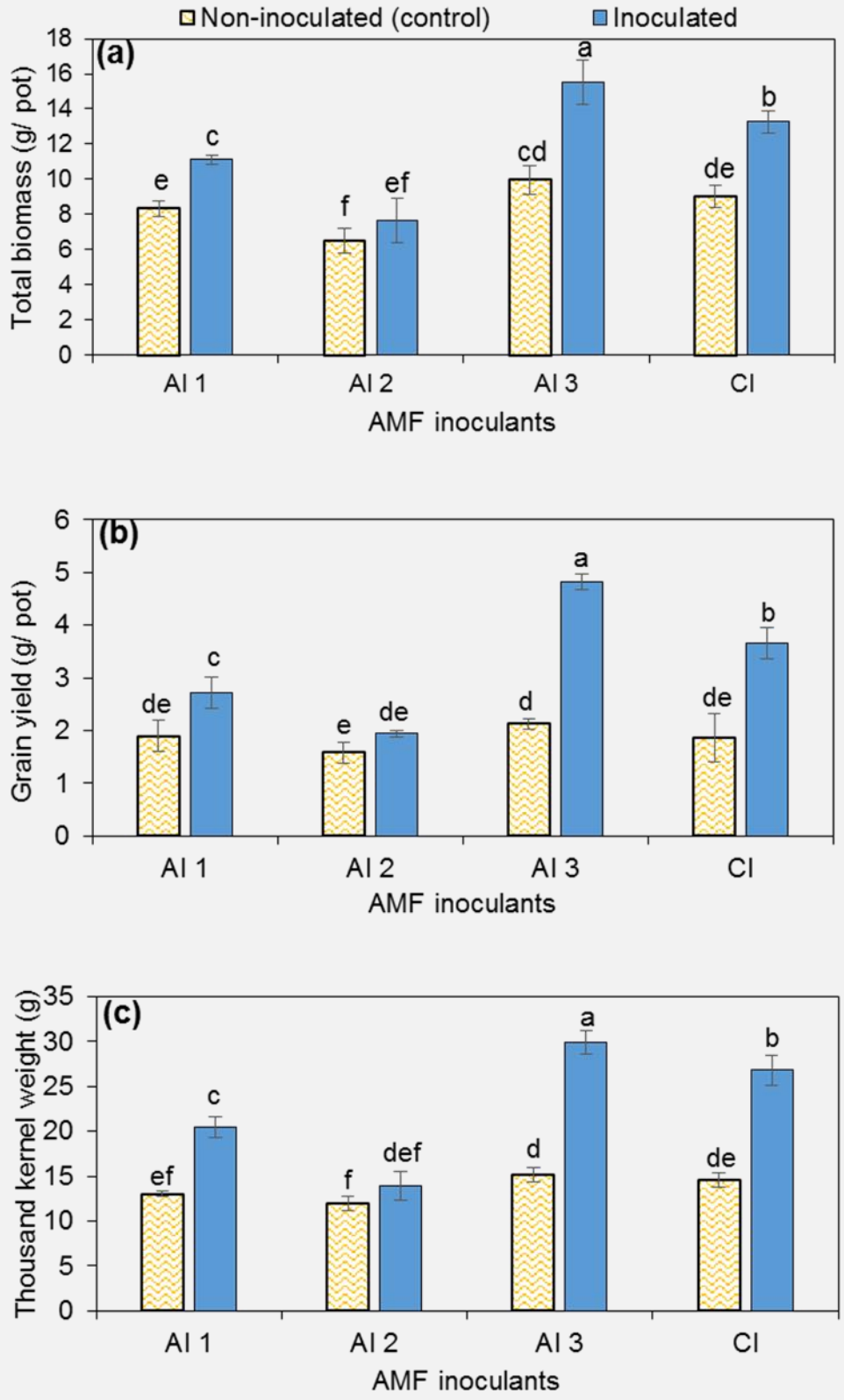

Figure 3. Effect of AMF inoculation on total biomass (a), grain yield (b) and thousand kernel weight (c) of hulless barley plants.

3.5. Mineral nutrition

AMF inoculation significantly influenced N, P, K, Cu, Fe and Zn concentrations in hulless barley plants ($p < 0.001$, Table 4). All mycorrhizal inoculants increased N contents as compared to controls, and there were no significant differences between the different AMF treatments (Table 4). Concerning P concentrations, the highest value was recorded in plants inoculated with CI and it was about (+130% greater) compared to its respective control (Table 4). However, only plants inoculated with AI2 showed

no significant differences in P content when compared to their respective controls (Table 4). In comparison to controls, K concentration was increased by 112, 162 and 123% with AI3, AI1 and CI, respectively (Table 4). Plant inoculated with AI1 showed the greatest Cu concentration and it was about 1.4-fold higher as compared to AI3 and CI. Also, Cu content was 2.5 and 2.9-fold higher in AI1 inoculated plants compared to those with AI2 and with controls, respectively (Table 4). Plants inoculated with AI3, AI1 and CI enhanced significantly Fe and Zn contents as compared to their respective controls. Compared to controls, AI3 inoculation enhanced Fe and Zn concentrations by 164 and 188%, respectively (Table 4).

Table 4. Effect of AMF inoculation on mineral content N, P, K, Cu, Fe and Zn of hulless barley straw.

AMF treatment	N (mg/ g DW)	P (mg/ g DW)	K (mg/ g DW)	Cu (µg/ g DW)	Fe (µg/ g DW)	Zn (µg/ g DW)
AI1	$1.56^{ab} \pm 0.18$	$0.54^{b} \pm 0.10$	$27.19^{a} \pm 3.27$	$14.77^{a} \pm 0.84$	$46.23^{c} \pm 1.40$	$28.98^{b} \pm 0.89$
control AI1	$1.39^{bc} \pm 0.08$	$0.17^{d} \pm 0.05$	$10.39^{c} \pm 1.37$	$5.10^{cd} \pm 0.70$	$23.70^{e} \pm 2.84$	$11.66^{e} \pm 0.79$
AI2	$1.59^{a} \pm 0.01$	$0.28^{cd} \pm 0.07$	$20.20^{b} \pm 2.77$	$5.93^{c} \pm 0.59$	$30.17^{d} \pm 3.67$	$25.26^{c} \pm 0.62$
control AI2	$1.23^{c} \pm 0.11$	$0.28^{cd} \pm 0.10$	$14.47^{c} \pm 0.73$	$4.03^{d} \pm 0.84$	$21.30^{e} \pm 3.10$	$10.54^{e} \pm 0.24$
AI3	$1.59^{a} \pm 0.14$	$0.50^{b} \pm 0.04$	$29.07^{a} \pm 1.57$	$10.53^{b} \pm 0.81$	$57.93^{a} \pm 3.26$	$34.82^{a} \pm 2.74$
control AI3	$1.35^{c} \pm 0.09$	$0.25^{cd} \pm 0.08$	$13.72^{c} \pm 2.05$	$4.90^{cd} \pm 0.95$	$21.97^{e} \pm 2.99$	$12.10^{de} \pm 0.91$
CI	$1.69^{a} \pm 0.06$	$0.74^{a} \pm 0.07$	$27.17^{a} \pm 3.63$	$10.37^{b} \pm 0.76$	$52.70^{b} \pm 2.98$	$28.34^{b} \pm 0.53$
control CI	$1.27^{c} \pm 0.10$	$0.32^{c} \pm 0.05$	$12.19^{c} \pm 3.29$	$2.47^{e} \pm 0.75$	$12.70^{f} \pm 2.52$	$14.01^{d} \pm 0.40$
ANOVA						
F values	7.469***	20.79***	26.97***	84.4***	98.51***	209.6***

* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.
Different letters in the same row indicate significant differences according to the Fisher's LSD test ($p < 0.05$).

4. Discussion

Hulless barley (*Hordeum vulgare* ssp *nudum* L.) has attracted increasing attention in the world as a feed and healthy human food. AMF have been extensively studied over the last decades and are associated with growth and nutrient enhancement of host plant (Smith and Smith 2011). In this study, we used three native AMF inoculants AI1 (*Funneliformis mosseae*, *Funneliformis geosporum* and *Scutellospora calospora*), AI2 (*Funneliformis mosseae* and *Funneliformis geosporum*) and AI3 (*Pacispora franciscana*, *Funneliformis mosseae*, *Funneliformis geosporum*, *Rhizophagus irregulare* and *Glomus tenebrosum*), and a commercial inoculum (*Glomus* sp.) to evaluate their capacity to improve crop productivity of hulless barley.

During this study, AMF inoculation improved mycorrhizal root colonization of hulless barley while the non-inoculated plants were not colonized. Our data showed that colonization rates ranging from 18.5 to 53.3%, varied significantly among AMF inoculants. It has been demonstrated by Grace et al. (2009) that barley plants were better colonized by *G. intraradices* (72%) and poorly by *G. geosporum* (19%). According to Graham and Abbott (2000), the difference in root colonization could be related to the AMF species. Our results revealed that AI3 allowed higher root colonization rate than other inoculants including the commercial one (CI). This result suggest that native AMF species had greater compatibility with hulless barley than commercial *Glomus* sp. These findings agree with those of Labidi et al. (2015), who found a higher root colonization rate in plants inoculated with native AMF species as compared to commercial *R. irregularis*. It has been well documented that native AMF had higher efficiency as compared to commercial inoculants (Berruti et al. 2016). This is could be related to the fact that native AMF species were more adapted to the local conditions in Mediterranean area (Querejeta et al. 2006).

Enhancement of plant growth by AMF inoculation has been reported for many plant species (Symanczik et al. 2018). In the present study, inoculation with a mixture of native AMF species increased significantly plant height of hulless barley compared with the control (non-inoculated). These results were in line with Quiñones-Aguilar et al. (2016) who found that plant growth was significantly enhanced in plants inoculated with native AMF consortia. Moreover, inoculation with native AMF species was found to increase plant biomass as well as grain yield and thousand kernel of hulless barley, which is in

agreement with previous study (Al-Karaki et al. 2004). The promotion of hulless barley biomass and yield is attributed to the capability of AMF to boost plant nutrients uptake (Smith and Smith 2011). The positive effect of AMF on the acquisition of mineral nutrients has been well documented (Clark and Zeto 2000), for P and N which are greatly increased after inoculation (Smith and Smith 2011). Similarly to previous research (Al-Karaki and Clark 1998), our results showed that inoculation with native and commercial inoculants improved P and N contents in barley straw as compared to non-inoculated plants. Increased N concentration in AMF inoculated plants was significantly related to higher chlorophyll contents, since chlorophyll molecules can trap N effectively (Peterson et al. 1993). Similarly, Zhu et al. (2003) had reported that colonisation by *G. intraradices* enhanced P concentrations in barley tissue. Also, Begum et al. (2019) had demonstrated that inoculation with *G. versiforme* increased significantly N and K content in maize. Increased K concentration may lead to an enhancement of the photosynthetic activity of barley (Ahanger and Agarwal 2017). On the other hand, inoculation with AMF (*R. irregularis*) improved the accumulation of micro-nutrient contents in host-plant (Gashgari et al. 2020). Our results showed that AMF inoculation significantly increased Zn concentration in hulless barley straw. It has been already demonstrated that Zn concentration in wheat straw was significantly enhanced by *R. intraradices* inoculation (Ma et al. 2019). This may be related to the ability of AMF to increase the translocation of Zn from roots to shoots (Kothari et al. 1991). Concerning iron and copper, and similarly to our findings, Lehmann and Rillig (2015) have demonstrated that AMF had a positive effect on Cu and Fe in different plant tissues. Overall, native inoculum (AI3) containing five AMF species performed better than commercial inoculum which could be linked to the well compatibility between these AMF species and hulless barley (Pellegrino et al. 2020).

5. Conclusion

In the present study, AMF inoculation enhanced the root colonization rate of hulless barley compared to the non-inoculated plants. Moreover, our results revealed that plant growth parameters were significantly higher in AMF inoculated plants. Indeed, inoculation with AI3 containing five native AMF species (*Pacispora franciscana*, *Funneliformis mosseae*, *Funneliformis geosporum*, *Rhizophagus irregulare* and *Glomus tenebrosum*) has the best effect on hulless barley, as illustrated by a higher plant growth, nutrient content, total biomass and grain yield. Further research is needed to understand the effectiveness of these native AMF species in field inoculation and under different environmental conditions. The application of native AMF species as biofertilizers could have great benefit for sustainable agriculture in Tunisian ecosystem.

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