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REGULAR ARTICLE

Strong increase of durum wheat iron and zinc content by field-inoculation with arbuscular mycorrhizal fungi at different soil nitrogen availabilities

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Abstract
Background and aims Since actual production of wheat often leads to human Fe and Zn deficiency, a better understanding of the potential of arbuscular mycorrhizal fungal (AMF) inoculation for micro-nutrient uptake of durum wheat is needed.
Methods Effects of AMF field inoculation and N availability were evaluated on an old and a modern durum wheat variety
Results Following AMF inoculation, the modern variety showed a higher increase of the early root colonization respect to the old one, whereas at maturity root colonization was decreased by N fertilization. In the old variety grain N concentration was increased by inoculation when plants were not fertilized and at the 40–0-40 N, whereas in the modern variety inoculation did not change N concentration. By contrast, in AMF inoculated plots the modern variety showed a higher increase of Fe and Zn in grain compared to the old variety. Accordingly, at harvest, the modern variety showed an higher increase of a molecular operational taxonomic unit affiliated to *Rhizophagus* compared to the old variety.
Conclusion The inoculated isolate is a good durum wheat colonizer and the modern variety showed higher responsiveness to inoculation in terms of N, Fe and Zn grain concentration respect to the old one.

Keywords Arbuscular mycorrhizal fungal (AMF) root colonization · Durum wheat · Grain yield · Micronutrient uptake · N fertilization · *Rhizophagus irregularis*

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Introduction

Population growth and increasing consumption of calorie- and meat-intensive diets are expected to double human food demand by 2050 (Tilman et al. 2011). For most major field crops, such as wheat, rice and maize, actual yield appears to be at 50–80% of yield potential, due to growth-limiting factors, i.e. water and nutrient availability (Mueller et al. 2012). Moreover, nutritional and nutraceutical quality of food is becoming increasingly important for agricultural production. For example, in many developed and developing countries, mineral deficiencies in iron (Fe) and zinc (Zn) are common and over 60% and 30% of the world population are Fe and Zn deficient, respectively (White and Broadley 2009).

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Arbuscular mycorrhizas (AM) are symbiotic associations established between soilborne fungi (*Glomeromycota*) and the roots of most terrestrial plant species, including most food crops (Smith and Read 2008). AM fungi (AMF) supply mineral nutrients to the plants in exchange for photosynthetically fixed carbohydrates (Bago et al. 2000). Beneficial effects of AMF may include, beside improved plant growth and yield, host plants' increased resistance to biotic (pathogens) and abiotic (drought, salinity, heavy metals) stresses and increased soil quality by improving soil aggregation and C sequestration (Augé 2001; Newsham et al. 1995; Rillig and Mummey 2006; Pellegrino et al. 2015a; Ciccolini et al. 2015). Therefore, AMF inoculants have been proposed as biofertilizers and biofortifiers in order to reduce yield constraints in sustainable agricultural systems (Fitter et al. 2011; Gianinazzi et al. 2010; He and Nara 2007).

Recently, the usefulness of AMF as biofortifier agents for the alleviation of nutrient deficiency in humans was evaluated across a range of crop species grown under varying conditions (Lehmann et al. 2014; Lehmann and Rillig 2015). The authors quantitatively analysed for Cu, Mn, Fe and Zn showing that AMF inoculation had a positive effect on Cu, Fe and Zn content in all tissue types, whereas the effect on Mn was limited. The positive effect of AMF on plant Cu, Fe and Zn uptake was modulated by soil texture and soil nutrient concentration, while Fe and Zn uptake was additionally affected by soil pH. In agricultural soils, the contribution of AMF to micronutrient nutrition of crops was also affected by agronomic practices, such as N and P fertilization (Hodge and Storer 2015; Lehmann and Rillig 2015).

A recent meta-analysis studying the responses of wheat (*Triticum* spp.) to AMF field inoculation indicated an increased grain yield, N and P (> 20%) and Zn (> 10%) content and a positive and strong correlation between AMF root colonization rate and grain yield, P and Zn concentration (Pellegrino et al. 2015b). However, these results were mostly obtained from trials on bread wheat in India, North America, China and Australia, but AMF field inoculation of durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.) was previously studied in a unique trial in India only (Behera and Rautaray 2010). On this background, there is a demand to study how AMF field inoculation impacts the nutrient uptake of durum wheat in major cultivation and production areas, such as the Mediterranean basin, where it is extensively and traditionally used to produce pasta (Ercoli et al. 2011, 2012).

In the Mediterranean basin, a huge genetic variability exists among durum wheat varieties, resulting in morphological and productive differences and distinct adaptability (Oliveira et al. 2012). Yield gains were attained during the last century through the modification of plant growth and assimilate partitioning among plant organs (Álvaro et al. 2008; Royo et al. 2007). Modern cultivars have a shorter growth-period from sowing to anthesis, a greater harvest index, more grains per unit area, but a lower number of health-promoting compounds (Dinelli et al. 2009, 2011) when compared to old cultivars, released before 1950. Early studies by Hetrick et al. (1992, 1993) demonstrated that old cultivars had stronger mycorrhizal dependency than modern cultivars, perhaps reflecting the fact that breeding was performed under high nutrient availability. By contrast, Singh et al. (2012) and Ellouze et al. (2015) did not confirm a higher mycorrhizal dependency of old cultivars, despite demonstrating a strong variability in the responsiveness among durum wheat genotypes, Thus, AMF inoculation effects on wheat must be understood in the context not only of nutrient availability and fertilization regimes, but also of the variety used, with potential strong difference in-between old and modern varieties.

The aim of this research was to evaluate the effects of field inoculation with *Rhizophagus irregularis* on an old and a modern durum wheat variety, with distinct assimilate distribution and putatively varying mycorrhizal dependency, grown at varying N fertilization regimes. The inoculation success was assessed by studying plant growth and yield, plant nutrient uptake during the growth cycle, AMF root colonization and molecular AMF diversity within roots.

Material and methods

Experimental field site

The experiment was conducted in 2013–2014 at the "Enrico Avanzi" Centre of Agro-Environmental Research of the University of Pisa (43° 41′ N, 10° 20′ E, 1 m above sea level, 5 km distance from sea and 0% slope), Italy. The soil was an alluvial loam, classified as *Typic Xerofluvent* according to the USDA soil taxonomy (Soil Survey Staff 1975) and as *Fluvisol* by FAO (IUSS

2006). Soil physical and chemical properties were: 39.4% sand (2 mm > ∅ > 0.02 mm); 40.5% silt (0.02 mm > ∅ > 0.002 mm); 20.1% clay (∅ < 0.002 mm); 8.4 pH (H_2O); 10.4 g kg^{-1} organic carbon (Walkley-Black); 1.5 g kg^{-1} total nitrogen (Kjeldahl method); 24.7 mg kg^{-1} available phosphorus (Olsen); 19.3 mg kg^{-1} DTPA-extractable Fe (Lindsay and Norvell 1978); 0.64 mg kg^{-1} DTPA-extractable Zn (Lindsay and Norvell 1978). Climate of the site is cold, humid Mediterranean (Csa) according to the Köppen–Geiger climate classification (Kottek et al. 2006). The 10-year averages (2004–2013) of mean annual maximum and minimum daily air temperatures are 20.6 and 8.6 °C, respectively, and annual precipitation is 940 mm (Vallebona et al. 2015). Over the last 10 years, during the period of the wheat cropping cycle (November–June) mean annual maximum and minimum air temperatures were 17.4 and 6.1 °C, with 724 mm annual rainfall. Before experimental set-up, the field site was conventionally cultivated with a sunflower (*Helianthus annuus* L.) - wheat (*Triticum* spp.) rotation. During durum wheat cropping cycle (November 2013–June 2014), mean maximum and minimum temperatures were 18.4 and 7.0 °C, respectively, while total precipitation was 881.2 mm and had a peak of 236 mm during the 2nd decade of January (Supplementary Fig. S1).

Experimental set-up and crop management

A full factorial experimental design with two durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.) varieties, five N fertilizer regimes and two AMF inoculation treatments with three replicates was arranged in a completely randomized design. The durum wheat varieties were Cappelli and Svevo. Cappelli (synonym of Senatore Cappelli) is an old tall variety derived from a North-African landrace (Jean Retifah), released in Italy in 1915 (D'Amato 1989). Svevo is a modern semi-dwarf variety that originated from a cross between a selection of the International Maize and Wheat Improvement Center (CIMMYT) and Zenith sib; it was released in Italy in 1996 and is widely used in local production (Dinelli et al. 2013). Cappelli was selected because it is considered the progenitor of many varieties of durum wheat (De Vita et al. 2007), while Svevo was selected because it represents one of the most cultivated modern varieties in Italy since its release (Arduini et al. 2014). However, no information about AMF colonization or responsiveness was available on these varieties. The N fertilizer regimes were split applications of N among pre-seeding, at 5th leaf unfolded stage (GS15) and at pseudo stem erection (GS30) as follows: 0–0-0, 0–40–40, 40–0-40, 40–40–0 and 40–40–40 kg N ha^{-1}. Growth stages were identified following the scale of Zadoks et al. (1974). Nitrogen fertilizer was applied as urea at all three crop growth stages (Supplementary Table S1). AMF inoculation treatment (+M) was compared to a mock inoculum (−M). AMF inoculation was performed using *Rhizophagus irregulari*s C. Walker and Schüßler (Schüßler and Walker 2010) (synonym *Glomus irregulare* Błaszk., Wubet, Renker& Buscot; earlier often named as *Glomus intraradices*). The inoculum was a dried spore-based inoculum (SYMPLANTA-001 research grade, Symplanta GmbH & Co. KG, Germany) with this isolate derived from the same culture line as DAOM197198 (Stockinger et al. 2009). *Rhizophagus irregulari*s spores and the carrier, a water-insoluble dry powder of calcined attapulgite clay, were distributed and incorporated into soil by harrowing at 5 cm depth, immediately before sowing. The rate of the application of the AMF inoculum was 9.3 g m^{-2}, corresponding to 8333 spores m^{-2}. Mock inoculation consisted of the distribution of calcined attapulgite clay powder at the same rate and timing as the AMF inoculation treatment.

Durum wheat was cultivated following the management techniques normally applied in the area. Soil tillage was performed in autumn before sowing through mouldboard ploughing (35 cm depth), disking (15 cm depth), and harrowing (20 cm depth). Phosphorus was applied pre-planting as mineral triple phosphate at a rate of 22 kg P ha^{-1}. Durum wheat was sown on November 4th 2013 (Supplementary Table S1), within the optimum planting time for wheat production in Central Italy. A rate of 400 viable seeds per m^2, in rows spaced 15 cm apart, was applied. Weed control was performed with a pre-emergence application of trifluralin, while no insecticide or fungicide was applied.

Sampling and morphological analyses

For all treatments, timing of growth stages such as 2 leaves unfolded (GS12), 5 leaves unfolded (GS15), pseudo stem erection (GS30), 1st node detectable (GS31), flag leaf ligule/collar just visible (GS39), and physiological maturity (GS90) were recorded

(Supplementary Table S1). At GS15 the number of plants per unit area was determined.

At stage GS12, three plants, randomly selected in each replicate plot, were excavated with their whole root system, while at GS31 and GS90 three random turfs were extracted (20 cm-depth) from each replicate plot and then combined. In the laboratory, roots were separated from soil by gently washing with tap water and oven dried at 65 °C up to constant weight for root measurements. At GS12, shoot and root dry weight and total root length were assessed. At GS12, GS31 and GS90 AMF root colonization rates were measured under a stereomicroscope (Olympus SZX 9, Olympus Optics, Tokyo, Japan) by the grid-line intersect method (McGonigle et al. 1990), after root clearing and staining, using lactic acid instead of phenol (Phillips and Hayman 1970). Sub-samples of roots of the modern and the old wheat variety from AMF inoculated and not inoculated plots under unfertilized conditions (N 0–0-0) collected at GS90 were stored at −80 °C for molecular analysis.

At GS31 and GS90, plants from a 1-m^2 area for each replicate plot were manually cut at ground level. At GS31 shoot dry weight was determined, while at GS90 plants were partitioned into straw, chaff and grain and number of spikes and number of fertile and total spikelets per spike were assessed. For dry weight determination, samples from all plant parts were oven dried at 65 °C up to constant weight. Mean kernel dry weight was also measured and number of kernels per unit area and harvest index (HI) was calculated. Spike fertility index was calculated following the method of Abbate et al. (2013), as the quotient between grain number m^{-2} and spike chaff dry weight m^{-2} at GS90.

At GS12 and GS31, Fe and Zn concentration in shoot and root were determined by atomic absorption spectrometry (Isaac et al. 1998), while at GS90 Fe and Zn concentration was assessed in grain. Grain was also analysed for N and P concentration by the Kjeldahl method and by the ammonium-molybdophosphoric blue colour method, respectively (Jones et al. 1991).

Molecular analysis

Genomic DNA from the sub-samples of roots collected at GS90 (n = 12) was extracted from 100-mg fresh-weight root samples, using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). DNA quality was checked on a ND-1000 spectrophotometer (NanoDrop Technology, Wilmington, DE). PCR amplification was performed using the primer pair AML1 and AML2 targeting a small subunit ribosomal RNA (SSU rRNA) gene fragment (Lee et al. 2008). The AML1/AML2 fragment (ca. 800 bp) was chosen for broad comparability with results on AMF communities obtained by applying AMF-specific primers targeting the SSU rRNA gene (Pellegrino et al. 2012; Kohout et al. 2014) although the method utilised reliably discriminates AMF diversity only at genus level (Krüger et al. 2012). AML1/AML2 PCR amplicons were generated in volumes of 20 μl with 0.5 U of HotStarTaq DNA Polymerase (Qiagen), 0.2 μM of each primer (AML1/AML2), 0.2 mM of each dNTP, 2 mM of $MgCl_2$ and 1× reaction buffer, using a S1000 Thermal CyclerTM (BIORAD, USA). The thermal cycler was programmed as follows: 95 °C for 2 min, 30 cycles at 95 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 10 min. Reaction yields were estimated by using a 1% agarose gel containing ethidium bromide (0.5 μg ml^{-1}). The QIAquick (Qiagen) purified PCR amplicons of DNA were ligated into the pGem®-T Easy vector (Promega) and transformed by XL10-Gold® Ultracompetent *Escherichia coli* cells (Stratagene, La Jolla, CA, USA). The structure and composition of the AM fungal communities were determined by sequencing the plasmids of 30 positive clones (ca. 800 bp) per clone library (a total of 12 libraries, one per each root replicate) using SP6 vector primers. Plasmids of clones (360) were purified by Wizard® Plus SV Minipreps (Promega) and sequenced using the BigDye® version 3.1 chemistry on a 3730XL Genetic Analyser automated sequencer (Applied Biosystems, Foster City, CA, USA) at the High-Throughput Genomics Unit in Seattle (WA, USA).

Statistics and data analyses

A reference alignment (open-access at https://sites.google.com/site/restomedpeatland/microbiology) and a reference tree (Supplementary Fig. S2) were computed based on sequences of morphologically characterized and described AMF species as listed in the Schüßler and Walker (2010) phylotaxonomic classification. The AMF origin of the newly generated sequences was determined using the Basic Local Alignment Search Tool (BLAST) search (Altschul et al. 1997) at NCBI and the detection of chimeric sequences was performed using USEARCH 6.0 (http://fungene.cme.msu.edu/FunGenePipeline/chimera_check/form.spr). The new sequences were aligned with

those of the reference alignment and with the closest matches from GenBank (open-access at https://sites.google.com/site/restomedpeatland/microbiology). Sequence alignments were performed using MUSCLE as implemented in MEGA7 (Kumar et al. 2016; http://www.megasoftware.net). *Corallochytrium limacisporum* sequence L42528 was used as outgroup. Phylogenetic trees were inferred by Neighbour-Joining (NJ) analysis (Saitou and Nei 1987) using MEGA7 and the Kimura 2-parameter model (Kimura 1980). Branch support bootstrap values are derived from 1000 bootstrap replicates. The phylograms were drawn by MEGA7 and edited by Adobe Illustrator CC 2017. The newly generated AMF sequences were assigned to molecular operational taxonomic units (MOTUs) on the basis of phylogenetic placement with a bootstrap value of 75 in the phylogram. AMF MOTU richness was calculated using Primer v6 (Clarke and Gorley 2006, http://www.primer-e.com).

All newly-generated sequences were submitted to the EMBL nucleotide sequence database (http://www.ebi.ac.uk/embl/).

Morphological data were analysed by three-way ANOVA, using variety (Var), N fertilization (N fert) and AMF inoculation (AMF inoc) as fixed factors. Data were ln- and arcsine-transformed when needed to fulfil the assumptions of the ANOVA, which was carried out according to the completely randomized design. Post-hoc Tukey-B significant difference test was used for comparison among treatments. Means and standard errors given in tables and figures are for untransformed data. Molecular data were analysed by non-parametric Kruskall-Wallis H test followed by the Mann-Whitney *U*-test as post-hoc, since the assumption of the ANOVA were not fulfilled even after the appropriate transformation. All the analyses were performed using the SPSS software package version 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Root growth and AMF root colonization

At the beginning of tillering (2 leaves unfolded, GS12; after the pre-seeding N fertilization), root dry weight was significantly affected by N fertilization (N fert), whereas total root length and AMF root colonization were significantly affected by the interaction Var x AMF inoc (Table 1). Root dry weight increased 1.5-fold following N fertilization and no statistically significant difference was detected among N regimes (Supplementary Table S2). While old and modern varieties (Cappelli and Svevo, respectively) showed similar total root length when not inoculated (−M), AMF inoculation increased the root length of the old variety by more than 80%, but not of the modern variety (Fig. 1a; Supplementary Table S2). AMF root colonization of the non-inoculated plants was higher in the modern (14.6%) than in the old variety (10.3%). Following inoculation, AMF root colonization increased by 70% (from 14.6% to 24.8%) for the modern variety and by 47% (from 10.3% to 15.1%) for the old variety (Fig. 1b; Supplementary Table S2). The total colonized root length (total root length x percentage AMF root colonization; data not shown) was significantly affected by AMF inoculation ($P < 0.001$), where it approximately doubled (+M: 9.5 ± 0.55 cm per plant; +M: 4.5 ± 0.55 cm per plant).

At the beginning of stem elongation (1st node detectable, GS31), AMF root colonization was significantly affected by the interaction Var x N fert x AMF inoc (Table 1). For both varieties, AMF root colonization increased with AMF inoculation at all N fertilizer regimes, with the exception of the highest rate, 40–40–40 kg N ha^{-1}, where root colonization did not change for the modern variety and slightly decreased for the old one (Fig. 2a; Supplementary Table S2).

At physiological maturity (GS90), AMF root colonization was significantly affected by N fert (Table 1). The highest colonization occurred without N fertilization (0–0-0 kg N ha^{-1}) or when N was distributed only topdressing (0–40–40 kg N ha^{-1}), while the lowest AMF root colonization occurred at the highest N fertilizer rate (40–40–40 kg N ha^{-1}) (Fig. 3a; Supplementary Table S2).

Arbuscular mycorrhizal fungal community diversity

The PCR amplicons (ca. 800 bp) obtained with the primer pair AML1/AML2 were used to construct clone libraries from 12 crude DNA extracts, obtained from roots of the AMF inoculated and non-inoculated modern and old wheat varieties under unfertilized conditions, sampled at GS90. About 90% of the clones contained the expected fragment length, which was sequenced from 30 positive clones per clone library (360 clones in total). The obtained sequences grouped into ten AMF MOTUs that were phylogenetically affiliated to two *Acaulospora* clades (MOTU Ac1PI and Ac2PI), one *Diversispora* (Di1PI), two *Scutellospora* (Scu1PI, Scu2PI), two *Funneliformis*

Table 1 *P*-values of three-way ANOVA to evaluate the effect of variety (Var), nitrogen fertilization (N fert) and arbuscular mycorrhizal fungal (AMF) inoculation (AMF inoc) on total root length and root dry weight at 2nd leaf unfolded stage (GS12), shoot dry weight at 1st node detectable (GS31), AMF root colonization at GS12, GS31, and physiological maturity (GS90) and on grain concentration of nutrients of durum wheat

Source of variation*	Total root length at GS12	AMF root colonization at GS12	Root dry weight at GS12	AMF root colonization at GS31	Shoot dry weight at GS31	AMF root colonization at GS90	N concentration	P concentration	Fe concentration	Zn concentration
Var*	**< 0.001**†	**< 0.001**	0.111	**< 0.001**	**< 0.001**	0.479	0.246	0.340	**0.004**	**0.007**
N fert*	0.359	0.115	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.026**	0.604	**0.002**
AMF inoc*	**< 0.001**	**< 0.001**	0.209	**< 0.001**	0.135	0.465	**0.018**	0.993	**< 0.001**	**< 0.001**
Var x N fert	0.597	0.624	0.257	**< 0.001**	0.051	0.840	**0.005**	0.146	0.720	**0.039**
N fert x AMF inoc	0.502	0.115	0.443	**< 0.001**	0.372	0.762	**< 0.001**	**0.034**	0.891	0.308
Var x AMF inoc	**< 0.001**	**0.001**	0.088	0.295	0.369	0.984	0.385	**0.002**	**0.016**	**0.003**
Var x N fert x AMF inoc	0.443	0.076	0.638	**0.002**	0.939	0.191	**0.011**	0.833	0.885	0.706

*Var, N fert and AMF inoc used as fixed factors; Var: Cappelli and Svevo are the old and the modern variety, respectively; N fert: 0–0-0, 0–40–40, 40–0-40, 40–40–0, 40–40–40 kg N ha^{-1} at pre-seeding, 5th leaf unfolded stage and pseudo stem erection, respectively; AMF inoc: *Rhizophagus irregularis* DAOM197198

† In bold statistically significant values ($P \leq 0.05$). Replicates field plots were three per treatment

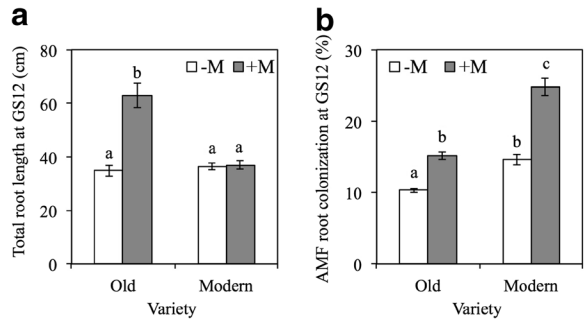

Fig. 1 Total root length (**a**) and arbuscular mycorrhizal fungal (AMF) root colonization (**b**) of an old (Cappelli) and a modern (Svevo) durum wheat variety inoculated with *Rhizophagus irregularis* (+M) and mock inoculated (-M). Plants were sampled at 2 leaf unfolded stage (GS12). Significant interactions variety (Var) x AMF inoculation (AMF inoc) are shown. Data are mean ± standard error (n=15) and are averaged over five nitrogen fertilization regimes

(Fu1PI, Fu2PI), two *Rhizophagus* (Rh1PI, Rh2PI) and one uncultured *Glomeromycota* (Glo1PI) clades (Supplementary Fig. S3). All ten MOTUs were retrieved from both, the AMF inoculated and the uninoculated modern and old varieties. The relative abundance of seven of them (Ac1PI, Ac2PI, Di1PI, Scu1PI, Scu2PI, Rh2PI, and Glo1PI) did not change among treatments (Table S4), but the relative abundance of Rh1PI, Fu1PI and Fu2PI was significantly affected by the interaction variety x AMF inoculation ($P < 0.001$). In uninoculated plants, the relative abundance of the *Rhizophagus* MOTU Rh1PI was ca. 4% and did not differ between varieties, whereas following AMF inoculation, the relative abundance of Rh1PI increased to 50% and 65% in the roots of the old and modern variety, respectively (Fig. 4a; Supplementary Table S3). The increase of Rh1PI following AMF inoculation was associated with significant decreases of the MOTUs Fu1PI and Fu2PI affiliated to *Funneliformis* ($P_{\text{AMF inoc}} < 0.001$) (Fig. 4b, c), which both decreased by 68%, averaged over the wheat varieties.

Plant growth, grain yield and yield components

At GS12, shoot dry weight did not vary between wheat varieties and was not affected by N fertilization and AMF inoculation (data not shown). At GS31, shoot dry weight varied between varieties ($P < 0.001$) and was affected by N fertilization ($P < 0.001$) (Table 1). It was 79% higher in the old variety when compared with the modern one and on average 90% higher in N-fertilized treatments than in the unfertilized control (Supplementary Table S4).

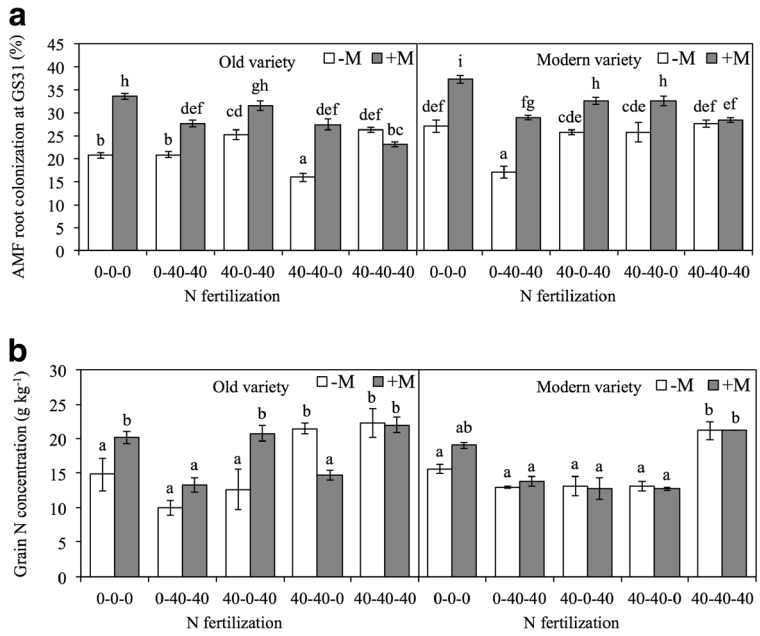

Fig. 2 Arbuscular mycorrhizal fungal (AMF) root colonization at 1 node detectable (GS31) (a) and grain nitrogen (N) concentration (b) at physiological maturity (GS90) of an old (Cappelli) and a modern (Svevo) durum wheat variety inoculated with *Rhizophagus irregularis* (+M) and mock inoculated (-M) and fertilized with five nitrogen (N) fertilization regimes (kg ha-1) at three crop growth stages. Significant interactions variety (Var) x N fertilization (N fert) x AMF inoculation (AMF inoc) are shown. Data are mean ± standard error (n=3)

Grain yield was significantly affected by N fert (Table 2). Grain yield was similar among all N fertilizer regimes but increased by an average of 74% when compared with the unfertilized control (Fig. 3b; Supplementary Table S4). Differences were due to significant variations in the number of kernels per spike, which increased when compared with the unfertilized control by 62%, 95%, 120%, 105% with 0–40–40, 40–0-40, 40–40–0, and 40–40–40 kg N ha^{-1}, respectively (Table 3; Supplementary Table S3). Significant differences between varieties were recorded in yield components (Table 3). The highest number of spikes per unit area was revealed in the modern variety (+33%), whereas the highest number of kernels per spike and mean kernel weight were recorded in the old variety (+16% and +19%, respectively) (Table 3; Supplementary Table S4).

Straw dry weight and Harvest Index (HI) varied between varieties, due to the different pattern of plant growth and assimilate distribution among plant organs (Table 2). Compared to the modern variety, the old variety had higher biomass allocation in straw (983.2 g m^{-2} t ha^{-1} vs 511.5 t ha^{-1}) (Table 3) and consequently lower HI (28.8% vs 40.5%) (Table 3; Supplementary Table S4). Like grain yield, straw dry weight was significantly increased by N fert (Table 2).

The number of fertile spikelets per spike varied between varieties and among N fertilization treatments (Table 2). It was 20% higher in the old variety compared

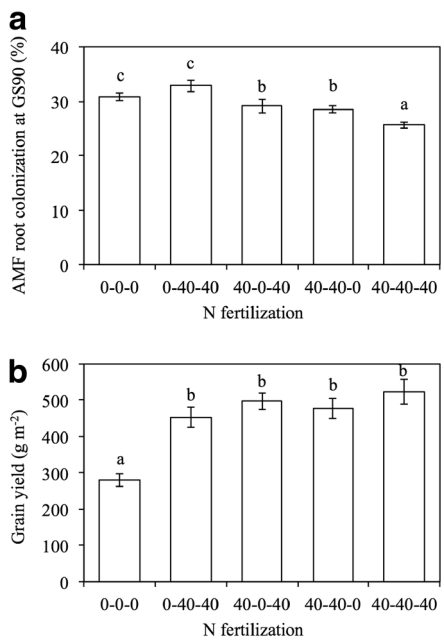

Fig. 3 Arbuscular mycorrhizal fungal (AMF) root colonization at physiological maturity (GS90) (a), grain yield (b) at physiological maturity (GS90) of durum wheat fertilized with five nitrogen (N) fertilization regimes (kg ha-1) at three crop growth stages. Significant N fertilization (N fert) mean effects are shown. Data are mean ± standard error (n=12) and are averaged over an old (Cappelli) and a modern (Svevo) durum wheat variety and both AMF inoculation treatments (inoculated with Rhizophagus irregularis; mock inoculated)

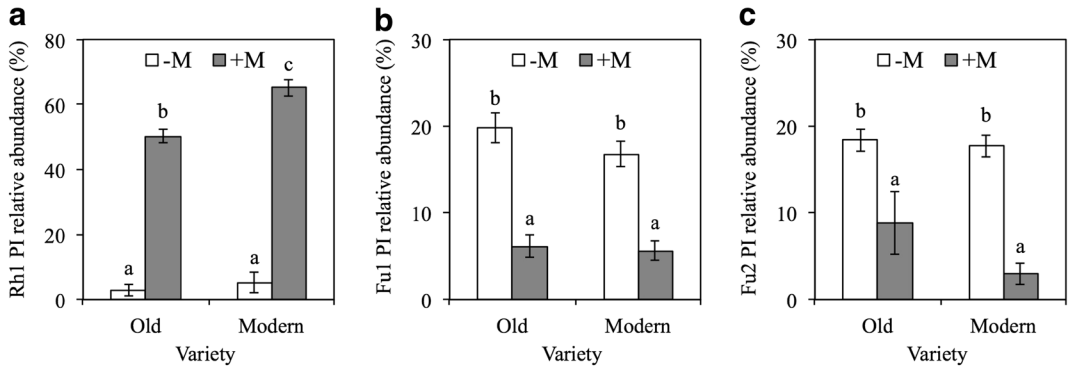

Fig. 4 Relative abundance (%) of molecular operational taxonomic units (MOTU) Rh1PI (a), Fu1PI (b) and Fu2PI (c) within the arbuscular mycorrhizal fungi (AMF) community in the roots of old and modern durum wheat varieties at physiological maturity (GS90) inoculated with *Rhizophagus irregularis* (+M) and mock inoculated (-M). Only significant changes of MOTUs due to AMF field inoculation and wheat variety are shown. Data are mean ± standard error (n=12)

to the modern one and increased with the increase of N fertilizer rate (Table 3; Supplementary Table S4). The spike fertility index varied between varieties (Table 2), being 44% higher in the modern variety compared with the old one (69.9 vs 48.6 number g^{-1}; Supplementary Table S4).

Plant nutrient and micronutrient concentrations

Iron and Zn concentrations in grain were both significantly affected by the interaction Var x AMF inoc (Table 1). AMF inoculation strongly increased Fe concentration in grain of both varieties, with +63% for the modern and +42% for the old variety (Fig. 5b; Supplementary Table S5). Grain zinc concentration after AMF inoculation experienced an even stronger increase, with +101% for the modern and +78% for the old variety (Fig. 5c; Supplementary Table S5). However, in shoots and roots at GS12 and GS31, Fe and Zn concentration was not affected by variety, N fertilization and AMF inoculation (data not shown).

Nitrogen concentration in grain was significantly affected by the interaction Var x N fert x AMF inoc (Table 1). In the old variety grain N concentration was increased by AMF inoculation when plants were not fertilized and at the 40–0-40 N fertilizer regime (+36% and +64%, respectively), whereas at 40–40–0 there was not and at 40–40–40 kg N ha^{-1} a negative effect (−32%) was detected (Fig. 2b). Grain N concentration in unfertilized plants inoculated by AMF (20.1 g kg^{-1}) was

Table 2 *P*-values of three-way ANOVA to evaluate the effect of variety (Var), nitrogen fertilization (N fert) and arbuscular mycorrhizal fungal inoculation (AMF inoc) on growth, yield and yield components of durum wheat

Source of variation*	Grain yield	Straw	HI	Number of spikes	Number of kernels per spike	Mean kernel weight	Number of fertile spikelets per spike	Spike fertility index
Var*	0.900†	**<0.001**	**<0.001**	**<0.001**	**0.014**	**<0.001**	**<0.001**	**<0.001**
N fert*	**<0.001**	**<0.001**	0.285	0.237	**<0.001**	0.066	**<0.001**	0.776
AMF inoc*	0.777	0.680	0.759	0.246	0.201	0.919	0.850	0.813
Var x N fert	0.298	0.078	0.241	0.182	0.138	0.278	0.054	0.363
N fert x AMF inoc	0.843	0.556	0.314	0.998	0.678	0.901	0.445	0.912
Var x AMF inoc	0.829	0.630	0.604	0.639	0.383	0.348	0.664	0.135
Var x N fert x AMF inoc	0.774	0.153	0.133	0.635	0.478	0.193	0.580	0.527

* Var, N fert and AMF inoc used as fixed factors; Var: Cappelli and Svevo are the old and the modern variety, respectively; N fert: 0–0-0, 0–40–40, 40–0-40, 40–40–0, 40–40–40 kg N ha^{-1} at pre-seeding, 5th leaf unfolded stage and pseudo stem erection, respectively; AMF inoc: *Rhizophagus irregularis* DAOM197198

† In bold statistically significant values ($P \leq 0.05$). Replicates field plots were three per treatment

Table 3 Straw, number of kernels per spike and number of fertile spikelets per spike of durum wheat. Variety (Var), nitrogen fertilization (N fert) and arbuscular mycorrhizal fungal inoculation (AMF inoc) mean values

Treatments	Shoot dry weight at GS31 (g m^{-2})	Straw (g m^{-2})	Number of kernels per spike	Number of fertile spikelets per spike
Var*				
Old	1637.2 b†	983.2 b	38.7 b	18.0 b
Modern	916.9 a	511.5 a	33.4 a	15.0 a
N fert*				
0–0-0	743.8 a	449.8 a	20.4 a	13.4 a
0–40–40	1508.7 b	759.4 b	33.0 b	15.5 b
40–0-40	1276.0 b	774.1 b	39.8 bc	17.2 c
40–40–0	1488.3 b	812.2 b	44.9 c	18.0 c
40–40–40	1368.5 b	941.2 b	42.0 c	18.3 c
AMF inoc*				
-M	1209.0 a	760.1 a	37.4 a	16.5 a
+M	1345.1 a	734.6 a	34.7 a	16.6 a

* Var, N fert and AMF inoc used as fixed factors; Cappelli and Svevo are the old and the modern variety, respectively; N fert: 0–0-0, 0–40–40, 40–0-40, 40–40–0, 40–40–40 kg N ha^{-1} at pre-seeding, 5th leaf unfolded stage and pseudo stem erection, respectively; −M: mock inoculated; +M: inoculated with *Rhizophagus irregularis* DAOM197198

† Values are means of 30, 12 and 30 observations for var., N fert and AMF inoc, respectively; within treatments Var, N fert and AMF inoc, values followed by different letters are significantly different at $P \leq 0.05$, according to the post-hoc Tukey-B test

similar to plants at the highest N fertilizer regime (40–40–40 kg N ha^{-1}) (22 g kg^{-1}).

Phosphorus concentration in grain was significantly affected by the interactions N fert x AMF inoc and Var x AMF inoc (Table 1). AMF inoculation increased P concentration in grain only when no N fertilizer was applied (0–0-0 kg N ha^{-1}) (Fig. 5a; Supplementary Table S5), by 54% when compared with the average of all other N fertilizer regimes and by 40% when compared with the non-inoculated control.

Discussion

In this field study it was shown that (i) AMF inoculation strongly increased grain Fe and Zn concentration of durum wheat under all N fertilizer regimes and (ii) modern and old varieties (genotypes) responded distinctly to AMF inoculation in term of root length, AMF root colonization and grain macro- and micronutrient contents. In addition, field AMF inoculation modified the relative abundance of three

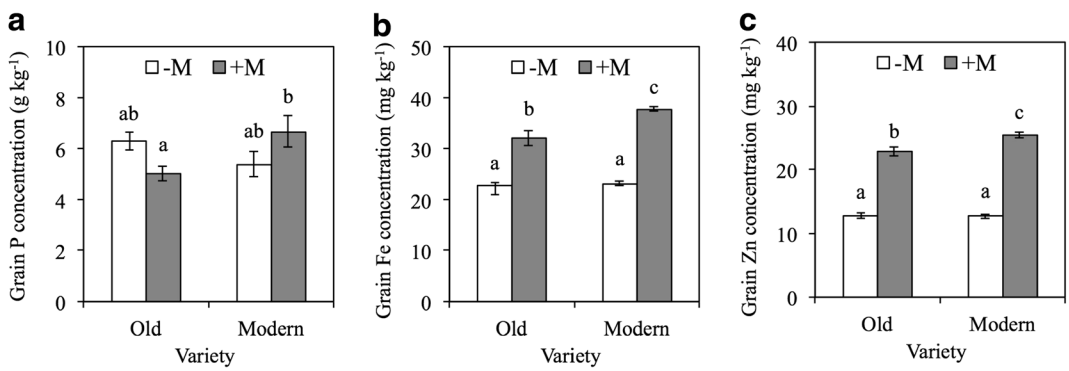

Fig. 5 Grain phosphorus (P) concentration (a), grain iron (Fe) concentration (b) and grain zinc (Zn) concentration (c) at physiological maturity (GS90) of an old (Cappelli) and a modern (Svevo) durum wheat variety inoculated with *Rhizophagus irregularis* (+M) and mock inoculated (-M). Significant interactions variety (Var) x AMF inoculation (AMF inoc) are shown. Data are mean ± standard error (n=15) and are averaged over five nitrogen fertilization regimes

AMF OTUs belonging to the genera *Rhizophagus* and *Funneliformis*.

Root growth and AMF root colonization

The increased root colonization at early crop development stages, after inoculation with *R. irregularis* DAOM197198, demonstrated the compatibility of durum wheat with the inoculated AMF in field conditions. These results agree with the unique record from field trials of durum wheat compatibility with native AMF (Gao et al. 2010) and with results from pot-grown durum and bread wheat inoculated with AMF (Al-Karaki and Al-Raddad 1997; Singh et al. 2012). Our results indicate that compatibility of field-grown durum wheat was higher than that of bread wheat (Mohammad et al. 1998). This difference may be related to genome (tetraploid vs hexaploid) and cultivar differences (Hetrick et al. 1993), but also to high soil mycorrhizal infection potential, low soil fertility and/or temperature during initial plant development (Hetrick and Bloom 1984; Hetrick et al. 1992). The increased root colonization due to AMF inoculation indicates efficient establishment of the inoculated *R. irregularis*, in agreement with data recorded at tillering and anthesis in field-grown bread wheat inoculated with *R. irregularis* [as *G. intraradices*] (Babana and Antoun 2006; Mohammad et al. 1998; Suri et al. 2011).

In non-inoculated conditions, in contrast to Siddique et al. (1990) reporting a higher root dry weight in old wheat varieties compared to modern ones at tillering, we found similar root length between the two genotypes studied here. However, the root system response to AMF inoculation strongly differed. At tillering, only the old variety responded to AMF with a strong increase in root length. An enhanced branching of secondary roots and a longer root system when colonized by AMF were previously observed in both monocotyledon and dicotyledon species, and these effects were attributed to an increasing proportion of inactive root apices, stimulating the development of new lateral or adventitious roots (Berta et al. 1990; Hodge et al. 2009). However, the lack of response of the modern variety to AMF in term of root length increase may be due to genetic variations determined by plant breeding and not to differences in root fibrousness (Hetrick et al. 1992; Mosse 1986; Siddique et al. 1990). Although for the old variety many root branches and fine roots were assessed at the beginning of tillering and were correlated to high AMF colonization (Hetrick et al. 1992), our results, similarly to Stöppler et al. (1990) and Zhu et al. (2001), did not allow to confirm the effect of breeding on AMF compatibility wheat.

At the starting of stem elongation, AMF root colonization of both, the old and modern varieties increased by inoculation at all N fertilization regimes, except at the highest rate. Although the effect of N fertilization on AMF root colonization of wheat was not previously studied, these data agree with the reduction of AMF root colonization due to N addition reported for sugar maple (*Acer saccharum* Marshall), common wormwood (*Artemisia vulgaris* L.), hawkweed oxtongue (*Picris hieracioides* L.), Canada bluegrass (*Poa compressa* L.) and field brome (*Bromu sjaponicus* Thunb.) (Blanke et al. 2011; Van Diepen et al. 2007). At physiological maturity, root colonization was affected only by N fertilization but no longer by the initial AMF inoculation. This might be explained by the availability of different N forms (e.g. NH_4^+, NO_3^-), which may change rhizosphere bacterial and fungal communities (Giagnoni et al. 2016), by negative impact of inorganic N fertilization on AMF biomass, as it was reported for wheat (Albizua et al. 2015), or simply by the fact that, in terms of root colonization, at physiological maturity the system became saturated (independent from initial infectious propagules concentration, including native ones), with colonization being dependent by the fertilization regimes only.

Arbuscular mycorrhizal fungal community diversity

Ten AMF molecular operational taxonomic units (MOTUs) could be defined from the roots of modern and old variety of durum wheat inoculated with AMF or not. The MOTUs were affiliated to *Acaulosporaceae*, *Diversisporaceae*, *Gigasporaceae* and *Glomeraceae*. The MOTU richness was higher than retrieved in previous work on wheat roots (Helgason et al. 1998; Daniell et al. 2001; Hijri et al. 2006). In detail, Helgason et al. (1998) and Daniell et al. (2001) retrieved six and five MOTUs, respectively, all affiliated to the *Glomeraceae*, including *Funneliformis mosseae* (as *Glomus mosseae*) and *Rhizophagus irregularis* (as *Glomus intraradices*), while Hijri et al. (2006) retrieved seven MOTUs affiliated to *Acaulosporaceae*, *Gigasporaceae*, *Glomeraceae* and *Paraglomeraceae*. The results of Helgason et al. (1998) and Daniell

et al. (2001) must be interpreted with care as the primers used (NS31/AM1) discriminate sequences from *Archeosporaceae* and *Paraglomeraceae*, which may explain some inconsistency when compared to our results and the ones of Hijri et al. (2006). The AML1/AML2 primer pair used here provides a better specificity and taxon coverage (Redecker 2000; Redecker et al. 2003; Lee et al. 2008).

In this study, at physiological maturity the relative abundance of the MOTU affiliated to *Rhizophagus* (Rh1PI) strongly increased in the AMF inoculated plants, for both wheat varieties, whereas the MOTUs Fu1PI and Fu2PI affiliated to *Funneliformis* decreased. Although the molecular marker utilized does not allow discriminating isolates of *R. irregularis*, the MOTU patterns indicates that the inoculated isolate *R. irregularis* DAOM197198 was successful as a root colonizer and competed with species from the genus *Funneliformis*. The successful inoculum establishment is supported by the inoculation-dependent increase in root colonization and the strongly increased in-grain Zn and Fe concentration. The use of other markers, such as the mitochondrial large subunit mtLSU (Börstler et al. 2008; Thiéry et al. 2010; Sýkorová et al. 2012), might allow specific detection *R. irregularis* isolates, but the use of such isolate specific markers would not have allowed to detect the simultaneous decrease of the *Funneliformis* MOTUs.

Plant growth, grain yield and yield components

Wheat productivity varies considerably according to the interaction between genotype and environment (Rozbicki et al. 2015). Regarding genotype, the target of Italian wheat breeding programs until the end of the 1970s was to increase grain yield by shortening the period from sowing to anthesis, by increasing the harvest index (HI) and by enhancing the number of grains per unit area (De Vita and Maggio 2006; De Vita et al. 2007). The old variety studied here differed from the modern one in term of crop cycle length, vegetative growth, yield components and spike fertility, although not in terms of grain yield. However, it was previously reported that it produces less grain yield than Svevo and other modern varieties at locations in Southern and Central Italy, at different N fertilization rates (Dinelli et al. 2013; Rascio et al. 2015; Stagnari et al. 2013). This inconsistency might be due to higher mean kernel weight (+20%) of the old variety under the pedoclimatic conditions of the present study, when compared to the modern one, compensating for a lower number of kernel per unit area. Grain filling in wheat is supported by transient photosynthesis and remobilization of stored reserves accumulated before anthesis (Blum 1998; Gebbing and Schnyder 1999). In this regard, the old variety had an increased capacity to store non-structural assimilates, as reflected by higher straw dry weight and a higher stay-green capability due to the longer growth cycle length (Palta et al. 1994; Royo et al. 2007).

Both durum wheat varieties responded positively and similarly to N fertilization (e.g., in terms of grain yield and straw weight), but the hypothesis of a lower dependency of N fertilization of old varieties could not be confirmed by our results (Hildermann et al. 2010; Stagnari et al. 2013). In addition, in line with Ercoli et al. (2013), the highest N fertilizer regime, based on three N applications, did not significantly increase grain yield when compared to regimes based on two N applications.

The highest as well as the intermediate N fertilization regimes with N application at pre-seeding all resulted in an about two-fold increase in number of kernel per spike and an about 30% increase in number of fertile spikelets per spike when compared to the unfertilized controls. The final number of wheat kernels per unit area is a consequence of an overproduction of primordia, that have the potential to become fertile florets and, later, grains. Indeed, environmental conditions and in particular N availability between the onset of stem elongation and the stage shortly after flowering affect the survival of the primordia and the fertilization of florets, and therefore the final number of kernel (Ferrante et al. 2012; Slafer et al. 2014).

Grain yield of durum wheat was not affected by AMF inoculation in our study, under N-fertilization, which agrees with other reports on AMF field inoculation of durum wheat (Behera and Rautaray 2010; Singh et al. 2012; Saia et al. 2015a). In contrast, for bread wheat, a 20% grain yield increase by AMF inoculation was reported (Pellegrino et al. 2015b).

Plant nutrient and micronutrient concentrations

Iron and Zn concentrations in durum wheat grain were strongly affected by AMF field inoculation in all fertilization treatments, for both varieties. This is in agreement with previous results (Cavagnaro 2008; Lehmann

et al. 2014; Lehmann and Rillig 2015; Pellegrino et al. 2015a, b; Zhang et al. 2016). The modern durum wheat variety showed a higher increases of grain Fe and Zn than the old variety. It has been reported that the ancestral wild wheat *Triticum turgidum spp. dicoccoides* (diploid) has an allele encoding a NAC transcription factor (*NAM-B1*) that is involved in Fe and Zn remobilization from leaves to grains during senescence. By contrast, modern wheat varieties (hexaploid) carry a non-functional *NAM-B1* allele, which leads to reduced grain Fe, Zn and protein content (Uauy et al. 2006). Our results indicate, that the AMF mediated higher grain content for Fe and Zn is not driven by this *NAM-B1* dependent remobilization, but by overall improved Fe and Zn nutrition of the plants. In the overall context of micronutrient malnutrition in many regions with wheat as dominant staple food, the strong increases of Fe and Zn concentrations by AMF inoculation observed in durum wheat is of great interest for the production of biofortified food. The role of AMF as crop biofortifiers might become even more important under the projection for the year 2050, where elevated CO_2 levels will lead to even lower concentration of Fe, Zn and protein in cereals, in particular wheat (Fe −5.1%, Zn −9.3% and protein −6.3%) (Myers et al. 2014).

We observed a significant interaction between AMF inoculation and N fertilization, by an AMF-mediated promotion of N uptake in unfertilized plots. In unfertilized treatments, an up-regulation of wheat ammonium and nitrate transporters due to AMF inoculation was recently reported (Duan et al. 2015; Saia et al. 2015b). In our study, grain N concentration of the AMF inoculated plants in the unfertilized control was similar to that of plants fertilized with the highest N rate.

Although numerous studies have shown a clear contribution of AMF to phosphate uptake (e.g., Li et al., 2006; Smith et al. 2011) and an AM-inducible Pi transporter gene was also reported for wheat (Duan et al. 2015), we did not observe increases in grain P concentration after AMF inoculation. This is in line with other results on grain P concentration of inoculated bread and durum wheat (Pellegrino et al. 2015a, b; Cabral et al. 2016), but is in contrast with reports showing increases in shoot P concentration at early developmental stages (Mohammad et al. 2004; Ellouze et al. 2015; Oliveira et al. 2016). To understand how AMF mediate wheat P uptake will still need further studies.

Conclusions

At harvest the altered pattern of AMF MOTUs abundance in the roots of inoculated durum wheat indicates the establishment and persistence of the inoculated AMF. Finally, the strongly increased Fe and Zn content in grain following AMF field inoculation, independently of the N fertilization regimes and the durum wheat variety, demonstrates the effect of application of AMF for micronutrient biofortification. Positive effects of the higher Fe and Zn content on human nutrition are most likely, but assessing their bioavailability will be needed for confirmation. An increased grain N content after AMF inoculation occurred only in unfertilized plots, indicating improved protein content only at low or moderate soil N availability. The highest N fertilization rate applied did not result in higher grain yield or quality compared to lower rates, but AMF root colonization was decreased. Thus, N fertilization management should be moderate to avoid the suppression of benefits provided by AMF. Finally, the significantly enlarged root system of the old durum wheat variety in response to AMF inoculation may have implications for water and nutrient uptake and pest resilience and could be considered a valuable trait in wheat breeding programmes.

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