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Field Inoculation of Bread Wheat with *Rhizophagus irregularis* under Organic Farming: Variability in Growth Response and Nutritional Uptake of Eleven Old Genotypes and A Modern Variety

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Abstract: Arbuscular mycorrhizal fungi (AMF) promote crop growth and yield by increasing N and P uptake and disease resistance, but the role of field AMF inoculation on the uptake of micronutrients, such as Fe and Zn, and accumulation in plant edible portions is still not clarified. Therefore, we studied the effect of field inoculation with *Rhizophagus irregularis* in an organic system on 11 old genotypes and a modern variety of bread wheat. Inoculation increased root colonization, root biomass and shoot Zn concentration at early stage and grain Fe concentration at harvest, while it did not modify yield. Genotypes widely varied for shoot Zn concentration at early stage, and for plant height, grain yield, Zn and protein concentration at harvest. Inoculation differentially modified root AMF community of the genotypes Autonomia B, Frassineto and Bologna. A higher abundance of *Rhizophagus* sp., putatively corresponding to the inoculated isolate, was only proved in Frassineto. The increase of plant growth and grain Zn content in Frassineto is likely linked to the higher *R. irregularis* abundance. The AMF role in increasing micronutrient uptake in grain was proved. This supports the introduction of inoculation in cereal farming, if the variable response of wheat genotypes to inoculation is considered.

Keywords: symbiotic fungi; arbuscular mycorrhizal fungi; common wheat; intraspecific variability; host benefit; micronutrient uptake

1. Introduction

Wheat (*Triticum* spp.) is one of the largest cereal crops in the world in terms of cultivated area and production [1]. In 2017, the agricultural area devoted to wheat was 220 million hectares and world production was 750 million tons. In Italy, the area cultivated with wheat is almost 2 million hectares and the production around 8 million tons. Wheat is the main source of energy and minerals, especially in developing countries, due to its high consumption in a variety of food products [2]. The mineral content and bioavailability of essential elements for humans is the primary determinant for the quality of wheat-based products. Therefore, low mineral content in grain of plants grown in soil depleted or with low availability of minerals can result in an insufficient dietary intake of essential elements [3].

Breeding efforts on wheat started in the early 20th century and were mainly focused on yield increase, while the content of essential elements was largely disregarded [4,5]. In Italy, a great contribution to breeding was given by Nazareno Strampelli, who carried out selection and intervarietal crossing with main objectives to increase grain yield and technological properties, as well as resistance

to pathogens and environmental stresses [6]. The new varieties were shorter and less prone to lodging, resistant to many diseases, with greater fertility and a shorter biological cycle [7]. The higher productive characteristics of those varieties determined a gradual abandonment of landraces that were replaced by cultivars based on single pure lines [8]. Thus, the breeding work that has been done to date has led to a narrowing of the genetic basis of the genus *Triticum*, which could potentially increase susceptibility and vulnerability to environmental stress, parasites and diseases [9,10]. Moreover, this determined a decrease in the concentration of essential nutrients, such as Fe and Zn, in grains [11,12].

In recent years, numerous studies have focused on the qualitative aspects of wheat production to face the widespread malnutrition due to the lack of nutrients in the diet, which leads to the development of pathologies [13–15]. There are several biofortification strategies for increasing the concentration of minerals in cereals, including selection for genotypes with high micronutrient use efficiency, intercropping with legumes, field application of micronutrients as chemical fertilizers, and utilization of beneficial microorganisms [12,16–19]. A meta-analysis studying the responses of wheat to field inoculation with arbuscular mycorrhizal fungi (AMF) indicated increases in yield (20%), N content (31%) and Zn concentration (12.8%) in the grain, and a positive correlation between AMF root colonization rate and grain yield and Zn concentration [20]. These results were confirmed by a field experiment on *Triticum turgidum* L. subsp. *durum* (Desf.) Husn (durum wheat), showing increases by 42–63% for Fe and by 78–101% for Zn due to inoculation [21]. Modern and old varieties responded distinctly to AMF inoculation, and the modern variety showed higher responsiveness in terms of root length, AMF root colonization and grain macro- and micro-nutrient contents. The mycorrhizal pathway of Zn uptake was recently quantified in a pot experiment, accounting for 24.3% and 12.7% of the total aboveground Zn content in bread wheat and barley, respectively [22].

In this work, we aimed to improve the micronutrient nutritional quality of wheat by exploiting genetic variation in micronutrient uptake and compatibility with AMF. We evaluated the effects of field inoculation with *Rhizophagus irregularis* on old wheat genotypes and on a modern variety, with distinct assimilate distribution and putatively varying mycorrhizal dependency. 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.

2. Materials and Methods

2.1. Experimental Field Site

The experiment was conducted in 2016–2017 in the Maremma Regional Park at the experimental farm Terre Regionali Toscane, Alberese, (42°40′ N, 11°0.6′ E), 39 m above sea level, 4.4 km distance from sea and 0% slope, Grosseto, Italy. The soil is an alluvial loam, classified as Eutric Cambisol [23]. Soil physical and chemical properties were: 42% sand (2 mm > ∅ > 0.02 mm); 11% silt (0.02 mm > ∅ > 0.002 mm); 47% clay (∅ < 0.002 mm); pH 8.2 (H_2O); 9.5 g kg^{-1} organic carbon (Corg) (Walkley-Black); 0.9 g kg^{-1} total nitrogen (N) (Kjeldahl method); 30.0 mg kg^{-1} available phosphorus (P) (Olsen); 432.0 mg kg^{-1} available K; 23.7 mg kg^{-1} DTPA-extractable Fe [24]; 1.0 mg kg^{-1} DTPA-extractable Zn [24]; 20.5 cation exchange capacity (meq L^{-1}). According to Agraval [25], soil DTPA-Fe and Zn critical limits for wheat growth are 5.0 and 0.8 mg kg^{-1}, respectively. Therefore, experimental plants were grown in soil conditions corresponding to medium availability for Fe and low availability for Zn. Climate of the site is cold, humid Mediterranean (Csa), according to the Köppen–Geiger climate classification [26]. The 10-year average (2009–2018) of mean annual maximum and minimum temperatures is 20.4 and 11.0 °C, respectively, and annual precipitation is 565 mm, with 367 mm during wheat cropping cycle from November to June [27]. During the experimental wheat cropping cycle (November 2016–June 2017), mean maximum and minimum temperatures were 17.8 and 8.3 °C, respectively, while total precipitation was 126.6 mm (Supplementary Figure S1).

2.2. Experimental Set-Up and Crop Management

A two-factor design with twelve bread wheat (*Triticum aestivum* L.) genotypes and two AMF inoculation treatments with three replicate plots was arranged as a split-plot design, with AMF inoculation as main plot factor and genotype as split-plot factor. Wheat genotypes included ten old, not dwarf and unregistered Italian genotypes (Andriolo, Autonomia B, Avanzi 3, Frassineto, Gentil Rosso, Grano Noè di Pavia, Inallettabile, Risciola, Sieve, Torrenova), one old semi-dwarf registered variety (Verna) and one modern dwarf registered variety (Bologna) of bread wheat. AMF inoculation treatment (+M) was compared to a mock inoculum (−M). AMF inoculation was performed using *Rhizophagus irregularis* C. Walker and A. Schüßler [28] (synonym *Glomus irregulare* Błaszk., Wubet, Renker and Buscot; earlier often named as *Glomus intraradices*). The inoculum was a dried spore-based inoculum (SYMPLANTA-001 research grade, Symplanta GmbH & Co. KG, Germany) derived from the same culture line as the sequenced DAOM197198 [29]. *Rhizophagus irregularis* spores and the carrier, a water-insoluble dry powder of calcined attapulgite clay, were distributed and incorporated into the soil by harrowing at 5 cm depth, immediately before sowing. The rate of the application of the AMF inoculum was 6.8 g m^{-2}, corresponding to 3444 spores m^{-2}. Mock inoculation consisted of the distribution of only the calcined attapulgite powder at the same rate and timing of the AMF inoculation treatment. Plots were 21 m^2 (7 × 3 m).

Bread wheat was cultivated following the management techniques normally applied in the area for organic agriculture, according to the Council Regulation No. 834/2007 [30]. The preceding crop was alfalfa (*Medicago sativa* L.). Soil tillage was performed in autumn before sowing through mouldboard ploughing (25 cm depth) and harrowing (15 cm depth). Organic fertilizer (NPK 3-3-3) at 1 t ha^{-1} was applied pre-planting. Bread wheat was sown on December 6th 2016 at the optimum planting time for wheat production in Central Italy [31]. A rate of 400 viable seeds per m^2, in rows spaced 18 cm apart, was applied. Lodging did not occur in any of the plots in this study. No herbicide, insecticide or fungicide were applied, following the protocols of organic agriculture.

2.3. Sampling and Morphological Analyses

At the end of tillering (GS26) [32], crop density (number of plants per unit area) was recorded. The dates of the growth stages, pseudostem erection (GS30) and physiological maturity (GS90), were recorded in all plots. The duration of growth periods from emergence (GS10) to GS30 was 87 d and from GS30 to GS90 was 104 d. At GS30, three plants, randomly selected in each replicate plot, were excavated with their root system (nine plants per treatment). Roots were separated from soil by gently washing with tap water and plants were divided into shoots and roots for dry weight and Fe and Zn determination in plant tissues. At GS90, three random turfs were extracted (20 cm soil depth) from each replicate plot and then combined. Roots were separated from soil by gently washing with tap water. At both stages, percentage of AMF root colonization was measured under stereomicroscope after root clearing and staining by the grid-line intersect method [33]. Specifically, we mounted the stained roots on microscope slides and covered with 40 × 22 mm coverslips. Four slides were prepared for each sample. Roots were aligned parallel to the long axis of the slides and observed by a light microscope (Leitz Wetzlar Microscope, Germany) at a magnification of ×200. Each slide was moved along the long axis eight times and the negative and positive intersections were counted by a grid-line intersect ocular. Negative intersections were those without fungal material in roots, whereas positive were those with arbuscules, vesicules or hyphae. A total of 200 intersections were evaluated for each slide.

At GS90, plants from a 1-m^2 area for each replicate plot were manually cut at ground level and number of spikes and plant height were assessed (n= 72). Plants were then partitioned into straw, chaff and grain. For dry weight determination, samples from all plant parts were oven dried at 65 °C, up to constant weight. Mean kernel dry weight was measured and number of kernels per spike was calculated. At GS30, Fe and Zn concentrations in shoots and roots were assessed by atomic absorption spectrometry following the method of Isaac et al. [34]. At GS90, grain was analyzed for Fe and Zn

concentrations [28] and for protein concentration (N Kjeldahl × 5.7) following the Kjeldahl method according to Bremner and Mulvaney [35].

2.4. Molecular Analysis

Genomic DNA was extracted from sub-samples of roots (50–70-mg fresh-weight root samples, GenUPTM Plant DNA Kit, Biotechrabbit, Germany) of three selected genotypes (Autonomia B, Frassineto and Bologna). Among the old genotypes, we selected Autonomia B and Frassineto with distinct grain yield and protein content [36] and adaptability to hilly and plain [37], and putatively varying mycorrhizal dependency. Samples were taken at GS90 on inoculated (+M) and not-inoculated (−M) plots (n = 18). DNA quality was checked by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). PCR amplification was performed using the primer pair NS31 and AML2, targeting the small subunit ribosomal RNA (SSU rRNA 18S) gene fragment [38,39]. Although the NS31/AML2 primer pair reliably discriminates AMF diversity only at genus level [40], the fragment was targeted due to the abundance in the public database of comparable Glomeromycota sequences [41,42]. NS31/AML2 PCR amplicons were generated in volumes of 20 µL with 0.5 U of HotStarTaq DNA Polymerase (Qiagen, Venlo, Netherlands), 10 µM of each primer (NS31⁄AML2), 0.2 mM of each dNTP, 1 mM of MgCl2 and 1x reaction buffer, using a S1000 Thermal CyclerTM (BIORAD, Hercules, CA, USA). The thermal cycler was programmed as follows: 95 °C for 2 min, 30 cycles at 95 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min and a final extension step at 72 °C for 5 min. Reaction yields were estimated by using a 1% agarose gel containing ethidium bromide (0.5 µg mL^{-1}). The QIAquick (Qiagen, Venlo, Netherlands) purified PCR amplicons of DNA were ligated into the pGem®-T Easy vector (Promega Corporation, WA, USA) 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 plasmid inserts of ca. 31 positive clones per clone library (a total of 18 libraries, one per each root replicate) using SP6 vector primers. Plasmids of clones (552) were purified by Wizard® Plus SV Minipreps (Promega Corporation, WA, USA) and sequenced by Sanger sequencing technique using the 3730XL Analyser automated sequencer (Life Technologies, Carlsbad, CA, USA) at the DNA Services of the University of Illinois in Chicago (USA).

2.5. Statistics and Data Analyses

The AMF origin of the newly generated sequences was determined by the Basic Local Alignment Search Tool (BLAST) search [43] at National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) and the detection of chimeric sequences was performed using Chimera Check version 11 [44]. The newly generated sequences were aligned with those of a reference alignment and with the closest matches from GenBank at NCBI. The reference alignment is a database composed by AMF sequences of morphologically characterized and described AMF species, listed in the phylotaxonomic classification [28]. Sequence alignment was performed using the algoritm ClustalW in MEGA7 [45]. *Corallochytrium limacisporum* sequence L42528 was used as outgroup, that is, a lineage that falls outside the clade studied, but closely related to that clade. The phylogenetic tree was inferred by Neighbor-Joining (NJ) analysis [46] using MEGA7 and the Kimura 2-parameter model [47]. Branch support bootstrap values are derived from 1000 bootstrap replicates. The phylogram was drawn by MEGA7 and edited with 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 ≥ 77. In addition, to assign taxonomic information to the identified MOTUs, the retrieved MOTUs were blasted against the MaarjAM database [48,49] and the closest virtual taxa were identified (similarity higher than 99%). AMF MOTU richness and Shannon and Simpson biodiversity indexes (H' and λ) were calculated using Primer v7 [50].

The phylogram was drawn by the interactive tree of life (ITOL) [51] and edited with Adobe Illustrator CC 2017. The relative abundance of the MOTUs were used for data input in ITOL for

building the pie charts, describing the AMF community structure in the inoculated and uninoculated wheat genotypes (Autonomia B, Frassineto and Bologna).

All newly-generated sequences were submitted to GenBank database under the submission SUB6855101 (accession numbers MN958910-MN959413).

Root colonization, plant growth and nutrient data, and AMF diversity indexes were analyzed by two-way ANOVA using inoculation and genotype as fixed factors, according to the split-plot design. Data were ln- and arcsine-transformed when needed to fulfil the assumptions of ANOVA. Post-hoc Tukey-B significant difference test was used for comparison among and between treatments. Means and standard errors given in tables and figures are for untransformed data. Analyses were performed using the SPSS software package version 21.0 (SPSS Inc., Chicago, IL, USA). Permutational analysis of variance (PERMANOVA) [52] was used to test the effect of inoculation and genotypes (Autonomia B, Frassineto and Bologna) on the AMF community composition and structure. The response data matrix was square-root transformed prior to the analysis in order to down-weight the importance of dominant taxa, and the Bray-Curtis index of dissimilarity was calculated to measure ecological distance. *P*-values were calculated using the Monte-Carlo test. Since PERMANOVA is sensitive to differences in multivariate location (average community composition of a group) and dispersion (within-group variability), the analysis of homogeneity of multivariate dispersion (PERMDISP) [53] was performed to check the homogeneity of dispersion among groups (beta-diversity) [54]. The principal coordinate analysis (PCO) was performed [43] for visualizing the most relevant patterns in the data. Analyses were performed using PRIMER 7 and PERMANOVA + software (Primer-e, Auckland, New Zealand) [50,55].

3. Results

3.1. AMF Root Colonization

At GS30, AMF root colonization was significantly affected by *R. irregularis* field inoculation (Figure 1a). In the inoculated plots, root colonization of all bread wheat genotypes was, on average, 14%, while that of the not inoculated ones was 9%. By contrast, at GS90, all genotypes were similarly colonized, irrespectively of the inoculation treatment (average: 15.8% ± 0.5) (Figure 1b).

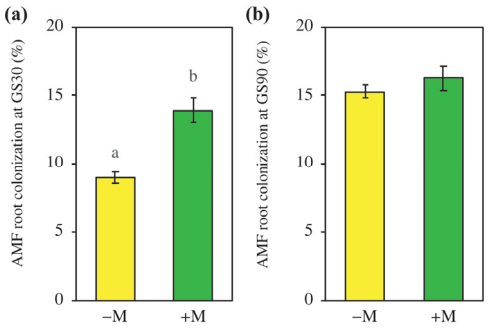

Figure 1. Effect of AMF inoculation on AMF root colonization at GS30 (**a**) and at GS90 (**b**). AMF inoculation and mock control are indicated as +M and −M, respectively.

3.2. Plant Growth and Micronutrient Uptake at GS30

At GS26, crop density was, on average, 350 plants m^{-2}. At GS30, shoot dry weight was not affected by wheat genotype or AMF field inoculation (average: 1.16 ± 0.04 g $plant^{-1}$) (Figure 2a). By contrast, root dry weight was significantly increased with inoculation (+53%) (Figure 2b). As a consequence, roots accounted for 86% and 91% of total plant in −M and +M, respectively. Although the response of genotypes to inoculation was not statistically significant, the roots of the modern variety, Bologna, together with the old genotype, Frassineto, showed the lowest biomass increase following inoculation (10–18%).

Shoot Fe concentration was not affected either by wheat genotype or AMF field inoculation (average: 374 ± 17.0 mg kg^{-1}) (Figure 3a,b). Conversely, shoot Zn concentration was significantly affected by both wheat genotypes and AMF field inoculation (Figure 3c,d). Autonomia B had the highest Zn concentration in shoots and Risciola the lowest (40.2 ± 1.6 mg kg^{-1} vs. 25.7 ± 2.0 mg kg^{-1}), while the other genotypes had intermediate values (Figure 3c). Moreover, shoot Zn concentration was increased with inoculation (+6.6%) (Figure 3d).

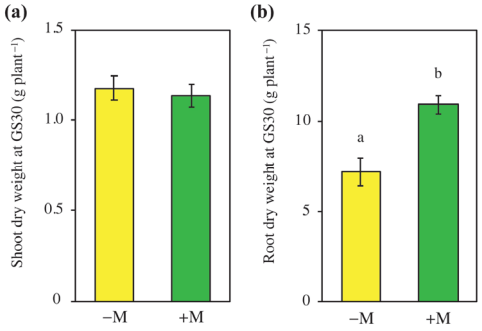

Figure 2. Effect of AMF inoculation on shoot dry weight (**a**) and root dry weight at GS30 (**b**). AMF inoculation and mock control are indicated as +M and −M, respectively.

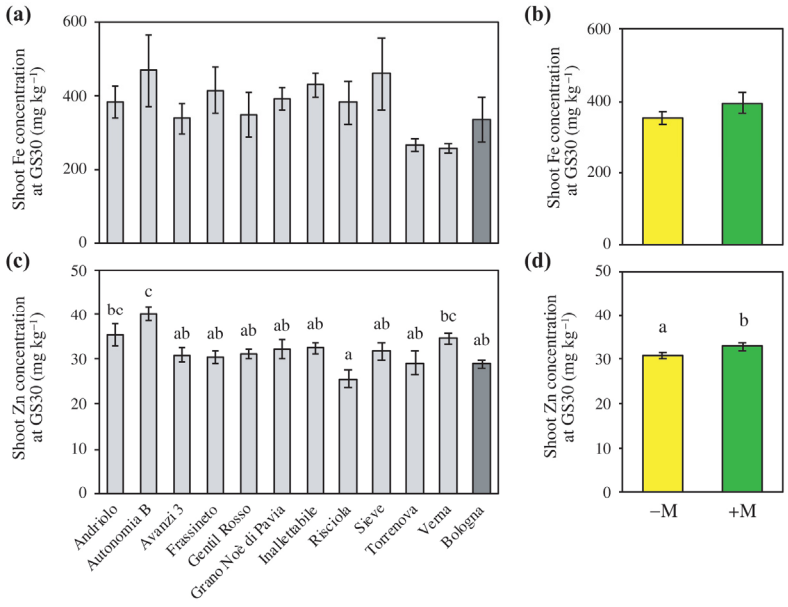

Figure 3. Effects of wheat genotype and AMF inoculation on shoot Fe concentration (**a**,**b**), and shoot Zn concentration at GS30 (**c**,**d**). AMF inoculation and mock control are indicated as +M and −M, respectively.

3.3. Plant Height, Yield and Yield Components at GS90

Plant height significantly varied among wheat genotypes (Figure 4a). The old genotype, Risciola, was the tallest (96.7 ± 2.1 cm), whereas the modern variety, Bologna, was the shortest (67.3 ± 1.2 cm). By contrast, AMF field inoculation did not significantly affect plant height. Grain yield varied among genotypes but was not affected by inoculation (Figure 4b). The modern variety, Bologna, had the highest grain yield (359 ± 15.9 g m^{-2}), whereas the old genotype, Grano Noè di Pavia, had the lowest (203 ± 10.7 g m^{-2}). The other wheat genotypes gave intermediate yield values.

Number of spikes per square meter and number of kernels per spike varied among genotypes and were significantly affected by AMF field inoculation (Figure 5a–d); whereas, mean kernel weight was only affected by wheat genotype (Figure 5e,f). The number of spikes per square meter was

highest in Bologna and lowest in Grano Noè di Pavia, whereas the other genotypes had intermediate values (Figure 5a). The number of kernels per spike was highest in Bologna and Sieve and lowest in Inallettabile (Figure 5c), while the mean kernel weight was highest in Risciola and lowest in Bologna (Figure 5e). The highest grain yield of Bologna was due to the combination of high number of spikes per square meter and number of kernels per spike and low mean kernel weight, whereas the lowest yield of Grano Noè di Pavia was due to low number of spikes and number of kernels per spike and medium-high mean kernel weight (Figure 5a,c,e). On average, the number of kernels per spike was by 35% lower in the old wheat genotypes compared to Bologna (Figure 5a).

Moreover, inoculation decreased the number of spikes per square meter (−13%) and increased the number of kernels per spike (+16%) (Figure 5b,d).

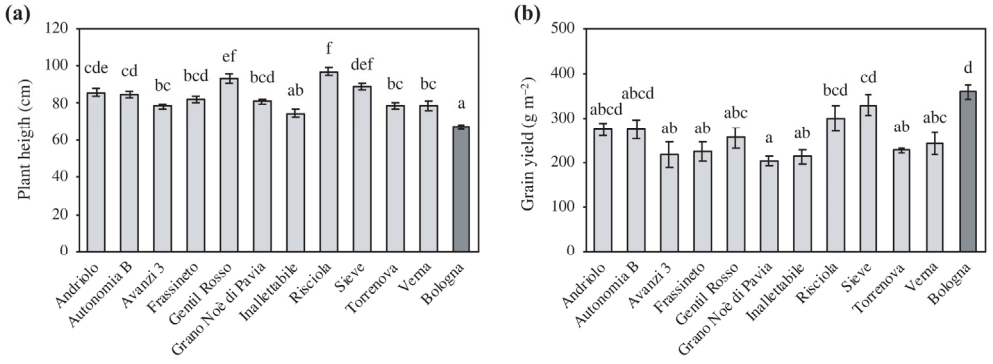

Figure 4. Effects of wheat genotype on plant height (**a**) and grain yield (**b**).

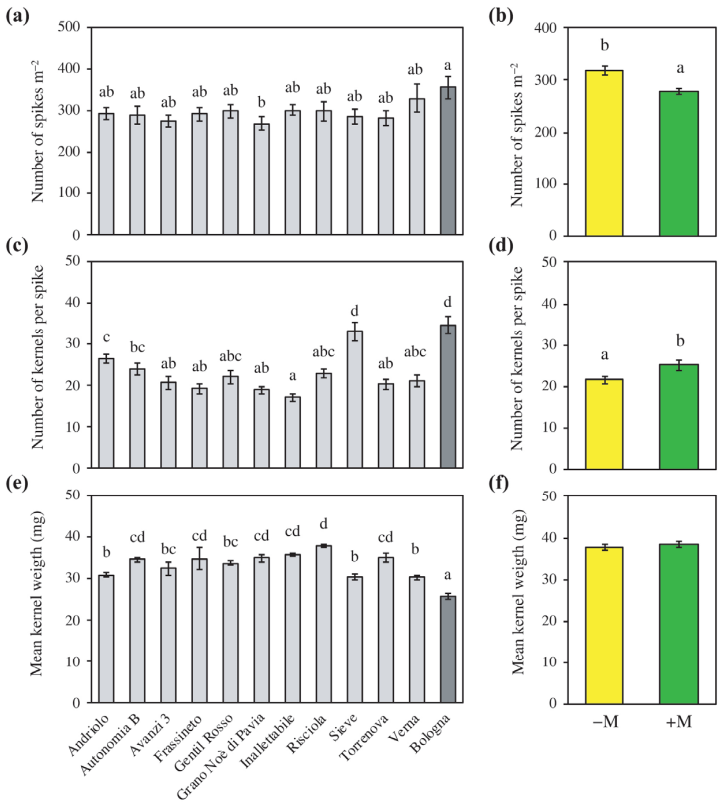

Figure 5. Effects of wheat genotype and AMF inoculation on number of spikes per m^{-2} (**a**,**b**), number of kernels per spike (**c**,**d**), and on mean kernel weight (**e**,**f**). AMF inoculation and mock control are indicated as +M and −M, respectively.

3.4. Micronutrient Uptake and Protein Content in Grain at GS90

Grain Fe concentration did not vary among wheat genotypes, but was strongly increased with AMF field inoculation (+24%) (Figure 6a,b). Conversely, grain Zn concentration was not modified by inoculation, but strongly varied among genotypes (Figure 6c,d). Andriolo, Gentil Rosso, Sieve and Verna had the highest Zn concentration (average: 55.4 mg kg^{-1}) and Bologna, the lowest (30.2 ± 0.9 mg kg^{-1}) (Figure 6c). Grain protein concentration was not modified by inoculation (Figure 6f), while among wheat genotypes, Verna showed the highest value (15.4%), whereas Autonomia B, Risciola and Bologna, the lowest ones (average: 12.2%) (Figure 6e).

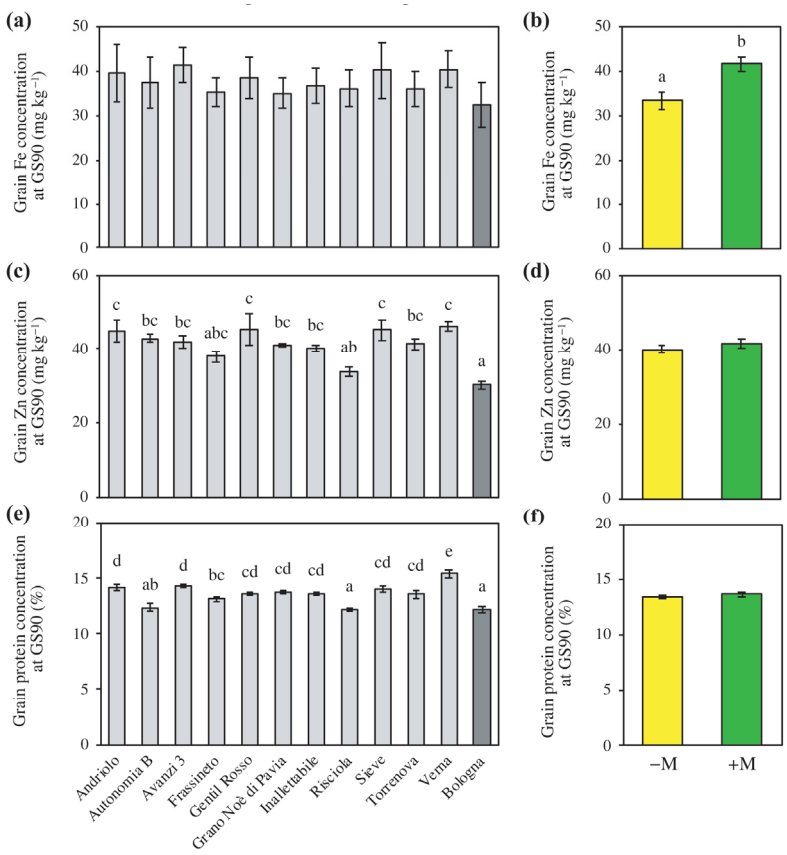

Figure 6. Effects of wheat genotype and AMF inoculation on grain Fe concentration (**a**,**b**), grain Zn concentration (**c**,**d**) and grain protein concentration at GS90 (**e**,**f**). AMF inoculation and mock control are indicated as +M and −M, respectively.

3.5. AMF Characterization within the Roots of Two Old Genotypes and the Modern Variety, Bologna

A total of 596 sequences were obtained and after the detection of chimeric sequences a total of 552 18S SSU rRNA sequences were obtained in 18 root samples (3 genotypes × 2 inoculations × 3 replicates). After blast against NCBI database, we found a total of 504 AMF sequences, corresponding to 27.9 mean number of sequences per sample. The number of sequences per treatment ranged from 19.0 (Frassineto +M) to 37.3 (Autonomia B +M) (Table S1, Figure S1). The sequences were grouped into six AMF molecular operational taxonomic units (MOTUs), which were phylogenetically affiliated to one Funneliformis sp. (Fun_Alb), two Rhizophagus sp. (Rhizo1_Alb and Rhizo2_Alb), one Glomus sp. (Glo_Alb), and two Claroideoglomus (Claro1_Alb, Claro2_Alb), belonging to Glomeraceae and Claroideoglomeraceae families (Figure 7, Figures S1 and S2). All total retrieved AMF sequences were succesfully found in the MaarjAM database. The correspondence between the retrieved MOTUs and the closest virtual taxa (similarity higher than 99%) after blast search against the MaarjAM database is shown in Table S1. The rarefaction curves showing the relationship between the number of sequences

and the number of AMF MOTUs found in the roots demonstrated that the sampling effort was sufficient since the curves reached the asymptote (results not shown).

The MOTU richness did not vary among wheat genotypes and inoculation treatments (on average, 1.8) (Table S2). Conversely, Shannon and Simpson biodiversity indexes (H' and λ) were null in Frassineto +M and Bologna +M, and were higher in the other genotypes, either inoculated or not. Averaged over genotype and inoculation treatments, H' and λ were 0.63 and 0.42.

Among the retrieved MOTUs, Rhizo1_Alb is the MOTU putatively corresponding to R. irregularis inoculated isolate and thus should be expected to occur abundantly in inoculated roots. The proportions of the sequences retrieved in uninoculated and inoculated genotypes for each MOTU was analyzed. In Autonomia B, Fun_Alb and Glo_Alb were retrieved in the roots of uninoculated (−M) and inoculated (+M) plots, respectively, whereas Rhizo1_Alb was found in similar abundance in −M and +M (47% and 53%, respectively) (Figure 6a). In Frassineto, Claro1_Alb and Claro2_Alb were only retrieved in −M, whereas Rhizo1_Alb was much higher in +M than −M (83% and 17%, respectively) (Figure 7b). In the modern genotype, Bologna, Rhizo2_Alb and Claro2_Alb were retrieved in −M, whereas Fun_Alb in +M. Rhizo1_Alb was not retrieved in this genotype, either inoculated or not (Figure 7c). Looking at the relative abundances of AMF MOTUs within roots, in uninoculated Autonomia B, we found 28% and 72% of Rhizo1_Alb and Fun_Alb, respectively, whereas inoculation promoted the presence of Glo_Alb (73%), while the relative abundance of Rhizo1_Alb did not change (Figure 7d). In uninoculated Frassineto, relative abundances of Rhizo1_Alb, Claro1_Alb and Claro2_Alb were 11%, 21% and 68%, respectively, whereas inoculation determined a shift to 100% Rhizo1_Alb. Finally, in uninoculated Bologna we found 59% and 41% of Rhizo2_Alb and Claro2_Alb, respectively, whereas inoculation determined a shift to 100% Fun_Alb.

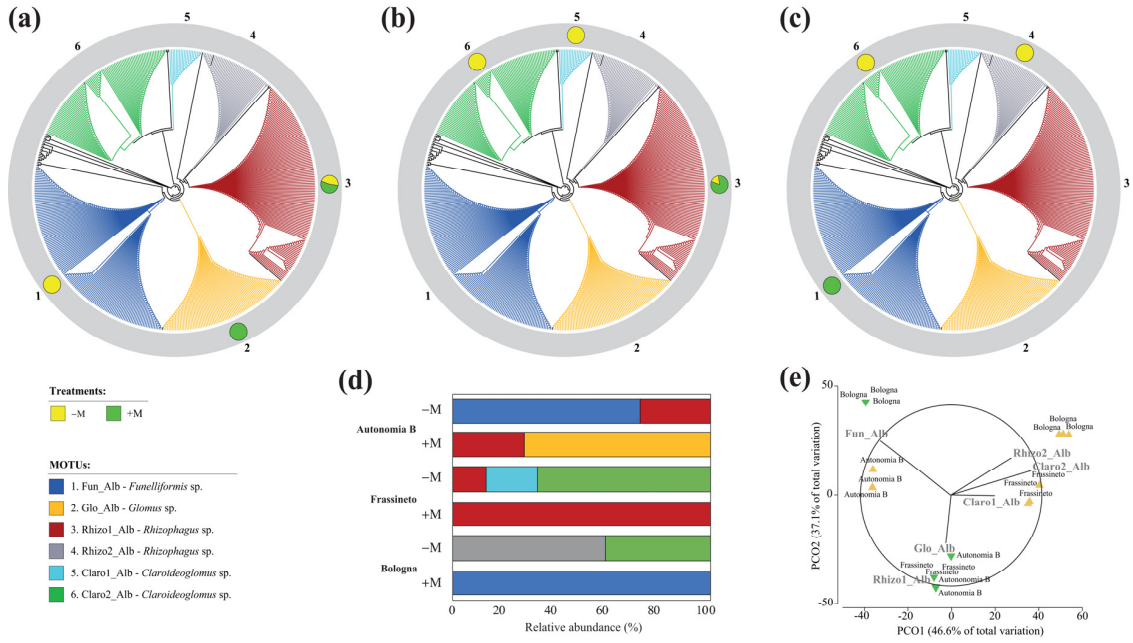

Figure 7. Neighbor-Joining trees of 504 AMF sequences retrieved in the uninoculated and inoculation roots of Autonomia B (**a**) Frassineto (**b**) and Bologna (**c**) and of 31 AMF reference sequences. For each MOTU, the proportion of sequences retrieved in the mock inoculated (−M) and inoculated roots (+M) are represented by the pie charts in (**a**–**c**). Relative abundances of AMF MOTUs found within the roots of Autonomia B, Frassineto and Bologna (**d**). Principal Coordinates analysis on the effect of AMF inoculation and wheat genotype on AMF community diversity. In the PCO biplot, the AMF MOTUs are displayed as arrows. The circle with a diameter is 1.0 and allows the reader to understand the scale of the vectors in the vector plot (**e**).

PERMANOVA results statistically confirmed the observed differences in composition shown in the bars of Figure 7d. AMF community composition and structure were significantly affected by AMF inoculation and wheat genotypes mean effects and their interaction, explaining 16, 27, and 56% of total variance, respectively (Table 1 and Table S3). In the PCO biplots, samples clustered into three groups (Figure 7e). Uninoculated Bologna and Frassineto showed a similar AMF community pattern, characterized by a high abundance of Rhizo2_Alb, Claro1_Alb and Claro2_Alb, whereas in inoculated Frassineto and Autonomia B, AMF communities were characterized by a high abundance of Rhizo1_Alb and Glo_Alb. Finally, uninoculated Autonomia B and inoculated Bologna showed similar AMF communities with a high abundance of Fun_Alb.

Table 1. Variation partitioning of the effects of wheat genotype and AMF inoculation on AMF community composition and structure.

	Explained Variance
AMF inoc [1]	15.67 [2]
Genotype	27.25
TIL × N fert	56.13

[1] PERMANOVA was performed following a split-plot design with AMF inoc as main-plot factor and genotype as subplot factor and with three replicate plots per treatment: AMF inoc (inoculated and mock inoculated) and genotype (Autonomia B, Frassineto and Bologna). [2] In bold statistically significant values in the PERMANOVAs ($P \leq 0.05$) (see Table S3).

4. Discussion

In this field study, it was shown that (i) field AMF inoculation increased AMF root colonization, root biomass and shoot Zn concentration at early growth stage and grain Fe concentration at harvest; (ii) bread wheat genotypes widely differed for shoot Zn concentration at early growth stage and for plant growth parameters, Zn and protein concentration at harvest; (iii) AMF inoculation differentially modified the AMF community composition and structure in the roots of the old genotypes, Autonomia B and Frassineto, and of the modern variety, Bologna; (iv) the establishment of the inoculated AMF isolate was proved only in Frassineto and its occurrence was associated with an increase of plant growth and grain Zn content.

4.1. AMF Root Colonization

At the end of tillering, the increased root colonization, following field inoculation with *R. irregularis*, demonstrated the compatibility of all wheat genotypes with the inoculated AMF taxa and its good competitive ability over the native AMF. These results agree with data recorded at tillering and anthesis in field-grown bread wheat inoculated with *R. irregularis* [as *G. intraradices*] or with an AMF polispecies inoculum [56–58]. Conversely, at physiological maturity, similarly to the results of Renaut et al. [59], root colonization was no longer affected by AMF inoculation, indicating that at this stage, the system became saturated (independent from initial infectious propagules concentration, including native ones).

4.2. Plant Growth and Micronutrient Uptake at GS30

Plants responded to AMF inoculation also with an enhancement of root size at the end of tillering (GS30). The enhancement was similar among old wheat genotypes and sligthly lower in the modern variety, Bologna. By contrast, at tillering, Siddique et al. [60] reported a higher root dry weight in old wheat varieties compared to modern ones. The variability in response of genotypes could be related to the presence of limiting conditions for soil nutrient availability. Indeed, some wheat genotypes are able to increase root development in conditions of nutrient deficiency, whereas under optimal nutrient conditions, this mechanism is not applied, as it determines an unnecessary energy cost for the plant. In this regard, Rengel and Graham [61] reported a differential response in terms of root

growth for Zn-inefficient and Zn-efficient genotypes, with increased root growth for Zn-inefficient genotypes and unchanged root growth for Zn-efficient genotypes. Therefore, in our study, we can suppose that all genotypes are Zn-efficient, since root dry weight did not change, despite plants grown at low availability of Zn.

Inoculation increased Zn concentration in shoots at GS30, suggesting an efficient establishment of the inoculated AMF. Conversely, in an experiment of field inoculation with the same AMF isolate, at early crop stages, Ercoli et al. [21] did not find any difference in Fe and Zn concentration in shoots and roots of durum wheat. This can be explained by multiple factors, such as soil Zn deficiency found in the present study, differential response of wheat species/varieties and a large compatibilty at early stages of bread wheat with the inoculated AMF isolate [22].

4.3. Plant Height, Grain Yield and Yield Components

The old genotypes differed from the modern variety, Bologna, in terms of crop height, grain yield and yield components. Specifically, old genotypes were generally less productive than Bologna. In most genotypes, the decrease of grain yield was due to the decrease in the number of kernels per spike, which was not counterbalanced by the increase of kernel size.

Wheat yield worldwide has shown a noteworthy increase during the 20th century, as a consequence of the adoption of better crop management practices and cultivars with higher yield potential [4,62,63]. In Italian wheat genotypes, yield gain due to breeding in the 20th century was about 2.5 Mg ha^{-1} (from 1 to 3.5 Mg ha^{-1}) [4], and this increase is in line with the difference recorded from the lowest yielding genotype, Grano Noè di Pavia, and the modern variety, Bologna.

The variations in yield components in the old genotypes compared to the modern variety are consistent with the modifications in plant morphology, resource partitioning and crop phenology induced by crop breeding [7]. The increase in the number of grains per unit area has been the main cause of genetic yield gain in bread wheat, and at least part of the increase in the number of grains per unit area can be attributed to a pleiotropic effect of the Rht-B1 dwarfing gene [64,65]. In Italian genotypes, it was estimated in 60 years, an increase of 0.14 grains spike^{-1} per year^{-1}, corresponding to 8.6 grains per spike [64]. Our data confirm this result, with the increase of 11.1 grains per spike in the modern variety, Bologna, compared to the average number of grains of the old genotypes. Ormoli et al. [66], comparing Italian local landraces cultivated in early 20th century to varieties released in 1997–2006, found a decrease in mean kernel weight (11%), while Alvaro et al. [64] found a similar decrease in mean kernel weight (7.3%), only in the near-apical spikelets comparing old to modern varieties.

AMF inoculation did not modify grain yield, owing to a decrease in the number of spikes per square meter and to an increase in the number of kernels per spike that led to unchanged grain number. Similarly, in the same pedoclimatic conditions, AMF inoculation to soil did not affect grain yield of durum wheat [21]. However, according to the synthesis of wordwide field experiments, wheat grain was increased by 20% with field AMF inoculation [20]. The disagrement about the yield benefits is likely to be due to the high availability of macro-nutrients, such as N and P, in our study. Indeed, high nutrient availability is generally negatively correlated with the output of the symbiosis [67,68]. The clayey soil texture can also explain the disagreement, since AMF are generally developing better in sandy soil. Moreover, since wheat yield is positively related to the rate of AMF root colonization [20], in our study, similar AMF root colonization at maturity is consistent with the lack of response of grain yield to AMF inoculation.

4.4. Plant Nutrient and Micronutrient Concentrations at Maturity

The present study showed a large variation among organically grown wheat genotypes for Fe, Zn and protein concentration in grain. The concentration of Fe and Zn across old genotypes ranged from 35.2 to 41.3 mg kg^{-1} for Fe and from 33.8 to 46.0 mg kg^{-1} for Zn. The modern variety, Bologna, compared to the old genotypes, had similar Fe concentration and lower Zn concentration in grain.

Protein concentration ranged from 14.3 to 16.8 % across old genotypes and was lower in Bologna than in most genotypes (14.4%).

In our experiment, plants were grown under organic management and in soil conditions corresponding to high availability for P, medium availability for Fe and low availability for Zn. In conditions of reduced availability of nutrients, the increases of Fe and Zn concentration in grain are mediated by morphological and physiological root traits, and depend on genotypes carrying gene encoding for efficient mineral uptake and translocation [69,70]. Thus, the differences observed among varieties for Zn concentration in grain suggest significant differences in the genes regulating for efficient mineral uptake and translocation to the developing grain.

The AM symbiosis is known for improving plant access to mineral nutrients, through the development of an extraradical mycelium, consisting of a complex and extensive network of hyphae spreading into the soil [71]. The active role of AMF in the uptake and transport of minerals needed for plant growth has been demonstrated in various plant/AMF isolate combinations (e.g., P [72,73]; N [74,75]; Zn [22,76]; Fe, Cu, Mn [19]. The effectiveness of the AM symbiosis for plant nutrient uptake was studied in bread wheat (*Triticum aestivum* L.) [77–79] and durum wheat (*Triticum durum* Desf.) [80]. Differences in the level of association of AMF with particular genotypes were found in four Canadian spring wheat cultivars [81] and in five genotypes of durum wheat, selected to represent five subpopulations of a collection of 93 diverse accessions from several countries [82]. Thus, the high variability of response according to the combination of genotype and fungus suggests that compatibility and dependency between plant and fungus are important factors for increasing grain nutrient content in wheat.

4.5. Arbuscular Mycorrhizal Fungal Community Diversity

In uninoculated conditions, two MOTUs were retrieved in the roots of Autonomia B and Bologna, belonging to two genera (*Funelliformis* and *Rhizophagus* in Autonomia B; *Rhizophagus* and *Claroideoglomus* in Bologna), whereas three MOTUs, belonging to two genera (*Rhizophagus* and *Claroideoglomus*), were retrieved in Frassineto. Several authors found similar numbers of AMF MOTUs within the roots of bread wheat [83–85], whereas a much higher number of MOTUs was found in durum wheat [18]. Given the low organic carbon content (SOC) found in the soil of our study, it is not surprising we retrieved a lower number of MOTUs than those found in other organic systems with high SOC [86,87].

Although this study is based on analyses at genus level and does not apply a next-generation sequencing approach, it seems to have captured distinctive differences in the AMF communities of the three wheat genotypes (Autonomia B, Frassineto and Bologna), as previously shown for several land-use types [88]. This is probably due to the adequate sampling of the root AMF communities of all genotypes. The approach of analyzing the AMF community structure within roots is confirmed as insightful and robust for revealing fungal-plant genotypes compatibility [89,90]. This approach was proved successful for AMF and other microbes in bulk and rhispheric soil [91–95].

In uninoculated conditions, a differential AMF pattern was found in the three wheat genotypes, supporting the strong effect of host plant identify on root AMF communities [91,96–98]. As above discussed, Singh et al. [82] and Ercoli et al. [21] found a large genetic variability in AMF compatibility and dependency in durum wheat. They also evidenced that the interaction between plant × AMF genotype affected grain and straw biomass production, nutrient straw content and nutrient uptake in grain (i.e., P, N, K, Fe, Zn and Cd).

Altough the applied SSU molecular markers do not allow discriminating the inoculated AMF isolate from the native AMF strains belonging to *Rhizophagus* genus, the strong increase of the relative abundance of a MOTU affiliated to *Rhizophagus* (Rhizo1_Alb; VTX00105) in inoculated Frassineto indicates that Rhizo1_Alb is likely to be the inoculated isolate. However, in Autonomia B, the Rhizo1_Alb was not modified, whereas in Bologna it was not retrieved. Similarly to our results, the same AMF isolate inoculated on two genotypes of durum wheat was found at physiological maturity within crop roots, by tracing the SSU region [21]. Moreover, the inoculated *R. irregularis* MUCL41833

isolate was retrieved at harvest only in one over two potato cultivars, tracing a more discriminant gene (mitochondrial large ribosomal subunit, mtLSU RNA gene) [99]. Previous works, tracing the SSU RNA gene, the internal transcriber spacer 2 (ITS2) and the large subunit (LSU), successfully discriminated between inoculated and native isolates, and found in *Medicago sativa* the inoculated *Funneliformis* isolates AZ225C and IMA1 at early crop growth stages, whereas two years after inoculation only AZ225C was retrieved [42]. Other authors did not detect, using the mtLSU marker, the inoculated AMF isolates [59] or could only detect minor effects in several crops [100]. The failure of detection of the inoculant might be due to the specificity of the markers or to the complex factors affecting field AMF inoculation, such as viability/infectivity of the inoculated isolates or incompatibilities with soil or host crop [101,102]. Moreover, the competition with other indigenous microorganisms or an inadequate localization of the inoculant might co-affect the establishment, persistence and efficiency of the inoculation. Overall, our results indicated that the increase of plant growth and grain Zn content of Frassineto is likely linked to the high *R. irregularis* abundance. However, inoculation per se can lead also to changes in some plant-soil-microbial parameters (e.g., root exudates, nutrient availability, microbial community shifts) [103,104] and these may determine some indirect effects, such as plant growth and micronutrient uptake. Therefore, Frassineto can be considered the wheat genotype mostly compatible with the inoculated AMF isolate. However, since we revealed strong changes in the native AMF community composition and structures following the inoculation with a non-native isolate, some concerns can be raised about long-term effects of species invasions that may cause agroecological damage [102,104].

5. Conclusions

Biofertilizers, such as AMF inoculants, are an alternative source to meet the nutrient requirements of wheat and are of great importance in organic agriculture. In this study, carried out in soil with Fe medium availability and Zn low availability, we demonstrated that wheat micronutrient content can be promoted by combined reliance on efficient genotypes and AMF field inoculation. Iron concentration in grain was increased with inoculation, whereas grain yield and Zn concentration did not vary. The increase of Fe and Zn in grain depends on genotypes carrying gene encoding for efficient mineral uptake and translocation or for high compatibility between plant and fungus. The old wheat genotypes could be a good source of genes for the enhancement of micronutrient content of grain and for the responsiveness to AMF inoculation. However, the variable response of wheat genotypes should be taken into consideration for the introduction of field AMF inoculation in the ordinary management techniques of cereal farms and for planning breeding strategies aiming to increase Fe and Zn in grain. Nevertheless, the invasiveness and dispersal of the inoculated AMF isolate and the changes of the AMF native communities may pose a threat to soil and plant biodiversity and ecosystem functions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/3/333/s1, Table S1: list of retrieved AM sequences and corresponding virtual taxa, Table S2: effect of AMF inoculation and bread wheat genotype on AMF richness and diversity indexes, Table S3: PERMANOVA of the effect of AMF inoculation and bread wheat genotype on AMF community structure in roots, Figure S1: collapsed NJ tree of retrieved and reference AMF sequences, Figure S2: NJ tree of retrieved and reference AMF sequences.

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