




Native mycorrhizal communities in maize roots as affected by plant genotype, starter fertilization and a seed-applied biostimulant

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Abstract

Background and aims One of the most promising strategies for sustainable intensification of crop production involves the utilization of beneficial root-associated microorganisms, such as plant growth-promoting bacteria and arbuscular mycorrhizal fungi (AMF). The aim of this study was to investigate whether a seed-applied biostimulant, based on the bacterial strain *Bacillus amyloliquefaciens* IT-45 and a plant polysaccharide extract, and crop enhancement tools, such as hybrids with contrasting early vigor and nitrogen (N) plus phosphorus (P) starter fertilization,

and their interactions, shape the communities of native root-colonizing AMF symbionts in maize.

Methods A factorial growth chamber experiment was set up with two maize genotypes in natural soil. Mycorrhizal colonization was evaluated after root staining. The diversity and composition of AMF communities were assessed by PCR-DGGE of the 18S rRNA gene and amplicon sequencing.

Results N and P fertilization determined a consistent reduction of AMF root colonization and, in combination of biostimulant, a reduction of AMF richness. The biostimulant alone generally did not affect AMF colonization or the community biodiversity. In addition the effect of the two factors were modulated by maize genotype. In all treatments, predominant AMF were represented by *Glomus* sp. and *Funneliformis mosseae*, while populations of the genus *Rhizoglomus* were rarely detected in biostimulant and NP fertilization treatments.

Conclusion The results of this study increase our understanding of how the biostimulant seed treatment may affect native AMF communities, depending on NP fertilization and maize genotype and may improve the implementation of innovative tools in sustainable and resilient agroecosystems.

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Introduction

In recent years, concerns have been raised on the consequences and limits of conventional intensive agriculture. Inorganic fertilizer use, which is related mainly to the application of plant macronutrients, nitrogen (N), phosphorus (P) and potassium (K), was estimated to reach 159.4 million tonnes worldwide in 2019, showing continuously increasing tendencies with an annual average growth of 1.5% until 2022 (FAO 2019). The use of inorganic fertilizers, although it supports a greater crop productivity, may negatively impact greenhouse-gas emissions (Robertson and Vitousek 2009), soil health, fertility, microbial diversity (Lazcano et al. 2013; Sun et al. 2015) and also induce soil acidification (Juo et al. 1995; Matsuyama et al. 2005; Guo et al. 2010), while it also enhances the eutrophication of water bodies (Carpenter et al. 1998; Withers and Haygarth 2007). Several studies, aimed at boosting the transition of agriculture towards sustainable intensification (The Royal Society 2009; Foley et al. 2011), focused on below-ground beneficial traits and improved nutrient-use efficiency of new plant varieties (Wissuwa et al. 2009; Veneklaas et al. 2012; Schmidt et al. 2016), as well as on the selection of soil microbial communities showing plant growth-promoting potentials to be used as biofertilizers, biostimulants and biopesticides (Berg 2009; Menendez and Garcia-Fraile 2017; Ke et al. 2021).

Among beneficial soil microbes, plant growth-promoting (PGP) bacteria, a group defined by its specific metabolic traits, are of great importance. Large populations of PGP bacteria live strictly associated with plant roots, in the rhizosphere and root endosphere, improving plant nutrient status and tolerance to numerous biotic and abiotic stresses via nutrient solubilization and mobilization, phytohormones, siderophores, antibiotics and protective enzymes production and systemic resistance induction (Berg 2009; Hayat et al. 2010; Mendes et al. 2013; Gouda et al. 2018). For their beneficial effects on plant growth, PGP bacteria are considered a promising tool as effectors of biostimulant and biofertilizer preparations, and their use begins to be more frequent in sustainable crop production (Zaidi et al. 2015; Ruzzi and Aroca 2015; Rouphael and Colla 2020). Among them, species of the genus *Bacillus* gained a specific attention in the research and development of novel microbial inocula, due to their vast functional diversity and endospore-forming

features – latter making them suitable for longer product shelf-life, comparable with that of conventional agrochemicals (Qiao et al. 2014; Amaresan et al. 2019; Borriss 2020). Strains of *Bacillus amyloliquefaciens*, in particular, were found to be potent and effective biostimulants and biocontrol agents in diverse crop species. In particular, such species possess many genes involved in root-bacteria interaction which allow them to efficiently colonize plant rhizosphere. The production of phytases present in several *B. amyloliquefaciens* strains, making organic phosphate available, improves the P supply for the plant. Moreover, their ability to modify the rhizosphere microbiota by stimulating beneficial microorganisms and decreasing fungal pathogens enhances their role in promoting plant growth (Qiao et al. 2014; Luo et al. 2022).

Arbuscular mycorrhizal fungi (AMF) represent another important group of beneficial soil microbes, given their key role in the enhancement of plant nutrition, health and product quality. AMF are non-host specific, obligate biotrophs that establish mutualistic symbioses with ca. 71% of land plants, including maize and other major food and industrial crops (Brundrett and Tedersoo 2018). Pre-symbiotic host recognition involves an intricate, yet not fully unveiled plant-AMF communication via molecular signals, such as strigolactones released by host roots, that trigger a differential hyphal branching (Giovannetti et al. 1993; Akiyama et al. 2005) and lipo-chitooligosaccharides released by fungal hyphae that lead to the expression of key genes allowing fungal root colonization (Maillet et al. 2011; MacLean et al. 2017). Following the establishment of the symbiosis, AMF obtain sugars and lipids from the host plant, and in return, AMF facilitate the uptake and transport of mineral macro- and micronutrients from the soil to the host plant via their extended extraradical mycelia (Giovannetti and Avio 2002; Smith and Read 2008; Luginbuehl et al. 2017). Moreover, AMF improve plant performance and health by increasing plant tolerance to biotic and abiotic stresses (Sikes et al. 2009; Bitterlich et al. 2018), and induce changes in the plant secondary metabolism leading to enhanced biosynthesis of health-promoting phytochemicals (Sbrana et al. 2014; Avio et al. 2018).

Maize (*Zea mays* L.) is one of the most important cereal crops worldwide with an annual average global grain production of more than 1.2 billion of tonnes (FAOSTAT 2022). The importance and benefits of AMF symbioses in maize have been demonstrated by

many studies, highlighting their roles in plant nutrition and health, and estimating their contribution to maize production at about one-third (Njeru et al. 2014; Mathur et al. 2018; Begum et al. 2019; Wang et al. 2020; Ramírez-Flores et al. 2020). Although most AMF species are considered generalist (Öpik et al. 2010), the most common AMF symbionts of maize are represented by *Glomus*, *Claroideoglomus*, *Funneliformis*, *Rhizophagus* and *Paraglomus* species that had a high frequency in maize roots and rhizosphere soils in contrasting agricultural conditions (Turrini et al. 2016; Wang et al. 2017; Higo et al. 2020; Alaux et al. 2021).

So far, many AMF utilised in agriculture, represented by commercial allochthonous inocula, failed to establish in field conditions (Farmer et al. 2007; Verbruggen et al. 2013; Berruti et al. 2017), therefore, the stimulation and preservation of native AMF communities could be a promising strategy to exploit their benefits. Native AMF communities of agricultural fields may be affected by many factors, e.g. soil tillage (Jansa et al. 2003; Higo et al. 2020), crop species, plant genotype (Oliveira et al. 2009; Turrini et al. 2016; Kavadia et al. 2020) and fertilization practices (Zhu et al. 2016a; Wang et al. 2017; Jeske et al. 2018), and also by bacterial inocula which were able to cause shifts in AMF communities (Dagher et al. 2020). Moreover, combinations of mineral fertilization and PGP microbial inoculation may strongly modulate crop-AMF interactions in maize (Sarabia et al. 2017). As a consequence, the possible interactions between autochthonous beneficial soil microbiota and microbial biostimulants should be considered. Previously, strains of *B. amyloliquefaciens* were found to express strong antifungal activities (Ji et al. 2013; Gu et al. 2017; Saechow et al. 2018; Kazerooni et al. 2021; Diabankana et al. 2022), which could inhibit native AMF, too. Actually, *B. amyloliquefaciens* BNMI22 inhibited soybean colonization by two AMF (Correa et al. 2009), while the strain FZB42 and another strain isolated from a commercial inoculant, showed mycorrhizal enhancing properties in specific co-inoculation experiments using various host plants (Xie et al. 2018; Wilkes et al. 2020).

The biostimulants applied to the seed, as well as the application of the NP starter fertilizer banded close to seed furrow at sowing and the use of cold-tolerant hybrids with high early vigor are some of the agronomic practices that can be applied in the first growth stages of maize cultivation in order to

enhance a rapid development of seedlings and young plants, with significant agronomic, productive and qualitative benefits (Blandino et al. 2022). Since these crop techniques could boost a plant response in the early growth stages, which determine a faster root system establishment with a more extended root volume and a higher root exudation of organic acids (Hund et al. 2008; Zhu et al. 2016b), the study of their impact on maize-AMF interaction, which occurs mainly in the first growth stages, is of great interest (Njeru et al. 2014). The goal of this study was to evaluate whether a seed-coating biostimulant based on the bacterial strain *B. amyloliquefaciens* IT-45 and a plant polysaccharide extract, and crop enhancement tools, such as hybrids with contrasting early vigor, and NP starter fertilization, and their interactions, shape the communities of native root-colonizing AMF symbionts in maize. To this aim, a factorial growth chamber experiment was set up in natural soil. We estimated the colonization potential of native AMF communities by Trypan blue staining of maize roots, and assessed the diversity and composition of root-colonizing AMF communities utilizing a culture-independent approach, i.e. PCR-DGGE (Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis) separation of the 18S ribosomal RNA (rRNA) gene and amplicon sequencing (Johnston-Monje and Lopez Mejia 2020).

Materials and methods

Microcosm experiment

A growth chamber experiment was set up with maize plants in order to investigate the effects of NP starter fertilization and a seed-applied microbial biostimulant and their interaction on the native AMF communities of an agricultural soil, considering their relationship with two plant genotypes. Natural, sub-alkaline silty loam soil (Typic Ustifluvents, USDA classification) was collected from the surface layer of a field at the experimental station of the University of Turin, Carmagnola, North-West Italy (44° 53' N, 7° 41' E; elevation 245 m). Physical and chemical properties of the soil are summarized in Table S1. The used soil is representative of the agronomic management of the conventional cereal farm of the growing area, in which maize and wheat are the most frequent crops,

previous crop debris were buried through ploughing each year and no manure, slurry or other amendments have been distributed.

The untreated soil was mixed thoroughly and filled in plastic pots with a volume of ca. 18 L each. Treatments were factorial combinations of different maize genotype, selecting two hybrids within the FAO 600 maturity class which showed a contrasting early vigor trait (LG 30600® ordinary or LG 31630® Rapid'START® high early vigor hybrid) (Limagrains Europe, Saint-Beauzire, France), NP starter fertilization (unfertilized control or sub-surface fertilized soils with diammonium phosphate [DAP]) and biostimulant seed treatment (no biostimulant or Starcover® [Limagrains Europe] biostimulant applied as a seed coating). Sub-surface NP starter fertilization was provided at a rate of 27 kg N ha⁻¹ and 30 kg P ha⁻¹ 10 cm deep in the hypothetical seed furrows, at 5 cm distance from the plantlets. The applied commercial biostimulant consisted of a phosphate mobilizing PGP bacteria, *B. amyloliquefaciens* IT-45 (Rise P®, Lallemand Plant Care, Castelmaurou, France) and the extract of a leguminous plant, *Cyamopsis psoraloides* (AgRHO® GSB30, Solvay, Clamecy, France), based on the polysaccharide hydroxypropyl guar hydroxypropyltrimonium chloride, which works as coating film to favor germination by channeling water from soil to seed. The experimental design was a completely randomized design with a factorial combination of two maize hybrids x two starter fertilization x two seed biostimulant treatments and three replicates; overall 24 pots have been prepared. In each pot, four maize seeds were sown, which were reduced to two seedlings after germination at 13 days after sowing (DAS). The pots were placed in a controlled growth chamber with 50% relative humidity range, 12 h photoperiod, 700 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR) and 14/17 °C (night/day) air temperature range (Table S2). Soil moisture content was maintained at water holding capacity by providing each pot with 0.57 L of water/week, corresponding to 10 mm of rainfall. Weed control was carried out manually. The experiment was terminated 49 DAS at 5-leaf stage.

Sample collection and preparation

At 49 DAS, plants were collected, and the total dry biomass was determined after oven drying at 105 °C

for 24 h. Data were expressed in grams per plant of dry weight (d.w.).

A 250 mg aliquot of clean fresh roots from plants were utilized for DNA extraction and successive molecular analyses. Aliquots of 0.5–1 g of fresh roots were utilized for Trypan blue staining in order to determine root colonization by AMF.

Determination of AMF colonization in roots

Percentage of mycorrhizal root length was determined after clearing and staining, as described in Turrini et al. (2017). Briefly, 0.5–1 g of root samples were cleared in 10% KOH in an 80 °C water bath for 15 min, neutralized in 2% aqueous HCl, and stained with 0.05% Trypan blue in lactic acid. The percentage of AMF colonization was calculated using a dissecting microscope (Wild/Leica, Wetzlar, Germany) at ×25 or ×40 magnification and the gridline intersect method (Giovannetti and Mosse 1980).

DNA extraction

250 mg root samples were homogenized in sterile mortars with liquid N₂ and then subjected to genomic DNA extraction using the DNeasy® Power Soil® Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. The extracted DNA was stored at –20 °C and subsequently used for the molecular analysis of root-colonizing AMF communities.

Molecular analysis of AMF community profiles with PCR-DGGE

For Denaturing Gradient Gel Electrophoresis (DGGE), a fragment of the AMF 18S rDNA was amplified in a semi-nested protocol. The first Polymerase Chain Reaction (PCR) was carried out using the primers NS31 (5'-TTG GAG GGC AAG TCT GGT GCC-3') and AM1 (5'-GTT TCC CGT AAG GCG CCG AA-3') (Eurofins, Ebersberg, Germany) (Simon et al. 1992; Helgason et al. 1998). Reaction mixes were prepared in a final volume of 50 μL, containing 1 μL of undiluted DNA extract from roots. Each reaction mixture contained 5 μL of ExTaq Buffer 10× (Takara Bio Inc., Kusatsu, Japan), 1.25 U of ExTaq (Takara Bio Inc.), 0.2 mM of each

dNTP (Takara Bio Inc.), 0.5 μM of both primers and 0.3 mg/mL Bovine Serum Albumin (BSA) (Promega Corp., Madison, WI, USA). The reaction was carried out in an iCycler-iQTM Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the following thermal cycles: initial denaturation at 94 °C for 1'; 35 cycles of denaturation – annealing – elongation at 94 °C for 30", at 66 °C for 30" and at 72 °C for 30", respectively; and final elongation at 72 °C for 5'.

The expected product was about 550 bp long. The presence of amplicons was confirmed by electrophoresis in 1.5% (w/v) agarose gels in 1×TBE buffer (Tris–borate-EDTA, pH 8.3) (AppliChem GmbH, Darmstadt, Germany) stained with 20,000×RealSafe Nucleic Acid Staining Solution (Durviz s.l., Valencia, Spain). A 100 bp DNA ladder (Thermo ScientificTM) was used as a molecular weight marker. DNA fragments were visualized using UV light (Uvitec Cambridge, Cambridge, UK), and captured as TIFF files using the UVI 1D v. 16.11 program (Uvitec Cambridge).

For the subsequent semi-nested PCR, the primers NS31 and Glo1 (5'-GCC TGC TTT AAA CAC TCT A-3') (Eurofins) were used (Cornejo et al. 2004). The primer NS31 had an additional GC-clamp (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG-3') on the 5' end to prevent complete DNA denaturation during the DGGE process. Reaction mixes were prepared in a final volume of 50 μL , containing 1 μL of 1:10 diluted DNA amplicon from the first reaction. The composition of the mixture and the thermal cycle were the same as above, except BSA was not added, and the annealing temperature was set to 56 °C. The expected product was about 230 bp long. The presence of amplicons was confirmed by agarose gel electrophoresis, as described above.

For the DGGE analysis, 20 μL of amplicon DNA was mixed with 20 μL of 2×loading dye, as described in Palla et al. (2020), and the mixture was separated in 8% (w/v) polyacrylamide 4 K (AppliChem GmbH) gels in the DCodeTM Universal Mutation Detection System (Bio-Rad). The urea-formamide denaturing gradient was 20–55%. Amplicon DNA from the biostimulant treated, 5-leaf stage sample of the high early vigor hybrid was loaded on both sides and in the middle of the gels as marker (M). Gels were run at 50 V for 16.5 h in 1×TAE buffer

(Tris–acetate-EDTA, pH 8.5) (AppliChem GmbH) at 60 °C. Subsequently, gels were stained in 1×TAE buffer with 10,000×diluted SYBR Gold Nucleic Acid Gel Stain (Thermo Fischer Scientific, Waltham, MA, USA) and visualized as previously described.

DGGE profile analysis

DGGE profiles were digitally processed with the BioNumerics software v. 8.1 (Applied Maths, St-Martens-Latem, Belgium) as reported in Palla et al. (2020). Similarities between DGGE profiles were calculated with Pearson's similarity coefficients applied on the lane patterns using the band-matching tool with 1% of optimization. The similarity coefficients were then used for generating dendrograms with the Unweighted Pair-Group Method With Arithmetic Mean (UPGMA) cluster analysis.

Based on the banding data, and treating each band as an individual operational taxonomic unit (OTU), six different diversity indices were calculated. Richness (S) indicated the number of OTUs detected in the sample. Shannon–Weaver's diversity (H_s) and Simpson's dominance (D) indices were calculated as $H_s = \sum_{i=1}^n -\frac{h_i}{H} \cdot \ln \frac{h_i}{H}$ and $D = \sum_{i=1}^n \frac{h_i \cdot (h_i - 1)}{H \cdot (H - 1)}$, where h_i was the peak intensity of a band and H was the sum of all peak intensities in a sample. Evenness (J_p) allowed to reveal the presence of dominant OTUs, calculated as $J_p = \frac{H_s}{\ln S}$. Hill 1 (H_1) and Hill 2 (H_2) numbers were computed as $H_1 = \frac{1}{D}$ and $H_2 = e^{H_s}$, respectively.

DGGE band sequencing

In order to identify major AMF taxa colonizing the roots of samples, the main bands of the DGGE profiles were cut from the gel for further molecular analysis. Bands were eluted in 50 μL UltraPureTM DNase/RNase-free distilled water (Invitrogen, Waltham, MA, USA) for one day at room temperature. Supernatants were diluted 1:100 and served as templates for PCR using the primers NS31 and Glo1 without GC-clamp, following the protocol described earlier. PCR products were then purified with the QIAquick[®] PCR Purification kit (Qiagen GmbH) according to the manufacturer's instructions. Purified amplicons were eluted in 50 μL H₂O and controlled in a 2% agarose gel to confirm product quality, and their concentration for dsDNA was estimated with an Eppendorf

Biophotometer (Eppendorf SE, Hamburg, Germany) measuring at $\lambda=260$ nm. Partial 18S rDNA amplicons were both 5' and 3'-end sequenced by Eurofins Genomics—Mix2Seq Custom DNA Sequencing Services (Ebersberg, Germany). Sequences were aligned and analysed as in Palla et al. (2020), using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) in the NCBI-GenBank (<https://www.ncbi.nlm.nih.gov/genbank>) and MaarjAM (<http://maarjam.botany.ut.ee>) (Öpik et al. 2010) databases, accessed in December, 2022. Related sequences were collected and aligned with the MUSCLE tool (Edgar 2004a, b) in the MEGA 11 software (Tamura et al. 2021). Phylogenetic trees were constructed using the neighbour-joining method based on Kimura's 2-parameter model (Kimura 1980) in MEGA 11 with 1000 bootstrap replicates. The DGGE band sequences were submitted to the NCBI-GenBank database under the accession numbers from OQ338371 to OQ338400.

Statistical analysis

All statistical analyses were carried out with the SPSS v. 25 software (IBM Corp., Armonk, NY, USA). Variances were submitted to Levene's test (p -value < 0.05 means variances are unequal) for testing variance homogeneity and data transformed, if needed. Data were assessed for normal distribution by Shapiro–Wilk test. Three-way ANOVAs (Analysis of Variance) were performed on the log transformed colonization and plant biomass values. Biodiversity indices of the ordinary hybrid showed heterogeneous variances even after transformation and were thus analyzed with a non-parametric test for unequal variances (two-ways Scheirer-Ray-Hare test). On the contrary, the early vigor maize hybrid was analysed with a two-way ANOVA. When interactions were significant (p -value < 0.05), a simple effects test was performed to determine the difference between groups at each level of each factor.

Results

Plant biomass and root colonization by AMF

Total plant biomass was strongly affected by starter fertilization, but a significant interaction between fertilization and biostimulant treatments was found

(p -value = 0.012, Table 1), since the fertilization increased maize total biomass after the addition of the biostimulant more than in its absence (+230% and +190%, respectively), in both hybrids (Table S3 and Fig. S1). In addition, in unfertilized plants the biostimulant did not increase maize biomass production. The high early vigor hybrid showed a significant larger biomass than the ordinary one (Table 1).

Average percent mycorrhizal colonization of maize by native AMF ranged between 5 and 54%, depending on the plant genotype, fertilizer, and biostimulant treatments (Table 1). A significant interaction among the three experimental factors was found (p -value = 0.027), showing that fertilization strongly reduced colonization compared to unfertilized plants, except for ordinary hybrid without biostimulant, as revealed by the pairwise comparisons of simple effects (Table S4 and Fig. S2). Application of biostimulant was effective only in the ordinary hybrid, which showed a reduced percent colonization, when associated to fertilization. The effect of genotype was significant depending on the fertilizer and biostimulant treatments. Actually, a higher level of colonization was observed in the early vigor maize hybrid compared to the ordinary hybrid, in the control treatment (unfertilized/No biostimulant), and when both fertilizer and biostimulant were applied (+146 and +98%, respectively).

Analysis of PCR-DGGE profiles

Arbuscular mycorrhizal fungal 18S rDNA fragments (ca. 230 bp) were successfully amplified in all root samples. The DGGE separation of amplicons revealed community profiles characterized by a varying number of bands of high intensity (Fig. 1). 5-leaf stage DGGE profiles were further investigated by cluster analysis (UPGMA), and biodiversity indices (S , H_s , D , J_p , H_1 , H_2) were estimated based on the banding patterns.

In the UPGMA cluster analysis (Fig. 2), within both maize genotypes, unfertilized samples clustered separately from those treated with NP fertilizer, showing similarities lower than 63.8% and 57.9% for the ordinary and the high early vigor hybrid, respectively. In the ordinary hybrid, a further division could be observed within the cluster of unfertilized samples, as Biostimulant and Unfertilized/No biostimulant samples grouped

Table 1 Effects of plant genotype, starter fertilization and biostimulant seed treatment on maize plant biomass and mycorrhizal colonization

Hybrid ¹	Treatment ²	Total plant biomass (g plant ⁻¹ d.w.) ± SD			Mycorrhizal colonization % ± SD		
Ordinary	Unfertilized/No biostimulant	0.89 ± 0.070			21.8 ± 6.0		
	Unfertilized/Biostimulant	0.97 ± 0.035			28.7 ± 3.0		
	NP/No biostimulant	2.77 ± 0.071			15.5 ± 5.9		
	NP + Biostimulant	3.49 ± 0.202			5.0 ± 2.3		
High early vigor	Unfertilized/No biostimulant	1.10 ± 0.042			53.6 ± 20.4		
	Unfertilized/Biostimulant	1.17 ± 0.199			36.9 ± 26.9		
	NP/No biostimulant	2.93 ± 0.194			14.4 ± 6.3		
	NP + Biostimulant	3.68 ± 0.222			9.9 ± 2.1		
ANOVA Results							
Source of variation		df	F	<i>p</i> -value	df	F	<i>p</i> -value
Hybrid		1	9.396	0.011	1	5.892	0.027
NP		1	1001.854	<0.001	1	50.210	<0.001
Biostimulant		1	21.034	0.001	1	6.110	0.025
Hybrid x NP		1	1.525	0.243	1	0.237	0.633
Hybrid x Biostimulant		1	0.017	0.898	1	0.004	0.948
NP x Biostimulant		1	8.960	0.012	1	3.614	0.075
Hybrid x NP x Biostimulant		1	0.022	0.885	1	5.815	0.028

The level of significance (*p*-value) is shown in the Table. For each treatment, the reported data are based on 3 observations ± standard deviation. ANOVA *p*-values of main effects and interactions, significant at *p* < 0.05, were highlighted with bold font

¹ Ordinary: LG 30600; High early vigor: LG 31630

² Unfertilized: no starter fertilization at sowing; NP: distribution of 27 kg N ha⁻¹ and 30 kg P ha⁻¹ as diammonium phosphate at sowing. No biostimulant: no maize biostimulant seed treatment; Biostimulant: maize seeds treated with *Bacillus amyloliquefaciens* IT-45 (Rise P®) and *Cyamopsis psoraloides* polysaccharide extract (AgRHO® GSB30)

separately, showing 72.4% similarity amongst them. In the high early vigor maize, no such separation was found, however, samples treated with NP + Biostimulant created a distinct cluster with 48.0% similarity to other samples and with relatively low (54.4%) internal similarities.

Concurrently, diversity indices were calculated from the community profiles. In the ordinary hybrid, two-ways Scheirer-Ray-Hare test revealed significant effects of NP fertilization on the richness index, showing higher biodiversity in unfertilized samples (Table 2). As for the high early vigor hybrid, two-ways ANOVA showed a significant interaction between NP fertilization and biostimulant on richness, Hill₁ and Hill₂ indices, so that NP + Biostimulant treatment resulted always in a clear reduction of AMF diversity.

DGGE amplicon sequencing and identification of the main AMF taxa

The main DGGE bands were excised from gels (Fig. 1) and sequenced in order to identify the relevant AMF genera, species and virtual taxa (VTX). According to the NCBI GenBank and MaarjAM databases, all partial 18S rDNA fragments belonged to a low number of AMF taxa in the family Glomeraceae, namely, to *Glomus* sp. (VTX00113, VTX00156), *Funneliformis mosseae* (VTX00067), *Rhizoglomus* sp. (VTX00115) and *Rhizoglomus intraradices* (VTX00114) (Table 3 and Fig. 3).

Virtual taxa of AMF were uniformly present in both maize hybrids, except for VTX00115 (*Rhizoglomus* sp.), which was occurring mainly in the high early vigor hybrid. In all treatments, VTX00067 (*F. mosseae*) appeared to be the predominant AMF

Fig. 1 PCR-DGGE profiles of the root AMF communities of two maize hybrids at the 5-leaf stage, treated or untreated with NP fertilization and with seed applied biostimulant. **a** ordinary hybrid. **b** high early vigor hybrid. The numbers indicate sequenced DNA fragments and the colored circles their relevant AMF genera, species (virtual taxa) affiliation. Marker (M)

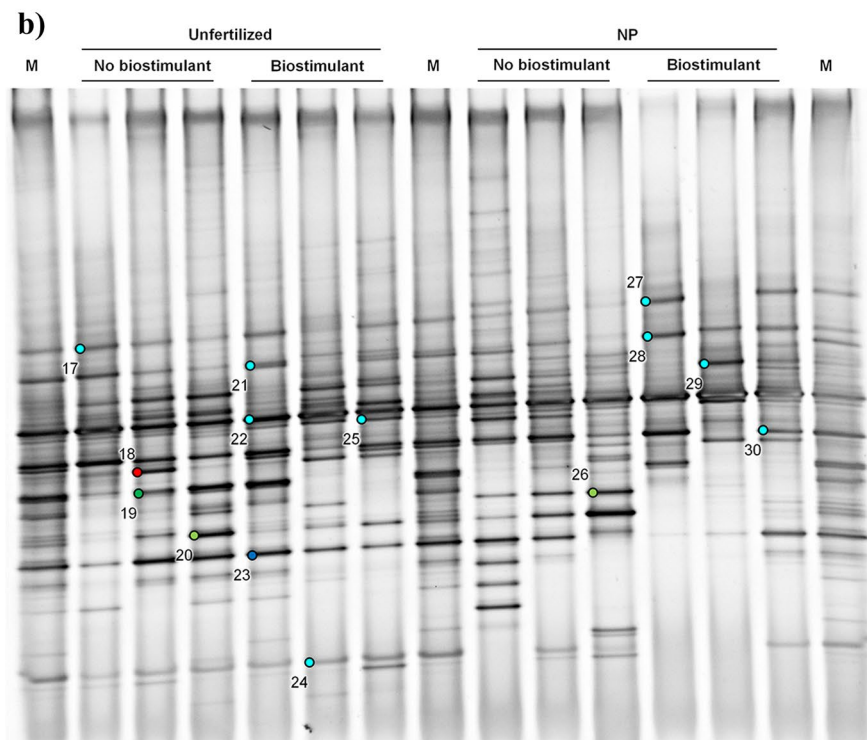
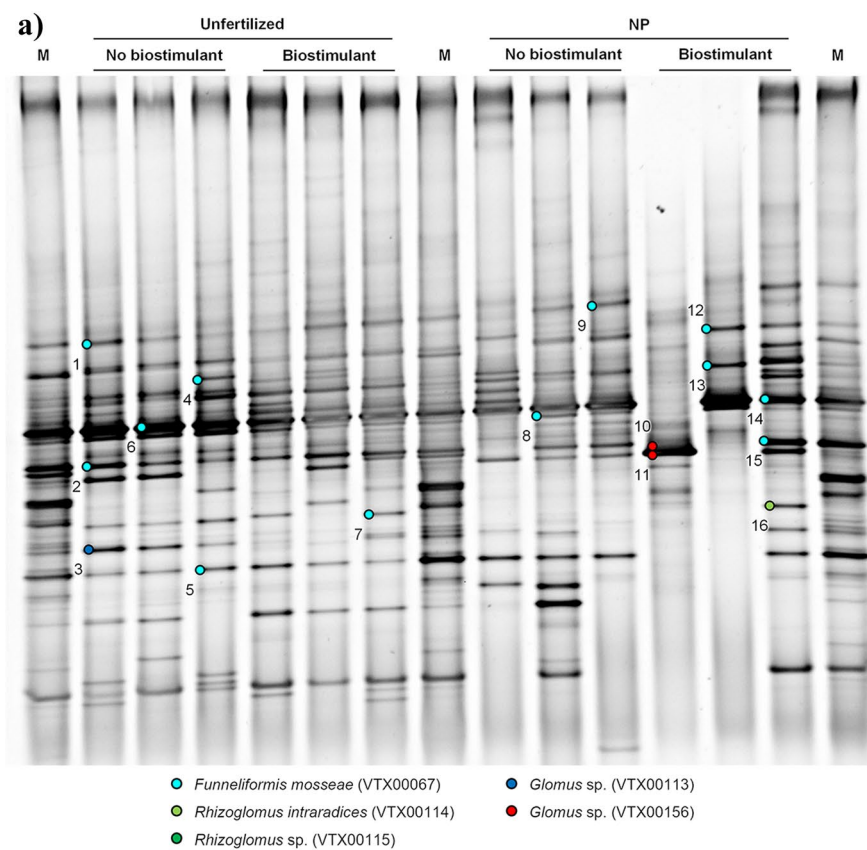
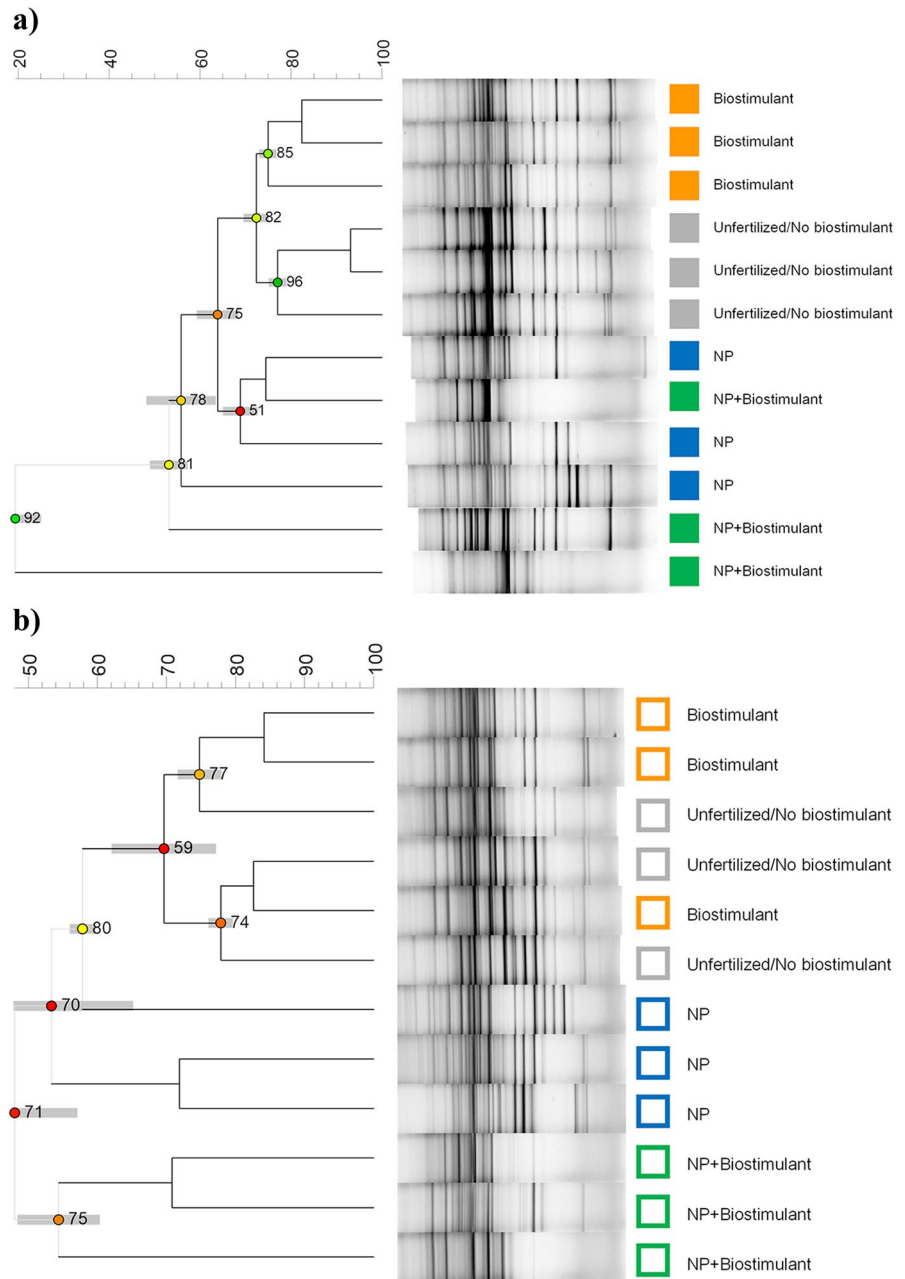


Fig. 2 Cluster analysis of AMF DGGE profiles indicating the relationships among samples, based on similarity, as shown by the numeric scale above each dendrogram, obtained by UPGMA (Unweighted Pair Group Method Using Arithmetic Average) analysis, using Pearson's similarity coefficients. Dendrograms are based on DGGE profiles of root-colonizing AMF communities in two maize hybrids at the 5-leaf stage, treated or untreated with NP fertilization and with seed-applied biostimulant. Closed symbols: Ordinary hybrid (a). Open symbols: High early vigor hybrid (b). No biostimulant: no seed application; Biostimulant: biostimulant seed treatment; NP: NP starter fertilization. Cophenetic correlation, expressing the consistency of clusters, is shown at each node by numbers and colored dots, ranging between green-yellow-orange-red, according to decreasing values. Standard deviation is shown at each node by a grey bar



colonizer of maize roots. Although the detected taxonomic diversity proved to be low in the samples, DNA fragments collected from different positions in the DGGE gels revealed a high intraspecific genetic diversity within VTX00067 (*F. mosseae*). Moreover, VTX00067 (*F. mosseae*) appeared to be resilient along all treatments, being frequently identified even in the NP+Biostimulant samples, while bands corresponding to VTX00115 (*Rhizoglyphus* sp.) and

VTX00114 (*Rhizoglyphus intraradices*) could be rarely detected in the double treatment.

Discussion

This study showed that mineral fertilization was the major driver of native AMF communities colonizing maize roots in an agricultural soil, while the

Table 2 Effects of starter fertilization and biostimulant seed treatment on diversity indices calculated from AMF 18S rDNA DGGE profiles of maize root samples at 5-leaf stage

Hybrid ¹	Treatment ²	Richness (S) \pm SD	Evenness (Jp) \pm SD	Hill 1 (H_1) \pm SD	Hill 2 (H_2) \pm SD	
Ordinary	Unfertilized/No biostimulant	13.7 \pm 0.58	0.94 \pm 0.003	10.58 \pm 0.497	11.81 \pm 0.520	
	Unfertilized/Biostimulant	12.7 \pm 1.53	0.96 \pm 0.009	10.74 \pm 1.680	11.51 \pm 1.553	
	NP/No biostimulant	10.0 \pm 2.0	0.97 \pm 0.014	8.77 \pm 1.182	9.27 \pm 1.533	
	NP + Biostimulant	6.7 \pm 6.35	0.88 \pm 0.189	5.76 \pm 6.005	6.06 \pm 6.162	
	Scheirer-Ray-Hare p -values					
	NP	0.041	0.109	0.078	0.078	
	Biostimulant	0.683	0.522	0.749	1.000	
High early vigor	NP fertilization \times Biostimulant	0.624	0.262	1.000	0.749	
	Unfertilized/No biostimulant	12 \pm 1 A ³ A ⁴	0.92 \pm 0.020	8.86 \pm 1.040 A ³ A ⁴	9.89 \pm 0.867 A ³ A ⁴	
	Unfertilized/Biostimulant	13 \pm 1 a A	0.95 \pm 0.038	10.48 \pm 2.050 a A	11.36 \pm 1.671 a A	
	NP/No biostimulant	13 \pm 1 A a	0.93 \pm 0.041	10.12 \pm 2.253 A a	11.11 \pm 1.867 A a	
	NP + Biostimulant	7 \pm 2 b b	0.93 \pm 0.044	5.89 \pm 2.175 b b	6.22 \pm 2.161 b b	
	ANOVA p -values					
	NP	0.011	0.948	0.176	0.082	
Biostimulant	0.011	0.689	0.277	0.121		
NP fertilization \times Biostimulant	0.002	0.517	0.031	0.012		

The level of significance (p -value) is shown in the Table. For each treatment, the reported data are based on 3 observations \pm standard deviation. Scheirer-Ray-Hare and ANOVA p -values of main effects and interactions, significant at p -value $<$ 0.05, were highlighted with bold font

¹ Ordinary: LG 30600; High early vigor: LG 31630

² Unfertilized: no starter fertilization at sowing; NP: distribution of 27 kg N ha⁻¹ and 30 kg P ha⁻¹ as diammonium phosphate at sowing. No biostimulant: no maize biostimulant seed treatment; Biostimulant: maize seeds treated with *Bacillus amyloliquefaciens* IT-45 (Rise P®) and *Cyamopsis psoraloides* polysaccharide extract (AgRH0® GSB30)

³ Capital and small letters indicate pair-wise differences of NP fertilization at each level of Biostimulant treatment, as assessed by simple effect tests

⁴ Capital and small letters indicate pair-wise differences of Biostimulant at each level of NP fertilization treatment, as assessed by simple effect tests

seed-applied biostimulant alone generally did not influence AMF colonization or community composition, which were negatively affected by the combination with NP fertilization. In addition the effect of the two factors were modulated by maize genotype.

Plant biomass and root colonization by AMF

As expected, plant biomass was positively affected by the fertilization treatment in both maize hybrids. Moreover, the biostimulant application, which alone had no effect, acted synergistically when the fertilization was applied, further increasing plant biomass. Such finding showed that in a very low fertility soil, as that used in this study, biostimulant was not able to boost plant nutrition, probably because the scarcity of nutrients enhanced the competition between plants

and native and inoculated microorganisms. On the contrary, when N and P were not limiting, the microbial component of the biostimulant may develop and utilize its PGP abilities, stimulating root growth and promoting the availability of N and P, thus enhancing plant growth more than the fertilization alone.

A synergistic effect was also observed when insoluble forms of phosphate were supplied together with phosphorus solubilizing bacteria and AMF (Singh and Kapoor 1998). In this case, such an effect was explained by the greater absorption of phosphorus made available by bacteria to AMF. However, when nutrients were not limiting, no synergistic effect was reported as inoculation with the strain *B. amyloliquefaciens* FZB42 and humic acids in mycorrhizal and organically (manure) fertilized maize plants had no effect on total biomass (Cozzolino et al. 2021).

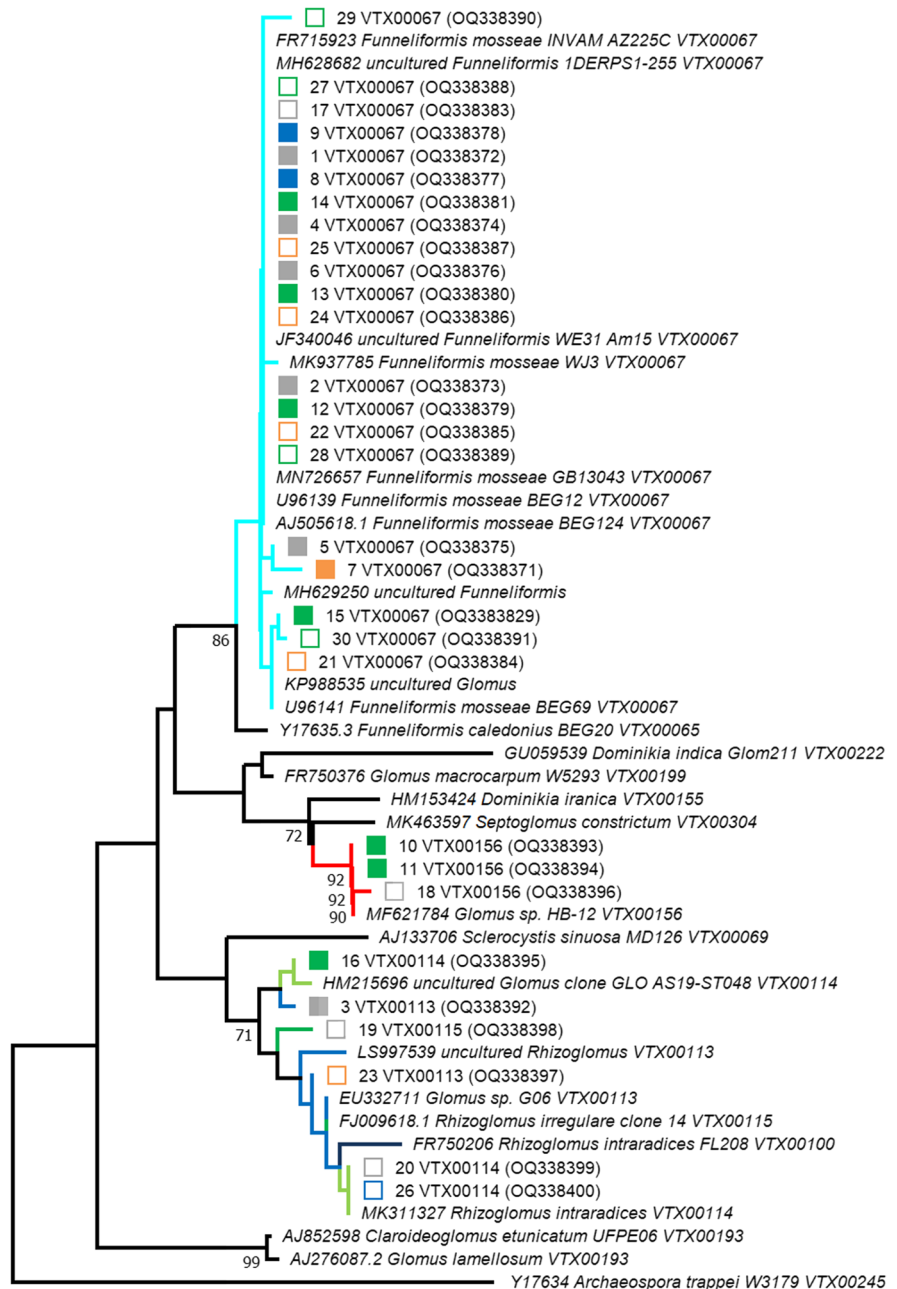
Table 3 Identification (identity %) of 5 leaf-stage root AMF sequences retrieved from the DGGE gel bands (marked in Fig. 1) with the existing 18S rRNA gene sequences (reported with their origin/isolation source) in NCBI–GeneBank and MaarjAM database, as obtained using BLASTN analysis

Fragment	Taxon name	Isolation source	Identity (%)	Accession number	Virtual taxa	Isolation source	Identity (%)	Accession number
<i>GenBank database</i>				<i>MaarjAM database</i>				
1	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	100.00	MN726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	100.00	JX461236.1
2	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	98.31	MN726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	98.70	JX461236.1
3	<i>Glomus</i> sp. clone GLO_AS19-ST048	unfertilized maize field	96.72	HM215696.1	VTX00113	<i>Anemone multifida</i> roots	95.60	LN825036.2
4	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	98.73	MN726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	98.30	JX461236.1
5	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	98.92	MN726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	98.90	JX461236.1
6	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	100.00	MN726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	100.00	JX461236.1
7	<i>Funneliformis</i> sp. clone WE31_Am15	<i>Cicer arietinum</i> root- ing soil	95.57	JF340046.1	VTX00067	<i>F. mosseae</i> BEG12 spores	95.70	JX461236.1
8	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	100.00	MN726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	100.00	JX461236.1
9	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	100.00	AM726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	100.00	JX461236.1
10	<i>Glomus</i> sp. HB-12	<i>Hedysarum scoparium</i> rhizos- phere	99.57	MF621784.1	VTX00156	<i>Stipa krylovii</i> roots	99.60	AB698603.1
11	<i>Glomus</i> sp. HB-12	<i>Hedysarum scoparium</i> rhizos- phere	100.00	MF621784.1	VTX00156	<i>Stipa krylovii</i> roots	100.00	AB698603.1
12	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	99.12	AM726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	99.10	JX461236.1
13	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	98.73	AM726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	99.10	JX461236.1
14	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	100.00	MN726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	100.00	JX461236.1
15	<i>Funneliformis mosseae</i> BEG69	<i>Zea mays</i> rhizos- phere	100.00	U96141.1	VTX00067	<i>F. mosseae</i> BEG69 spores	100.00	U96141.1

Table 3 (continued)

Fragment	GenBank database	Isolation source	Identity (%)	Accession number	Virtual taxa	MaarjAM database	Identity (%)	Accession number
	Taxon name					Isolation source		
16	<i>Glomus</i> sp. clone GLO_AS19-ST048	unfertilized maize field	98.40	HM215696.1	VTX00114	olive plantation soil	97.90	LT836959.1
17	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	99.16	AM726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	99.20	JX461236.1
18	<i>Glomus</i> sp. HB-12	<i>Hedysarum scoparium</i> rhizosphere	99.14	MF621784.1	VTX00156	<i>Stipa krylovii</i> roots	99.10	AB698603.1
19	Uncultured <i>Rhizoglomus</i> sp.	roots	95.90	LS997539.1	VTX00115	<i>Veronica chamaedrys</i> roots	94.70	HG976108.1
20	<i>Rhizoglomus intraradices</i> (formerly <i>Rhizophagus intraradices</i>)	soil	100.00	MK311327.1	VTX00114	<i>Verbena officinalis</i> roots	100.00	FN429367.1
21	<i>Funneliformis mosseae</i> clone Q1AZ225CN89	<i>Medicago sativa</i> roots	100.00	OL412381.1	VTX00067	<i>F. mosseae</i> BEG12 spores	99.60	JX461236.1
22	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	99.58	AM726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	99.60	JX461236.1
23	<i>Glomus</i> sp. G06	<i>Glycine max</i> roots	98.98	EU332711.1	VTX00113	<i>Araucaria araucana</i> roots	99.50	LN825034.2
24	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	96.23	AM726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	99.20	JX461236.1
25	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	100.00	MN726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	100.00	JX461236.1
26	<i>Rhizoglomus intraradices</i> (formerly <i>Rhizophagus intraradices</i>)	soil	98.33	MK311327.1	VTX00114	<i>Verbena officinalis</i> roots	99.10	FN429367.1
27	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	100.00	MN726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	100.00	JX461236.1
28	<i>Funneliformis mosseae</i> clone AZ225C/2-5	<i>Medicago sativa</i> roots	99.48	FR715923.1	VTX00067	<i>F. mosseae</i> BEG12 spores	100.00	JX461236.1
29	<i>Funneliformis mosseae</i> GB13043	<i>Prunus sargentii</i> roots	96.95	MN726657.1	VTX00067	<i>F. mosseae</i> BEG12 spores	97.80	JX461236.1
30	<i>Funneliformis mosseae</i> clone Q1AZ225CN89	<i>Medicago sativa</i> roots	100.00	OL412381.1	VTX00067	<i>F. mosseae</i> BEG69 spores	100.00	U96141.1

Fig. 3 Affiliation of the sequences retrieved from DGGE gel bands of the root AMF communities of two maize hybrids at the 5-leaf stage, treated or untreated with NP fertilization and with seed applied biostimulant (marked in Fig. 1), with the sequences of the NS31-Glo1 18S rRNA gene retrieved in gene banks. Phylogenetic analysis was inferred by using the neighbour-joining method. The evolutionary distances were computed using the Kimura's 2-parameter model. Bootstrap (1000 replicates) values below 70 are not shown. Evolutionary analyses were conducted in MEGA 11. The DNA sequences retrieved in this work are indicated by their corresponding band number and their accession number. The MaarjAM database Virtual Taxa of each sequence are also shown. Black branches refer to taxonomic reference species sequences retrieved from databases only. Colors are used for sequences obtained in this work and for their related sequences from GenBank. Branches of different colors correspond to different AMF species (virtual taxa)



Similar results on soybean plants were obtained by Correa et al. (2009) using the strain *B. amyloliquefaciens* BNM122 and different AMF.

Root colonization by AMF was negatively influenced by the NP fertilization treatment, which was consistent with previous scientific evidence. Indeed, AMF symbioses were severely affected by chemical

P fertilization in durum wheat (Tang et al. 2016) and maize (Liu et al. 2016). In particular, maize root colonization by native AMF species was negatively affected by P fertilization, which was accompanied by a significant decrease in fungal phosphatase activities (Wang et al. 2017; Lang et al. 2022). Moreover, increasing rates of mineral N input decreased AMF

biomass in maize field soils (Jeske et al. 2018), while long-term NP fertilization caused drastic reductions in AMF spore production in a maize monoculture (Bhadalung et al. 2005).

Inorganic fertilizers have been long known for their potential negative impacts on AMF symbioses, presumably via modulations in rhizodeposition patterns, ultimately affecting host recognition and colonization. Actually, both N and P fertilizations were shown to down-regulate the production and root excretion of strigolactones, an important signalling factor in the establishment of AMF symbioses (Akiyama et al. 2005; Yoneyama et al. 2013). On the other hand, morphological changes in the root system should not be overlooked, as in maize, they were shown to respond to P fertilization more consistently than root physiological parameters (Wen et al. 2017). Several authors suggested that increased levels of plant available P in the rhizosphere may imbalance the costs and benefits of AMF symbioses, affecting plant/AMF interactions and making the host plants less reliant on P mobilized by potential AMF partners (Guttay and Dandurand 1989; Treseder 2004; Ji and Bever 2016; Konvalinková et al. 2017; Liu et al. 2020).

In our work, the application of biostimulant in unfertilized plants did not cause any significant differences in AMF colonization rate. Conversely, the findings of Sarabia et al. (2017), showed that the inoculation with a yeast based biostimulant (*Cryptococcus flavus*) negatively affected AMF colonization in maize plants under P limitation, while Xie et al. (2018) reported mycorrhiza helper effects of the strain *B. amyloliquifaciens* FZB42 in diverse host plants.

Interestingly, our results showed that in fertilized plants the biostimulant differently affected root colonization rate depending on the hybrid, as the ordinary hybrid showed an additional reduced percentage of mycorrhization while the hearily vigor hybrid did not change its colonization rate. Similar variable findings are reported in previous works. Ben Zineb et al. (2022), co-inoculating barrel medic with P-solubilizing *Pseudomonas corrugata* SP77 and *Rhizophagus irregularis* EEZ58 reported no effect of AMF root colonization when the soil was amended with triple super phosphate. In another experiment, Cozzolino et al. (2021) did not find significant increase in maize plants inoculated or not with *B. amyloliquifaciens* FZB42, while Sarabia et al. (2017), reported

that maize root colonization by AMF was positively affected by the inoculation with the biostimulant yeast *Candida railenensis*. Similarly, a positive effect was reported on wheat inoculated with a commercial strain of *B. amyloliquifaciens* (Wilkes et al. 2020). On the contrary, but accordingly with our findings obtained with the ordinary hybrid, a negative effect was found in soybeans inoculated with the strain *B. amyloliquifaciens* BNM122 (Correa et al. 2009).

These different results may be ascribed to the different compatibility between AMF community and biostimulants utilized in the different studies. For instance, different capability of phytohormone production among strains may affect a stronger or weaker induction of root or hyphal growth essential for the plant nutrient uptake (Bradáčová et al. 2019). In addition, it is known that a decrease in percentage root colonization may have no effect on the plant response (Nahar et al. 2020; Watts-Williams et al. 2019): in the experiment of Correa et al. (2009), the reduction of root colonization without a decrease of plant biomass was observed after the addition of *B. amyloliquifaciens* BNM122, in P and N non limiting condition.

In this work, some variations between the two hybrids were not unexpected, as root- and rhizosphere-related traits, such as rhizodeposition and root architecture, important in the recruiting of root-associated microbiota, may vary among different plant genotypes (Hu et al. 2018; Kerstens et al. 2021; Semchenko et al. 2021). By contrast, in other studies, no significant effects of maize genotype on colonization by native AMF were reported (Londoño et al. 2020; Meyer et al. 2021).

AMF community diversity and composition assessed by PCR-DGGE and band sequencing

All biodiversity indices, except for the Evenness, showed similar trends within the two hybrids, although the presence of interaction between NP treatment and Biostimulant was statistically significant only for the hearily vigor hybrid. The lack of the effect of the biostimulant in the ordinary hybrid may be due to the low number of replicates and large variation among NP + Biostimulant replicates. Generally, the Richness of the AMF communities was low and the distribution of the species, as differently represented by Evenness, Hill₁ and Hill₂ indices, was quite uniform showing small changes among

treatments. Cluster analysis of the DGGE profiles of root-colonizing AMF communities revealed that the inorganic fertilization had an important effect on the AMF diversity in both maize genotypes. Our findings are consistent with previous data obtained from maize roots and rhizosphere. Actually, in field-grown maize roots, AMF community composition was greatly altered along a P amendment gradient, while the overall AMF diversity was not necessarily decreasing (Wang et al. 2017). Lang et al. (2022) described negative tendencies in the AMF biodiversity and substantial changes in the AMF community structure of maize rhizosphere, as affected by increasing mineral P input. Nevertheless, N fertilization was also reported to have a strong impact on maize root and rhizosphere AMF diversity (Borriello et al. 2012; Zeng et al. 2021). Moreover, increasing levels of long-term NPK fertilization caused significant decreases in the biodiversity and major shifts in the composition of soil AMF communities in a conventional maize field, principally correlated to soil available P (Liu et al. 2020).

As the significant alterations in AMF biodiversity under NP fertilization were coupled with a major decrease in the root colonization rates, we suppose that the primary mechanisms controlling plant symbiotic status and symbiotic partner selection are the same, i.e. changes in root exudation patterns, signal molecule deposition and root architecture, which may be modulated by the plant genotype (Nouri et al. 2021). Several works reported that P or N stress stimulated plants to modify root morphology (Zhu et al. 2006) and root exudation patterns which may reduce carbon allocation to AMF enhancing competition within fungal species (Wang et al. 2021; Knecht et al. 2016).

The biostimulant seed coating expressed less influence on AMF diversity than fertilization treatment. Biostimulant treated samples clearly clustered separately from Unfertilized/No biostimulant samples in the ordinary hybrid, while in fertilized treatments showed a slight but significant decrease in the AMF community richness of the high early vigor hybrid. Currently, there are only a few studies to compare with our results. Consistently with our data obtained from the high early vigor maize, a *Bradyrhizobium* sp. inoculum significantly altered the native AMF assemblages in cowpea roots (Omirou et al. 2016). Moreover, the diversity and composition of root-colonizing

native AMF communities were significantly affected by a proteobacterial inoculum, depending on the host plant species (Dagher et al. 2020).

Genetic identification of excised DGGE bands revealed that all retrieved 18S rDNA fragments affiliated to the family Glomeraceae, with a low taxonomic diversity, represented by species belonging to the genera *Glomus*, *Funneliformis* and *Rhizoglomus*. Low AMF diversity in arable soils is not unusual, as reported by many authors (Daniell et al. 2001; Hijri et al. 2006; Qin et al. 2015; Peyret-Guzzon et al. 2016; Lang et al. 2022). In line with our results, several studies revealed that AMF taxa colonizing field-grown maize roots affiliated mainly with the family Glomeraceae (Borriello et al. 2012; Higo et al. 2020; Alaux et al. 2021; Zeng et al. 2021), and high abundance of the genus *Funneliformis* was found in the rhizosphere and endosphere of maize grown in the field (Turrini et al. 2016; Lang et al. 2022). The high frequency of *Funneliformis mosseae* ribotypes in our samples may be explained by the generalist nature of this species, that makes it well adapted to disturbed and agricultural environments (Öpik et al. 2006; Rosendahl et al. 2009; Oehl et al. 2010). Moreover, *F. mosseae* is able to tolerate soil disturbance, such as tillage, due to its highly interconnected extraradical mycelium which can re-establish hyphal connections afterwards (Helgason et al. 1998; Simard and Durall 2004; Avio et al. 2013). In contrast to our data, VTX00067 (*F. mosseae*), appearing in all our samples, was found to reach a considerably higher abundance in native AMF communities present in the roots and rhizosphere of unfertilized maize plants, in respect to those treated with different levels of N fertilization (Zeng et al. 2021).

Among the identified species, *F. mosseae* appeared to be the most resilient taxon, colonizing maize roots even in the NP+Biostimulant treatments. The high intraspecific diversity of *F. mosseae* found in our experimental soil may have played an important role in its genetic adaptation to the different chemical and biological treatments, together with its genotypic and phenotypic plasticity (Koch et al. 2006; Ehinger et al. 2012). On the other hand, *Rhizoglomus* related sequences were poorly represented in NP+Biostimulant treated maize roots. Interestingly, Ben Zineb et al. (2022) reported possible competition between P-solubilizing bacteria and *Rhizoglyphus irregularis* EEZ58 in the presence

of mineral P supplies. Although the cited experiment was conducted in sterile substrates, thus lacking the complexity of native AMF communities, our data suggest that *B. amyloliquefaciens* IT-45 and AMF may compete for P-mobilization in NP-fertilized soils, which could result in the exclusion of certain susceptible taxa from the maize roots.

In conclusion, this study showed significant interactions between conventional NP fertilization and a seed applied biostimulant containing the PGP strain *B. amyloliquefaciens* IT-45, as affecting abundance (root colonization) and diversity (richness and composition) of native AMF communities in maize. A possible explanation of this behaviour may be ascribed to the very low level of nutrients in our soil which could hinder the growth of AMF and *B. amyloliquefaciens* IT-45, due to the competition for limited resources, as observed by the lack of growth in maize plants. When N and P are externally supplied, the biostimulant application can determine a better utilization of available nutrients by the plants with different observed effects on the AMF community.

Present findings improved our knowledge on the effects of biostimulants on beneficial root-associated microorganisms, as affected by plant genotypes and fertilization, with the aim to implement the use of innovative tools in sustainable and resilient agroecosystems.

Author contribution M.A., M.B. and M.G. conceived and designed the experiments. M.B. and L.C. performed the agronomic experiment; G.U., A.G., C.C., I.P. and A.T. performed molecular and data analysis. L.A. and G.U. performed statistical analyses. M.A., M.G., A.T. and G.U. wrote the paper. All authors read and approved the final manuscript. This paper was part of G.U.'s doctoral thesis work at the University of Pisa.

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Data availability Sequencing data generated during this study are available in the NCBI-GenBank database under the accession numbers from OQ338371 to OQ338400.

Declarations

Competing interest The authors declare they have no financial interests.

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