Chapter 5
Microbial Socialization Highlights the AMF Effect

Teresa Dias, Cristina Cruz, Ajit Varma, Juliana Melo, Patrícia Correia, and Luís Carvalho

Abstract Arbuscular mycorrhizal fungi (AMF) are recommended as biofertilizers for sustainable agriculture. So far, most researchers have investigated the effects of AMF on plant growth under highly controlled conditions with sterilized soil. However, it is still poorly documented how the biotic context alone shapes AMF's impact on host plant performance. We inoculated maize (*Zea mays* ssp. *mays*) seedlings with five commercial inoculants of arbuscular mycorrhizal fungi (AMF—*Claroideoglomus claroideum*, *Funneliformis mosseae*, *Gigaspora* sp, *Rhizophagus irregularis* and *Scutellospora* sp.). Plants were pot-cultivated for 9 weeks using soil which had been used for maize monocropping in the field. Since we wanted to focus on the impact of belowground interactions (plant-AMF-soil microbes) alone, we compared sterilized versus non-sterilized soil. AMF inoculation was successful, despite an abundant native AMF communities. As hypothesized: (i) the soil biotic context controlled AMF's benefits on maize growth; (ii) AMF's benefits depend on the isolate identity; and (iii) *C. claroideum*, *F. mosseae* and *Gigaspora* sp. overruled soil legacy effects of maize monocropping. We found little to no effects of AMF inoculation on maize growth and nutrients acquisition when plants were grown in sterilized soil. AMFs benefits to their host plants could not be explained by improved nutrition alone because interaction with the remainder soil microbes also differed between inoculated AMF. The results demonstrate that the soil biotic context and AMF isolate identity should be taken into consideration when applying AMF inoculants in agriculture.

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5.1 Introduction

The ongoing human population growth and changing consumption patterns affect food demand and quality, livestock and fibre production, energy use (fossil- and bio-fuel), and land use management (Rockström et al. 2009). As a result, food demand is forecasted to double by 2050 but the environmental footprint must be reduced (for the EU, see Directive 2009/128/EC). This creates an urgent need for cleaner agronomic practices capable of boosting crop yields while alleviating environmental impacts (Dias et al. 2015).

Monocropping is responsible for significant crop yield losses via negative plant-soil feedbacks (or feedbacks). Feedbacks occur because plants 'culture' their interacting soil microbes, which may affect their own growth (e.g. seed germination, seedling survival, individual growth, vegetative propagation and seed production- Bonanomi et al. 2005) and demography as well as that of other plant species (Bever et al. 1997; Bever 2003; van der Putten et al. 2013). Feedbacks can be positive or negative (Bever et al. 1997; Bever 2003). Since increased nutrient availability and plant density shift plant-microbe interactions from mutualistic to neutral or parasitic (Anacker et al. 2014), negative feedbacks in agriculture are well-known since ancient times (Dias et al. 2015). Consequently, so is manipulating plant-microbe interactions in agriculture, namely through crop rotations. Still nowadays, manipulating biotic interactions (e.g. plant-animal, plant-microbe, microbe-microbe) to provide the desired services and thus reduce or eliminate the need for external inputs is fundamental to a cleaner agricultural production. The challenge is to favor positive interactions, while reducing the negative ones (Shennan 2008).

In line with this perspective, there is a steadily growing appreciation of the vital role of soil life in agricultural sustainability (Bender et al. 2016), including plant symbiotic associations. One approach is the use of biofertilizers (i.e. a product containing soil microbes applied to plants to promote their growth- Herrmann and Lesueur, 2013). Among these products, those based on mycorrhizae (the widespread symbioses between fungi and plant roots- Smith and Read 2008) are of special interest because mycorrhizae commonly overrule negative feedbacks on plant growth (Fitzsimons and Miller 2010). Almost all important crops (e.g. maize, wheat, soybean) form associations with arbuscular mycorrhizal fungi (AMF), which are therefore an intricate component of the agrosystem. Examples of AMF's role in agrosystems include pathogen suppression, pollination enhancement, herbivore protection and improved water relations (Verbruggen and Kiers 2010). Despite its enormous potential, the application of AMF has not been fully adopted by farmers so far (Berruti et al. 2016).

AMF generally form mutualisms with plants by trading soil resources and other benefits (e.g. protection from pathogens and stress factors), for photosynthates (Smith and Read 2008). But not all AMF partnerships are equally beneficial for plants; neutral and parasitic AMF symbioses also occur (Johnson et al. 2008). Furthermore, since AMF are obligate biotrophs (Smith and Read 2008), AMF are

often applied in experiments (pot and field trials) and agricultural practices without having in consideration the specificity of the AMF inoculants, compatibility with the target environment and competition with other soil organisms (Berruti et al. 2016). In fact, inoculant production is much more determined by the easiness of growing one isolate than by its effects on plant performance (above a certain positive impact).

Not much is known on how the biotic and abiotic contexts shape biotic interactions, and affect feedback magnitude and direction (Agrawal et al. 2007). AMF are a good model for studying how contextual frameworks affect symbioses, because both biotic and abiotic contexts influence how AMF impact host plant performance (Hoeksema et al. 2010). Given the increasing evidence that non-mycorrhizal soil microbes significantly impact the formation and outcome of the mycorrhizal symbiosis (Frey-Klett et al. 2007), we focused on how the biotic context alone shapes AMF's impact on host plant performance. We chose *Zea mays* subsp. *mays* L. because it is: (i) a fast-growing crop with great economic and nutritional importance worldwide (Ranum et al. 2014); (ii) significantly affected by negative feedbacks (e.g. in the early 1980s, maize monocropping reduced production by 10–15%—http://corn.agronomy.wisc.edu/AA/A014.aspx); and (iii) highly dependent on AMF (Aquino et al. 2015). Since maize is a fast-growing and highly nutrient-demanding crop, we hypothesize that:

1. Subjecting maize to soil legacy effects of maize monocropping will result in negative feedback on plant biomass and nutrients acquisition;
2. Inoculation with AMF will overrule soil legacy effects of maize monocropping.

Negative feedbacks can, non-exclusively, be due to: release of allelopathic compounds by organic matter decomposition (Bonanomi et al. 2005; van de Voorde et al. 2012), nutrient depletion (Bonanomi et al. 2005) and changes in soil microbial communities (including accumulation of pathogens and parasites) (Bever et al. 1997). Since we wanted to focus on the impact of belowground interactions (plant-AMF-soil microbes) alone, from the several feedback approaches (Brinkman et al. 2010; van der Putten et al. 2013), we compared sterilized versus non-sterilized soil. Although decomposition of maize straw releases compounds that may enhance or reduce pathogenicity (Javaid 2008) and affects the following crop (Qi et al. 2015), as far as we know, maize is not auto-allelopathic. To exclude nutrient depletion we used a very poor soil, and to overcome autoclaved-induced increases in nutrients availability (Berns et al. 2008), plants were supplemented weekly with readily available nutrients (Brinkman et al. 2010). Therefore, differences in plant growth between the sterilized and non-sterilized soil treatments will describe the feedback, while differences between AMF isolate treatments will describe interactions of each AMF with the soil microbes (Frey-Klett et al. 2007).

5.2 Experimental Protocol

5.2.1 Experimental Design

Our experimental design consisted of two factors: AMFs inoculation and soil sterilization. The design was fully factorial resulting in 12 treatments with 6 replicates (pots) each. To test if the nutritional benefit to their host plant (symbiont quality) varied between AMF species, we assessed plant response to five AMF species: *Claroideoglomus claroideum*, *Funneliformis mosseae*, *Gigaspora* sp., *Rhizophagus irregularis* and *Scutellospora* sp. To test if symbiont quality was soil biotic community context dependent, we assessed plant response to the presence/absence of a stable soil microbial community (plant-soil feedback). Using soil collected from a maize field in northern Portugal (Vagos, Aveiro—38°29′N−9°1′W) ensured the pre-training of the soil so that there was no need to include a training phase in our experiment.

The soil, at the sampling time, contained 0.4% organic matter, 2.2% humic susbtances, 0.1% total N, 182 ppm total P and 77 ppm K, and had pH (H_2O) 6.5. Available N was 37 ppm while available P and K were 8 and 40 ppm respectively. Soil was mostly composed of sand (>70%), while clay and sand accounted for <30%. Given that mycorrhization is often negatively affected by high nutrient availability, soil was mixed with sterilized river sand in a 1:4 proportion to dilute soil's nutrients. Both sand and soil (only for the sterilized soil treatment) were autoclaved at 121 °C for 1.1 atm for 60 min. Soil and sand were autoclaved three times in consecutive days and then left untouched for a week.

Maize (*Zea mays* L.) seeds (Syngenta) were put under running tap water to remove the antifungal coating and were then sterilized by being placed in ethanol 70% (v/v) for 1 min, then in sodium hypochlorite 2.5% (v/v) for 10 min, and then washed in sterilized distilled water. After sterilization the seeds were germinated in sterilized (70% alcohol) trays containing autoclaved perlite for 5 days and then transferred to the pots. The maize seedlings were planted in 20-cm diameter, 3 L pots (previously sterilized with 70% alcohol) containing the 1-soil: 4-sand mixture. Inoculation was performed a week after seedling transplant.

For each AMF species, six pots were each seeded with 20 g of AMF inoculum containing ~250 AMF spores; an additional six pots were used as controls.

Plants were watered daily with 100 mL of tap water except on the days when they would be supplied with nutrient solution. All plants were fertilised with 100 mL of a 1⁄4 strength Hoagland's solution (1.5 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.5 mM $NH_4H_2PO_4$; 0.25 mM $MgSO_4$; 50 μM KCl; 25 μM H_3BO_3; 2 μM $MnSO_4$; 2 μM $ZnSO_4$; 0.5 μM $CuSO_4$; 0.5 μM $(NH_4)_6Mo_7O_{24}$; 20 μM FeNaEDTA) every week, which represented the weekly addition of 5.6 mg N; 1.6 mg P; 6.0 mg K; 4.0 mg Ca; 0.6 mg Mg; 0.8 mg S; 27.5 μg B; 177.5 μg Cl; 3.2 μg Cu; 112 μg Fe; 11 μg Mn; 33.6 μg Mo; and 13.1 μg Zn. Plants were grown for 9 weeks, between

July and September 2012, in a greenhouse under a non-sterile environment, with natural light (~15 h day/9 h night), maximum photosynthetic active radiation between 600 and 1000 μmol m^{-2} s^{-1}, and ambient temperature between 17–40°C. Pots were randomized once a week.

5.2.2 Harvest and Analysis

At harvest, maize plants were separated into roots and shoots, and dried at 60 °C until constant mass. Maize shoots were analyzed for macro (nitrogen—N, phosphorus—P, potassium—K, calcium—Ca, magnesium—Mg and sulphur—S) and micronutrients (boron—B, chromium—Cr, copper—Cu, iron—Fe, manganese—Mn, molybdenum—Mo, nickel—Ni and zinc—Zn). The dried plant material was ground into powder using a ball mill (Retsch MM 2000). N concentrations in the plant material were determined using an elemental analyzer (EuroVector) by combustion—DCT (Rodrigues et al. 2009) while the concentrations of all the other nutrients was determined using Inductively Coupled Plasma—Optical Emission Spectroscopy (ICP-OES—Spectro Ciros CCD, Spectro, Germany). We calculated shoot nutrient contents by combining shoot biomass and the respective concentrations. The natural abundance of ^{13}C and ^{15}N in the maize shoots was determined using mass spectrometry (IRMS, Micromass-GV Instruments, UK) and the expressions: $\delta^{13}C = (R\ sample/R\ standard - 1) \times 1000$, where R is the ratio $^{13}C/^{12}C$, in the sample and in the standard and $\delta^{15}N = (R\ sample/R\ standard - 1) \times 1000$, where R is the ratio $^{15}N/^{14}N$, in the sample and in the standard.

To control for effective mycorrhization of the AMF inocula, we evaluated roots' mycorrhizal colonization on plants grown in the sterilized soil: segments of 1 cm length cut 1–2 cm above the root apices. These root segments were stained (Koske and Gemma 1989), and mycorrhizal colonization was evaluated on quadrilateral plaques in accordance with Giovannetti and Mosse (1980) as presence or absence. Another sample of or root tips was used to characterize the microbial community on the root surface and inside the roots (including endophytes) but only for the plants that were hypothesized to suffer negative feedback (those grown in the non-sterilized soil). For that root tips from each of the six replicates per treatment were collected, bulked together in the same proportion, and stored at −20 °C until analysis. DNA was extracted using the GeneMATRIX Plant & Fungi DNA Purification Kit (EURx, Poland). DNA amplification and molecular identification of microorganisms was carried out by sequencing the PCR amplified 16SrRNA gene sequence for prokaryotes (Case et al. 2007) and LO/LOR for fungi (Delgado unpublished). The operational taxonomic units (OTUs) were identified to at least the phylum level.

5.2.3 Calculations and Statistics

Feedback was calculated according to Kardol et al. (2007) as follows:

$$\text{Feedback} = \frac{(\text{Value non sterilized treatment} - \text{Average value sterilized treatment})}{\text{Average value sterilized treatment}}$$

The effect of soil sterilization on plant biomass and on nutrient contents was tested separately using a two-way ANOVA, with soil and AMF treatments as fixed factors. Then, differences between sterilized and non-sterilized soil were analyzed by Student's *t*-test ($p < 0.05$). The effect of the AMF treatments on feedbacks on plant biomass and on nutrient contents was tested separately using a one-way ANOVA, with treatment as fixed factor. Bonferroni post hoc multiple comparisons tested for differences ($p < 0.05$) in feedbacks on plant biomass and on nutrient contents between treatments. Finally, to identify the microbial groups that most contributed to distinguish the microbial communities inhabiting maize roots of plants grown in the non-sterilized soil we used a PCA. For this analysis, the number of sequences per phylum of one sample per each of the six AMF treatments were pooled (n = 6). Preliminary analyses were performed to ensure there was no violation of the assumptions regarding the tests' application. SPSS software, version 23.0, was used for all tests.

5.3 Salient Observations

Only non-inoculated (control) plants grown in sterilized soil were not mycorrhized; plants from all other treatments (including control plants grown in non-sterilized soil) were mycorrhized (data not shown). Despite molecular analysis of the root segments confirmed the presence of the inoculated AMF, for plants grown in the non-sterilized soil it was not possible to conclude whether mycorrhization was done by the inocula or by native AMF.

Control plants grown in sterilized soil accumulated more root, shoot and total biomass than those grown in non-sterilized soil (Fig. 5.1 and Table 5.1). Inoculation with *Rhizoglomus irregularisradices* or *Scutellospora* sp. did not cancel the negative soil feedback (i.e., negative impact of non-sterilized soil) on biomass, while inoculation with *Claroideoglomus claroideum*, *Funneliformis mosseae* and *Gigaspora* sp. enabled maize plants growing in non-sterilized soil to accumulate as much root, shoot and total biomass as those growing in sterilized soil. Since shoot biomass was highly correlated with total biomass (r = 0.98; $p = 0.000$), the impacts of AMF and soil sterilization on plant nutrients were assessed on the shoots. Again, inoculation with *R. irregularisradices* or *Scutellospora* sp did not cancel the negative soil feedback (i.e., negative impact of non-sterilized soil) on nutrients, while inoculation with *C. claroideum*, *F. mosseae* and *Gigaspora* sp

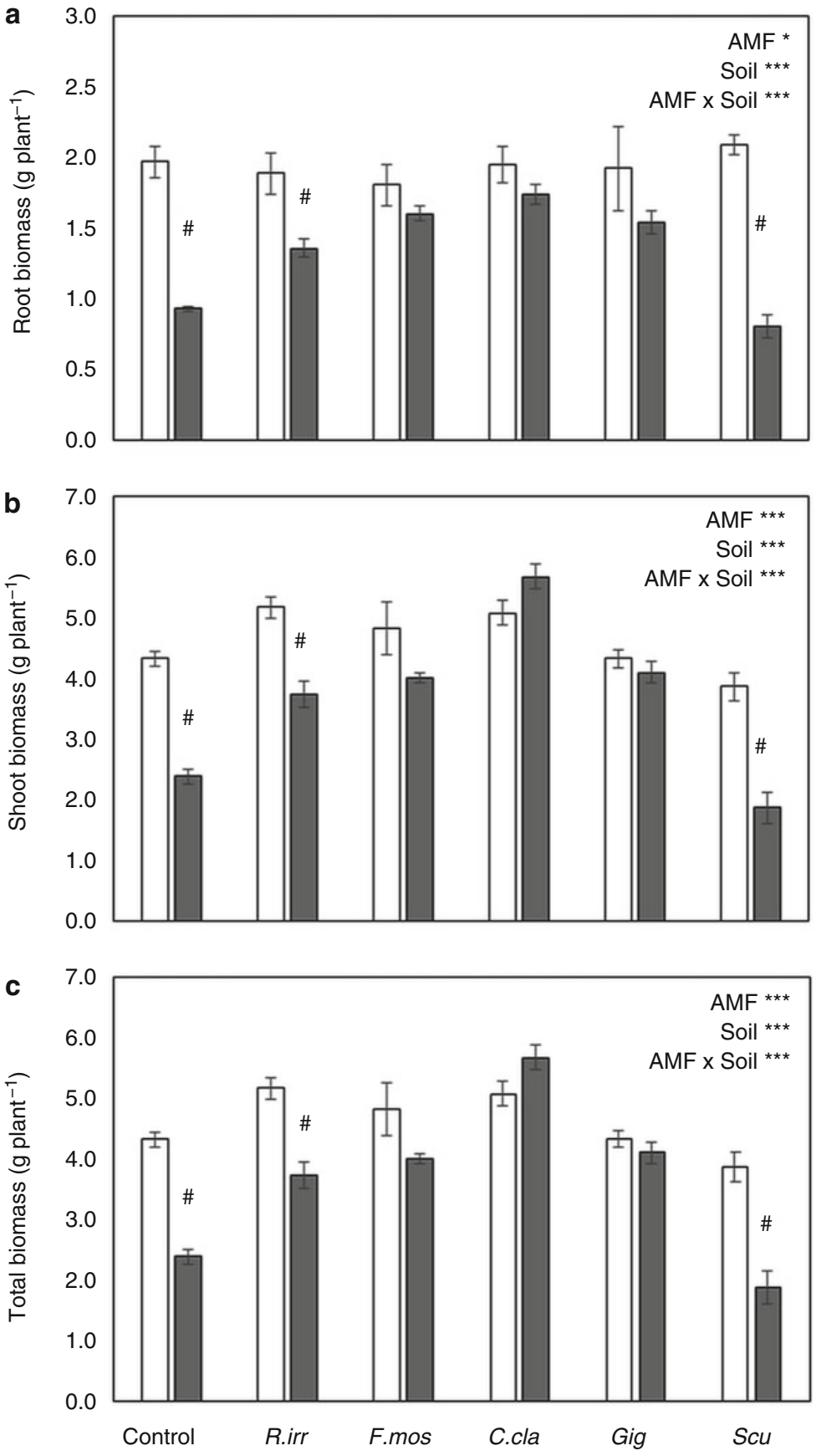

Fig. 5.1 Impact of AMF inoculation and soil sterilization on root (**a**), shoot (**b**) and total plant biomass (**c**). *$p < 0.05$; **$p < 0.01$; ***$p < 0.000$. # shows significant differences between sterilized and non-sterilized soil at the 5% level. Bars are the mean ± 1SE (n = 6)

Table 5.1 Impact of AMF inoculation on soil feedback on plant biomass and on shoot macro- and micronutrients contents

Feedback on:	Control	*C. claroideum*	*F. mosseae*	*Gigaspora* sp.	*R. irregularis*	*Scutellospora* sp.
Root biomass	-0.5 ± 0.0^{c}	-0.1 ± 0.0^{ab}	-0.1 ± 0.0^{a}	-0.1 ± 0.0^{ab}	-0.3 ± 0.0^{b}	-0.7 ± 0.1^{d}
Shoot biomass	-0.5 ± 0.1^{c}	0.1 ± 0.0^{a}	-0.1 ± 0.1^{b}	-0.1 ± 0.0^{ab}	-0.3 ± 0.0^{c}	-0.7 ± 0.1^{d}
Total biomass	-0.5 ± 0.0^{cd}	0.0 ± 0.0^{a}	-0.1 ± 0.1^{ab}	-0.1 ± 0.0^{ab}	-0.3 ± 0.0^{bc}	-0.7 ± 0.1^{d}
Macro**	-0.4 ± 0.0^{bc}	0.0 ± 0.1^{a}	-0.2 ± 0.0^{ab}	-0.1 ± 0.0^{a}	-0.2 ± 0.1^{ab}	-0.5 ± 0.1^{c}
N**	-0.3 ± 0.0^{c}	0.1 ± 0.1^{a}	-0.2 ± 0.0^{c}	0.1 ± 0.1^{ab}	-0.2 ± 0.1^{bc}	-0.4 ± 0.1^{c}
P**	-0.5 ± 0.0^{ab}	-0.1 ± 0.1^{a}	-0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	-0.2 ± 0.1^{ab}	-0.6 ± 0.1^{b}
K**	-0.3 ± 0.0^{ab}	0.0 ± 0.1^{a}	-0.1 ± 0.0^{a}	-0.2 ± 0.1^{a}	-0.2 ± 0.1^{ab}	-0.5 ± 0.1^{b}
Ca**	-0.6 ± 0.0^{bc}	-0.3 ± 0.1^{a}	-0.4 ± 0.0^{ab}	-0.2 ± 0.1^{a}	-0.3 ± 0.1^{a}	-0.7 ± 0.1^{c}
Mg**	-0.5 ± 0.0^{cd}	-0.2 ± 0.1^{ab}	-0.3 ± 0.0^{bc}	0.0 ± 0.1^{a}	-0.3 ± 0.1^{bc}	-0.6 ± 0.1^{d}
S**	-0.4 ± 0.0^{bc}	-0.2 ± 0.0^{ab}	-0.3 ± 0.0^{abc}	-0.1 ± 0.1^{a}	-0.2 ± 0.0^{ab}	-0.6 ± 0.1^{c}
Micro	-0.3 ± 0.1	1.1 ± 1.1	0.5 ± 0.3	-0.5 ± 0.3	-0.8 ± 0.0	-0.3 ± 0.3
B**	-0.6 ± 0.0^{bc}	-0.2 ± 0.0^{a}	-0.3 ± 0.0^{a}	-0.1 ± 0.1^{a}	-0.4 ± 0.1^{ab}	-0.7 ± 0.1^{c}
Cu	-0.8 ± 0.1	-0.7 ± 0.1	-0.6 ± 0.1	-0.7 ± 0.2	-0.5 ± 0.3	-0.8 ± 0.1
Fe	0.0 ± 0.1	2.3 ± 2.0	1.2 ± 0.5	-0.5 ± 0.3	-0.8 ± 0.0	-0.2 ± 0.4
Mn**	-0.8 ± 0.0^{c}	-0.6 ± 0.0^{bc}	-0.5 ± 0.0^{ab}	-0.3 ± 0.1^{a}	-0.7 ± 0.0^{bc}	-0.8 ± 0.1^{c}
Mo	-0.3 ± 0.1	-0.2 ± 0.2	0.4 ± 0.2	0.3 ± 0.4	0.2 ± 0.2	-0.4 ± 0.2
Ni	1.0 ± 1.0	5.6 ± 2.9	18.7 ± 12.0	1.4 ± 1.2	0.9 ± 1.3	0.7 ± 0.6
Zn*	-0.6 ± 0.0^{ab}	-0.4 ± 0.1^{ab}	-0.4 ± 0.1^{ab}	-0.4 ± 0.1^{ab}	-0.2 ± 0.2^{a}	-0.7 ± 0.1^{b}

*$p < 0.05$; **$p < 0.01$. Different letters show significance at the 5% level. Values are the mean ± 1SE (n = 6 for biomass and four for nutrients)

enabled maize plants growing in non-sterilized soil to accumulate as much nutrients as those growing in sterilized soil. Therefore, two clusters became evident in terms of AMF's impact on plant biomass and nutrients: (i) inoculation with *R. irregularisradices* and *Scutellospora* sp resulted in a negative feedback, within the same range as that of the control; and (ii) inoculation with *C. claroideum*, *F. mosseae* and *Gigaspora* sp. overruled the negative soil feedback.

Analysis of roots' microbial community growing in the non-sterilized soil showed that the inoculated AMFs were present in the roots and so were other many other eukaryotes and prokaryotes (data not shown). Principal component analysis (PCA) of the number of sequences of eukaryotes and prokaryotes detected in these roots showed that the first two components explained 76% of the variation (Fig. 5.2). PC1, which explained 35% of the variation, was associated with higher number of bacterial phyla sequences (inoculation with *C. claroideum*, *R. irregularis* and *Scutellospora* sp.), and in the opposite direction, to the number of

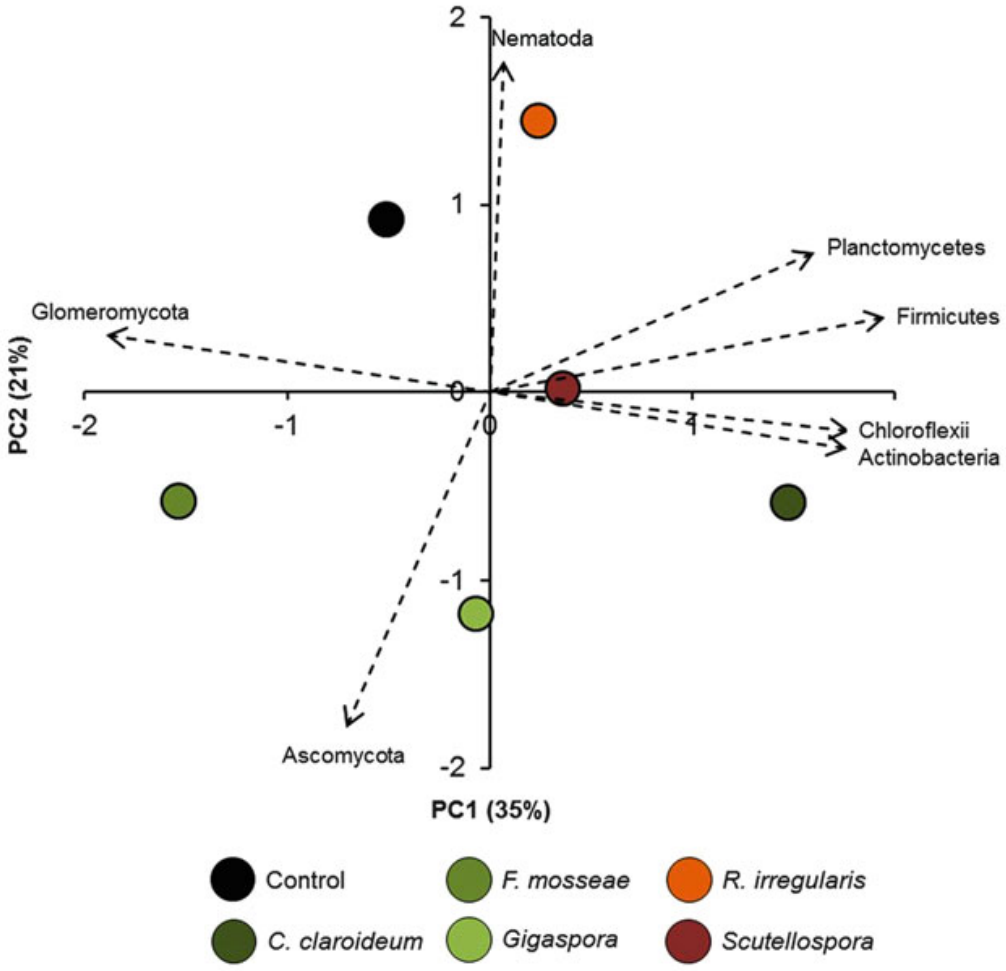

Fig. 5.2 Principal component analysis (PCA) of the root microorganisms (# sequences per phyllum) in the different AMF inoculation in the non-sterilized soil. Symbols represent one bulk sample per treatment; PC1 explains 35% of the variance in the roots microbial community data, PC2 explains 21%. The microbial phylla most responsible for the variations in root microbial community composition (loadings > 0.8) were presented by vectors

Glomeromycota sequences (Control and inoculation with *Gigaspora* sp. and *F. mosseae*). By contrast, PC2, which explained 21% of the variation, grouped the treatments according to the feedbacks on biomass and on nutrients: maize roots from the treatments where plants suffered negative feedback on biomass (Control and inoculated with *R. irregularis* and *Scutellospora* sp.) were associated with higher number of Nematoda sequences, while those where plants did not suffer negative feedback (inoculated with *C. claroideum*, *F. mosseae* and *Gigaspora* sp.) were associated with higher number of Ascomycota sequences. Since PC1 and PC2 contributed in similar ways to explain the variation it is difficult to identify which microbial group(s) would be a particularly strong explanatory gradient influencing roots microbial communities.

5.4 Interpretation of Data

Our study allowed simultaneous examination of plant response to both whole-soil communities and mycorrhizal fractions and showed that: (i) the soil biotic context controls AMF's benefits on maize growth; (ii) AMF's benefits depend on the isolate identity; and (iii) *C. claroideum*, *F. mosseae* and *Gigaspora* sp. overrule soil legacy effects of maize monocropping.

5.4.1 AMF Benefits Depended on the Soil Biotic Context

As expected, the soil legacy effects of maize monocropping resulted in negative feedbacks on plant biomass and nutrient contents (Fig. 5.1 and Table 5.1). The feedbacks on biomass and nutrients we observed resulted from both soil microbes (e.g. bacteria, mycorrhizal or pathogenic fungi) (Kardol et al. 2007) and soil fauna (e.g. nematodes) (Voorde et al. 2012). Both the potential impacts of nutrient depletion (Bonanomi et al. 2005) and of increased nutrient availability due to autoclaving (Berns et al. 2008) were excluded from our study by using a very poor soil and supplying plants with readily available nutrients. Furthermore, the effects of the other growth promoting additives in the tested inocula (e.g. bacteria) were also ruled out from our study by pooling all the additives of each inoculant and adding the same amount of that common extract to each pot (including the controls). So, at time zero the only difference between the treatments was indeed the presence (or absence in the controls) of a certain AMF isolate. Therefore, all the differences observed must be related with the activity of the inoculated AMF: (i) directly on nutrient uptake; and/or (ii) indirectly through distinct interactions with the rhizospheric microbes.

Sterilized and non-sterilized soil differed in soil microbes, including pathogens and parasites (Bever et al. 1997), which interacted differently with the inoculated AMFs. As a result, the plants grown in the sterilized soil grew more than those

grown in the non-sterilized soil, and they also contained more macronutrients (Table 5.1) that are the 'building blocks' of biomass. Surprisingly, and contrary to most studies, we found little to no effects of AMF inoculation on maize growth and nutrients acquisition (Fig. 5.1 and Table 5.1) when the microbes pre-trained by maize monocropping were eliminated by soil sterilization. However, some studies also report a lack of AMF benefits for plants grown in very poor sterilized soils (Ceulemans et al. 2017), likely reflecting severe plant nutrient limitation, together with a lack of 'alternative' nutrient sources to be scavenged by AMF. Non-exclusively, the lack of AMF benefits highlights that mycorrhizal effects can range from fully mutualistic to parasitic interactions, depending on a complex interplay of both partners' identity (Reynolds et al. 2006; Janouskova et al. 2013).

5.4.2 AMF Benefits Depend on the Isolate Identity

Despite an abundant native AMF community that mycorrhized control plants grown in non-sterilized soil, AMF inoculation was successful as shown by the lower biomass and lower nutrient contents in the plants that were not AMF-inoculated (Fig. 5.1 and Table 5.1). These results are in agreement with other studies on AMF inoculation (Vosatka 1995; Kohl et al. 2016). But not all inoculated AMFs conferred benefits to their host plants (van der Heijden et al. 1998; Hart and Reader 2002), which could not be explained by improved nutrition alone because interaction with the remainder soil microbes also differed between inoculated AMF. Due to distinct socialization strategies between inoculated AMFs and the remainder soil microbes and fauna (Fig. 5.2), inoculation with *R. irregularis* and especially with *Scutellospora* sp. did not overrule the soil legacy effects of maize monocropping while inoculation with *C. claroideum*, *F. mosseae* and *Gigaspora* sp. did cancel the negative feedbacks (Fig. 5.1 and Table 5.1).

Maize plants inoculated with *Scutellospora* sp suffered feedback on root and shoot biomass even more negative than that under control conditions (Fig. 5.1 and Table 5.1) thus suggesting mycorrhizal colonization. Since mycorrhized plants experience an initial growth depression compared to non-mycorrhized (Hart and Reader 2002), and *Scutellospora*'s growth is very slow it is possible that *Scutellospora*'s benefits would need longer than the experiment's duration to manifest. This may have implications for the use of this AMF species in crops with short life cycle.

In the absence of the soil legacy effects of maize monocropping (sterilized soil), the plants that presented bigger shoots were those inoculated with *R. irregularis* (Fig. 5.1). However, since plants grew less in the non-sterilized soil than in the sterilized soil, and roots accumulated the most nematodes (Fig. 5.2), inoculation with *R. irregularis* did not overrule the soil legacy effects of maize monocropping (Fig. 5.1). In arable fields, nematode population densities in the upper soil layer can reach 10^7 m^{-2}, the equivalent of 2.0 kg C and 0.25 kg N ha^{-1}. Bacterivores often dominate this fauna, particularly rhabditid and cephalobid species (Bouwman et al.

1996), which were the most abundant nematodes in *R. irregularis* roots. This suggests that nematodes, and possibly other parasites and pathogens decreased *R. irregularis*' efficiency in acquiring nutrients.

By contrast, the roots of plants inoculated with *C. claroideum*, *F. mosseae* and *Gigaspora* sp. accumulated the least nematodes (Fig. 5.2), which is in agreement with other studies (e.g. (Sasanelli et al. 2009; Affokpon et al. 2011). Even though we cannot infer which mechanism(s) caused pathogen protection (changes in root architecture, activation of plant defense mechanisms, competition for infection sites and improved nutrient status, Wehner et al. 2011), the soil legacy effects of maize monocropping was overruled (Fig. 5.1 and Table 5.1). AMFs' role in improving the growth and nutrition of the plant host is widely documented and recognized (Dias et al. 2015) for P (Kothari et al. 1991; van der Heijden et al. 2006, 2008), N (Cruz et al. 2007; Correa et al. 2014, 2015) and micronutrients (Kothari et al. 1991; Liu et al. 2000; Balakrishnan and Subramanian 2012). AMF improve plant nutrition by scavenging 'alternative' nutrient sources that otherwise would not be accessible to plant roots (Smith and Read 1997) and/or by acting as a 'pipeline' of plant-derived C to other soil microorganisms, trading the carbon for nutrients and transferring the nutrients to the plant (Nuccio et al. 2013). Our data does not support the hypothesis that AMF were scavenging 'alternative' nutrient sources since shoot ^{15}N, an integrative indicator of the N source (Ariz et al. 2015), did not change (data not shown). Instead, our data suggest that *C. claroideum*, *F. mosseae* and *Gigaspora* sp. simply extended the root system and thereby took up more nutrients (Smith and Read 1997), and enhanced their host's competitive success against free-living soil microbes (Schimel and Bennett 2004).

5.5 Conclusions

Unlike former observations that AMF are not beneficial in agricultural fields, our results demonstrate that AMF inoculation in field soils can enhance growth of maize irrespective of the pre-established microbial community, being able to compete successfully with indigenous AMF (Kohl et al. 2016). We confirmed clear biological consequences of belowground socialization of AMF with remainder soil microbial communities (biotic context) on plant growth. Furthermore, this effect was AMF species-dependent under a more-structured and stable soil microbial community (i.e., non-sterilized soil) but not under a recently assembled soil microbial community (i.e., sterilized soil), where AMF had little to no effect.

Rhizophagus intraradices, *R. irregularis* and *Funneliformis mosseae* are very generalist symbionts that can colonize a large variety of host plants, survive long-term storage, are geographically distributed all over the world, and can be easily and massively propagated, which makes these species suitable for premium inoculum components. However, our data shows that other AMF (*C. claroideum* and *Gigaspora* sp) may be equally or even more beneficial and should be further assessed for their application in agriculture.

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