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Enhancing ecosystem services in sustainable agriculture: biofertilization and biofortification of chickpea (*Cicer arietinum* L.) by arbuscular mycorrhizal fungi.

Authors: Elisa Pellegrino, Stefano Bedini

Regular manuscript

Dear Editor,

We revised the manuscript following the reviewer suggestions and comments. We tested the PERMANOVA as multivariate method of data analysis alternative to RDA. Our data allowed both analyses and both the analyses gave the same picture. Therefore, we preferred to maintain the RDA analysis because it allows also to discriminate among the levels of the treatments. We hope that now our manuscript can be finally accepted for publication.

Yours sincerely,

Elisa Pellegrino

RESPONSE TO REVIEWER

REVIEWER: 1

1) We appreciate the comment of the reviewer about the choice of the multivariate data analysis method. We checked our dataset on the basis of his two main questions:

1. "A limitation of RDA is that it should be used when multiple response variables are normally distributed and if they are intercorrelated, they are correlated in a linear fashion. Is this true?"

- YES, our response variables were all log- or arcsen transformed to fulfil the assumption of normality. In addition, we did a Detrended Correspondence Analysis (DCA) for checking linearity or unimodality behaviour of the response variables (MS-rev: lines 240-244). This method is based on determining the length of gradients describing response variable variation and the results indicated a short variational gradients (< 4) and, hence that the linear relations are a good assumption for our response data (Leps & Smilauer 2003, p. 28).

2. "You state a concern that PERMANOVA may not be reliable if there are differences in multivariate dispersion (the multivariate equivalent of unequal variance). Do your data suffer from this problem?

- NO, actually, our data do not suffer from differences in multivariate dispersion. We checked this by drawing a triplot (samples, response and environmental variables).

Taking into consideration that on the basis of the above answers (YES/NO) both analyses could have been done, we also performed a PERMANOVA and a PCO ordination (see Table and Figure below). Actually, both the analyses gave the same picture (see below Table and Figure). Therefore, we preferred to maintain the RDA analysis because it allows also the discrimination among the levels of the treatments.

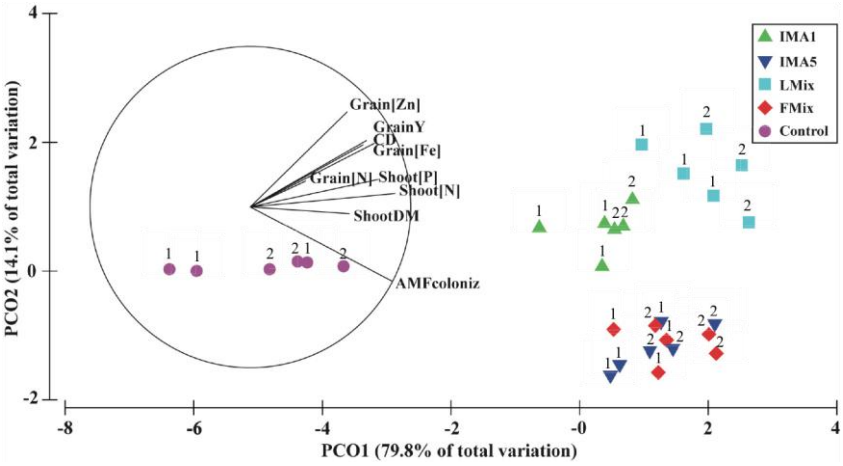
Results of PERMANOVA analyses on the effects, at harvest, of inoculation with arbuscular mycorrhizal (AM) fungi (AMF inoc), sowing time (S time) and their interactions on chickpea (*Cicer arietinum* L.) AM fungal colonisation, plant growth, yield, yield components, plant nutrient uptakes and grain biofortification.

Source of variation	df [a]	SS	MS	Pseudo-*F*	*P* (perm)
AMF inoc [b]	4	234.830	58.706	65.110	**0.001**
S time	1	8.326	8.326	9.235	**0.002**
AMF inoc x S time	4	2.063	0.516	0.572	0.748
Residual	20	18.033	0.902		
Total	29	263.250			

permutation).

[b] Two-way PERMANOVA: AMF inoc, fixed factor; S time, fixed factor. AMF inoc: two single, foreign AMF (*Funneliformis mosseae*, IMA1; *Rhizophagus irregularis,* IMA5); a dual strain inoculum, foreign mixture (FMix): IMA1 + IMA5; a trap-culture-enriched locally-sourced AM fungal community (local mixture = LMix) and a mock inoculum as control; S time: autumn (October) and spring (March).

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



1= Autumn sowing; 2 = Spring sowing.

Minor comments

We changed through the text all instances "at autumn or spring sowing" to "in the autumn or spring sowing". All suggested minor changes were done.

HIGHLIGHTS

- AMF field inoculation increased chickpea root colonization, yield & nutrient uptake
- AMF improved chickpea nutritional value by protein, Fe & Zn grain biofortification
- Local AMF were more effective than foreign for yield & grain N content
- Local AMF inoculum was the most efficient in Fe and Zn grain biofortification

Enhancing ecosystem services in sustainable agriculture: biofertilization and biofortification of chickpea (*Cicer arietinum* L.) by arbuscular mycorrhizal fungi

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) establishing beneficial symbiosis with most crop plants have gained a growing interest as agro-ecosystem service providers able to sustain crop productivity and quality. In this study we tested the agronomic relevance of field-inoculated locally sourced and foreign inocula on chickpea (*Cicer arietinum* L.), one of the most important worldwide grain legumes. The foreign AMF *Funneliformis mosseae* and *Rhizophagus irregularis* were used as single and dual species inocula. Crop growth and productivity, plant nutrient uptakes and protein, Fe and Zn grain biofortification were assessed under a rainfed low-input cropping system after autumn and spring sowings. Uni- and multivariate analyses of data showed that AM fungal field inoculation increased chickpea AM fungal root colonization as well as plant biomass and yield. In addition, AMF were also effective in improving the nutritional value of grain by protein, Fe and Zn biofortification. The locally sourced AM fungal inoculum was more efficient then the foreign ones in Fe and Zn grain biofortification and, in the spring sowing treatment, also in improving yield and grain protein content. These findings enhance our understanding of the field potential role of AMF showing that a mycorrhiza-friendly approach in agriculture may have great potential in biofertilization of crops and biofortification of foods.

Key words:

arbuscular mycorrhizal (AM) fungal field inoculation, biofertilization, biofortification, functional diversity, *Funneliformis mosseae*, *Rhizophagus irregularis*, crop yield and quality improvement, local AM fungi (AMF), foreign AMF, sowing time.

1. Introduction

Over the last 20 years, low-input and organic agriculture has increased worldwide to preserve agro-ecosystem functionality (Altieri, 1999; Lotter, 2003; Crowder et al., 2010; Postma-Blaauw et al., 2010). The central pillar of such an agriculture is a systemic 'holistic' approach to cropping system management, which is based on the use of the ecosystem services, in order to achieve sustainable yield and crop quality together with high energy efficiency and low environmental impact (Pimentel et al., 2005; Moonen and Bàrberi, 2008). In this view, soil microorganisms, such as arbuscular mycorrhizal (AM) fungi (AMF, phylum *Glomeromycota*), representing a key interface between plant hosts and soil mineral nutrients, have gained a growing interest as *ecosystem engineers* and *biofertilizers* (Gianinazzi et al., 1990; Gianinazzi and Vosátka, 2004; Fitter et al., 2011).

AMF, which could represent 10% or more of the soil microbial biomass, establish a mutual symbiosis with the majority (approx. 80%) of land plant species and agricultural crops (Smith and Read, 2008). They supply mineral nutrients to the plants, mainly phosphate, in exchange for photosynthetically fixed carbon (Bago et al., 2000; Hodge et al., 2010). As an effect of the symbiosis, AMF improve agriculture productivity by enhancing plant growth (Koide, 1991), seed production (Shumway and Koide, 1994) and protecting plants from root pathogenic fungi (Newsham et al., 1995; Linderman, 2000) and drought (Augé, 2001). Moreover, AMF have a direct effect on the ecosystem, driving the structure of plant communities (van der Heijden et al., 1998a,b) and ameliorating the quality of soil by improving its aggregation and organic carbon content (Miller and Jastrow, 1990; Rillig and Mummey, 2006; Bedini et al., 2009).

Although the presence of AMF is widespread in agricultural soils, field experiments showed that a further addition of AMF by inoculation can positively affect plant the root colonization

and increase the crop productivity (McGonigle, 1988; Lekberg and Koide, 2005; Lehmann et al., 2012).

Beside yield, AMF, due to their role in plant nutrition, could also enhance crop quality not only by enrichment in macronutrients (i.e., N and P) (Karandashov and Bucher, 2005; Veresoglou et al., 2012), but also in micronutrients (White and Broadley, 2009; He and Nara, 2007; Antunes et al., 2012). Micronutrient deficiency is a major issue affecting health of billions of people all over the world. Actually, because of the intensity of crop production, agricultural soils have became more and more depleted in micronutrients and, as a consequence, yield mineral element contents are decreasing, compromising the nutritional value of food. Indeed, biofortification, the increase of the concentrations and/or bioavailability of mineral elements in produce, is considered a promising strategy for tackling micronutrient malnutrition, especially in developing countries (White and Broadley, 2009).

AM fungal field inoculation studies have been mostly based on the use of selected, foreign AM fungal isolates (Clarke and Mosse, 1981; Edathil et al., 1996; Meyer et al., 2005). However, due to different affinities between host plants and AMF (van der Heijden et al., 1998a; Klironomos, 2003; Munkvold et al., 2004; Avio et al., 2006), the use of a single AM fungal strain is likely not optimal for all crops (Koomen et al., 1987; Jansa et al., 2008, 2009; Smith et al., 2000; Koide, 2000; Maherali and Klironomos, 2007). Moreover, because interactions among different AMF are not always synergistic (Koide, 2000; Jansa et al., 2008, 2009), AM fungal inocula have to be evaluated also in the field, where a local community is present. As an alternative, the use of an inoculum based on locally sourced AMF may be a suitable choice because of a better adaptation to the prevailing conditions (Lambert et al., 1980) and also because they could avoid the ecological risks of the introduction of foreign species (Schwartz et al., 2006). Actually, some studies showed higher or similar plant growth and nutritional performances of locally sourced AMF compared to foreign selected ones

(Caravaca et al., 2003, 2005; Requena et al., 2001; Tchabi et al., 2010; Pellegrino et al., 2011a).

In the present study we evaluated the effectiveness of the inoculation of locally sourced and foreign AMF on chickpea (*Cicer arietinum* L.), cultivated under a rainfed low-input system. This crop is one of the most ancient pulse crops domesticated in the Middle East around 7500 years ago (Maiti and Wesche-Ebeling, 2001) and one among the five most important worldwide grain legumes with an annual production of about 9.4 million tons (FAOSTAT, 2010). This crop is also largely cultivated in the Mediterranean area, where to benefit from the rainfall season, autumn sowing has been proposed as an alternative to the traditional spring one (Frenkel et al., 2010).

Despite several studies reported the beneficial effects of AMF on shoot concentrations of mineral elements, such as iron (Fe) and zinc (Zn) (Tarafdar and Rao, 1997; Al-Karaki, 2000; Ryan and Angus, 2003; Cavagnaro, 2008; Ortas, 2012), the effectiveness of AM fungal inoculation on agricultural produce is still not resolved (Antunes et al., 2012). Starting from the evidence that chickpea positively responds to single foreign AM fungal inocula (Singh and Tilak, 1989; Weber et al., 1993; Clark and Zeto, 2000; Tufenkci et al., 2005), in the present study the field effectiveness of the inoculation of locally sourced AMF on chickpea (*Cicer arietinum* L.), cultivated under a rainfed low-input system was evaluated. The response of autumn and spring-sown chickpea in terms of growth, yield and nutritional profile has been assessed and compared with the effects of two foreign strains of the AM fungal species *Funneliformis mosseae* and *Rhizophagus irregularis*, used as single or dual species inocula.

2. Material and methods

2.1. Fungal and plant material

The AMF used were: *Funnelliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüβler, isolate IMA1 = BEG12 from UK (collector B. Mosse) and *Rhizophagus irregularis* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüβler, isolate IMA5 from Italy (collector M. Giovannetti) (new classification by Schüßler and Walker, 2010; the species were formerly known as *Glomus mosseae* and *Glomus intraradices*), used as single or dual species inocula (foreign mixture (FMix) = IMA1 + IMA5) and a locally sourced inoculum (LMix) consisting of AMF originating from the field site. The trap-culture-enriched locally sourced AM fungal inoculum was composed by: *Acaulospora cavernata* (syn. *Acaulospora scrobiculata*), *A. spinosa*, *Acaulospora spp.* (*syn. A. rugosa*), *Diversispora spurca*, *Funneliformis coronatum* (syn. *Glomus coronatum*), *Claroideoglomus etunicatum* (syn. *Glomus etunicatum*), *Funneliformis geosporum* (syn. *Glomus geosporum*), *F. mosseae*, *Glomus* spp., *Rhizophagus clarus* (syn. *Glomus clarum*), *R. irregularis*, *Scutellospora aurigloba* and *S. calospora* and *Septoglomus viscosum* (*syn. Glomus viscosum*) (Pellegrino, 2007; Redecker et al., 2013). The plant species used was chickpea (*Cicer arietinum* L.) cv. Sultano.

2.2. Experimental field site

The experiment was settled at the Rottaia Experimental Centre of the University of Pisa, Italy (43°30'86"N - 10°19'00"E). The soil is a sandy loam (66.1% sand, 24.4% silt and 9.5% clay) with 8.1 g kg^{-1} soil organic carbon (Walkley-Black), pH(H_2O) of 8.4 and the following total nutrient concentrations: 0.7 g kg^{-1} N (Kjeldahl), 36.0 mg kg^{-1} P and 14.6 mg kg^{-1} available P (Olsen). Climatic conditions are typically Mediterranean with rainfall mainly concentrated in autumn and spring (mean 948 mm $year^{-1}$) and mean monthly temperature ranging from 11 °C in February to 30 °C in August (mean of 14.5 °C $year^{-1}$). Before experimental setup, the field site has been uncultivated for about three years.

2.3. Mycorrhizal potential of the experimental soil

Number and infectivity of AM fungal soil propagules of the experimental site were evaluated by mycorrhizal index, AM fungal spore density and mycorrhizal infection potential (MIP). Mycorrhizal index (calculated as percentage of AM fungal colonized root length) was determined by examining the roots of 15 naturally occurring mycotrophic plants (*Bellis perennis* L. , *n* = 3; *Calendula arvensis* L., *n* = 5; *Daucus carota* L., *n* = 5; *Papaver rhoeas* L. *n* = 1 *and Plantago lanceolata*, *n* = 1). The percentage of AM fungal colonization was assessed, after root clearing and staining, using lactic acid instead of phenol (Phillips and Hayman, 1970) by the gridline intersect method (Giovannetti and Mosse, 1980). The AM fungal spores density was estimated in 50 g of soil from ten core samples by wet-sieving and decanting, followed by sucrose centrifugation (Sieverding, 1991) and spore number was assessed under a Wild dissecting microscope (Leica, Milano, Italy). The MIP was evaluated as follows: *Lactuca sativa* L. seeds were sown in 50 mL sterile plastic tubes filled with 40 mL of 15 soil samples taken up to a depth of 30 cm using a soil corer and air-dried. Six replicate plastic tubes were used for each soil sample. After emergence, *L. sativa* plants were thinned to three. After two week's growth, plants were removed from tubes and root systems were cleared and stained as above, then mounted on microscope slides and examined under a Reichert-Jung (Vienna, Austria) Polyvar microscope. Root length and AM fungal colonized root length were measured using a grid eyepiece. Number of infection units (hyphal entry points at the roots) and number of entry points were assessed at magnifications of x125-500 and verified at a magnification of x1250.

2.4. AM fungal inoculum production

The AM fungal inocula required for the field inoculation were produced in 18 L pots filled with soil and Terragreen (calcinated clay, OILDRI, Chicago, IL, USA) (1:1, by volume), as potting substrate. The soil used for the potting substrate was collected at the Rottaia Experimental Centre and was a sandy loam (54.5% sand, 30.1%; silt and 15.3% clay) with 12.8 g kg^{-1} soil organic carbon (Walkley-Black), pH(H_2O) of 8.0 and the following total nutrient concentrations: 1.3 g kg^{-1} N (Kjeldahl), 469.5 mg kg^{-1} P, 14.6 mg kg^{-1} available P (Olsen) and 149.6 mg kg^{-1} extractable K. The potting substrate was steam-sterilized (121° C for 25 min, on two consecutive days) to kill naturally occurring AMF. The single species inocula (IMA1 and IMA5) were produced by inoculating each pot with 500 g of crude inoculum. The dual species foreign inoculum (FMix) was obtained by mixing equal quantities of the two single species inocula (250 g IMA1 and 250 g IMA5). The crude inocula were obtained from pot cultures maintained in the collection of the Soil Microbiology Laboratory of the Department of Agriculture, Food and Environment, University of Pisa, Italy. The locally sourced inoculum (LMix) was produced by inoculating the pots, containing the steam-sterilized potting substrate of soil and Terragreen, with 500 g of soil from the Rottaia field site. In addition a mock inoculum, free of AMF, was prepared to treat the control plots (Control). The mock inoculum was produced by adding to the steam-sterilized potting substrate 500 g of a sterilized mixture of equal quantities (about 170 g each) of each of the two single species crude inocula (IMA1and IMA5) and of the Rottaia field soil. The inocula were produced in greenhouse using maize (*Zea mays* L.) as host plant (10 plants per each pot). Finally, to ensure a common microflora, all pots received 1.5 L of soil filtrate, obtained by filtering, through Whatman no. 1 filter paper, a mixture of each single species inoculum and of the Rottaia field soil. Pots were supplied with deionized water (irrigation cycle: 4 days) replaced, after two month's growth, by half-strength Hoagland's solution (Hoagland and Snyder, 1933). After four month's growth, maize plants were harvested and the roots removed from the pots. The roots and the potting substrate were air-dried. The roots were,

then, cut and mixed with the potting substrate and stored in polyethylene bags at 4° C until field use. The MIP of the AM fungal inocula showed values of infection units ranging from 1.10 ± 0.34 to 2.89 ± 0.54 cm^{-1} root length (mean ± S.E.) in IMA5 and IMA1, respectively (Pellegrino et al., 2011a).

2.5. Experimental set-up

A two-factor design was applied with the inoculum treatment (IMA1, IMA5, FMix, LMix and the control) and the sowing time (autumn and spring sowing) as factors and three replicate plots. Plots (2.5 m x 1.5 m) were dug (10 cm depth) and harrowed (5 cm depth) and then inoculated with 5.3 kg $plot^{-1}$ of crude inoculum (mock inoculum for the control) along the rows. Plots were sown on October 2004 (autumn sowing) (n = 15) and on March 2005 (spring sowing) (n = 15) with 40 x 10 cm row spacing and 10 cm border spacing (8 rows $plot^{-1}$) in order to obtain a plant density of 25 plants m^{-2}. Three seeds were placed in each planting position and, after the emergence, the seedlings were thinned to one. Chickpea plants were harvested at the drying of seed pods on June 2005. During the crop cycle, plots were manually kept weed-free.

2.6. Measurements

One month after emergence, the percentage of AM fungal root colonization was assessed, after root clearing and staining, using lactic acid instead of phenol (Phillips and Hayman, 1970) by the gridline intersect method (Giovannetti and Mosse, 1980) on a random sample (n = 6) of chickpea plants from each plot. At harvest, 10 plants from each replicate were cut at ground level and air-dried. AM fungal root colonization, shoot dry matter, collar diameter and grain yield were measured. Shoot and seed N and shoot P concentrations were assessed using

the Kjeldahl method and the sulphuric/perchloric acid digestion using the photometric method (Jones et al., 1991), respectively. Fe and Zn seed concentrations were detected by inductively coupled plasma-mass spectrometric (ICP-MS) analysis. The acid mineralization was done on 0.5 g dry samples. Samples were digested for 10 min into a microwave-digesting machine with a solution composed by 6 ml HNO_3 65% and 1 ml H_2O_2 33%. The resulting solutions were filtered and diluted with ultra-pure water to a 25 ml volume and then analyzed with ICP-MS analysis.

2.7. Statistics and data analyses

Data were analyzed by two-way (inoculum treatment and sowing time as fixed factors) or one-way ANOVA (inoculum treatment as factor). Data were ln- and arcsine-transformed when needed to fulfill the assumptions of the ANOVA, which was carried out according to the completely randomized design. Multiple comparisons within the one-way ANOVAs were done with orthogonal contrasts. All the analyses were performed by the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Means and standard errors (S.E.) given in tables are for untransformed data.

To test the null hyphotheses about differences between groups on the basis of multiple response variables, along with the univariate methods, we also analyzed the data set with a multivariate constrained approach. Redundancy analyses (RDA) (van den Wollenberg, 1977) were used to investigate, for each sowing time, the influence of the different inoculation treatments (used as explanatory variables) on plant growth, yield, plant nutrient uptake, grain biofortification variables and AM fungal colonization (used as response variables). Since the response variables were in different measurement units and the length of the gradient of the detrended correspondence analysis (DCA) was lower than four, we utilized the RDA linear method (Lepš and Šmilauer, 2003). All data were log- or arcsen-transformed, centered and

standardized by the response variables. Monte Carlo permutation tests were performed using 499 random permutations (unrestricted permutation) in order to determine the statistical significance of the relations between the whole set of inoculation treatments and the response variables. RDA analyses were done by Canoco for Windows v. 4.5 (ter Braak and Šmilauer, 2002). The biplots were drawn by CanoDraw for Windows.

3. Results

3.1. Mycorrhizal potential of the experimental soil and chickpea AM fungal root colonization

In the experimental soil, AM fungal root colonization of the selected plant species was 7.1 ± 1.6%, spore density was 3.1 ± 0.4 spores g^{-1} soil**,** while the MIP test values were: root colonization, 1.3 ± 0.5%; infection units, 0.27 ± 0.07 cm^{-1} root length; entry points, 0.13 ± 0.03 cm^{-1} root length.

One month after emergence, the percentages of colonized root length of control plants in the autumn and spring sowing treatments were 10.1 ± 1.1 and 12.6 ± 0.6%, respectively, and lower than those of mycorrhizal treatments that ranged, in the autumn sowing, from 21.0 ± 1.1% to 33.7 ± 8.4% in IMA5 and FMix, respectively, and, in the spring sowing, from 25.2 ± 0.5% to 40.9 ± 2.3% in IMA5 and IMA1, respectively (data not shown).

At harvest, mycorrhizal root colonization was significantly affected by AM fungal inoculation (Table 1). In the autumn sowing treatment, the degree of root colonization ranged from 20.4% to 69.7%, while in the spring sowing treatment from 25.1% to 71.7% in control and FMix, respectively (Fig. 1a). At both sowing times, significant differences were also observed between IMA1 and IMA5 and between FMix and LMix (Table 1). In detail, orthogonal contrasts showed that FMix consistently colonized chickpea more than LMix and single strain inocula (Table 1).

3.3. Plant growth

One month after emergence no differences among treatments were observed in root (data not shown) and shoot dry matter. In the autumn sowing treatment, shoot dry matter ranged from 0.29 ± 0.03 to 0.36 ± 0.01 g plant^{-1} in IMA5 and control, respectively, while in the spring one it ranged from 0.32 ± 0.02 to 0.41 ± 0.07 g plant^{-1} in IMA5 and in IMA1, respectively.

At harvest, shoot dry matter of spring-sown plants was significantly affected by AM fungal inoculation (Table 1). In the spring sowing treatment, inoculated chickpea showed in average 25% higher plant biomass than control (Fig. 1b). In both sowing times, larger plant collar diameters were observed in AM fungal treatments with respect to controls (Table 1). In addition, in the spring sowing treatment, LMix inoculated plants showed larger collar diameter than the FMix ones (Table 1; Fig. 1c).

In the spring sowing, shoot dry matter per plant showed a strong correlation with the percentage of AM fungal colonized root length (R = 0.724, $F_{1,13}$ = 14.326, P = 0.002).

3.4. Yield

As regards yield, in both sowing times, AM fungal inoculation consistently induced better plant responses compared with control (Table 1). Host benefit, calculated, in both sowing times, for each AM fungal inoculation treatment as [((mycorrhizal treatment – control)/control) x 100], was 38%, 52%, 69% and 79% in the autumn sowing treatment and 41%, 52%, 86% and 93% in the spring one for IMA5, FMix, IMA1 and LMix, respectively. In the spring sowing, LMix chickpea showed higher values of grain yield per plant respect to FMix (Table 1). In detail, LMix inoculated plants showed 27% higher grain yield than the

FMix ones (Fig. 1d). Moreover, in the spring sowing treatment a significant better performance of IMA1 when compared to IMA5 was also observed (32%) (Table 1; Fig. 1d).

3.5. Nutrient uptake and grain biofortification

In both sowing times, N and P uptakes, evaluated by shoot nutrient concentrations, were affected by AM fungal inoculation (Table 2). On the basis of N and P shoot concentrations, in the autumn sowing treatment host benefits were on average 106% and 48%, respectively, while in the spring sowing treatment were 110% and 45%, respectively. No significant difference was observed among inoculated treatments, except for shoot N concentration between IMA1 and IMA5 (Table 2). Interestingly, in both sowing times, N and P shoot concentrations were mostly strongly correlated with the percentage of colonized root length (autumn sowing: shoot N and P concentrations R = 0.781, $F_{1,13}$ = 20.310, P = 0.001 and R = 0.678, $F_{1,13}$ = 11.031, P = 0.006, respectively; spring sowing: shoot N and P concentrations R = 0.793, $F_{1,13}$ = 22.042, P < 0.001 and R = 0.589, $F_{1,13}$ = 6.904, P = 0.021, respectively).

As regards grain, N concentrations in the spring sowing treatment were affected by AM fungal inoculation (Table 2), with a host benefit of 6 % in average. Interestingly, lower grain N concentrations were observed in FMix in comparison with single AM fungal inoculated plants (Table 2).

Fe and Zn seed concentrations were significantly higher in inoculated plants than controls, both in the autumn and spring sowing treatments (Table 3). In detail, Fe and Zn increases were of 5% and 16%, respectively. Interestingly, LMix induced higher concentrations of the analyzed micronutrients in comparison with FMix. These increases, considering both sowing times, were of 4% and 21% for Fe and Zn uptakes, respectively. LMix resulted the most efficient inoculum for biofortification, raising the micronutrient level of about 8% and 36% respect to the control for Fe and Zn, respectively (Table 3).

3.4. Effect of sowing time

The two-way ANOVAs showed no differences in AM fungal root colonization between sowing times (S time) or interaction between AM fungal inoculation (AMF inoc) and S time, both one month after emergence ($P = 0.738$ and $P = 0.483$, respectively) and at harvest (Table 4). No differences between S times or interactions were detected in shoot and root dry weights one month after emergence (data not shown).

By contrast, at harvest, shoot dry matter and collar diameter were significantly affected by the sowing time (Table 4; Fig. 1b,c). No differences in shoot and grain nutrient uptake between S times and no interaction between AMF inoc and S time were detected (Table 5). Interestingly, grain Fe concentrations were significantly affected by S time, showing higher values in the spring sowing treatment than in the autumn one (Tables 3, 5).

3.5. Main patterns of chickpea traits as affected by AMF

RDA, in line with the univariate tests, showed that AM fungal inoculation explained 62.9% and 69.4% of the whole variance in the autumn and spring sowing treatments, respectively, and that its effect on the response variables was significant ($P = 0.002$). In both sowing treatments, the Monte Carlo permutation test showed that control was significantly different from the other treatments ($P = 0.002$) and LMix from the FMix ($P = 0.002$). In addition, in the spring sowing, we also observed significant differences between IMA1 and IMA5/FMix ($P = 0.008$). The biplots also show that collar diameter, grain yield, shoot P concentration were the most discriminating variables between AM fungal treatments and the control in the autumn sowing treatment (Fig. 2a), whereas grain yield, shoot dry matter and collar diameter were the most discriminating in the spring one (Fig. 2b). The biplots of the autumn and spring

sowing treatment also show that grain Zn and Fe concentrations and AM fungal colonization were highly discriminative between LMix and the other AM fungal treatments (Fig. 2a,b).

4. Discussion

In the present study data showed that: (i) AM fungal field inoculation increased chickpea AM fungal root colonization as well as yield and plant nutrient uptake; (ii) AM fungal inoculation was effective in improving the nutritional value of chickpea grain by protein, Fe and Zn grain biofortification; (iii) locally sourced AM fungal inoculum was the most efficient in Fe and Zn grain biofortification; (v) in the spring sowing treatment, local AM fungal inoculation was more effective than foreign inocula in improving yield and grain N content.

4.1. Mycorrhizal potential of the experimental soil and chickpea AM fungal root colonization

All AM fungal inocula promoted a rapid increase of the chickpea root colonization that, already one month after emergence, was higher compared to control plots. Since AM fungal inoculation success is expected to be negatively related to the amount of the active AM fungal propagules already present in the soil (Abbott and Robson, 1982, 1991; Gianinazzi and Vosátka, 2004), the higher AM fungal colonization observed in the inoculated plants compared with controls indicated that the mycorrhizal infection potential (MIP) of the soil at the beginning of the experiment was low or sub-optimal. These low MIP values, in line with the those observed in other agricultural soils (Purin et al., 2006; Di Bene et al., 2011; Pellegrino et al., 2011a; 2012; Di Bene et al., 2013; Gosling et al., 2006; Bedini et al., 2013), confirm the detrimental effects of agricultural practices on AM fungal infectivity (Kabir et al., 2005; Plenchette et al., 2005). In addition, the success of the inoculation in terms of AM

fungal root colonization indicates that the MIP assay is appropriate as preliminary test in the planning of an effective AM fungal inoculation treatment.

At harvest, AM fungal root colonization of the inoculated chickpea was up to three-fold respect to the controls. Such colonization increases are in line with previous trials on field AM fungal inoculation of chickpea (Singh and Tilak, 1989; Weber et al., 1993; Saini et al., 2004) and of other crop plants such as lucerne (*Medicago sativa* L.), sorghum (*Sorghum bicolor* L.) and white clover (*Trifolium repens* L.) (Hayman and Mosse, 1979; Bagyaray et al., 1979; Rangeley et al., 1982; Hayman, 1984; Pellegrino et al., 2011a; 2012) and strongly support that the colonization of mycorrhizothrophic crops can be effectively improved by field inoculation. Although, all the inocula were effective in raising the level of AM fungal colonization of chickpea, at harvest, we observed differences between the two foreign isolates when individually inoculated. Actually, the higher colonization by IMA5 compared to IMA1 is consistent with previous observations both in microcosm (Avio et al., 2006) and field conditions (Pellegrino et al., 2011a) and confirms *R. irregularis* (IMA5) as a good competitor for field inoculation (Alkan et al., 2006; Douds et al., 2011).

Interestingly, the linear orthogonal contrast analysis indicated that IMA1 and IMA5 were more efficient in boosting AM fungal colonization when used as dual than as single inocula, suggesting an additive or synergistic effect. However, since the level of root colonization of IMA5 is almost the same of FMix, such an effect is probably due to a dominance of IMA5 respect to IMA1. Moreover, the higher root colonization of IMA1/IMA5 dual species respect to the locally sourced inoculum confirms that selected AM fungal species may be more effective in the establishment, survival and spreading of the symbiosis within roots of crops (Sýkorová et al., 2012). However, it should be taken in account that although the colonization effectiveness could be a positive trait for commercial inocula, a high aggressiveness of foreign AMF could determine shifts in composition, structure and functionality of the whole

local soil microbial community (Mummey et al., 2009; Koch et al., 2011; Sýkorova et al., 2012; Veresoglou et al., 2012).

4.2. Plant growth

Contrary to our expectation, spring-sown chickpea showed higher plant growth parameters in comparison with the autumn-sown one. In Mediterranean areas, autumn sowing is considered more convenient because the winter rainy season may assure a better crop growth and yield (Singh, 1997). However, especially in the northern part of the Mediterranean areas, it may also result in a higher exposure to the cold and to pathogen diseases such as aschochyta blight that could cause loss of productivity (Nene, 1981). Actually, we observed differences between the two sowing date on the aboveground chickpea biomass that was effectively increased by AM fungal field inoculation only in spring-sown chickpea. AM fungal inoculation resulted much less effective in the autumn sowing treatment respect to spring one (8% *vs* 25%, respectively) where, inoculation benefits, were consistent with previous chickpea field inoculation trials (Singh and Tilak, 1989; Weber et al., 1993; Zaidi et al., 2003).

It is also should be noted that the chickpea AM fungal benefit was lower than what was observed in other pulses such as Egyptian clover and lucerne (mean increases of 93% and 83%, respectively) (Pellegrino et al., 2011a, 2012). Taking into account the main role of AMF in plant P nutrition, this lower responsiveness may be due to chickpea minor P requirements (30-80 Kg ha^{-1} P_2O_5; Foti e Abbate, 2000; Saccardo et al., 2001) in comparison with other fodders (about 120 Kg ha^{-1} P_2O_5 per year; Masoni et al., 1993). Similarly, the lack of any significant effect on biomass one month after plant emergence may still depend on the large chickpea seed reserves, which could sustain the early growth of the plant (Weber et al., 1993) and thus masking the effect of AM symbiosis.

Despite observing significant differences in root AM fungal colonization, no interspecific differences in plant biomass were detected between IMA1 and IMA5. This may depend on the effects of co-occurring soil AMF as well as on the functional diversity of the two isolates (Klironomos, 2000, 2003; Smith et al., 2004).

In line with plant biomass benefits, collar diameter responded positively to AM fungal inoculation. Collar diameter was strongly correlated with N and P uptakes. Since no correlations were observed with shoot dry matter (data not shown), the increase of the collar diameter appears to be associated to a better crop nutritional status of the inoculated plants. Considering that collar diameter is a key trait for plant stress resistance, we can argue that AM fungal inoculation might lead to a stronger resistance of the crop and to lower yield losses due to plant laid down and/or to pathogen attacks.

4.3. Yield

Overall, chickpea productivity was in line with the mean production reported in previous works (Singh et al., 1989; Weber et al., 1993; Zaidi et al., 2003). In this trial, AM fungal field inoculation was very effective in improving chickpea yield, with increases of about 75% in comparison with the controls. Such increase was much higher of that obtained by Singh and Tilak (1989), who, using *Glomus versiforme* as field inoculum, observed increases of about 11% and in contrast with Weber et al. (1993) and Zaidi et al. (2003) who did not register any yield difference. However, similar large benefits were observed for *Trifolium alexandrinum* in the same pedo-climatic conditions by Pellegrino et al. (2011a), who reported seed production increments of about 77% with the same AM fungal isolates and by Saini et al. (2004), who obtained large yield chickpea increases (109%) using a dual inoculum of AMF and rhizobia. This variability on yield benefit may depend on differences in plant-fungal genotype compatibilities (van der Heijden and Sanders, 2002) or on different climatic and soil

conditions, which may also include soil mycorrhizal infection potential and rhizobia populations. However, even if the actual impact of AMF on the productivity of agricultural systems seems to be variable, our results indicate that, in the appropriate pedo-climatic conditions and agronomical managements, AM fungal field inoculation can be very effective in improving the yield of grain legumes. This indicates also that, although the modern cultivars of some of the most important crops, such as wheat, seem to be much less responsive to AMF compared to chickpea and other pulses (Ryan and Kirkegaard, 2012), a redesign of agricultural systems in order to enhance the benefits of AM fungal ecosystem services, even considering the additional cost of the inocula, can result in an improved plant growth and yield that could benefit crop economics, in particular in organic and low-input farming systems.

It is noteworthy that we observed better overall yield performances of chickpea inoculated with the local inoculum compared to the foreign. In this regard, local AMF may be preferable for field inoculation and on-farm inoculum production because of their better adaptation to local prevailing conditions (Dodd et al., 1983; Requena et al., 2001; Caravaca et al., 2003; Douds et al., 2011). Moreover, foreign AM fungal inocula may have negative ecological consequences interfering with the local microbial communities and thus altering agro-ecosystem functions (Schwartz et al., 2006; van der Heijden et al., 2008).

4.4. Nutrient uptake and grain biofortification

Inoculated chickpea plants showed a higher shoot N and P concentration when compared to the controls. A better nutritional status of AM fungal inoculated chickpea plants was already observed by Weber et al. (1992, 1993) and Zaidi et al. (2003) and confirms the key role that AMF can play in plant nutrient uptake even in agricultural conditions.

Beside the positive effect on shoot N and P, AMF inoculation was effective in raising Fe and Zn grain concentrations. To the best of our knowledge, this is the first experiment showing that AMF inoculation enhance the nutritional value of chickpea. Actually, AM fungal inoculation determined increases in Fe and Zn concentration of about 5% and 16%, respectively respect with controls. This may be due to the higher AM fungal root colonisation observed in the inoculated chickpea and, consequently, to a larger hyphal network that could have enhanced the transfer and uptake of trace elements (Audet and Charest, 2007). In addition, the larger occurrence of AMF could have determined an acidification of the rhizosphere due to the higher release of organic acids and phenolic compounds with the increases of soil Fe availability (White and Broadley, 2009; Antunes et al., 2012). Consistently, we observed higher grain Fe concentrations in the spring sowing treatment than in the autumn one, in line with the larger AM fungal root colonization of spring-sown chickpea.

Beside micronutrient, in the spring sowing treatment, AM fungal inoculation had a significant biofortification effect also on protein (N concentration x 6.25; Antongiovanni, 2004), enriching the chickpea grain total protein content about four percent (from 17% to 21%). Also for what concern proteins, we can speculate that the higher grain protein contents observed in the inoculated plants could be related to the larger AM fungal hyphal network that has been shown to increase the inorganic and organic soil N mobilization (Harrison et al., 2002; Hodge et al., 2001; Govindarajulu et al., 2005) along with a more efficient N fixation by symbiotic rhizobial bacteria due to the better P nutritional status of the inoculated plants compared with controls.

Interestingly, when considering the different inocula, the data suggest that AM fungal biofortification benefits may also depend on other factors than the root colonization rates, such as the functional properties of the different AMF. Actually, in the spring sowing treatment, grain protein, Fe and Zn concentration values of the NMix inoculated plants were

higher than those observed in FMix, despite FMix showed a higher AM fungal colonization indicating that, when considering crop productivity and yield quality, local AMF could be more convenient for field inoculation than foreign strains.

5. Conclusion

The results obtained in this experiment show that AM fungal field inoculation could be an effective tool to improve the cultivation of chickpea by boosting its productivity and grain nutritional quality. Since chickpea is a basic food for millions of people, AM fungal inoculation, in particular if based on on-farm inoculum production and on local AM fungal strains, could represent a valid biofertilization and biofortification strategy able to benefit not only the economics of the cultivation, but also the healthiness of the agricultural produces. The responses obtained by chickpea to AM fungal field inoculation are a further indication that the potential of a more mycorrhiza-friendly approach in agriculture could be great. However, more research is needed to understand the effectiveness of different AM fungal inoculation strategies in industrial scale agricultural systems and with different crops and soil conditions.

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References

Abbott, L.K. and Robson A.D., 1982. The role of vesicular- arbuscular rnycorrhizal fungi in agriculture and the selection of fungi for inoculation. Australian Journal of Agriculture Research 33, 389-408.

Abbott, L.K., Robson, A.D., 1991. Field management of VA mycorrhizal fungi. In: Keister, D.L., Cregan, P.B. (Eds.), The Rhizosphere and Plant Growth, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 355-362.

Al-Karaki, G.N., 2000. Growth of mycorrhizal tomato and mineral acquisition under salt stress. Mycorrhiza 10, 51-4.

Alkan, N., Gadkar, V., Yarden, O., Kapulnik, Y., 2006. Analysis of quantitative interactions between two species of arbuscular mycorrhizal fungi, *Glomus mosseae* and *G. intraradices*, by real-time PCR. Applied Environmental Microbiology 72, 4192-4199.

Altieri, M.A., 1999. The ecological role of biodiversity in agroecosystems. Agriculture Ecosystems Environment 74, 19-31.

Antongiovanni, M., 2004. Nutrizione degli Animali in Produzione Zootecnica, Edagricole, Bologna.

Antunes, P.M., Franken, P., Schwarz, D., Rillig, M.C., Cosme, M., Scott, M., Hart, M.M., 2012. Linking soil biodiversity and human health: do arbuscular mycorrhizal fungi contribute to food nutrition. In: Wall, D.H. et al., (Eds.) Soil Ecology and Ecosystem Services, Oxford University Press, Oxford, UK, pp. 153-172.

Audet, P., Charest, C., 2007. Dynamics of arbuscular mycorrhizal symbiosis in heavy metal phytoremediation: meta-analytical and conceptual perspectives. Environmental Pollution 147, 609-614.

Augé, R.M., 2001. Water relations, drought and VA mycorrhizal symbiosis. Mycorrhiza 11, 3-42.

Avio, L., Pellegrino, E., Bonari, E., Giovannetti, M., 2006. Functional diversity of arbuscular mycorrhizal fungal isolates in relation to extraradical mycelial networks. New Phytologist 172, 347-357.

Bago, B., Pfeffer, P.E., Shachar-Hill, Y., 2000. Carbon Metabolism and Transport in Arbuscular Mycorrhizas. Plant Physiology 124, 949-957.

Bagyaraj, D.J., Manjunath, A., Patil, R.B., 1979. Interaction between a vesicular-arbuscular mycorrhiza and rhizobium and their effects on soybean in the field. New Phytologist 82, 141-145.

Bedini, S., Pellegrino, E., Avio, L., Pellegrini, S., Bazzoffi, P., Argese, E., Giovannetti, M., 2009. Changes in soil aggregation and glomalin-related soil protein content as affected by the arbuscular mycorrhizal fungal species *Glomus mosseae* and *Glomus intraradices*. Soil Biology and Biochemistry 41, 1491-1496.

Bedini, S., Avio, L., Sbrana, C., Turrini, A., Migliorini, P., Vazzana, C., Giovannetti, M., 2013. Mycorrhizal activity and diversity in a long-term organic Mediterranean agroecosystem. Biology and Fertility of Soils, doi:10.1007/s00374-012-0770-6.

Caravaca, F., Alguacil, M.M., Figueroa, D., Barea, J.M., Roldán, A., 2003. Re-establishment of *Retama sphaerocarpa* as a target species for reclamation of soil physical and biological properties in a semiarid Mediterranean land. Forest Ecolology and Managment 182, 49-58.

Caravaca, F., Alguacil, M.M., Barea, J.M., Roldán, A., 2005. Survival of inocula and native AM fungi species associated with shrubs in a degraded Mediterranean ecosystem. Soil Biology and Biochemistry 37, 227-233.

Cavagnaro, T. R., 2008. The role of arbuscular mycorrhizas in improving plant zinc nutrition under low soil zinc concentrations: A review. Plant and Soil 304, 315–325.

Clarke, C., Mosse, B., 1981. Plant growth responses to vesicular-arbuscular mycorrhiza. XII. Field inoculation responses of barley at two soil P levels. New Phytologist 87, 695-703.

Clark, R.B., Zeto, S.K., 2000. Mineral acquisition by arbuscular mycorrhizal plants. Journal of Plant Nutrition 23, 867-902.

Crowder, D.W, Northfield, T.D., Strand, M.R., Snyder, W.E., 2010. Organic agriculture promotes evenness and natural pest control. Nature 466, 109-112.

Di Bene, C., Pellegrino, E., Tozzini, C., Bonari, E. 2011. Changes in soil quality following poplar short-rotation forestry under different cutting cycles. Italian Journal of Agronomy 6, 28-35.

Di Bene, C, Pellegrino, E., Debolini, M., Silvestri, N., Bonari, E., 2013. Multi-parameter approach to assess short- and long-term effect of olive mill wastewater land spreading on soil quality. Soil Biology and Biochemistry 56, 21-30.

Dodd, J., Krikun, J., Haas, J., 1983. Relative effectiveness of indigenous populations of vesicular-arbuscular mycorrhizal fungi from four sites in the Negev. Israel Journal of Botany 32, 10-21.

Douds, D.D., Nagahashi, G., Wilson, D.O., Moyer, J. 2011. Monitoring the decline in AM fungus populations and efficacy during a long term bare fallow. Plant and Soil 342, 319-326.

Edathil, T.T., Manian, S., Udaiyan, K., 1996. Interaction of multiple VAM fungal species on root colonization, plant growth and nutrient status of tomato seedlings (*Lycopersicon esculentus* Mill.). Agriculture, Ecosystems and Environment 59, 63-68.

FAOSTAT, 2010. http://faostat3.fao.org.

Fitter, A.H., Helgason, T., Hodge, A., 2011. Nutritional exchanges in the arbuscular mycorrhizal symbiosis: implications for sustainable agriculture. Fungal Biology Review 25, 68-72.

Foti, S., Abbate, V., 2000. Spring sowing (*Cicer arietinum* L.). In: Baldoni, R., Giardini, L. (Eds.), Coltivazioni Erbacee. Cereali e proteaginose. Pàtron Editore, Bologna, Italy.

Frenkel, O., Peever, T.L., Chilvers, M., Ozkilinc, H., Can, C., Abbo, S., Shtienberg, D., Sherman, A., 2010. Ecological genetic divergence of the fungal pathogen *Didymella rabiei* on sympatric wild and domesticated *Cicer* spp. (Chickpea). Applied and Environmental Microbiology 76, 30-39.

Gianinazzi, S., Vosatka, M., 2004. Inoculum of arbuscular mycorrhizal fungi for production systems, science meets business. Canadian Journal of Botany 82, 1264-1271.

Gianinazzi, S., Trouvelot, A., Gianinazzi-Pearson, V., 1990. Role and use of mycorrhizas in horticultural crop production. Advances in Horticulture Sciences 4, 25-30.

Giovannetti, M., Mosse, B., 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytologist 84, 489-500.

Gosling, P., Hodge, A., Goodlass, G., Bending, G.D., 2006. Arbuscular mycorrhizal fungi and organic farming. Agriculture, Ecosystems and Environment 113, 17-35.

Govindarajulu, M., Pfeffer, P.E., Jin, H., Abubaker, J., Douds, D.D., Allen, J.W., Bücking, H., Lammers, P.J., Shachar-Hill, Y, 2005. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435, 819-823.

Harrison, M.J., Dewbre, G.R., Liu, J., 2002. A phosphate transporter from Medicago *truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. The Plant Cell 14, 2413-2429.

Hayman, D.S., Mosse, B., 1979. Improved growth of white clover in hill grassland by mycorrhizal inoculation. Annals of Applied Biology 93, 141-149.

Hayman, D.S., 1984. Methods for evaluating and manipulating vesicular-arbuscular mycorrhiza. In: J Lynch, M., Grainger, J.M. (Eds.), Microbiological Methods for Environmental Biotechnology, Academic Press, London, UK, pp. 95-117.

He, X., Kazuhide Nara, K., 2007. Element biofortification: can mycorrhizas potentially offer a more effective and sustainable pathway to curb human malnutrition? Trends in Plant Science 12, 331-333.

Hoagland, D.R., Snyder, W.C., 1933. Nutrition of strawberry plants under controlled conditions. Proceeding of the American Society for Horticulture Science 30, 288-296.

Hodge, A., Campbell, C.D., Fitter, A.H., 2001. An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature 413, 297-299.

Hodge, A., Helgason, T., Fitter, A., 2010. Nutritional ecology of arbuscular mycorrhizal fungi. Fungal Ecology 3, 267-273.

Jansa, J., Smith, F.A., Smith, S.E., 2008. Are there benefits of simultaneous root colonization by different arbuscular mycorrhizal fungi? New Phytologist 177, 779-789.

Jansa, J., Thonar, C., Frossard, E., 2009. Enhancement of symbiotic benefits through manipulation of the mycorrhizal community composition. Aspect of Applied Biology 98, 9-15.

Jones Jr., J.B., Wolf, B., Mills, H.A., 1991. Plant analysis handbook II: a practical sampling, preparation, analysis, and interpretation guide. Micro-Macro Publishing Inc., Athens, USA.

Kabir, Z., 2005. Tillage or no-tillage: Impact on mycorrhizae. Canadian Journal of Plant Science 85, 23-29.

Karandashov, V., Bucher, M., 2005. Symbiotic phosphate transport in arbuscular mycorrhizas. Trends in Plant Science 10, 22-29.

Klironomos, J.N., 2000. Host-specificity and functional diversity among arbuscular mycorrhizal fungi. In: Bell, C.R., Brylinsky, M., Johnson-Green, P. (Eds.), Proceedings of the 8th International Symposium on Microbial Ecology, Atlantic Canada Society from Microbial Ecology, Halifax, Canada, pp. 845-851.

Klironomos, J.N., 2003. Variation in plant response to native and exotic arbuscular mycorrhizal fungi. Ecology 84, 2292-2301.

Koch, A.M., Antunes, P.M., Barto, E.K., Cipollini, D., Mummey, D.L., Klironomos, J.N. 2011. The effects of arbuscular mycorrhizal (AM) fungal and garlic mustard introductions on native AM fungal diversity. Biological Invasions 13, 1627-1639.

Koide, R.T., 1991. Nutrient supply, nutrient demand and plant response to mycorrhizal infection. New Phytologist 117, 365-386

Koide, R.T. 2000. Functional complementarity in the arbuscular mycorrhizal symbiosis New Phytologist 147, 233-235.

Koomen, I., Grace, C., Hayman, D.S., 1987. Effectiveness of single and multiple mycorrhizal inocula on growth of clover and strawberry plants at two soil pHs. Soil Biology and Biochemistry19, 539-544.

Lambert, D.H., Cloe, H., Baker, D.E., 1980. Variation in the response of Alfa alfa clones and cultivars of mycorrhiza and phosphorus. Crop Science 20, 615-618.

Lekberg Y, Koide RT., 2005. Is plant performance limited by abundance of arbuscular mycorrhizal fungi? A meta-analysis of studies published between 1988 and 2003. New Phytologist 168, 189-204.

Lehmann, A., Barto, E.K., Powell, J.R., Rillig, M.C., 2012. Mycorrhizal responsiveness trends in annual crop plants and their wild relatives-a meta-analysis on studies from 1981 to 2010. Plant and Soil 355, 231-250.

Lepš, J, Šmilauer, P., 2003. Multivariate analysis of ecological data using CANOCO. Cambridge University Press, Cambridge, UK.

Linderman, R.G., 2000. Effects of mycorrhizas on plant tolerances to diseases. In: Kapulnik, Y., Douds, D.D. (Eds.), Arbuscular mycorrhizas: physiology and function. Kluwer, Dordrecht, The Netherlands, pp. 345-365.

Lotter, D.W., 2003. Organic agriculture. Journal of Sustainable Agriculture 21, 59-128.

Maherali, H., Klironomos J.N., 2007. Influence of phylogeny on fungal community assembly and ecosystem functioning. Science 316, 1746-1748.

Maiti, R., Wesche-Ebeling P., 2001. Advances in chickpea science. Enfield: Science Publishers Inc.

Masoni, A., Ercoli, L., Bonari, E., 1993. Coltivazioni foraggere. Servizio Editoriale Universitario, Pisa, Italy.

McGonigle, T.P., 1988. A numerical analysis of published field trials with vesicular-arbuscular mycorrhizal fungi. Functional Ecology 2, 473-478.

Meyer, A.H., Valentine, A.J., Botha, A., Archer, E., Louw, P.J.E., 2005. The occurrence and infectivity of arbuscular mycorrhizal fungi in inoculated and uninoculated rhizosphere soils of two-year-old commercial grapevines. South African Journal of Enology and Viticulture 25, 90-94.

Miller, R.M., Jastrow, J.D., 1990. Hierarchy of root and mycorrhizal fungal interactions with soil aggregation. Soil Biology and Biochemistry 22, 579-584.

Moonen, C., Bàrberi, P., 2008. Functional biodiversity: An agroecosystem approach. Agriculture, Ecosystems and Environment 127, 7-21.

Mummey, D.L., Antunes, P.M., Rillig, M.C. 2009. Arbuscular mycorrhizal fungi pre-inoculant identity determines community composition in roots. Soil Biology and Biochemistry 41, 1173-1179.

Munkvold, L., Kjøller, R., Vestberg, M., Rosendahl, S., Jakobsen, I., 2004. High functional diversity within species of arbuscular mycorrhizal fungi. New Phytologist 164, 357-364.

Nene, 1981. A review of Ascochyta blight of chickpea. Tropical Pest Management 28, 17-33.

Newsham, K.K., Fitter, A.H., Watkinson, A.R., 1995. Multi-functionality and biodiversity in arbuscular mycorrhizas. Trends in Ecology and Evolution 10, 407-411.

Ortas, I., 2012. The effect of mycorrhizal fungal inoculation on plant yield, nutrient uptake and inoculation effectiveness under long-term field conditions. Field Crops Research 125, 35-48.

Pellegrino, E., Bedini, S., Avio, L., Bonari, E., Giovannetti, M., 2011a. Field inoculation effectiveness of native and exotic arbuscular mycorrhizal fungi in a Mediterranean agricultural soil. Soil Biology and Biochemistry 43, 367-376.

Pellegrino, E., Di Bene, C., Tozzini, C., Bonari, E., 2011b. Impact on soil quality of a 10-year-old short-rotation coppice poplar stand compared with intensive agricultural and uncultivated systems in a Mediterranean area. Agriculture Agroecosystems and Environment 140, 245-254.

Pellegrino, E., Turrini, A., Gamper, H.A., Cafà, G., Bonari, E., Young, J.P.W., Giovannetti, M., 2012. Establishment, persistence and effectiveness of arbuscular mycorrhizal fungal inoculants in the field revealed using molecular genetic tracing and measurement of yield components. New Phytologist 194, 810-822.

Pellegrino E., 2007. Mycorrhizas for the sustainable management of agro-ecosystems. Field studies on molecular and functional biodiversity of indigenous and introduced arbuscular mycorrhizal fungi. PhD thesis. University of Pisa, Pisa, Italy.

Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assesment of infection. Transactions of the British Mycological Society 55, 158-160.

Pimentel, D., Hepperly, P., Hanson, J., Douds, D., Seidel, R., 2005. Environmental, energetic and economic comparisons of organic and conventional farming systems. Bioscience 55, 573-582.

Plenchette, C., Clermont-Dauphin, C., Meynard, J., Fortin, J., 2005. Managing arbuscular mycorrhizal fungi in cropping systems. Canadian Journal of Plant Science 85, 31-40.

Postma-Blaauw, M.B., De Goede, R.G.M., Bloem, J., Faber, J.H., Brussaard, L., 2010. Soil biota community structure and abundance under agricultural intensification and extensification. Ecology 91, 460-473.

Purin, S., Filho, O.K., Stürmer, S.L., 2006. Mycorrhizae activity and diversity in conventional and organic apple orchards from Brazil. Soil Biology and Biochemistry 38, 1831-1839.

Rangeley, A., Daft, M.J., Newbould, P., 1982. The inoculation of white clover with mycorrhizal fungi in unsterile hill soils. New Phytologist 92, 89-102.

Redecker, D., Schüßler, A., Stockinger, H., Stürmer, S.L., Morton, J.B., Walker, C., 2013. An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (Glomeromycota). Mycorrhiza, doi 10.1007/s00572-013-0486-y.

Requena, N., Perez-Solis, E., Azcón-Aguilar, C., Jeffries, P., Barea, J.M., 2001. Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. Applied and Environmental Microbiology 67, 495-498.

Rillig, M.C., Mummey, D.L., 2006. Mycorrhizas and soil structure. New Phytologist 171, 41-53.

Ryan, M.H., Angus, J.F., 2003. Arbuscular mycorrhizae in wheat and field pea crops on a low P soil: increased Zn-uptake but no increase in P-uptake or yield. Plant and Soil 250, 225-239.

Ryan, M.H., Kirkegaard, J.A., 2012. The agronomic relevance of arbuscular mycorrhizas in the fertility of Australian extensive cropping systems. Agriculture, Ecosystems and Environment 163, 37-53.

Saccardo, F., Crinò, P., Giordano, I., 2001. Spring sowing (*Cicer arietinum* L.). In: Ranalli, P. (Eds.), Leguminose e Agricoltura Sostenibile - Specie da granella e cover crops, Calderini Edagricole, Bologna, Italy, pp. 555-590.

Saini, V.K., Bhandarib, S.C., Tarafdara, J.C., 2004. Comparison of crop yield, soil microbial C, N and P, N-fixation, nodulation and mycorrhizal infection in inoculated and non-inoculated sorghum and chickpea crops. Field Crops Research 89, 39-47.

Schüßler, A., Walker, C., 2010. The Glomeromycota: a species list with new families and new genera. The Royal Botanic Garden, Edinburgh & Kew, UK; Botanische Staatssammlung Munich, Munich, Germany; Oregon State University, Oregon, USA.

Schwartz, M.W., Hoeksema, J.D., Gehring, C.A., Johnson, N.C., Klironomos, J.N., Abbott, L.K., Pringle, A., 2006. The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecology Letters 9, 501-515.

Shumway, D.L., Koide, R.T., 1994. Reproductive responses to mycorrhizal colonization of *Abutilon theophrasti* Medic. plants grown for two generations in the field. New Phytologist 128, 219-224.

Sieverding, E., 1991. Vesicular-arbuscular mycorrhiza management in tropical agrosystems. GTZ, Eschborn, Germany.

Singh, K.B., 1997. Chickpea (*Cicer arietinum* L.). Field Crops Research 53, 161-170.

Singh, M., Tilak, K.V.B.R., 1989. Field response of chickpea to inoculation *with Glomus versiforme*. Plant and Soil 119, 281-284.

Smith, S.E., Read, D.J., 2008. Mycorrhizal Symbiosis, 3rd ed. Academic Press, Amsterdam, The Netherlands.

Smith, F.A., Jakobsen, I., Smith, S.E., 2000. Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula*. New Phytologist 147, 357-366.

Smith, S., Smith, F.A., Jakobsen, I., 2004. Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New Phytologist 162, 511-524.

Sýkorová, Z., Börstler, B., Zvolenská, S., Fehrer, J., Gryndler, M., Vosátka, M., Redecker, D., 2012. Long-term tracing of *Rhizophagus irregularis* isolate BEG140 inoculated on *Phalaris arundinacea* in a coal mine spoil bank, using mitochondrial large subunit rDNA markers. Mycorrhiza 22, 69-80.

Tarafdar, J.C., Rao, A.V., 1997. Response of arid legumes to VAM fungal inoculation. Symbiosis 22, 265-274.

Tchabi, A., Coyne, D., Hountondji, F., Lawouin, L., Wiemken, A., Oehl, F., 2010. Efficacy of indigenous arbuscular mycorrhizal fungi for promoting white yam (*Dioscorea rotundata*) growth in West Africa. Applied Soil Ecology 45, 92-100.

ter Braak, C.J.F., Šmilauer, P., 2002. CANOCO Reference Manual and CanoDraw for Windows User's guide: Software for Canonical Community Ordination (version 4.5). Microcomputer Power, Ithaca, USA.

Tufenkci, S., Sönmez, F., Sensoy, R.I.G., 2005. Effects of arbuscular mycorrhiza fungus inoculation and phosphorus and nitrogen fertilization on some plant growth parameters and nutrient content of chickpea. Journal of Biological Sciences 5, 738-743.

van den Wollenberg, A.L., 1977. Redundancy analysis. An alternative for canonical correlation analysis. Psycometrika 42, 207-219.

van der Heijden, M.G.A., Boller, T., Wiemken, A., Sanders, I.R., 1998a. Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79, 2082-2091.

van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf- Engel, R., Boller, T., Wiemken, A., Sanders, I.R., 1998b. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396, 72-75.

van der Heijden, M.G.A., Sanders, I.R., 2002. Mycorrhizal ecology. Springer-Verlag, Heidelberg, Germany.

van der Heijden, M.G.A., Bardgett, R.D., Van Straalen, N.M., 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11, 296-310.

Veresoglou, S.D., Chen, B., Rillig, M.C., 2012. Arbuscular mycorrhiza and soil nitrogen cycling. Soil Biology and Biochemistry 46, 53-62.

Weber, E., 1992. Role of vesicular arbuscular mycorrhizae in the mineral nutrition of chickpea (*Cicer arietinum* L.) grown in Northern Syria, Verlag Ulrich E. Grauer, Wendlingen, Germany.

Weber, E., Saxena, M.C., George, E., Marschner, H., 1993. Effect of vesicular-arbuscular mycorrhiza on vegetative growth and harvest index of chickpea grown in northern Syria. Field Crops Research 32: 115-128.

White, P.J., Broadley, M.R., 2009. Biofortification of crops with seven mineral elements often lacking in human diets - Iron, zinc, copper, calcium, magnesium, selenium and iodine. New Phytologist 182, 49-84.

Zaidi, A., Khan, Md.S., Amil, Md., 2003. Interactive effect of rhizotrophic microorganisms on yield and nutrient uptake of chickpea (*Cicer arietinum* L.). European Journal of Agronomy 19, 15-21.

CAPTIONS

Figure 1 Arbuscular mycorrhizal (AM) fungal root colonization, shoot dry matter, collar diameter and grain yield of chickpea (*Cicer arietinum* L.) plants inoculated with the two different single, foreign AM fungi *Funneliformis mosseae* (IMA1) or *Rhizophagus irregularis* (IMA5), a dual strain inoculum [foreign mixture (FMix): IMA1 + IMA5], a trap-culture-enriched locally-sourced AM fungal community (local mixture = LMix) and a control (mock inoculum = C). Plants were sampled at harvest (June). Black rectangles: autumn sowing, plants sown in October; white rectangles: spring sowing, plants sown in March.

Figure 2 Redundancy Analysis biplots based on plant growth, yield, plant nutrient uptake, grain biofortification and arbuscular mycorrhizal (AM) fungal colonization of chickpea (*Cicer arietinum* L.) plants (used as response variable) sown in October [(a) autumn sowing] and in March [(b) spring sowing] and inoculated with the two different single, non-native AM fungi *Funneliformis mosseae* (IMA1) or *Rhizophagus irregularis* (IMA5), the dual strain inoculum [foreign mixture (FMix): IMA1 + IMA5], a trap-culture-enriched locally-sourced AM fungal community (local mixture = LMix) and a control (mock inoculum = Control) (used as explanatory variables). Solid arrows represent plant growth and yield variables: collar diameter, CD; shoot dry matter per plant, ShootDM; grain yield per plant, GrainY. Plant nutrient uptake and grain biofortification are represented by dashed arrows: grain Fe concentration, Grain[Fe]; grain Zn concentration, Grain[Zn]; grain N concentration, Grain[N]; shoot N concentration, Shoot[N]; shoot P concentration, Shoot[P]. AM fungal colonization (AMFcoloniz) is represented by dotted arrow. The 1st and 2nd axes accounted for 62.9% and 69.4% of the total variance explained by all canonical axes for the autumn (a) and the spring sowing treatment (b), respectively.

Table 1

P-values of linear orthogonal contrasts for arbuscular mycorrhizal (AM) fungal root colonization, shoot dry matter, collar diameter and grain yield of cickpea (*Cicer arietinum* L.) plants inoculated with the two different single, foreign AM fungi *Funneliformis mosseae* (IMA1) or *Rhizophagus irregularis* (IMA5), a dual strain inoculum [foreign mixture (FMix): IMA1 + IMA5], a trap-culture-enriched locally-sourced AM fungal community (local mixture = LMix) and a control (mock inoculum = C). Autumn sowing, plants sown in October; Spring sowing, plants sown in March.

	AM fungal root colonization (%) [a]	Shoot dry matter [a,b] (g plant^{-1})	Collar diameter [a] (mm)	Grain yield (g plant^{-1})
Treatment comparisons (*P-values of linear orthogonal contrasts*)				
Autumn sowing				
C vs M [c]	**<0.001** [d]	0.234	**0.001**	**0.004**
FMix vs Single [e]	**0.008**	0.564	0.613	0.992
FMix vs LMix	**<0.001**	0.946	0.057	0.242
F. mosseae IMA1 vs *R. irregularis* IMA5	**0.001**	0.726	0.532	0.144
Spring sowing				
C vs M	**<0.001**	**0.003**	**<0.001**	**0.001**
FMix vs Single	**0.002**	0.111	0.448	0.483
FMix vs LMix	**<0.001**	0.930	**0.004**	**0.049**
F. mosseae IMA1 vs *R. irregularis* IMA5	**<0.001**	0.389	0.624	**0.029**

[a] At harvest (June).

[b] Leaf and stem dry weight.

[c] M, AM fungal inocula.

[d] In bold statistically significant values accoring to the linear orthogonal contrats ($P \leq 0.05$).

[e] Single, single AM fungal inocula.

Table 2
Shoot and grain N and shoot P concentrations of chickpea (*Cicer arietinum* L.) plants inoculated with the two different single, foreign arbuscular mycorrhizal (AM) fungi *Funneliformis mosseae* (IMA1) or *Rhizophagus irregularis* (IMA5), a dual strain inoculum [foreign mixture (FMix): IMA1 + IMA5], a trap-culture-enriched locally-sourced AM fungal community (local mixture = LMix) and a control (mock inoculum = C). Plants were sampled at harvest (June). Autumn sowing, plants sown in October; Spring sowing, plants sown in March.

	Shoot N (%)	Shoot P (%)	Grain N (%)	Shoot N (%)	Shoot P (%)	Grain N (%)
	Autumn sowing			Spring sowing		
C	0.98 ± 0.07 [a]	0.25 ± 0.03	2.75 ± 0.03	0.93 ± 0.08	0.25 ± 0.03	2.76 ± 0.02
FMix	1.94 ± 0.06	0.35 ± 0.02	2.77 ± 0.09	1.87 ± 0.06	0.35 ± 0.01	2.81 ± 0.06
LMix	2.09 ± 0.04	0.40 ± 0.01	2.84 ± 0.14	1.94 ± 0.03	0.36 ± 0.02	2.92 ± 0.10
IMA1	2.13 ± 0.05	0.35 ± 0.03	2.89 ± 0.08	2.05 ± 0.07	0.39 ± 0.02	3.01 ± 0.01
IMA5	1.91 ± 0.00	0.38 ± 0.01	2.88 ± 0.08	1.96 ± 0.03	0.35 ± 0.00	2.95 ± 0.04
Treatment comparisons (P-*values of linear orthogonal contrasts*)						
C vs M [b]	**<0.001** [c]	**0.001**	0.381	**<0.001**	**0.001**	**0.022**
FMix vs Single [d]	0.234	0.506	0.314	0.077	0.552	**0.029**
FMix vs LMix	0.063	0.134	0.606	0.372	0.730	0.191
F. mosseae IMA1 vs *R. irregularis* IMA5	**0.010**	0.260	0.938	0.282	0.154	0.462

[a] Values are means ± SE of three replicate plots per treatment.
[b] M, AM fungal inocula.
[c] In bold statistically significant values accoring to the linear orthogonal contrats ($P \leq 0.05$).
[d] Single, single AM fungal inocula.

Table 3

Grain Fe and Zn concentrations of chickpea (*Cicer arietinum* L.) plants inoculated with the two different single, foreign AM fungi *Funneliformis mosseae* (IMA1) or *Rhizophagus irregularis* (IMA5), a dual strain inoculum [foreign mixture (FMix): IMA1 + IMA5], a trap-culture-enriched locally-sourced AM fungal community (local mixture = LMix) and a control (mock inoculum = C). Plants were sampled at harvest (June). Autumn sowing, plants sown in October; Spring sowing, plants sown in March.

	Fe ($\mu g\ g^{-1}$)	Zn ($\mu g\ g^{-1}$)	Fe ($\mu g\ g^{-1}$)	Zn ($\mu g\ g^{-1}$)
	Autumn sowing		Spring sowing	
C	472.67 ± 5.36 [a]	23.57 ± 0.90	487.33 ± 2.19	23.33 ± 0.88
FMix	492.33 ± 1.45	26.27 ± 0.09	500.33 ± 2.60	26.33 ± 0.18
LMix	512.33 ± 1.45	32.67 ± 0.88	523.67 ± 6.64	31.10 ± 0.63
IMA1	484.33 ± 4.70	25.07 ± 0.26	494.33 ± 2.03	25.67 ± 0.09
IMA5	488.67 ± 5.49	25.40 ± 0.38	504.00 ± 2.08	24.60 ± 0.15
Treatment comparisons (P-*values of linear orthogonal contrasts*)				
C vs M [b]	**0.001** [c]	**<0.001**	**0.001**	**<0.001**
FMix vs Single [d]	0.275	0.354	0.796	0.078
FMix vs LMix	**0.006**	**<0.001**	**0.001**	**<0.001**
F. mosseae IMA1 vs *R. irregularis* IMA5	0.475	0.732	0.085	0.182

[a] Values are means ± SE of three replicate plots per treatment.

[b] M, AM fungal inocula.

[c] In bold statistically significant values accoring to the linear orthogonal contrats ($P \leq 0.05$).

[d] Single, single AM fungal inocula.

Table 4

Effects of inoculation with arbuscular mycorrhizal (AM) fungi (AMF inoc), sowing time (S time) and their interactions on chickpea (*Cicer arietinum* L.) AM fungal colonization, plant growth, yield and yield components at harvest.

Factors [a]	AM fungal colonization	Shoot dry matter [b]	Collar diameter	Grain yield [c]
AMF inoc [d]	**< 0.001** [e]	**0.007**	**< 0.001**	**< 0.001**
S time	0.095	**0.013**	**< 0.001**	0.473
AMF inoc x S time	0.801	0.193	0.794	0.941

[a] Two single, foreign AM fungi (*Funneliformis mosseae*, IMA1; *Rhizophagus irregularis* IMA5); a dual strain inoculum, foreign mixture (FMix): IMA1 + IMA5; a trap-culture-enriched locally-sourced AM fungal community (local mixture = LMix) and a mock inoculum as control; S time: autumn (October) and spring (March).

[b] Leaf and stem dry weigth.

[c] Measurements per plant.

[d] Two-way ANOVAs: AMF inoc, fixed factor; S time, fixed factor.

[e] *P*-values of the two-way ANOVAs: in bold statistically significant values ($P \leq 0.05$). Replicates field plots were three per treatment.

Table 5

Effects of inoculation with arbuscular mycorrhizal (AM) fungi (AMF inoc), sowing time (S time) and their interactions on chickpea (*Cicer arietinum* L.) plant nutrient uptakes and grain biofortification.

Factors [a]	Shoot		Grain		
	N [b]	P	N	Fe	Zn
AMF inoc [c]	**< 0.001** [d]	**< 0.001**	0.071	**< 0.001**	**< 0.001**
S time	0.098	0.764	0.182	**< 0.001**	0.158
AMF inoc x S time	0.506	0.326	0.942	0.859	0.569

[a] Two single, foreign AMF (*Funneliformis mosseae*, IMA1; *Rhizophagus irregularis* IMA5); a dual strain inoculum, foreign mixture (FMix): IMA1 + IMA5; a trap-culture-enriched locally-sourced AM fungal community (local mixture = LMix) and a mock inoculum as control; S time: autumn (October) and spring (March).

[b] Concentrations.

[c] Two-way ANOVAs: AMF inoc, fixed factor; S time, fixed factor.

[d] *P*-values of the two-way ANOVAs: in bold statistically significant values ($P \leq 0.05$). Replicates field plots were three per treatment.

Figure

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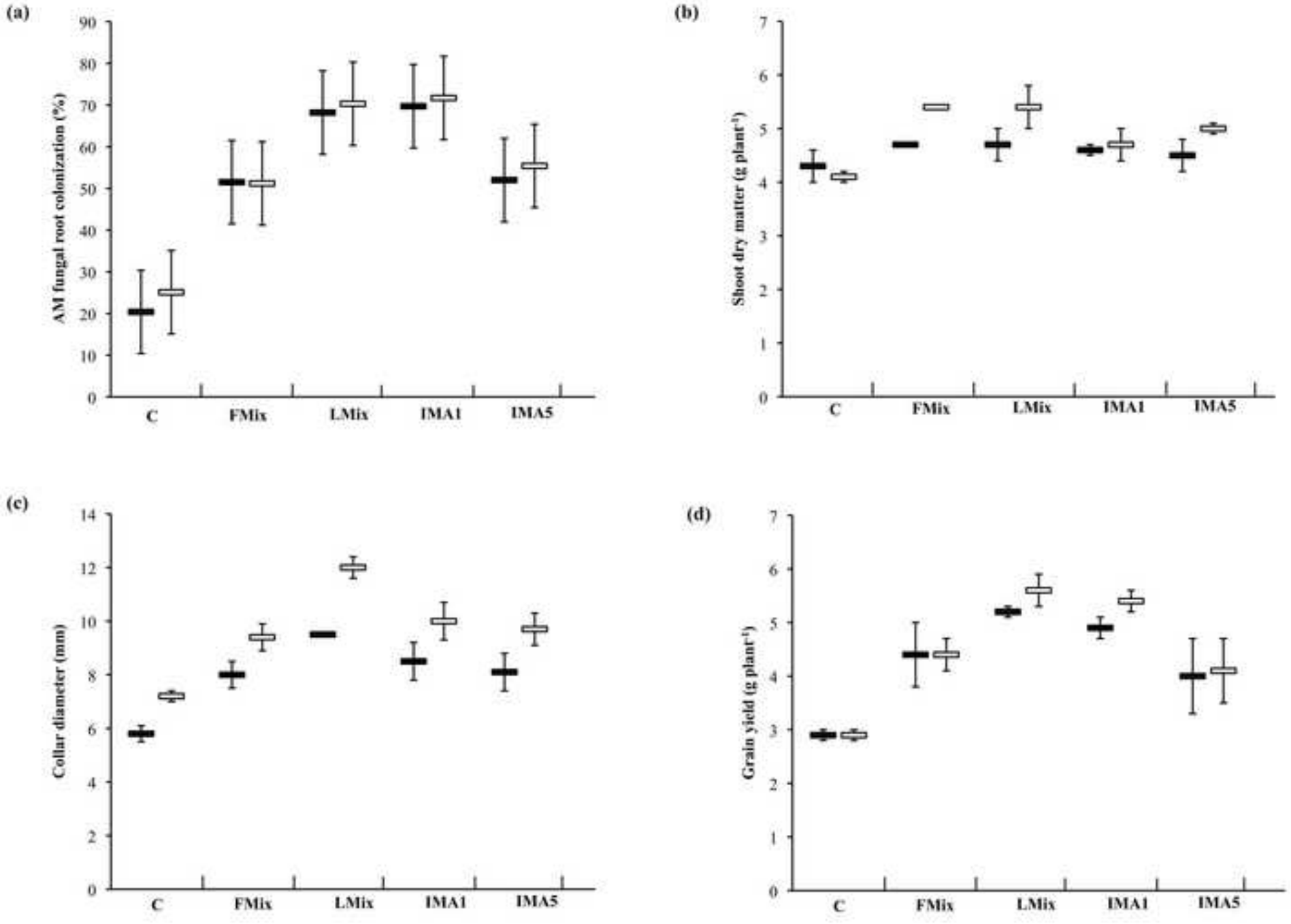

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