Is host crop identity a stronger determinant than cover cropping for arbuscular mycorrhizal fungal communities colonizing maize and soybean roots?

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Background: Understanding the role of communities of arbuscular mycorrhizal fungi (AMF) in agricultural systems is imperative for enhancing crop production. The key variables influencing change in AMF communities are the type of cover crop species or the type of subsequent host crop species. However, how maize and soybean performance is related to the diversity of AMF communities in cover cropping systems remains unclear. We therefore investigated which cover cropping or host identity is the most important factor in shaping AMF community structure in subsequent crop roots using an Illumina Miseq platform amplicon sequencing.

Methods: In this study, we established three cover crop systems (Italian ryegrass, hairy vetch, and brown mustard) or bare fallow prior to planting maize and soybean as cash crops. After cover cropping, we divided the cover crop experimental plots into two subsequent crop plots (maize and soybean) to understand which cover cropping or host crop identity is an important factor for determining the AMF communities and diversity both in maize and soybeans.

Results: We found that most of the operational taxonomic units (OTUs) in root samples were common in both maize and soybean, and the proportion of common generalists in this experiment for maize and soybean roots was 79.5% according to the multinomial species classification method (CLAM test). The proportion of OTUs specifically detected in only maize and soybean was 9.6% and 10.8%, respectively. Additionally, the cover cropping noticeably altered the AMF community structure in the maize and soybean roots. However, the differentiation of AMF communities between maize and soybean was not significantly different.

Discussion: Our results suggest cover cropping prior to planting maize and soybean may be a strong factor for shaping AMF community structure in subsequent maize and soybean roots rather than two host crop identities. Additionally, we could not determine the suitable rotational combination for cover crops and subsequent maize and soybean crops to improve the diversity of the AMF communities in their roots. However, our findings may have implications for understanding suitable rotational combinations between cover crops and subsequent cash crops and further research should investigate in-depth the benefit of AMF on cash crop performances in cover crop rotational systems.

1 **Title:** Is host crop identity a stronger determinant than cover cropping for arbuscular mycorrhizal

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14 **Abstract**

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16 agricultural systems is imperative for enhancing crop production. The key variables influencing

17 change in AMF communities are the type of cover crop species or the type of subsequent host

18 crop species. However, how maize and soybean performance is related to the diversity of AMF

19 communities in cover cropping systems remains unclear. We therefore investigated which cover

20 cropping or host identity is the most important factor in shaping AMF community structure in

21 subsequent crop roots using an Illumina Miseq platform amplicon sequencing.

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23 and brown mustard) or bare fallow prior to planting maize and soybean as cash crops. After

24 cover cropping, we divided the cover crop experimental plots into two subsequent crop plots

25 (maize and soybean) to understand which cover cropping or host crop identity is an important

26 factor for determining the AMF communities and diversity both in maize and soybeans.

27 **Results**: We found that most of the operational taxonomic units (OTUs) in root samples were

28 common in both maize and soybean, and the proportion of common generalists in this

29   experiment for maize and soybean roots was 79.5% according to the multinomial species

30   classification method (CLAM test). The proportion of OTUs specifically detected in only maize

31   and soybean was 9.6% and 10.8%, respectively. Additionally, the cover cropping noticeably

32   altered the AMF community structure in the maize and soybean roots. However, the

33   differentiation of AMF communities between maize and soybean was not significantly different.

34   **Discussion**: Our results suggest cover cropping prior to planting maize and soybean may be a

35   strong factor for shaping AMF community structure in subsequent maize and soybean roots

36   rather than two host crop identities. Additionally, we could not determine the suitable rotational

37   combination for cover crops and subsequent maize and soybean crops to improve the diversity of

38   the AMF communities in their roots. However, our findings may have implications for

39   understanding suitable rotational combinations between cover crops and subsequent cash crops

40   and further research should investigate in-depth the benefit of AMF on cash crop performances

41   in cover crop rotational systems.

43   **Introduction**

44   Growing cover crops can be an effective technique in crop rotations to enhance soil health and

45   suppress weed populations (Snapp et al., 2005; Clark, 2008). Cover crops can cover fallow

46   periods between cash crops that may be vulnerable to weed establishment or erosion. Short-

47   season cover crops are also utilized and overwintered (live until spring) in temperate regions to

48   reduce soil erosion, increase soil organic matter (Snapp et al., 2005; García-González et al.,

49   2018), and enhance the biomass of plant growth-promoting microorganisms, such as phosphate

50   solubilizing fungi and arbuscular mycorrhizal fungi (AMF) (Lehman et al., 2012; Higo et al.,

51   2013; Karasawa & Takahashi, 2015; García-González et al., 2016).

52   It is well known that AMF can improve host plant phosphorus (P) uptake and growth

53   performance (Smith & Read, 2008). These nutritional benefits of AMF can be enhanced through

54   appropriate agricultural management (Gosling et al., 2006; Kahiluoto et al., 2012; Ryan &

55   Graham, 2018). Indeed, many previous studies have indicated that AMF benefits agricultural

56   crops, such as maize (*Zea mays*) and soybean (*Glycine max*), by increasing mycorrhization and

57 AMF hyphal abundance in soil during early growth to enhance P uptake as well as maize and

58 soybean yield (Gavito & Miller, 1998; Isobe et al., 2014). Additionally, agricultural practices

59 may positively or negatively impact AMF taxonomic and functional community composition

60 (Ohsowski et al., 2014; Xiang et al., 2016; Cofré et al., 2017; Xu et al., 2017; Zhao et al., 2017;

61 Berruti et al., 2018; García de León et al., 2018; Higo et al., 2018b). Indeed, intensive

62 agricultural practices, such as tillage, mono-cropping, seasonal fallow periods, and inorganic

63 nutrient application, reduce both AMF populations and AMF benefits for field crops (Lehman et

64 al., 2012; Higo et al., 2018a). On the contrary, adding cover crops during the winter period is

65 also an effective technique in increasing indigenous AMF abundance in soil for following crops

66 (Higo et al., 2015a). Thus, the introduction of cover crops, such as ryegrass (*Lolium multiflorum*),

67 wheat (*Triticum aestivum*), mustards and oilseed rape (*Brassicaceae*) or leguminous crops,

68 including hairy vetch (*Vicia villosa* Roth.) and clovers (*Trifolium*), in crop rotations in temperate

69 agricultural systems is essential to reduce seasonal fallow and thus provides many benefits for

70 subsequent crops and soil fertility (Karasawa & Takahashi, 2015; Higo et al., 2018b). Given

71 these facts regarding AMF in cover crop rotational systems, it is important to understand which

72 agricultural management, such as cover crop species and crop rotation, positively or negatively

73 impacts individual AMF taxa and community structures in roots and soil.

74 However, what remains poorly understood and unclear is which factor, such as cover

75 cropping or host crop identity, is driving increases in cash crop performance and AMF diversity.

76 Little is also known about how cover cropping and host identity are linked to the diversity of

77 AMF community structure, and the effectiveness of AMF in cover crop rotational systems to

78 improve the robustness and reliability of agricultural management. Additionally, the best

79 combination of cover crops and subsequent cash crops (maize and soybean) in rotations to

80 increase the diversity or to alter the structure of AMF communities remains unknown. Thus, the

81 goal of this research was to determine 1) which cover cropping or host identity is the most

82 important factor in shaping AMF community structure in subsequent crop roots, and 2) how

83 cover cropping and host identity alter the belowground communities of AMF in two different

84 types of subsequent maize and soybean crops.

85

Materials and methods

Experimental design

88 A field experiment of cover crop rotation was established at Nihon University, in Kanagawa,

89 Japan (35°22'N 139°27'E). The soil at the field site is classified as a volcanic ash soil

90 (Allophonic andosol). In this study, we examined the impact of cover cropping and host crop

91 type on the diversity of AMF communities colonizing roots of subsequent crops. We established

92 cover cropping treatments in rotation with maize (*Zea mays* L., cv: Snow Dent 125 Wakaba) or

93 soybean (*Glycine max* (L.) Merr., cv: Enrei). Three cover crop treatments including Italian

94 ryegrass (*Lolium multiflorum* Lam., cv: Akatsuki), hairy vetch (*Vicia villosa* Roth., cv:

95 Mamekko), and brown mustard (*Brassica juncea* (L.) Czern & Coss., cv: Karajin) or bare fallow

96 was used to cover the soil surface during winter and the fallow period in annual cropping

97 systems in a temperate region of Japan. There were three replicate plots per treatment arranged in

98 a randomized complete block design. Each plot had an area of 9 m² (3 m × 3 m). The Italian

99 ryegrass, hairy vetch, and brown mustard were sown in rows, spaced 30 cm apart, in the cropped

100 treatment on November 9, 2016. The ryegrass seeds were sown at 400 kg ha^{-1} (because the rate

101 of emergence in our research field was low) with N (ammonium sulfate) and K_2O (potassium

102 chloride) application rates of 100 kg ha^{-1}. The hairy vetch seeds were sown at 80 kg ha^{-1} with N

103 (ammonium sulfate) and K_2O (potassium chloride) application rates of 50 and 100 kg ha^{-1},

104 respectively. Brown mustard seeds were sown at 80 kg ha^{-1} with N and K_2O application rates of

105 100 kg ha^{-1}. The tops of the cover crops were cut close to the ground and removed on April 24,

106 2017. For fallow, weeds were manually removed during the winter period. Soil biochemical

107 properties of the field after cultivation of cover crops are shown in Table S1.

108   We used a split-plot design to divide the 3 m × 3 m of the cover crop experimental plots

109 into 3.0 m × 1.5 m plots for the two subsequent cropping plots (maize and soybean) (Fig. S1).

110 Then, both maize and soybean cropping plots were replicated three times in 3 m × 3 m plots. The

111 maize and soybean seeds were sown at a spacing of 60 cm × 15 cm on May 29, 2017. In

112 soybeans, the N and K_2O application rates were 50 and 100 kg ha^{-1}, respectively. In maize, the N

113   and K$_2$O application rates were 150 and 100 kg ha^{-1}, respectively. No P fertilizer was applied in

114   both maize and soybean cropping in this study.

115

116   **Soil and root sampling and root staining**

117   The soil samples were randomly taken from ten points using a soil core sampler (0−20 cm depth,

118   4 cm diameter) in each replicate and pooled to one composite sample on April 24, 2017. Maize

119   and soybean root samples were taken at the 6 weeks after sowing on July 10, 2017. The root

120   samples in both maize and soybean were collected from nine plants (to a depth of 15 cm, the

121   diameter of 20 cm) per replicate. The root samples were maintained at −80°C for DNA

122   extraction and measurement of AMF colonization. We stained the root samples with a 5% (w/v)

123   black ink-vinegar solution (Vierheilig et al., 1998). The AMF root colonization in the maize and

124   soybean was measured according to Giovannetti & Mosse (1980).

125

126   **Measurement of the aboveground plant biomass of maize and soybean**

127   The aboveground plant parts of eight plants in soybean and maize were cut close to the ground at

128   the 6 weeks after sowing and were randomly sampled on July 10, 2017. The aboveground maize

129   and soybean plant biomass were measured in all plots. The aboveground plant biomass by maize

130   and soybeans were determined after the samples were oven dried at 80°C for 48 h.

131

132   **DNA extraction from root samples**

133   Genomic DNA was extracted from 100 mg of fresh root samples using DNA suisui-P kit (RIZO,

134   Tsukuba, Japan) according to the manufacturer's instructions. Briefly, root samples were soaked

135   directly in liquid nitrogen, and then the root samples were crushed using a beads smasher MS-

136   100 (TOMY, Tokyo, Japan). After crushing the roots, 360 μl of DNA suisui-P lysis solution and

137   40 μl of prepared additives with the DNA extraction kit were put into 1.5-mL tubes. Up to 400 μl

138   of phenol: chloroform (1:1, v/v) was added to the tubes and mixed vigorously using a vortex

139   mixer (Vortex-Genie 2, Scientific Industries, Tokyo). The samples were centrifuged at 15,000

140   rpm for 10 min, at room temperature. After the centrifugation, 200 μl of supernatant was

141 transferred to a clean tube (1.5-mL) and 200 µl (equal volume) of 2-propanol was added. After

142 that, the samples were centrifuged at 15,000 rpm for 10 min, at room temperature. After this

143 centrifugation, the supernatant was discarded and added 800µl of 70% ethanol into the 1.5-mL

144 tube, and then this was centrifuged at 15,000 rpm for 10 min, at 4°C. The supernatant was

145 discarded and the remaining pellet was dried. We added 50 µl of Tris-EDTA (TE) buffer [1 mM

146 Tris (pH 8.0), 0.1 mM EDTA (pH 8.0)] to dissolve the pellet, and this solution served as

147 template DNA for polymerase chain reaction (PCR). The genomic DNA pellet was stored at

148 −30°C until use in the PCR.

149

150 **Polymerase chain reaction (PCR) amplification**

151 The fragments in the fungal small subunit ribosomal DNA (SSU rDNA) were amplified using

152 the first-round PCR. The universal primer NS31 (Simon et al., 1992) and the fungus-specific

153 primer AM1 (Helgason et al., 1998) were used in the first PCR round to amplify the 5' end of the

154 SSU rDNA region for Glomeromycotina. To decrease variations in the PCR process, samples

155 were amplified in triplicate (Polz and Cavanaugh, 1998) using the fusion primer set in a PCR at

156 10 µl per subsample. The PCR was performed in 10 µl reaction mixtures with each containing 2×

157 of reaction buffer, 0.4 µM of forward and reverse primers (10 µM), 1 U of Taq DNA polymerase

158 (KOD multi & Epi, Toyobo, Japan), and 1 µl of template DNA using a Mastercycler ep Gradient

159 (Eppendorf, Hamburg, Germany). The first-round PCR was performed according to the PCR

160 cycle of Liang et al. (2008). The first PCR products were diluted 10-fold and used as templates

161 for the second-round PCR using the primers AMV4.5NF and AMDGR (Lumini et al., 2010).

162 The second-round PCR was performed in 10 µl reaction mixtures that each contained 2× of

163 reaction buffer, 0.3 µM of forward and reverse primers (10 µM), 1 U of Taq DNA polymerase

164 (KOD multi & Epi, Toyobo, Japan), and 1 µl of template DNA. The second-round PCR protocol

165 was composed of an initial treatment at 94°C for 2 min; 45 cycles of treatments at 98°C for 10 s

166 and at 60°C for 10 s. Gel electrophoresis separated amplicons on 1% agarose gel, and the

167 approximately 300 bp DNA amplicons were visualized by staining with ethidium bromide.

168

169   **Illumina MiSeq sequencing and molecular diversity of AMF communities in roots**

170   Three independent PCR products were pooled together and purified using NucleoSpin Gel and

171   PCR Clean-up kit (Macherey-Nagel, Duren, Germany) to reduce potential early-round PCR

172   errors, and quantified using UV spectrophotometry (DS-11 NanoPad, DeNovix Inc., USA). The

173   purified PCR amplicons were normalized before an Illumina MiSeq platform sequencing. The

174   amplicons were paired-end (PE) sequenced on the Illumina MiSeq platform (Bioengineering Lab

175   Co., Ltd, Kanagawa, Japan). In total, 24 sequencing libraries were constructed and independently

176   sequenced. Sequence read processing was performed using QIIME version 1.9.1 (Caporaso et al.,

177   2010).

178   The reads were truncated at any site that received an average quality score <20 over a 40 bp

179   sliding window, and the truncated reads shorter than 40 bp were discarded using FASTX-Toolkit.

180   Then, PE reads were assembled according to their overlap sequence with a minimum overlap

181   length of 10 bp, while reads that could not be assembled were discarded. The clean sequences

182   were analyzed using the FLASH (Fast length adjustment of short reads). Chimeric sequences

183   were identified and removed using UCHIME in USEARCH. Operational taxonomic unit (OTU)

184   grouping was performed using Silva and NCBI Genbank at 97% similarity. The representative

185   sequences were checked against NCBI GenBank. The raw sequence data are available in the

186   DNA Data Bank of Japan (DDBJ) (DDBJ Sequence Read Archive, DRA007103). Additionally,

187   we performed a rarefaction analysis using the *vegan* package in R 3.5.0.

188

189   **Statistical analysis**

190   A generalized linear model (GLM) was used to determine the effects of host identity and cover

191   cropping on each parameter in this study in the *multcomp* package in R 3.5.0. Data for the

192   significance of differences between subsequent host crop types among cover crop treatments

193   were assessed using Student's t-test. Differences among means were assessed using Tukey's test

194   (P-values < 0.05) using the *multcomp* package in R 3.5.0. In addition, the amplicons are

195   relatively short (<400 bp), the fungal community that was obtained after pyrosequencing, was

196   highly a good reflection of the fungal composition in the sample (Ihrnark et al., 2012). Moreover,

197   Hill numbers (or the effective number of species) have been increasingly used to quantify the

198   species/taxonomic diversity of an assemblage because they represent an intuitive and statistically

199   rigorous alternative to other diversity indices (Chao et al. 2014; Hsieh et al., 2016). Thus,

200   diversity of AMF OTU communities were measured based on the first three Hill numbers, such

201   as species richness, Shannon diversity (the exponential of Shannon entropy), and Simpson

202   diversity (the inverse Simpson concentration) using the "*renyi*" function the *vegan* package in R

203   3.5.0.

204   Additionally, the differences in the AMF community structures among host crop type and

205   cover cropping was also examined by a distance-based redundancy analysis (db-RDA) in the

206   *vegan* package in R 3.5.0 to analyze the relationship of cover crops and subsequent host crop

207   type with respect to AMF community structures. The environmental variable of cover cropping

208   and host crop type was coded as a dummy variable (0 and 1). Goodness-of-fit statistics (R^2) of

209   measured factors fitted to the db-RDA ordination of the AMF community structures were

210   calculated using the "*envfit*" function in the *vegan* package with P-values based on 999

211   permutations (Oksanen, 2018). Differences in the AMF community structures between maize

212   and soybean were also determined by non-metric multidimensional scaling (NMDS) with the

213   "*metaMDS*" function. A permutational multivariate analysis of variance (PERMANOVA) was

214   performed with 999 permutations using the "*Adonis*" function in the *vegan* package in R 3.5.0 to

215   investigate if AMF community structures differed significantly between cover crops and

216   subsequent host crop type. The "Bray-Curtis" metric of AMF community structure was used in

217   the PERMANOVA and NMDS analysis (Chase et al., 2011).

218   Furthermore, we performed an analysis based on the multinomial species classification

219   method (CLAM test) with the "*clamtest*" function of the *vegan* package in R 3.5.0 (Chazdon et

220   al., 2011) to understand AMF OTUs showing a preference for maize or soybean. The CLAM test

221   uses a multinomial model based on estimated species relative abundance in two habitat or host

222   species (maize and soybean). The multinomial model implemented in the test minimizes biases

223   due to different sampling intensities between the two habitats or host species being compared.

224   The method permits a robust statistical classification of habitat specialists and generalists,

225 without excluding rare species a priori (Chazdon et al. 2011). In this study, the model classifies

226 species into one of four groups: (1) generalists; (2) maize specialists; (3) soybean specialists; and

227 (4) too rare species.

228   In the diversity indices, db-RDA, PERMANOVA, NMDS, and CLAM test, we rarefied our

229 dataset down to the lowest OTU abundance to compare evenly between samples regardless of

230 sequencing depth (i.e. samples with greater sequencing depth can allow for greater detection of

231 low-abundance taxa compared with more shallowly sequenced samples).

232

233 **Results**

234 **AMF colonization and plant growth in maize and soybean roots**

235 Overall, our results showed that cover cropping impacted AMF colonization in maize roots,

236 whereas differences in AMF colonization of soybean were not impacted by cover cropping (Fig.

237 1). We found that AMF colonization of maize under Italian ryegrass and hairy vetch treatments

238 was significantly higher than that of brown mustard and bare fallow treatments. In soybean, a

239 similar tendency in AMF colonization with regard to maize cropping was observed. However, no

240 significant differences in AMF colonization of soybean were found among cover cropping

241 treatments.

242   According to Figure 2, the aboveground plant biomass in maize did not vary among cover

243 crop treatments in this study (Fig. 2A). The aboveground plant biomass under Italian ryegrass

244 and brown mustard treatments tended to be higher than that of hairy vetch and bare fallow

245 treatments. On the contrary, the aboveground plant biomass of soybean in the Italian ryegrass

246 plot was more than double than those of hairy vetch and bare fallow treatments (Fig. 2B).

247 Additionally, the aboveground plant biomass of soybeans in the brown mustard treatment was

248 higher than those of hairy vetch and bare fallow treatments, as the brown mustard is a non-host

249 crop.

250

251 **General sequencing information and taxonomic richness**

252   In this study, a total of 1,087,375 paired-end sequences were obtained from the 24 libraries using

253   the AMV4.5NF/AMDGR primer set. Of these, 873,458 sequences belonged to Glomeromycotina

254   (corresponding to 80.3% of the total). We showed the OTU distributions of the obtained

255   sequences in root samples (Table S2). In total, 83 OTUs were found in the AMF communities

256   based on 97% similarity after the rarefaction process (Fig. 3). The AMF OTUs were also

257   classified into one of four AMF families including *Glomeraceae* (average relative abundance:

258   60.7%), *Gigasporaceae* (22.0%), *Acaulosporaceae* (16.2%) and uncultured *Glomeromycotina* in

259   roots (1.1%).

260   Additionally, the OTU richness in maize after cultivation of brown mustard or bare fallow

261   was higher compared with that from roots after cultivation of Italian ryegrass and hairy vetch,

262   but this was not a significant difference (Fig. 4A). On the contrary, the Shannon (*H'*) and

263   Simpson (*1/D*) indices in hairy vetch were significantly lower than those of other cover cropping

264   treatments (Fig. 4B and 4C). In terms of other cover cropping treatments, no significant

265   differences in the Shannon and Simpson indices were found (Fig. 4B and 4C). However, in

266   soybean after cultivation of brown mustard or bare fallow, the OTU richness and Shannon index

267   was lower compared with those from roots after cultivation of Italian ryegrass and hairy vetch

268   (Fig. 4A and 4B). Moreover, at each parameter between maize and soybean roots, no significant

269   difference was found (Fig. 4A-4C).

270

271   **Structures of AMF communities in the two different subsequent crops**

272   Our results showed that the relative abundance of each AMF OTU and family tended to be

273   different among cover cropping treatments when we compared the AMF communities in maize

274   with that in soybean (Fig. S2A and S2B). Moreover, we found that the structure of AMF

275   communities in Italian ryegrass versus hairy vetch treatments, and brown mustard versus bare

276   fallow treatments in maize tended to shape a similar community structure. In Italian ryegrass and

277   hairy vetch treatments, *Glomeraceae* was predominant. On the contrary, *Gigasporaceae* and

278   *Acaulosporaceae* were predominant in brown mustard and bare fallow treatments. In soybean,

279   the structure of AMF communities in brown mustard versus bare fallow treatments also tended to

280   shape a similar community structure (Fig. S2A). In hairy vetch, brown mustard and bare fallow

281   treatments, *Glomeraceae* were predominant, on the contrary, *Gigasporaceae* and

282   *Acaulosporaceae* were predominant only in Italian ryegrass treatment. Additionally, we found

283   that *Glomeracea*e was detected at a high frequency in both maize (56.2%) and soybean roots

284   (62.2%) regardless of cover cropping treatments (Fig. S2A). *Gigasporaceae* was more abundant

285   in maize (25.0%) compared with in soybean (20.6%), and the relative abundance of

286   *Acaulosporaceae* in the maize (17.7%) was larger than that in soybean (16.2%). Among the

287   OTUs of *Glomeraceae*, the OTUs of uncultured *Glomus* (Accession No., LT217508; LT723917;

288   LT217431) were detected at a much higher frequency in both maize (28.8%; 8.5%; 8.3%,

289   respectively) and soybean roots (22.4%; 18.4%; 13.5%, respectively) (Table S4 and Fig. S2B).

290   In turn, the OTU of *Rhizophagus irregularis* (HF968834) was also detected at a higher frequency

291   in both maize (5.6%) and soybean roots (3.4%). Among the OTUs of *Gigasporaceae*, the OTU

292   of *Cetraspora pellucida* (KX879059) was highly detected at a much higher frequency in both

293   maize (19.5%) and soybean roots (16.1%). Additionally, the OTU of uncultured *Acaulospora*

294   (LN890608) was detected at a much higher frequency in both maize (13.5%) and soybean roots

295   (12.5%) among the OTUs of *Acaulosporaceae*.

296

297   **Host preference of AMF communities in the two different subsequent crops**

298   The AMF OTUs in various taxonomic lineages were also classified as "generalists," commonly

299   found in both subsequent crop species according to the multinomial species classification method

300   (CLAM) test (Table S3 and S4). The proportion of common generalists in roots in this

301   experiment among Italian ryegrass, hairy vetch or brown mustard versus bare fallow was 77.1%,

302   68.8% and 18.8%, respectively (Fig. 5A-5C). The proportion of detected OTUs that occurred

303   specifically in only ryegrass, hairy vetch or brown mustard was 8.4%, 12.5%, and 33.8%,

304   respectively. Moreover, detected OTUs that occurred specifically in only bare fallow measured

305   4.8%, 12.5%, and 41.2%, respectively, when comparing with Italian ryegrass, hairy vetch or

306   brown mustard treatments. Additionally, most of the OTUs in root samples were common

307   between maize and soybean, and the proportion of common generalists in this experiment for

308 maize and soybean roots was 79.5% (Fig. 6). Detected OTUs that occurred specifically in only

309 maize and soybean were 9.6% and 10.8%, respectively.

310

311 **Relationships between AMF communities of cover cropping and subsequent host crops**

312 We used distance-based redundancy analysis (db-RDA) to identify the relationships among AMF

313 communities in maize and soybean roots with that in cover crop management (Fig. 7A and 7B).

314 The db-RDA trends clearly showed that the cover cropping noticeably altered the AMF

315 community structure in the maize and soybean roots. In maize, the ordination diagram indicates

316 that Italian ryegrass ($R^2 = 0.787$, $P = 0.003$), hairy vetch ($R^2 = 0.721$, $P = 0.01$) and bare fallow

317 ($R^2 = 0.492$, $P = 0.028$) contributed significantly to the variation in AMF root communities (Fig.

318 7A). However, brown mustard ($R^2 = 0.064$, $P = 0.737$) did not contribute to the variation in the

319 AMF root communities. In soybean, the ordination diagram indicates that Italian ryegrass ($R^2 =$

320 0.558, $P = 0.046$) and bare fallow ($R^2 = 0.533$, $P = 0.039$) contributed significantly to the

321 variation in the AMF root communities (Fig. 7B). However, hairy vetch ($R^2 = 0.410$, $P = 0.076$)

322 and brown mustard ($R^2 = 0.475$, $P = 0.055$) did not contribute to the variation in the AMF root

323 communities. A permutational multivariate analysis of variance (PERMANOVA) was also

324 carried out to examine the effect of cover cropping on the AMF root communities in maize and

325 soybean. The PERMANOVA showed that cover cropping significantly affected the AMF root

326 community structure (maize; $F = 4.647$, $P < 0.05$, soybean; $F = 6.339$, $P < 0.001$) (Fig. 7A and

327 7B).

328   Additionally, we used non-metric multidimensional scaling (NMDS) to identify the

329 differences in the AMF communities between maize and soybean (Fig. 8). The differentiation of

330 AMF communities between the two subsequent host crop species was not statistically significant

331 (host crop; $F = 1.238$, $P > 0.05$, cover cropping; $F = 2.048$, $P > 0.05$). However, we found that

332 the interaction with host crop species and cover cropping on the differences in the AMF

333 communities in the maize and soybean roots was significantly different (interaction; $F = 8.565$, P

334 < 0.001).

335

336   **Discussion**

337   **Impact of cover cropping on AMF root colonization**

338   Previous studies have shown that the introduction of cover crops or fallow affects AMF

339   colonization of subsequent cash crops (Isobe et al., 2014; Karasawa & Takahashi, 2015; Higo et

340   al., 2018a). Indeed, in the current study, brown mustard and bare fallow treatments significantly

341   affected the AMF colonization of subsequent maize; however, these treatments did not affect the

342   AMF colonization of subsequent soybean (Fig. 1), which is a finding that is in partial agreement

343   with the results of previous studies (Karasawa et al., 2002; Karasawa & Takebe, 2012). Some

344   previous findings also indicated that the introduction of *Brassicaceae* plants reduced AMF root

345   colonization in subsequent crops during early growth stages compared with the introduction of

346   host crops (Sorensen et al., 2005; Koide & Peoples, 2012). Because brown mustard (a member of

347   *Brassicaceae*) does not form symbiotic relationships with AMF in its roots due to the release of

348   antifungal compounds, such as mustard oils or isothiocyanates. Indeed, antifungal compounds,

349   such as isothiocyanates, decompose in soil owing to microbial effects and responses to organic

350   matter (Morra & Kirkegaard, 2002). Schreiner and Koide (1993) showed that extracts from

351   *Brassicaceae* roots reduced AMF spore germination after 5 and 7 days. However, AMF spore

352   germination recovered to normal conditions after about 14 days, thereby suggesting that

353   isothiocyanates and mustard oils may have an antimicrobial effect on soil microorganisms,

354   including AMF spores. In fact, the maize and soybean growth performance in the early growth

355   stage, even after brown mustard treatment, did not reduce with decreasing AMF root

356   colonization (Fig. 2A and 2B), although brown mustard is a non-host crop, in agreement with the

357   results of Higo et al. (2018b). Thus, adding brown mustard or fallow in a rotation with maize and

358   soybean may not necessarily have negative impacts on the performance of subsequent maize and

359   soybean in early growth stages.

360

361   **Distribution of AMF communities in maize and soybean roots**

362   Our results showed that the representatives of *Glomeraceae* (including a genus of *Glomus* and

363   *Rhizophagus*) were found to be the main family, although *Gigasporaceae* (including a genus of

364   *Cetraspora*, *Gigaspora*, and *Racocetra*), *Acaulosporaceae* (including a genus of *Acaulospora*)

365   and uncultured *Glomeromycotina* were found in both maize and soybean roots (Fig. S2). These

366   results are similar to the findings of published studies confirming that the AMF taxa of

367   *Glomeraceae* are the most abundant in the AMF communities of maize (Isobe et al., 2011; Wang

368   et al., 2017; Higo et al., 2018b) and soybean roots (Higo et al., 2014, 2018a; García de León et

369   al., 2018; Faggioli et al., 2019). The AMF taxa of *Glomeraceae* are particularly predominant in

370   roots and arable soils (Senés-Guerrero et al., 2016; Xiang et al., 2016; Xu et al., 2017; Zhao et al.,

371   2017) because they are better adapted to disturbed environments than other families in addition

372   to having high sporulation rates for rapid recovery (Oehl et al., 2003). Moreover, *Glomeraceae*

373   colonize throughout fragments of hyphae or mycorrhizal roots, thereby forming a hyphal

374   anastomosis (Giovannetti et al., 1999), and therefore possessing the ability to reconstruct a

375   network after mechanical disruption. However, *Gigasporaceae* of AMF propagates throughout

376   spore dispersal or infection from an intact hypha (Biermann & Linderman, 2006; Schalamuk &

377   Cabello, 2010). Thus, these factors promote the survival and spread of *Glomeraceae* family

378   members in agricultural ecosystems, and the occurrence of this phenomenon can also be the

379   result of adaptation to agroecological environments.

380   Additionally, Clavel et al. (2011) indicated that in general, taxa of generalists are thought to

381   be related to taxa of specialists with undisturbed habitats and disturbed habitats. Johnson (1993)

382   has reported that AMF have different niches and are well known to prefer inhabiting different

383   soils. Host plant types and environmental filters may favor AMF taxa that grow better in soils

384   (Dumbrell et al., 2010). In fact, Börstler et al. (2008) and Séry et al. (2018) suggested that a

385   *Glomeraceae* such as the generalist, *R. irregularis*, has been observed in many different types of

386   fields and can have a high tolerance for environmental filters such as tillage. On the contrary,

387   Gottshall et al. (2017) reported that AMF taxa of *Gigasporaceae* were affected by agricultural

388   management practices with a low level of soil disturbance. Faggioli et al. (2019) also suggested

389   that abundance of *Gigasporaceae* can rely on no-tillage practice. Additionally, intensive

390   agricultural practices may reduce the taxa richness and change the AMF community structures

391   according to favoring certain AMF taxa and disfavouring other AMF taxa (Gottshall et al., 2017).

392 This phenomenon shows the shift in the occurrence of generalist and specialist AMF taxa with

393 respect to a range of host plant species or habitat (Helgason et al., 2007; Oehl et al., 2010). Thus,

394 the fluctuation in abundance of AMF taxa (generalists, specialists and rare taxa) as a result of

395 cover cropping or host crops (maize and soybean) (Fig. 5 and 6, Fig. S2, Table S3 and S4) could

396 link to the preference of host identity or inhabiting preferable agricultural practices such as cover

397 cropping among AMF taxa in maize and soybean roots.

398   Additionally, there are few but increasing evidence of the spatial distribution of AMF

399 communities in soil at small scales using molecular techniques. For example, Mummey and

400 Rillig (2008) also indicated the difference in the spatial distribution of AMF communities at a

401 small (1 m x 1 m) scale in a temperate grassland. Wolfe et al. (2007) also found that the spatial

402 variation of AMF communities to be within a 2 m x 2 m scale. In this study, the 3 m x 3 m scale

403 of our research plots may be small for performing experiments to determine the distribution of

404 AMF communities in agricultural practices. However, we considered that the spatial distribution

405 of specific AMF communities of our study responded to the presence of the crop in our crop

406 rotational system, in accordance with the findings of previous studies (Wolfe et al., 2007;

407 Mummey & Rillig, 2008). Taken together, our research plot scale is complete enough to

408 compare the diversity of AMF communities with the impact of cover cropping and host identity.

409

410 **Impact of cover cropping on the diversity of AMF communities**

411 Although the benefits of a host plant may differ according to individual AMF taxa, there are little

412 data with respect to how the diversity of AMF communities varies with cover cropping practices.

413 To date, Higo et al. (2014) have demonstrated that the diversity of AMF communities colonizing

414 subsequent soybean roots in a cover crop rotations system is clearly changed by rotation year,

415 suggesting that climate or other environmental filters are more important than winter cover

416 cropping. Furthermore, Higo et al. (2018b) and Turrini et al. (2016) showed that a difference in

417 the structure of AMF communities colonizing subsequent maize roots in a cover crop rotation is

418 independent of the identity of preceding crops. However, these studies used separate plots for the

419 cover crop rotation experiment to assess the effect of cover crops on the AMF communities in

420   maize or soybean roots. Thus, we examined three cover crop treatments (Italian ryegrass, hairy

421   vetch, and brown mustard) and bare fallow for the diversity of AMF colonizing both subsequent

422   maize and soybean roots. We found that hairy vetch before planting maize had a negative impact

423   on the diversity of AMF in roots of maize, but not for that of soybean (Fig. 4B and 4C). In

424   soybeans, brown mustard before planting soybean had a negative impact on the diversity of

425   AMF in roots. Additionally, the shift of AMF communities in both maize and soybean was

426   obvious from the results of db-RDA, which showed how cover cropping changed the AMF

427   community structure in both subsequent crop roots (Fig. 6A and 6B), in agreement with the

428   observations of a previous study (Higo et al., 2018a, 2018b). These findings indicate that cover

429   crop species may induce a shift in the diversity and structure of AMF communities in soils, and

430   the differences in the soil AMF communities after the different types of cover cropping may

431   change the diversity of AMF communities in subsequent crops.

432

433   **Impact of host identity on the diversity of AMF communities**

434   Several studies have reported that host preference between host plants and AMF taxa may impact

435   AMF community structure (Helgason et al., 2002; Yang et al., 2012). We expected that if host

436   identity is a strong factor in determining AMF communities compared with cover cropping in the

437   split-plot of our experiment, the AMF community structure associated with maize and soybean

438   roots after cover cropping would be distinct host AMF community structures between maize and

439   soybean roots. This would hold true even if different cover crop treatments were to shape a

440   specific assemblage of AMF taxa in soils before planting maize and soybean in our cover crop

441   rotation. However, our NMDS and PERMANOVA result demonstrated that host identity did not

442   change the AMF community structure of maize and soybean roots (Fig. 8). Furthermore, host

443   identity did not impact the richness, diversity, or structure of the AMF community in the roots of

444   maize and soybean at the early growth stage, and this finding was somewhat unexpected.

445   Gosling et al. (2013) reported that soil P concentration had a greater impact on host identity. On

446   the contrary, abiotic environmental filters, such as land-use type (Bainard et al., 2014), growing

447   season (Higo et al., 2015b), and soil P (Bainard et al., 2014), appear to override the impact of

448   host identity if their impacts are large enough compared with host identity.

449   Additionally, Isobe et al. (2011) have shown that the AMF community structure in soybeans

450   grown in cool and temperate regions are drastically different, thus suggesting climate condition,

451   such as growth temperature, can also be a strong determinant in shaping AMF community

452   structures. To date, only a few studies have also shown that land-use type and host identity have

453   interactive effects on root AMF community structure in grasslands (Vályi et al., 2015; Ciccolini

454   et al., 2016) and agricultural fields (Higo et al., 2015b, 2016; Ciccolini et al., 2016), whereas

455   interactions between land use and other factors, such as soil biochemical properties, have also

456   been reported in temperate areas (Jansa et al., 2014; Ciccolini et al., 2015). Indeed, a specific

457   reason for why there was a significant interaction in the AMF communities between maize and

458   soybean, was uncertain in this study (Fig. 8). Thus, we hypothesize that one possible explanation

459   for the consistency in AMF communities between both subsequent crops may be that the impacts

460   of specific root exudate patterns in both maize and soybean on the host selectivity of AMF taxa

461   may be weakened due to planting maize and soybean within the same plot, or may be weakened

462   at a too early growth stage, as suggested by Higo et al. (2015b) and Borrell et al. (2016).

463   However, how and whether the selectivity of the host plant impacts the pattern of AMF

464   communities in agricultural systems, remains unknown. Thus, further investigation into the

465   relationships among a pattern of AMF communities would be required to better understand

466   whether and how host selectively impacts the AMF communities in cover crop rotational

467   systems.

468

469   **Conclusions**

470   In conclusion, cover cropping can be an important factor for shaping AMF communities in maize

471   and soybean roots rather than their host identity in a cover crop rotation. In addition, different

472   cover crop species may have different influences on the diversity of AMF community structure

473   in both maize and soybean roots. Additionally, interactions of cover cropping and host identity

474   may partially relate to shaping the diversity and structure of AMF communities in maize and

475 soybean roots in this study. These differences in the AMF community structure may relate to

476 maize and soybean production in cover crop rotational systems. However, we still need to clarify

477 whether and how host identity interacts with the diversity and structure of AMF communities

478 associated with maize and soybean in cover cropping systems. This knowledge will give useful

479 information on appropriate cover crop choices in cover crop rotational systems.

480

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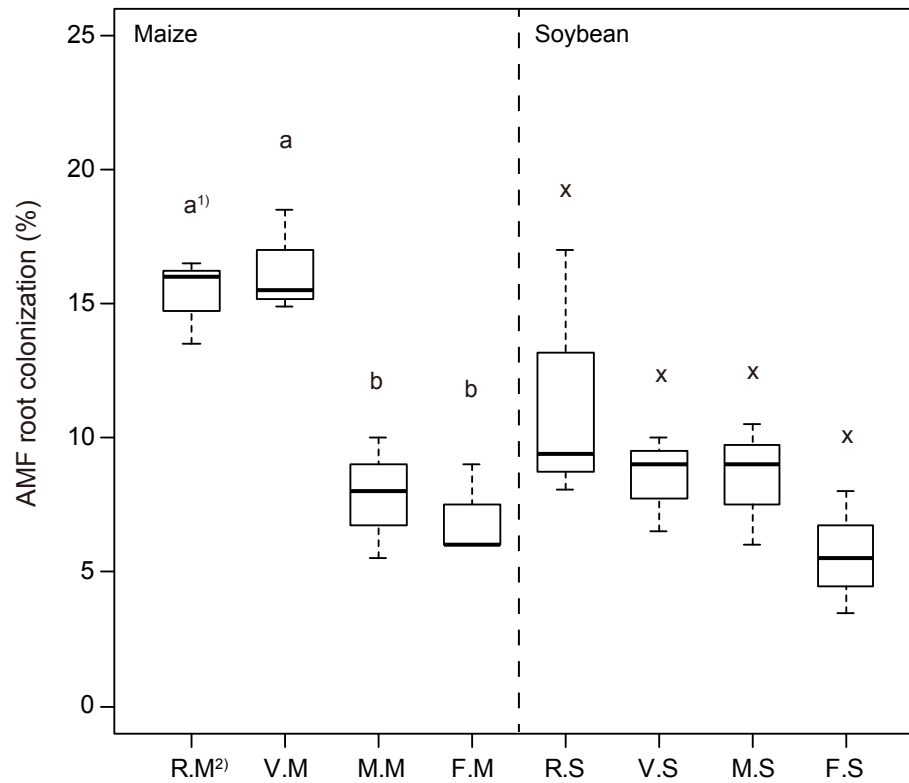
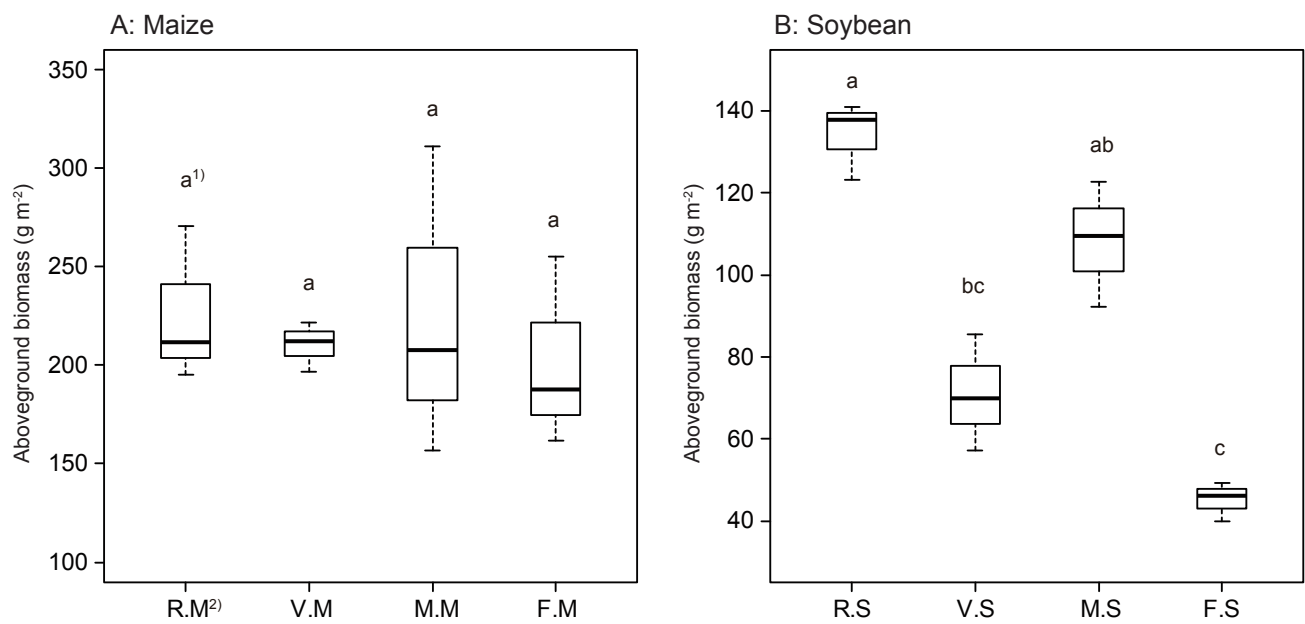
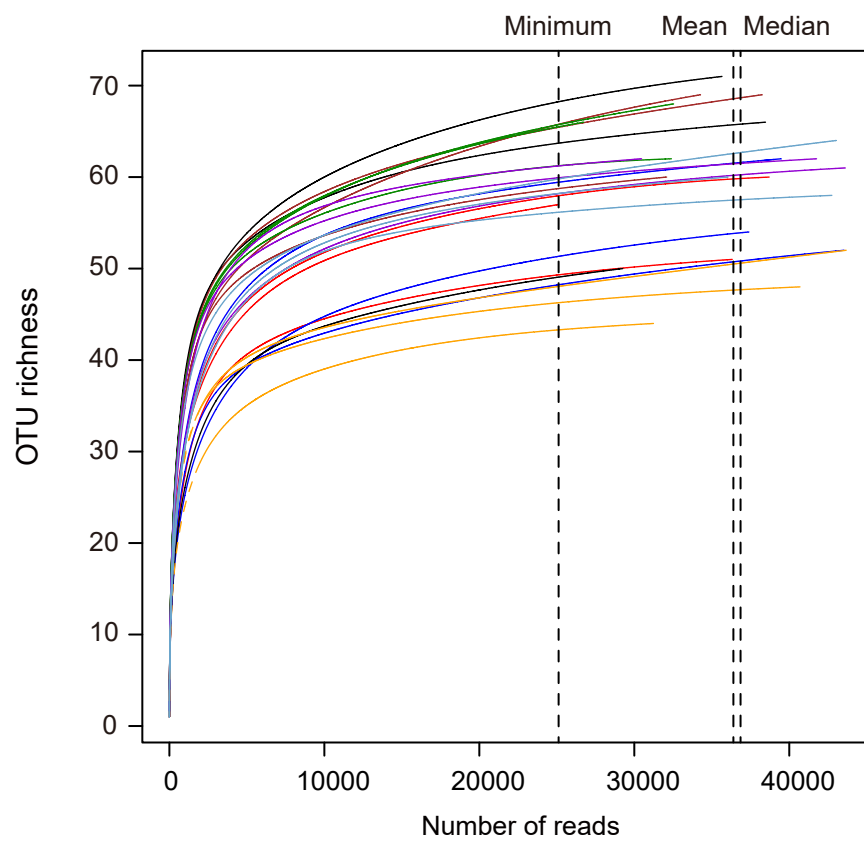
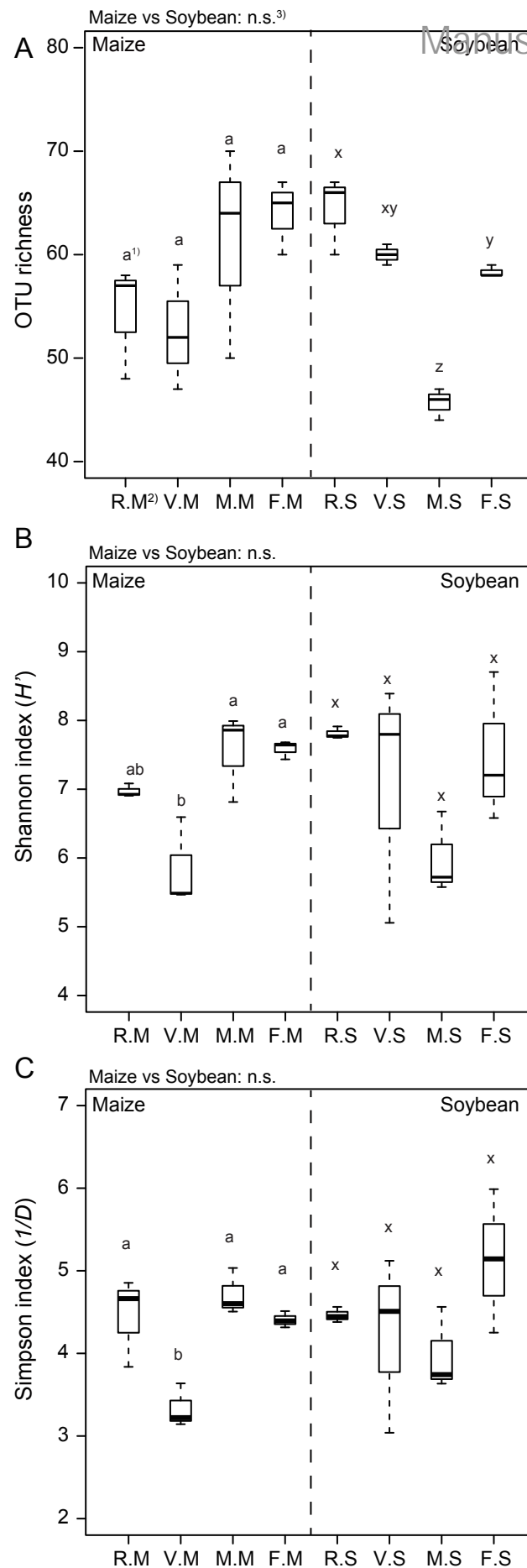
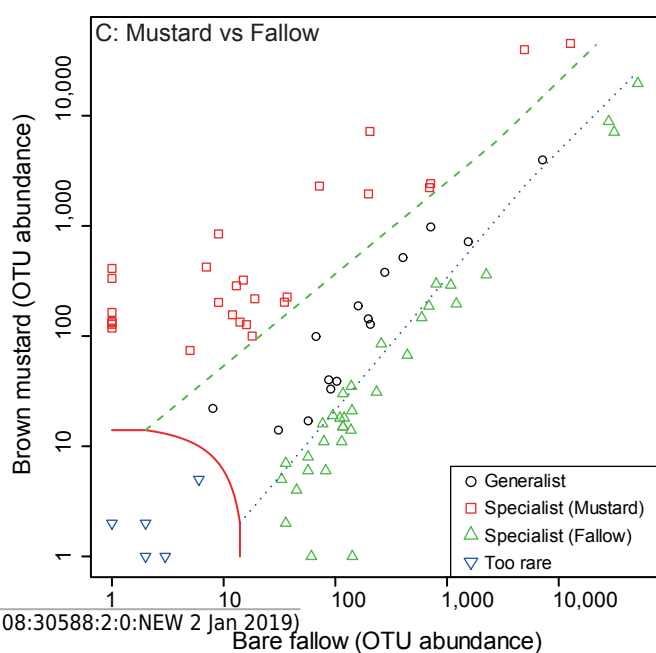
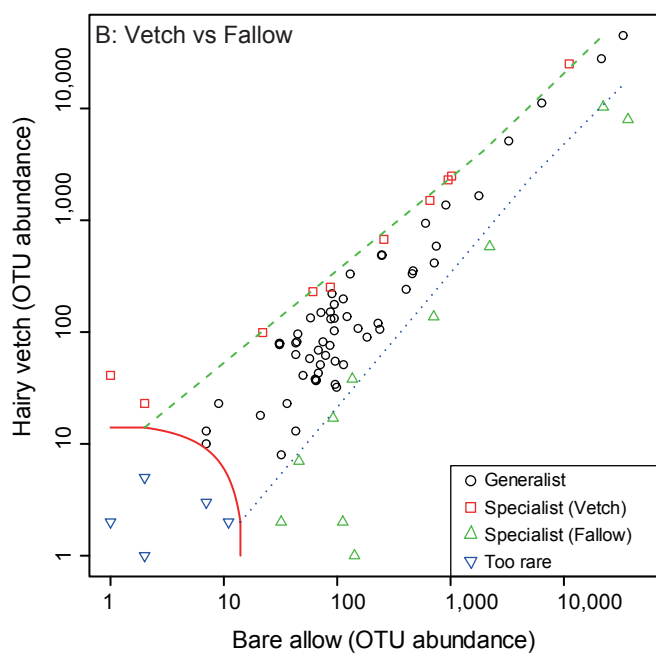
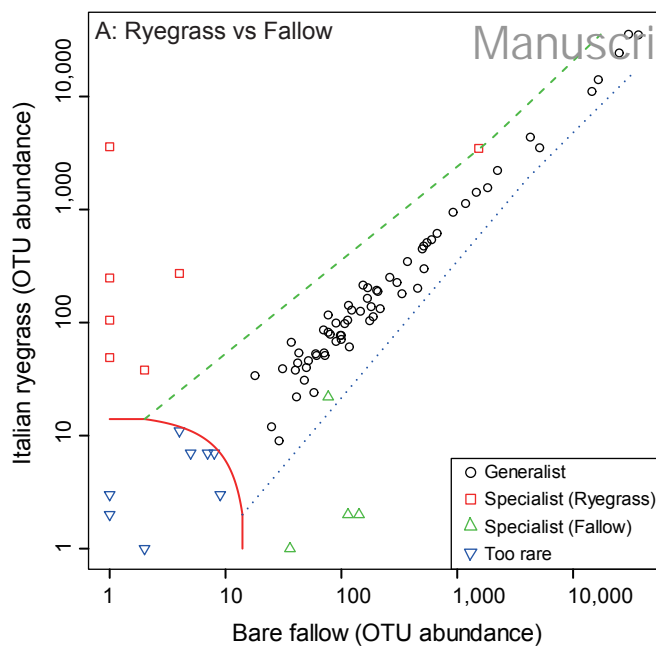
Boxplots illustrating differences in group averages in root colonization of arbuscular mycorrhizal fungi (AMF) in maize and soybean at 6 weeks after sowing.

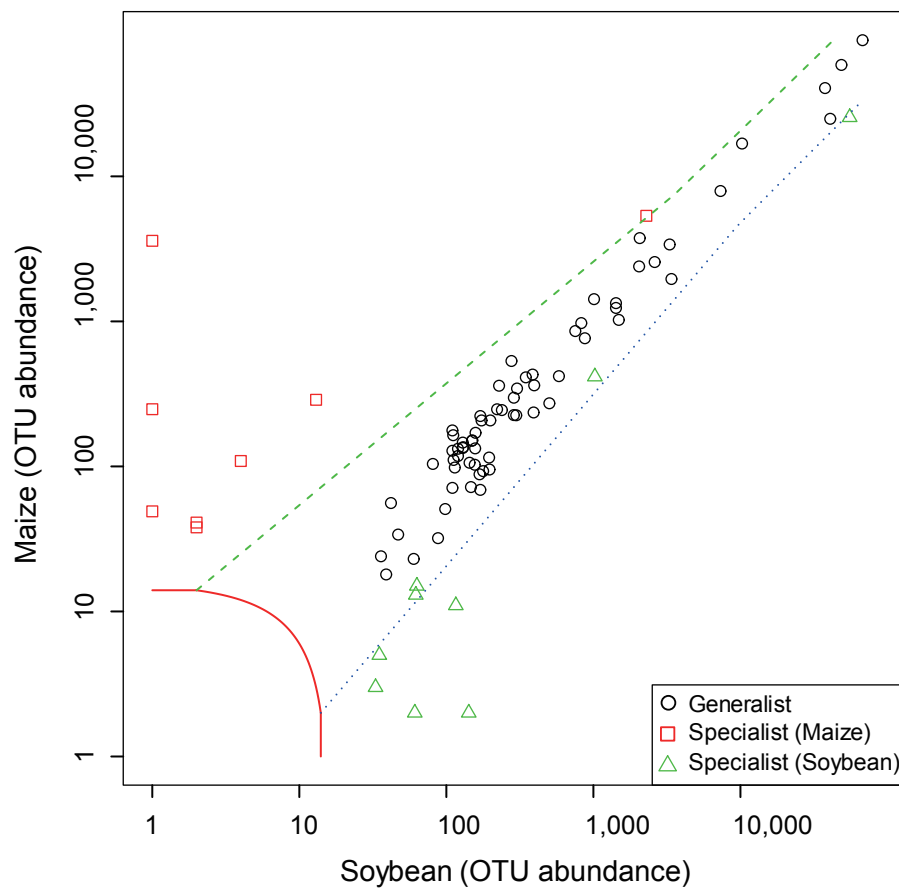
Bold horizontal lines represent median values; box margins ± SE and vertical lines represent minimum and maximum values of the groups. 1) Different letters in the maize or soybean plots among the cover crop treatments show a significant difference according to Tukey's test at the 5% level. 2) R.M, V.M, M.M and F.M show the AMF root colonization of maize after cultivation of Italian ryegrass, hairy vetch, brown mustard cropping or bare fallow, respectively. R.S, V.S, M.S and F.S show the AMF root colonization of soybean after cultivation of Italian ryegrass, hairy vetch, brown mustard cropping or bare fallow, respectively.

Boxplots illustrating differences in group averages in aboveground plant biomass of maize and soybean at 6 weeks after sowing.

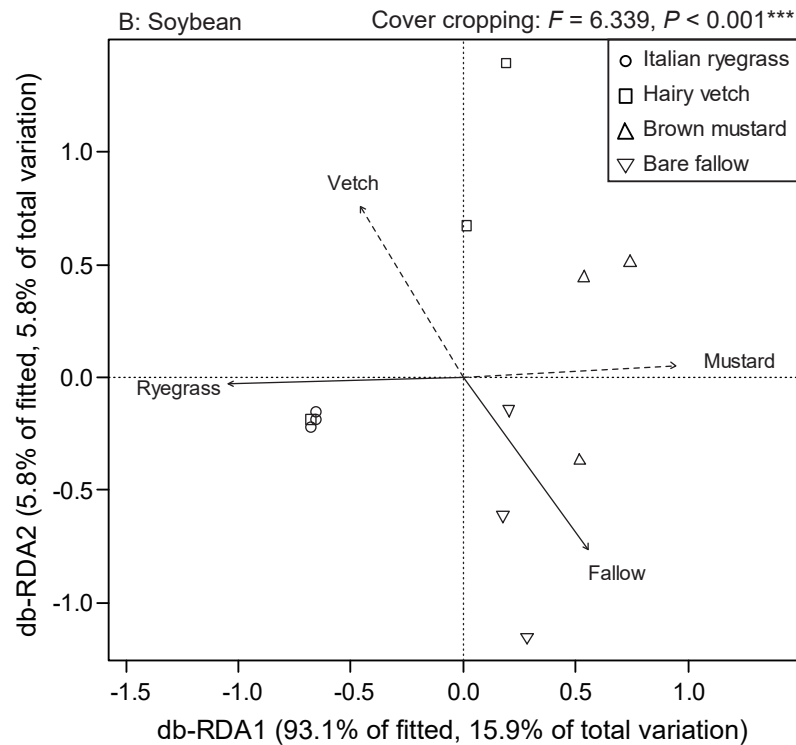
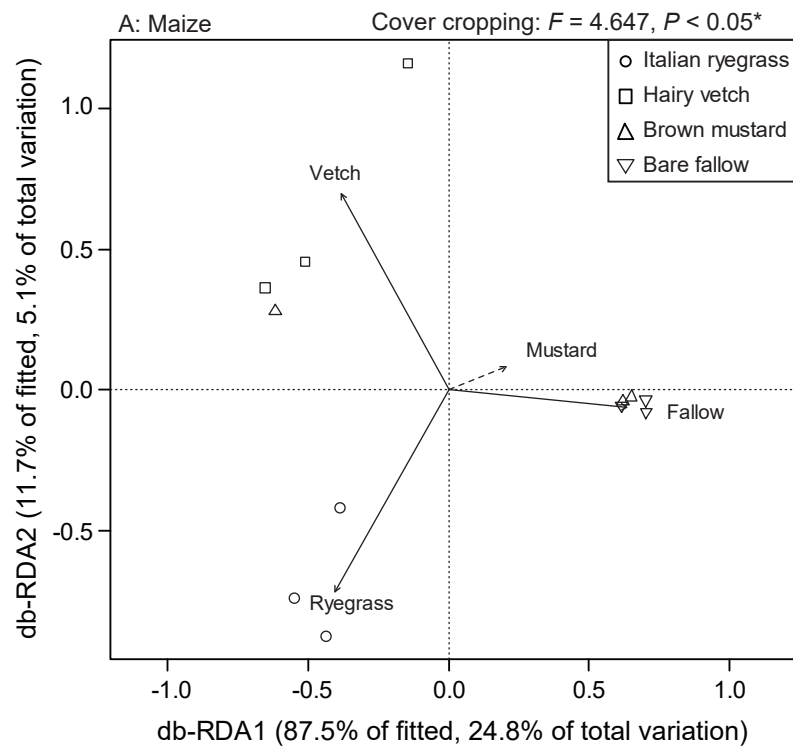
Bold horizontal lines represent median values; box margins ± SE and vertical lines represent minimum and maximum values of the groups. 1) Different letters in the maize or soybean plot among the cover crop treatments show a significant difference according to Tukey's test at the 5% level. 2) R.M, V.M, M.M and F.M show the aboveground plant biomass of maize after cultivation of Italian ryegrass, hairy vetch, brown mustard cropping or bare fallow, respectively. R.S, V.S, M.S and F.S show the aboveground plant biomass of soybean after cultivation of Italian ryegrass, hairy vetch, brown mustard cropping or bare fallow, respectively. A = Maize, B = Soybean.

Rarefaction curves showing the amplicon sequencing depths in the maize and soybean root samples in this study.

The dashed lines show a minimum (25,116 sequences), mean (36,394 sequences) and median (36,857 sequences) number of obtained sequences in this study. The operational taxonomic units (OTUs) were compared across samples when samples were rarefied at 25,116 sequences.

Boxplots illustrating differences in group averages regarding (A) operational taxonomic unit (OTU) richness, (B) Shannon index (*H'*) and (C) Simpson index (*1/D*) in the roots of maize and soybean at 6 weeks after sowing.

Bold horizontal lines represent median values; box margins ± SE and vertical lines represent minimum and maximum values of the groups. 1) Different letters in the maize or soybean plot among the cover crop treatments show a significant difference according to Tukey's test at the 5% level. 2) R.M, V.M, M.M and F.M show the OTU diversity of maize after Italian ryegrass, hairy vetch, brown mustard cropping or bare fallow, respectively. R.S, V.S, M.S and F.S show the OTU diversity of soybean after Italian ryegrass, hairy vetch, brown mustard cropping or bare fallow, respectively.

Screening of generalists and specialists of arbuscular mycorrhizal fungal communities in root samples among different cover cropping treatments.

The operational taxonomic units (OTUs) of arbuscular mycorrhizal fungi (AMF) commonly detected from both Italian ryegrass, hairy vetch or brown mustard and bare fallow treatments (circle), and those preferentially found from Italian ryegrass, hairy vetch or brown mustard (square) or bare fallow treatments (diamond). Rare AMF OTUs (triangle) were classified according to a multinomial species classification method (CLAM) test. A: Italian ryegrass versus bare fallow treatment, B: hairy vetch versus bare fallow treatment, and C: brown mustard versus bare fallow treatment.

Screening of generalists and specialists of arbuscular mycorrhizal fungal communities in root samples between maize and soybean plots.

The operational taxonomic units (OTUs) of arbuscular mycorrhizal fungi (AMF) commonly detected from both maize and soybean (circle) samples; those preferentially found from maize (square) or soybean (diamond) samples were classified according to a multinomial species classification method (CLAM) test.

Distance-based redundancy analysis (db-RDA) biplot showing the relationship among the AMF communities and cover cropping in maize (A) and soybean (B).

Solid lines indicate significant effects, and dashed lines indicate non-significant effects.

Non-metric multidimensional scaling (NMDS) plot showing differences in the arbuscular mycorrhizal fungal communities between maize (closed) and soybean (open) root samples.

Circles in the NMDS plot are 95% confidence ellipses of maize (solid line) and soybean (dashed line).