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1 ***Trichoderma* enriched compost, BCAs and potassium phosphite control Fusarium wilt of lettuce**
2 **without affecting soil microbiome at genus level**

3

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16

17 **Abstract**

18

19 *Fusarium oxysporum* f. sp. *lactucae* (Fol) is the causal agent of Fusarium wilt of lettuce, one of the most
20 troublesome diseases affecting lettuce worldwide. Chemical control strategies are inadequate due to
21 limited fungicide availability and consumer interest in organic vegetable production. Alternative control
22 strategies, such as biological control agents (BCAs), suppressive compost, and resistance inducers, have
23 been intensively studied to test their ability to reduce pathogen attacks. Research has been recently
24 focused on the influence of BCAs on the rhizosphere microbiota, which plays a critical role in soil
25 suppressiveness. In this work, three strategies of integrated pest management (IPM) were tested against
26 Fol attacks in two fields for two consecutive years: (i) a compost enriched with *Trichoderma*, (ii) a
27 combination of *T. gamsii* + *T. asperellum*, *Bacillus amyloliquefaciens* and potassium posphite and (iii) a
28 combination of *T. polysporum* + *T. atroviride*. The rhizosphere microbiota was characterized by high-
29 throughput sequencing of bacterial and eukaryotic rRNA gene markers. Obtained results indicated IPM
30 strategies statistically reduced disease severity, in both fields and years, from 50% to 70% compared to
31 untreated controls. An increased crop yield compared to untreated controls was also observed.
32 Predominant phyla were Proteobacteria, Firmicutes and Actinobacteria for bacteria, and Ascomycota for
33 fungi. However, microbiota populations were not affected by any of the treatments, nor were significant
34 differences observed when the soil microbial community was compared to that of untreated controls.
35 Conversely, large differences were observed when comparing the two fields and years, indicating an
36 important microbial buffering effect triggered by the soil.

37

38 **Keywords:** microbiota, rhizosphere, biocontrol agents, resistance inducers, seed born pathogen,
39 *Fusarium oxysporum* f. sp. *lactucae*.

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41

42 **1. Introduction**

43 Lettuce is cultivated on 20,000 ha of agricultural land in Italy (ISTAT 2020). As an intensive crop, lettuce
44 yields are threatened by the presence of *Fusarium oxysporum* f. sp. *lactucae* (Fol), the causal agent of
45 Fusarium lettuce wilt. It is one of the most dangerous diseases affecting this crop worldwide (Matheron
46 and Gullino 2012; Gullino *et al.*, 2019). Characteristic symptoms of Fol infection are yellowing of leaves,
47 slow growth accompanied by brown or red streaks of the vascular system, and wilting to death.
48 Significant economic losses can occur if the disease is not properly managed (Matheron and Gullino
49 2012). In any case, Fol management is difficult for two reasons: (i) it spreads rapidly and easily as it is
50 seed-borne (Garibaldi *et al.*, 2004a) and (ii) has the ability to distinguish new races, in fact four are
51 already known and reported (Fujinaga *et al.*, 2001; 2003; Matheron and Gullino 2012; Gilardi *et al.*,
52 2017). Chemical control is not considered a sufficient strategy due to the reduced availability of soil
53 fumigants, the need to reduce chemical treatments to avoid environment and lettuce contamination, and
54 the increasing consumer demand for organic vegetable production. For these reasons, it is important to
55 develop and test new alternative control strategies against this pathogen. The use of biological control
56 agents (BCAs), organic amendments and resistance inducers are among the most studied (Bonanomi *et*
57 *al.*, 2007, 2010; Gilardi *et al.*, 2019, 2020). BCAs can act directly against pathogens via antibiosis,
58 parasitism or predation, or indirectly by colonizing the rhizosphere environment and using resources
59 more efficiently than pathogens (Pal and Gardener 2006). Several BCAs are registered in Europe against
60 soil-borne disease (Bardin and Pugliese, 2020). *Bacillus amyloliquefaciens* (former *subtilis*) QST713,
61 which is registered in Europe and Italy, is used to control *Pythium ultimum* and *Rhizoctonia solani*, but
62 also *Botrytis cinerea* and powdery mildews, via antibiosis and induced resistance (Bardin and Pugliese,
63 2020). *Trichoderma* strains are good soil and rhizosphere colonizers, and *Trichoderma gamsii* ICC080,
64 *T. asperellum asperellum* ICC012, *Trichoderma polysporum* IMI206039, and *Trichoderma atroviride*

65 IMI206040 are applied towards a broad spectrum of plant pathogens (EFSA, 2013; Bardin and Pugliese,
66 2020). *Trichoderma gamsii* ICC080 and *T. asperellum asperellum* ICC012 are registered in Europe and
67 Italy, while *Trichoderma polysporum* IMI206039 and *Trichoderma atroviride* IMI206040 are registered
68 in Europe.

69 Organic amendments, and composts, have been described for decades as suppressive against various
70 vascular pathogens, including Fol (Pugliese *et al.*, 2015; Gilardi *et al.*, 2016; De Corato *et al.*, 2018a,
71 2018b; Bonanomi *et al.*, 2018). Many studies have demonstrated that their microbiota plays an important
72 role in suppressive action (Reuveni *et al.*, 2002; Tilston *et al.*, 2002; Papatotiriou *et al.*, 2013; De Corato
73 *et al.*, 2019). Their mechanisms of action are similar to those of BCAs, but the complexity and richness
74 of the compost microbiota suggest that those different mechanisms could be used and act synergistically.
75 Composts can also be enriched with BCAs to enhance their suppressive activity, as it has been indicated
76 that this technique could be the most promising to achieve long-term suppressiveness against vascular
77 pathogens (Pugliese *et al.*, 2011; Bonanomi *et al.*, 2018; Gilardi *et al.*, 2019). A compost enriched with
78 *Trichoderma virens* TW2 demonstrated to be effective in controlling Fusarium wilt on vegetable crops
79 (Gilardi *et al.*, 2019; Cucu *et al.*, 2019 and 2020b).

80 Resistance inducers are compounds of various origins that have been shown to activate the plant immune
81 system (Walters *et al.*, 2009; Akram and Anjum 2011; Alexandersson *et al.*, 2016). BCAs, and by
82 extension composts, have been shown to activate the induction of resistance in several pathosystems
83 (Compant *et al.*, 2005; Ongena *et al.*, 2007; Pieterse *et al.*, 2014; Akram *et al.*, 2015; Bellini *et al.*, 2021).

84 Integrated pest management (IPM) strategies are combinations of different measures based on the
85 principle of synergy between them to control pathogen attacks (Barzman *et al.*, 2015).

86 Soil microbial activities are responsible for nutrient transformation, decomposition of organic matter,
87 protection against pathogens and contribute to soil structure (Bowles *et al.*, 2014). The complex of
88 microorganisms inhabiting the rhizosphere can strengthen plants and protect them from both biotic and

89 abiotic stresses (Nihorimbere *et al.*, 2010; Chaudary *et al.*, 2021a), in fact, the rhizosphere microbiota
90 population is one of the greatest influences on plant and soil health (Berendsen, 2012; Kumari *et al.*,
91 2020; Chaudary *et al.*, 2022). The role of rhizosphere microbiota population is even more important for
92 the protective effect against vascular pathogens that invade plant tissues through the root system, as is
93 the case with Fol (Hubband and Gerik 1993; Gordon, 2017). The application of organic amendments and
94 BCAs may cause a shift in the composition and diversity of the rhizosphere microbiota, leading to a
95 change in soil suppressiveness. The combination of plant growth promoting rhizobacteria such as
96 *Bacillus* sp. and nanocompounds was demonstrated to have a strong influence on the composition of
97 rhizospheric microbiota on maize and to increase bacterial diversity and richness (Chaudary *et al.*,
98 2021b). The role of the rhizosphere microbiota in plant health is well established but it is not clear how
99 microorganisms applied as treatments interact with those already present in the soil and their ability to
100 establish permanent colonization. Studying the rhizosphere microbiota with amplicon-based sequencing
101 is a powerful tool (Simmons *et al.*, 2018; Elsayed *et al.*, 2020; Chaudary *et al.*, 2021b) to understand the
102 effects of IPM strategies on microbial populations at the end of the crop cycle and to determine whether
103 the protective effect guaranteed by the strategies is related to the shift in these populations. The key
104 difference between the conventional methods, i.e., Sanger sequencing and high-throughput sequencing
105 (HTS), is the sequencing volume. While the Sanger method sequences a single DNA fragment at a time,
106 HTS is massively parallel and sequences millions of fragments simultaneously per run. This process
107 enables the simultaneous sequencing of hundreds to thousands of genes. HTS also provides greater
108 discovery power to detect novel or rare variants with deep sequencing. Currently, HTS is used not only
109 for universal gene analysis but also for functional microbes (Hou *et al.*, 2018; Sultana *et al.*, 2019)
110 because it provides considerable information, is fast and easy to use, and is relatively inexpensive.
111 Furthermore, HTS was already applied and compared to conventional methods for studying the impact

112 of BCAs and biofertilizers on soil and rhizosphere microbial community (Cucu *et al.*, 2020a; Bellini *et*
113 *al.*, 2021).

114 In the present work, and based on previous studies (Gilardi *et al.*, 2016; 2019), three IPM strategies were
115 developed by using compost enriched with *T. virens* TW2 and the combinations of different BCAs
116 (*Trichoderma* spp. and *B. amyloliquefaciens*) alone or with potassium phosphite. Lettuce rhizosphere
117 composition and diversity were investigated for both bacteria (16S rRNA) and fungi (ITS) using Illumina
118 amplicon-based sequencing. The objectives of the work were: i) to evaluate the efficacy of the three
119 selected IPM strategies against lettuce Fusarium wilt under commercial and experimental fields with
120 different soils and ii) to study the rhizosphere microbiota of treated and untreated plants at genus/class
121 level.

122

123 **2. Material and Methods**

124

125 *2.1. Plants material and experimental design*

126 Field trials were conducted on two farms: a commercial farm in Moretta (CN, Italy) and an experimental
127 farm in Carmagnola (TO, Italy). The experiments were conducted under 360 m² and 64 m² plastic
128 tunnels, respectively, in Moretta and Carmagnola. The farm in Moretta (sand:silt:clay 56:19:25, pH 7.12
129 and 1.37% organic matter) had a natural infestation of Fol race 1, which causes significant crop losses in
130 susceptible lettuce cultivars (Gilardi *et al.*, 2019). The soil in Carmagnola (sand:silt:clay 68.16:10.7:21.1,
131 pH 8.2 and 0.94% organic matter) has no history of intensive lettuce cultivation nor evidence of natural
132 occurrence of the pathogen. For these reasons, the soil in Carmagnola was artificially infested with a
133 virulent strain of Fol race 1 coded as MYA-3040 (ATCC), previously isolated from lettuce wilted plants
134 in Italy, from the Agroinnova collection (Garibaldi *et al.*, 2002). Soil infestation was carried out as
135 follows: i) the pathogen was cultured in sterilized wheat kernels left at 23°C for two weeks, then ii) 100

136 g/m² of the colonized wheat kernels were incorporated into the soil at a depth of 10-15 cm using a rake.
137 The experiments were repeated in two consecutive years (2019 and 2020, see Table S1) with the lettuce
138 cultivar 'Voluski', classified as moderately susceptible to Fol (Gilardi *et al.*, 2017). The trial Carmagnola
139 2020 was done about 1 month later compared to the previous year, due to weather conditions and intense
140 rainfall. Trials lasted 33-41 days according to weather conditions. In both fields (Carmagnola and
141 Moretta), two-week-old lettuce plants were transplanted in a mulched soil at a density of 16 plants/m²,
142 with a randomized design of treatments and untreated controls, with four replicates to avoid the side
143 effect. Plants were irrigated with a drip irrigation system and grown in accordance with standard
144 cultivation practices in the region.

145

146 2.2. Integrated pest managements treatments

147 The three IPM strategies tested in this experiment were selected from those described in previous works
148 (Gilardi *et al.* 2016; 2019), choosing the best combination for the control of Fol in greenhouse and field
149 conditions. The strategies were designed and applied starting at the nursery level as follows: (i) a compost
150 enriched with *T. virens* TW2 (ANT's compost M; AgriNewTech s.r.l., Italy), (ii) a combination of
151 commercial BCAs *B. amyloliquefaciens* (former *subtilis*) QST713 (accession number CP025079,
152 Serenade Max, 15.6%, Bayer Crop Science, Italy), *T. gamsii* ICC080 (accession number IMI 392151) +
153 *T. asperellum asperellum* ICC012 (accession number IMI 392716) (Remedier, 2+2%, Isagro Ricerca,
154 Milan, Italy), and potassium phosphite (Alexin, 95PS, P₂O₅ 52%, K₂O 42%, Massò, Spain), and (iii) a
155 commercial mixture of *T. polysporum* IMI206039 (accession number IMI 206039, ATCC 20475) and *T.*
156 *atroviride* IMI206040 (accession number IMI 206040, ATCC 20476) (Binab solution 1+1%, BINAB
157 Bio-innovation AB, Helsingborg, Sweden). *T. virens* TW2 (accession number MZ222411) was isolated
158 from compost (Gilardi *et al.*, 2019; Cucu *et al.*, 2019, 2020a and 2020b; Bellini *et al.*, 2021). To simplify,
159 the tested IPM strategies are coded here as: i) Comp_Tricho, ii) Bac_Tricho and iii) Tricho. The products

160 Bac_Tricho and Tricho were applied as soil drench in the nursery. Comp_Tricho was used at sowing
161 (T0) at a dosage of 400 g/100 seedlings and applied in the field immediately before transplanting at a
162 dosage of 1 kg/m² for Moretta. In Carmagnola, Comp_Tricho was applied at a dosage of 0.5 kg/m²,
163 considering the soil mixture, the possibility of applying the treatment with the rototiller (in Moretta field)
164 or manually (in Carmagnola field), and because of the smaller size of the tunnel, which allows less deep
165 diffusion. The type, dosage and timing of application of the three strategies are shown in Table 1.

166

167 2.3. Lettuce yield and disease assessment

168 Yield and disease were evaluated at the end of each experiment. Sixteen plants per replicate were visually
169 evaluated, and dissected to assign rating scale 0 to 4 (Garibaldi *et al.*, 2004b), for a total of 64 plants for
170 each experimental thesis. Disease severity data were calculated as follows:

$$171 DS_{0-100} = \frac{\left(\frac{\sum N_{plants} \cdot Rating\ scale_{0-4} \cdot 100}{Total\ N\ recorded\ plants} \right)}{4}.$$

172 The same 64 plants were weighed at the end of the trials to measure their fresh weight. The data were
173 then analysed using SPSS 26 software by performing one-way analysis of variance (ANOVA) and
174 *Tukey's post hoc* tests to determine the statistical values of differences at the value of $\alpha = 0.05$.

175

176 2.4. Soil collection and DNA extraction

177 Rhizosphere samples were collected at the end of the experiment as follows: roots were shaken to avoid
178 any excess soil, and the remaining particles adhering root surface were collected in sterile vials for an
179 amount of 100 g from each plant. Three biological samples were collected from each treatment in both
180 fields and years. Each sample was made unifying the rhizosphere soil of five plants randomly chosen
181 inside each replicate. In total 48 samples were collected.

182 Microbial DNA extraction was performed using the “EZNA soil DNA kit” (Omega Bio-Tek, Norcross,
183 GA), following manufacturer’s instructions. DNA concentration was assessed by using a NanoDrop 2000
184 spectrophotometer (Thermo Fisher Scientific, Waltham MA). Amplicon based sequencing was
185 performed using V3 – V4 region (16S rRNA) for bacterial community (primers: 341F -
186 CCTAYGGGRBGCASCAG, 806R – GGACTACNNGGGTATCTAAT) and ITS2 region for fungal
187 community (primers: ITS3 - GCATCGATGAAGAACGCAGC, ITS4-
188 TCCTCCGCTTATTGATATGC). The analysis was done by Novogene using Illumina NovaSeq 6000
189 platform (Cambridge Science Park, Cambridge, CB4 0FW, United Kingdom).

190

191 2.5. Sequence data analysis

192 Demultiplexed fastq files were processed using the DADA2 pipeline (version 1.16.0) (Callahan *et al.*,
193 2016) in R software (4.0.4) (Team 2016). The resulting taxonomic units are referred to as amplicon
194 sequence variants (ASVs) and the identified Operational Taxonomic Units (OTUs) were 50. For bacterial
195 sequences, forward and reverse reads were trimmed to 250 bp and primer sequences were removed using
196 the following filter parameters: maxN = 0, maxEE for both reads = 2, truncQ = 2 (MaxEE corresponded
197 to the maximum expected errors, TruncQ represented the parameter that truncates reads on the first
198 occurrence of a quality score less than or equal to two, and MaxN was the maximum number of 'N' bases
199 accepted). Nearly 4 million reads were used to estimate the error rates by learnErrors function. Sequences
200 were dereplicated using derepFastq with default parameters and exact sequence variants were resolved
201 using the dada algorithm. The RemoveBimeraDenovo function was then used to remove chimeric
202 sequences. Fungal sequences were preliminary trimmed using Cutadapt software (Martin 2011) to
203 remove adapter sequences and low-quality ends (<Q20). For both the bacterial and fungal datasets, reads
204 with more than three errors in the forward reads and five errors in the reverse reads were removed.
205 Taxonomy was then assigned using assignTaxonomy based on the SILVA (v132) and UNITE (v7)

206 databases for bacterial and fungal communities, respectively (Quast *et al.*, 2013; Nilsson *et al.*, 2019).
207 Raw sequences are available at the National Centre for Biotechnology Information (NCBI), under
208 accession number PRJNA781120 with the title “BCAs application for soil microbiome”.

209

210 2.6. Statistical analysis and data visualization

211 Plotting was performed using PRIMER 7 software (Primer-E Ltd, Plymouth; UK). Alpha diversity
212 metrics were calculated. Heatmaps were created to assess variation in community composition at lowest
213 taxonomic levels including the most frequent 50 ASVs of both bacterial and fungal communities for all
214 the samples. In heatmaps, to assess the behaviour of group of variables according to IPM strategies,
215 clustering was made according index of association. Differently, samples were ordered according to
216 non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity matrices. The
217 significance of changes in composition of the two communities analysed were tested by PERMANOVA
218 (999 permutations, Table S2), using the treatments, field, and year as fixed factors. The significance of
219 variation in the alpha diversity metrics of the two communities was assessed using the ANOVA test, and
220 specific changes in IPM strategies assessed by *post-hoc* Tukey test. The level of significant differences
221 was assessed at $\alpha < 0.05$. All statistical analyses were performed using R software. Furthermore,
222 functional group variation for the fungal community were analysed, identifying putative fungal
223 functional groups as well as their trophic modes using FUNGuild (Nguyen *et al.*, 2016). Co-occurrence
224 networks incorporating communities containing bacteria and fungi were based on single ASV and
225 generated using only the 50 most frequent ASVs for each bacteria and fungi. The pairwise correlations
226 between the ASVs were calculated using the Spearman correlation in R (version 3.3.2 and Hmisc package
227 4.0–1). Based on the statistical analysis, only strong and significant correlations were considered
228 (Spearman’s $r > 0.6$ or $r < -0.6$ and $p < 0.05$). The network visualization was made using Cytoscape
229 (version 3.8.2). Each edge represents correlation, and each node represents an ASV. A set of integrative

230 metrics were calculated and compared to describe the network topology. For example, the average
231 number of neighbours explains the complex pairwise connections and the average path length describes
232 node distribution. Pearson's correlation was used to identify the correlations between disease severity
233 and yield production and the first 50 most abundant ASVs for bacteria and fungi.

234 **3. Results**

235

236 *3.1. Disease assessment and yield*

237 In both years, the disease severity (DS) recorded in the control plots always showed a statistically higher
238 infection rate compared to the treated ones in Carmagnola (DS 34.4-43.3) and Moretta (DS 43.3-40.0),
239 with the only exception for the Comp_Tricho and Tricho strategies in the trial conducted in 2020 at the
240 Carmagnola experimental farm (Fig. 1a). At the Carmagnola site, Comp_Tricho, Bac_Tricho, Tricho and
241 the control provided DS of 17, 12.5, 14.3 and 34.4 in 2019 and 21.7, 10, 20.8 and 43.3 in 2020,
242 respectively; while at the Moretta site DS of 11, 13, 13.1 and 43.3 in 2019 and 24.4, 14.4, 18.1 and 40 in
243 2020 were recorded. Except for the case in Carmagnola in 2020, which had a problem with water runoff
244 due to the intense summer rains, all treatments showed a fresh weight between 3.7 and 5.3 kg/m², while
245 the controls ranged between 1.4 and 4.4 kg/m² (Fig. 1b). In Moretta, all treated plots had statistically
246 higher yield compared to the untreated control in 2020. The same trend was observed in Carmagnola in
247 2020 with the treatments Comp_Tricho and Bac_Tricho IPM compared to the control. The efficacies of
248 both treatments and yield production are shown in Figure 1.

249

250 *3.2. Microbial diversity*

251 Illumina amplicon-based sequencing (on 16S rRNA and ITS fragments) revealed that the composition
252 of the microbiota in the rhizosphere, expressed as diversity indices (number of species, number of reads,
253 and Shannon index), showed no statistical differences between samples for both bacterial and fungal
254 communities (Fig. 2-3).

255

256 3.3. *Rhizosphere associated bacteria*

257 The bacterial community showed a dominance of five phyla: Proteobacteria, Chloroflexi, Firmicutes,
258 Acidobacteria and Actinobacteria (Fig. 4a; Table S3) in all samples. There were observable differences
259 only between different trials, indicating some kind of site- and year-specific pattern. No similarities were
260 observed between the same treatments in different years or fields, nor were there any significant
261 differences between treated plants and controls. Moretta 2020 had the most similar pattern for the
262 bacterial community. In Moretta 2019 and in Carmagnola 2019, Comp_Tricho and control plants had a
263 similar rhizosphere bacterial community. Overall, no major differences were observed between controls
264 and treatments. Plotting the 50 most abundant bacterial community ASVs at genus level (Fig. S3) showed
265 that the greatest differences in community composition existed between the two fields. Specifically, most
266 bacterial ASVs were found in similar abundance in both Carmagnola and Moretta, while some ASVs
267 showed some specificity for one or the other field, such as *Rodopseudomonas*, *Enterobacteriaceae*,
268 *Paenisporosarcina*, which were found more frequently in the Carmagnola field, and *Blautia*,
269 *Faecalibacterium*, *Escherichia* and *Ralstonia*, which were more frequent in Moretta. Bacteria of the
270 genus *Bacillus*, which were slightly more abundant in Carmagnola, were the most abundant ASV, which
271 underlines the analysis. This genus was not more abundant in the plants treated with Bac_Tricho.

272

273 3.4. *Rhizosphere associated fungi*

274 A similar situation was observed for the fungal community (Fig. 4b; Table S3), where Ascomycota
275 occupied 75 to 90% of the observed phyla, followed by Basidiomycota, Mucoromycota, and
276 Aphelidiomycota, with variable proportions. There was also no clear separation between the rhizosphere
277 of the treated plants and that of the control among the fungi. More in detail, Carmagnola 2019 had a clear
278 pattern of similarity between samples, in Carmagnola 2020 there was a higher proportion of
279 Mucoromycota and Aphelidiomycota in Bac_Tricho, in Tricho and in the control samples. In Moretta

280 2019, there was the highest proportion of Basidiomycota (about 20%) in the Bac_Tricho treatment, while
281 Moretta 2020 had the highest proportion of unassigned sequences in the Tricho treated plants. The
282 heatmap of the 50 most abundant ASVs at genus level in the fungal community (Fig. S4) showed that
283 few of them dominated over the others. Ascomycota, for example, was the most abundant, with a greater
284 presence in the Carmagnola field compared to the Moretta field. In terms of fungi, there was also a
285 common core of ASVs between the two sites, but also some differences: *Fusarium* and *Chaetomium*
286 were more abundant in Moretta, while *Rhizopus* was slightly more abundant in Carmagnola.
287 *Trichoderma* did not show a greater predominance in the rhizosphere of the treated plants compared to
288 the control plants.

289

290 3.5. Bacterial and fungal distribution and co-occurrence

291 Considered at the whole community level with nMDS analysis, both bacteria and fungi showed no spatial
292 segregation as a function of treatments. The ordination of the bacterial community (Fig. 5a) clearly
293 separated by field and year, while that of the fungi (Fig. 5b) followed the site. Pearson correlations (Fig.
294 S2) between bacterial or fungal communities and disease severity or yield production for the Carmagnola
295 and Moretta fields generally showed no strong correlations for any individual ASV, except for
296 Carmagnola and yield in the bacterial community. The correlation basis network (Fig. 6) showed that the
297 Carmagnola and Moretta fields had different interactive structure when the community of bacteria and
298 fungi were analysed together. In the Moretta field, the correlations can be explained as more open, fungi
299 and bacteria seemed to co-occur and co-exclude each other, while in Carmagnola the trend was more
300 closed, as fungi seemed to interact only with each other and bacteria did the same. Figures (6a and 6b)
301 highlighted some genera that were considered more important for the experiments. *Fusarium* and
302 *Trichoderma* did not correlate in a strong way either in Carmagnola or in Moretta. *Bacillus*, which

303 dominated the population of both fields, showed few correlations in the Moretta field and had none in
304 the Carmagnola field.

305 *3.6. Fungal functional guilds*

306 The fungal community was analysed to identify the different guilds (Fig. 7). Twenty-one main guilds
307 were found, with a predominance of plant pathogens, animal pathogens, fungal parasites, endophytes and
308 wood saprophytes. In terms of phyla composition, there was no clear pattern identifying the treatments
309 in the different trials, nor was there a consistent difference between treated and control plants. The tricho
310 treatment of Carmagnola 2019 and the Bac_Tricho treatment of Moretta 2019 showed a greater number
311 of guilds compared to all other treatments. Seven trophic modes were found (Fig. S1) with dominance
312 for pathotroph-saprotroph-symbiotroph in Moretta (both 2019 and 2020) and increased abundance of
313 pathotroph-saprotroph and saprotroph-symbiotroph for Carmagnola field in both years.

314

315 **4. Discussion**

316 *4.1. Fusarium wilt control by IPM strategies*

317 The introduction of environmentally friendly strategies to control lettuce Fusarium wilt is extremely
318 important, as is a better understanding of how they behave under real farm conditions. In this work, the
319 effectiveness of three IPM strategies against Fol was tested under commercial and experimental
320 conditions in tunnels. The three IPM strategies tested showed a great ability to statistically reduce disease
321 severity in both fields and years, from 50% to 70% compared to untreated controls. This is in agreement
322 with preliminary experiments conducted in both greenhouse and field conditions (Cucu *et al.*, 2019;
323 Gilardi *et al.*, 2019). Also yield of treated lettuce plants significantly increased in 2020 trials, while in
324 2019 yields were not different from untreated control (Figure 1). This is in accordance with previous

325 publication (Gilardi *et al.*, 2016), in which it was clearly demonstrated that the fresh weight reduction of
326 the lettuce grown in the inoculated and treated soil was not significantly influenced by disease severity.

327

328 4.2. Impact on rhizosphere microbiota

329 At the phylum level, the microbiota data showed that there was no detectable treatment effect at the end
330 of the experiment. In fact, there was no clear differentiation between treated plants and the corresponding
331 controls, in contrast to a previous work where the same compost applied against *Phytophthora capsici*
332 protected *Cucurbita pepo* by altering its rhizosphere composition (Bellini *et al.*, 2020). In this case, the
333 experiment was conducted under greenhouse/pot conditions, using steam-sterilized peat as substrate,
334 which was a system less complex than soil in terms of microbial diversity. Some work reported a change
335 in rhizosphere microbiota when different BCAs were used, but only in pot systems (Liu *et al.*, 2021) or
336 when plant growth promoting bacteria were applied along with nanocomposites (Kumari *et al.*, 2020;
337 Chaudary *et al.*, 2022). While, in agreement with our results, Cucu *et al.*, (2020a) reported that field
338 application of BCAs did not alter the rhizosphere microbiome of *Cucurbita pepo* grown in agricultural
339 soil.

340

341 4.3. Impact on bacterial microbial diversity

342 Most ASVs were similar, but some differences were observed between the two fields. Proteobacteria,
343 Chloroflexi, Firmicutes, Acidobacteria and Actinobacteria were predominant phyla, and *Bacillus* was the
344 most abundant ASV for bacteria, which is consistent with the literature (Amin *et al.*, 2015). All
345 predominant phyla play an important role in ecological and metabolic functioning of the soil.
346 Proteobacteria are mainly involved in decomposition, nitrogen fixation, and humus formation
347 (Chaudhary *et al.*, 2021b). Chloroflexi and Acidobacteria are considered green chlorophototrophic
348 bacteria that can use chlorosomes for light-harvesting (Thweatt *et al.*, 2019). Abundance of Chloroflexi

349 was reported in wheat planted soil (Liu *et al.*, 2017), and increased with the application of nanozeolite
350 under wheat cultivation (Khaliq *et al.*, 2019) and of nanocompounds under maize cultivation (Chaudhary
351 *et al.*, 2021b). Firmicutes and *Bacillus* are considered important in plant growth promotion, facilitating
352 plant nutrient acquisition, in biocontrol of plant pathogens, as well as in the phytoremediation of heavy
353 metals (Amaresan *et al.*, 2020). Actinobacteria populations of soil participate in the decomposition of
354 organic matter, promote plant growth and are producers of secondary metabolites, which can be exploited
355 for disease suppression (Amaresan *et al.*, 2020). The Moretta field had a greater abundance of
356 *Gemmatimonadaceae*, *Blautia* and *Bifidobacterium* than the Carmagnola field. These bacterial ASVs
357 have been reported to be involved in chitin degradation and could be affected by fertilization and the use
358 of sludge amendments in the soil (Vo *et al.*, 2017; Hui *et al.*, 2020), which is consistent with the fact that
359 Moretta is a commercial farm subject to more intensive management and therefore conventional
360 fertilization. Moreover, the abundances of *Blautia* and *Bifidobacterium* could also be explained by the
361 difference in pH between the two fields, as the two bacterial genera have a neutral pH optimum (Cui *et*
362 *al.*, 2021; Liu *et al.*, 2021). Some of the ASVs found in greater presence in Carmagnola soil (such as
363 Enterobacteriaceae, *Pantoea* and *Lactococcus*) have been associated with the ability to degrade simple
364 sugar molecules (Degelmann *et al.*, 2009; Teuber *et al.*, 2006) and this may indicate a richer presence of
365 these molecules in this field compared to Moretta.

366

367 4.4. Impact on fungal microbial diversity

368 The 50 most abundant ASVs of fungi showed that the greatest dominance was in Ascomycota, which
369 was expected since this phylum is predominant in agricultural soils (Ma *et al.*, 2013; Egidi *et al.*, 2019).
370 *Fusarium* was very abundant in the Moretta field, as expected based on the history of Fol infection at
371 this site. *Chaetomium* was also more abundant in Moretta, again a genus containing species that degrade
372 cellulose-rich substrates, such as components in soil, straw, or wood (Aru *et al.*, 1997). Neither

373 *Trichoderma* nor *Bacillus* was found in greater abundance in the rhizosphere of plants treated with these
374 BCAs, even if BCAs and compost were applied together. Accordingly, very low Pearson correlation
375 coefficients were found between the 50 most abundant ASVs with yield production and disease severity.
376 Two-dimensional nMDS analyses showed that there was no treatment effect on the spatial distribution
377 of bacterial and fungal communities. Bacteria segregated by site and year, while for the fungal
378 community segregation was only observed for site. Network analyses of the two fields confirmed that
379 genera such as *Fusarium*, *Trichoderma*, and *Bacillus* were not involved in large co-occurrences or co-
380 exclusions; however, some differences were highlighted between the two fields, again suggesting that
381 the microbiota compositions of the rhizosphere samples at genus/class levels were mainly influenced by
382 soil type than by experimental treatments. Both in the present work and in Cucu *et al.* (2020) a standard
383 microbiome analysis was done at the genus / class level. Therefore, there might be shifts at the species
384 or strain level and natural strains may have replaced the applied strains. This aspect should be further
385 investigated in future research.

386 Nevertheless, a clear protective effect of the treatments against *Fusarium* wilt in lettuce was observed.
387 Apart from changing the rhizosphere microbial community, several mechanisms have been proposed to
388 explain the observed disease suppression, including the release of fungitoxic compounds (Blok *et al.*,
389 2000; Larkin & Griffin, 2006). Moreover, the induction of resistance may also have played a central role
390 in these experiments by activating the molecular pathways that can protect the plant from pathogen
391 attacks. Many studies have reported the ability of *Trichoderma* spp. to activate the immune system of the
392 plants they encounter (Fontenelle *et al.*, 2011; Ramírez-Cariño *et al.*, 2020; Sawant *et al.*, 2020). Indeed,
393 in a previous work with the *Capsicum annuum* - *Phytophthora capsici* pathosystem (Bellini *et al.*, 2021),
394 it was shown that the same *Trichoderma* TW2-enriched compost used in the first IPM strategy and
395 potassium phosphite (used in the second IPM strategy) activate systemic acquired resistance.

396

397 **5. Conclusion**

398 This study showed that the three IPM strategies tested here were able to reduce disease severity caused
399 by Fol in two different soils under commercial tunnel conditions in two consecutive years. The
400 rhizospheric microbiota at genus/class levels was not driven by the treatments done in nursery, but it was
401 shaped by the autochthonous soil microbial populations. Induction of resistance may have also been
402 involved in the protective effect of the treatments. Further studies should be considered to evaluate
403 whether lettuce resistance pathways can be activated by these IPM strategies, the effects on rhizospheric
404 microbiota at species/strain levels, and possible correlation of soil type and soil pH on microbial
405 community and disease suppression by BCAs.

406

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415

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- 619

620 **Table 1.** Coding names, technical formulation, dosage and timing of administration of the three IPM
 621 strategies used in this work for in fields experiments. T0 refers to the day of sowing, the other timing
 622 here presented are referred in days. Dosage column refers to the amount of commercial product used to
 623 treat seedling or soil. Comp_Tricho was given as compost as such, while Bac_Tricho and Tricho were
 624 given in a volume of 1 l per treatment.

Treatment	Formulation	Commercial name	Dosage	Number of applications	Timing	Application
Comp_Tricho	Ant Compost + <i>Trichoderma virens</i> TW2	ANT's CM	400 g/100 seedlings	2	T0	sowing
			1 kg/m ² for Moretta; 0.5 kg/m ² for Carmagnola		Immediately before transplant	field
Bac_Tricho	<i>Bacillus amyloliquefaciens</i> (former subtilis) QST 713	Serenade MAX	8 ml/l	2	T0	sowing
					T10	nursery
	<i>Trichoderma gamsii</i> icc 080 + <i>T. asperellum</i> <i>asperellum</i> icc 012	Remedier	2.5 g/l	2	T5	sowing
					T15	nursery
Potassium phosphite	Alexin	2.5 g/l	2	T0	sowing	
				T15	nursery	
Tricho	<i>Trichoderma polysporum</i> IMI 206039 + <i>Trichoderma atroviride</i> IMI 206040	Binab solution	1.7 g/l	3	T0	nursery
					T7	nursery
					T15	nursery

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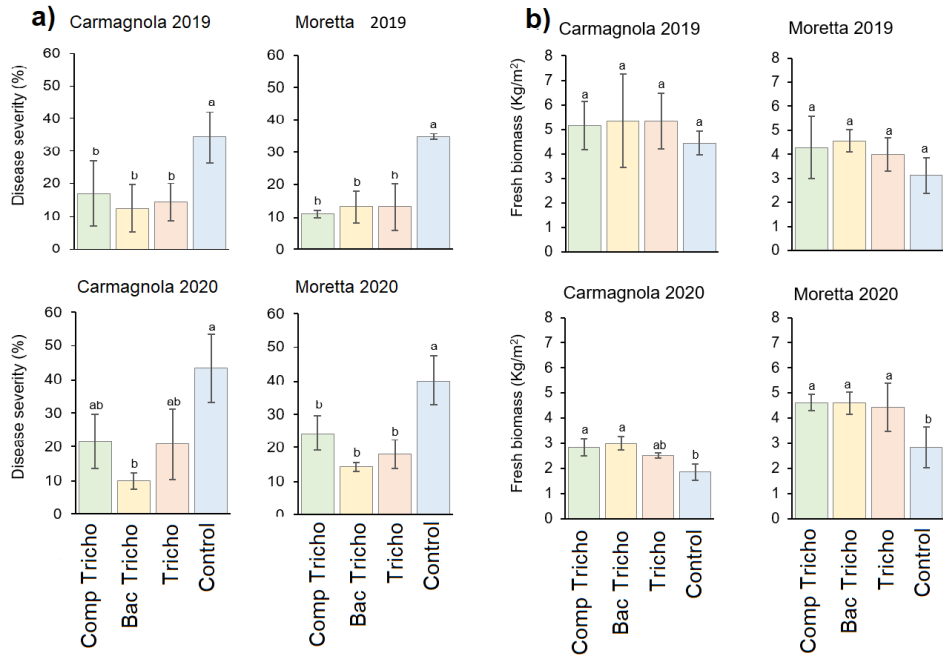
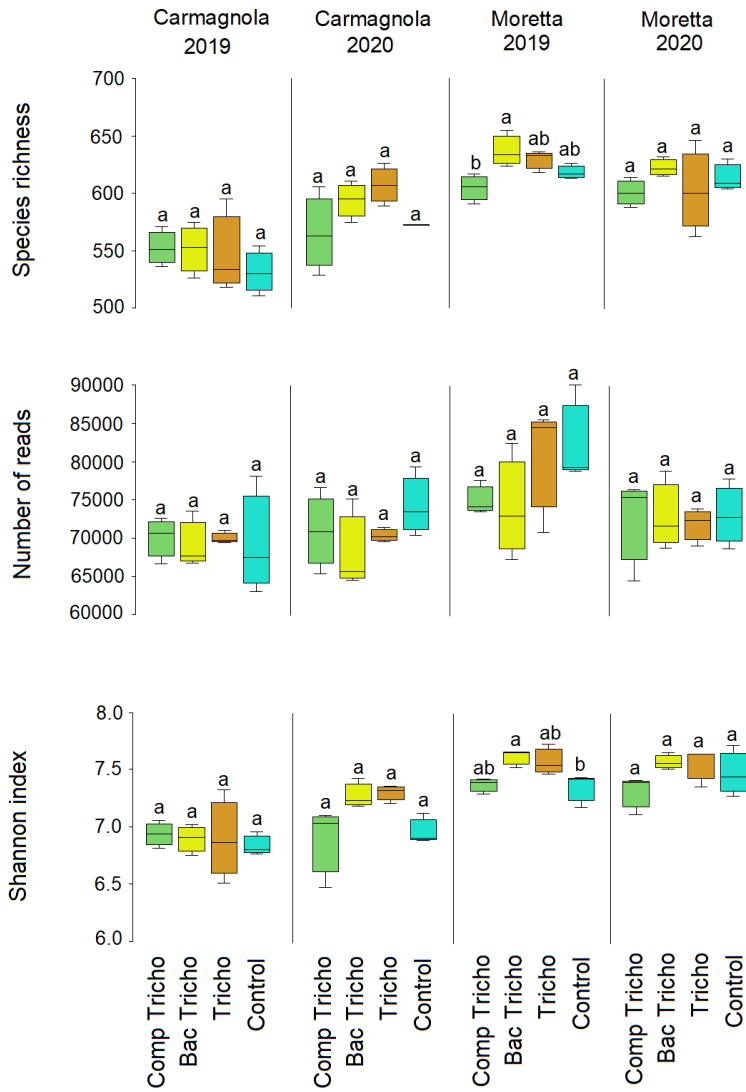
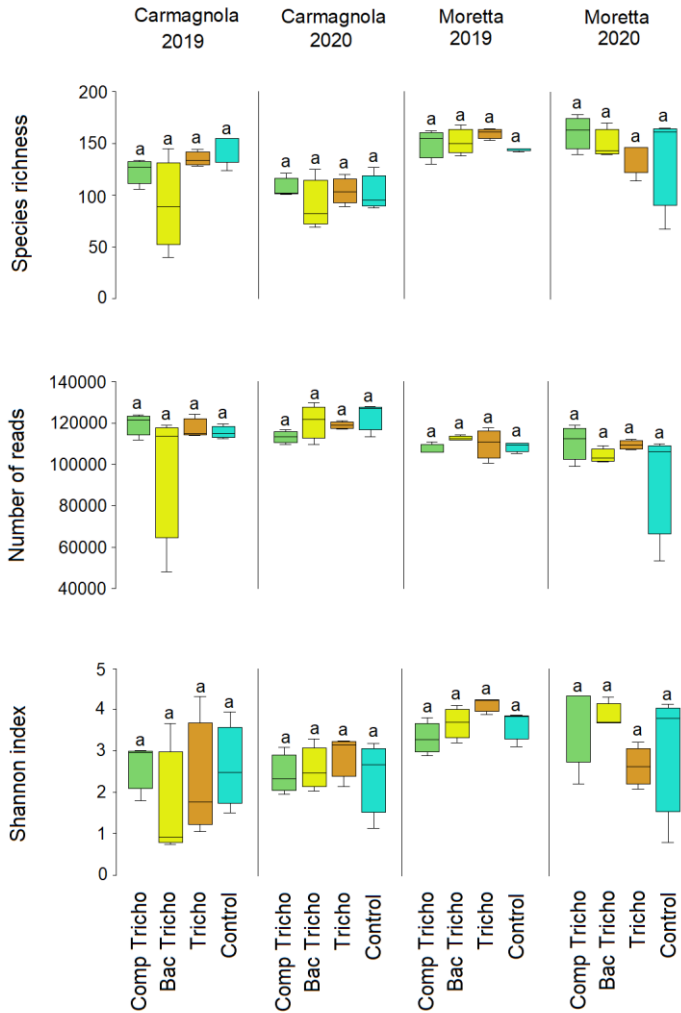


Fig 1. (a) Efficacy of the three IPM strategies to reduce *Fusarium oxysporum* f. sp. *lactucae* disease on lettuce plants expressed as disease severity (%) and (b) productivity expressed as fresh biomass (Kg/m²). Evaluation made at the final survey. Different letters indicate statistical differences between the four thesis, as obtained with the ANOVA test and Tukey's post hoc test (p<0.05).



654
 655 **Fig 2.** Box and whisker plots showing distribution of diversity indices, number of species, number of
 656 reads and Shannon Index, for bacteria community, for each treatment divided per site and year of
 657 rhizosphere sampling.

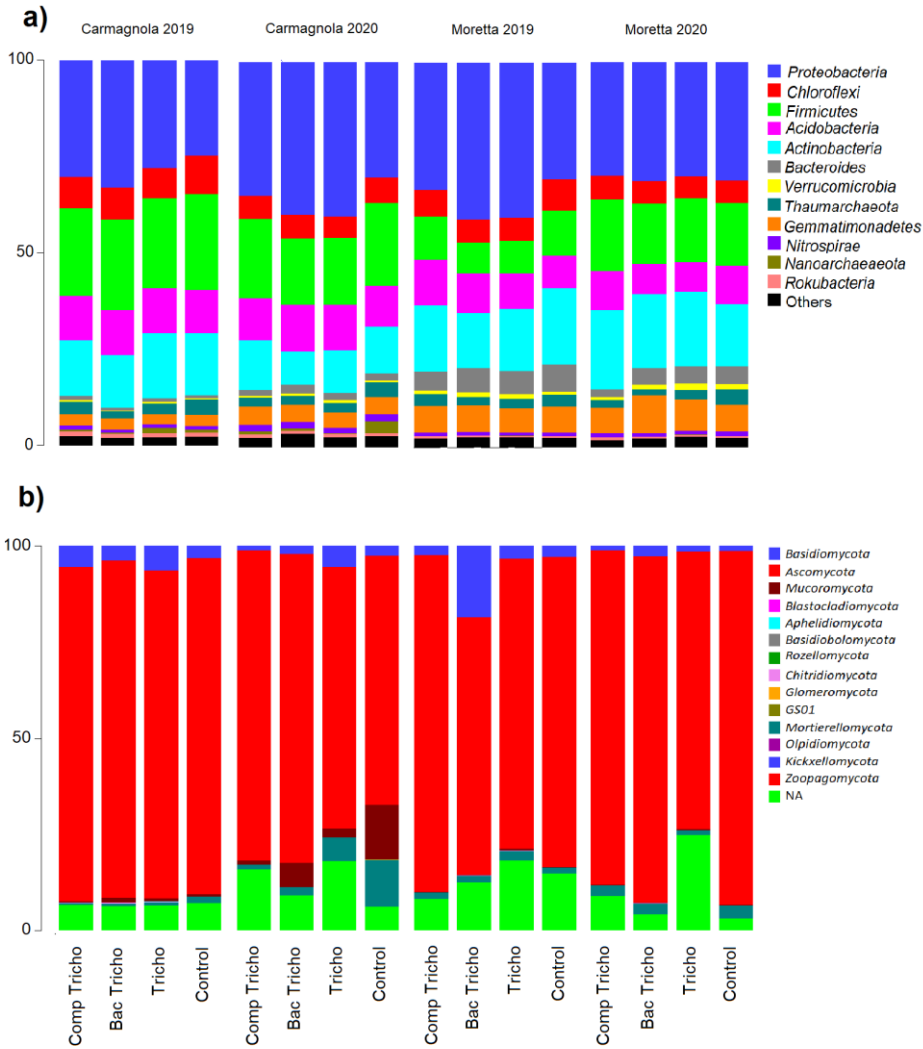
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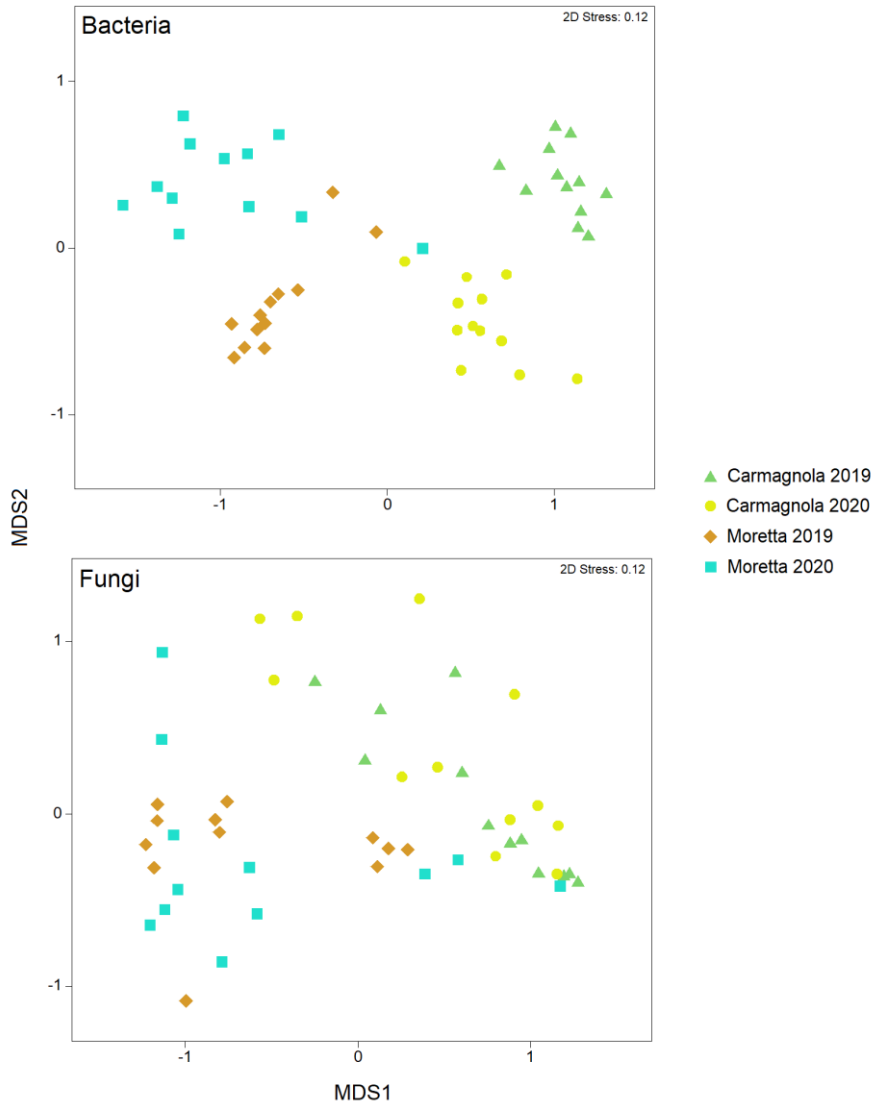
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660 **Fig 3.** Box and whisker plots showing distribution of diversity indices, number of species, number of
 661 reads and Shannon Index, for fungal community, for each treatment divided per site and year of
 662 rhizosphere sampling.

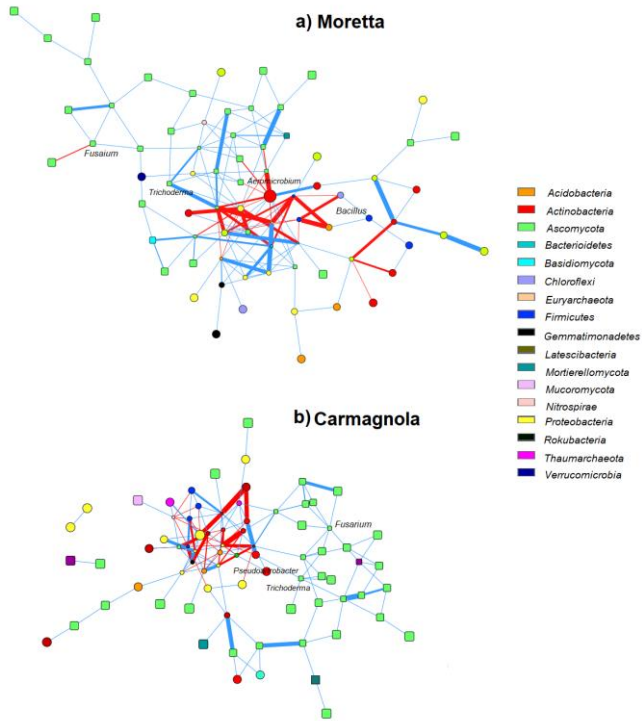
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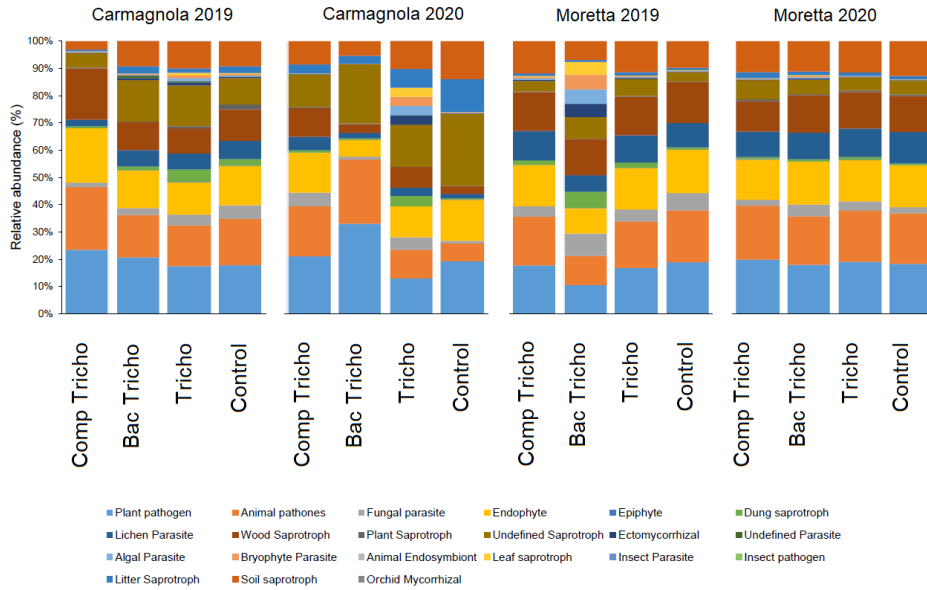
664
 665 **Fig 4.** Stacked bar chart of relative abundance for (a) bacterial and (b) fungal communities at phylum
 666 level. Data are averaged among three biological replicates for each treatment and are here presented
 667 grouped for site and year.
 668



669
670 **Fig 5.** Bi-dimensional Non-metric MDS of the bacterial and fungal communities in the rhizosphere
671 collected at the end of the trials. Data are averaged for year and site of sampling, following the colour
672 coding of the legend.



673
 674 **Fig 6.** Correlation base network analysis showing potential interactions between bacterial and fungal
 675 families in (a) Moretta and (b) Carmagnola fields. The lines connecting nodes (edges) represent positive
 676 (blue) or negative (red) co-occurrence relationship. The intensity of the colour and the length of the edges
 677 represent the strength of correlation. Square nodes correspond to fungal ASVs and circle nodes
 678 correspond to bacterial ASVs. The connection stands for a strong (Spearman's $\rho > 0.6$ and $\rho < -0.6$) and
 679 significant ($P\text{-value} < 0.05$) correlation. The size of each node is proportional to the ASV relative
 680 abundance, only the top 50 ASVs were kept. The nodes were coloured by phylum level. Data of the years
 681 and the treatments were averaged.



682
 683 **Fig 7.** Stacked bar chart of relative abundance of fungal guilds based on FunGuilds. Data are averaged
 684 among three biological replicates for each treatment and are here presented grouped for site and year.

685 **Appendix A. Supplementary materials**

686

687 **Table S1.** Schedule of the trials conducted in 2019 and in 2020 in both farm and average temperatures
688 registered.

year	Farm	Sowing	Transplantation	Trial end	Field trial duration (days)
2019	Carmagnola	06/5/2019	30/05/2019	10/07/2019	41
2020		26/06/2020	21/07/2020	24/08/2020	34
2019	Moretta	06/5/2019	29/05/2019	01/07/2019	33
2020		07/05/2020	01/06/2020	06/07/2020	36

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691 **Table S2.** Result of Permanova significance test across treatments during the two years of the
692 experiment, in two different fields. Treatments were used as the fixed factor (N° of permutation 999).
693 Here reported main significant variation between the studied treatments for fungal and bacterial
694 communities. Test of significance is based on Bray-Curtis similarity values. Significance level is fixed
695 for p-value below 0.05.

696

	Fungal community	Bacterial community
Permutation N	999	999
Total sum of squares	8.067	3.435
Within-group sum of squares	4.092	1.104
F	2.073	4.504
p	0.004*	0.001*

697 (*Significance level is at 0.05)

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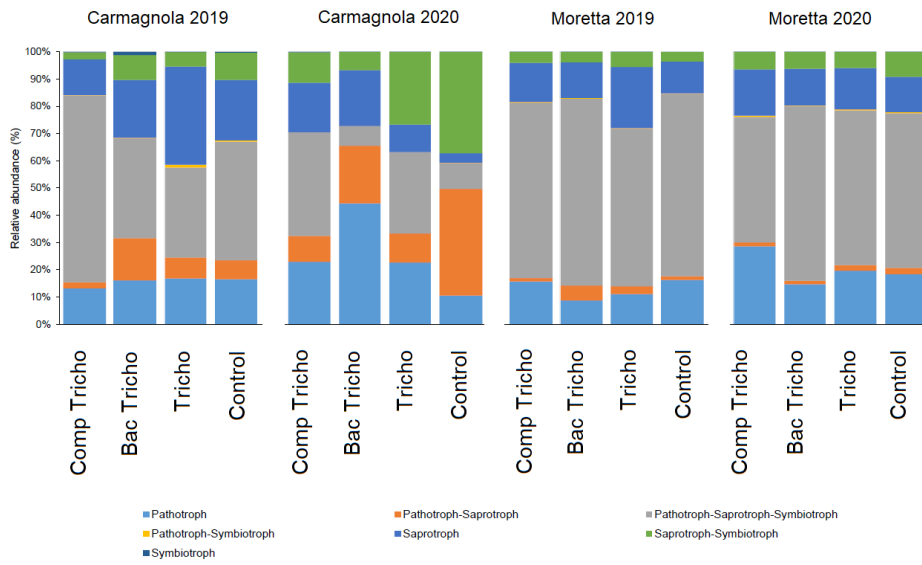
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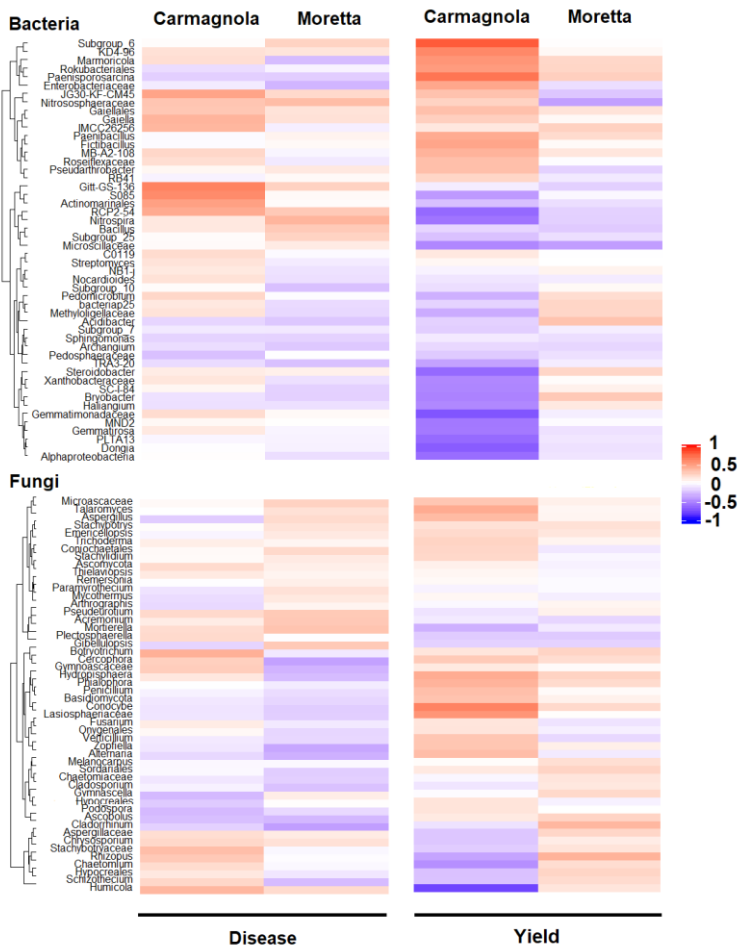
702 **Table S3.** Result of amplicon sequence analysis in the bacterial and fungal community for each sample
703 collected from rhizosphere at the end of the trials.

Commentato [MP1]: Excel file

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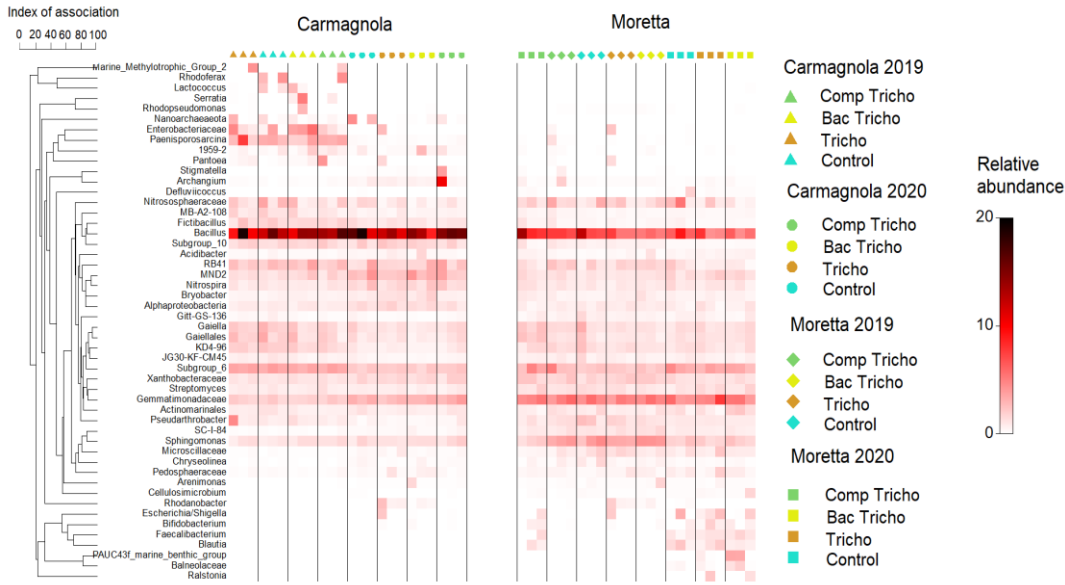
706
707 **Fig S1.** Stacked bar chart of relative abundance of fungal trophic mode. Data are averaged among
708 biological replicates for each treatment and are here presented grouped for site and year.



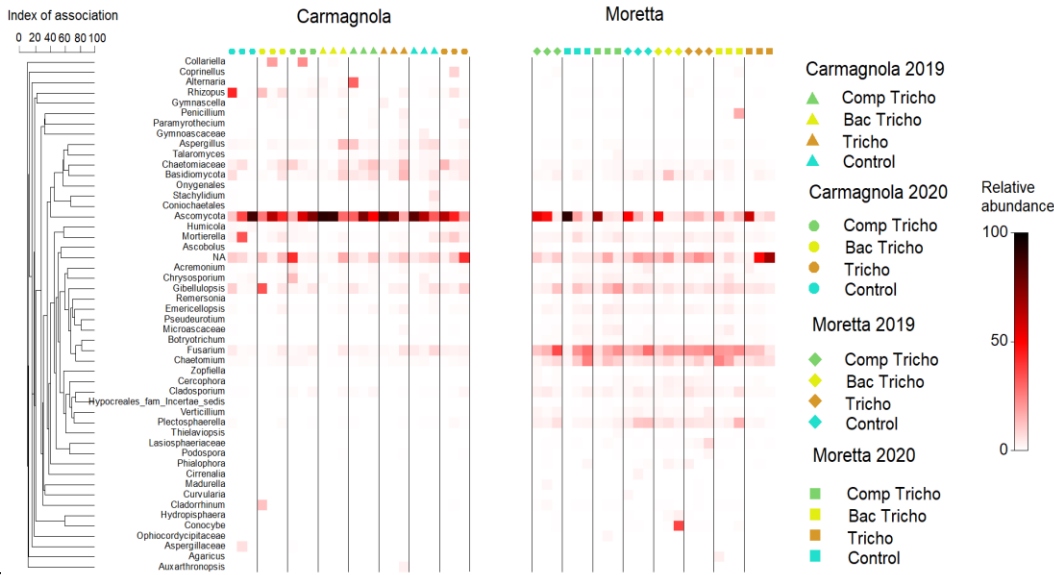
709
 710 **Fig S2.** Heatmap of Pearson's correlation between the 50 most frequent amplicon sequence variant
 711 (ASV) in the bacterial and fungal community and the disease index and growth (fresh biomass). Data are
 712 expressed as average of every sample per each site.

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714



716 **Fig S3.** Heatmap showing relative abundance of the 50 most frequent amplicon sequence variant (ASV)
 717 in the bacterial community for each sample collected from rhizosphere at the end of the trials.
 718 Hierarchical clustering of variables is based on an association index. Samples follow the colour coding
 719 of the legend.



720
721 **Fig S4.** Heatmap showing relative abundance of the 50 most frequent amplicon sequence variant (ASV)
722 in the fungal community for each sample collected from rhizosphere at the end of the trials. Hierarchical
723 clustering of variables is based on an association index. Samples follow the colour coding of the legend.
724