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Title

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

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Abstract

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

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

1. Introduction

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

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

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

Resistance inducers are compounds of various origins that have been shown to activate the plant immune system (Walters *et al.*, 2009; Akram and Anjum 2011; Alexandersson *et al*., 2016). BCAs, and by extension composts, have been shown to activate the induction of resistance in several pathosystems (Compant *et al*., 2005; Ongena *et al*., 2007; Pieterse *et al*., 2014; Akram *et al*., 2015; Bellini *et al*., 2021). Integrated pest management (IPM) strategies are combinations of different measures based on the principle of synergy between them to control pathogen attacks (Barzman *et al*., 2015).

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

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

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

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

2. Material and Methods

2.1. Plants material and experimental design

Field trials were conducted on two farms: a commercial farm in Moretta (CN, Italy) and an experimental farm in Carmagnola (TO, Italy). The experiments were conducted under 360 m^2 and 64 m^2 plastic tunnels, respectively, in Moretta and Carmagnola. The farm in Moretta (sand:silt:clay 56:19:25, pH 7.12 and 1.37% organic matter) had a natural infestation of Fol race 1, which causes significant crop losses in susceptible lettuce cultivars (Gilardi *et al.*, 2019). The soil in Carmagnola (sand:silt:clay 68.16:10.7:21.1, pH 8.2 and 0.94% organic matter) has no history of intensive lettuce cultivation nor evidence of natural occurrence of the pathogen. For these reasons, the soil in Carmagnola was artificially infested with a virulent strain of Fol race 1 coded as MYA-3040 (ATCC), previously isolated from lettuce wilted plants in Italy, from the Agroinnova collection (Garibaldi *et al.*, 2002). Soil infestation was carried out as follows: i) the pathogen was cultured in sterilized wheat kernels left at 23°C for two weeks, then ii) 100

g/m^2 of the colonized wheat kernels were incorporated into the soil at a depth of 10-15 cm using a rake. The experiments were repeated in two consecutive years (2019 and 2020, see Table S1) with the lettuce cultivar 'Voluski', classified as moderately susceptible to Fol (Gilardi *et al.*, 2017). The trial Carmagnola 2020 was done about 1 month later compared to the previous year, due to weather conditions and intense rainfall. Trials lasted 33-41 days according to weather conditions. In both fields (Carmagnola and Moretta), two-week-old lettuce plants were transplanted in a mulched soil at a density of 16 plants/m^2, with a randomized design of treatments and untreated controls, with four replicates to avoid the side effect. Plants were irrigated with a drip irrigation system and grown in accordance with standard cultivation practices in the region.

2.2. Integrated pest managements treatments

The three IPM strategies tested in this experiment were selected from those described in previous works (Gilardi *et al.* 2016; 2019), choosing the best combination for the control of Fol in greenhouse and field conditions. The strategies were designed and applied starting at the nursery level as follows: (i) a compost enriched with *T. virens* TW2 (ANT's compost M; AgriNewTech s.r.l., Italy), (ii) a combination of commercial BCAs *B. amyloliquefaciens* (former *subtilis*) QST713 (accession number CP025079, Serenade Max, 15.6%, Bayer Crop Science, Italy), *T. gamsii* ICC080 (accession number IMI 392151) + *T. asperellum asperellum* ICC012 (accession number IMI 392716) (Remedier, 2+2%, Isagro Ricerca, Milan, Italy), and potassium phosphite (Alexin, 95PS, P_2O_5 52%, K_2O 42%, Massò, Spain), and (iii) a commercial mixture of *T. polysporum* IMI206039 (accession number IMI 206039, ATCC 20475) and *T. atroviride* IMI206040 (accession number IMI 206040, ATCC 20476) (Binab solution 1+1%, BINAB Bio-innovation AB, Helsingborg, Sweden). *T. virens* TW2 (accession number MZ222411) was isolated from compost (Gilardi *et al.*, 2019; Cucu *et al.*, 2019, 2020a and 2020b; Bellini *et al.*, 2021). To simplify, the tested IPM strategies are coded here as: i) Comp_Tricho, ii) Bac_Tricho and iii) Tricho. The products

Bac_Tricho and Tricho were applied as soil drench in the nursery. Comp_Tricho was used at sowing (T0) at a dosage of 400 g/100 seedlings and applied in the field immediately before transplanting at a dosage of 1 kg/m^2 for Moretta. In Carmagnola, Comp_Tricho was applied at a dosage of 0.5 kg/m^2, considering the soil mixture, the possibility of applying the treatment with the rototiller (in Moretta field) or manually (in Carmagnola field), and because of the smaller size of the tunnel, which allows less deep diffusion. The type, dosage and timing of application of the three strategies are shown in Table 1.

2.3. Lettuce yield and disease assessment

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

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

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

2.4. Soil collection and DNA extraction

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

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

2.5. Sequence data analysis

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

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

2.6. Statistical analysis and data visualization

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

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

3. Results

3.1. Disease assessment and yield

In both years, the disease severity (DS) recorded in the control plots always showed a statistically higher infection rate compared to the treated ones in Carmagnola (DS 34.4-43.3) and Moretta (DS 43.3-40.0), with the only exception for the Comp_Tricho and Tricho strategies in the trial conducted in 2020 at the Carmagnola experimental farm (Fig. 1a). At the Carmagnola site, Comp_Tricho, Bac_Tricho, Tricho and 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, 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 2020 were recorded. Except for the case in Carmagnola in 2020, which had a problem with water runoff due to the intense summer rains, all treatments showed a fresh weight between 3.7 and 5.3 kg/m^2, while the controls ranged between 1.4 and 4.4 kg/m^2 (Fig. 1b). In Moretta, all treated plots had statistically higher yield compared to the untreated control in 2020. The same trend was observed in Carmagnola in 2020 with the treatments Comp_Tricho and Bac_Tricho IPM compared to the control. The efficacies of both treatments and yield production are shown in Figure 1.

3.2. Microbial diversity

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

259 *3.3. Rhizosphere associated bacteria*

260 The bacterial community showed a dominance of five phyla: Proteobacteria, Chloroflexi, Firmicutes,
261 Acidobacteria and Actinobacteria (Fig. 4a; Table S3) in all samples. There were observable differences
262 only between different trials, indicating some kind of site- and year-specific pattern. No similarities were
263 observed between the same treatments in different years or fields, nor were there any significant
264 differences between treated plants and controls. Moretta 2020 had the most similar pattern for the
265 bacterial community. In Moretta 2019 and in Carmagnola 2019, Comp_Tricho and control plants had a
266 similar rhizosphere bacterial community. Overall, no major differences were observed between controls
267 and treatments. Plotting the 50 most abundant bacterial community ASVs at genus level (Fig. S3) showed
268 that the greatest differences in community composition existed between the two fields. Specifically, most
269 bacterial ASVs were found in similar abundance in both Carmagnola and Moretta, while some ASVs
270 showed some specificity for one or the other field, such as *Rodopseudomonas*, *Enterobacteriaceae*,
271 *Paenisporosarcina*, which were found more frequently in the Carmagnola field, and *Blautia*,
272 *Faecalibacterium*, *Escherichia* and *Ralstonia*, which were more frequent in Moretta. Bacteria of the
273 genus *Bacillus*, which were slightly more abundant in Carmagnola, were the most abundant ASV, which
274 underlines the analysis. This genus was not more abundant in the plants treated with Bac_Tricho.
275
276 *3.4. Rhizosphere associated fungi*
277 A similar situation was observed for the fungal community (Fig. 4b; Table S3), where Ascomycota
278 occupied 75 to 90% of the observed phyla, followed by Basidiomycota, Mucoromycota, and
279 Aphelidiomycota, with variable proportions. There was also no clear separation between the rhizosphere
280 of the treated plants and that of the control among the fungi. More in detail, Carmagnola 2019 had a clear
281 pattern of similarity between samples, in Carmagnola 2020 there was a higher proportion of
282 Mucoromycota and Aphelidiomycota in Bac_Tricho, in Tricho and in the control samples. In Moretta

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

3.5. Bacterial and fungal distribution and co-occurrence

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

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

308 *3.6. Fungal functional guilds*

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

317

318 **4. Discussion**

319 *4.1. Fusarium wilt control by IPM strategies*

320 The introduction of environmentally friendly strategies to control lettuce Fusarium wilt is extremely
321 important, as is a better understanding of how they behave under real farm conditions. In this work, the
322 effectiveness of three IPM strategies against Fol was tested under commercial and experimental
323 conditions in tunnels. The three IPM strategies tested showed a great ability to statistically reduce disease
324 severity in both fields and years, from 50% to 70% compared to untreated controls. This is in agreement
325 with preliminary experiments conducted in both greenhouse and field conditions (Cucu *et al*., 2019;
326 Gilardi *et al.*, 2019). Also yield of treated lettuce plants significantly increased in 2020 trials, while in
327 2019 yields were not different from untreated control (Figure 1). This is in accordance with previous

328 publication (Gilardi *et al.,* 2016), in which it was clearly demonstrated that the fresh weight reduction of
329 the lettuce grown in the inoculated and treated soil was not significantly influenced by disease severity.
330
331 *4.2. Impact on rhizosphere microbiota*
332 At the phylum level, the microbiota data showed that there was no detectable treatment effect at the end
333 of the experiment. In fact, there was no clear differentiation between treated plants and the corresponding
334 controls, in contrast to a previous work where the same compost applied against *Phytophthora capsici*
335 protected *Cucurbita pepo* by altering its rhizosphere composition (Bellini *et al*., 2020). In this case, the
336 experiment was conducted under greenhouse/pot conditions, using steam-sterilized peat as substrate,
337 which was a system less complex than soil in terms of microbial diversity. Some work reported a change
338 in rhizosphere microbiota when different BCAs were used, but only in pot systems (Liu *et al*., 2021) or
339 when plant growth promoting bacteria were applied along with nanocomposites (Kumari et al., 2020;
340 Chaudary et al., 2022). While, in agreement with our results, Cucu *et al.,* (2020a) reported that field
341 application of BCAs did not alter the rhizosphere microbiome of *Cucurbita pepo* grown in agricultural
342 soil.
343
344 *4.3. Impact on bacterial microbial diversity*
345 Most ASVs were similar, but some differences were observed between the two fields. Proteobacteria,
346 Chloroflexi, Firmicutes, Acidobacteria and Actinobacteria were predominant phyla, and *Bacillus* was the
347 most abundant ASV for bacteria, which is consistent with the literature (Amin *et al.*, 2015). All
348 predominat phyla play an important role in ecological and metabolic functioning of the soil.
349 Proteobacteria are mainly involved in decomposition, nitrogen fixation, and humus formation
350 (Chaudhary *et al.,* 2021b). Chloroflexi and Acidobacteria are considered green chlorophototrophic
351 bacteria that can use chlorosomes for light-harvesting (Thweatt *et al*., 2019). Abundance of Chloroflexi

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

4.4. Impact on fungal microbial diversity

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

376 *Trichoderma* nor *Bacillus* was found in greater abundance in the rhizosphere of plants treated with these
377 BCAs, even if BCAs and compost were applied together. Accordingly, very low Pearson correlation
378 coefficients were found between the 50 most abundant ASVs with yield production and disease severity.
379 Two-dimensional nMDS analyses showed that there was no treatment effect on the spatial distribution
380 of bacterial and fungal communities. Bacteria segregated by site and year, while for the fungal
381 community segregation was only observed for site. Network analyses of the two fields confirmed that
382 genera such as *Fusarium*, *Trichoderma*, and *Bacillus* were not involved in large co-occurrences or co-
383 exclusions; however, some differences were highlighted between the two fields, again suggesting that
384 the microbiota compositions of the rhizosphere samples at genus/class levels were mainly influenced by
385 soil type than by experimental treatments. Both in the present work and in Cucu *et al.* (2020) a standard
386 microbiome analysis was done at the genus / class level. Therefore, there might be shifts at the species
387 or strain level and natural strains may have replaced the applied strains. This aspect should be further
388 investigated in future research.
389 Nevertheless, a clear protective effect of the treatments against Fusarium wilt in lettuce was observed.
390 Apart from changing the rhizosphere microbial community, several mechanisms have been proposed to
391 explain the observed disease suppression, including the release of fungitoxic compounds (Blok et al.,
392 2000; Larkin & Griffin, 2006). Moreover, the induction of resistance may also have played a central role
393 in these experiments by activating the molecular pathways that can protect the plant from pathogen
394 attacks. Many studies have reported the ability of *Trichoderma* spp. to activate the immune system of the
395 plants they encounter (Fontenelle *et al*., 2011; Ramírez-Cariño *et al.*, 2020; Sawant *et al*., 2020). Indeed,
396 in a previous work with the *Capsicum annuum* - *Phytophthora capsici* pathosystem (Bellini *et al*., 2021),
397 it was shown that the same *Trichoderma* TW2-enriched compost used in the first IPM strategy and
398 potassium phosphite (used in the second IPM strategy) activate systemic acquired resistance.
399

5. Conclusion

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

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

Treatment	Formulation	Commercial name	Dosage	Number of applications	Timing	Application
Comp_Tricho	Ant Compost + *Trichoderma virens* 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	*Bacillus amyloliquefaciens* (former subtilis) QST 713	Serenade MAX	8 ml/l	2	T0	sowing
					T10	nursery
	Trichoderma gamsii icc 080 + *T. asperellum asperellum* 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	*Trichoderma polysporum* IMI 206039 + *Trichoderma atroviride* IMI 206040	Binab solution	1.7 g/l	3	T0	nursery
					T7	nursery
					T15	nursery

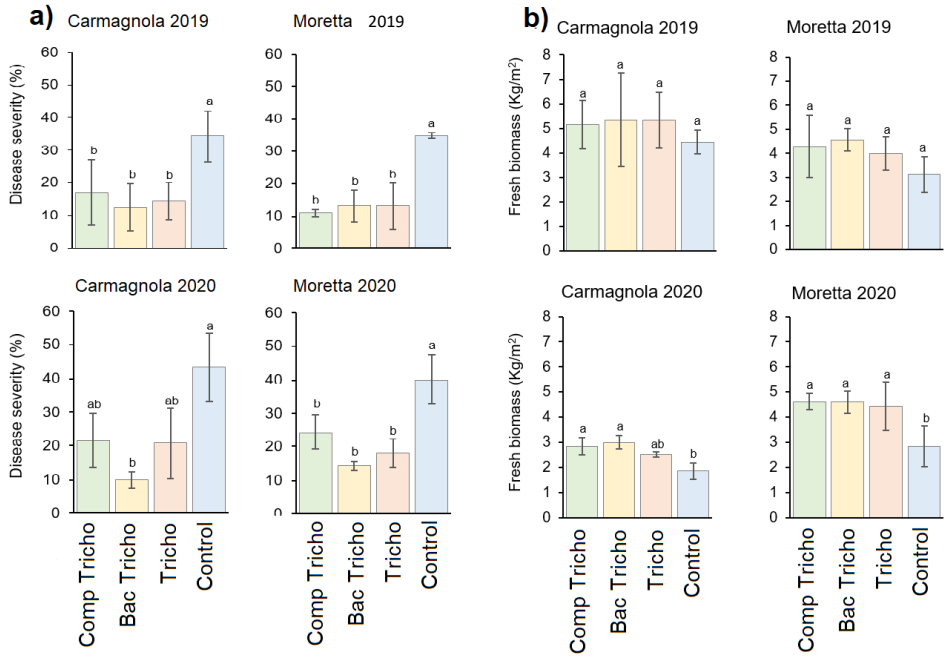


Fig 1. (a) Efficacy of the three IPM strategies to reduce *Fusarium oxysporum* f. sp. *lactucae* disease on lettuce plants expressed as disease severity (%) and (b) productivity expressed as fresh biomass (Kg/m^2). Evaluation made at the final survey. Different letters indicate statistical differences between the four thesis, as obtained with the ANOVA test and Tukey's post hoc test ($p<0.05$).

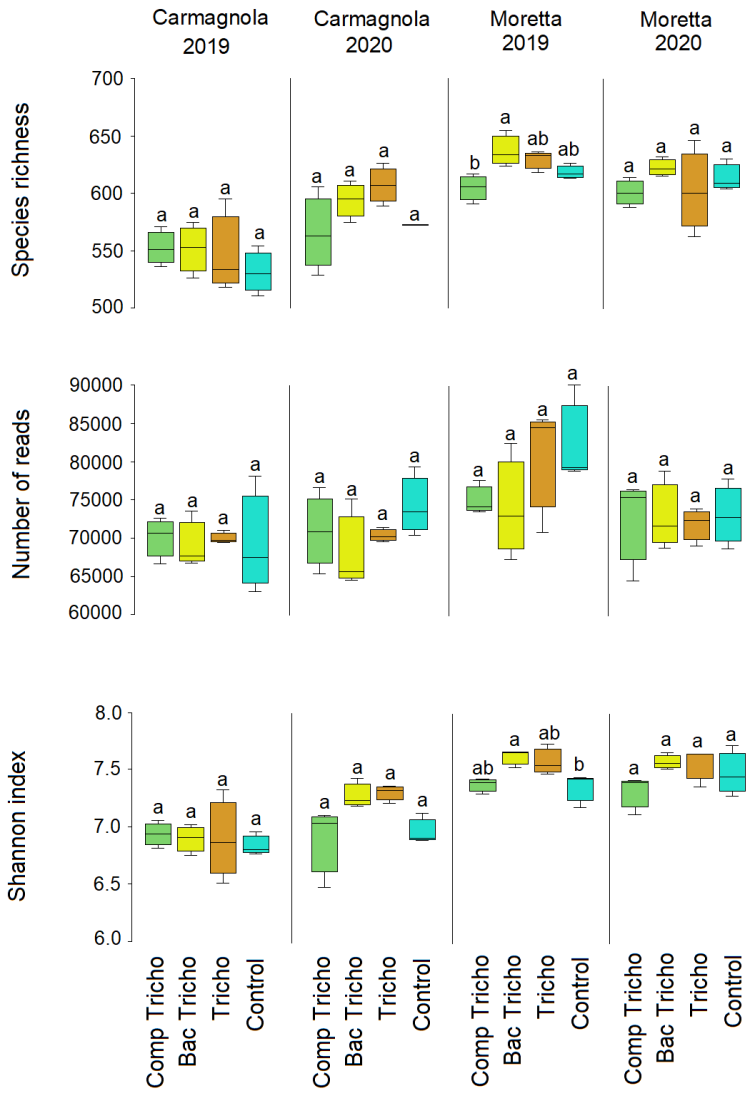


Fig 2. Box and whisker plots showing distribution of diversity indices, number of species, number of reads and Shannon Index, for bacteria community, for each treatment divided per site and year of rhizosphere sampling.

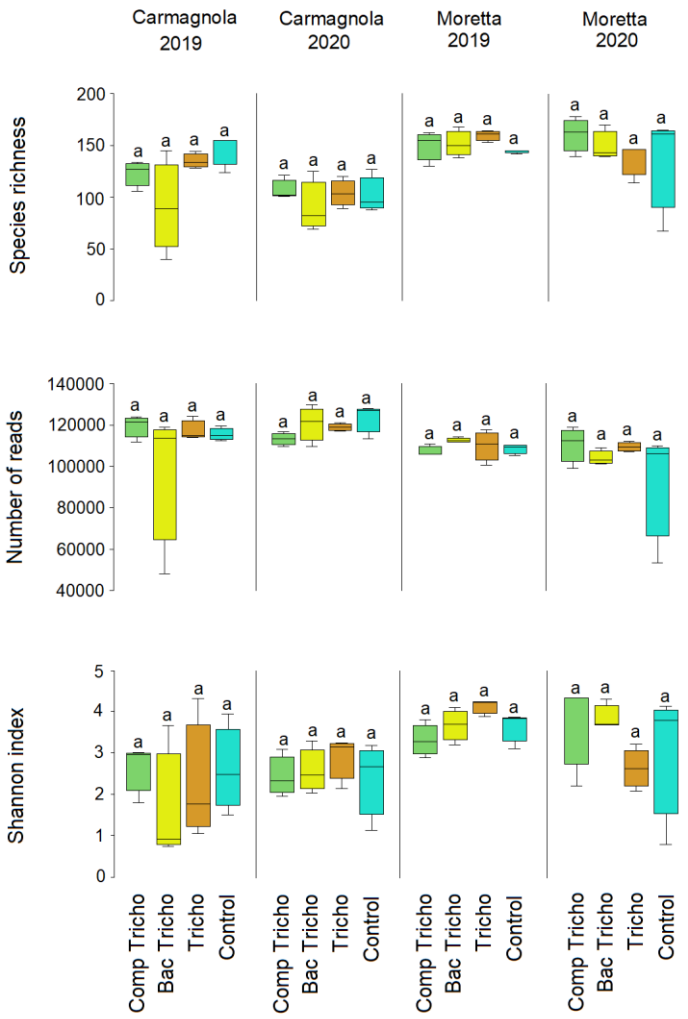


Fig 3. Box and whisker plots showing distribution of diversity indices, number of species, number of reads and Shannon Index, for fungal community, for each treatment divided per site and year of rhizosphere sampling.

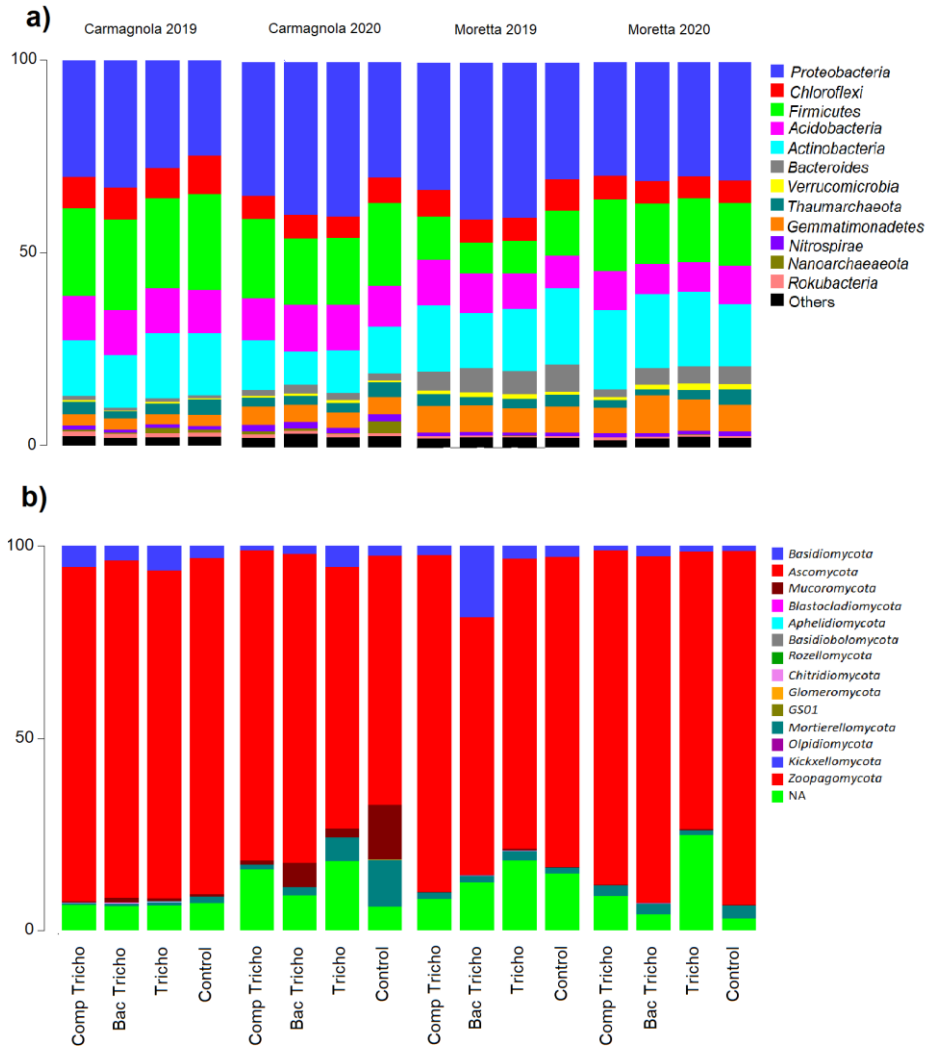


Fig 4. Stacked bar chart of relative abundance for (a) bacterial and (b) fungal communities at phylum level. Data are averaged among three biological replicates for each treatment and are here presented grouped for site and year.

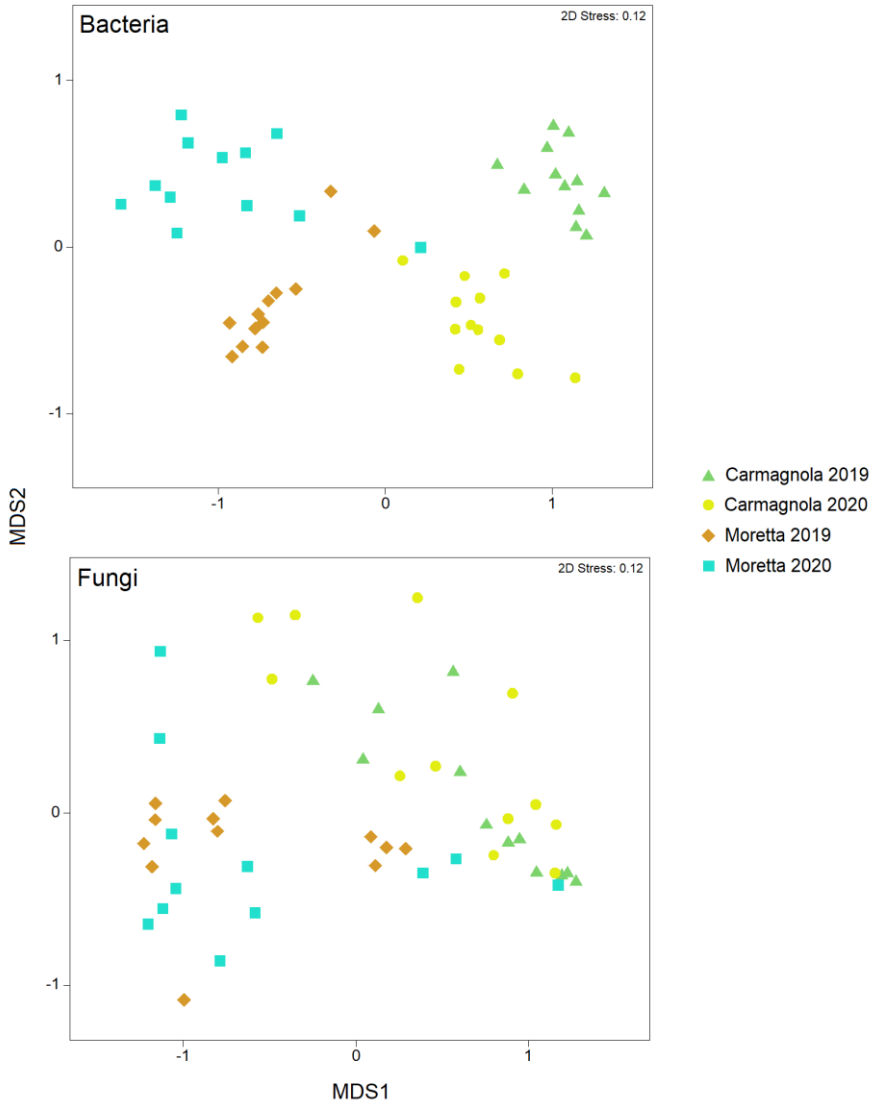


Fig 5. Bi-dimensional Non-metric MDS of the bacterial and fungal communities in the rhizosphere collected at the end of the trials. Data are averaged for year and site of sampling, following the colour coding of the legend.

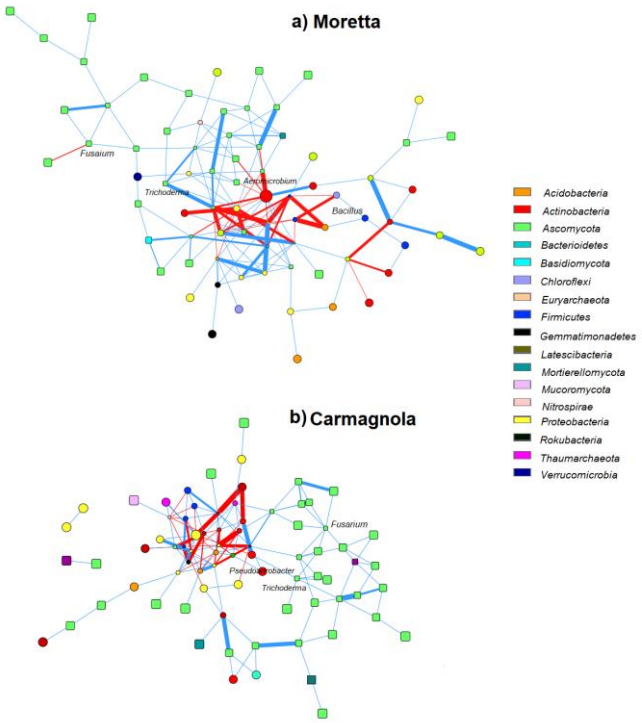
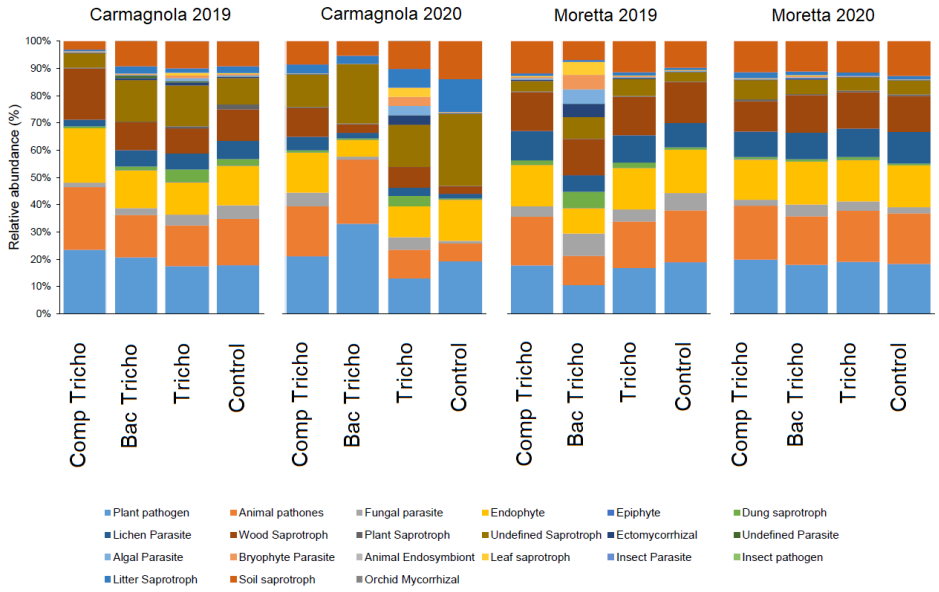


Fig 6. Correlation base network analysis showing potential interactions between bacterial and fungal families in (a) Moretta and (b) Carmagnola fields. The lines connecting nodes (edges) represent positive (blue) or negative (red) co-occurrence relationship. The intensity of the colour and the length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



685

686 **Fig 7.** Stacked bar chart of relative abundance of fungal guilds based on FunGuilds. Data are averaged

687 among three biological replicates for each treatment and are here presented grouped for site and year.

Appendix A. Supplementary materials

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

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

	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*

(*Significance level is at 0.05)

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

Commentato [MP1]: Excel file

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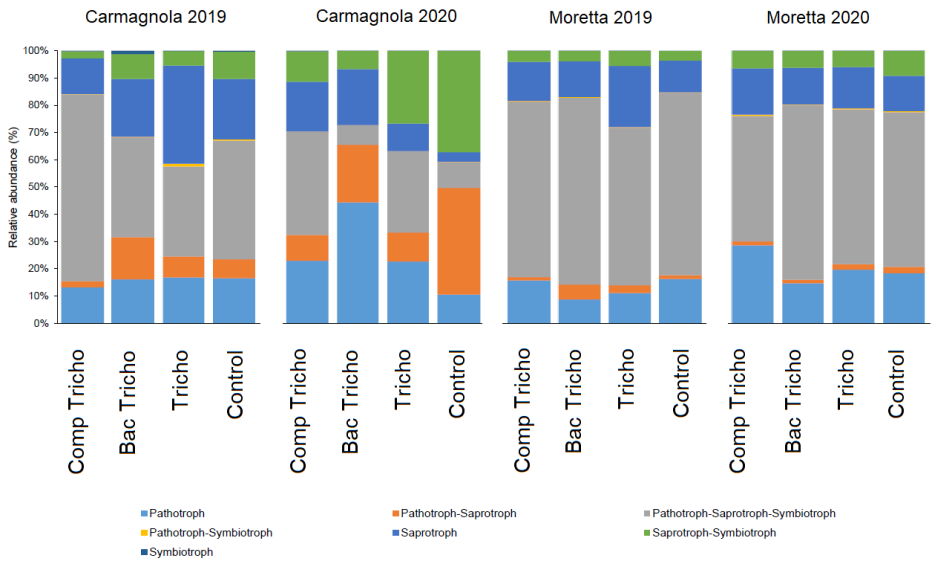
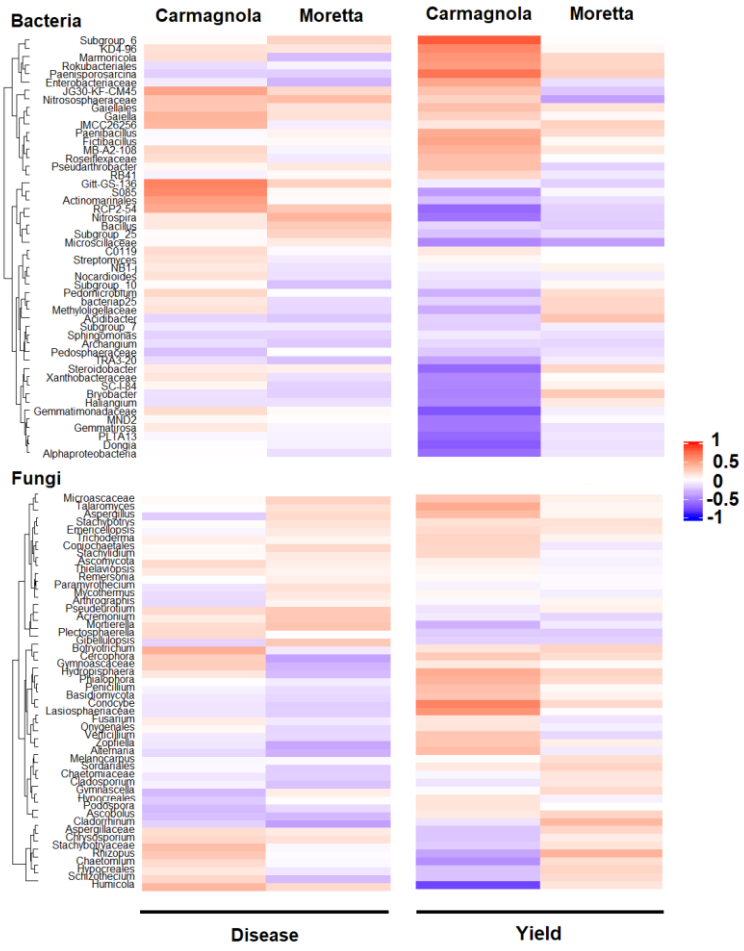


709

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



712

713 **Fig S2.** Heatmap of Pearson's correlation between the 50 most frequent amplicon sequence variant

714 (ASV) in the bacterial and fungal community and the disease index and growth (fresh biomass). Data are

715 expressed as average of every sample per each site.

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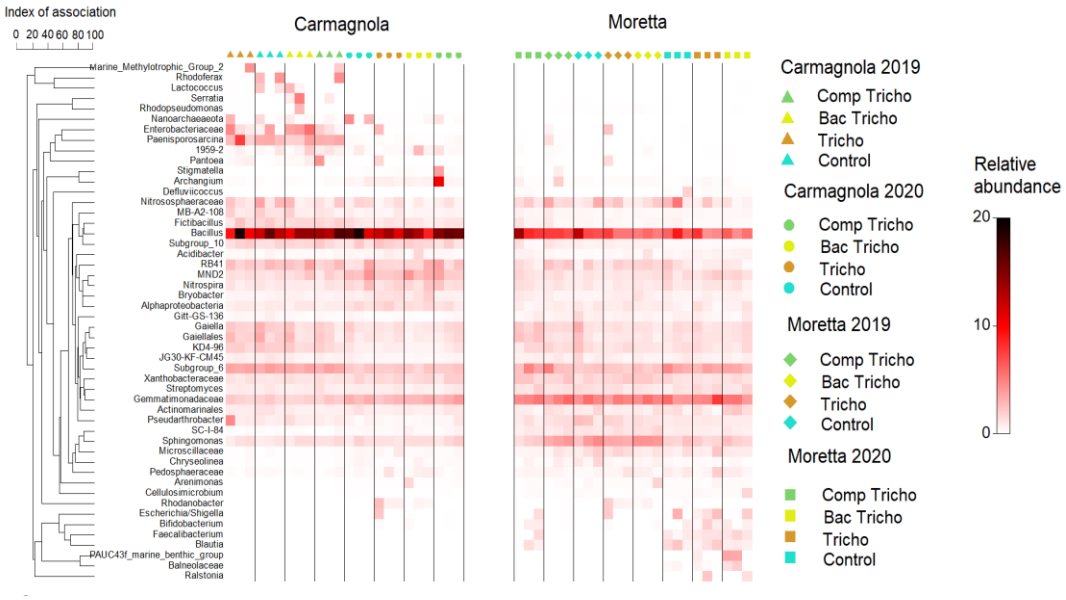

719 **Fig S3.** Heatmap showing relative abundance of the 50 most frequent amplicon sequence variant (ASV)
720 in the bacterial community for each sample collected from rhizosphere at the end of the trials.
721 Hierarchical clustering of variables is based on an association index. Samples follow the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

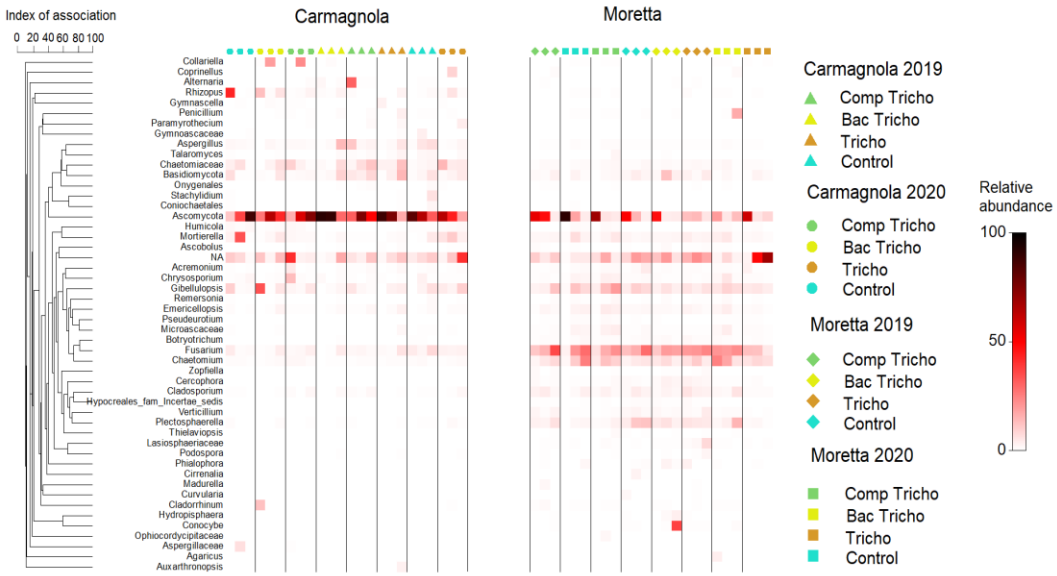

Fig S4. Heatmap showing relative abundance of the 50 most frequent amplicon sequence variant (ASV) in the fungal community for each sample collected from rhizosphere at the end of the trials. Hierarchical clustering of variables is based on an association index. Samples follow the colour coding of the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