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Effects of biocontrol agents and compost against *Phytophthora capsici* of zucchini and the impact on the rhizosphere microbiota

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Abstract

Four different biocontrol agents (BCA’s) and two composts have been used in this study to evaluate their efficiency against Phytophthora blight of zucchini caused by *Phytophthora capsici*. The effects of the BCA’s and composts on the zucchini rhizosphere’s microbiota were examined at the end of experimental trials from 2016 and 2017 in two farms under natural and artificially infested soils.

Next generation amplicon sequencing technology and quantitative Polymerase Chain Reaction - qPCR as targeted and untargeted approaches respectively, have been used. The abundance of *P. capsici*, measured for all the treatments over both years, decreased with respect to the untreated control, and it may be assumed that direct pathogen-beneficial microorganism interaction occurred. All treatments reduced the disease incidence by at least 50% in both sites under naturally or artificially infested soil and at both sampling times. The disease index was in accordance with the pathogen abundance. The development of microbiota and mycobiota was not affected by the BCA’s, thus indicating that the tested treatments did not interact negatively with microbial communities, which in turn suggested a slow but good adaptation of the biocontrol agents to the environment. The combination of targeted and untargeted approaches may help to understand the effect of different BCA’s on the development of *Phytophthora capsici*.

Keywords: *Phythophthora capsici*; BCA’s; disease suppression; Next Generation Sequencing
1. Introduction

Zucchini (*Cucurbita pepo* L.) is a very important crop from economically point of view (Jeffrey 2001; Paris and Maynard, 2008). The covered growing area in northern Italy is approximately 5,473 ha, with 1,326,811 tons of total production in open fields and under greenhouse conditions (ISTAT, 2017). This crop is often subjected to attacks by several soil-borne pathogens, one of which, *Phytophthora capsici* (Leon.), is a very serious agent of crown and root rot of cucurbit as well as of other crops (Hausbeck and Lamour, 2004; Lamour et al., 2012). Symptoms caused by *P. capsici* on zucchini have been observed extensively throughout Italy and are very serious in open fields (Garibaldi and Gullino, 2010).

The ability to develop fungicide resistance (Hausbeck and Lamour, 2004; Ploetz et al., 2002; Tamietti and Valentino, 2001), the existence of several physiological races and the lack of resistant cultivars (Sy et al., 2008; da Costa Ribeiro and Bosland, 2012), as well as the decreasing availability of fumigants (Colla et al., 2012) are making the disease control very difficult. Thus, there is a need for evolving environmentally friendly stratagems (Parra and Ristaino, 2001) such as the biological control. *Trichoderma* genus is generally known to have a good biocontrol activity (Howell, 2003; Harman 2000; Harman et al., 2004; Verma et al., 2007; Lorito et al., 2010; Lorito and Woo, 2015a; Woo et al., 2014). Antagonistic isolates of *Trichoderma* spp. have in fact been screened for their ability to control chili blight caused by *P. capsici* (Vinale et al., 2008; Lombardi et al., 2018). For example, *T. harzianum* and *T. asperellum* have resulted to be effective against *P. capsici* in different pathosystems (Ezziyyani et al., 2007; Segarra et al., 2007, 2013; Jiang et al., 2016). *Bacillus* genus have also shown the ability to reduce the presence of a variety of soil-borne phytopathogenic fungi and oomycetes, including *P. capsici* (Lee et al., 2008; Zhang et al., 2010). Also other microorganisms have been assessed for potentially control *P. capsici*, including *Streptomyces* spp. (Sang et al., 2008; Nguyen et al., 2012), *Paenibacillus* spp. (Kim et al., 2009, Naing et al., 2014), *Clitocybe nuda* (Chen et al., 2012) and *Aspergillus* spp. (Kang and Kim, 2004). In addition, organic amendments, such as composts, have shown a suppressive activity and positive
effect on the management of Phytophthora root and crown rot of bell pepper (Chae et al., 2006; Gilardi et al., 2013; Kim et al., 1997; Nunez-Zofio et al., 2011; Sang et al., 2010).

Biological control is often based on the rhizosphere competence which includes soil environment colonization of biocontrol agents (BCA’s) alone, or mixed with composts (Garibaldi et al., 1989; Gupta et al., 2014; Lorito and Woo, 2015b; Philippot et al., 2013; Wallenstein, 2017; Bonanomi et al., 2018). Since rhizosphere represents the pivotal interface for plant roots and the beneficial microorganisms which have a very import role in plant health, understanding how BCA’s and compost applications influence them under field conditions over several seasons of monoculture, may be helpful for the development of practices to suppress pathogens and for the success and fitness of the crop in the agro system.

In this study, the effectiveness of BCA’s commercially available (Bacillus subtilis QST713 and Trichoderma gamsii ICC 012 + Trichoderma asperellum ICC 080) and experimental (Trichoderma sp. TW2 and a mixture of Trichoderma sp. FC7 and FC8), together with compost, has been evaluated under field conditions and over two years, in naturally and artificially infested soil conditions. The following objectives were proposed: i) the estimation of the pathogen and disease reduction following the use of BCA’s and compost as treatments applied in pre-planting stage; ii) the evaluation of the extent of rhizosphere colonization by BCAs; iii) the evaluation of BCA’s and compost impact may have on the rhizosphere microbiota.

2. Materials and Methods

2.1. Layout of the field experiments

The experiments were carried out under field conditions in Northern Italy in one experimental farm at Carmagnola (44°8′55.188"N 7°6′37.457"E) as well as in a commercial farm, at Moretta (44°45′49.75"N 7°32′29.18"E). The soils in both sites are silty loam mixed, mesic, Entisoi Typic Ustifluvent (Carmagnola) and Entisoi Aquic Udifluvent (Moretta) (Soil Survey Staff, 2010). The principal difference between the sites is their pH, which is almost neutral for Moretta (pH = 6.85)
and alkaline (pH = 7.89) for Carmagnola. The studied soil sites are characterized by silt:sand:clay in a ratio of 60:30:10% and 1.5% organic matter at Moretta, with Phytophthora capsici naturally infested soil, and by silt:sand:clay 64:24:12% and 1.5% organic matter at Carmagnola, artificially infested with a selected pathogenic isolate of P. capsici obtained from the crown tissues of affected zucchini plants (100 g pathogen biomass per square meter).

In both trials carried out in the experimental farm from Carmagnola, a highly virulent strain of P. capsici (PHC1/16) obtained from infected zucchini plants in a field in Northern-Italy was used. The isolate was propagated by inoculation of a colonized agar–plug on to a sterile mixture of wheat-hemp kernels (2:1 v/v) in a 1-L flask kept at room temperature in the dark (Gilardi et al., 2016). The 15-day-old culture of the pathogen was mixed into the soil before planting the treated and untreated seedlings. In order to achieve a uniform soil infestation, the pathogen biomass obtained was incorporated in the soil 7 days before planting at 100 g/m² by rototilling at a depth of 1–20 cm. Two trials were also carried out in the Moretta commercial farm, naturally infested with the pathogen. The site of the trials was selected because had a history of zucchini cultivation with 20 to 40% of plant losses depending the years.

The field trials were conducted over two consecutive years, that is, 2016 and 2017 in order to evaluate the efficacy of soil treatments applied in pre-planting starting from the nursery against P. capsici with different biocontrol agents (BCA’s) such as Bacillus subtilis QST713 (Serenade, Bayer Crop Science, Italy) and Trichoderma gamsii ICC 012 + Trichoderma asperellum ICC 080 (Remedier, ISAGRO, Italy), which are commercially available. Experimental BCA’s have been tested as well: Trichoderma sp. TW2 (AgriNewTech, Italy) (Cucu et al., 2018; 2020) and a mixture of Trichoderma sp. FC7 and FC8 isolated from suppressive soilless (Agroinnova, Italy) (Garibaldi et al., 2003). In addition, two composts, produced from green waste from a dynamic industrial treatment system, were used: a green compost (Ant’s Compost V - CV, AgriNewTech, Italy) and the same compost inoculated with Trichoderma sp. TW2 (Ant’s Compost M - CM, AgriNewTech, Italy) according to Pugliese et al., (2011). An untreated control was used to monitor P. capsici
development, while metalaxyl-M (Ridomil gold, 480 g L, Syngenta Crop Protection, Italy) as
reference chemical control at 0.48 g a.i. L, label dose concentration, was applied by soil drenching
before planting. Summarized details of these treatments can be found in Table 1.

Zucchini seeds (cv. Ortano, Syngenta) were sown in 40-plug trays (3.4 cm diameter pots, 4 L of
soil, 53 × 42 cm surface) containing a peat mixture substrate.

Three soil applications with BCAs were carried out on the plug trays between sowing and
transplanting (at the first true leaf stage) in a commercial nursery under glasshouse conditions at
temperatures of 20-24°C. The BCAs were applied by spraying them onto the soil surface in a high
volume of water (500 ml tray⁻¹), using a 1 L capacity hand sprayer. The composts were mixed at
20% v/v at the tray level and immediately before sowing (T0).

The zucchini plants (15-day old) were transplanted at a density of 3 plants per square meter, drip
irrigated and grown according to the cultural practices adopted by commercial growers in the
region. The plots were arranged in a completely randomized block design, with five replicates per
treatment corresponding to 15 plants per replicate in trials carried out in 2016 and 20 plants per
replicate in trials carried out in 2017. The final disease rating was established 62 to 82 days after
transplanting on each plot by visually estimate the severity of Phytophthora crown rot symptoms of
all the plants present in each plot (Table 2). According to Padley et al. (2008), the disease severity
(DS) scale was as follows: 0 = healthy plant, 25% = initial leaf chlorosis, 50% = severe leaf
chlorosis and initial symptoms of wilting during the hottest hours of the day, 75% = severe wilting
and severe symptoms of leaf chlorosis, 100% = plant totally wilted, leaves completely necrotic.

Symptoms were visually estimated at the final evaluation for each plant using a scale rate as follow
from 0 to 5.: 0=no symptoms, healthy plants; 1=1 to 30% leaves slight wilted (midpoint 15%); 2=31
to 50% foliar wilting with crown lesion (midpoint 40%); 3=51 to 70% of plant is partially collapsed
(midpoint 60%); 4=71 to 90% of plant is collapsed (midpoint 80%): 5=over 90% dead plant
(midpoint 95%). Disease severity was expressed by using the formula $\frac{\sum(n^\circ \text{ plants} \times x \ 0-5)}{(\text{total}
no \text{ of \ plants \ recorded})} \times 0-5$ corresponding to the value reported.
2.2. Rhizosphere soil sampling

The influence of soil-treatments with BCA’s and compost on indigenous microbial communities was studied at the rhizosphere level by collecting samples from both experimental sites at the end of the trials (November 2016 and 2017), as described elsewhere (Cucu et al., 2018). Briefly, one composite rhizosphere sample per plot consisted of the roots of five randomly selected zucchini plants. The roots were shaken vigorously to remove the soil particles not tightly adhering to them. The rhizosphere soil was gently scraped off with sterile forceps and transferred into sterile sampling bags. Each treatment consisted of three biological replicates, which were kept on ice. The samples were then placed in plastic bags, kept on ice and transported to the laboratory.

2.3. Microbial community analysis

2.3.1. Rhizosphere soil DNA extraction

A NucleoSpin® Soil kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used, according to the manufacturer’s instructions, for the rhizosphere soil genomic DNA extraction. Extraction was conducted on 0.6 g sample material. DNA quantity and purity were measured using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and standardized at 10 ng μl⁻¹.

2.3.2 Microbial structure

Amplicon target sequencing

Total genomic DNA extracted from the rhizosphere samples was used as template to amplify the V3-V4 region of 16S rRNA (primers 16S-F (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′) and 16S-R (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′)) (Klindworth et al., 2013) as well as the D1 domain of the 26S rRNA gene (primers LS2-MF 5′-GAGTCGAGTTGTGTTGGAAT-3′ NL4R 5′-GGTCCGTGTTTCAAGACGG-3′) (Mota-Gutierrez,...
et al., 2018). The PCR mix was done in a total volume of 25 μl as follows: 12.5 μl of the 2 × Kapa HiFi HotStart ReadyMix Taq (Roche, Milan, Italy), 5 μl of each primer (1 μM) and 2.5 μl DNA as a template.

The PCR mix was subjected to the following amplification cycling: 95°C for 3 minutes; 30 cycles of: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and then 72°C for 10 minutes. Amplicons were purified according to the Metagenomic sequencing library preparation of Illumina. Nextera XT Index Kit (Illumina Inc. San Diego, CA) was used based on manufacturer’s instructions, to tag each sample. After the 2nd clean up stage, the amplicons were quantified using a QUBIT dsDNA Assay kit and equimolar amounts of amplicons from different samples, belonging to the same target gene, were pooled. The pooled libraries (16S and 26S) were run on an Experion workstation (Biorad, Milan, Italy) for a quality analysis prior to sequencing. The denaturated pool library (with 0.2 N NaOH) was combined with 20% (v/v) of denatured 12 pM PhiX, prepared according to the Illumina guidelines. The sequencing was performed with a MiSeq Illumina instrument (Illumina), with V3 chemistry, and 250 bp paired-end reads were generated, according to the manufacturer’s instructions.

Bioinformatics analysis

After sequencing, FLASH software (Magoc et al., 2011) was used to join the paired end reads. Any joined reads shorter than 300 bp were discarded using PRINSEQ. The data were analyzed through QIIME 1.9.0 software (Caporaso et al., 2010). Centroid sequences were used from each Operational Taxonomic Unit (OTU) cluster for the 16S data to assign a taxonomy against the Greengenes 16S rRNA gene database, version 2013, while the in-house database from Mota-Gutierrez et al. (2018) was used for the 26S data.

For avoiding biases, due to different sequencing scales, each dataset was reduced at the lowest number of reads: the 26S data were rarefied at 11808 sequences, while the 6285 sequence was chosen for the 16S data. Contamination of chloroplasts was removed from the 16S OTU tables.
The 26S OTUs tables generated through QIIME showed the highest taxonomy resolution for the species or genus. The order or class level was instead used for the 16S data. The taxonomy was double-checked using the BlastN search tool (http://www.ncbi.nlm.nih.gov/blast/) to confirm the taxonomy assignment.

The data on microbial communities structure were analyzed using the R software (www.r-project.org). The number of different taxa was estimated by sample coverage and microbial α-diversity assessed by Chao1 index. The taxa richness and evenness was calculated by Shannon diversity index, using the diversity function of the vegan package of R (Dixon, 2003). Beta diversity of 16S data used the phylogenetic distances to compare samples associated with multivariate statistical analysis (Principal coordinate analysis) based on Weighted and Unweighted UniFrac distance matrixes. Anosim and Adonis statistical test, through the vegan function in the R environment were used for the 16S data to find any differences between samples, using the phylogenetic distances matrix. A non-parametric Pairwise Wilcoxon test was used, when appropriate, to determine any significant differences in the alpha diversity index or in OTU abundance. Principal component analysis (PCA), based on OTU abundance, was performed and the results were plotted using the dudi.pca function of the made4 package of R. Hierarchical clustering heatplots were obtained, through the made4 package, using the weighted pair group method together with the arithmetic mean (WPGMA) method. Spearman’s pairwise correlations were assessed between the taxa and chemical parameters using the R package psych. The P values were adjusted for multiple testing using the Benjamini-Hochberg procedure, which assesses the false discovery rate (FDR). Box plots were used to represent the interquartile range between the first and the third quartiles, with the error bars showing the lowest and the highest values.

2.3.3 Microbial abundance (quantitative PCR - qPCR)

qPCR assays were used to determine the abundance of *P. capisci* (Lan et al., 2013), *Bacillus* spp. (Gao et al., 2011) and *Trichoderma* spp. (Hagn et al., 2007) with the StepOnePlus™ Real-Time
PCR System (Applied Biosystems, Foster City, CA, USA). Fungal chitinase *chiA* gene, and the nitrifiers (i.e., bacterial ammonia monooxygenase *amoA* gene - AOB and archaeal *amoA* gene – AOA) have been also evaluated. Table S1 contains a description of the primer sets and amplification details.

The standards for the qPCR assays were generated as described by Cucu et al. (2017) from amplicons of each target gene, purified (Invisorb Fragment CleanUp, Stratec Molecular GmbH, Berlin, Germany) and ligated in Strata-Clone PCR cloning vector pSC-A (Strataclone PCR Cloning Kit, Agilent Technologies Inc.); the products of the ligation were then transformed into StrataClone SoloPack competent cells (Agilent Technologies Inc.). The specificity of the amplicons used as qPCR standards was controlled and the Plasmid DNA was isolated (GenElute™ Plasmid Miniprep Kit, Sigma-Aldrich, St. Louis, MO, USA) from standard clones and quantified as described above.

*P. capsici*, BCA’s, and the bacterial and archaeal *amoA* abundances were determined in a final volume of the qPCR reaction mixtures of 25 μl, while the *chiaA* gene abundance in 20 μl. 10 ng DNA template were used for all quantification reactions. As described earlier by Cucu et al. (2018), the qPCR cocktail had a 1x Power SYBR green master mix (Applied Biosystems), 0.12 μM of each primer (Table S1) for *P. capsici* 0.32 μM for the BCA’s, AOB and AOA and 0.4 μM for the *chiA* gene.

The quantifications were done with three replicates across plates, while standards were run in duplicate and in 10-fold serial dilutions. Prior quantifications, the best DNA dilution was tested to reduce any inhibitory reactions. Amplification efficiency ranged from 96% (BCA’s and *chiA* gene) to 95.3% and 99.1% for AOB and AOA, respectively tor the nitrifiers. The $R^2$ was always $\geq 0.98$.

Melting curves of the amplicons were generated to guarantee the specificity of the reaction and then the amplification products were checked on 1% agarose gel. Always the size of the amplicons was as previously reported by the given protocols (see Table S1). StepOne™ software, version 2.2 (Applied Biosystems) was used to calculate the gene copy number and the data were normalized and presented in tables as log copy / g dw$^{-1}$.
The data on gene abundance and on the chemical properties of the soil were subjected to a linear mixed model, with time considered as the random variable, using the Statistical Analysis Software program (SAS V 9.2, SAS Institute Inc., North Carolina, USA). The impact of different treatments on the abundance of the considered genes, on the rhizosphere chemical properties and on disease severity were checked out. The Levene test was used to verify the homogeneity of variance, and Shapiro-Wilk test was chosen to check the normality on the residuals; the DS data were arcsin transformed when not normally distributed, while the data from microorganism abundance were log-transformed, and normality checked again. Univariate ANOVA (SPSS 24.09) was used for the data set analyses, and the means were separated by Tukey’s multiple comparison test (p = 0.05). The statistical analysis included the treatment, year and treatment × year. A least square means comparison between treatments was conducted using Tukey’s range test (p < 0.05). Pearson linear correlation analyses were conducted, and the results were visualized to establish linearity in the SAS COR procedure in order to relate the abundance of the target genes (dependent variables) to the chemical properties of the soil (independent variables).

2.4. Geo-chemical characterization of the rhizosphere samples

The fresh rhizosphere samples were homogenized, passed through a 2 mm sieve, stored at 4°C and analyzed within 24 h. All the samples were split into two. One part was analysed at the Regional Chemistry Laboratory (Turin, Italy) for humidity, pH, total N (TN), inorganic N as nitrate (NO$_3^-$) and ammonium (NH$_4^+$), total phosphorus (Pt) and available phosphorus (Pav), while the second part was used for molecular investigations. The method for measuring pH was in water suspensions with solid: liquid ratio of 1:2.5. A Leco TruSpect CN automatic analyzer was used for the total nitrogen (TN) quantification. Inorganic nitrogen, ammonium (NH$_4^+$) and nitrate (NO$_3^-$) using standard colorimetric techniques by means of a continuous flow auto-analyzer (Alliance Evolution II). The total phosphorus (Pt) was determined by means of ‘ICP Varian mod. Liberty LR’, after microwave based digestion with hydrogen.
peroxide, hydrochloric acid and nitric acid, filtration and dilution. The available P (Pav) was determined by means of the molybdenum blue method (Olsen et al., 1954), modified for continuous flow colorimetric analysis (Alliance Evolution II).

3. Results

3.1 Disease severity (DS)

The obtained results showed that the application of BCAs and certain organic matter can effectively suppress soil pathogens and reduce the disease. The disease severity (DS) in the non-treated control plots was higher in Carmagnola, where the soil was artificially infested with the pathogen, than in Moretta for both years (Table 2). During 2016, all the treatments based BCAs and compost significantly reduced the disease from 48 to 62% in the Carmagnola site and from 52 to 80% in the Moretta site, compared to the untreated plots (Table 2). In 2017, the artificial infestation of soil with the pathogen at the Carmagnola site produced a greater disease severity (DS 78% in the untreated control) resulting in a significant Phytophthora crown rot reduction between 32 to 49% provided by all the treatments, apart from the Trichoderma spp. T and TW2 treatments. While, in the Moretta site, under a low disease pressure (DS 28% in the untreated control) all the tested treatments provided a significant disease reduction from 50 to 82%. The reference chemical treatment based metalaxyl-M was the most effective, reducing the DS by 56-78% in Carmagnola and by 94-99% in Moretta in both years. The efficacy of the two composts (CV and CM) was statistically similar to that of the chemical fungicide in both locations, reducing the DS by 56-59% and 45-49% in Carmagnola and by 69-80% and 73-82% in Moretta, respectively. (Table 2).

3.2. Microbial diversity

The microbiota and mycobiota diversity in the two sites for the two different years was characterized by 16S and 26S rRNA gene sequencing, obtained from gDNA extracted directly from the rhizosphere soil samples. The DNA concentration which ranged between 82-100 ng/g dw\(^{-1}\) for...
Moretta and 55 - 83 ng/ g dw for Carmagnola, in both years, had the ratio of the absorbance at 260 nm and 280 nm of ~1.8, assessing a “pure” DNA. Overall, 1,907,342 and 3,849,188 high quality reads were analyzed for 16S and 26S, respectively. The alpha-diversity indexes (Shannon, Chao1 diversity index) and the number of species observed on the basis of OTUs of 97% identity, are shown in Figure 1A (16S) and Figure 1B (26S). Anosim and Adonis statistical test performed on 16S as well as 26S showed no difference for the different BCA’s treatments and composts in the two soils in the two years (P>0.05).

As far as the 16S data are concerned, the Carmagnola 2017 soil displayed a significantly higher microbial diversity and richness (P < 0.001) than Carmagnola 2016 and than the other site. Moreover, the Moretta 2017 samples showed higher richness than the Moretta 2016 ones (P < 0.05) - see Figure 1A.

As for the mycobiota (Figure 1B), it was possible to observe that the Chao1 index (an estimator of species richness) and the number of observed OTUs were higher in the Moretta samples than in the Carmagnola ones (P < 0.001). However, no differences were observed between Carmagnola 2016 and Carmagnola 2017 or between Moretta 2016 and Moretta 2017. It should be pointed out that the different treatments did not affect the microbial diversity.

Using a Principal Coordinate analysis (PCoA, Fig. 2) of the weighted UniFrac distance matrix, we observed a clear separation of the microbiota between Carmagnola 2016 vs. Carmagnola 2017 and Moretta 2016 vs. Moretta 2017 (ANOSIM statistical test P < 0.001) for the beta diversity calculation of the 16S data. No differences were observed between Carmagnola 2016 and Moretta 2016. Samples from these two sites from the same year clustered together and were well separated from the others (Fig. 2).

A similar trend was observed for the mycobiota populations. The principal component analysis (PCA), based on the 26S OTU table, clearly showed a separation of Carmagnola 2017 from the other site, while a distribution of the other samples was less marked (ANOSIM statistical test P <
0.001) (Figure S1). No differences were observed when the different soil treatments were taken into
account.

3.2.a Microbiota composition

The relative abundances of the bacterial taxa were analyzed at a class level to show the
development of the microbiota over time, in both years, and across sites. A total of 34 classes, with
a relative abundance of 0.2% in at least 10 samples, were observed (Table 3). When the
Carmagnola site was considered in 2016 and 2017 (Table 3), the microbiota were dominated by the
presence of Actinobacteria (median value of 6 and 13% of the relative abundance, respectively),
Alphaproteobacteria (11 and 13%), Bacilli (5 and 7%), Betaproteobacteria (6 and 4%), Chloroflexi
(3 and 5%), Deltaproteobacteria (5 and 4%), Phycisphaerae (5 and 3%), Planctomycetia (9 and
6%) and Thermoleophilia (3 and 6%) - see Table 3.

The same trend was observed for the Moretta samples (Table 4), where Actinobacteria showed a
relative abundance, with a median value of 6 and 13% in 2016 and 2017, respectively;
Alphaproteobacteria (12 and 12%), Bacilli (3 and 7%), Betaproteobacteria (5 and 4%), Chloroflexi
(3 and 5%), Deltaproteobacteria (5 and 4%), Gammaproteobacteria (7 and 4%), Phycisphaerae (6
and 4%), Planctomycetia (5 and 7%) and Thermoleophilia (2 and 6%).

We observed a specific microbiota signature among the variables from a the pairwise comparison of
the sites and the years. In particular, Actinobacteria, Bacilli and Chloroflexi were found to be
associated (FDR < 0.05) with Carmagnola 2016 and Moretta 2016; Betaproteobacteria,
Deltaproteobacteria and Phycisphaerae with Carmagnola 2017 and Moretta 2017;
Alphaproteobacteria was observed to be associated with Carmagnola 2016 and
Gammaproteobacteria with Moretta 2017 (Figure S2).

3.2.b Mycobiota composition
A total of 58 taxa, with a relative abundance of 0.2% in at least 10 samples, were observed (Tables S2 and S3). When only the main fungi that occurred with a higher percentage than 5% were taken into account, the presence of *Alternaria tenuissima* (median values of 1 and 9% of the relative abundance in 2016 and 2017, respectively), *Cladosporium cladosporioides* (median value sof 4 and 9%), *Fusarium oxysporum* (6 and 3%), Leotiomycetes (19 and 2%) and *Pseudeurotium zonatum* (28 and 1%) was observed in the Carmagnola site in 2016 and 2017 (Table S2).

The Moretta samples (Table S3) were dominated by the presence of *Aspergillus oryzae* (median values of 5 and 1% of the relative abundance in 2016 and 2017, respectively), Leotiomycetes (13 and 23%) and *Pseudeurotium zonatum* (34 and 35%).

A specific mycobiota signature between the sites and the years was observed from the pairwise comparison (Figure S3, FDR < 0.05). In particular, it was observed that *Alternaria tenuissima* and *Cladosporium cladosporioides* were associated with the Carmagnola site in 2017, *Aspergillus oryzae* and *Fusarium oxysporum* with the Carmagnola site in 2016, Leotiomycetes were associated with both sites in 2016 and 2017 and *Pseudeurotium zonatum* was associated with the Carmagnola site in 2016 and Moretta 2016 (Figure S3, FDR < 0.05).

### 3.3. Microbial abundance

#### 3.3a Abundance of soil-borne *P. capsici* by means of qPCR

All the treatments resulted in a significant pathogen reduction (p < 0.05), compared to the untreated control (C), in both the Moretta and Carmagnola sites, in both years. The quantity of the pathogen in the Moretta rhizosphere samples was lower after the RM treatment in both years, while it was lower in the Carmagnola site after the RM treatment in 2016 and 2017 respectively (Table 5).

#### 3.3b Abundance of antagonists N- cycling genes by means of qPCR

All the treatments resulted in a significant boost of *Bacillus* spp. and *Trichoderma* spp., compared to the untreated control - C. The *Bacillus* spp. abundance was higher after the *Bacillus* - SM
treatment and lower after the *Trichoderma* sp. - T treatment and *Trichoderma* sp. - TW2 in the Moretta samples in 2016 and 2017 respectively, while the *Bacillus* spp. abundance was higher after the *Bacillus* - SM treatment and lower after the *Trichoderma* sp. - TW2 treatment for the Carmagnola counterparts after both years (Table 6).

*Trichoderma* spp. abundance significantly enhanced after all the *Trichoderma* sp. treatments, compared with the untreated control - C for both sites and both years (Table 6). The same trend was observed for *chiA* gene abundance, which significantly enhanced after all the *Trichoderma* spp. treatments, compared with the untreated control - C and the *Bacillus* treatment - SM in Moretta samples after both years. However, the enriched compost – CM had the highest abundance of the *chiA* gene. Carmagnola samples were characterised by the same situation, but with the highest abundance of *chiA* gene after *Trichoderma* sp. - TW2 in 2016 and enriched compost – CM in 2017 (Table 7).

The ammonia-oxidizing bacterial (AOB) and archaeal (AOA) gene abundance was significantly influenced by treatments. Both sites were characterized by the increase of AOB and AOA abundance in all treatments comparing with the untreated control. In general, the abundance of AOB was higher in rhizosphere samples from Carmagnola site, while the abundance of AOA was higher in rhizosphere samples from Moretta site (Table S4 and Table S5).

3.4. Chemical properties

The pH values of rhizosphere samples generally were lower for Moretta site than for Carmagnola ones and ranged from between 5.36 to and 6.23 and 6.20 to 7.78 respectively. The untreated control was characterized by higher $\text{NH}_4^+$, $\text{NO}_3^-$ concentrations than all the treatments. On the other hand, the Pt and Pav contents were lower (Table S6).

3.5. Correlations

3.5 a. Spearman’s pairwise correlations assessed between the taxa and chemical parameters
By plotting the correlation between chemical determination, microbiota and mycobiota in Carmagnola samples (Figure S4A; FDR < 0.05) the results showed more relationships between the taxa and TN, NO$_3^-$ and NH$_4^+$ with respect to Moretta samples (Figure S4B; FDR < 0.05). By plotting the correlation between chemical determination, microbiota and mycobiota in Moretta samples for both years (Figure S4B; FDR < 0.05) the results showed more relationships between the taxa and pH as well as between the taxa and the pathogen abundance.

b. Pearson correlations between *Bacillus* and *Trichoderma* abundances and chemical parameters

In general, the correlations with pH were positive, with r coefficients ranging from 0.55 to 0.85 (p < 0.001) for the rhizosphere samples from Moretta e Carmagnola sites. The correlations were negative between *Bacillus* and Pt and Pav and positive for *Trichoderma* (Table S7).

4. Discussion

Four biocontrol agents and two compost were chosen to control soil borne pathogens as *P. capsici*. It is well known that their effect is relative, depending on the plant host, involved pathogen species and the characteristics of the applied treatments (Bonanomi et al., 2006). Even more, the interactions among beneficial microorganisms newly introduced into the soil system, as single strains or in microbial consortium, with indigenous rhizosphere microbial populations and the plant usually vary with the environmental conditions (Mendes et al., 2013).

The results of the field experiments showed that the all treatments reduced the disease severity and were particularly effective against the pathogen, in comparison to the untreated control, (Heydari and Pessarakli, 2010; Noble and Coventry 2005; Pugliese et al., 2015). The findings were in line with the fact that pathogens populations in the soils could be reduced by biocontrol agents (Huang et al., 2011; Wei et al., 2011) and suppressive compost (Cucu et al., 2018). Similarly, previous studies showed several bacilli strains being effective against Phytophthora blight caused by *P.*
Capsici on squash (Zhang et al., 2010; Gilardi et al., 2015) and on pepper (Sang et al, 2013). Even more, the mixture of organic substrates and BCA’s may induce the suppression of plant pathogens through different mechanisms. Blaya et al. (2013), Cao et al. (2011) and Hoitink et al. (2006) hypothesized the presence of direct interactions as competition for nutrients and space, the production of antibiotics, mycoparasitism and indirect interactions, such as systemic and acquired resistance (ISR and SAR). Nevertheless, the disease severity was greater at higher pH conditions which was in agreement with previous studies involving P. capsici as pathogen (Muchovej et al., 1980).

Through the use of the targeting approach, a reduction in P. capsici abundance was observed as a result of all the applied treatments, thereby assuming that a direct pathogen-BCA’s interaction occurred. This effect could be due to antibiosis and/or mycoparasitism process induced by Trichoderma spp. hydrolytic enzyme secretion, which may have caused the hydrolysis of the pathogen cell wall, or antibiotic compound production (Woo et al., 2006, Lorito and Woo, 2015a). A higher abundance of Trichoderma spp. and of the chiA gene in all the treatments was observed highlighting the positive effect of Trichoderma spp. when used as a biocontrol agent or in combination with organic material as pointed out by Lorito et al. (2010), Hermosa et al. (2012), and Cucu et al. (2018). This result in corroboration with good relationships with rhizosphere chemical parameters evidenced the fact that Trichodema spp. readily transfers and absorbs soil nutrients (e.g., phosphate - Tandon et al., 2018) and is more efficient and competitive that many other fungi. In consequence, it can therefore be used effectively to improve the soil structure and promote the establishment and maintenance of other beneficial microorganisms (Benitez et al., 2004). The targeting analyses showed also a good feedback of Bacillus spp. after all the treatments. The same response was observed by Cucu et al. (2018) in a Fusarium oxysporum - lettuce pathosystem, evidencing a good feedback of indigenous rhizosphere Bacillus populations. In general, Bacillus spp. was found to be extremely effective in plant disease suppression as it forms a stable and extensive biofilm (Bais et al. 2004) composed of secreted antifungal compounds, such as surfactin,
bacillomycin and microlactin, which protect plants from attack by soil-borne pathogens (Bais et al. 2004; Yuan et al. 2012). Even more, Yang et al. (2012) and Sid et al. (2003) reported that most species of Bacillus were able to inhibit the mycelia growth of *P. capsici* effectively “*in vitro*”.

Through the use of the un-targeting approach (amplicon based sequencing), it was shown also that the different BCA’s and compost treatments did not affect the composition of the resident microbiota and mycobiota. This implied that the used treatments did not have any effect on the total microbial communities reflecting the great ecological stability of the rhizosphere (Thébault and Fontaine, 2010). In turn suggested a slow but good adaptation of the BCA’s to the environment. Anyway, the observed differences among sampling dates may suggest that the composition of the soil microbial community might have been altered by differences in temperature and precipitation, as response of the microbial groups to environmental variables.

*Actinobacteria, Bacilli* and *Chloroflexi* were the most abundant bacterial phyla in both sites in 2016, while *Betaproteobacteria, Deltaproteobacteria* and *Phycisphaerae* were observed in both of the studied sites in 2017; *Alphaproteobacteria* was associated with the Carmagnola site in 2016, while *Gammaproteobacteria* was associated with the Moretta site in 2017. Soil microbial communities structure was shaped by a multitude of factors, including environmental parameters as suggested by the correlation analyses of soil chemical properties namely TN, NO$_3^-$-N, NH$_4^+$-N available P and the relative abundances of different microbial populations across the considered sites, especially for the Carmagnola rhizosphere soil. This observed differences could be attributed to the difference in the soil pH, as it is generally considered as the best predictor of the microbial community composition and diversity (Fierer and Jackson, 2006). The similarity of the chemical and physical characteristics of the individual soil sites permitted to consider independently the pH effects. This was confirmed by the abundance data of populations of ammonia-oxidizing bacteria and archaea. The results showed an elevated amount of ammonia-oxidizing bacteria, mainly *Betaproteobacteria*, with respect to the untreated control especially at higher pH of Carmagnola site, in line with what previously reported (Nicol et. al., 2008). On the opposite a higher abundance of archaeal ammonia
oxidizers was observed in samples from Moretta site this suggesting a potential niche differentiation
between the ammonia oxidizers as indicated also by the relationships with chemical properties
(Figure S4A and B). Although the increase of ammonia-oxidizing bacteria and archaea abundance
with respect to the untreated control may suggest a possible contribution of these microorganisms to
the disease reduction, further research in regard must be taken in consideration.

The Moretta site showed a predominance of relationships between the pH and microbial taxa
(Figure S4B). Prior to the use of NGS, Fierer and Jackson (2006), using Terminal Restriction
Length Polymorphism, identified a maximum of microbial diversity for a neutral pH. Rousk et al.
(2010) extended this finding using experimental plots. Sequences that were classified as belonging
to Gammaproteobacteria were shown to correlate negatively with pH, a result that was in
contradiction with what Postma et al. (2011) reported. On the other hand, the relative abundance of
Actinobacteria was found to be positively correlated with pH. Lauber et al. (2009) also reported
clear shifts in the bacterial-phylum level, with the relative abundance of Actinobacteria increasing
as the pH value increased.

The soil artificially infested with *P. capsici* showed a somewhat increased abundance of
Actinobacteria. Ros et al. (2016) observed the same phenomenon after seedling infection by *P.
nicotianae*. This result, together with good correlations with the soil properties (Figure S4A),
indicated that the highlighted microbial communities could play a strategic role in the organic
materials decomposition and in the nutrient cycle, as evidenced by Steger et al. (2003). Although
these microorganisms have a great ability to produce antibiotic-like compounds (McKellar and
Nelson, 2003), the results indicated that they did not have a positive influence on plant disease
suppression in the case of an artificial infestation since no relationship with *P. capsici* was found.

The opposite was observed for the natural infestation, result evidenced by correlations between
Actinobacteria and pathogen. Thampi and Bhai, (2017) reported some Actinobacteria strains as
potential antagonists against *P. capsici*, after their isolation and characterization from rhizosphere
samples of black pepper. However, these results might be contradictory as Sutherland and
Papavizas (1991) reported, in a very early “in vitro” study, that *Actinobacteria*, which infested oospores of *P. capsici*, were not effective under greenhouse conditions and didn’t control the crown rot of pepper. Cuesta et al. (2012) also reported that the presence of *Actinobacteria* is typical of suppressive substrates. Bonanomi et al., (2010) concluded that disease suppression was only correlated with *Actinobacteria* in a small number of experiments. However, the here presented results suggested that enlarged populations of Actinomycetes might lead to a favourable environment for biocontrol strains against *P. capsici* on zucchini.

*Bacillus* was found as some of the most abundant genera in all the treatments, the sequencing results being supported by the quantitative analyses. The observed relatively high abundance of *Bacilli* phyla was in accordance with several other studies which reported the potential antagonism of *Bacillus* species against *P. capsici*. For example, Lee et al., (2008), on the basis of 16S rDNA sequencing, showed that the most effective isolates with antagonistic activity against *P. capsici* were *Bacillus subtilis*. In addition, differences were observed between the studied sites regarding the relationships between the soil parameters and the pathogen, results which indicated that the presence of *Bacillus* was induced by a natural infestation with the pathogen at a neutral pH. This may explain the effectiveness of *Bacillus* spp. in the presence of a native pathogen. The *Bacillus* treatment was also effective in the case of artificially infested soil, as evidenced by Li et al. (2019). The good correlations between the relative abundance of *Bacillus* and the soil properties in the artificially infested soil indicated the important effects of *Bacillus* spp. on nutrient availability and plant growth, as already described by Charest at al. (2005) and Garcia et al. (2011).

The interaction between *P. capsici* – BCA’s strains at the rhizosphere level showed the presence of *Alphaproteobacteria* enrichment and, in particular, the more relative abundance of *Bradyrizobium*, *Mesorhizobium*, *Hypomicrobium*, thus indicating that they may be involved in the disease suppression of *P. capsici*. As pointed out by Compant et al., (2010), rhizobia may play a very important role in biocontrol, due to the secretion of antibiotics and hydrogen cyanide (HCN), and also due to the production of siderophores.
The fungal community was characterised by the abundant presence of Ascomycota after all the treatments administered including the compost ones, as also shown by Blaya et al., (2016) and Langarica et al., (2014). The variation of the mycobiota at a phylum level was probably principally due to the type of substrate rather than to the pathogen infection. It should be considered that different genera, such as Trichoderma spp. and Fusarium spp., were abundant in all the treatments, independently of the pathogen infection. Trichoderma, Fusarium and Cladosporium species have been considered as biological control agents (Kaur et al., 2010, Lopez-Mondeja et al., 2010) and can be speculated to have contributed to the disease suppression to a great extent. The fungal species belonging to Leotiomycetes (Wang et al., 2006) have the potential to improve nutrient acquisition and combat pathogenic taxa, maintaining cooperative metabolic associations with other species. Species of Aspergillus and Pseudoritium, the potential phosphate-solubilising fungal genera, were also detected in this study. Nevertheless, further research must be considered to better understand the functional composition of fungal community as a wide range of fungi may be involved effectively in the pathogen control. In addition, the temperature, soil moisture and soil type must be taken into account as this are among the most import factors that affect the composition and structure of a microbial community (Spadaro and Gullino, 2005; Cregger et al., 2015).

However, to better understand the effects of the different used treatments, it is necessary to focus on the functional mechanism induced by the interaction of the pathogen with the BCA’s newly introduced into the system and dictated by the chemistry soil parameters, particularly the pH. Hence, further studies are necessary considering several soil types characterized by contrasting physico-chemical parameters.

Conclusions and outlook

The use of Bacillus subtilis and Trichoderma spp., and two different composts has been effective in reducing P. capsici abundance and the disease severity at both experimental sites in naturally and
artificial infested soils. In addition, the results have shown that, in general, the *Bacillus subtilis* and *Trichoderma* spp. based treatments resulted in a good feedback of the resident *Trichoderma* spp. and *Bacillus* spp. communities from rhizosphere. After applied BCA’s treatments, the antagonists established itself in soil together with the same resident populations used the nutrients from the organic matter which boosted their population and made them more competitive in the soil. The disease suppression was probably BCA’s functionally induced and not controlled by changes in the microbial structure or diversity. The differences in the rhizosphere bacterial community in both sites were not attributed to the treatments, but rather to the soil pH and to the origin of the pathogen infection. On the other hand, the fungal community was found to depend on the type of rhizosphere as substrate, but was not affected by the modality of plant infection (e.g., naturally or artificially).

However, the complex phenomenon of disease suppressiveness of the studied soils cannot simply be ascribed to a single bacterial or fungal taxon, or group, but is most likely governed by microbial consortia. These results suggested that the next-generation sequencing approaches is useful to investigate microbial interactions, as essential knowledge may be acquired on the impact that beneficial microorganisms have on non-targeted microbial communities in the rhizosphere, while controlling the pathogen. Thus, the modern approach of this study has highlighted and confirmed the potential of *Bacillus subtilis* and *Trichoderma* spp. used alone or in microbial consortia in combination also with organic material, as biocontrol agents for a sustainable management of Phytophthora crown rot of zucchini, since the disease severity was reduced without causing any significant change in the resident microbial communities under the considered field conditions.

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Conflict of Interest

The authors declare that they have no conflict of interest. Massimo Pugliese declares he has a financial interest (shareholder) in the company AgriNewTech that provided some of the products tested in this study.

References


Figure 1 Boxplots of the α-diversity measures of microbiota (Panel A) and mycobiota (Panel B) of the soils. Boxplot display Chao1 index, estimating the number of different taxa, Shannon diversity index evaluating the taxa richness as well as number of OTUs observed. The bars are color-coded according to the site and the sampling year. Individual points and brackets represent the richness estimate and the theoretical standard error range, respectively.

Figure 2 Principal coordinate analysis of the Unweighted UniFrac distances of the 16S rRNA gene sequence data. The samples are color-coded according to the site and the sampling year.