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Influence of different biological control agents and compost on the rhizosphere and soil total and nitrification driving microbial communities in a lettuce – *Fusarium oxysporum* f.sp. *lactucae* pathosystem

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Abstract

The response of rhizosphere and bulk soil indigenous total microbial communities focusing on nitrifying prokaryotes was evaluated after the application of different biological control agents (BCAs) (*Bacillus subtilis*, *Trichoderma asperellum* + *Trichoderma gamsii*, *Trichoderma W2*, *Pseudomonas putida*) and compost in controlling lettuce Fusarium wilt. Experiments were conducted *"in situ"* over two lettuce cropping seasons. Total fungal, bacterial and archaeal microbial populations, as well as the ammonia oxidizing groups (i.e., AOB, AOA) and the pathogen, *Fusarium oxysporum* f.sp. *lactucae* (FOL) have been screen out using quantitative polymerase chain reaction (qPCR) method. The BCAs - like communities and different antifungal functional genes (fungal chitinase *chiA*, and bacterial 2,4-diacetylphloroglucinol - *phlD* and HCN synthase - *hcnAB* abundances have been also assessed. Quantitative data were corroborated with the disease index, the potential nitrification activity and chemical parameters of the soil.

The application of BCAs and compost resulted in a significantly lower disease severity than the untreated control, and Fusarium wilt was reduced by as much as 60%. This result was confirmed by significant negative correlations between abundance of BCA-like microbial population and disease severity. The BCAs and compost treatments did not negatively affect the microbial communities at the rhizosphere or bulk soil level. The presence of FOL in the untreated control resulted in a niche differentiation of the nitrifiers. Overall, the observed decreased FOL abundance, the increased abundance of inoculum-like populations and the active response of nitrifiers community pointed out the sustainability of the used treatments which achieved an important level of disease suppression.

Keywords: BCAs; disease suppression; nitrifying prokaryotes**;** *chiA* gene, *phlD* gene; *hcnAB* gene

1. Introduction

Lettuce is cultivated and consumed by the whole world and its production exceeded 24 million tons in 2014 (FAO, 2014). Lettuce crops are grown intensively in Mediterranean regions. Spain, Italy and France are the three most important producing countries, with 35, 21, and 13%, respectively, of lettuce production in Europe in 2010 (Eurostat, 2012). *Fusarium oxysporum* f. sp. *lactucae* (FOL) is a soil-borne, host-specific lettuce pathogen that has been observed in Italy since 2002 (Garibaldi et al., 2002), which causes serious economic losses (Matheron & Gullino, 2012). Its management has been investigated intensively through different methods, such as solarization (Matheron and Porchas, 2010) or the application of different organic amendments (Franceschini et al., 2016; Gilardi et al., 2016). However, the seed transmission of this pathogen makes soil disinfection only partially effective (Garibaldi et al., 2004; Gilardi et al., 2007). For these reasons, the use of biological control agents (BCAs), based on antagonistic microorganisms or different composts (e.g., compost with inoculated BCA, green compost), is considered an effective and sustainable strategy (Barrière et al., 2014; Pugliese et al., 2015). Species of the *Trichoderma* genus (*T. harzianum, T. viridi)* have been shown to be efficient "biofungicides", as they are involved in the suppression of different pathogens, including *Fusarium oxysporum* f. sp. *lactucae* (Gilardi et al., 2007, 2013; Innocenti et., 2015). *Pseudomonas* spp. is known to be responsible for the "natural" suppressiveness of some soils against soil-borne pathogens (e.g., *Pythium ultimum*, *Rhizoctonia solani*) (Weller et al., 2002; 2007), and the same phenomenon has also been observed for various *Bacillus* species (e.g., *B. subtilis, B. thuringiensis, B. amyloliquefaciens*) (Cawoy et al., 2011; Kloepper et al., 2004). Many studies have been conducted on the effects of BCAs and compost on disease control (Bonanomi et al. 2007; Berg, 2009; Pane et al. 2013). The presence of BCAs candidates in compost, belonging to the *Trichoderma*, *Pseudomonas* and *Bacillus* genera, has also been demonstrated (Pugliese et al., 2008).

BCAs are known to be aggressive colonizers that indirectly affect the functional guilds in the rhizosphere environment (Gupta et al., 2012). However, their introduction into a new soil system is a complex process which may disturb the indigenous soil microorganisms that play crucial roles in

the essential nutrient cycles, especially nitrogen (N) cycle. A critical component of the microbially driven N cycle is the nitrification process, encoded by *amoA* as key genes that controls the availability of soil nitrates (Nicol et al., 2008; Zhang et al., 2013). N supply and form affect lettuce plant defense and FOL pathogenicity (Orr and Nelson, 2018). Nevertheless, little information are available regarding the impact of BCAs and compost may have on ammonia-oxidizing bacteria or on the archaea that harbor the *amoA* gene (AOB and AOA) (Musyoki et al., 2014; Zimmermann et al., 2016), and in consequence on the soil N form which may further control FOL suppression. Hence, there is a critical need to study the potential side - effects of the applied treatments on both AOB and AOA.

The primary objective of this study was to explore the direct impact of BCAs and compost treatments *"in situ"* on the abundance of total fungi, bacteria and archaeal communities, as well as on the ammonia oxidizing groups (i.e., AOB, AOA) in the FOL affected lettuce rhizosphere and bulk soil. Moreover, fungal and bacterial recruitment upon pathogen infection, has been assessed by studying three anti-pathogen genes that are usually correlated with disease suppression: the *Trichoderma* based chitinase *chiA* gene, and two well-described bacterial antifungal genes: *phlD* (coding for 2,4-diacetylphloroglucinol - 2,4-DAPG) and *hcnAB* (coding for hydrogen cyanide - HCN).

2. Materials and Methods

2.1. Layout of the experiments

The experiments were carried out in tunnels (6 x 60 m) on a commercial farm in Moretta (Cuneo, Northern Italy), which had a history of several lettuce cycles prior to the beginning of this study. The soil was characterized as a silty loam soil (silt : sand : clay 60 : 30 : 10 %, with neutral pH and 1.5 % organic matter) naturally infested with the FOL pathogen.

The lettuce cultivar 'Novelsky', which belongs to the batavia type and is moderately susceptible to Fusarium wilt was used (Gilardi et al., 2017). The experimental trials were carried out over two years, 2016 and 2017, in order to test the efficacy of pre-planting treatments with different commercially available *Bacillus subtilis*: Serenade Max – SM, QST713, Bayer Crop Science, Italy and *Trichoderma gamsii* + *Trichoderma asperellum*: Remedier – RM ISAGRO, Italy) and experimental BCAs (*Trichoderma, TW2,* AgriNewTech, Italy and *Pseudomonas putida* - Pp, Agroinnova, Italy) (Table 1) against lettuce wilting. In addition, two composts (Ant's Compost V and Ant's Compost M; AgriNewTech, Italy), produced from green wastes, in a dynamic industrial treatment system, were used (Table 1): one green compost - CV and the same compost inoculated with *Trichoderma TW2* - CM. An untreated control was used to monitor Fusarium wilt development. Summarized details of these treatments can be found in Table 1.

Lettuce seeds were sown in 100-plug trays (3.4 cm diameter pots, 4 l of soil, 53 x 42 cm surface) filled with a peat mixture substrate.

Four substrate (i.e., peat) treatments with BCAs were carried out on the plug tray between sowing and transplanting (at the first true leaf stage) in a commercial nursery; incubation until transplanting was conducted under greenhouse conditions at a temperature of 22-24°C (Table 1). The BCAs were applied by spraying them onto the peat surface in a high volume of water (500 ml/tray) using a 1 l capacity hand sprayer. The compost products were distributed in a thin layer of 8 g/plug at sowing (T0) over the surface of 53 x 42 cm plug trays, then mixed with the soil immediately before transplanting (T20). Azoxystrobin (Ortiva, 23.2% a. i., Syngenta Crop Protection, Italy) was used as the reference chemical treatment and was applied once as a soil drench at transplanting.

Twenty day old lettuce plants were transplanted (at T20) at a density of 24 plants/m^2, drip irrigated and grown according to the cultural practices adopted by commercial growers in the region. Each treatment had four biological replicates arranged in a completely randomized block design. The plants were monitored weekly for symptoms development, and the data were recorded starting from the appearance of the first symptoms (yellow leaves and reduced growth). The final disease rating was carried out 48-53 days after transplanting on 16 plants/treatment by dissecting each plant. 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. Lettuce yield was measured at the end of the trials by collecting 12 plants per treatment, and determining the fresh weight of the plants.

2.2. Rhizosphere and bulk soil sampling

The influence of soil-treatments with BCAs and compost on indigenous microbial communities was studied at the rhizosphere and bulk soil levels by collecting samples at the end of the trials (the end of September 2016 and 2017). One composite rhizosphere sample per plot consisted of the roots of five randomly selected lettuce plants. The roots were shaken vigorously to remove the soil particles that were not tightly adhering on 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. Soil bulk samples were taken also in three replicates (0-15 cm soil depth) per treatment. Each replicate consisted of five sub-replicates collected from the same plots as the rhizosphere samples. The replicates were sampled in a W - shaped sampling pattern, to obtain a good bulk soil representation and homogeneity. The samples were then placed in plastic bags, kept on ice and transported to the laboratory.

The fresh rhizosphere and bulk soil samples were homogenized, passed through a 2 mm sieve and stored at 4°C. All the samples were split into two parts: one part was sent to the Regional Chemistry

Laboratory (Turin, Italy) for further geochemical analysis: humidity, pH, total N (TN), inorganic N as nitrate (NO_3^-) and ammonium (NH_4^+), total organic carbon (TOC), total phosphorus (TP) and available phosphorus (AP). The second part was used for enzymatic measurements (i.e., potential nitrification activity – PNA) and further microbiological and molecular investigations.

2.3. Molecular analyses

2.3.1. Rhizosphere and bulk soils DNA extraction

A NucleoSpin® Soil kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used, according to the manufacturer's instructions, for the rhizosphere and bulk soil genomic DNA extraction. Extraction was conducted on fresh samples (250-500 mg of sample material). DNA quantity and purity were measured spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

2.3.2 Microbial abundance (quantitative PCR (qPCR) assays)

The abundance of the bacterial and archaeal 16S rRNA genes, fungal 18S rRNA genes and of five functional genes (i.e., bacterial ammonia monooxygenase *amoA* gene - AOB, archaeal *amoA* gene - AOA, fungal chitinase *chiA* gene, and bacterial 2,4-diacetylphloroglucinol *phlD* and HCN synthase *hcnAB* genes) was determined by means of qPCR, using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) for the rhizosphere and bulk soil DNA samples. In addition, the abundance of FOL and BCAs - like microorganisms (i.e., *Bacillus*, *Trichoderma* and *Pseudomonas*) was also investigated. A description of the primer sets and amplification details are given in Table 2.

The primers for fungal chitinase *chiA* gene quantification were designed as follows: DNA from rhizosphere and bulk soil samples from the treatments with *Trichoderma* sp. was used to amplify the fungal chitinase *chi42* gene, according to the Nguyen HL et al., (2001) protocol, with CHIf/CHIr primers, which generally amplify a 1450 bp sequence. Four new primer pairs targeting

an internal fragment of the *chi42* gene, were designed using Gen Script (http://www.genscript.com/tools.html#biology), considering relatively high annealing temperatures (≥ 60 °C) and smaller amplicon sizes than 200 bp to reduce generation of artifacts and to achieve appropriate reaction efficiencies during qPCR. The accuracy of the primers was confirmed using BLAST of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov); preliminary tests (data not shown) were carried out to assess their specificity, and the best primer pair was then chosen. The designed primer pair (chiaxF (ACCCTGCCGATGACACTCAG)::chiaxR (GGCAGCGATGGAGAGAAGGA) generated an amplicon of 122 bp, with the following sequence: 5'ACCCTGCCGATGACACTCAGGCCACCAACATGGTTCTTCTGCTCAAGGAGATCCGAT CTCAACTAGATGCCTATGCTGCGCAATACGCTCCAGGCTACCACTTCCTTCTCTCCATC GCTGCC-3'.

Amplicons from each target gene were generated for the standard preparation, 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 ligation products were then transformed into StrataClone SoloPack competent cells (Agilent Technologies Inc.). The specificity of the clones used as qPCR standards was checked via sequencing, at LGC Genomics GmbH (Berlin, Germany) and through BLAST analysis. Plasmid DNA was isolated (GenElute™ Plasmid Miniprep Kit, Sigma-Aldrich, St. Louis, MO, USA) from standard clones and quantified as described above.

As assessed in preliminary tests and in a qPCR assay optimization, the final volume of the qPCR reaction cocktails was 25 µl for the 16S and 18S rRNA genes, the bacterial and archaeal *amoA* gene, and the FOL and BCA - like microorganisms quantification, and it was 20 µl for the *chiaA*, *phlD* and *hcnAB* genes. All the quantifications were conducted with a 10 ng DNA template, except for the bacterial 16S rRNA gene, for which a 5 ng DNA template was used (Cucu et al., 2017). The reaction mixtures contained 1x Power SYBR green master mix (Applied Biosystems), 0.12 µM of

each oligonucleotide (Table 2) for the total bacteria, archaea, fungi and FOL, 0.15 μM for the *phlD* and *hcnAB* genes, 0.32 μM for the AOB, AOA and BCA - microorganisms and 0.4 μM for the *chiA* gene.

All the considered genes were quantified in triplicate across plates, while standards were run in duplicate in 10-fold serial dilutions. The optimal dilution of the DNA extracts was tested to compensate for any inhibitory reactions by the organic compounds (e.g., humic acids) co-extracted during the DNA isolations. In all experiments, negative controls containing no-template DNA were subjected to the same qPCR procedure to detect and exclude any possible contamination. Amplification efficiency ranged from 96%, (archaeal 16S rRNA, fungal 18S rRNA, BCAs and their functional genes) to 103% (bacterial 16S rRNA). For the nitrifiers, the amplification efficiency were 95.3% and 99.1% for AOB and AOA, respectively. The R2 was always ≥ 0.98. Melting curves of the amplicons were generated to ensure that the fluorescence signals originated from specific amplicons and not from primer dimers or other artifacts. This was confirmed by checking the amplification products on 1% agarose gel. Gene copy numbers were calculated with StepOne™ software, version 2.2 (Applied Biosystems). The data were normalized and presented as log copies g^{-1} dry soil.

2.4. Chemical properties of rhizosphere and bulk soil samples

In short, the pH values were measured in water suspensions at a solid: liquid ratio of 1:2.5. The total organic carbon (TOC) and total nitrogen (TN) were quantified using a Leco Tru Spect CN automatic analyzer. Ammonium (NH_4^+) and nitrate (NO_3^-) were measured colorimetrically, by means of a continuous flow auto-analyzer (Alliance Evolution II), using standard colorimetric techniques. The total phosphorus (TP) was determined by means of 'ICP Varian mod. Liberty LR', after microwave digestion with hydrogen peroxide, hydrochloric acid and nitric acid, filtration and dilution. The available P (AP) was extracted using sodium bicarbonate and determined by means of

the molybdenum blue method (Olsen et al., 1954), modified for continuous flow colorimetric analysis (Alliance Evolution II).

The potential nitrification activity (PNA) was determined according to the procedures described by Hoffmann et al. (2007).

2.5. Statistical analyses

The data on disease index, gene abundance, chemical properties of the soil and potential nitrification activity were subjected to a linear mixed model, with year considered as the random variable, using R software (Software R 3.0.1, R foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org). The effects of different BCAs and compost on the abundance of the studied genes, on the chemical properties of the soil and on disease severity were evaluated. All data were subjected to a Levene test to check for the homogeneity of variance and normality was tested on the residuals using the Shapiro-Wilk test; when not normally distributed data from disease severity (DS) were arcsin transformed while, the data from microorganisms abundance were log-transformed and normality was checked again. The means were separated by Bonferroni test. The statistical analysis included treatment rhizosphere soil, bulk soil, year, treatment × year.

Pearson's linear correlation coefficients were calculated for assessing the relations between microbial gene abundance, disease severity and PNA, as well as between *Pseudomonas*, *Bacillus*, *Trichoderma* and FOL abundance and soil chemical properties.

Linear regression between total microbial gene abundance and the functional genes of N cycle (bacterial *amoA*, archaeal *amoA*, *chiA* gene) with soil chemical data were calculated to evaluate how much of their abundance is explained by variation in the soil chemical data.

3. Results

3.1. Disease severity and plant biomass

The lettuce plants in the non-treated control plots showed an average disease severity DS of up to 60% at the end of both trials (Table 3). The BCA treated plots provided significantly lower disease severities than the untreated ones, and statistically similar results to one application of azoxystrobin, reducing Fusarium wilt by 47.2 to 58.5% in the two years, respectively. As a consequence, the fresh weight of the lettuce was significantly higher in all the treatments compared to control, without statistically differences among them (Table 3). No significant difference was observed between the disease severity data. A significant time effect for fresh weight of the lettuce between trials was noted ($p < 0.001$). However, the *Pseudomonas* and *Trichoderma* treatments were less effective in the second trial.

3.2. Microbial abundance

3.2.1. FOL abundance

In general, FOL abundance assessed by gene copy quantification was significantly higher in the bulk soil than in the rhizosphere soil. Almost all the treatments resulted in a significant reduction of pathogen - associate gene copies compared to the untreated control - C, in both the rhizosphere and bulk soil at the end of both trials (2016 and 2017 respectively) (Table 4). At the end of first trial (2016), Serenade max - SM - *Bacillus* treatment was not significantly different compared with the untreated control - C at the rhizosphere level, while at the end of the second trial (2017) the *Pseudomonas* - Pp treatment was not significantly different compared with the untreated control – C. The interaction treatment × year was not significant (Table 4).

3.2.2. Total microbial population abundance

At the end of both trials (2016 and 2017 respectively) the number of gene copy of bacterial, archaeal and fungal populations showed an important homogeneity, with no significant interaction treatment × year. Therefore the data were presented as average of all values from both trials.

Overall, at the end of both trials (2016 and 2017 respectively) the bacterial 16S rRNA abundance was significantly higher at the bulk soil level than at the rhizosphere level (Figure 1a). The effect of BCAs treatments was more evident in the rhizosphere; the Remedier - RM - *Trichoderma* spp. and *Pseudomonas* - Pp resulted in a lower bacterial abundance than the other treatments but not significantly different than the untreated control - C, while the Serenade max - SM - *Bacillus* treatment resulted in a significantly higher bacterial abundance than the untreated control - C. However, the bacterial community abundance was more or less stable at the soil level over all the treatments (Figure 1a). The archaeal 16S rRNA gene copy number in the rhizosphere followed almost the same trend as the overall bacterial counterpart. The green compost - CV and *Trichoderma* W2 - TW2 treatments resulted in slightly higher archaeal abundance in the bulk soil samples than the untreated control - C (Figure 1b). The fungal 18S rRNA abundance was significantly higher in the rhizosphere than in the bulk soil samples, in all the treatments as well as in the untreated control - C. Remedier - RM - *Trichoderma* spp. inoculation resulted in a slightly higher fungal abundance than the untreated control - C, while the total fungal community in the soil samples was more abundant in the Serenade max - SM - *Bacillus*, *Pseudomonas* - Pp and green compost - CV treatments (Figure 1c).

3.2.3. Functional gene abundance

In general, the ammonia-oxidizing bacterial (AOB) gene abundance was significantly influenced by treatments and was significantly higher in the bulk soil than in the rhizosphere samples (Table 5). After the first trial, 2016, the AOB gene in the bulk soil samples was more abundant in the untreated control - C, while the lower abundance was observed after Serenade max - SM - *Bacillus* treatment. At the rhizosphere soil level the Serenade max- SM - *Bacillus*, green compost - CV and

Trichoderma W2 – TW2 treatments resulted in a highest AOB gene abundance while no significant difference was observed between Remedier - RM-*Trichoderma* spp., *Pseudomonas* – Pp and the untreated control - C. After the second trial, 2017, the same trend of AOB abundance was observed at the bulk soil level, while at the rhizosphere soil level the Remedier - RM-*Trichoderma* spp. and *Pseudomonas* – Pp treatments resulted in the lowest AOB abundance. The interaction treatment × year was not significant. (Table 5).

In general, the ammonia-oxidizing archaeal (AOA) gene abundance was significantly influenced by treatments and was significantly higher in the bulk soil than in the rhizosphere soil samples (Table 5).

All the treatments resulted in higher archaeal *amoA* gene abundance than the untreated control - C, at both rhizosphere and bulk soil levels for both trials, 2016 and 2017 respectively. After the first trial, 2016, the green compost - CV treatment resulted in the highest AOA abundance than the other treatments at the bulk soil level, while the Serenade max - SM – *Bacillus* and *Trichoderma* W2 – TW2 treatments resulted in the highest abundance than the other treatments at the rhizosphere soil level. After the second trial, 2017, the green compost - CV and the Remedier - RM-*Trichoderma* spp treatments resulted in the highest AOA abundance at the bulk soil level, while the Serenade max - SM – *Bacillus* treatment resulted in the highest AOA abundance at the rhizosphere level. The interaction treatment × year was not significant.

In general, the *chiA* gene (*Trichoderma* based chitinase) abundance was significantly influenced by treatments (Table 6). After the first trial, 2016, the abundance of the *chiA* gene was higher in the rhizosphere soil samples for the *Trichoderma* TW2 – TW2 and Remedier - RM-*Trichoderma* spp treatments. However the Remedier - RM-*Trichoderma* spp was not statistically different compared with the inoculated compost – CM. The *Pseudomonas* - Pp and the green compost - CV treatments resulted in the lowest *chiA* gene abundance compared with the other treatments, but not significantly different compared with the untreated control - C. At the bulk soil level, the *chiA* gene was more abundant in the Remedier - RM-*Trichoderma* spp, *Pseudomonas* - Pp and green compost

- CV treatments comparing with the other treatments and with the untreated control - C (Table 6). After the second trial, 2017, all the treatments at the rhizosphere soil level resulted in significant differences between the *chiA* gene abundances. The highest *chiA* gene abundance was observed in the *Trichoderma* W2 - TW2 treatment. Also at the bulk soil level all the treatments resulted in higher *chiA* abundance compared with the untreated control - C. The highest *chiA* gene abundance was observed in the *Pseudomonas* - Pp and green compost - CV treatments.

The abundance of the *phlD* gene was significantly higher in the rhizosphere than in the bulk soil samples for all the treatments and for the untreated control - C (Table 6). After the first trial, 2016, *Pseudomonas* - Pp and the green compost - CV treatments resulted in the highest *phlD* abundance at the rhizosphere soil level, while the same treatments resulted in the lowest *phlD* abundance at the bulk soil level. After the second trial, 2017, *Pseudomonas* - Pp treatment resulted in the highest *phlD* abundance at the rhizosphere soil level. At the bulk soil level, the highest abundance was observed after *Trichoderma* W2 - TW2 treatment, while *Pseudomonas* - Pp and the green compost – CV treatments resulted in a lower *phlD* gene abundance than the untreated control - C.

The abundance of the *hcnAB* gene was significantly influenced by treatments and it was significantly higher in the rhizosphere than in the bulk soil samples for all the treatments and for the untreated control - C (Table 6). After the first trial, 2016, the highest *hcnAB* gene abundance was observed after *Pseudomonas* - Pp , inoculated compost – CM and *Trichoderma* W2 – TW2 treatments at the rhizosphere level. At the soil level the *hcnAB* gene abundance was not significantly different compared with the untreated control –C, with the exception of *Pseudomonas* - Pp and the green compost - CV treatments which resulted in the lowest *hcnAB* gene abundance. After the second trial, 2017, the *hcnAB* gene abundance was highest after the inoculated compost - CM treatment and in the untreated control - C was noted the lowest *hcnAB* gene abundance at the rhizosphere level. At the soil level the *hcnAB* gene abundance was higher in the untreated control C and the *Trichoderma* W2 – TW2 treatment while the lowest *hcnAB* gene abundance was noted after *Pseudomonas* – Pp and the green compost – CV treatments.

3.2.4. BCAs - like microorganisms abundance

Overall, at the end of both trials (2016 and 2017 respectively) the BCA applications resulted in an increase in the indigenous populations of *Bacillus, Trichoderma* and *Pseudomonas* in all the treatments, compared to the untreated control - C (Table 7). No significant interaction treatment × year was observed. In general, the abundance of *Bacillus* sp. was significantly higher for the *Bacillus* - SM treatment than the untreated control - C and all the other treatments in both the rhizosphere and bulk soil. However, the inoculated compost treatment - CM also resulted in an increased resident *Bacillus* abundance in rhizosphere and bulk soils after both trials. Nevertheless, the *Trichoderma* TW2 - TW2 treatment showed similar levels to those of the untreated control - C. Significant interactions bulk × rhizosphere were observed (Table 7).

The inoculation with the *Pseudomonas* - Pp significantly increased the overall *Pseudomonas* community in both the rhizosphere and bulk soils, compared to the untreated control - C and the other treatments. Significant interactions bulk × rhizosphere were observed (Table 7).

The inoculation with the *Trichoderma* - based formulate significantly increased the overall *Trichoderma* community in both the rhizosphere and bulk soils, compared to the untreated control - C. After both trials, the highest abundance of *Trichoderm*a was observed in the rhizosphere after *Trichoderma* TW2 - TW2 treatment and in the bulk soil after the green compost – CV treatment. After the second trial, 2017, a highest *Trichoderma* abundance was also observed after the inoculated compost - CM treatment at the bulk soil level. The Serenade max - SM – *Bacillus* application resulted in somewhat lower *Trichoderma* abundance than all the other BCA applications. Significant interactions bulk × rhizosphere were observed (Table 7).

3.3. Chemical properties

Generally for both trials, 2016 and 2017 respectively, pH was significantly lower ($p < 0.05$) at the rhizosphere level than at the bulk soil level, with values ranging from between 5.70 and 6.43 for the rhizosphere and between 6.34 and 6.77 for the bulk soil samples. The concentrations of TN, NH_4^+, NO_3^- and TOC were generally significantly higher in the rhizosphere than in the bulk soil samples. The untreated control - C was characterized by higher NH_4^+, NO_3^- and TOC concentrations than all the treatments. On the other hand, the highest TP and AP contents were observed in the bulk soil samples (Table S1). No significant treatment × year interaction was found.

3.4. Potential Nitrification Activity (PNA)

The PNA measurements generally exhibited significantly higher values at the bulk soil level than in the rhizosphere level. No significant treatment × year interaction was observed, therefore the data were presented as the average of all data from both trials, 2016 and 2017 respectively. The PNA in the bulk soil was significantly ($p < 0.05$) higher in the untreated control - C, followed by the inoculated compost - CM and the *Pseudomonas* - Pp treatments. The lowest value was observed after both, Serenade max - SM – *Bacillus* and Remedier - RM-*Trichoderma* spp treatments. The PNA in the rhizosphere was significantly lower ($p < 0.05$) in the untreated control - C than in the other treatments. (Figure 2).

3.5. Correlations between the levels of total microbial communities (16S bacteria, 16S archaea, 18S fungi), the functional genes (fungal *chiA* gene, bacterial and archaeal *amoA* genes, bacterial *phlD* and *hcnAB* genes), the *Bacillus*, *Pseudomonas*, *Trichoderma* population levels and FOL abundances, DS and PNA.

In general the correlations were negative, with r coefficients ranging from -0.37 to -0.5 ($p < 0.001$) at the rhizosphere level and from -0.3 to -0.6 ($p < 0.001$) at the bulk soil level (Table 8). Very strong positive correlations were observed between FOL and DS, as well as between the nitrification rate - PNA and DS. Strong negative correlations were recorded at the rhizosphere and

bulk soil levels especially between AOB and DS at the soil level, and between AOA and PNA at the soil level (Table 8).

3.6. Correlations between *Bacillus, Pseudomonas, Trichoderma* population levels and chemical properties in rhizosphere (rhizo) and soil samples

In general the correlations were significant only with respect to AP, TP, NO_3^- and TOC, with r values ranging from -0.5 to 0.8 (Table 9).

3.7. Regressions between indigenous microbial communities and soil chemical properties

Variance in the total bacterial abundance was explained by alterations of NO_3^- and TP at the rhizosphere level (Table S2). Changes in the total archaeal abundance were explained by alterations of NO_3^- and TP at the rhizosphere and soil level respectively. Changes in the total fungal community were explained by alterations of TP and TOC at the rhizosphere level and TP at the soil level. The variance in *chiA* gene abundance was explained by alteration of TN including NO_3^- and NH_4^+ at both rhizosphere and soil level. AOA abundance changes were explained by TN, NO_3^- and TOC at the rhizosphere level and by NO_3^- at the soil level. However, a greater percentage of AOB was explained also by NO_3^- (Table S2).

4. Discussion

In the present study, rhizosphere and soil microbial community changes have been investigated monitoring total bacterial, archaeal and fungal highly distinctive genes abundance as indicators of the general alterations that may take place as a result of plant treatments and the infection of lettuce by FOL. In this context, the potential impact of various BCA and compost treatments on the total indigenous microbial communities and on the functional genes that controls nitrogen availability was assayed.

4.1. Disease severity, FOL *abundance and plant biomass*

The obtained results were in line with previous studies showing that the application of BCAs and certain organic matter can effectively suppress soil pathogens (Zhang et al., 2008; Luo et al., 2009; Wu et al., 2009; Ling et al., 2010; Cao et al., 2011; Lang et al., 2011; Yang et al., 2011; Gilardi et al., 2016). The application of different BCAs and compost significantly reduced wilting by suppressing FOL abundance compared to the untreated control. This result was corroborated by the significant positive correlations ($p < 0.001$) that were observed between disease severity and FOL abundance. These findings were supported by previous studies that showed that *Fusarium* abundance in the cucumber and banana rhizosphere was reduced after BCA treatments (Qiu et al., 2012; Shen et al., 2015; Fu et al., 2017). Furthermore, FOL abundance was very low after the treatment with inoculated compost (CM), thus indicating that an effective disease control could be achieved with organic amendments, as previously pointed out (Bonanomi et al., 2010). Similar results were obtained in a previous study (Pugliese et al., 2011), confirming that inoculation of compost with *Trichoderma* strains can improve the suppressive activity of the substrate. The application of compost may have increased the competition for resources and the interactions between antagonist species of compost and soil, and this may have resulted in a reduction in the FOL abundance and in the potential activity of the pathogen (Bonilla et al., 2012; Larkin et al., 2015).

4.2. Ecological fitness of the BCA - like microorganisms and their effect on the total indigenous prokaryotes

Our results have evidenced an excellent feedback of the BCA - *like microorganisms* after the BCAs were introduced into the soil, as inoculated plantlets or inoculated compost. This was highlighted by significant negative relationships ($p < 0.001$) between the BCA – like populations and Fusarium wilt severity, which suggested not only the ecological fitness of inoculated microorganisms, but also their efficiency against FOL. Most probably, the efficiency of biological control of FOL was based on mechanisms as saprophytic competition for nutrients, parasitic competition for infection

sites and induced resistance in host cells (Haas and Defago, 2005; Nel et al., 2006; Srinivasan et al., 2009). Even more, the good relationships between the BCAs - like microorganisms and soil nutrients highlighted the role of the antagonists in solubilising certain compounds (nitrogen, phosphorus, potassium, iron) (Gyaneshwar et al., 2002; Richardson et al., 2009) contributing to the disease suppression.

The application of BCA - based formulates resulted in a subsequent boost in abundance of the respective microbial populations (i.e., *Pseudomonas*, *Trichoderma* and *Bacillus*). A remarkable result of this study concerns the high abundance that was observed in the rhizosphere samples of the *phlD* and *hcnAB* genes known to encode the production of antifungal compounds such as 2,4-diacetylphloroglucinol (DAPG) and hydrogen cyanide (HCN) (Dudenhoffer et al., 2016). We speculated that *Pseudomonas spp* (e.g., *P. fluorescens*) abundance would have increased in the rhizosphere environment upon pathogen infection, as these bacteria are strong root colonizer (Looper et al., 2012). This may explain the lower observed abundance of the total *Pseudomonas* community compared with other treatments. This was probably due to the action of antifungal metabolites against FOL, but also against other microorganisms that generally colonize the rhizosphere. The *chiA* gene abundance confirmed the potential antagonistic activity of the inoculated *Trichoderma* strain TW2, but also of the inoculated compost. In addition, a significant relationship of *chiA* gene abundance with the total and especially with ammonium N ($p < 0.001$) content was observed (Table S2). This result highlighted the involvement of fungal population in remineralization of nutrients during the decomposition of chitin (Gould et al., 1981) with important contribution to nitrification process.

Overall, we did not observe any negative side-effects of BCA application on the total microbial communities, thus indicating that the microorganisms introduced into the soil system may have favoured the resident microbial populations through positive interactions. Our results were in line with previous reports (Ghini et al., 2000; Gullino et al., 1995; Mezzalama et al., 1998). Contrary to our expectations, a higher total fungal abundance was observed in the rhizosphere than in the bulk

soil. The boost in the fungal community in all the treatments and in the untreated control was probably supported by the elevated level of organic carbon (Table S1). This may be linked to a competitive potential, based on an increased root exudation, which favours rhizosphere colonization by fungi, including FOL. In this context, Strange (2005) showed that fungi mobilize nutrients through the production of growth regulators. This may explain the observed positive correlation between AP and the total fungal community in both the rhizosphere and bulk soil. On the other hand, a lower archaeal population level was observed in the rhizosphere, probably as a result of the apparently lower pH. Moreover, the archaeal community may also have been lower due to a lower growth rate and competitiveness than the bacteria and fungi (Karlsson et al., 2012). In addition, in the present study, the archaeal community was negatively correlated ($p < 0.05$) with the disease severity, thus suggesting a likely competition for nutrients with FOL. Thus may underline the possible ability of archaea to contribute to the suppression of lettuce Fusarium wilt development. In a previous study, Mendez et al. (2013) described a relationship between archaeal community composition and soil suppressiveness to *Rhizoctonia* damping-off. However, further investigations are needed to clarify whether archaea play an important role in the effective protection of lettuce against FOL.

4.3. Ammonia oxidizing archaea and bacteria

The archaeal nitrifiers (AOA) were more abundant in the soil samples than their bacterial counterparts. In fact, a lower AOA abundance was observed in the rhizosphere than in the bulk soil, which may be a result of a high organic carbon content and a lower pH (Wessén et al., 2010; Bates et al., 2011). In addition, a low ammonia environment represents the key factor that determines a niche separation of AOA and AOB in acidic/neutral soils. A strong positive relationship was observed between the AOB and TN at the rhizosphere level, and this resulted in a dominance of bacterial nitrifiers and their resilience to lower pH compared to AOA (Wessén et al., 2010). The result supports the finding of Valentine (2007), who showed that all archaea, and their nitrifiers

components in particular, in general have a higher competitive advantage under reduced organic resource conditions. Accordingly, we observed a higher AOA abundance in the soil samples, which indicated that the archaeal nitrifiers, and in general the entire archaeal community, were probably adapted to the decomposition of recalcitrant organic matter, as described earlier on by Cucu et al., (2017).

Overall, the effect of different applied treatments resulted in the increase of nitrifiers abundance with respect to the untreated control thus suggesting that BCAs may not have negative ecological impacts on other groups of microorganisms. Comparing with all treatments,, a higher AOB abundance was observed in the bulk soil of the untreated and FOL infested control, while the opposite was true for the AOA in the rhizosphere, thus suggesting that the sole presence of FOL may has induced a differentiation, in the rhizosphere and bulk soil, among the indigenous prokaryotes involved in the nitrification process. Hence, the AOB abundance was not reduced in the control samples, probably as a result of a niche differentiation among nitrifiers at the rhizosphere and bulk soil levels induced by the FOL presence, rather than the commonly acknowledged direct resource competition with AOA (Nicole et al., 2008; Wessén et al., 2010; Musyoki et al., 2015). Additionally, a strong negative relationship between the nitrification rate and disease severity was observed at the bulk soil level. However, competition for resources may exist between AOA abundance and FOL. This hypothesis was corroborated by the low nitrification rate that was observed at the rhizosphere level especially in the untreated control. Our findings indicated that nitrification may play an important role in disease control when environmental conditions for nitrifiers are favourable. Similarly, Fujiwara et al., (2013) showed that, in a multiple parallel mineralization system, rhizosphere microbiota suppressed the Fusarium wilt disease of lettuce by affecting the morphological characteristics of *F. oxysporum*. However, further research is needed to obtain a better understanding of the promoting effect of nitrifiers after the application of BCAs.

Conclusions and outlook

Our results have shown that the application of different biological control agents and compost resulted in a significant reduction in the Fusarium wilt of lettuce crops, without any significant differences in the degree of disease control among treatments. On the basis of the presented results, it is possible to conclude that the applied treatments (based on *Bacillus*, *Pseudomonas* and *Trichoderma)* did not affect negatively the density of resident microbial communities (i.e., bacteria archaea and fungi) or the nitrifying prokaryotes. Nevertheless, the archaeal nitrifiers had a more dynamic response after the treatments. Hence, more investigations are needed to study the influence of BCA and compost treatments on archaeal communities. In addition, our results have shown that lettuce plants could favour bacterial species which are good lettuce rhizosphere colonizers and may act as antagonists.

Although no negative effects of BCAs were observed in this study on the no-target rhizosphere or on the soil microbial biomass, their application requires further evaluations, including the consideration of a broader range of soils with different physico-chemical properties, as well as additional soil-borne pathogens and different plant species/cultivars, as they may have various effects on the microbial communities and processes in the rhizosphere and bulk soil (Soderberg et al., 2002; Rasche et al., 2006). In addition, the long-term effect on the same crop should be evaluated in order to fully understand the legacy and the results of the interactions of BCAs and compost with microbial biomass and activity. Although this study was focused on the potential functionality of selected microbial populations in terms of abundance it can be assumed that the introduction of different microbial strains or strain combinations could also influence the structure and composition of the overall microbial communities. Therefore, advanced studies on the potential effects of BCAs and organic matrices on the composition and biomass of non-target microbial populations in the crop rhizosphere and bulk soil, using molecular fingerprinting techniques, are still needed.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Captions

Figure 1 Abundance of bacterial (a), archaeal (b) 16S rRNA genes and fungal (c) 18S rRNA genes in rhizosphere and bulk soil between different treatments* and the untreated control (SM - Serenade Max; RM - Remedier; Pp - *Pseudomonas*; CM – Green compost plus *Trichoderma* TW2; CV - green compost; TW2 - *Trichoderma* TW2; C - control) (n = 6, means±standard errors). Different letters above bars indicate significant differences between treatments in rhizosphere (uppercase letters) and bulk soil (lowercase letters)

Figure 2 Potential Nitrification Activity (PNA) in the rhizosphere and bulk soil after different treatments* and the untreated control (SM - Serenade Max; RM - Remedier; Pp - *Pseudomonas*; CM - Green compost plus Trichoderma TW2; CV - green compost; TW2 - *Trichoderma* TW2; C - control) (n = 6, means±with standard errors). Different letters above bars indicate significant differences between treatments in rhizosphere (uppercase letters) and bulk soil (lowercase letters)

*Serenade max – SM: *Bacillus subtilis* ; Remedier – RM : *Trichoderma asperellum* + *T. gamsii*; *Pseudomonas putida* – Pp; ANT'S COMPOST M – CM: Green compost + *Trichoderma* TW2; ANT'S COMPOST V – CV: Green compost; Trichoderma sp. – TW2

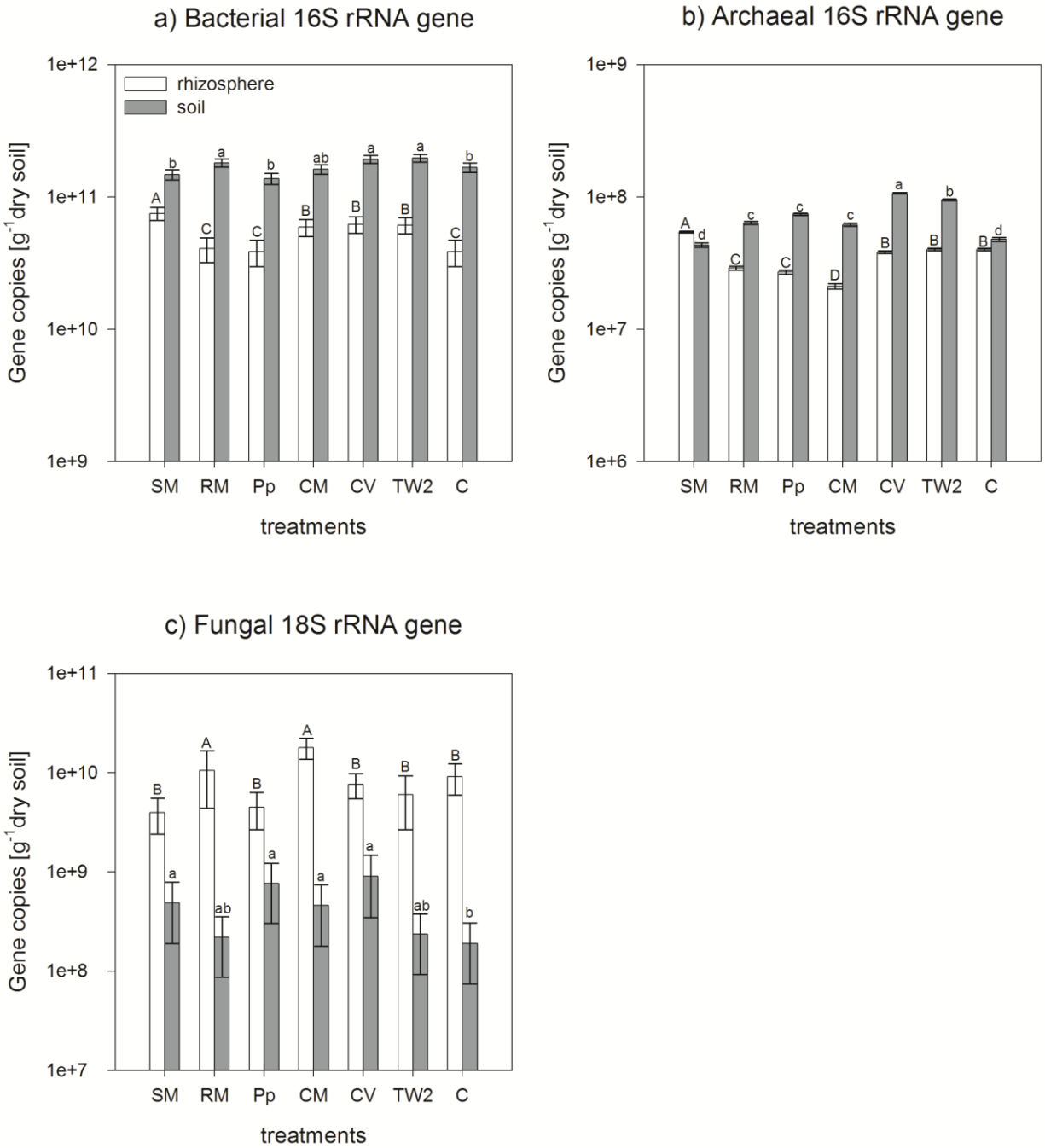
a) Bacterial 16S rRNA gene
rhizosphere
soil
Gene copies [g-1dry soil]
1e+12
1e+11
1e+10
1e+9
SM RM Pp CM CV TW2 C
treatments
b) Archaeal 16S rRNA gene
Gene copies [g-1dry soil]
1e+9
1e+8
1e+7
1e+6
SM RM Pp CM CV TW2 C
treatments
c) Fungal 18S rRNA gene
Gene copies [g-1dry soil]
1e+11
1e+10
1e+9
1e+8
1e+7
SM RM Pp CM CV TW2 C
treatments

Figure1

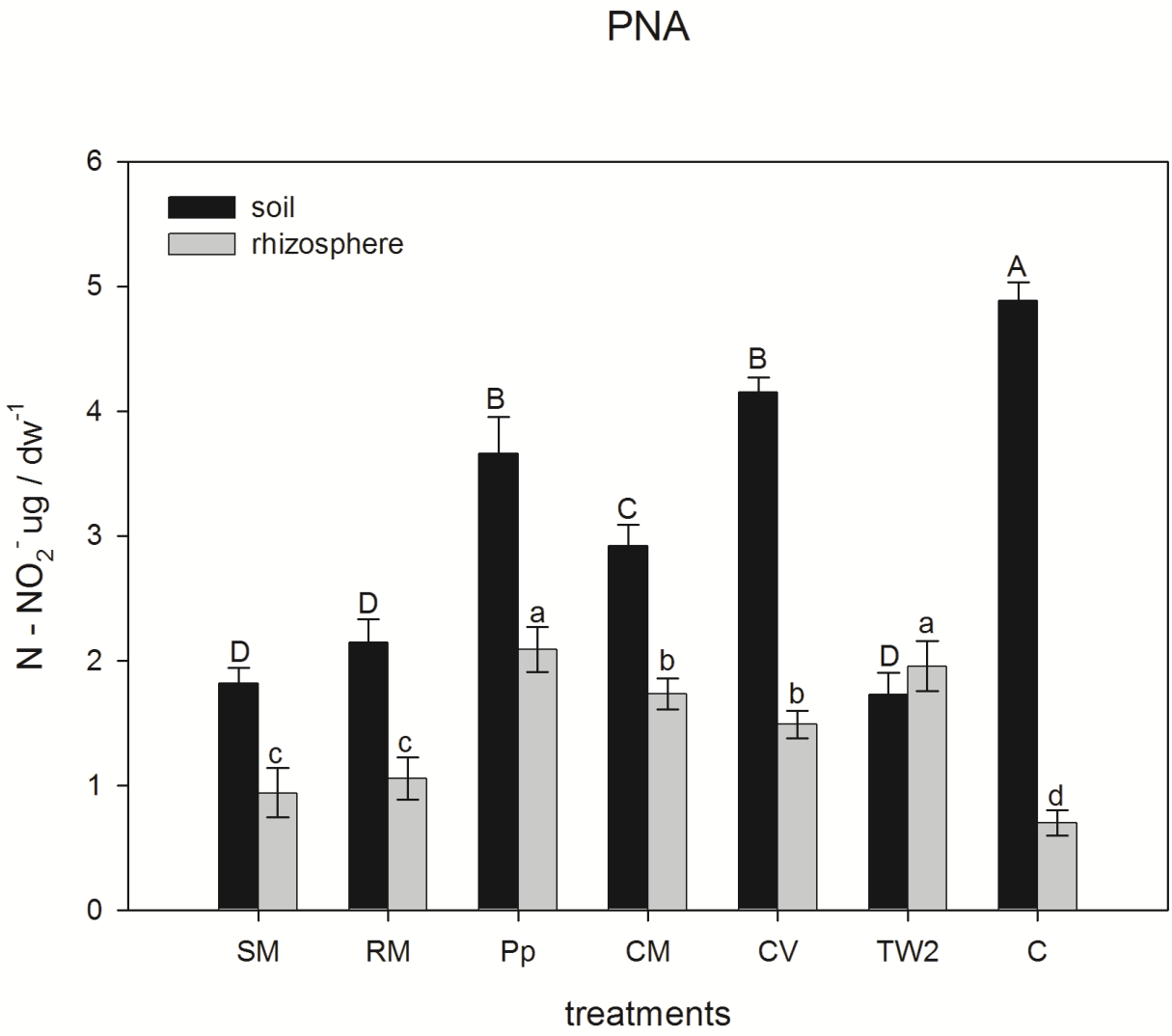
PNA
soil
rhizosphere
N - NO2- ug / dw-1
0
1
2
3
4
5
6
D
c
D
c
B
a
C
b
B
b
D
a
A
d
SM
RM
Pp
CM
CV
TW2
C
treatments

Figure2

Supplementary material

Table S1 Chemical properties of the rhizosphere (rhizo) and bulk soil for all the treatments and the untreated control at the end of trial 1 (2016) and trial 2 (2017). Values are given as average (n = 3). Different letters within a row show significant differences between treatments and the untreated control.

Property	Serenade Max		Remedier		Pseudomonas		Compost M		Compost V		Trichoderma		Control	
	Rhizo	Bulk soil	Rhizo	Bulk soil	Rhizo	Bulk soil	Rhizo	Bulk soil	Rhizo	Bulk soil	Rhizo	Bulk soil	Rhizo	Bulk soi
2016														
pH	5.76±0.1^{d}	6.34±0.1^{d}	6.01±0.2^{b}	6.67±0.2^{b}	5.98±0.2^{c}	6.73±0.1^{a}	6.23±0.1^{a}	6.61±0.1^{b}	5.92±0.2^{c}	6.59±0.2^{b}	6.03±0.2^{b}	6.6±0.1^{b}	5.80±0.1^{d}	6.53±0.1
TN %	0.57±0.2^{d}	0.21±0.05^{a}	0.50±0.2^{d}	0.19±0.08^{a}	0.27±0.3^{f}	0.21±0.04^{a}	0.34±0.1^{e}	0.21±0.05^{a}	0.83±0.1^{b}	0.20±0.03^{a}	0.62±0.3^{c}	0.20±0.08^{a}	1.02±0.1^{a}	0.20±0.0
NH_4^+(mg/kg)	2.9±0.9^{d}	0.23±0.09^{b}	1.6±0.6^{e}	0.39±0.2^{a}	2.07±0.8^{c}	0.27±0.1^{b}	1.13±0.5^{f}	0.29±0.1^{b}	5.29±0.1^{b}	0.33±0.1^{b}	0.6±0.1^{g}	0.36±0.1^{a}	37.55±1.7^{a}	0.42±0.1
NO_3^-(mg/kg)	18.06±1.2^{g}	5.91±1.9^{f}	22.65±1.8^{e}	8.51±1.6^{e}	39.28±1.9^{b}	22.86±1.7^{a}	30.0±0.1^{d}	16.83±0.1^{c}	36.0±0.1^{c}	14.30±0.1^{d}	19.15±0.1^{f}	20.58±0.1^{b}	46.43±0.1^{a}	8.62±0.1
TOC %	19.89±0.1^{c}	1.87±0.1^{a}	13.82±0.1^{d}	1.90±0.1^{a}	20.51±0.1^{c}	1.90±0.1^{a}	7.21±0.1^{e}	1.96±0.1^{a}	31.22±0.1^{b}	1.90±0.1^{a}	19.34±0.1^{c}	1.89±0.1^{a}	43.36±0.1^{a}	1.71±0.1
TP (mg/kg)	906±1.5^{f}	1262.4±2.1^{b}	1020±1.2^{d}	1337.3±0.1.5^{a}	1296±1.2^{b}	1339.7±1.2^{a}	1401±1.6^{a}	1264.9±2.1^{b}	949±1.5^{e}	1254±2.1^{b}	1191±2.2^{c}	1238±2.5^{b}	679±1.5^{g}	1237±2.1
AP (mg/kg)	124.4±0.2^{b}	150.2±2.7^{c}	118.5±0.5^{c}	152.7±2.3^{b}	129.3±0.7^{a}	156.2±2.1^{a}	125.9±0.1^{b}	158±2.31^{a}	93.3±0.2^{d}	159.7±1.8^{a}	113.5±0.1^{c}	156.6±2.6^{a}	83.5±0.1^{e}	159.5±2.
2017														
pH	5.70±0.1^{b}	6.56±0.1^{c}	6.1±0.23^{a}	6.88±0.1^{a}	5.70±0.2^{b}	6.77±0.1^{b}	6.43±0.1^{a}	6.76±0.1^{b}	5.85±0.1^{b}	6.74±0.2^{b}	5.98±0.2^{b}	6.9±0.2^{a}	5.7±0.1^{b}	6.55±0.1
TN %	0.67±0.1^{d}	0.12±0.07^{c}	0.47±0.1^{e}	0.21±0.05^{b}	0.33±0.2^{f}	0.32±0.04^{a}	0.35±0.1^{f}	0.19±0.05^{b}	0.97±0.2^{b}	0.12±0.05^{c}	0.79±0.3^{c}	0.18±0.05^{b}	1.20±0.2^{a}	0.18±0.0
NH_4^+(mg/kg)	2.59±0.5^{c}	0.25±0.1^{d}	2.6±0.17^{e}	0.41±0.1^{b}	2.9±0.5^{d}	0.25±0.2^{c}	2.27±0.2^{f}	0.3±0.1^{c}	4.43±0.4^{b}	0.27±0.2^{b}	0.5±0.2^{g}	0.41±0.1^{b}	33.76±1.2^{a}	0.56±0.1
NO_3^-(mg/kg)	18.31±1.2^{e}	6.35±1.5^{f}	25.83±1.2^{d}	7.67±1.5^{e}	45.28±1.6^{a}	25.55±1.5^{a}	36.0±1.2^{c}	19.91±1.2^{c}	39.0±1.3^{b}	12.47±1.2^{d}	19.21±1.2^{e}	22.47±1.4^{b}	51.29±1.2^{c}	5.37±1.2
TOC %	21.35±2.1^{d}	2.56±1.2^{b}	15.79±2.3^{e}	3.2±1.2^{a}	22.34±2.1^{d}	2.2±1.2^{c}	13.33±2.2^{f}	2.85±2.4^{a}	27.46±2.2^{c}	2.3±2.3^{c}	32.34±2.3^{b}	1.89±1.2^{d}	56.36±1.3^{a}	1.71±2.1
TP (mg/kg)	1023±1.5^{d}	1357±2.1^{a}	980±1.2^{d}	975±2.1^{d}	1136±1.4^{c}	1238±1.2^{b}	1521±2.1^{a}	1132.9±1.5^{c}	1178±2.1^{c}	1376±1.2^{a}	1275±2.1^{b}	1231±1.2^{b}	53±2.1^{e}	1129±1-6
AP (mg/kg)	112.9±0.1^{c}	176.1±2.1^{b}	131.4±0.5^{b}	164.6±2.2^{b}	131.7±0.5^{a}	175.4±2.1^{b}	131.2±0.1^{b}	187±2.1^{a}	112±0.2^{c}	198.5±2.1^{a}	99.5±0.2^{c}	153±2.1^{c}	76±0.1^{d}	171±2.2

Table S2 - Linear regressions (R^2 correlation coefficients, n=21) between microbial abundance and chemical properties in rhizosphere (rhizo) and bulk soil samples

Property	16S bacteria		16S archaea		18S fungi		*chiA gene*		AOA		AOB	
	rhizo	bulk soil	rhizo	bulk soil	rhizo	bulk soil	rhizo	bulk soil	rhizo	bulk soil	rhizo	bulk soil
pH	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
TN	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	0.38*	0.673*	0.77***	ns	0.876	ns
NH_4^+	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	0.754***	0.85***	*ns*	*ns*	*ns*	*ns*
NO_3^-	0.656***	ns	0.778***	0.697**	ns	ns	0.68***	0.50**	0.79***	0.777***	0.65**	0.856***
Ptot	0.50**	*ns*	*ns*	*ns*	0.679***	0.95***	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Pav	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
TOC	*ns*	*ns*	*ns*	*ns*	6.975***	*ns*	*ns*	*ns*	0.76***	*ns*	*ns*	*ns*

Significance levels: not significant-ns: $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Tables

Table 1 – General information on the trials and timing of the operations carried out in 2016 (trial 1) and 2017 (trial 2)

Treatment	Microorganism - active ingredient	Dosage	Tray treatment	Plot Treatment
Serenade max - SM	*Bacillus subtilis* QST 713	$2.9X10^{10}$ cells/L water	T5; T10; T15;T20*	-
Remedier - RM	*Trichoderma asperellum + T gamsii*	$1.2X10^{6}$ cells/L water	T5; T10; T15;T20	-
Pseudomonas putida - Pp	FC7B+ FC8B +FC9B	1x10^7 (cells /ml water)	T5; T10; T15;T20	-
ANT'S COMPOST M - CM	Green compost + *Trichoderma* TW2	8g/seedling;1kg/0.2m^3 of soil	T0	- T20
ANT'S COMPOST V - CV	*Green compost*	8 g/seedling; 1kg/0.1m^3 of soil	T0	- T20
Trichoderma sp. - TW2	*Trichoderma* strain TW2	1x10^7 (cells /ml)	T5; T10; T15; T20	
Ortiva	Azoxystrobin	0.19 g a.i./Lwater		T20
Untreated control - C	-	-		

*Treatments: T0 at sowing; T5: 5 days after sowing; T10: 10 days after sowing; T15: 15 days after sowing; T 20: 20 days after sowing and immediately before transplanting.

Table 2 Description of the primer sets and amplification details used for the quantitative PCR.

Target group	Primer (reference)	Amplification details
Fusarium oxysporum f.sp. *lactucae* (FOL)	FLA0001F (Shimazu et al., 2005) FLA0001R (Shimazu et al., 2005)	40 cycles 95°C 15s, 60°C 60s, 72°C 45s
All bacteria (16S rRNA gene)	Eub338 (Lane 1991) Eub518 (Muyzer et al. 1993)	40 cycles 95°C 30s, 55°C 35s, 72°C 45s
All archaea (16S rRNA gene)	340F (Gantner et al., 2011) 1000R (Gantner et al., 2011)	40 cycles 95°C 30s, 57°C 30s, 72°C 30s
All fungi (18S rRNA gene)	FR1 (Vainio and Hantula, 2000) 390FF (Vainio and Hantula, 2000)	45 cycles 95°C 30s, 50°C 30s, 70°C 60s
Ammonia oxidizing bacteria (AOB*)*	AmoA-1f (Rotthauwe et al. 1997) AmoA-2r (Rotthauwe et al. 1997)	45 cycles 95°C 30s, 57°C 45s, 72°C 45s, 78°C 20s
Ammonia oxidizing archaea (AOA)	Arch-amoAf (Francis et al., 2005) Arch-amoAr (Francis et al.,2005)	45cycles 95°C 30s, 53°C 45s, 72°C 45s, 78°C 20s
chiA gene	chiaxf (this study) chiaxr (this study)	35cycles 95°C 15s, 59.9°C 30s, 70°C 30s
phlD gene	BPF2 (McSpadden Gardeneret al., 2001) BPR4 (McSpadden Gardeneret al., 2001)	40 cycles 95°C 15s, 60°C 45s, 72°C 45s, 78°C 20s
hcnAB gene	PM2 (Svercel, Duffy & Défago 2007) PM-26R (Svercel, Duffy & Défago 2007)	40 cycles 95°C 15s, 60°C 45s, 72°C 45s, 78°C 20s
Bacillus	Forward *B. subtilis* (Gao et al., 2011) Reverse *B. subtilis* (Gao et al., 2011)	40 cycles 95°C 30s, 60°C 60s, 80°C 10s
Trichoderma	uTf (Hagn et al., 2007) uTr (Hagn et al., 2007)	35 cycles 95 °C 30 s, 55.5 °C 30 s, 72 °C 30 s
Pseudomonas	Pse435F (Bergmark et al., 2012) Pse686R (Bergmark et al., 2012)	40 cycles 95°C 30s, 60°C 60s, 80°C 10s

Table 3 Effect of the preventative soil treatments with BCAs and compost on disease severity and fresh weight at the end of trials 1 and 2, 2016 and 2017, respectively

Treatment	Microorganism - active ingredient	Disease severity* %				Fresh weight g/12 plants			
		2016		2017		2016		2017	
Serenade max - SM	*Bacillus subtilis* QST 713	26.9	±3.6[a]	22.1	±3.3[ab]	3135.6	±128.9[a]	2727.2	±371.5[ab]
Remedier - RM	*Trichoderma asperellum + T. gamsii*	27.7	±3.2[a]	29.5	±7.0[ab]	2731.0	±79.0[a]	2868.4	±348.7[ab]
Pseudomonas putida - Pp	FC7B+ FC8B +FC9B	28.1	±2.9[a]	35.2	±6.3[ab]	3090.7	±49.6[a]	1528.0	±67.2[bc]
ANT'S COMPOST M - CM	Green compost + *Trichoderma* TW2	25.3	±3.3[a]	13.0	±4.7[a]	3256.3	±267.6[a]	3766.0	±481.6[a]
ANT'S COMPOST V - CV	*Green compost*	32.0	±4.1[a]	29.0	±5.3[ab]	3323.9	±146.7[a]	3747.6	±130.1[a]
Trichoderma sp. - TW2	*Trichoderma* strain TW2	32.2	±2.7[a]	36.4	±3.3[b]	2815.7	±78.6[a]	1917.6	±134.7[bc]
Ortiva	Azoxystrobin	24.8	±4.0[a]	19.8	±2.3[ab]	3014.0	±185.3[a]	3360.4	±273.0[a]
Untreated control - C	-	61.0	±3.5[b]	61.7	±4.1[c]	1745.7	±93.0[b]	770.8	±243.1[c]

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*Sixteen plants/treatment were evaluated for disease severity using the rating scale: 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.

Table 4 Abundance of *F. oxysporum* f.sp. *lactucae* (FOL) in the rhizosphere and bulk soil after different treatments* and in the untreated control at the end of trial 1 (2016) and trial 2 (2017)

Treatment	Rhizosphere 2016		Bulk soil 2016		Rhizosphere 2017		Bulk soil 2017	
	FOL (logcopy DNA $^{-1}$)							
Serenade Max - SM	6.359	a	6.711	c	6.345	b	6.796	b
Remedier - RM	5.626	c	6.758	c	5.692	c	6.828	b
P. putida - Pp	6.034	b	7.117	b	6.179	b	6.959	a
ANT'S COMPOST M - CM	5.423	d	6.086	d	5.243	d	6.119	c
ANT'S COMPOST V - CV	5.897	c	6.769	c	5.897	c	6.821	b
Trichoderma sp. - TW2	6.122	b	5.950	d	6.292	b	5.983	c
Untreated control - C	6.445	a	7.298	a	7.635	a	7.073	a
P(F) Treat	*0.000*							
P (F) Year	*0.000*							
*P(F) Treat*Year*	*ns*							
*P(F) Rhizo*Bulk*	*0.001*							

Values represent marginal means

*Serenade max – SM: *Bacillus subtilis* ; Remedier – RM : *Trichoderma asperellum* + *T. gamsii*; *Pseudomonas putida* – Pp; ANT'S COMPOST M – CM: Green compost + *Trichoderma* TW2; ANT'S COMPOST V – CV: Green compost; Trichoderma sp. – TW2

Table 5 Abundance of bacterial (AOB) and archaeal (AOA) *amoA* genes in the rhizosphere and bulk soil after different treatments* and in the untreated control at the end of trial 1 (2016) and trial 2 (2017)

Treatment	Rhizosphere 2016		Bulk soil 2016		Rhizosphere 2017		Bulk soil 2017	
	AOB (logcopy DNA $^{-1}$)							
Serenade Max - SM	7.417	a	7.374	c	7.493	a	7.383	c
Remedier - RM	7.139	c	7.653	b	7.192	c	7.741	b
P. putida - Pp	7.045	c	7.628	b	7.086	c	7.660	b
ANT'S COMPOST M - CM	7.236	b	7.680	b	7.320	b	7.715	b
ANT'S COMPOST V - CV	7.407	a	7.656	b	7.446	a	7.693	b
Trichoderma sp. - TW2	7.396	a	7.703	b	7.410	a	7.698	b
Untreated control - C	7.039	c	7.951	a	7.341	b	7.854	a
P(F) Treat	*0.000*							
P (F) Year	*0.000*							
*P(F) Treat*Year*	*ns*							
*P(F) Rhizo*Bulk*	*0.007*							
	AOA (logcopy DNA $^{-1}$)							
Serenade Max - SM	7.057	a	8.829	c	7.100	a	8.834	b
Remedier - RM	6.653	b	8.744	c	6.826	b	8.830	b
P. putida - Pp	6.415	c	8.958	b	6.653	c	8.985	b
ANT'S COMPOST M - CM	6.613	b	8.823	c	6.690	c	8.828	b
ANT'S COMPOST V - CV	6.721	b	9.163	a	6.800	b	9.224	a
Trichoderma sp. - TW2	6.974	a	9.042	b	6.844	b	9.123	a
Untreated control - C	5.799	d	8.118	d	5.839	d	8.422	c
P(F) Treat	*0.000*							
P (F) Year	*0.000*							
*P(F) Treat*Year*	*0.015*							
*P(F) Rhizo*Bulk*	*0.006*							

Values represent marginal means

*Serenade max – SM: *Bacillus subtilis* ; Remedier – RM : *Trichoderma asperellum* + *T. gamsii*; *Pseudomonas putida* – Pp; ANT'S COMPOST M – CM: Green compost + *Trichoderma* TW2; ANT'S COMPOST V – CV: Green compost; Trichoderma sp. – TW2

Table 6 Abundance of *chiaA*, *phlD*, and *hcnAB* genes in the rhizosphere and bulk soil after different treatments* and the untreated control at the end of trial 1 (2016) and trial 2 (2017)

Treatment	Rhizosphere 2016		Bulk soil 2016		Rhizosphere 2017		Bulk soil 2017	
	chiA gene (logcopy DNA $^{-1}$)							
Serenade Max - SM	4.528	c	4.406	b	4.572	d	4.539	b
Remedier - RM	4.761	ab	4.632	a	4.815	c	4.513	b
P. putida - Pp	4.317	d	4.612	a	4.482	d	4.635	a
ANT'S COMPOST M - CM	4.905	b	4.337	b	5.080	b	4.358	c
ANT'S COMPOST V - CV	4.292	d	4.566	a	4.334	e	4.621	a
Trichoderma sp. - TW2	5.676	a	4.433	b	5.711	a	4.520	b
Untreated control - C	4.327	d	4.385	b	3.915	f	4.284	d
P(F) Treat	*0.000*							
P (F) Year	*0.000*							
*P(F) Treat*Year*	*ns*							
*P(F) Rhizo*Bulk*	*ns*							
	phlD gene (logcopy DNA $^{-1}$)							
Serenade Max - SM	4.952	c	2.627	a	4.842	c	2.613	b
Remedier - RM	4.864	c	2.662	a	4.726	c	2.642	b
P. putida - Pp	5.371	a	2.166	b	5.554	a	2.198	c
ANT'S COMPOST M - CM	4.814	c	2.756	a	4.852	c	2.663	b
ANT'S COMPOST V - CV	5.119	a	2.280	b	5.159	b	2.249	c
Trichoderma sp. - TW2	5.248	b	2.957	a	5.260	b	2.942	a
Untreated control - C	4.950	c	2.786	a	3.959	d	2.621	b
P(F) Treat	*ns*							
P (F) Year	*0.000*							
*P(F) Treat*Year*	*ns*							
*P(F) Rhizo*Bulk*	*0.013*							
	hcnAB gene (logcopy DNA $^{-1}$)							
Serenade Max - SM	6.319	b	2.613	a	6.482	b	2.556	b
Remedier - RM	3.734	d	2.642	a	3.754	e	2.670	b
P. putida - Pp	6.707	a	2.198	b	6.555	b	2.155	c
ANT'S COMPOST M - CM	6.910	a	2.663	a	6.940	a	2.643	b
ANT'S COMPOST V - CV	5.601	d	2.249	b	5.636	c	2.227	c
Trichoderma sp. - TW2	6.810	a	2.942	a	6.391	b	2.892	a
Untreated control - C	4.950	c	2.791	a	4.864	d	2.811	a
P(F) Treat	*ns*							
P (F) Year	*0.000*							
*P(F) Treat*Year*	*ns*							
*P(F) Rhizo*Bulk*	*0.016*							

Values represent marginal means

*Serenade max – SM: *Bacillus subtilis* ; Remedier – RM : *Trichoderma asperellum* + *T. gamsii*; *Pseudomonas putida* – Pp; ANT'S COMPOST M – CM: Green compost + *Trichoderma* TW2; ANT'S COMPOST V – CV: Green compost; Trichoderma sp. – TW2

Table 7 Abundance of *Bacillus*, *Pseudomonas*, and *Trichoderma* genes in the rhizosphere and bulk soil after different treatments* and the untreated control at the end of trial 1 (2016) and trial 2 (2017)

Treatment	Rhizosphere 2016		Bulk soil 2016		Rhizosphere 2017		Bulk soil 2017	
	Bacillus (logcopy DNA $^{-1}$)							
Serenade Max - SM	5.039	a	5.394	a	5.087	a	5.477	a
Remedier - RM	4.249	c	4.109	d	4.194	b	4.130	d
P. putida - Pp	3.989	c	4.320	c	3.980	c	4.331	c
ANT'S COMPOST M - CM	4.702	b	4.790	b	4.308	b	4.814	b
ANT'S COMPOST V - CV	3.982	c	4.428	c	3.990	c	4.228	c
Trichoderma sp. - TW2	3.654	d	3.908	d	3.641	d	3.898	e
Untreated control - C	3.615	d	3.871	d	3.617	d	3.879	e
P(F) Treat	*0.000*							
P (F) Year	*0.000*							
*P(F) Treat*Year*	*ns*							
*P(F) Rhizo*Bulk*	*0.000*							
	Pseudomonas (logcopy DNA $^{-1}$)							
Serenade Max - SM	4.295	b	3.928	c	4.337	b	3.902	c
Remedier - RM	4.249	b	4.109	c	4.194	b	4.148	c
P. putida - Pp	4.599	a	4.506	a	4.921	a	4.781	a
ANT'S COMPOST M - CM	4.781	a	4.301	b	4.837	a	4.515	b
ANT'S COMPOST V - CV	4.292	b	3.566	d	3.880	c	4.554	b
Trichoderma sp. - TW2	3.654	c	3.908	c	3.735	c	3.681	d
Untreated control - C	3.495	c	3.234	e	3.435	d	3.252	e
P(F) Treat	*0.000*							
P (F) Year	*0.000*							
*P(F) Treat*Year*	*ns*							
*P(F) Rhizo*Bulk*	*0.000*							
	Trichoderma (logcopy DNA $^{-1}$)							
Serenade Max - SM	3.988	d	2.928	c	3.936	d	2.902	d
Remedier - RM	4.249	c	3.109	bc	4.345	d	3.252	c
P. putida - Pp	3.989	cd	3.320	b	3.992	d	3.507	b
ANT'S COMPOST M - CM	4.905	b	3.337	b	5.001	b	4.621	a
ANT'S COMPOST V - CV	4.292	c	3.566	a	4.775	c	4.668	a
Trichoderma sp. - TW2	5.676	a	3.433	b	5.658	a	4.418	b
Untreated control - C	3.327	e	2.384	d	4.207	d	3.293	d
P(F) Treat	*0.000*							
P (F) Year	*0.000*							
*P(F) Treat*Year*	*ns*							
*P(F) Rhizo*Bulk*	*0.000*							

Values represent marginal means

*Serenade max – SM: *Bacillus subtilis* ; Remedier – RM : *Trichoderma asperellum* + *T. gamsii*; *Pseudomonas putida* – Pp; ANT'S COMPOST M – CM: Green compost + *Trichoderma* TW2; ANT'S COMPOST V – CV: Green compost; Trichoderma sp. – TW2

Table 8 Pearson's correlation coefficient between microbial gene abundance, disease severity (DS) and potential nitrification activity (PNA) assesses in rhizosphere (rhizo) and bulk soil samples

Microbial abundance	DS		PNA	
	rhizo	bulk soil	rhizo	bulk soil
FOL	0.73***	0.57***	ns	-0.7***
16S Bacteria	ns	0.23		
16S Archaea	-0.35	-0.24		
18S Fungi	ns	-0.39**		
AOB	-0.45**	0.75***	-0.31	-0.5***
AOA	-0.67***	-0.65***	-0.5***	0.87***
Bacillus	-0.38**	-0.38*		
Pseudomonas	-0.44***	-0.35*		
Trichoderma	ns	-0.65***		
chiA gene	ns	-0.46**		
phlD gene	-0.7***	-0.5**		
hcnAB gene	-0.67***	-0.76**		
PNA	0.253	-0.6***		

Significance levels: not significant-ns: $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$

Table 9 Pearson's correlation coefficient between, *Bacillus*, *Pseudomonas*, *Trichoderma*, FOL population gene abundance and chemical properties assessed in rhizosphere (rhizo) and bulk soil samples

Property	*Bacillus*		*Pseudomonas*		*Trichoderma*		FOL	
	rhizo	bulk soil	rhizo	bulk soil	rhizo	bulk soil	rhizo	bulk soil
pH	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
TN	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
NH_4^+	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
NO_3^-	-0.5	ns	ns	0.60**	-0.57**	ns	ns	ns
PT	-0.80***	-0.50**	0.60***	*ns*	0.6**	ns	-0.5**	-0.76**
AP	0.50**	-0.60**	0.75***	0.40*	0.55**	0.60**	0.565**	0.438*
TOC	-0.6**	0.4 *	-0.70***	0.60**	-0.75***	0.76***	0.657***	0.543**

Significance levels: not significant-ns: $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$