Influence of different biological control agents and compost on total and nitrification-driven microbial communities at rhizosphere and soil level in a lettuce - Fusarium oxysporum f. sp. lactucae pathosystem

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2 **and nitrification driving microbial communities in a lettuce – *Fusarium oxysporum* f.sp.**

3 ***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) (*Bacillu*s *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.

1. Introduction

52   Lettuce is cultivated and consumed by the whole world and its production exceeded 24 million tons

53   in 2014 (FAO, 2014). Lettuce crops are grown intensively in Mediterranean regions. Spain, Italy

54   and France are the three most important producing countries, with 35, 21, and 13%, respectively, of

55   lettuce production in Europe in 2010 (Eurostat, 2012). *Fusarium oxysporum* f. sp. *lactucae* (FOL) is

56   a soil-borne, host-specific lettuce pathogen that has been observed in Italy since 2002 (Garibaldi et

57   al., 2002), which causes serious economic losses (Matheron & Gullino, 2012). Its management has

58   been investigated intensively through different methods, such as solarization (Matheron and

59   Porchas, 2010) or the application of different organic amendments (Franceschini et al., 2016;

60   Gilardi et al., 2016). However, the seed transmission of this pathogen makes soil disinfection only

61   partially effective (Garibaldi et al., 2004; Gilardi et al., 2007). For these reasons, the use of

62   biological control agents (BCAs), based on antagonistic microorganisms or different composts (e.g.,

63   compost with inoculated BCA, green compost), is considered an effective and sustainable strategy

64   (Barrière et al., 2014; Pugliese et al., 2015). Species of the *Trichoderma* genus (*T. harzianum, T.

65   viridi)* have been shown to be efficient "biofungicides", as they are involved in the suppression of

66   different pathogens, including *Fusarium oxysporum* f. sp. *lactucae* (Gilardi et al., 2007, 2013;

67   Innocenti et., 2015). *Pseudomonas* spp. is known to be responsible for the "natural" suppressiveness

68   of some soils against soil-borne pathogens (e.g., *Pythium ultimum*, *Rhizoctonia solani*) (Weller et

69   al., 2002; 2007), and the same phenomenon has also been observed for various *Bacillus* species

70   (e.g., *B. subtilis, B. thuringiensis*, *B. amyloliquefaciens*) (Cawoy et al., 2011; Kloepper et al., 2004).

71   Many studies have been conducted on the effects of BCAs and compost on disease control

72   (Bonanomi et al. 2007; Berg, 2009; Pane et al. 2013). The presence of BCAs candidates in compost,

73   belonging to the *Trichoderma, Pseudomonas* and *Bacillus* genera, has also been demonstrated

74   (Pugliese et al., 2008).

75   BCAs are known to be aggressive colonizers that indirectly affect the functional guilds in the

76   rhizosphere environment (Gupta et al., 2012). However, their introduction into a new soil system is

77   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

104 2.1. Layout of the experiments

105 The experiments were carried out in tunnels (6 x 60 m) on a commercial farm in Moretta (Cuneo,

106 Northern Italy), which had a history of several lettuce cycles prior to the beginning of this study.

107 The soil was characterized as a silty loam soil (silt : sand : clay 60 : 30 : 10 %, with neutral pH and

108 1.5 % organic matter) naturally infested with the FOL pathogen.

109 The lettuce cultivar 'Novelsky', which belongs to the batavia type and is moderately susceptible to

110 Fusarium wilt was used (Gilardi et al., 2017). The experimental trials were carried out over two

111 years, 2016 and 2017, in order to test the efficacy of pre-planting treatments with different

112 commercially available *Bacillus subtilis*: Serenade Max – SM, QST713, Bayer Crop Science, Italy

113 and *Trichoderma gamsii + Trichoderma asperellum*: Remedier – RM ISAGRO, Italy) and

114 experimental BCAs (*Trichoderma, TW2,* AgriNewTech, Italy and *Pseudomonas putida* - Pp,

115 Agroinnova, Italy) (Table 1) against lettuce wilting. In addition, two composts (Ant's Compost V

116 and Ant's Compost M; AgriNewTech, Italy), produced from green wastes, in a dynamic industrial

117 treatment system, were used (Table 1): one green compost - CV and the same compost inoculated

118 with *Trichoderma TW2* - CM. An untreated control was used to monitor Fusarium wilt

119 development. Summarized details of these treatments can be found in Table 1.

120   Lettuce seeds were sown in 100-plug trays (3.4 cm diameter pots, 4 l of soil, 53 x 42 cm surface)

121 filled with a peat mixture substrate.

122 Four substrate (i.e., peat) treatments with BCAs were carried out on the plug tray between sowing

123 and transplanting (at the first true leaf stage) in a commercial nursery; incubation until transplanting

124 was conducted under greenhouse conditions at a temperature of 22-24°C (Table 1). The BCAs were

125 applied by spraying them onto the peat surface in a high volume of water (500 ml/tray) using a 1 l

126 capacity hand sprayer. The compost products were distributed in a thin layer of 8 g/plug at sowing

127 (T0) over the surface of 53 x 42 cm plug trays, then mixed with the soil immediately before

128 transplanting (T20). Azoxystrobin (Ortiva, 23.2% a. i., Syngenta Crop Protection, Italy) was used as

129 the reference chemical treatment and was applied once as a soil drench at transplanting.

130 Twenty day old lettuce plants were transplanted (at T20) at a density of 24 plants/m^2, drip irrigated

131 and grown according to the cultural practices adopted by commercial growers in the region. Each

132 treatment had four biological replicates arranged in a completely randomized block design. The

133 plants were monitored weekly for symptoms development, and the data were recorded starting from

134 the appearance of the first symptoms (yellow leaves and reduced growth). The final disease rating

135 was carried out 48-53 days after transplanting on 16 plants/treatment by dissecting each plant. The

136 disease severity (DS) scale was as follows: 0 = healthy plant, 25% = initial leaf chlorosis, 50% =

137 severe leaf chlorosis and initial symptoms of wilting during the hottest hours of the day, 75% =

138 severe wilting and severe symptoms of leaf chlorosis; 100% = plant totally wilted, leaves

139 completely necrotic. Lettuce yield was measured at the end of the trials by collecting 12 plants per

140 treatment, and determining the fresh weight of the plants.

141

142 2.2. Rhizosphere and bulk soil sampling

143 The influence of soil-treatments with BCAs and compost on indigenous microbial communities was

144 studied at the rhizosphere and bulk soil levels by collecting samples at the end of the trials (the end

145 of September 2016 and 2017). One composite rhizosphere sample per plot consisted of the roots of

146 five randomly selected lettuce plants. The roots were shaken vigorously to remove the soil particles

147 that were not tightly adhering on them. The rhizosphere soil was gently scraped off with sterile

148 forceps and transferred into sterile sampling bags. Each treatment consisted of three biological

149 replicates, which were kept on ice. Soil bulk samples were taken also in three replicates (0-15 cm

150 soil depth) per treatment. Each replicate consisted of five sub-replicates collected from the same

151 plots as the rhizosphere samples. The replicates were sampled in a W - shaped sampling pattern, to

152 obtain a good bulk soil representation and homogeneity. The samples were then placed in plastic

153 bags, kept on ice and transported to the laboratory.

154 The fresh rhizosphere and bulk soil samples were homogenized, passed through a 2 mm sieve and

155 stored at 4°C. All the samples were split into two parts: one part was sent to the Regional Chemistry

156   Laboratory (Turin, Italy) for further geochemical analysis: humidity, pH, total N (TN), inorganic N

157   as nitrate (NO_3^-) and ammonium (NH_4^+), total organic carbon (TOC), total phosphorus (TP) and

158   available phosphorus (AP). The second part was used for enzymatic measurements (i.e., potential

159   nitrification activity – PNA) and further microbiological and molecular investigations.

160

161   2.3. Molecular analyses

162   2.3.1. Rhizosphere and bulk soils DNA extraction

163   A NucleoSpin® Soil kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used,

164   according to the manufacturer's instructions, for the rhizosphere and bulk soil genomic DNA

165   extraction. Extraction was conducted on fresh samples (250-500 mg of sample material). DNA

166   quantity and purity were measured spectrophotometrically using a NanoDrop ND-1000 (NanoDrop

167   Technologies, Wilmington, DE, USA).

168

169   2.3.2 Microbial abundance (quantitative PCR (qPCR) assays)

170   The abundance of the bacterial and archaeal 16S rRNA genes, fungal 18S rRNA genes and of five

171   functional genes (i.e., bacterial ammonia monooxygenase *amoA* gene - AOB, archaeal *amoA* gene -

172   AOA, fungal chitinase *chiA* gene, and bacterial 2,4-diacetylphloroglucinol *phlD* and HCN synthase

173   *hcnAB* genes) was determined by means of qPCR, using a StepOnePlus™ Real-Time PCR System

174   (Applied Biosystems, Foster City, CA, USA) for the rhizosphere and bulk soil DNA samples. In

175   addition, the abundance of FOL and BCAs - like microorganisms (i.e., *Bacillus*, *Trichoderma* and

176   *Pseudomonas*) was also investigated. A description of the primer sets and amplification details are

177   given in Table 2.

178   The primers for fungal chitinase *chiA* gene quantification were designed as follows: DNA from

179   rhizosphere and bulk soil samples from the treatments with *Trichoderma* sp. was used to amplify

180   the fungal chitinase *chi42* gene, according to the Nguyen HL et al., (2001) protocol, with

181   CHIf/CHIr primers, which generally amplify a 1450 bp sequence. Four new primer pairs targeting

182 an internal fragment of the *chi42* gene, were designed using Gen Script

183 (http://www.genscript.com/tools.html#biology), considering relatively high annealing temperatures

184 (≥ 60 °C) and smaller amplicon sizes than 200 bp to reduce generation of artifacts and to achieve

185 appropriate reaction efficiencies during qPCR. The accuracy of the primers was confirmed using

186 BLAST of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov);

187 preliminary tests (data not shown) were carried out to assess their specificity, and the best primer

188 pair was then chosen. The designed primer pair (chiaxF

189 (ACCCTGCCGATGACACTCAG)::chiaxR (GGCAGCGATGGAGAGAAGGA) generated an

190 amplicon of 122 bp, with the following sequence:

191 5'ACCCTGCCGATGACACTCAGGCCACCAACATGGTTCTTCTGCTCAAGGAGATCCGAT

192 CTCAACTAGATGCCTATGCTGCGCAATACGCTCCAGGCTACCACTTCCTTCTCTCCATC

193 GCTGCC-3'.

194 Amplicons from each target gene were generated for the standard preparation, purified (Invisorb

195 Fragment CleanUp, Stratec Molecular GmbH, Berlin, Germany), and ligated in Strata-Clone PCR

196 cloning vector pSC-A (Strataclone PCR Cloning Kit, Agilent Technologies Inc.); the ligation

197 products were then transformed into StrataClone SoloPack competent cells (Agilent Technologies

198 Inc.). The specificity of the clones used as qPCR standards was checked via sequencing, at LGC

199 Genomics GmbH (Berlin, Germany) and through BLAST analysis. Plasmid DNA was isolated

200 (GenElute™ Plasmid Miniprep Kit, Sigma-Aldrich, St. Louis, MO, USA) from standard clones and

201 quantified as described above.

202 As assessed in preliminary tests and in a qPCR assay optimization, the final volume of the qPCR

203 reaction cocktails was 25 µl for the 16S and 18S rRNA genes, the bacterial and archaeal *amoA*

204 gene, and the FOL and BCA - like microorganisms quantification, and it was 20 µl for the *chiaA*,

205 *phlD* and *hcnAB* genes. All the quantifications were conducted with a 10 ng DNA template, except

206 for the bacterial 16S rRNA gene, for which a 5 ng DNA template was used (Cucu et al., 2017). The

207 reaction mixtures contained 1x Power SYBR green master mix (Applied Biosystems), 0.12 µM of

208   each oligonucleotide (Table 2) for the total bacteria, archaea, fungi and FOL, 0.15 μM for the *phlD*

209   and *hcnAB* genes, 0.32 μM for the AOB, AOA and BCA - microorganisms and 0.4 μM for the *chiA*

210   gene.

211   All the considered genes were quantified in triplicate across plates, while standards were run in

212   duplicate in 10-fold serial dilutions. The optimal dilution of the DNA extracts was tested to

213   compensate for any inhibitory reactions by the organic compounds (e.g., humic acids) co-extracted

214   during the DNA isolations. In all experiments, negative controls containing no-template DNA were

215   subjected to the same qPCR procedure to detect and exclude any possible contamination.

216   Amplification efficiency ranged from 96%, (archaeal 16S rRNA, fungal 18S rRNA, BCAs and their

217   functional genes) to 103% (bacterial 16S rRNA). For the nitrifiers, the amplification efficiency

218   were 95.3% and 99.1% for AOB and AOA, respectively. The R2 was always $\geq$ 0.98. Melting curves

219   of the amplicons were generated to ensure that the fluorescence signals originated from specific

220   amplicons and not from primer dimers or other artifacts. This was confirmed by checking the

221   amplification products on 1% agarose gel. Gene copy numbers were calculated with StepOne™

222   software, version 2.2 (Applied Biosystems). The data were normalized and presented as log copies

223   g^{-1} dry soil.

224

225   2.4. Chemical properties of rhizosphere and bulk soil samples

226   In short, the pH values were measured in water suspensions at a solid: liquid ratio of 1:2.5. The total

227   organic carbon (TOC) and total nitrogen (TN) were quantified using a Leco Tru Spect CN

228   automatic analyzer. Ammonium (NH_4^+) and nitrate (NO_3^-) were measured colorimetrically, by

229   means of a continuous flow auto-analyzer (Alliance Evolution II), using standard colorimetric

230   techniques. The total phosphorus (TP) was determined by means of 'ICP Varian mod. Liberty LR',

231   after microwave digestion with hydrogen peroxide, hydrochloric acid and nitric acid, filtration and

232   dilution. The available P (AP) was extracted using sodium bicarbonate and determined by means of

233 the molybdenum blue method (Olsen et al., 1954), modified for continuous flow colorimetric

234 analysis (Alliance Evolution II).

235 The potential nitrification activity (PNA) was determined according to the procedures described by

236 Hoffmann et al. (2007).

237

238 2.5. Statistical analyses

239 The data on disease index, gene abundance, chemical properties of the soil and potential

240 nitrification activity were subjected to a linear mixed model, with year considered as the random

241 variable, using R software (Software R 3.0.1, R foundation for Statistical Computing, Vienna,

242 Austria, http://www.R-project.org). The effects of different BCAs and compost on the abundance of

243 the studied genes, on the chemical properties of the soil and on disease severity were evaluated. All

244 data were subjected to a Levene test to check for the homogeneity of variance and normality was

245 tested on the residuals using the Shapiro-Wilk test; when not normally distributed data from disease

246 severity (DS) were arcsin transformed while, the data from microorganisms abundance were log-

247 transformed and normality was checked again. The means were separated by Bonferroni test. The

248 statistical analysis included treatment rhizosphere soil, bulk soil, year, treatment $\times$ year.

249 Pearson's linear correlation coefficients were calculated for assessing the relations between

250 microbial gene abundance, disease severity and PNA, as well as between *Pseudomonas*, *Bacillus*,

251 *Trichoderma* and FOL abundance and soil chemical properties.

252 Linear regression between total microbial gene abundance and the functional genes of N cycle

253 (bacterial *amoA*, archaeal *amoA*, *chiA* gene) with soil chemical data were calculated to evaluate how

254 much of their abundance is explained by variation in the soil chemical data.

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258 **3. Results**

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259   3.1. Disease severity and plant biomass

260   The lettuce plants in the non-treated control plots showed an average disease severity DS of up to

261   60% at the end of both trials (Table 3). The BCA treated plots provided significantly lower disease

262   severities than the untreated ones, and statistically similar results to one application of azoxystrobin,

263   reducing Fusarium wilt by 47.2 to 58.5% in the two years, respectively. As a consequence, the fresh

264   weight of the lettuce was significantly higher in all the treatments compared to control, without

265   statistically differences among them (Table 3). No significant difference was observed between the

266   disease severity data. A significant time effect for fresh weight of the lettuce between trials was

267   noted ($p < 0.001$). However, the *Pseudomonas* and *Trichoderma* treatments were less effective in

268   the second trial.

269

270   3.2. Microbial abundance

271   *3.2.1. FOL abundance*

272   In general, FOL abundance assessed by gene copy quantification was significantly higher in the

273   bulk soil than in the rhizosphere soil. Almost all the treatments resulted in a significant reduction of

274   pathogen - associate gene copies compared to the untreated control - C, in both the rhizosphere and

275   bulk soil at the end of both trials (2016 and 2017 respectively) (Table 4). At the end of first trial

276   (2016), Serenade max - SM - *Bacillus* treatment was not significantly different compared with the

277   untreated control - C at the rhizosphere level, while at the end of the second trial (2017) the

278   *Pseudomonas* - Pp treatment was not significantly different compared with the untreated control –

279   C. The interaction treatment × year was not significant (Table 4).

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284   *3.2.2. Total microbial population abundance*

285 At the end of both trials (2016 and 2017 respectively) the number of gene copy of bacterial,

286 archaeal and fungal populations showed an important homogeneity, with no significant interaction

287 treatment × year. Therefore the data were presented as average of all values from both trials.

288 Overall, at the end of both trials (2016 and 2017 respectively) the bacterial 16S rRNA abundance

289 was significantly higher at the bulk soil level than at the rhizosphere level (Figure 1a). The effect of

290 BCAs treatments was more evident in the rhizosphere; the Remedier - RM - *Trichoderma* spp. and

291 *Pseudomonas* - Pp resulted in a lower bacterial abundance than the other treatments but not

292 significantly different than the untreated control - C, while the Serenade max - SM - *Bacillus*

293 treatment resulted in a significantly higher bacterial abundance than the untreated control - C.

294 However, the bacterial community abundance was more or less stable at the soil level over all the

295 treatments (Figure 1a). The archaeal 16S rRNA gene copy number in the rhizosphere followed

296 almost the same trend as the overall bacterial counterpart. The green compost - CV and

297 *Trichoderma* W2 - TW2 treatments resulted in slightly higher archaeal abundance in the bulk soil

298 samples than the untreated control - C (Figure 1b). The fungal 18S rRNA abundance was

299 significantly higher in the rhizosphere than in the bulk soil samples, in all the treatments as well as

300 in the untreated control - C. Remedier - RM - *Trichoderma* spp. inoculation resulted in a slightly

301 higher fungal abundance than the untreated control - C, while the total fungal community in the soil

302 samples was more abundant in the Serenade max - SM - *Bacillus*, *Pseudomonas* - Pp and green

303 compost - CV treatments (Figure 1c).

304

305 *3.2.3. Functional gene abundance*

306 In general, the ammonia-oxidizing bacterial (AOB) gene abundance was significantly influenced by

307 treatments and was significantly higher in the bulk soil than in the rhizosphere samples (Table 5).

308 After the first trial, 2016, the AOB gene in the bulk soil samples was more abundant in the

309 untreated control - C, while the lower abundance was observed after Serenade max - SM - *Bacillus*

310 treatment. At the rhizosphere soil level the Serenade max- SM - *Bacillus*, green compost - CV and

311   *Trichoderma* W2 – TW2 treatments resulted in a highest AOB gene abundance while no significant

312   difference was observed between Remedier - RM-*Trichoderma* spp., *Pseudomonas* – Pp and the

313   untreated control - C. After the second trial, 2017, the same trend of AOB abundance was observed

314   at the bulk soil level, while at the rhizosphere soil level the Remedier - RM-*Trichoderma* spp. and

315   *Pseudomonas* – Pp treatments resulted in the lowest AOB abundance. The interaction treatment ×

316   year was not significant. (Table 5).

317   In general, the ammonia-oxidizing archaeal (AOA) gene abundance was significantly influenced by

318   treatments and was significantly higher in the bulk soil than in the rhizosphere soil samples (Table

319   5).

320   All the treatments resulted in higher archaeal *amoA* gene abundance than the untreated control - C,

321   at both rhizosphere and bulk soil levels for both trials, 2016 and 2017 respectively. After the first

322   trial, 2016, the green compost - CV treatment resulted in the highest AOA abundance than the other

323   treatments at the bulk soil level, while the Serenade max - SM – *Bacillus* and *Trichoderma* W2 –

324   TW2 treatments resulted in the highest abundance than the other treatments at the rhizosphere soil

325   level. After the second trial, 2017, the green compost - CV and the Remedier - RM-*Trichoderma*

326   spp treatments resulted in the highest AOA abundance at the bulk soil level, while the Serenade

327   max - SM – *Bacillus* treatment resulted in the highest AOA abundance at the rhizosphere level. The

328   interaction treatment × year was not significant.

329   In general, the *chiA* gene (*Trichoderma* based chitinase) abundance was significantly influenced by

330   treatments (Table 6). After the first trial, 2016, the abundance of the *chiA* gene was higher in the

331   rhizosphere soil samples for the *Trichoderma* TW2 – TW2 and Remedier - RM-*Trichoderma* spp

332   treatments. However the Remedier - RM-*Trichoderma* spp was not statistically different compared

333   with the inoculated compost – CM. The *Pseudomonas* - Pp and the green compost - CV treatments

334   resulted in the lowest *chiA* gene abundance compared with the other treatments, but not

335   significantly different compared with the untreated control - C. At the bulk soil level, the *chiA* gene

336   was more abundant in the Remedier - RM-*Trichoderma* spp, *Pseudomonas* - Pp and green compost

337 - CV treatments comparing with the other treatments and with the untreated control - C (Table 6).

338 After the second trial, 2017, all the treatments at the rhizosphere soil level resulted in significant

339 differences between the *chiA* gene abundances. The highest *chiA* gene abundance was observed in

340 the *Trichoderma* W2 - TW2 treatment. Also at the bulk soil level all the treatments resulted in

341 higher *chiA* abundance compared with the untreated control - C. The highest *chiA* gene abundance

342 was observed in the *Pseudomonas* - Pp and green compost - CV treatments.

343 The abundance of the *phlD* gene was significantly higher in the rhizosphere than in the bulk soil

344 samples for all the treatments and for the untreated control - C (Table 6). After the first trial, 2016,

345 *Pseudomonas* - Pp and the green compost - CV treatments resulted in the highest *phlD* abundance at

346 the rhizosphere soil level, while the same treatments resulted in the lowest *phlD* abundance at the

347 bulk soil level. After the second trial, 2017, *Pseudomonas* - Pp treatment resulted in the highest

348 *phlD* abundance at the rhizosphere soil level. At the bulk soil level, the highest abundance was

349 observed after *Trichoderma* W2 - TW2 treatment, while *Pseudomonas* - Pp and the green compost

350 – CV treatments resulted in a lower *phlD* gene abundance than the untreated control - C.

351 The abundance of the *hcnAB* gene was significantly influenced by treatments and it was

352 significantly higher in the rhizosphere than in the bulk soil samples for all the treatments and for the

353 untreated control - C (Table 6). After the first trial, 2016, the highest *hcnAB* gene abundance was

354 observed after *Pseudomonas* - Pp , inoculated compost – CM and *Trichoderma* W2 – TW2

355 treatments at the rhizosphere level. At the soil level the *hcnAB* gene abundance was not

356 significantly different compared with the untreated control –C, with the exception of *Pseudomonas*

357 - Pp and the green compost - CV treatments which resulted in the lowest *hcnAB* gene abundance.

358 After the second trial, 2017, the *hcnAB* gene abundance was highest after the inoculated compost -

359 CM treatment and in the untreated control - C was noted the lowest *hcnAB* gene abundance at the

360 rhizosphere level. At the soil level the *hcnAB* gene abundance was higher in the untreated control C

361 and the *Trichoderma* W2 – TW2 treatment while the lowest *hcnAB* gene abundance was noted after

362 *Pseudomonas* – Pp and the green compost – CV treatments.

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363

364   3.2.4. BCAs - like microorganisms abundance

365   Overall, at the end of both trials (2016 and 2017 respectively) the BCA applications resulted in an

366   increase in the indigenous populations of *Bacillus, Trichoderma* and *Pseudomonas* in all the

367   treatments, compared to the untreated control - C (Table 7). No significant interaction treatment $\times$

368   year was observed. In general, the abundance of *Bacillus* sp. was significantly higher for the

369   *Bacillus* - SM treatment than the untreated control - C and all the other treatments in both the

370   rhizosphere and bulk soil. However, the inoculated compost treatment - CM also resulted in an

371   increased resident *Bacillus* abundance in rhizosphere and bulk soils after both trials. Nevertheless,

372   the *Trichoderma* TW2 - TW2 treatment showed similar levels to those of the untreated control - C.

373   Significant interactions bulk $\times$ rhizosphere were observed (Table 7).

374   The inoculation with the *Pseudomonas* - Pp significantly increased the overall *Pseudomonas*

375   community in both the rhizosphere and bulk soils, compared to the untreated control - C and the

376   other treatments. Significant interactions bulk $\times$ rhizosphere were observed (Table 7).

377   The inoculation with the *Trichoderma* - based formulate significantly increased the overall

378   *Trichoderma* community in both the rhizosphere and bulk soils, compared to the untreated control -

379   C. After both trials, the highest abundance of *Trichoderm*a was observed in the rhizosphere after

380   *Trichoderma* TW2 - TW2 treatment and in the bulk soil after the green compost – CV treatment.

381   After the second trial, 2017, a highest *Trichoderma* abundance was also observed after the

382   inoculated compost - CM treatment at the bulk soil level. The Serenade max - SM – *Bacillus*

383   application resulted in somewhat lower *Trichoderma* abundance than all the other BCA

384   applications. Significant interactions bulk $\times$ rhizosphere were observed (Table 7).

385

386

387   3.3. Chemical properties

388   Generally for both trials, 2016 and 2017 respectively, pH was significantly lower (p < 0.05) at the

389   rhizosphere level than at the bulk soil level, with values ranging from between 5.70 and 6.43 for the

390   rhizosphere and between 6.34 and 6.77 for the bulk soil samples. The concentrations of TN, NH_4^+,

391   NO_3^- and TOC were generally significantly higher in the rhizosphere than in the bulk soil samples.

392   The untreated control - C was characterized by higher NH_4^+, NO_3^- and TOC concentrations than all

393   the treatments. On the other hand, the highest TP and AP contents were observed in the bulk soil

394   samples (Table S1). No significant treatment × year interaction was found.

395

396   3.4. Potential Nitrification Activity (PNA)

397   The PNA measurements generally exhibited significantly higher values at the bulk soil level than in

398   the rhizosphere level. No significant treatment × year interaction was observed, therefore the data

399   were presented as the average of all data from both trials, 2016 and 2017 respectively. The PNA in

400   the bulk soil was significantly (p < 0.05) higher in the untreated control - C, followed by the

401   inoculated compost - CM and the *Pseudomonas* - Pp treatments. The lowest value was observed

402   after both, Serenade max - SM – *Bacillus* and Remedier - RM-*Trichoderma* spp treatments. The

403   PNA in the rhizosphere was significantly lower (p < 0.05) in the untreated control - C than in the

404   other treatments. (Figure 2).

405

406   3.5. Correlations between the levels of total microbial communities (16S bacteria, 16S archaea, 18S

407   fungi), the functional genes (fungal *chiA* gene, bacterial and archaeal *amoA* genes, bacterial *phlD*

408   and *hcnAB* genes), the *Bacillus, Pseudomonas, Trichoderma* population levels and FOL

409   abundances, DS and PNA.

410   In general the correlations were negative, with r coefficients ranging from -0.37 to -0.5 (p < 0.001)

411   at the rhizosphere level and from -0.3 to -0.6 (p < 0.001) at the bulk soil level (Table 8). Very

412   strong positive correlations were observed between FOL and DS, as well as between the

413   nitrification rate - PNA and DS. Strong negative correlations were recorded at the rhizosphere and

16

414 bulk soil levels especially between AOB and DS at the soil level, and between AOA and PNA at the

415 soil level (Table 8).

416

417 3.6. Correlations between *Bacillus, Pseudomonas, Trichoderma* population levels and chemical

418 properties in rhizosphere (rhizo) and soil samples

419 In general the correlations were significant only with respect to AP, TP, NO_3^- and TOC, with r

420 values ranging from -0.5 to 0.8 (Table 9).

421

422 3.7. Regressions between indigenous microbial communities and soil chemical properties

423 Variance in the total bacterial abundance was explained by alterations of NO_3^- and TP at the

424 rhizosphere level (Table S2). Changes in the total archaeal abundance were explained by alterations

425 of NO_3^- and TP at the rhizosphere and soil level respectively. Changes in the total fungal

426 community were explained by alterations of TP and TOC at the rhizosphere level and TP at the soil

427 level. The variance in *chiA* gene abundance was explained by alteration of TN including NO_3^- and

428 NH_4^+ at both rhizosphere and soil level. AOA abundance changes were explained by TN, NO_3^- and

429 TOC at the rhizosphere level and by NO_3^- at the soil level. However, a greater percentage of AOB

430 was explained also by NO_3^- (Table S2).

431

432 **4. Discussion**

433 In the present study, rhizosphere and soil microbial community changes have been investigated

434 monitoring total bacterial, archaeal and fungal highly distinctive genes abundance as indicators of

435 the general alterations that may take place as a result of plant treatments and the infection of lettuce

436 by FOL. In this context, the potential impact of various BCA and compost treatments on the total

437 indigenous microbial communities and on the functional genes that controls nitrogen availability

438 was assayed.

439

440 *4.1. Disease severity,* FOL *abundance and plant biomass*

17

441   The obtained results were in line with previous studies showing that the application of BCAs and

442   certain organic matter can effectively suppress soil pathogens (Zhang et al., 2008; Luo et al., 2009;

443   Wu et al., 2009; Ling et al., 2010; Cao et al., 2011; Lang et al., 2011; Yang et al., 2011; Gilardi et

444   al., 2016). The application of different BCAs and compost significantly reduced wilting by

445   suppressing FOL abundance compared to the untreated control. This result was corroborated by the

446   significant positive correlations (p < 0.001) that were observed between disease severity and FOL

447   abundance. These findings were supported by previous studies that showed that *Fusarium*

448   abundance in the cucumber and banana rhizosphere was reduced after BCA treatments (Qiu et al.,

449   2012; Shen et al., 2015; Fu et al., 2017). Furthermore, FOL abundance was very low after the

450   treatment with inoculated compost (CM), thus indicating that an effective disease control could be

451   achieved with organic amendments, as previously pointed out (Bonanomi et al., 2010). Similar

452   results were obtained in a previous study (Pugliese et al., 2011), confirming that inoculation of

453   compost with *Trichoderma* strains can improve the suppressive activity of the substrate. The

454   application of compost may have increased the competition for resources and the interactions

455   between antagonist species of compost and soil, and this may have resulted in a reduction in the

456   FOL abundance and in the potential activity of the pathogen (Bonilla et al., 2012; Larkin et al.,

457   2015).

458

459   *4.2. Ecological fitness of the BCA - like microorganisms and their effect on the total indigenous*

460   *prokaryotes*

461   Our results have evidenced an excellent feedback of the BCA - *like microorganisms* after the BCAs

462   were introduced into the soil, as inoculated plantlets or inoculated compost. This was highlighted by

463   significant negative relationships (p < 0.001) between the BCA – like populations and Fusarium

464   wilt severity, which suggested not only the ecological fitness of inoculated microorganisms, but

465   also their efficiency against FOL. Most probably, the efficiency of biological control of FOL was

466   based on mechanisms as saprophytic competition for nutrients, parasitic competition for infection

467   sites and induced resistance in host cells (Haas and Defago, 2005; Nel et al., 2006; Srinivasan et al.,

468   2009). Even more, the good relationships between the BCAs - like microorganisms and soil

469   nutrients highlighted the role of the antagonists in solubilising certain compounds (nitrogen,

470   phosphorus, potassium, iron) (Gyaneshwar et al., 2002; Richardson et al., 2009) contributing to the

471   disease suppression.

472   The application of BCA - based formulates resulted in a subsequent boost in abundance of the

473   respective microbial populations (i.e., *Pseudomonas*, *Trichoderma* and *Bacillus*). A remarkable

474   result of this study concerns the high abundance that was observed in the rhizosphere samples of the

475   *phlD* and *hcnAB* genes known to encode the production of antifungal compounds such as 2,4-

476   diacetylphloroglucinol (DAPG) and hydrogen cyanide (HCN) (Dudenhoffer et al., 2016). We

477   speculated that *Pseudomonas spp* (e.g., *P. fluorescens*) abundance would have increased in the

478   rhizosphere environment upon pathogen infection, as these bacteria are strong root colonizer

479   (Looper et al., 2012). This may explain the lower observed abundance of the total *Pseudomonas*

480   community compared with other treatments. This was probably due to the action of antifungal

481   metabolites against FOL, but also against other microorganisms that generally colonize the

482   rhizosphere. The *chiA* gene abundance confirmed the potential antagonistic activity of the

483   inoculated *Trichoderma* strain TW2, but also of the inoculated compost. In addition, a significant

484   relationship of *chiA* gene abundance with the total and especially with ammonium N ($p < 0.001$)

485   content was observed (Table S2). This result highlighted the involvement of fungal population in

486   remineralization of nutrients during the decomposition of chitin (Gould et al., 1981) with important

487   contribution to nitrification process.

488   Overall, we did not observe any negative side-effects of BCA application on the total microbial

489   communities, thus indicating that the microorganisms introduced into the soil system may have

490   favoured the resident microbial populations through positive interactions. Our results were in line

491   with previous reports (Ghini et al., 2000; Gullino et al., 1995; Mezzalama et al., 1998). Contrary to

492   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

519   components in particular, in general have a higher competitive advantage under reduced organic

520   resource conditions. Accordingly, we observed a higher AOA abundance in the soil samples, which

521   indicated that the archaeal nitrifiers, and in general the entire archaeal community, were probably

522   adapted to the decomposition of recalcitrant organic matter, as described earlier on by Cucu et al.,

523   (2017).

524   Overall, the effect of different applied treatments resulted in the increase of nitrifiers abundance

525   with respect to the untreated control thus suggesting that BCAs may not have negative ecological

526   impacts on other groups of microorganisms. Comparing with all treatments,, a higher AOB

527   abundance was observed in the bulk soil of the untreated and FOL infested control, while the

528   opposite was true for the AOA in the rhizosphere, thus suggesting that the sole presence of FOL

529   may has induced a differentiation, in the rhizosphere and bulk soil, among the indigenous

530   prokaryotes involved in the nitrification process. Hence, the AOB abundance was not reduced in the

531   control samples, probably as a result of a niche differentiation among nitrifiers at the rhizosphere

532   and bulk soil levels induced by the FOL presence, rather than the commonly acknowledged direct

533   resource competition with AOA (Nicole et al., 2008; Wessén et al., 2010; Musyoki et al., 2015).

534   Additionally, a strong negative relationship between the nitrification rate and disease severity was

535   observed at the bulk soil level. However, competition for resources may exist between AOA

536   abundance and FOL. This hypothesis was corroborated by the low nitrification rate that was

537   observed at the rhizosphere level especially in the untreated control. Our findings indicated that

538   nitrification may play an important role in disease control when environmental conditions for

539   nitrifiers are favourable. Similarly, Fujiwara et al., (2013) showed that, in a multiple parallel

540   mineralization system, rhizosphere microbiota suppressed the Fusarium wilt disease of lettuce by

541   affecting the morphological characteristics of *F. oxysporum*. However, further research is needed to

542   obtain a better understanding of the promoting effect of nitrifiers after the application of BCAs.

543

544   **Conclusions and outlook**

545 Our results have shown that the application of different biological control agents and compost

546 resulted in a significant reduction in the Fusarium wilt of lettuce crops, without any significant

547 differences in the degree of disease control among treatments. On the basis of the presented results,

548 it is possible to conclude that the applied treatments (based on *Bacillus*, *Pseudomonas* and

549 *Trichoderma)* did not affect negatively the density of resident microbial communities (i.e., bacteria

550 archaea and fungi) or the nitrifying prokaryotes. Nevertheless, the archaeal nitrifiers had a more

551 dynamic response after the treatments. Hence, more investigations are needed to study the influence

552 of BCA and compost treatments on archaeal communities. In addition, our results have shown that

553 lettuce plants could favour bacterial species which are good lettuce rhizosphere colonizers and may

554 act as antagonists.

555 Although no negative effects of BCAs were observed in this study on the no-target rhizosphere or

556 on the soil microbial biomass, their application requires further evaluations, including the

557 consideration of a broader range of soils with different physico-chemical properties, as well as

558 additional soil-borne pathogens and different plant species/cultivars, as they may have various

559 effects on the microbial communities and processes in the rhizosphere and bulk soil (Soderberg et

560 al., 2002; Rasche et al., 2006). In addition, the long-term effect on the same crop should be

561 evaluated in order to fully understand the legacy and the results of the interactions of BCAs and

562 compost with microbial biomass and activity. Although this study was focused on the potential

563 functionality of selected microbial populations in terms of abundance it can be assumed that the

564 introduction of different microbial strains or strain combinations could also influence the structure

565 and composition of the overall microbial communities. Therefore, advanced studies on the potential

566 effects of BCAs and organic matrices on the composition and biomass of non-target microbial

567 populations in the crop rhizosphere and bulk soil, using molecular fingerprinting techniques, are

568 still needed.

569

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575 Conflict of interest

576 The authors declare that they have no conflict of interest.

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Figure Captions

799 Figure 1 Abundance of bacterial (a), archaeal (b) 16S rRNA genes and fungal (c) 18S rRNA genes

800 in rhizosphere and bulk soil between different treatments* and the untreated control (SM - Serenade

801 Max; RM - Remedier; Pp - *Pseudomonas*; CM – Green compost plus *Trichoderma* TW2; CV -

802 green compost; TW2 - *Trichoderma* TW2; C - control) (n = 6, means±standard errors). Different

803 letters above bars indicate significant differences between treatments in rhizosphere (uppercase

804 letters) and bulk soil (lowercase letters)

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807 Figure 2 Potential Nitrification Activity (PNA) in the rhizosphere and bulk soil after different

808 treatments* and the untreated control (SM - Serenade Max; RM - Remedier; Pp - *Pseudomonas*;

809 CM - Green compost plus Trichoderma TW2; CV - green compost; TW2 - *Trichoderma* TW2; C -

810 control) (n = 6, means±with standard errors). Different letters above bars indicate significant

811 differences between treatments in rhizosphere (uppercase letters) and bulk soil (lowercase letters)

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813 *Serenade max – SM: *Bacillus subtilis* ; Remedier – RM : *Trichoderma asperellum* + *T. gamsii*; *Pseudomonas putida* – Pp; ANT'S COMPOST M –

814 CM: Green compost + *Trichoderma* TW2; ANT'S COMPOST V – CV: Green compost; Trichoderma sp. – TW2

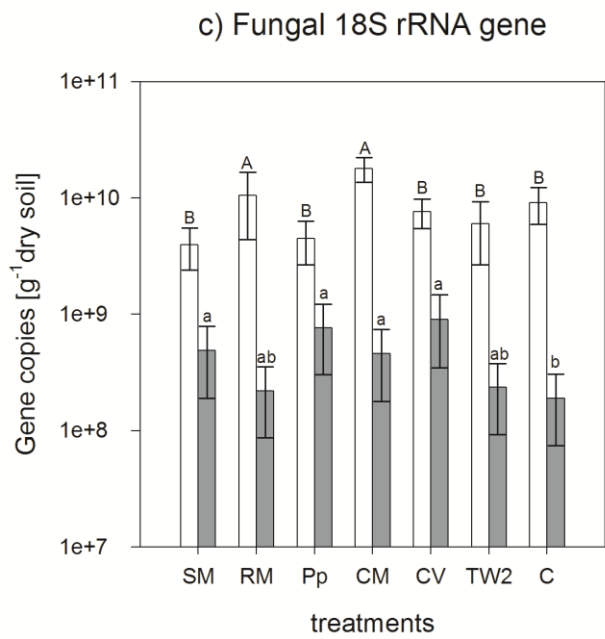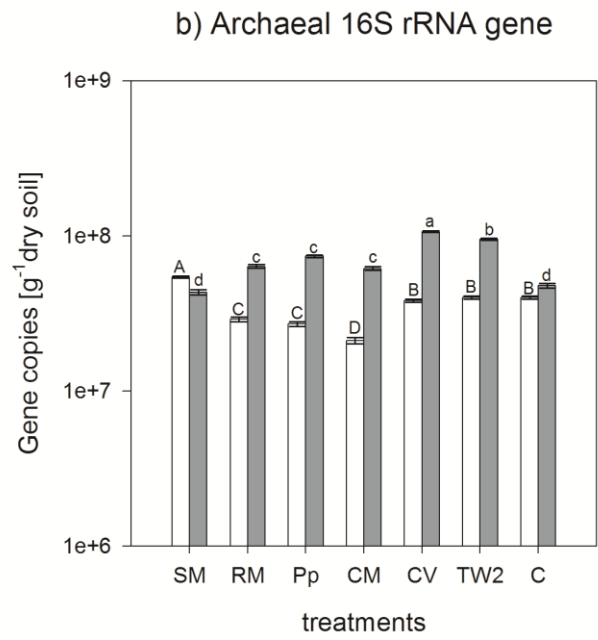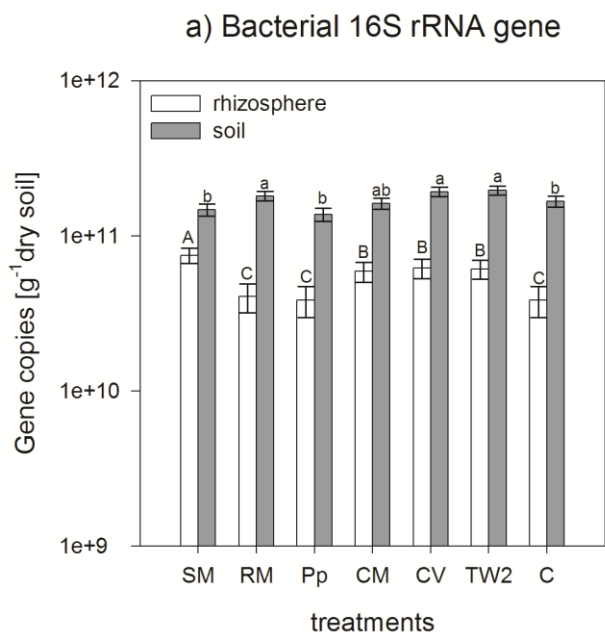815

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a) Bacterial 16S rRNA gene

b) Archaeal 16S rRNA gene

c) Fungal 18S rRNA gene

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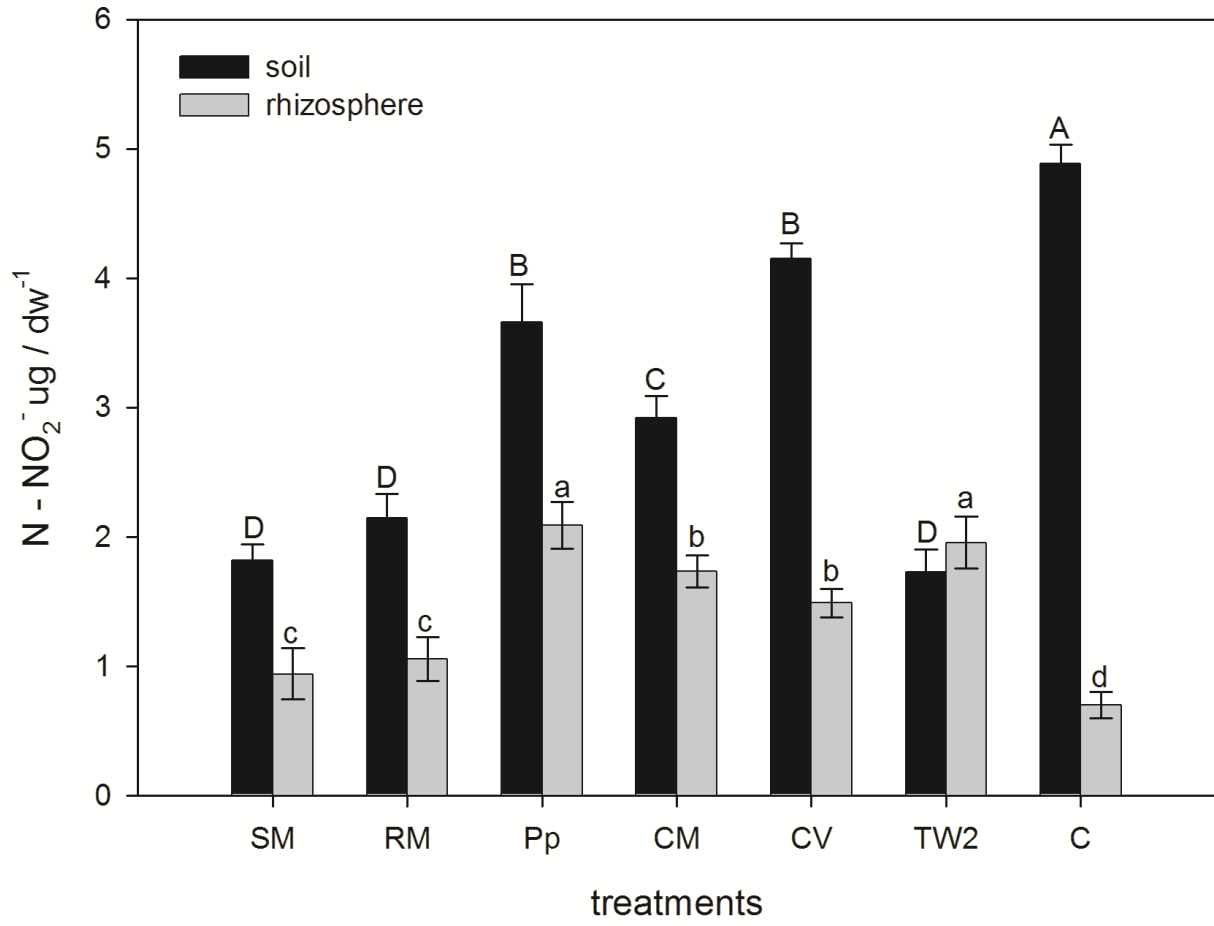839

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PNA

846   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 soil
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	$1337.3\pm0.1.5^a$	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	$1237\pm2.$
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	$159.5\pm2.$
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	$1129\pm1\text{-}$
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.

883 Tables

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

885 and 2017 (trial 2)

Treatment	Microorganism - active ingredient	Dosage	Tray treatment	Plot Treatment
Serenade max - SM	*Bacillus subtilis* QST 713	2.9×10^{10} cells/L water	T5; T10; T15;T20*	-
Remedier - RM	*Trichoderma asperellum + T gamsii*	1.2×10^{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	-	-		

886 *Treatments: T0 at sowing;   T5: 5 days after sowing; T10: 10 days after sowing; T15: 15 days

887 after sowing; T 20: 20 days after sowing and immediately before transplanting.

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891   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

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896 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

897 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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900 *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

901 wilting during the hottest hours of the day, 75 = severe wilting and severe symptoms of leaf chlorosis; 100 = plant totally wilted, leaves completely necrotic.

902 Table 4 Abundance of *F. oxysporum* f.sp. *lactucae* (FOL) in the rhizosphere and bulk soil after

903 different treatments* and in the untreated control at the end of trial 1 (2016) and trial 2 (2017)

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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*							

905

906 Values represent marginal means

907 *Serenade max – SM: *Bacillus subtilis* ; Remedier – RM : *Trichoderma asperellum* + *T. gamsii*; *Pseudomonas putida* – Pp; ANT'S COMPOST M –

908 CM: Green compost + *Trichoderma* TW2; ANT'S COMPOST V – CV: Green compost; Trichoderma sp. – TW2

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921 Table 5 Abundance of bacterial (AOB) and archaeal (AOA) *amoA* genes in the rhizosphere and

922 bulk soil after different treatments* and in the untreated control at the end of trial 1 (2016) and trial

923 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*							

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925

926 Values represent marginal means

927 *Serenade max – SM: *Bacillus subtilis* ; Remedier – RM : *Trichoderma asperellum + T. gamsii*; *Pseudomonas putida* – Pp; ANT'S COMPOST M –

928 CM: Green compost + *Trichoderma* TW2; ANT'S COMPOST V – CV: Green compost; Trichoderma sp. – TW2

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934 Table 6 Abundance of *chiaA*, *phlD*, and *hcnAB* genes in the rhizosphere and bulk soil after different

935 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							

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938 Values represent marginal means

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940 *Serenade max – SM: *Bacillus subtilis* ; Remedier – RM : *Trichoderma asperellum + T. gamsii*; *Pseudomonas putida* – Pp; ANT'S COMPOST M –

941 CM: Green compost + *Trichoderma* TW2; ANT'S COMPOST V – CV: Green compost; Trichoderma sp. – TW2

942 Table 7 Abundance of *Bacillus*, *Pseudomonas*, and *Trichoderma* genes in the rhizosphere and bulk

943 soil after different treatments* and the untreated control at the end of trial 1 (2016) and trial 2

944 (2017)

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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*							

946

947 Values represent marginal means

948 *Serenade max – SM: *Bacillus subtilis* ; Remedier – RM : *Trichoderma asperellum + T. gamsii*; *Pseudomonas putida* – Pp; ANT'S COMPOST M –

949 CM: Green compost + *Trichoderma* TW2; ANT'S COMPOST V – CV: Green compost; Trichoderma sp. – TW2

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951 Table 8 Pearson's correlation coefficient between microbial gene abundance, disease severity (DS)

952 and potential nitrification activity (PNA) assesses in rhizosphere (rhizo) and bulk soil samples

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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***		

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

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974 Table 9 Pearson's correlation coefficient between, *Bacillus*, *Pseudomonas, Trichoderma*, FOL

975 population gene abundance and chemical properties assessed in rhizosphere (rhizo) and bulk soil

976 samples

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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**

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979
980 Significance levels: not significant-ns: p>0.05; *p<0.05; **p<0.01; ***p<0.001

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