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Influence of plant genotype on the cultivable fungi associated to tomato rhizosphere and roots in different soils

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1	Influence of plant genotype on the cultivable fungi associated to tomato rhizosphere and
2	roots in different soils
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20 Abstract

21 Rhizosphere and root-associated microbiota are crucial in determining plant health and in 22 increasing productivity of agricultural crops. To date, research has mainly focused on the 23 bacterial dimension of the microbiota. However, fungi play a key role in soil ecosystems, 24 being involved in symbiosis, plant pathogenicity, or biocontrol. Consequently, interest in 25 the mycobiota is rapidly increasing. In this work, we examined the effect of plant genotype, soil, and of the pathogen Fusarium oxysporum f. sp. lycopersici (Fol) on the cultivable 26 27 component of rhizosphere and root-associated mycobiota of tomato plants. Resistant 28 (Heinz 1706) and susceptible (Moneymaker) varieties were cultivated on two soils diverse 29 for their history, geographical origin, physical and chemical characteristics, (identified as 30 A and B throughout the work), under glasshouse conditions. Isolated fungi were identified 31 by morphological and molecular approaches. The lower diversity was retrieved from the 32 combination soil A/Moneymaker, where Fusarium, Trichoderma, and Penicillium were the 33 most represented genera. Differences were found when comparing the rhizosphere to the 34 roots, which in general displayed a lower number of species. The structure of the cultivable 35 mycobiota was significantly affected by the soil type in the rhizosphere as well as by the 36 plant genotype within the roots (NPERMANOVA, p<0.05). The addition of *Fol* to Heinz 37 1706 changed the community structure, particularly in soil A, where *Penicillium* spp. and 38 *Fusarium* spp. were the dominant responding fungi. Overall, the results indicated that i) 39 soil type and plant genotype affect the fungal communities; ii) plant roots select few species 40 from the rhizosphere; and iii) the fungal community structure is influenced by the pathogen. 41

42 Keywords: Mycobiota, Fusarium wilt, Plant Genotype, Soil Type

44 **1. Introduction**

In plants, a microbiota is an interactive microorganism community associated with the plant rhizosphere and roots, which plays a crucial role in influencing plant health (Abd-Elsalam et al. 2010; Mendes et al. 2011). Likewise, the microbial community is affected by both plant and soil type; specific members of the microbiota are stimulated or repressed by chemical exudates released in the rhizosphere, the root-surrounding soil region (Berendsen et al. 2012).

51 Vegetable and ornamental crops are often attacked by several soilborne pathogens, 52 resulting in economic losses. Tomato (Lycopersicum esculetum) is a popular and 53 economically relevant culture and has been proposed as a model for studying plant-54 pathogens interactions, since its productivity can be limited by a number of diseases caused 55 by viruses, bacteria and fungi (Arie et al. 2007). One of the major soilborne pathogens that 56 endangers tomato crops worldwide is *Fusarium oxysporum*, the causal agent of Fusarium 57 wilt, which is capable of affecting a variety of crops species. F. oxysporum has been 58 subdivided in over 120 morphologically undistinguishable *formae speciales*, depending on 59 the host plant (Michielse and Rep 2009), further classified into physiological races on the basis of cultivar specificity (Di Pietro et al. 2003). To date, management of wilt disease 60 61 relies mainly on soil disinfestation and use of resistant cultivars. However, several 62 compounds have been banned or limited in their use. As for the use of resistant cultivars, 63 new more virulent races frequently arise to overcome the host resistance (Kinkel et al. 64 2011). Therefore, due to the possible alternatives in disease control (Fravel et al. 2003; 65 Mazzola 2002, 2004), the search for potential biocontrol agents is intensifying.

66 The microbial community *in toto* (bacteria, fungi, pseudofungi and protozoa) is
67 considered to be crucial for plant protection and novel discoveries are necessary to improve
68 crop quality and yield. As supported by a number of studies, several factors including the

69 plant species, the plant genotype and the soil type are capable of shaping the rhizosphere 70 microbiota (Hardoim et al. 2011; Inceoglu et al. 2012; Philippot et al. 2013). Considering 71 the fact that plant resistance represents one of the strategies to overcome vascular diseases, 72 several studies have been conducted on a number of crops in order to clarify the effects of 73 resistant and susceptible cultivars on microbial communities (An et al. 2011; Azad et al. 74 1987; Nallanchakravarthula et al. 2014; Yao and Wu 2010). The soil microbial community has been demonstrated to be significantly affected by the plant genotype, indicating a role 75 76 of the rhizosphere microorganisms in conferring resistance to pathogens (An et al. 2011; 77 Inceoglu et al. 2012; Nallanchakravarthula et al. 2014).

Along with the rhizosphere microorganisms, the so-called "endophytes" which are associated to the plant tissues, are a relevant component of the root microbiome. The endophytic community, as the rhizospheric community, is important for plant growth and is influenced by plant and soil factors, and microbial features responsible for the survival of endophytes within the roots (Gaiero et al. 2013; Turner et al. 2013).

Understanding the rules that drive formation of a plant microbiome and identifying its components is a crucial point to increase productivity and reduce pathogen attacks. To date, several studies have mainly focused on the bacterial microbiota (Bulgarelli et al. 2013; Chaparro et al. 2014; Inceoglu et al. 2012; Spence et al. 2014; Turner et al. 2013), while a void has still to be filled on the fungal community and its function, although research on this topic is rapidly increasing (Nallanchakravarthula et al. 2014; Nam et al. 2015; Yao and Wu 2010).

Tomato is known to differentially respond to beneficial (Salvioli et al. 2012), pathogenic and biocontrol fungi (Spadaro and Gullino 2005) and genotypes with different features provide an unprecedented model to investigate the network of interactions taking place belowground. With the present work, we intended to shed a light on the cultivable

94 component of the mycobiota associated to tomato plant, clarifying how the soil and the 95 plant genotype can determine its shaping. In addition, we aimed to assess whether the 96 presence of a fungal pathogen could modify the structure of the rhizosphere and root 97 associated fungal community. Finally, the availability of fungal cultures (both from 98 rhizhosphere and roots) would offer valuable tools to investigate the functionality of the 99 fungal communities with the intent of reconstructing specific tomato microbiomes; to this 100 aim, cultivable fungi only were considered in this work.

101

102 2. Materials and methods

103 **2.1. Plant cultivars, experimental soils and plant growth**

104 Two cultivars of tomato and two different soils were used in this study. The 105 cultivars Heinz 1706 and Moneymaker, were selected as resistant (R) and susceptible (S) 106 to *Fusarium oxysporum* f. sp. *lycopersici*, respectively (Huang and Lindhout 1997; 107 Ozminkowski 2004). Two soils, A and B, were collected in Northern Italy and chosen on 108 the basis of their different history, physical and chemical characteristics which were 109 determined by AgroBio Lab (Rutigliano, Italy) with accredited methods for pH, structure, 110 organic carbon, total nitrogen, mineral composition, and conductivity (Table 1). Soil A was 111 cultivated with vegetables since 1980 while soil B was taken from a field where wheat was 112 cultivated for 15 years and later the soil was set aside for ten years (no crops were grown). 113 Tomato seeds of both cultivars were sown in plug trays (80 plugs/tray) containing 114 peat-perlite substrate and were watered daily. Following, three 14 days old tomato 115 seedlings were transplanted in 2 L pots containing either soil A or soil B. Three pots were 116 prepared for each treatment. Plants were maintained for 4 weeks under glasshouse 117 conditions (temperature ranging between 26°C and 28°C; automatic watering and shading).

- 118 In order to evaluate the influence of a soilborne pathogen on the mycobiota of the
- 119 resistant cultivar, *Fusarium oxysporum* f. sp. *lycopersici (Fol)* was inoculated in both soils.
- 120 Prior to seedling transplant the two soils were mixed with Fol in form of talc powder
- 121 (Srinivasan et al. 2009) at the final rate of 3×10^4 chlamydospores mL⁻¹ of soil.
- 122 **2.2. Isolation and identification of cultivable fungi**
- 123 **2.2.1. Sample collection**
- 124 Following careful removal of the aboveground plant, rhizospheric soils derived
- 125 from Heinz 1706 and Moneymaker tomato plants cultivated in soil A and B, were treated
- 126 as described by Lundberg et al. (Lundberg et al. 2012). Briefly, loose soil was removed
- 127 from the roots by gently shaking and patting with sterile gloves. Roots were placed in sterile
- 128 50 mL tubes containing 25 mL phosphate buffer and vortexed to release most of the
- 129 rhizospheric soil. To remove large debris, the turbid solution was filtered into a new 50 mL
- 130 tube and centrifuged for 15 min at 3,500 g. The supernatant was discarded and the loose
- 131 pellets containing microorganisms was resuspended and transferred to 1.5 ml tubes.
- 132 Following centrifugation at 10,000 g for 5 min, pellets were processed further, as described
- 133 in the next section. In parallel, the root systems were transferred to clean sterile tubes, and
- 134 serially washed until the buffer was clear after vortexing.
- 135 **2.2.2. Isolation of fungi from the rhizosphere**
- 136 Rhizosphere samples were analysed by soil dilution plate method on two agar media,
- 137 as follows. A phosphate buffer dilution of 10^{-4} was prepared from about 1 g of fresh soil
- 138 obtained from roots serial washing. One mL of the final dilution was mixed with 30 ml of
- 139 Malt Extract Agar (MEA) or Komada's medium (selective for Fusarium spp.),
- 140 supplemented with antibiotics (streptomycin, 0.015 g L^{-1} ;chloramphenicol, 0.05 g L^{-1}) and
- 141 placed in 15 cm diameter Petri dishes.

142 For each plant and medium, three replicates were performed. Following incubation 143 at 24°C in the dark for 7-10 days, colony forming units (CFU) were counted by visual 144 observation and isolated in pure culture. The fungal load (CFU per g of dry weight) was then calculated both for the total mycoflora and for each species or morphotype. 145 146 2.2.3. Isolation of roots associated fungi For the isolation of endophytic fungi, ten 0.5 cm specimens for each cleaned plant 147 148 root, were sonicated in sterile distilled water at low intensity five times for 30 seconds and 149 placed in 15 cm Petri dishes containing MEA or Komada. Three replicates were performed

150 for each plant and medium. Samples were incubated in the dark at 24°C and colony growth

- 151 was monitored over time up to 30 days. Colonies isolation and count were accomplished
- 152 following the methods described above.
- 153 **2.2.4. Morphological and molecular identification**

154 Morphological identification of each strain was achieved according to the relevant 155 taxonomic keys (Domsch et al. 1980; Kiffer and Morelet 1997; von Arx 1981) and 156 confirmed by sequencing the appropriate DNA region (ITS, α -actin, β -tubulin). Genomic 157 DNA of all strains was extracted from about 100 mg of mycelium scraped from PDA Petri 158 dishes using the NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA), according to 159 the manufacturer's instructions. The quality and quantity of DNA samples was measured 160 with the ND-1000 Spectrophotometer NanoDropH (Thermo Scientific, Wilmington, 161 Germany). DNA extracts were stored at -20°C. The ITS sequences were amplified using the primer pair ITS1/ITS4 (White et al. 1990). For those strains morphologically identified 162 163 as Aspergillus spp. and Penicillium spp., amplification of the β -tubulin gene was performed 164 using primers Bt2a/Bt2b (Geiser et al. 1998; Glass and Donaldson 1995; Samson et al. 165 2004), while molecular identification of species belonging to the genus *Cladosporium* spp. 166 was inferred through the analysis of the α -actin gene using the primer pair ACT-

512F/ACT-783R (Carbone and Kohn 1999). Reaction mixtures consisted of 30 ng genomic
DNA, 1 μM each primer, 1 U Taq DNA Polymerase (Qiagen, Chatsworth, CA, USA), 10x
buffer, and 200 μM each dNTP. DNA amplifications were performed using a T-Gradient
thermal cycler (Biometra, Göttingen, Germany) with the following profile: 95°C for 5 min;
35 cycles: 95°C for 40 sec, 55°C (58°C for Bt2a/Bt2b) for 45 sec, 72°C for 50 sec; 72°C
for 8 min. PCR products were purified and sequenced at Macrogen Europe Laboratory
(Amsterdam, The Netherlands).

To confirm pathogen inoculation, genomic DNA of all *Fusarium oxysporum* isolates was subjected to microsatellite screening by using the core sequence of the microsatellite M-13 as a primer (Abd-Elsalam et al. 2010; Asran-Amal et al. 2005). Amplicons were separated on 1.5% agarose gel stained with 5 μ L 100 mL⁻¹ ethidium bromide and a GelPilot 1 kb plus DNA Ladder was used; images were acquired with a Gel Doc 1000 System (Bio-Rad, Hercules, CA, USA) and fingerprints were analysed against the positive control used for inoculum preparation (*Fol*) using Bionumerics 7.1 software.

181 Representative strains of each species isolated in pure culture during this work are
182 preserved at Mycotheca Universitatis Taurinensis (MUT). The Accession numbers of the
183 sequences deposited in GenBank are: KR709174-KR709205, KR856498-KR856506,
184 KT013225-KT013243, KT030798.

185 **2.3. Disease suppression assay**

In light of the findings that the organisms isolated included species, which may act as biological control agents, the two soils were tested for disease suppression potential. Steamed peat (30 min at 70 °C) served as control. Following soil inoculation with *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), five 14 days old tomato seedlings of both cultivars were transplanted in 2 L pots. *Fol* was supplied in a talc powder form at a concentration of 3×10^4 chlamydospores mL⁻¹ of soil, as previously described. Four pots per treatment were set 192 up. Plants were maintained under glasshouse conditions with temperature ranging between 193 26°C and 28°C. Symptoms started to be visible 14 days after artificial inoculation. Disease 194 development was monitored weekly, and a disease index ranging from 0 to 100 was 195 assigned throughout the experiments (0 = healthy plant; 25 = slight leaf chlorosis; 50 =196 severe leaf chlorosis, growth reduction and initial symptoms of wilting; 75 = severe wilting 197 symptoms, leaf chlorosis and strong growth reduction; 100 = dead plant). Symptomatic 198 plants showed brown or black streaks in the vascular system. The final disease index was 199 evaluated 30 days after transplant, and a stem dissection confirmed the presence of Fol, 200 revealed by discoloration of the vascular system. The experiment was repeated three times.

201 **2.4. Statistical analysis**

202 NPMANOVA and Biodiversity analyses were performed using PAST
203 (PAleontological STatistics) software for data analysis in ecology (Huang et al. 2013)
204 available on the Web (http://folk. uio.no/ohammer/past/).

Statistical significance on the total fungal load (CFU g⁻¹ dwt) was inferred by 205 206 applying the analysis of variance (ANOVA), Bonferroni post hoc test (p<0.05), using 207 GraphPad Prism 5 software. In order to avoid sampling size effects, the number of CFU 208 per sample was normalized by randomly subsampling to the lowest number of CFU 209 among samples; the relative abundance of species was used for NPMANOVA analysis 210 using Bray-Curtis dissimilarity indices. Subsampling was achieved by means of *rrarefy* 211 function in the R package vegan (Oksanen et al. 2013). Biodiversity of fungal communities 212 was compared in four conditions (SA, Susceptible cultivar/soil A; SB, Susceptible cultivar/ 213 soil B; RA, Resistant cultivar/ soil A; RB, Resistant cultivar/soil B) by applying the 214 Shannon (H, which incorporates species richness and species evenness) and Simpson (D, 215 which incorporates species richness and abundance) diversity indices, which were 216 statistically compared (Mann-Whitney test; p<0.05).

217 **3. Results**

218 **3.1. Soils features**

219 Soil A and soil B were collected in Northern Italy (Liguria and Piedmont 220 respectively) and analysed for the respective chemical features. Both soils presented a 221 similar soil texture, with a high proportion of sand, followed by clay and silt. A sandy clay 222 loam texture was observed in both cases. The pH was similar, although slightly higher in 223 soil B. Organic carbon was much higher in soil A while the total nitrogen was similar 224 between the two soils. Soil A was characterized by a high conductivity and a high presence 225 of minerals, including potassium, sodium and iron. Finally, magnesium was high in both 226 soils (Table 1).

227 **3.2.** Direct counts of fungal communities in soil and roots

The total fungal load for cultivable rhizospheric fungi ranged from 3.68×10^5 to 228 6.50×10^6 CFU g⁻¹ dwt, with significant differences among the samples (Fig. 1A). The 229 230 highest fungal load was found in the soil A cultivated with the susceptible cultivar (6.50 x)231 10⁶ CFU g⁻¹ dwt), while a significant reduction was present in soil B for both cultivars (3.68 x 10⁵ CFU g⁻¹ dwt and 6.48 x 10⁵ CFU g⁻¹ dwt for susceptible and resistant, 232 233 respectively). A significant difference between the two cultivars was observed only in soil A (6.50 x 10⁶ CFU g⁻¹ dwt and 1.48 x 10⁶ CFU g⁻¹ dwt for susceptible and resistant, 234 235 respectively). As for the endophytic fungi, the fungal load changed significantly only when 236 the resistant cultivar was grown on the soil B (Fig. 1B).

A total of 84 fungal entities, belonging to 40 genera, were identified from soil of tomato plants. The highest number of species was isolated from the rhizosphere (81 species belonging to 39 genera). As for the roots, 24 species belonging to 15 genera ("endophytic fungi" throughout the work) were detected; of these, 22 were in common with the rhizosphere, while *Myrothecium verrucaria* and *Setophoma terrestris* were exclusively isolated from roots. The majority of the species belonged to the Ascomycota, whereas one
single Basidiomycota (*Irpex lacteus*) was recovered from the rhizosphere of soil B. Seven
Zygomycota were also detected (Table S1).

The statistical analysis of the normalized data relative to the fungal abundance in the rhizosphere, showed that the soil had a strong effect on the variance (NPERMANOVA; p<0.05) while in the roots both soil and genotype, and the combination of these two factors, influenced significantly the structure of the mycobiota (Table 2).

249 Diversity of fungal communities was also compared. The Shannon biodiversity 250 index was significantly lower (p<0.05) when soil A was cultivated with Moneymaker for 251 both the rhizospheric and endophytic species (Table 3). Moreover, a significant higher 252 diversity was detected in the resistant cultivar/soil A for the rhizosphere and in the 253 susceptible cultivar/soil B within the roots. The genera with the highest load in the 254 rhizosphere were Fusarium, Gibellulopsis, Penicillium, Phoma, Pyrenochaetopsis, 255 Sarocladium, and Trichoderma, whereas in the roots were Fusarium and Trichoderma. The 256 genera with the highest load in soil A were Fusarium spp., Phoma spp., Pyrenochaetopsis 257 decipiens, Sarocladium strictum, and Trichoderma spp., while in soil B were Trichoderma 258 spp., *Penicillium* spp., *Sarocladium* strictum and *Fusarium* spp. (Fig. 2 A & B, Table S1). 259 As shown in the Venn diagrams (Fig. S1), four rhizospheric species (Trichoderma 260 harzianum, Sarocladium strictum, Trichoderma longibrachiatum, and Penicillium 261 carneum) were common to all treatments (SA, SB, RA, RB). In soil A, the highest number 262 of both total (34 vs 19) and exclusive (15 vs 5) species was recorded on the resistant cultivar, 263 while in soil B the number of total (24 vs 26) and exclusive (10 vs 10) species was almost 264 identical between the resistant and susceptible cultivar. Finally, the number of species in 265 common between the two cultivars was almost identical in both soils (10 and 11, 266 respectively). When considering the endophytic fungi, none of the species isolated was

shared among the four treatments. Interestingly, *Trichoderma harzianum* was the only organism common to three treatments (SA, RA, RB) (Fig. S1B). Furthermore, the percentage of species exclusive for one of any treatment was higher in the roots (86% exclusive vs 14% shared) while, in the rhizosphere, 59% of the species were unique and 41% were common to at least two conditions (Table S1).

272 **3.3. Effect of pathogen addition on the fungal community**

Fingerprints images obtained from M-13 microsatellite amplification of all *Fusarium oxysporum* isolates were analysed against the positive control used for inoculum preparation (*Fol*). *Fol* was re-isolated from the rhizosphere and roots of tomato planted on both soils (Fig. 3).

277 The addition of the pathogen generally did not have a significant influence on the 278 load and fungal diversity in the rhizosphere in both soils (Fig. 4A; Table 3). However, a 279 deeper analysis revealed that, following the inoculation of the pathogen, soil A responded 280 with a significant increment of *Fusarium* spp., *Penicillium* spp., and *Trichoderma* spp. in 281 the rhizosphere (Fig. 5A) and of *Fusarium* spp. and *Zygomycetes* spp. in the roots (Fig. 282 5B). As for soil B, the number of total CFU increased only in endophytic fungi (Fig. 4B). 283 In addition, few species (Acremonium crotocinigenum, Aspergillus fumigatus, 284 Cladosporium oxysporum, Doratomyces stemonitis, Penicillium griseofulvum, Penicillium 285 spinulosporum) were detected only in the presence of Fol, suggesting a change in the 286 mycoflora composition.

287

288 **3.4. Disease suppression assay**

Considering the results described above, a disease suppression assay was performed
in order to evaluate whether the species retrieved in soil A could reduce the incidence of
Fusarium wilt. The assessment of a disease index showed that only the susceptible cultivar

(Moneymaker) developed wilt disease, which showed a reduction both in soil A (2.8 %)
and soil B (27.1 %) in comparison to steamed peat (57.2%) which was used as a control.
No disease symptoms appeared in the resistant cultivar Heinz 1706, as expected. Due to
the high variability however, the difference between the two soils was not significant, even
though a clear trend was observed.

297

298

5. Discussion

Taking in consideration multiple parameters (plant genotype, soil, pathogen presence) we demonstrate that soil is the major driving force in shaping the cultivable mycobiota, where *Fusarium, Penicillium, Sarocladium* and *Trichoderma* genera resulted dominant. Alternatively, a genotype effect and a lower fungal diversity were found among fungi with an endophytic profile different from the more diverse rhizospheric fungi.

304 In terms of quantitative evaluation, a significantly higher fungal load for the 305 susceptible cultivar was measured in the rhizosphere of soil A, although the lower diversity 306 observed could be due to a soil/cultivar synergic effect. Our results are similar to those 307 recently reported by Nallanchakravarthula et al. (2014), who assessed the influence of soil 308 type and cultivars on the rhizosphere and root mycobiota of strawberry, demonstrating a 309 stronger effect of soil respect to the plant genotype. It could reasonably be argued that a 310 variation in fungal diversity may be due to different physical-chemical properties of the 311 soils, although this can not completely explain the differences observed in soil A between 312 the susceptible (>CFU) and resistant cultivars (Fig. 1A). This aspect was taken into 313 consideration in determining the structure of fungal communities in suppressive and 314 conducive soils to Rhizoctonia solani, clarifying that it was not as crucial as the one 315 associated to suppression abilities (Penton et al. 2014), thus supporting the hypothesis that 316 the difference observed is due to the mycotic community specific for each soil.

The effect of plant genotype on fungal population was not significant when soil B was evaluated (Fig. 1A), in contrast with the observations on the rhizosphere of different cultivars of cucumber, where a higher number of CFU was associated with the cultivar susceptible to Fusarium wilt (Yao and Wu 2010). The opposite behaviour of the pair Moneymaker/Heinz 1706 which appears to be clear in soil A, vanished in soil B suggesting a synergism between the factors soil and genotype.

323 Regarding the endophytes, significant differences were detected in the resistant 324 cultivar when grown on soil A(>CFU) or soil B (<CFU) (Fig 1B). This may be explained 325 considering the fact that in soil A a wider fungal population might penetrate the roots of 326 the resistant cultivar for the following reasons: (i) the abundance of the species in soil A is 327 slightly higher, (ii) some of these species may be involved in biological control processes, 328 (iii) none of the species present in the rhizosphere compromise the resistant cultivar; 329 consequently the root colonization would not affect the plant health and the resistance 330 properties. In addition, some species appear to be recruited by the resistant genotype 331 cultivated on soil B (i.e. Penicillium spp. and Trichoderma spp.). A soil effect is evident, 332 since a similar trend, although not significant, can be noticed in the susceptible cultivar, 333 probably as a consequence of a higher root colonization due to poor resistant mechanisms 334 of the plant.

When a random subsampling was applied to normalize the number of CFU with the intent of avoiding sampling size artifacts, a genotype effect on the fungal community was evident only within the roots, while a dominant role was played in the rhizosphere by the soil. This may be explained by hypothesizing that in the rhizosphere the difference inferred by the soil is prevalent to such an extent that the genotype factor appears to be trivial. Besides, it has to be considered that roots are the first plant organs to come physically in contact with the microbiome belowground, thus disclosing the importance of

342 the plant genotype in selecting different microorganisms (Lakshmanan et al. 2014). To this 343 respect, diverse genetic profiles of Arabidopsis thaliana have been proved to influence the 344 production and secretion of phytohormones, which in turn modulate the assemblage of the 345 endophytic bacterial community (Lebeis et al. 2015). Species of Penicillium (e.g. P. 346 canescens and P. rubens) and Paecilomyces marquandii have been found almost 347 exclusively in the rhizosphere of the susceptible plants grown in soil A. These species, 348 together with others belonging to the genera Chaetomium, Gliocladium, Penicillium, 349 Paecilomyces, Sporothrix and Trichoderma are known for their biocontrol properties 350 (Paulitz and Belanger 2001; Punja and Utkhede 2003). Thus, the genotype of tomato plants 351 may be important for the selection of a pool of useful organisms naturally present in a soil. 352 This hypothesis may explain the lower diversity observed in this condition. Interestingly, 353 the addition of the pathogen Fol to soil A, evaluated on the resistant cultivar only, is 354 associated to a significant increase in CFU of Fusarium spp., Penicillium spp. and 355 Trichoderma spp. in the rhizosphere, as reported by a number of studies (Berg et al. 2005; 356 Rivera et al. 2009). The ability of a soil to contain a disease is usually ascribed to the effects 357 of a number of microorganisms and the three genera retrieved include species largely 358 recognised as biocontrol agents. Several studies attest the antagonistic activity of species 359 of Penicillium spp. (e.g. P. canescens, P. funiculosum, P. oxalicum and P. rubens (Chen et 360 al. 2006; Larena et al. 2003; Nicoletti et al. 2007; Radhakrishnan et al. 2013; Sabuquillo et 361 al. 2006), Trichoderma spp. (Balasubramanian et al. 2014; Dubey et al. 2007; Nel et al. 362 2006b) and non pathogenic Fusarium spp. (Aime et al. 2013; Fuchs et al. 1999; Nel et al. 363 2006a, b) against Fusarium wilt. The suppression of a soilborne disease is generally 364 accomplished through the secretion of plant growth promoting metabolites, such as indole 365 acetic acid (Radhakrishnan et al. 2013), production of antibiotics, competition for nutrients, mycoparasitism and induction of plant defence reactions (Vos et al. 2014). Presumably, the 366

367 presence of *Fol* allows the plant to draw in its vicinity those beneficial fungal species 368 naturally present in a soil which are capable of conferring protection. For instance, *P*. 369 *canescens*, known for the production of fungitoxic secondary metabolites strongly 370 increases its load in the presence of *Fol* (Nicoletti et al. 2007). Therefore, a relation between 371 a pathogenic species and potential biocontrol taxa should be considered, as also proposed 372 by Vujanovic et al. (2007), who conducted a survey on fungal species associated with black 373 spruce trees.

374 A different situation occurred in the roots: *Penicillium* spp. and *Trichoderma* spp. 375 were not recovered, contrary to Fusarium spp. (which can be only partly ascribed to the 376 presence of the inoculum) and to the fast growing Zygomycetes, which can dominate an 377 environment free of other competitors. Among the F. oxysporum strains isolated from the 378 roots of plants cultivated on soil A, beside Fol, a number of strains with different genetic 379 profiles, which may be both pathogenic and/or antagonists, were detected. Non pathogenic 380 as well as pathogenic F. oxysporum can colonize tomato roots (Bao and Lazarovits 2001) 381 as demonstrated for the well-studied biocontrol strain F. oxysporum 47 (Fo47), whose artificial root inoculation has been proved to increase the expression of genes encoding 382 383 extracellular proteins potentially involved in the salicylic acid-dependent plant defence 384 pathway (Aime et al. 2013). Finally, a change in the composition of the fungal community 385 can be asserted, since few species are present or absent only in this condition.

In light of these findings, it was reasonable to assess whether soil A had the potential to prevent Fusarium wilt in tomato. To this aim, a disease suppression assay was performed, and a decrease of the disease incidence was recorded in both soils in comparison to the steamed peat. Every natural soil possesses the ability to counteract a disease to different degrees, depending on the biotic components (Mazzola 2004). However, the disease index in soil A was slightly lower than soil B, although due to the high variability

392 observed a statistical significance was not inferred. The observed disease reduction may be 393 due to the presence of antagonistic bacteria and/or fungi; however, clarifying to which 394 extent these organisms contribute to the feature displayed by soil A, was not the purpose 395 of this work and further detailed studies will be necessary.

396 In conclusion, this work shows a major driving force of the soil type in shaping the 397 rhizosphere mycobiota in tomato plants, while a significant role of the genotype was 398 additionally found within the roots. Future studies will broaden the number of tested soils 399 in order to extrapolate common features underlying soil ability to control a disease. In 400 addition, the presence of the soilborne pathogen Fol is remarkable, since genera that may 401 include agents of biocontrol positively respond to its presence particularly in the soil A. 402 However, it must be considered that only cultivable fungi were contemplated, which could 403 not completely account for the reported observations; a combined approach merging 404 metagenomics and culturomics studies could unfold the entire scenario, as many cultured 405 species fail to be identified by PCR-based methods only (Gouba et al. 2013, 2014).

406

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- 411
- 412

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TABLES

- **Table 1.** Physical and chemical characteristics of the soils considered in this study.

•		Soil A	Soil B
	Origin	Albenga (SV), Liguria,	Rosta (TO), Piedmont,
		northern Italy	northern Italy
	Geographical	44.067171 N, 8.212949 E	45.074190 N, 7.461910 E
	coordinates		
	pН	7.22	7.60
	sand:silt:clay (%)	60.0:10.7:29.3	60.0:16.7:23.3
	C organic (%)	3.18	0.60
	N total(‰)	1.68	1.54
	Ca (mg/Kg)	3903.80	4036.90
	Mg (mg/Kg)	726.00	469.40
	K (mg/Kg)	834.20	116.00
	Na(mg/Kg)	895.20	149.40
	P (mg/Kg)	16.90	10.20
	B (mg/Kg)	0.70	1.00
	Fe (mg/Kg)	93.70	19.60
	Conductivity		
-	(mS/cm)	9.90	0.46
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Table 2. NPMANOVA analysis of Bray-Curtis dissimilarities in the rhizospheric (A) and18in the endophytic (B) fungal community structure in relation to soil type, genotype and19their interaction (p<0.05). SSquares = sum of squares; df = degrees of freedom; MSquares20= mean sum of squares; F = value by permutation; p = p value based on 9999 permutations.

21 A.

Permutation N: 9999	SSquares	df	MSquares	F	р
Genotype	0.20288	1	0.20288	2.27	0.0798
Soil	0.4797	1	0.4797	5.3671	0.0005
Interaction	0.40047	1	0.40047	4.4807	0.0033
Residual	0.71502	8	0.089377		
Total	1.7981	11			

23 B.

Permutation N: 9999	SSquares	df	MSquares	F	р
Soil	0.7946	1	0.7946	3.3601	0.0013
Genotype	1.1468	1	1.1468	4.8496	0.0001
Interaction	0.7449	1	0.7449	3.1501	0.0022
Residual	1.8919	8	0.2364		
Total	4.5783	11			

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Table 3. *t-test* evaluating the effect of the soil type, genotype, and pathogen addition (+ *Fol*) on the rhizospheric (A) and endophytic (B) fungal diversity (Shannon index). t = t
score; df = degrees of freedom; p = p value.

- 36 A.
- 37

Genotype	t	р		38
SA vs. RA	-0.16	0.8	ns	39
SB vs. RB	0.73	0.5	ns	07
Soil				40
SA vs. SB	2.99	0.04	*	41
RA vs. RB	3.51	0.02	*	40
+ Fol	t	р		42
RAF vs. RA	0.98	0.3	ns	43
RBF vs. RB	-0.78	0.4	ns	
				44

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46 B.

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Genotype	t	р		48
SA vs. RA	-0.083	0.93	ns	49
SB vs. RB	3.71	0.02	*	
Soil				50
SA vs. SB	-7.0	0.002	**	51
RA vs. RB	-0.80	0.47	ns	F 2
+ Fol	t	р		52
RAF vs. RA	-0.11	0.91	ns	53
RBF vs. RB	-0.94	0.39	ns	_ .
				54

55 ** P< 0.01; * P< 0.05; ns non significant

CAPTION TO ILLUSTRATIONS

Figure 1. Fungal total load (CFU per gram of soil dry weight) detected in the rhizhosphere (A) and in the roots (B) of tomato plants cultivated on the two soils. SA (Susceptible cultivar/ soil A), SB (Susceptible cultivar/soil B), RA (Resistant cultivar/soil A), RB (Resistant cultivar /soil B). Results are expressed as mean \pm s.e. and analyzed through one-way analysis of variance (ANOVA), Bonferroni post hoc (p < 0.05).

Figure 2. Diversity (relative abundance in percentage) in the rhizosphere (A) and in the roots (B) of species contributing to the differences observed in all treatments. SA (Susceptible cultivar/ soil A), SB (Susceptible cultivar/soil B), RA (Resistant cultivar/soil A), RB (Resistant cultivar /soil B). (Resistant cv/ Conducive soil), RAF (Resistant cv/soil A/*Fol*), RBF (Resistant cv/soil B/*Fol*).

Figure 3. DNA fingerprinting profiles generated from genomic DNA of 20 *Fusarium oxysporum* isolates with the microsatellite primer M13. Twenty μ L of PCR products were separated by electrophoresis on a 1.5% agarose gel for 3 h at 45 V/cm². M = 1kb DNA ladder; 1 = *Fusarium oxysporum f. sp. lycopersici* used for the inoculum; 2 – 20 *Fusarium oxyporum* isolates from both rizospheric and root samples. Arrows indicate the pathogen retrieved in the samples.

Figure 4. Effect of the addition of *Fol* on the fungal total load (CFU per gram of soil dry weight) in the rhizosphere and in the roots for the treatment indicated. RA (Resistant/soil A), RAF (Resistant/soil A/*Fol*), RB (Resistant/soil B), RBF (Resistant/soil B/*Fol*). Results are expressed as mean \pm s.e. and analyzed through one-way analysis of variance (ANOVA), Bonferroni post hoc (p < 0.05).

Figure 5. Effect of the addition of *Fol* on the load (CFU per gram of soil dry weight) of single genera/groups in the rhizosphere and in the roots for the treatment indicated. Results are expressed

as mean \pm s.e. and analyzed through unpaired *t-test* RA (Resistant/soil A) vs RAF (Resistant/soil A/*Fol*) (capital letters) and RB (Resistant/soil B) vs RBF (Resistant/soil B/*Fol*) (lower case letters).

Figure 1. Number of total CFU per gram of dry soil detected in the rhizhosphere (A) and
in the roots (B) of tomato plants cultivated on the two soils. SA (Susceptible cultivar/ soil
A), SB (Susceptible cultivar/soil B), RA (Resistant cultivar/soil A), RB (Resistant
cultivar /soil B). Results are expressed as mean ± s.e. and analyzed through one-way
analysis of variance (ANOVA), Bonferroni post hoc (p < 0.05).

6 A.















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26 B.





- Aspergillus niger
- Cladosporium spp.
- Cylindrocarpon didymum
- Fusarium spp.
- Gliocladium virens
- Myrothecium verrucaria
- Penicillium spp.
- Pyrenochaetopsis decipiens
- Sarocladium strictum
- Setophoma terrestris
- Trichoderma spp.
- Zygomycetes spp.
- Not identified

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Figure 3. DNA fingerprinting profiles generated from genomic DNA of 20 *Fusarium oxysporum* isolates with the microsatellite primer M13. Twenty μ L of PCR products were separated by electrophoresis on a 1.5% agarose gel for 3 h at 45 V/cm². M = 1kb DNA ladder; 1 = *Fusarium oxysporum f. sp. lycopersici* used for the inoculum; 2 – 20 *Fusarium oxyporum* isolates from both rizospheric and root samples. Arrows indicate the pathogen retrieved in the samples.



Figure 4. Effect of the addition of *Fol* on the total fungal load of the rhizosphere and in
the roots for the treatment indicated. RA (Resistant/soil A), RAF (Resistant/soil A/*Fol*),
RB (Resistant/soil B), RBF (Resistant/soil B/*Fol*). Results are expressed as mean ± s.e.
and analyzed through one-way analysis of variance (ANOVA), Bonferroni post hoc (p <
0.05).

67 A.



68

69 B.



- 72 Figure 5. Effect of the addition of *Fol* on single genera/groups in the rhizosphere and in
- 73 the roots for the treatment indicated. Results are expressed as mean \pm s.e. and analyzed
- 74 through unpaired t-test RA (Resistant/soil A) vs RAF (Resistant/soil A/Fol) (capital
- 75 letters) and RB (Resistant/soil B) vs RBF (Resistant/soil B/Fol) (lower case letters).







