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Running Head: Root and rhizosphere mycobiota of tomato

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Abstract

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

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

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).

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

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

plant species, the plant genotype and the soil type are capable of shaping the rhizosphere microbiota (Hardoim et al. 2011; Inceoglu et al. 2012; Philippot et al. 2013). Considering the fact that plant resistance represents one of the strategies to overcome vascular diseases, several studies have been conducted on a number of crops in order to clarify the effects of resistant and susceptible cultivars on microbial communities (An et al. 2011; Azad et al. 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 of the rhizosphere microorganisms in conferring resistance to pathogens (An et al. 2011; 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

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

2. Materials and methods

2.1. Plant cultivars, experimental soils and plant growth

Two cultivars of tomato and two different soils were used in this study. The cultivars Heinz 1706 and Moneymaker, were selected as resistant (R) and susceptible (S) to *Fusarium oxysporum* f. sp. *lycopersici*, respectively (Huang and Lindhout 1997; Ozminkowski 2004). Two soils, A and B, were collected in Northern Italy and chosen on the basis of their different history, physical and chemical characteristics which were determined by AgroBio Lab (Rutigliano, Italy) with accredited methods for pH, structure, organic carbon, total nitrogen, mineral composition, and conductivity (Table 1). Soil A was cultivated with vegetables since 1980 while soil B was taken from a field where wheat was cultivated for 15 years and later the soil was set aside for ten years (no crops were grown).

Tomato seeds of both cultivars were sown in plug trays (80 plugs/tray) containing peat-perlite substrate and were watered daily. Following, three 14 days old tomato seedlings were transplanted in 2 L pots containing either soil A or soil B. Three pots were prepared for each treatment. Plants were maintained for 4 weeks under glasshouse conditions (temperature ranging between 26°C and 28°C; automatic watering and shading).

In order to evaluate the influence of a soilborne pathogen on the mycobiota of the resistant cultivar, *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) was inoculated in both soils. Prior to seedling transplant the two soils were mixed with *Fol* in form of talc powder (Srinivasan et al. 2009) at the final rate of 3×10^4 chlamydospores mL^{-1} of soil.

2.2. Isolation and identification of cultivable fungi

2.2.1. Sample collection

Following careful removal of the aboveground plant, rhizospheric soils derived from Heinz 1706 and Moneymaker tomato plants cultivated in soil A and B, were treated as described by Lundberg et al. (Lundberg et al. 2012). Briefly, loose soil was removed from the roots by gently shaking and patting with sterile gloves. Roots were placed in sterile 50 mL tubes containing 25 mL phosphate buffer and vortexed to release most of the rhizospheric soil. To remove large debris, the turbid solution was filtered into a new 50 mL tube and centrifuged for 15 min at 3,500 *g*. The supernatant was discarded and the loose pellets containing microorganisms was resuspended and transferred to 1.5 ml tubes. Following centrifugation at 10,000 *g* for 5 min, pellets were processed further, as described in the next section. In parallel, the root systems were transferred to clean sterile tubes, and serially washed until the buffer was clear after vortexing.

2.2.2. Isolation of fungi from the rhizosphere

Rhizosphere samples were analysed by soil dilution plate method on two agar media, as follows. A phosphate buffer dilution of 10^{-4} was prepared from about 1 g of fresh soil obtained from roots serial washing. One mL of the final dilution was mixed with 30 ml of Malt Extract Agar (MEA) or Komada's medium (selective for *Fusarium* spp.), supplemented with antibiotics (streptomycin, 0.015 g L^{-1};chloramphenicol, 0.05 g L^{-1}) and placed in 15 cm diameter Petri dishes.

For each plant and medium, three replicates were performed. Following incubation at 24°C in the dark for 7-10 days, colony forming units (CFU) were counted by visual 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.

2.2.3. Isolation of roots associated fungi

For the isolation of endophytic fungi, ten 0.5 cm specimens for each cleaned plant root, were sonicated in sterile distilled water at low intensity five times for 30 seconds and placed in 15 cm Petri dishes containing MEA or Komada. Three replicates were performed for each plant and medium. Samples were incubated in the dark at 24°C and colony growth was monitored over time up to 30 days. Colonies isolation and count were accomplished following the methods described above.

2.2.4. Morphological and molecular identification

Morphological identification of each strain was achieved according to the relevant taxonomic keys (Domsch et al. 1980; Kiffer and Morelet 1997; von Arx 1981) and confirmed by sequencing the appropriate DNA region (ITS, α-actin, β-tubulin). Genomic DNA of all strains was extracted from about 100 mg of mycelium scraped from PDA Petri dishes using the NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA), according to the manufacturer's instructions. The quality and quantity of DNA samples was measured with the ND-1000 Spectrophotometer NanoDropH (Thermo Scientific, Wilmington, 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 as *Aspergillus* spp. and *Penicillium* spp., amplification of the β-tubulin gene was performed using primers Bt2a/Bt2b (Geiser et al. 1998; Glass and Donaldson 1995; Samson et al. 2004), while molecular identification of species belonging to the genus *Cladosporium* spp. 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 µL 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.

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

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

up. Plants were maintained under glasshouse conditions with temperature ranging between 26°C and 28°C. Symptoms started to be visible 14 days after artificial inoculation. Disease development was monitored weekly, and a disease index ranging from 0 to 100 was assigned throughout the experiments (0 = healthy plant; 25 = slight leaf chlorosis; 50 = severe leaf chlorosis, growth reduction and initial symptoms of wilting; 75 = severe wilting symptoms, leaf chlorosis and strong growth reduction; 100 = dead plant). Symptomatic plants showed brown or black streaks in the vascular system. The final disease index was evaluated 30 days after transplant, and a stem dissection confirmed the presence of *Fol*, revealed by discoloration of the vascular system. The experiment was repeated three times.

2.4. Statistical analysis

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

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

3. Results

3.1. Soils features

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

3.2. Direct counts of fungal communities in soil and roots

The total fungal load for cultivable rhizospheric fungi ranged from 3.68 x 10^5 to 6.50 x 10^6 CFU g^{-1} dwt, with significant differences among the samples (Fig. 1A). The highest fungal load was found in the soil A cultivated with the susceptible cultivar (6.50 x 10^6 CFU g^{-1} dwt), while a significant reduction was present in soil B for both cultivars (3.68 x 10^5 CFU g^{-1} dwt and 6.48 x 10^5 CFU g^{-1} dwt for susceptible and resistant, respectively). A significant difference between the two cultivars was observed only in soil A (6.50 x 10^6 CFU g^{-1} dwt and 1.48 x 10^6 CFU g^{-1} dwt for susceptible and resistant, respectively). As for the endophytic fungi, the fungal load changed significantly only when 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).

Diversity of fungal communities was also compared. The Shannon biodiversity index was significantly lower ($p<0.05$) when soil A was cultivated with Moneymaker for both the rhizospheric and endophytic species (Table 3). Moreover, a significant higher diversity was detected in the resistant cultivar/soil A for the rhizosphere and in the susceptible cultivar/soil B within the roots. The genera with the highest load in the rhizosphere were *Fusarium*, *Gibellulopsis*, *Penicillium*, *Phoma*, *Pyrenochaetopsis*, *Sarocladium*, and *Trichoderma*, whereas in the roots were *Fusarium* and *Trichoderma*. The genera with the highest load in soil A were *Fusarium* spp., *Phoma* spp., *Pyrenochaetopsis decipiens*, *Sarocladium strictum*, and *Trichoderma* spp. , while in soil B were *Trichoderma* spp., *Penicillium* spp., *Sarocladium strictum* and *Fusarium* spp. (Fig. 2 A & B, Table S1).

As shown in the Venn diagrams (Fig. S1), four rhizospheric species (*Trichoderma harzianum*, *Sarocladium strictum*, *Trichoderma longibrachiatum*, and *Penicillium carneum*) were common to all treatments (SA, SB, RA, RB). In soil A, the highest number of both total (34 vs 19) and exclusive (15 vs 5) species was recorded on the resistant cultivar, while in soil B the number of total (24 vs 26) and exclusive (10 vs 10) species was almost identical between the resistant and susceptible cultivar. Finally, the number of species in common between the two cultivars was almost identical in both soils (10 and 11, 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).

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).

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

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

292 (Moneymaker) developed wilt disease, which showed a reduction both in soil A (2.8 %)
293 and soil B (27.1 %) in comparison to steamed peat (57.2%) which was used as a control.
294 No disease symptoms appeared in the resistant cultivar Heinz 1706, as expected. Due to
295 the high variability however, the difference between the two soils was not significant, even
296 though a clear trend was observed.
297
298 **5. Discussion**
299 Taking in consideration multiple parameters (plant genotype, soil, pathogen
300 presence) we demonstrate that soil is the major driving force in shaping the cultivable
301 mycobiota, where *Fusarium, Penicillium, Sarocladium* and *Trichoderma* genera resulted
302 dominant. Alternatively, a genotype effect and a lower fungal diversity were found among
303 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.

317 The effect of plant genotype on fungal population was not significant when soil B
318 was evaluated (Fig. 1A), in contrast with the observations on the rhizosphere of different
319 cultivars of cucumber, where a higher number of CFU was associated with the cultivar
320 susceptible to Fusarium wilt (Yao and Wu 2010). The opposite behaviour of the pair
321 Moneymaker/Heinz 1706 which appears to be clear in soil A, vanished in soil B suggesting
322 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.
335 When a random subsampling was applied to normalize the number of CFU with
336 the intent of avoiding sampling size artifacts, a genotype effect on the fungal community
337 was evident only within the roots, while a dominant role was played in the rhizosphere by
338 the soil. This may be explained by hypothesizing that in the rhizosphere the difference
339 inferred by the soil is prevalent to such an extent that the genotype factor appears to be
340 trivial. Besides, it has to be considered that roots are the first plant organs to come
341 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,
366 mycoparasitism and induction of plant defence reactions (Vos et al. 2014). Presumably, the

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

A different situation occurred in the roots: *Penicillium* spp. and *Trichoderma* spp. were not recovered, contrary to *Fusarium* spp. (which can be only partly ascribed to the presence of the inoculum) and to the fast growing Zygomycetes, which can dominate an environment free of other competitors. Among the *F. oxysporum* strains isolated from the roots of plants cultivated on soil A, beside *Fol*, a number of strains with different genetic profiles, which may be both pathogenic and/or antagonists, were detected. Non pathogenic as well as pathogenic *F. oxysporum* can colonize tomato roots (Bao and Lazarovits 2001) 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 extracellular proteins potentially involved in the salicylic acid-dependent plant defence pathway (Aime et al. 2013). Finally, a change in the composition of the fungal community 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

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

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

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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, northern Italy	Rosta (TO), Piedmont, northern Italy
Geographical coordinates	44.067171 N, 8.212949 E	45.074190 N, 7.461910 E
pH	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

Table 2. NPMANOVA analysis of Bray-Curtis dissimilarities in the rhizospheric (A) and in the endophytic (B) fungal community structure in relation to soil type, genotype and their interaction ($p<0.05$). SSquares = sum of squares; df = degrees of freedom; MSquares = mean sum of squares; F = value by permutation; p = p value based on 9999 permutations.

A.

Permutation N: 9999	**SSquares**	**df**	**MSquares**	**F**	**p**
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			

B.

Permutation N: 9999	**SSquares**	**df**	**MSquares**	**F**	**p**
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			

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.

A.

Genotype	*t*	*p*	
SA vs. RA	-0.16	0.8	ns
SB vs. RB	0.73	0.5	ns
Soil			
SA vs. SB	2.99	0.04	*
RA vs. RB	3.51	0.02	*
+ *Fol*	*t*	*p*	
RAF vs. RA	0.98	0.3	ns
RBF vs. RB	-0.78	0.4	ns

B.

Genotype	*t*	*p*	
SA vs. RA	-0.083	0.93	ns
SB vs. RB	3.71	0.02	*
Soil			
SA vs. SB	-7.0	0.002	**
RA vs. RB	-0.80	0.47	ns
+ *Fol*	*t*	*p*	
RAF vs. RA	-0.11	0.91	ns
RBF vs. RB	-0.94	0.39	ns

** 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 ± 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 µL of PCR products were separated by electrophoresis on a 1.5% agarose gel for 3 h at 45 V/cm^2. 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.

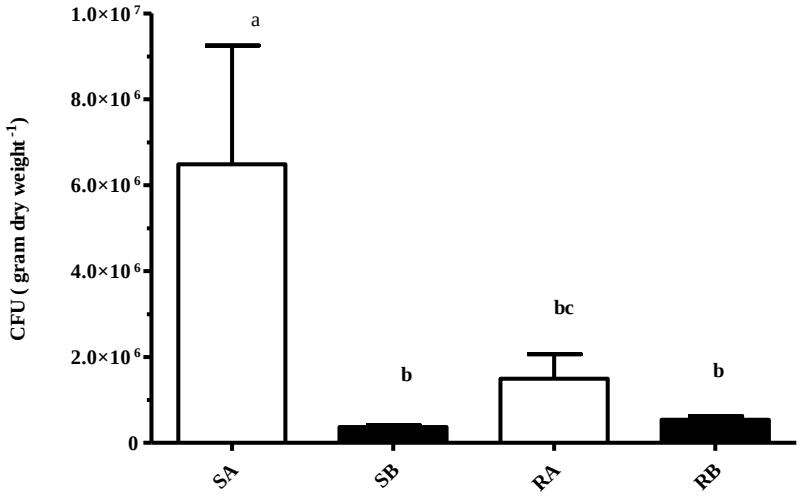
Figure 4. Effect of the addition of *Fol* on the fungal total load (CFU per gram of soil dry weight) in the rhizosphere and in the roots for the treatment indicated. RA (Resistant/soil A), RAF (Resistant/soil A/*Fol*), RB (Resistant/soil B), RBF (Resistant/soil B/*Fol*). Results are expressed as mean ± 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

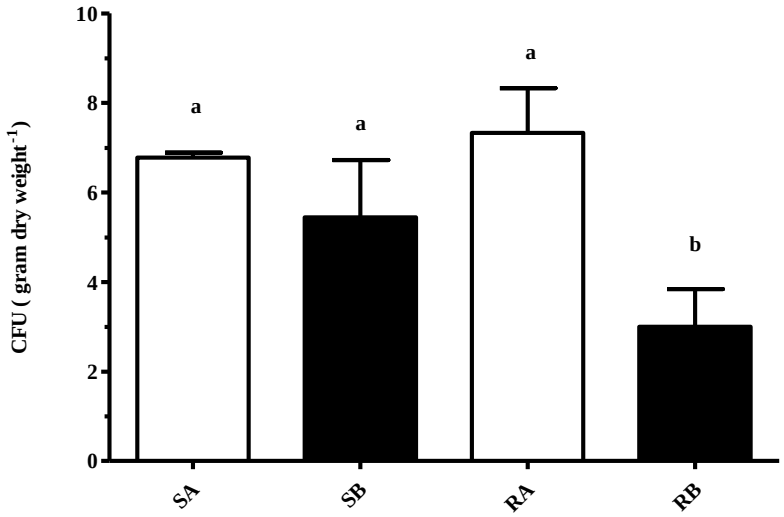
as mean ± s.e. and analyzed through unpaired *t-test* RA (Resistant/soil A) vs RAF (Resistant/soil A/*Fol*) (capital letters) and RB (Resistant/soil B) vs RBF (Resistant/soil B/*Fol*) (lower case letters).

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 ($p < 0.05$).

A.



B.



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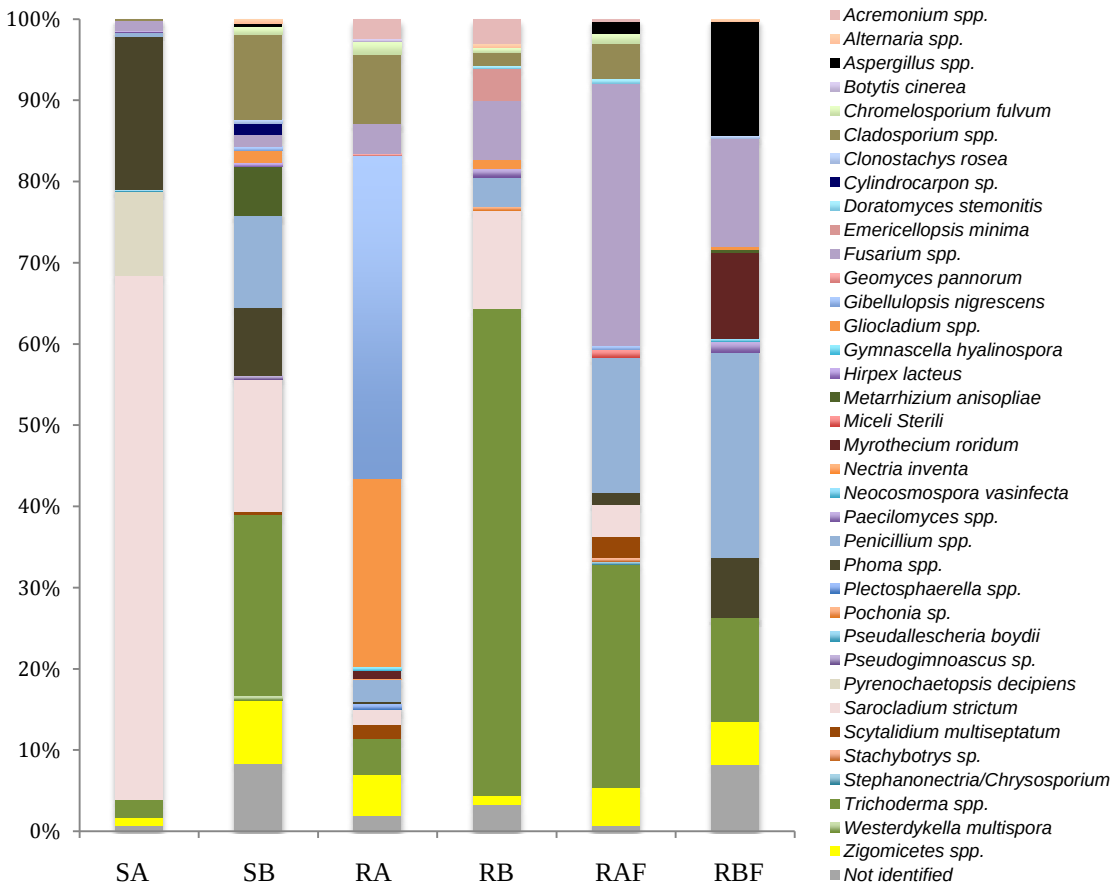
A.



B.

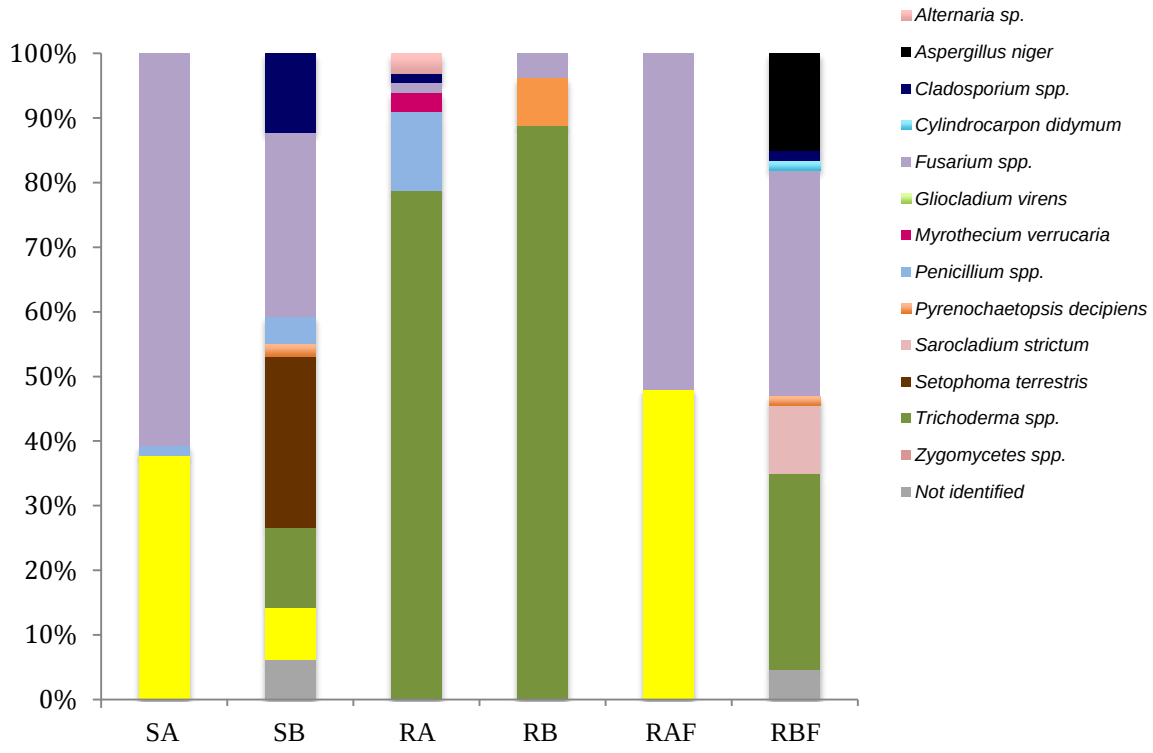

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
SA
SB
RA
RB
RAF
RBF
Alternaria sp.
Aspergillus niger
Cladosporium spp.
Cylindrocarpon didymum
Fusarium spp.
Gliocladium virens
Myrothecium verrucaria
Penicillium spp.
Pyrenochaetopsis decipiens
Sarocladium strictum
Setophoma terrestris
Trichoderma spp.
Zygomycetes spp.
Not identified

Figure 3. DNA fingerprinting profiles generated from genomic DNA of 20 *Fusarium oxysporum* isolates with the microsatellite primer M13. Twenty μL of PCR products were separated by electrophoresis on a 1.5% agarose gel for 3 h at 45 V/cm^2. 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.

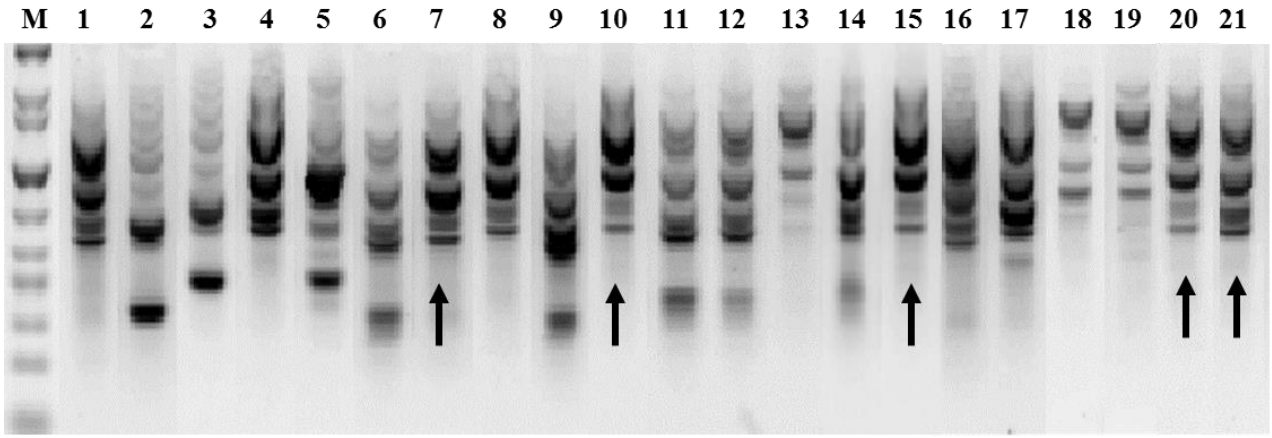
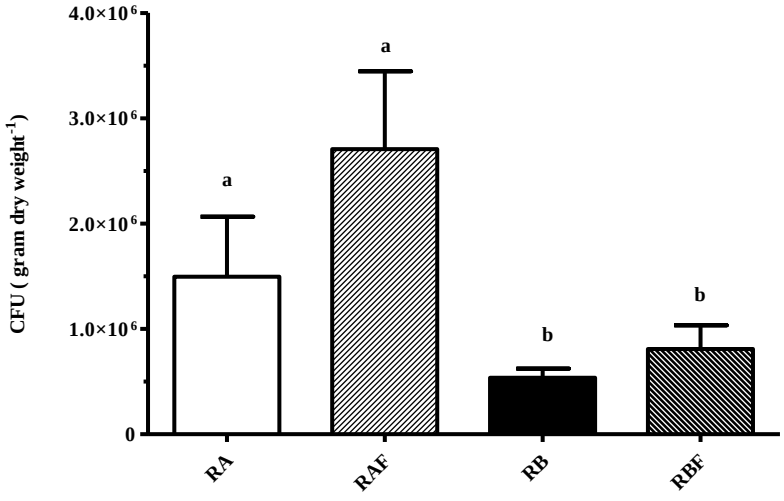


Figure 4. Effect of the addition of *Fol* on the total fungal load of the rhizosphere and in the roots for the treatment indicated. RA (Resistant/soil A), RAF (Resistant/soil A/*Fol*), RB (Resistant/soil B), RBF (Resistant/soil B/*Fol*). Results are expressed as mean ± s.e. and analyzed through one-way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

A.



B.

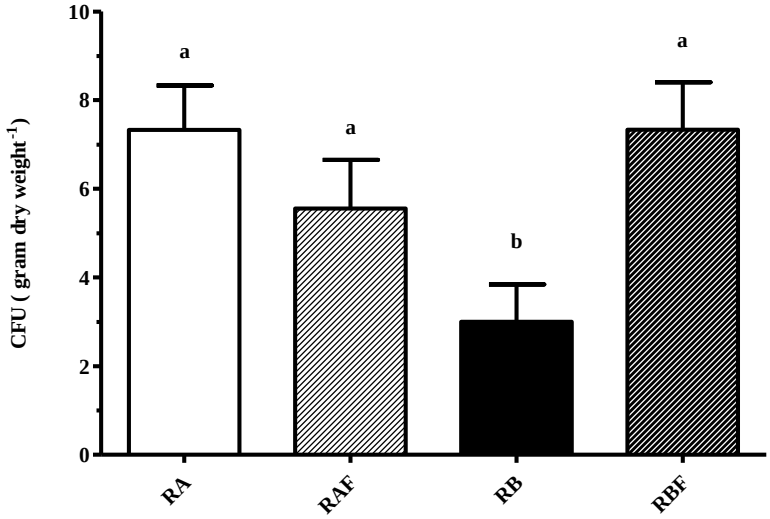
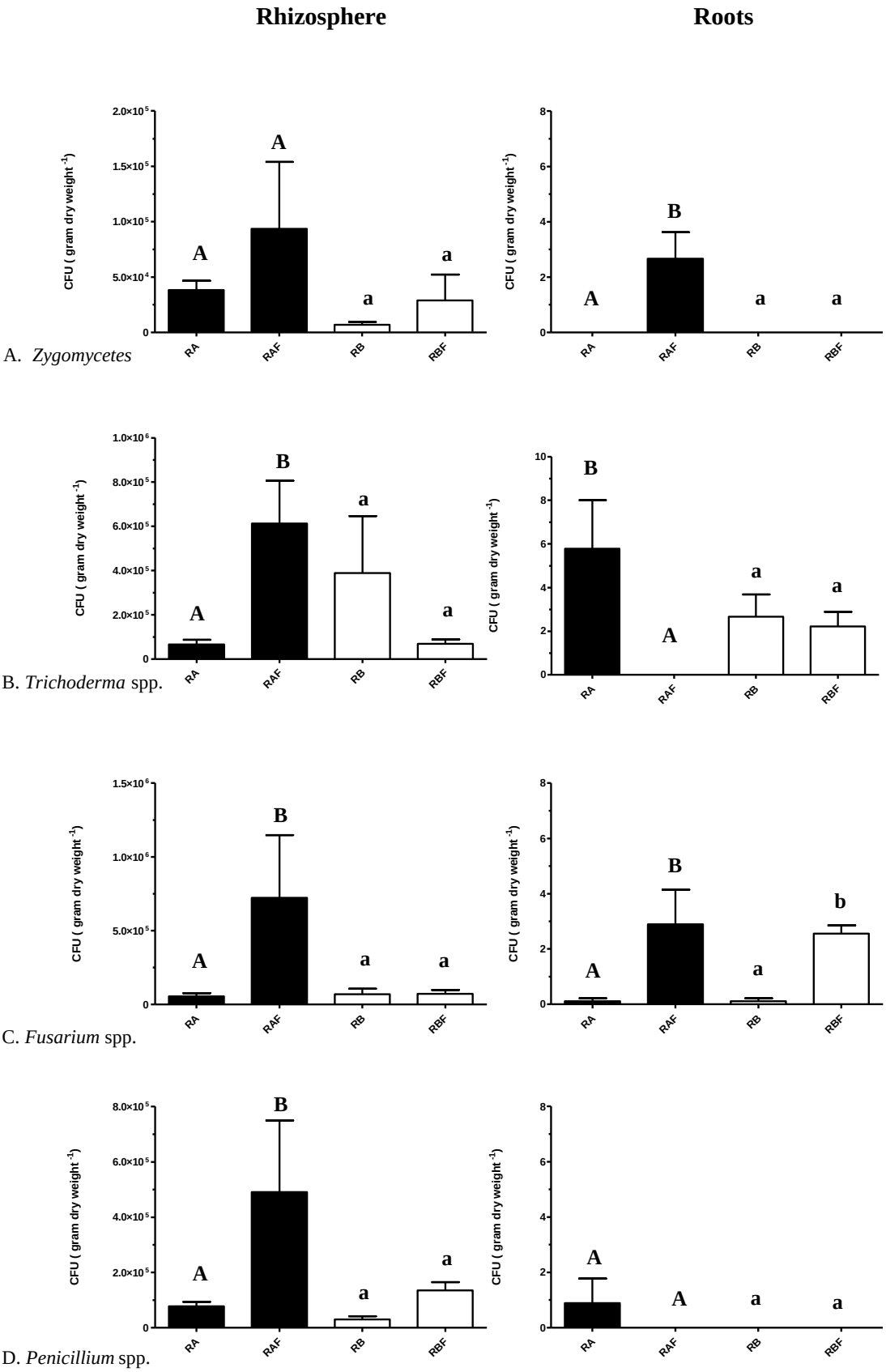


Figure 5. Effect of the addition of *Fol* on single genera/groups in the rhizosphere and in the roots for the treatment indicated. Results are expressed as mean ± s.e. and analyzed through unpaired *t-test* RA (Resistant/soil A) vs RAF (Resistant/soil A/*Fol*) (capital letters) and RB (Resistant/soil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



A. *Zygomycetes*

B. *Trichoderma* spp.

C. *Fusarium* spp.

D. *Penicillium* spp.

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