Soil microbiome analysis in an ESCA diseased vineyard☆

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ABSTRACT

The soil microbiome is linked to the microbial ecosystem of aboveground plant tissues and it is able to modulate and stimulate plant responses. The community composition, i.e. diversity and abundance, is influenced by several factors such as agronomical practices, agrochemical practices and geographical location. For the first time, we present here the investigation of the microbial community related to the soil of a long-established cultivated vineyard using the meta-barcoding approach. Specifically, we analyzed the bacterial and fungal communities of the bulk soils associated with esca-symptomatic and asymptomatic vines. Results showed no significant differences in richness between the two types of samples. Conversely, we observed that esca-related pathogens and grapevine trunk disease (GTD) pathogens were more abundant in the bulk soils of symptomatic plants, suggesting that the soil could represent an important source of inoculum. We also identified two fungal genera, *Curvularia* and *Coprinosis*, which are exclusive to the soil associated with asymptomatic plants. Moreover, Actinobacteria, a well-known group of bacteria symbionts, are over-represented in asymptomatic soils. Further studies are needed to expand the knowledge about these microorganisms, since they could have a role in controlling the development and/or spread of esca pathogens.

1. Introduction

Worldwide, the decline of vineyards associated with esca syndrome and grapevine trunk diseases (GTDs) is becoming an issue of increasing concern for viticulture, leading to economic losses and threatening the final product quality (Scheck et al., 1998; Mugnai et al., 1999; Bertsch et al., 2009). Esca is a chronic and complex wood disease in which multiple pathogens simultaneously or sequentially colonize plant tissues (Bertsch et al., 2013), causing mild to severe symptoms. One of the best-known symptoms is tiger-striped leaves (Viala, 1926), in which interveinal discoloration and scorching of leaves is observed. This symptom was initially described as the lighter form of esca and therefore as the chronic form (Surico, 2009), which is thought to be due to both the activity of phytotoxic metabolite secreted by the invading fungi and by the degrading activity of fungal enzymes (Mugnai et al., 1999; Andolfi et al., 2011). The most severe symptom is apoplexy, in which the diseased plants display a sudden wilting, the dieback of one or more shoots accompanied by leaf drop and the withering of fruit clusters, followed by plant death (Mugnai et al., 1999). A number of fungal species are associated with esca syndrome, but two tracheomycotic fungi, both belonging to the Ascomycota phylum, are considered to be essential for syndrome development: *Phaeomoniella chlamydospora* (Phaeomoniellales: *Phaeomoniellaceae*) and *Phaeoacremonium minimum* (Diaporthales: *Togniniaceae*) (Crous et al., 1996; Crous and Gams, 2000; Fischer, 2006; Kuntzmann et al., 2010). In addition, the white rot fungus *Fomitiporia mediterranea* (belonging to the Basidiomycota phylum) and *Botryosphaeriaceae* species, e.g. *Neofusicoccum parvum*, seem to play an important role in chronic wood disease development (Cloete et al., 2014; Abou-Mansour et al., 2015).

Several multidisciplinary studies were performed to understand both fungal and plant behaviours during syndrome development. In more detail, the best studied fungus to date is *P. minimum*, for which it is well known to produce several phytotoxic secondary metabolites (Bruno and Sparapano, 2006a, 2006b); in addition, it is able to secrete cell-wall-degrading enzymes (Valtaud et al., 2009). Despite the generic description of the pathogen, significant differences in virulence were reported among the different *P. minimum* isolates studied, suggesting the possibility of a plastic genome (Tegli et al., 2000) and an efficient heterothallic reproductive system (Rooney-Latham et al., 2005). Moreover, a recent study investigated the genome plasticity of different fungal isolates through the use of high throughput sequencing techniques, revealing the presence of genomic structural variation that

Fig. 1. Relative abundances of bacterial orders (a) and genera (b) in bulk soils of asymptomatic and symptomatic vines detected in each of the biological replicate. Only orders or genera representing at least the 1% over the total number of classified amplicons were retained.

Table 1
Average abundances of bacterial orders calculated for Asymptomatic and Symptomatic bulk soils samples. Student *t*-test were conducted to evaluate differences between the two groups.

	Asymptomatic		Symptomatic		
	Percentage	SD	Percentage	SD	
Order					
Acidimicrobiales	2.90	± 0.65	2.72	± 0.08	
Acidobacteriales	3.86	± 0.71	2.23	± 0.29	*
Alteromonadales	2.27	± 0.41	2.67	± 0.51	
Bacillales	1.17	± 0.21	2.62	± 0.51	*
Burkholderiales	10.46	± 0.95	12.54	± 0.49	
Caulobacterales	1.29	± 0.24	0.94	± 0.16	
Chitinophagales	2.74	± 0.21	2.58	± 0.65	
Chthoniobacterales	2.43	± 0.80	4.64	± 0.91	
Cytophagales	3.91	± 0.56	3.52	± 0.82	
Flavobacteriales	1.06	± 0.48	1.31	± 0.22	
Gaiellales	3.06	± 0.26	2.72	± 0.52	
Gemmatimonadales	1.32	± 0.33	0.91	± 0.17	
Micrococcales	1.02	± 0.18	1.14	± 0.02	
Micromonosporales	2.35	± 0.12	1.02	± 0.15	**
Myxococcales	13.70	± 0.91	13.34	± 0.96	
Nevskiales	2.80	± 0.60	2.27	± 0.21	
Nitrosomonadales	0.98	± 0.18	1.29	± 0.32	
Planctomycetales	7.20	± 0.84	5.72	± 0.64	
Propionibacteriales	1.50	± 0.28	0.93	± 0.10	*
Pseudomonadales	1.29	± 0.40	2.29	± 0.33	*
Pseudonocardiales	1.23	± 0.47	0.76	± 0.27	
Rhizobiales	10.67	± 0.44	11.82	± 0.78	*
Rhodospirillales	3.54	± 0.18	3.25	± 0.58	
Solibacterales	1.65	± 0.19	1.08	± 0.07	*
Solirubrobacterales	1.94	± 0.29	1.16	± 0.25	
Sphingomonadales	2.82	± 1.00	1.24	± 0.12	
Streptomycetales	1.42	± 0.11	0.81	± 0.12	**
Verrucomicrobiales	7.57	± 1.01	10.47	± 0.45	
Xanthomonadales	1.85	± 0.22	2.04	± 0.55	

*p < 0.05.
**p < 0.01.

impacted blocks of genes involved in virulence and secondary metabolism (Massonnet et al., 2018). On the plant side, some more details are known, especially about the physiological changes occurring during the pathogenesis, which represent complex and diverse responses in different plant organs, such as berries, leaves and stems (Fontaine et al., 2016). Berries are not only affected by significant decreases in catechin, epicatechin, anthocyanins, and sugar, but also by an increase of tartaric acid, malic acid, and mineral levels (Calzarano et al., 2008; Lorrain et al., 2012), leading to decreased product quality. In leaves, besides the evident symptoms, foliar physiology has been reported to be strongly affected by both stomatal closure and alteration of the photosynthetic apparatus, leading to: 1) reduced total chlorophyll content, 2) decreased CO_2 assimilation and 3) reduced Photosystem II quantum yield (Petit et al., 2006; Magnin-Robert et al., 2011). Moreover, looking at the general physiological state of the plant, water transport was also impaired due to xylem dysfunction, which leads to a dramatic loss of water transport, that in turn causes a considerable decline of leaf gas exchange and water use efficiency (Edwards et al., 2007a, 2007b; Pouzoulet et al., 2014). Furthermore, despite an accumulation of antimicrobial compound in leaves of symptomatic plants, it was recently demonstrated that phytoalexins are not involved in pathogens inhibition under natural conditions (Calzarano et al., 2018).

Due to the complexity of esca syndrome, over the last few years efforts have been made to decipher the molecular mechanisms involved in the pathogenesis, with the advent of modern molecular techniques, such as gene expression analysis and transcriptomics (Camps et al., 2010; Czemmel et al., 2015). To the best of our knowledge, only one study has looked deeply at grapevine trunk pathogens (GTPs) by means of a wider point of view, demonstrating the possibility to study the community of associated microorganisms and the plant responses using a new bioinformatics pipeline for the analysis of metatranscriptomics data (Morales-Cruz et al., 2018). On the other hand, despite the recent interest in meta-omics sciences, the decreasing cost of high-throughput sequencing services, and the increase of new user-friendly computational methodologies, little is known about the microbial community

Table 2
Average abundances of bacterial genera calculated for Asymptomatic and Symptomatic bulk soils samples. Student *t*-test were conducted to evaluate differences between the two groups.

	Asymptomatic			Symptomatic			
	Percentage		SD	Percentage		SD	
GENUS							
Acidobacterium	2.99	±	0.93	1.36	±	0.31	*
Azospirillum	2.06	±	0.26	2.16	±	0.70	
Bacillus	1.04	±	0.40	4.15	±	1.94	*
Bradyrhizobium	1.65	±	1.31	1.99	±	0.95	
Candidatus Solibacter	2.60	±	0.56	1.74	±	0.42	
Chryseolinea	2.59	±	0.64	2.71	±	0.59	
Chthoniobacter	2.14	±	0.64	5.02	±	0.94	*
Flavobacterium	2.25	±	0.92	3.06	±	0.72	
Gaiella	7.47	±	0.33	6.18	±	1.38	
Gemmata	3.55	±	1.13	2.26	±	0.40	
Hyphomicrobium	2.15	±	0.40	2.99	±	0.57	
Ilumatobacter	2.10	±	1.03	2.10	±	0.25	
Lysobacter	2.95	±	0.15	2.84	±	0.86	
Mesorhizobium	1.25	±	0.29	1.81	±	0.82	
Niastella	1.25	±	0.95	1.84	±	0.61	
Nitrospira	2.64	±	1.03	1.80	±	0.81	*
Nocardioides	2.91	±	0.73	1.29	±	0.15	*
Ohtaekwangia	3.55	±	1.11	2.94	±	0.52	
Pedomicrobium	3.51	±	1.02	4.98	±	0.72	
Pedosphaera	4.65	±	0.82	7.33	±	0.46	*
Pseudomonas	2.80	±	1.26	4.51	±	0.96	
Solirubrobacter	3.42	±	0.73	1.88	±	0.71	
Sphingomonas	6.19	±	2.44	1.87	±	0.37	*
Stenotrophobacter	2.21	±	0.81	0.87	±	0.31	
Steroidobacter	5.11	±	0.37	3.72	±	0.54	*
Streptomyces	2.82	±	0.28	1.62	±	0.18	**
Terrimicrobium	2.67	±	1.24	4.19	±	0.83	
Terrimonas	1.94	±	0.53	1.87	±	0.27	
Thiobacter	7.07	±	0.12	11.37	±	1.20	*
Variovorax	3.28	±	1.15	2.47	±	0.25	
Vicinamibacter	7.20	±	0.48	5.09	±	0.75	

*$p < 0.05$.
**$p < 0.01$.

associated with symptomatic or asymptomatic plants. To date, little is documented that describes the microbiome in grape plants with esca symptoms using high throughput sequencing. For example, the bacterial communities in symptomatic plants have been observed and the results about the potential role(s) of bacterial species in disease development were not as informative as expected (Bruez et al., 2015). New information needs to be unearthed within the scope of the holobiont concept (Martins et al., 2013; Vandenkoornhuyse et al., 2015; Perrone et al., 2017); it has been shown that grapevine growth and survival are significantly impacted by the associated microflora (Compant et al., 2011). In addition, grape associated microorganisms are able to influence plant physiological responses, which in turn is believed to impact the organoleptic properties of wine, contributing to what is known as the *terroir* (Verginer et al., 2010; Bokulich et al., 2014).

Soil microbial communities have a crucial role in nutrient recycling, soil fertility, and carbon sequestration (Fierer, 2017). They are able to greatly influence the productivity of agricultural systems forming complex and dynamic associations, which can range from mutualistic to commensal to pathogenic (Newton et al., 2010), and for these reasons they are also referred to as the second genome of plants (Berendsen et al., 2012). The impact that soil microbial communities can have on plant health is evident in disease-suppressive soils. Pathogens grow saprophytically in soils to reach the host plant and infect its tissues; prior to reaching the plant, pathogens must interact with the rhizosphere-associated microbial community, which can suppress the ability of pathogens to invade plant tissues (Schroth and Hancock, 1982). The ability of the suppressive soils is related to the microbial composition,

and it is enhanced by agricultural practices that promote microbial diversity in soils (Cook, 2014), but in some cases can depend upon a single microorganism. Specifically, it was recently reported that a single soil bacterial species is able to suppress a tomato fungal pathogen, and if transplanted to susceptible plants, it can suppress disease symptoms (Kwak et al., 2018). For these reasons, it is important to investigate the microbial community and decipher relationships occurring between microorganisms and the grape rhizosphere, which could help to define the best agronomical strategies on the topic of a sustainable viticulture.

In the present study, we characterized the bacterial and fungal communities associated with the bulk soils of a long-established cultivated vineyard in the Veneto region. We used the amplicon sequencing technique applied on variable regions of the 16 S ribosomal RNA (rRNA) gene for bacteria and the internal transcribed spacer (ITS) of rRNA polycistronic gene of fungi. The vineyard was selected because it was subjected to a high percentage of esca-symptomatic vines (more than 50% of plants displaying the tiger stripe symptoms), and because plants were singularly monitored for several years. We present here, for the first time, the comparisons of microorganism community compositions associated with bulk soils of symptomatic and asymptomatic vines in the same vineyard.
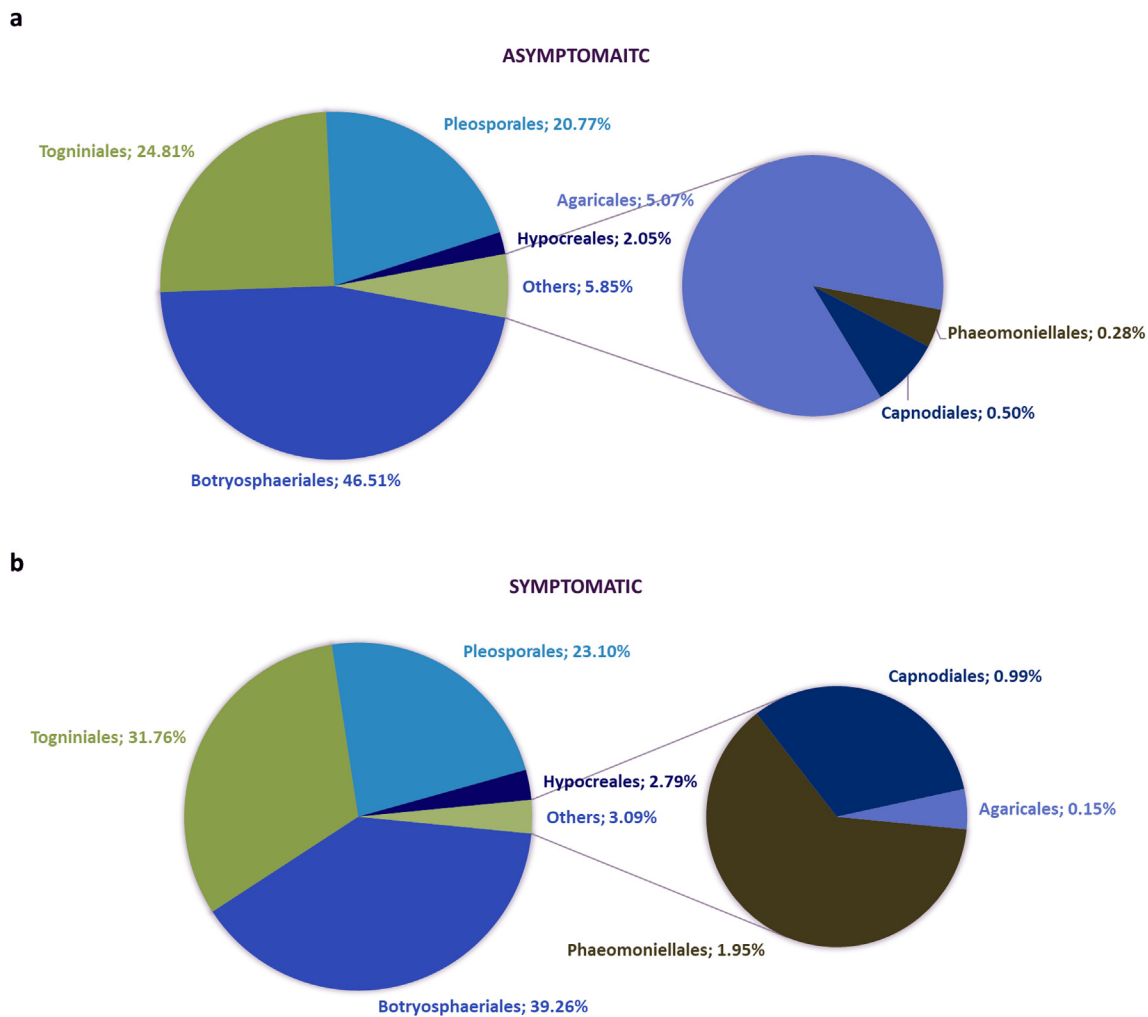
2. Material and methods

2.1. Vineyard location and sampling

Our study was conducted in the experimental vineyard of the CREA - Research Centre for Viticulture and Enology in Spresiano (TV) in Veneto region, Italy. The elevation is 56 m a.s.l, and the area is characterized by a warm temperature climate as reported in the Köppen and Geiger climate zones (http://koeppen-geiger.vu-wien.ac.at/alps.htm). The region is one of the most intensive wine producing areas of northern Italy (90000 ha of vineyard) and decline associated to esca syndrome generally appears ten to twelve years after planting (Manici et al., 2017). Plant replacement is the most applied control system and is thought to be the best agronomical practice to limit the spread of diseases (Bruno and Sparapano, 2007; Ogawa, 2016). Soil was sandy-loamy, with 8.2 pH, 0.18% total nitrogen, and 67 and 177 mg/kg exchangeable phosphorous and potassium, respectively with a soil carbon content ranging from 1.2 to 2.4%.

Bulk soil samples were collected in mid-September 2017 at a depth of 30–40 cm under canopy of adult vines (cultivar Glera, grafted on Selection Oppenheim 4 – SO4 rootstock) asymptomatic for ESCA syndrome or displaying tiger striped leaves (considered as symptomatic). Plants were selected based on the sanitary data monitored in the last four vegetative seasons, selecting continuous asymptomatic plants and vines displaying symptoms at the collection date and more than once over the monitoring period. To avoid differences in soil composition, plants were chosen in couple, one symptomatic and one asymptomatic, close to each other (adjacent or in front). Soil samples surrounding the roots of 9 plants for each condition (500 g for each sampling point) were taken and mixed to obtain a homogeneous sample of about 4.5 kg (bulk soil of asymptomatic plants = As, bulk soil of symptomatic plants = Sy). Three subsamples of 200 g for each were randomly selected and stored at −80 °C in sterile 50 ml tubes until processing.

2.2. DNA isolation and sequencing

Total nucleic acid were obtained from 1 g of soil following a previous reported protocol (Angel, 2012). DNA was then cleaned using the commercial kit E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's protocols yielding 3–5 μg of DNA per extraction quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). DNA integrity was evaluated by electrophoresis on a 1% agarose gel in 1x TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 7.5) stained with Red Safe Nucleic Acid

a

ASYMPTOMAITC

Togniniales; 24.81%
Pleosporales; 20.77%
Agaricales; 5.07%
Hypocreales; 2.05%
Others; 5.85%
Botryosphaeriales; 46.51%
Phaeomoniellales; 0.28%
Capnodiales; 0.50%

b

SYMPTOMATIC

Togniniales; 31.76%
Pleosporales; 23.10%
Capnodiales; 0.99%
Hypocreales; 2.79%
Others; 3.09%
Agaricales; 0.15%
Phaeomoniellales; 1.95%
Botryosphaeriales; 39.26%

Fig. 2. Relative average abundances of fungal orders in bulk soils of asymptomatic (a) and symptomatic (b) vines. The top 10 representative orders for each condition were retained.

Staining Solution (Labotaq, Sevilla, Spain) and then visualized under UV light. Three biological replicates for each condition were obtained and used as independent samples.

Illumina tag screening of the V3−V4 hypervariable regions of the 16 S rRNA gene was performed on the DNA by Macrogen Inc. (South Korea), using primers 341f and 785r to build the bacterial amplicon libraries (Kuczynski et al., 2012). The primer ITS3-ITS4 were used to amplify the highly variable spacers ITS2 of the rDNA fungal operon (Lindahl et al., 2013) by Macrogen, Inc. (South Korea). Sequencing of both bacterial and fungal libraries were done with the MiSeq Illumina apparatus.

2.3. Metaphylogenomic analyses, taxonomic distributions and statistical analyses

The obtained raw data were first subjected to strict quality control with PrinSeq v0.20.4 (Schmieder and Edwards, 2011) and then processed in Qiime 2 (Caporaso et al., 2010). For fungi, cleaned reads were then subjected to accurately trim using the hidden Markov models (HMMs), implemented in ITSxpress (Rivers et al., 2018), created for fungi and 17 other groups of eukaryotes to identify the start and stop sites for the ITS region. Briefly, the software allows to distinguish true sequences from sequencing errors (quite frequent in the amplicon sequencing technique), then sequences are clustered into operational taxonomy units (OTU's) by sorting reads by abundance and then clustered in a greedy fashion at 97% percent identity. Trimmed sequences

are then analyzed with DADA2 (Callahan et al., 2016), which models and corrects Illumina-sequenced amplicon errors. Sequence variants are then taxonomically classified through the UNITE database (we selected the reference database built on a dynamic use of clustering thresholds) (Abarenkov et al., 2010).

For bacteria, DADA2 was used for quality filtering, chimera removal, error-correction and sequence variant calling with reads truncated at 260 bp, resulting to a quality score above 20. Obtained feature sequences were summarized and annotated using the RDP classifier (Cole et al., 2013) trained to the full length 16 S database retrieved from the curated NCBI database.

To statistically test differences in the relative abundances of microbial taxa (family for bacteria or genera for fungi) the DESeq2 R package was used (McMurdie and Holmes, 2014). Non-parametric multivariate analysis (PERMANOVA) and non-metric multidimensional scaling (NMDS) was calculated among both the fungal and bacterial diversity in As and Sy bulk soils communities using PAST (Hammer et al., 2001).

3. Results

3.1. Sequencing information

Raw reads obtained from the MiSeq apparatus were cleaned from adaptor and quality filtered using a Macrogen Inc. in-house script based on the Illumina conversion software package bcl2fastq v1.8.4. After the

Table 3
Average abundances of fungal orders and genera calculated for Asymptomatic and Symptomatic bulk soils samples. Student *t*-test were conducted to evaluate differences between the two groups.

	Asymptomatic			Symptomatic			
	Percentage		SD	Percentage		SD	
Order							
Botryosphaeriales	46.51	±	2.20	39.26	±	2.42	*
Togniniales	24.81	±	1.35	31.76	±	1.14	*
Pleosporales	20.77	±	0.95	23.10	±	0.69	*
Hypocreales	2.05	±	0.52	2.79	±	1.77	
Phaeomoniellales	0.28	±	0.03	1.95	±	0.72	*
Capnodiales	0.50	±	0.02	0.99	±	0.09	**
Agaricales	5.07	±	2.58	0.15	±	0.11	*
Genus							
Neofusicoccum	51.27	±	0.37	41.46	±	1.25	**
Phaeoacremonium	25.54	±	0.52	33.91	±	1.97	**
Camarosporium	16.44	±	2.25	18.02	±	1.62	*
Arxiella	1.70	±	1.27	0.12	±	0.14	
Alternaria	1.15	±	0.90	0.92	±	0.26	
Fusarium	0.92	±	0.10	1.16	±	1.11	
Cladosporium	0.46	±	0.05	0.82	±	0.03	**
Paraconiothyrium	0.32	±	0.05	0.40	±	0.04	
Phaeomoniella	0.30	±	0.04	2.02	±	0.75	*
Mortierella	0.27	±	0.11	0.69	±	0.38	
Pithomyces	0.07	±	0.05	0.48	±	0.66	
Curvularia	1.26	±	1.20	0.00	±	0.00	***
Coprinopsis	0.30	±	0.23	0.00	±	0.00	***

*p < 0.05.
**p < 0.01.
***p < 0.001.

reads were cleaned, sequences ranged from 290 to 302 bp. The total number of reads per sample for 16 S sequencing were 96291, 82003, 90187, and 85294, for bulk soil of As samples; and 74420 and 74812 for bulk soil of Sy samples. The total number of reads per sample for ITS sequencing were 81638, 82526, and 84833 for bulk soil of As and 86297, 82921, 88227 for bulk soil of Sy. A further summary of sequencing data is reported in Table S1. The species accumulation curve tended to saturation when increasing the number of samples, indicating that sequencing depth was sufficient (Fig. S1).

3.2. Bacterial community diversity and composition

The bacterial community was first analyzed at the order level; we retained only orders with a comprehensive relative abundance above 1%. For As samples, 47794, 41438, and 45383 amplicons were taken, and 43593, 37405, and 37794 amplicons were taken for Sy samples. Order Myxococcales is the most abundant order in both As and Sy samples, followed by Rhizobiales, Burkholderiales, Verrucomicrobiales and Planctomycetales, which, when taken all together, account for about 50% of identified amplicons (Fig. 1a). Statistical analysis revealed that Rhizobiales, Pseudomonadales, and Bacillales are more abundant in Sy samples than in As samples. Conversely, orders Acidobacteriales, Micromonosporales, Solirubrobacterales, Propionibacteriales, and Streptomycetales are more abundant in As samples than in Sy samples (Table 1).

Bacterial genera were then analyzed, retaining only the ones with a comprehensive relative abundance above a fixed cut-off of 1%; for As samples, 32101, 28016, and 31332 amplicons were retained after filtering; for Sy samples, 28466, 24910, and 24939 amplicons were retained after filtering. At the genera level, bacteria accounting for at least 50% of the identified amplicons were different in As and Sy samples. Specifically, bulk soil data showed the presence of three genera (*Gaiella*, *Vicinamibacter* and *Thiobacter*), each representing about the 7% of amplicons, followed by *Sphingomonas*, *Steroidobacter*, *Pedosphaera*, *Gemmata*, *Ohtaekwangia*, *Pedomicrobium*, and

Solirubrobacter. The ten genera mentioned above, taken all together, represent about 50% of the identified amplicons (Fig. 1b). Data obtained from Sy samples revealed *Thiobacter* as the most abundant genera (accounting for an average of more than 11% of reads), followed by *Pedosphaera*. *Gaiella*, *Vicinamibacter, Chthoniobacter, Pedomicrobium, Pseudomonas, Terrimicrobium,* and *Bacillus* all together accounted for at least the 50% of identified amplicons. Analysis of abundances in the two groups showed that *Sphingomonas, Steroidobacter, Acidobacterium, Nocardioides, Streptomyces* and *Nitrospira* genera were more abundant in As samples. However, *Thiobacter, Pedosphaera* and *Chthoniobacter* genera were more abundant in Sy samples (Table 2).

3.3. Fungal community diversity and composition

To analyze the fungal community diversity, we first focused on the abundances at the order level to determine whether any diversity was detectable. Orders with a comprehensive abundance of at least 1% (in both As or Sy samples) were retained; to perform the comparative analysis (after filtering) for As samples, 69909, 67758, and 72760 reads were taken into account; for Sy samples, 77648, 68294, and 70181 reads were taken into account. Data showed that, in both Sy and As samples, Botryosphaeriales is the most abundant order, followed by Togniniales and Pleosporales, together representing more than the 90% of identified reads (Fig. 2a and b). When we compared the two conditions, we observed a slight diversity in abundances; as reported in Table 3, Botryosphaeriales is a significantly more abundant order in As samples with respect to Sy samples. Conversely, Togniniales and Pleosporales display an inverted pattern, being more abundant in Sy samples than in As samples. Moreover, Phaeomoniellales and Capnodiales also occurred more in Sy samples than in As samples. Interestingly, the Agaricales order accounted for about 5% of amplicons in As samples, but it was detected in only 0.15% of Sy samples.

For the genera level, we selected the top 10 representatives for each condition: for As, 60178, 62565 and 67141, amplicons were retained after filtering, and, for Sy, 73192, 65729 and 65712 amplicons were retained after filtering. Data reported in Fig. 3a and b mirrored what was observed in the analysis of order abundances; *Neofusicoccum*, a genera belonging to the Botryosphaeriales order, is the most abundant in both As and Sy samples, followed by *Phaeoacremonium* (order Togniniales) and *Camarosporium* (order Pleosporales), which all together represented more than the 90% of identified amplicons. Similarly, the relative genera abundances (Table 3) are mirrors of those of the orders: *Neofusicoccum* is more abundant in As samples than in Sy samples, and *Phaeoacremonium* and *Camarosporium* are more abundant in Sy samples than in As samples. Notably, *Phaeomoniella* significantly accumulated in Sy samples at a rate more than 6 times that of As samples. In addition, the *Cladosporium* genera accumulate at higher level in Sy samples with respect to As samples. Moreover, two genera are exclusive to As samples: *Curvularia* (order Pleosporales), accounting for about 1.26%, and *Coprinopsis* (order Agaricales), accounting for 0.3% of identified amplicons.

3.4. Community structure

Community structure is represented by two factor: the diversity and the complexity of taxa present in each condition. Diversity indices (Chao, Shannon, Simpson and Fisher), representing species richness and evenness, were calculated for both bacterial and fungi in As or Sy bulk soil (Table 4). No significant differences were detected between bulk soil of esca-symptomatic and asymptomatic vine-related samples. Results indicate that the fungal and bacterial community was not affected in composition, as also attested to by the permutational multivariate analysis of variance (PERMANOVA) for the fungal communities (F = 4.06 and p = 0.094) and bacterial communities (F = 6.42 and p = 0.091).

To better understand differences occurring between the microbial

Fig. 3. Relative average abundances of fungal genera in bulk soils of asymptomatic (a) and symptomatic (b) vines. The top 10 representative genera for each condition were retained.

Table 4

Richness estimators and diversity indices for fungal (ITS) and bacterial (16 S) communities sampled in the bulk soil of ESCA asymptomatic (As) and ESCA symptomatic (Sy) vines. Statistical Student's *t*-test (p < 0.05) was conducted to detect significant differences.

Sample	Chao				Shannon				Simpson				Fisher			
	Value		SD	p	Value		SD	p	Value		SD	p	Value		SD	p
As_ITS	77.92	±	1.91	0.16	1.72	±	0.11	0.38	0.75	±	0.02	0.35	8.45	±	0.19	0.12
Sy_ITS	82.08	±	2.55		1.80	±	0.15		0.77	±	0.03		8.99	±	0.31	
As_16 S	139.28	±	4.50	0.71	2.53	±	0.03	0.08	0.69	±	0.01	0.16	16.08	±	0.47	0.28
Sy_16 S	140.75	±	1.56		2.46	±	0.01		0.68	±	0.00		16.79	±	0.38	

communities of the two conditions tested, we analyzed some general features related to each of the community structures. First, we determined the exclusive orders or genera in both the fungal and bacterial community using Venn diagrams (Fig. 4). After such analysis for the fungal community, we detected 40 orders: 4 exclusive to the As samples, 9 exclusive to the Sy soils and 27 shared between the two conditions. Focusing on the 102 bacterial orders identified, diagram showed

that 4 of them were exclusive to As samples, 9 were exclusive to Sy samples, and 89 were shared between the two conditions. Repeating the same analysis at genera level, we obtained 22 fungal genera and 59 bacterial genera exclusive to the asymptomatic samples, 28 fungal genera and 66 bacterial genera detected only in symptomatic-related bulk soil samples, and 57 fungal genera and 237 bacterial genera shared among all the samples. Taken together, among the samples analyzed,
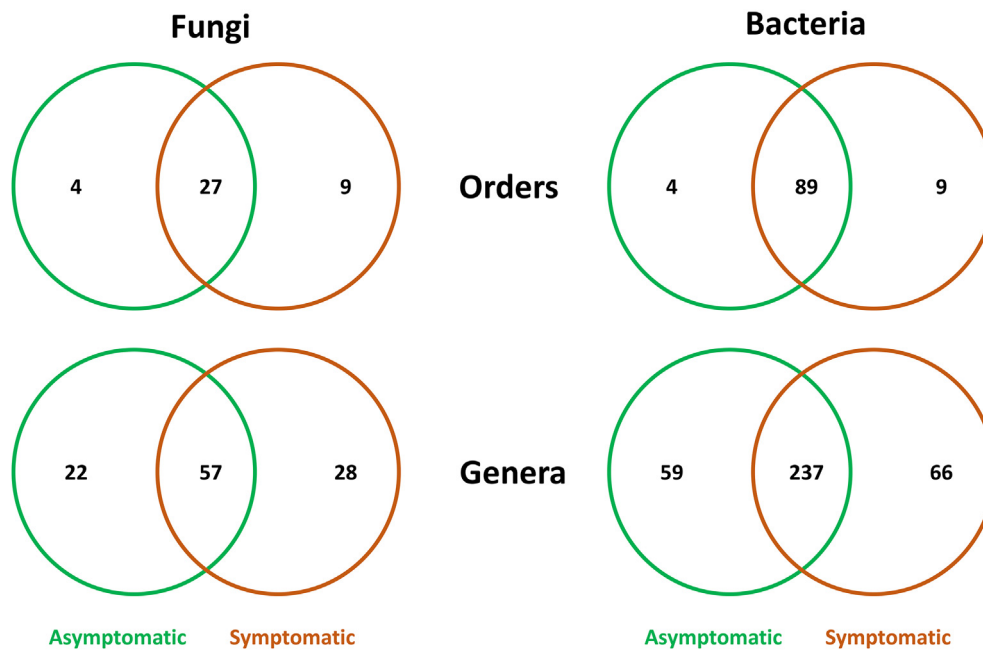
Fungi

Bacteria

Orders

Fungi Orders: 4, 27, 9

Bacteria Orders: 4, 89, 9

Genera

Fungi Genera: 22, 57, 28

Bacteria Genera: 59, 237, 66

Asymptomatic **Symptomatic**   **Asymptomatic** **Symptomatic**

Fig. 4. Venn-diagram illustrating the overall community composition, with number of exclusive and shared orders and genera detected in both fungi and bacteria communities.

107 and 362 genera were detected for fungal and bacterial communities, respectively.

In addition, after the bioinformatics classification of amplicons of both the 16 S and ITS regions were considered, we summarized and reduced the dataset of each biological replicate to a bi-dimensional scaling using a Bray-Curtis distance matrix and plotting the results in corresponding non-metric multidimensional scaling (NMDS). As reported in Fig. 5, for each community analyzed, the three biological replicates for each condition tended to cluster together, similarly for fungal, bacterial or the general microbial communities.

4. Discussion

The aims of our work were to investigate the microbial community associated with the bulk soil of esca-symptomatic and asymptomatic vines, focusing on the richness and abundance of esca-related pathogens as well as the community structure of both the conditions tested. We started from a vineyard where ≈ 20-year-old grapevine plants of cv. Glera were cultivated. Here, plants were visually monitored individually over a four-year period to check their sanitary status. Interestingly, in our work, the diversity and richness of the two conditions (As and Sy) in the soil samples did not differ one from each other (Table 1). This is possibly because, as already stated in another work, the major components shaping the soil microbial composition are related to environmental factors such as soil physio-chemical composition, climate, cropping practices, geographical area and grapevine cultivars present (Berendsen et al., 2012; Manici et al., 2017). In addition, as recently reported, the plant microbiome compositions (endophytes in particular) are often similar to those found in the associated soil and play key roles in influencing growth and sanitary status and consequently ensuring the balance in plant ecosystems (Vandenkoornhuyse et al., 2015). The complexity of grapevine microbiomes is still under scrutiny and is not fully understood; the plant-microbial interactions and the possibility of shaping the soil microbiome in response to several biotic and/or abiotic stimuli are assertions challenging the scientific community. To add a further level of complexity, in a vineyard, the agricultural practices are many and diverse during the seasons, influencing soil characteristics, with effects on the grapevine root system, which in turn impacts microbial community

assemblages (Vega-Avila et al., 2015; Marasco et al., 2018). Accordingly, it is important to study the microbial community and determine if any connection between the sanitary status of vines and the associated soil microbiome exists, particularly in an economically important disease syndrome such as esca, where several pathogens act together to influence the entire microbial community that seems to have crucial roles in syndrome development (Elena et al., 2018).

It is well known that bacteria form complex associations with plants, which range from mutualistic to pathogenic, thus playing crucial roles in plant health status (Kogel et al., 2006; Newton et al., 2010). They can also trigger direct or indirect responses, activating defense mechanisms or producing metabolites against pathogens (Compant et al., 2010; Bhattacharyya and Jha, 2012). In addition, the community composition of soil acts as the microbial reservoir, able to drive the composition of the microbial community associated with the aboveground organs and influencing in this way the *terroir* of the vineyard (Zarraonaindia et al., 2015). To the best of our knowledge, only one work has taken into account the bacterial community associated with wood tissues of esca-symptomatic vines, highlighting some difference when compared to the asymptomatic ones (Bruez et al., 2015). Interestingly, we found that Bacillales order, and hence the *Bacillus* genus, is more abundant in the bulk soil of symptomatic plants, accordingly to what was observed by Bruez et al. in their work focusing on the above ground tissues. To date, the *Bacillus* genus hosts several species that are known for their ability to inhibit plant fungal pathogens (Kai et al., 2007; Chen et al., 2008; Ongena and Jacques, 2008), to promote plant growth (Idriss et al., 2002), or to induce systemic resistance (Choudhary and Johri, 2009).

Conversely, the *Streptomyces* genus, belonging to the Actinobacteria phylum, was over-represented in the bulk soil of our asymptomatic plants. Similarly to the *Bacillus* genera, the Actinobacteria are known for their antagonistic activity, inducing the activation of plant defense pathways (Conn et al., 2008), or suppressing diseases and inducing plant growth (Palaniyandi et al., 2013). Aside from this, it is still unclear how these last two genera accumulate preferentially in the bulk soil of symptomatic or asymptomatic plants (or in plant tissues), and how this can affect the microbial community composition. In addition, we speculate that the isolation and characterization of *Streptomyces* genera surrounding the asymptomatic plants could result in interesting
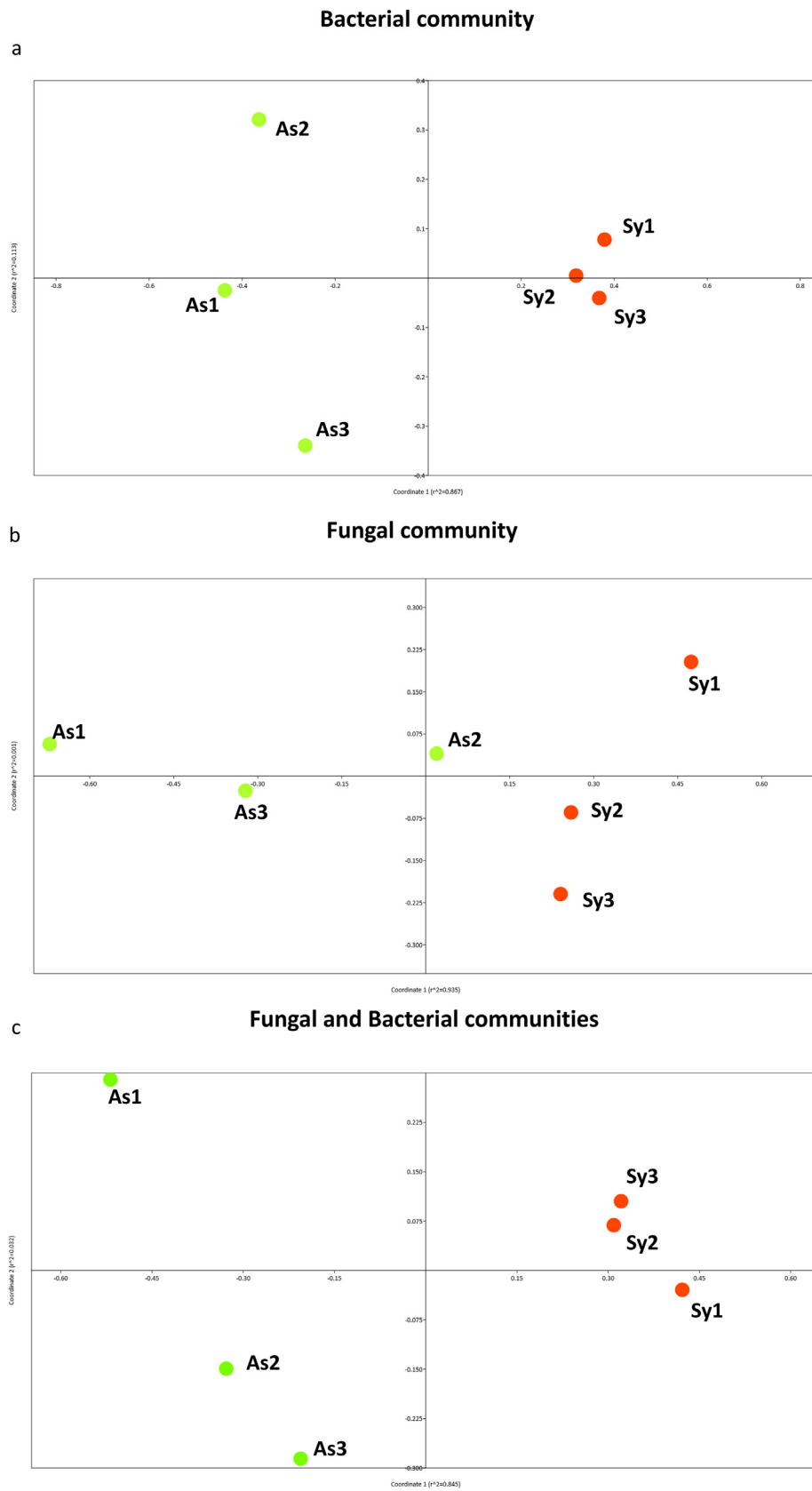
Fig. 5. NMDS algorithm based on Bray-Curtis distances matrixes were used to reduce into a bi-dimensional scaling data obtained for bacteria (a), fungi (b) and the overall microbial community (c).

potential prospects to control esca, because of the already reported ability of Actinobacteria to contain other grape pathogens such as *Botrytis cinerea* (Loqman et al., 2009), and also to reduce the presence of GTPs in soil (Álvarez-Pérez et al., 2017).

On one hand, grapevine trunk diseases (GTDs), such as esca, Petri, or many others, are becoming a significant problem for grapevine cultivation worldwide (Bertsch et al., 2013; Úrbez-Torres et al., 2013); however, little is known about their molecular and biochemical features, especially from the aspect of the microbial community composition. The complexity of GTDs is due to peculiar characteristics specific to the latent infections, during which plants do not show symptoms for years (Marchi, 2001; Savocchia et al., 2007). The exact moment at which plants become infected is still under debate, but all the authors agree to the fact that a wound is necessary for the pathogen to invade plant tissues. For example, several studies infer that the pruning practices are the main cause of infection, since wounding represents the preferential entry site (Mugnai et al., 1999; Rolshausen et al., 2010; Mondello et al., 2018), which is probably linked to the production of a gel rich in pectins as a plant reaction to cutting. The latter is a perfect substrate for the development of fungi such as *P. minimum* and *P. chlamydospora* (Sun et al., 2008). Another proposed entry site was reported as grafting; grapevine rootstock mother plants and propagation processes of grapevine plants were indicated as important sources of inoculum for fungal trunk pathogens (Aroca et al., 2010; Gramaje and Armengol, 2011).

Among routes of entry, a few authors proposed the possibility of the soil as a source of inoculum, which could lead to new infections (Whiteman et al., 2003; Giménez-Jaime et al., 2006). Specifically, it has been demonstrated that *P. minimum* and *P. chlamydospora* were not detectable in grafted plants until transplanting in open-field and growth during summer (Giménez-Jaime et al., 2006). Specifically, the authors showed that in the case of young vines during the summer season without the presence of pruning or similar wounds, the pathogens are somehow capable of invading plant tissues. Furthermore, in other works, it was also demonstrated that pathogens can easily move from soil to the upper plant tissues, because of several dispersal mechanisms, including rain and wind (Madden, 1997; Bock et al., 2012). Coupling this evidence with the finding of our work in which *Neofusicoccum* and *Phaeoacremonium* are the two most abundant fungal genera, accounting for Botryosphaeria canker and esca syndrome, respectively, we can speculate that soil could represent a primary source of inoculum, at least for these pathogens independent from entry site mechanisms.

An effective protocol to control the spread of GTPs still does not exist and when plants become symptomatic, the most widespread practice is single plant replacement (Bruno and Sparapano, 2007; Becker and Oberhofer, 2009; Ogawa, 2016). If our work can represent a supporting approach for viticulturists to counteract yield and quality losses, it is also possible that the fungal communities associated with soil and roots are not different before and after vine replacement (Manici et al., 2017). To date, different scientific papers are reporting an increasing trend of GTDs in the last decades, which are now reaching worrying proportions in the main grape producing countries (Mugnai et al., 1999; Larignon et al., 2009; Bertsch et al., 2013).

Integrating the observations that long-established grape-cultivated soils overcome the ability of plants to shape the microbial community associated with the rhizosphere with the data collected in our study, we can speculate that replacing symptomatic plants can mitigate the economic losses caused by GTDs, but the newly transplanted plants would be surrounded by a bulk soil enriched in GTPs. Furthermore, it is worth noting that GTPs are not obligate biotrophs, are not strictly associated with the root system and are impossible to be eradicated with the simple replacement of symptomatic plants. Specifically, if we look at the comparison between the bulk soils related to symptomatic and asymptomatic plants we observe, in Sy samples, an enrichment of *Phaeoacremonium* and *Phaeomoniella* genera, known to be involved in esca disease. Moreover, the reduction and/or disappearance of some

other genera, as for example *Curvularia* and *Coprinopsis*, could play a role in the overall balance of the microbial community, by containing the pathogenic genera (simply by competing with them) or stimulating plants to respond to fungal infections. Especially in the case of *Curvularia*, it has already been demonstrated that it can establish a positive symbiotic relationship, as reported for *Curvularia protuberata* and the host plant *Dichanthelium languinosum* (Marquez et al., 2007). Some efforts have been already done to identify possible biological agents able to induce resistance and/or tolerance against esca syndrome (Mondello et al., 2018), but to date the soil microbial community has not been analyzed. In light of our results, more information is needed to elucidate the role of some bacterial and fungal genera in shaping the microbial community and also to deepen the understanding of the interactions occurring with the surrounding plants.

5. Conclusions

In conclusion, we reported here for the first time a comprehensive evaluation of the soil microbiome in a long-established cultivated vineyard affected by esca syndrome, highlighting the differences in the abundance of fungal and bacterial communities in the soil surrounding symptomatic and asymptomatic plants. Moreover, the observed results suggest that soil can represent a reservoir of pathogens; hence, new transplanted vines, taking place of the symptomatic ones in an infected vineyard, could be exposed to a high source of pathogens. We suggest that the establishing of a suppressive microbial community could represent a potential alternative protocol for disease restraint.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.04.014.

Author contributions

L.N. and W.C. designed the experimental system, carried out the wet lab experiments, analyzed data and wrote the manuscript draft. A.Z., M.G. and F.G. helped to design the experiments, contributed to the writing and carefully revised the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest. This article does not contain any studies with human or animal participants.

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