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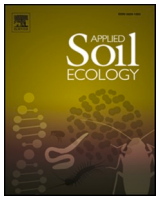

In grapevine decline, microbiomes are affected differently in symptomatic and asymptomatic soils

Romain Darriaut [a], Joseph Tran [a], Guilherme Martins [b,c], Nathalie Ollat [a], Isabelle Masneuf-Pomarède [b,c], Virginie Lauvergeat [a,*]

[a] EGFV, Univ. Bordeaux, Bordeaux Sciences Agro, INRAE, ISVV, F-33882 Villenave d'Ornon, France
[b] Univ. Bordeaux, Bordeaux INP, INRAE, OENO, UMR 1366, ISVV, Villenave d'Ornon, France
[c] Bordeaux Sciences Agro, Bordeaux INP, INRAE, OENO, UMR 1366, ISVV, Gradignan, France

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ABSTRACT

Grapevine decline affects viticulture worldwide. It is caused by a wide range of individual and combined biotic and abiotic factors. Some declines remain unexplained because they are not associated with known pathological symptoms or mineral dysregulation. Vineyard microbiological quality is an important area of study, since grapevine-associated microbiome primarily originates in vineyard soils and determines host health and development. To understand the decrease of growth and yield, and the high mortality of plants in vineyards affected by these declines, a multisite study investigated soil microbial communities. Spatial (terroir: two distinct geographical locations) and temporal (season: autumn and spring) dimensions were added to the inter-row soil status factor (S for areas with symptomatic vines and AS for those with asymptomatic vines). The microbiomes of AS and S soils were analyzed using high-throughput sequencing based on the bacterial 16S rRNA gene, the fungal ITS1 region (Internal Transcribed Spacer), or the fungal 18S rRNA gene for *Glomeromycota* family. Geographical location was the strongest driver of bacterial and fungal microbial communities, while the seasonal factor primarily influenced bacterial community. Based on metabarcoding analysis, symptomatic soils presented enriched bacterial taxa that can be potentially beneficial for grapevine growth. In addition, fungal diversity and richness, including *Glomeromycota* division, were greater in symptomatic soils. Fungal genera associated with grapevine diseases were detected across all conditions, with higher abundances in symptomatic soils. These findings reveal that vineyard soils affected by unexplained decline are a potential source of both fungal pathogens and beneficial microorganisms.

1. Introduction

Important microbial dynamics take place in the soil and drastically influence the health of surrounding plants through direct and indirect processes (Wei et al., 2019; Trivedi et al., 2020). Environmental factors, such as salinity, drought, agricultural practices, and crop systems, shape the soil microbial communities that affect these dynamics (Hariharan et al., 2017; Delgado-Baquerizo et al., 2018; Schimel, 2018; Zhang et al., 2019a). Microbial dialogues are therefore modulated by different stressors and can be categorized as beneficial, commensal, or negative interactions. In addition to these environmental factors, temporal and spatial variations greatly influence the composition of the soil microbiome (Nuccio et al., 2020).

Pathogen infection can modulate the microbiome diversity and alter host responses and functionalities, resulting in microbiome dysregulation. Microbiome dysbiosis refers to a disequilibrium in a microbial profile, such as the microbiome composition, that deviates from a healthy one (i.e., asymptomatic). This dysbiosis might cause the pathogen predisposition to affect the host immune system (Berg et al., 2020). The term dysbiosis is commonly used in the medical field, where the high or low relative abundance of a taxon is a marker used to diagnose and treat disease (Levy et al., 2017). At the same time, the gut

Abbreviations: AS, Asymptomatic; GTD, Grapevine trunk disease; LEfSe, Linear discriminant analysis effect size; NMDS, Nonmetric multidimensional scaling; OTU, Operational taxonomic unit; PERMANOVA, Permutational analysis of variance; S, Symptomatic.

* Corresponding author.
E-mail addresses: joseph.tran@inrae.fr (J. Tran), guilherme.martins@agro-bordeaux.fr (G. Martins), nathalie.ollat@inrae.fr (N. Ollat), isabelle.masneuf@agro-bordeaux.fr (I. Masneuf-Pomarède), virginie.lauvergeat@inrae.fr (V. Lauvergeat).

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microbiome could be compared to the rhizosphere microbiome, as these specific niches are essential for nutrition, immunity, and pathogen resistance in individuals (Berendsen et al., 2012). This microbiome dysregulation has been observed in plants, including apple rootstock affected by apple replant disease and the surrounding bulk soil (Balbín-Suárez et al., 2021).

Biological control strategies rely primarily on specific strains that demonstrate defensive responses to plant pathogens. However, studies suggest that the higher the microbiome diversity, the higher the chances of generating beneficial functional features for plants, such as resistance to phytopathogens and plant-growth promoting traits (Wei et al., 2015; Saleem et al., 2019; Hu et al., 2020). In this context, soil biological quality refers to the ability of soil to exhibit a broad range of diversity and quantity of microorganisms involved in ecosystem processes (Loreau and de Mazancourt, 2013). Some keystone taxa are known to trigger such processes, particularly in litter degradation, nitrogen fixation, soil remediation, and plant nutrient solubilization. Consequently, the absence or low presence of any of these taxa would have a negative impact on soil quality and thus plant health. In addition, the removal of keystone taxa might alter stability of soil microbiome and increase the soil dysbiosis with further depletion of microbial communities (Herren and McMahon, 2018).

Soil microbial communities are usually identified and characterized using DNA metabarcoding technology (Francioli et al., 2021). Amplicon-based sequencing is one of the preferred tools in microbial ecology due to the decreasing cost of high-throughput sequencing, improved computational methods, and growing BLAST databases. This methodology has been applied in all grapevine compartments, including berries (Zhang et al., 2019b), leaves (Wei et al., 2018), bark (Vitulo et al., 2019), rhizosphere (Berlanas et al., 2019), and roots (Carbone et al., 2021). The most targeted regions are the 16S rRNA gene for bacteria and ITS or 18S rRNA gene for fungal communities. In addition to soil bacteria and fungi, the most studied microorganisms in vineyards are the *Glomeromycota* fungi, which are known for their mycorrhizal association with roots, forming a mutualistic symbiosis that might contribute to grapevine development. These Arbuscular Mycorrhizal Fungi (AMF) contribute to the mineral nutrition of the plant (mainly phosphorus) and the defense processes against pathogens (Trouvelot et al., 2015; Scandellari, 2017).

To address grapevine health, most of the microbiome research has been conducted on grapevine trunk diseases (GTD) such as esca complex, Botryosphaeria dieback, and Petri disease. These widespread diseases cause grapevine decline with easily recognizable foliar symptoms (Gramaje et al., 2018). Studies in this area have primarily focused on the rhizosphere (Saccà et al., 2019) and wood (Fotios et al., 2021) interfaces. Relatively little research has been conducted on bulk soil in vineyards in relation to grapevine fitness (Nerva et al., 2019; Geiger et al., 2022) and still less on bulk soil microbiome from vineyards experiencing unexplained decline.

A previous analysis demonstrated the dysregulation of microbial and functional profiles of vineyard soils affected by unexplained grapevine decline (Darriaut et al., 2021). Four vineyards in the Bordeaux region of France were studied, each with localized areas of decline symptoms (S areas), including low vigour and high plant mortality, compared with other normal-growing areas (AS areas) in the same plot. Two vineyards were located in the Haut-Médoc terroir and the two others in the Graves region. Soil physicochemical parameters and the presence or lack viruses in the plants failed to explain these declines. The soil was sampled in the autumn and spring, and microbial analysis based on cultivable methods revealed higher enzymatic activities, higher levels of cultivable bacteria and 16S gene copies, as well as lower levels of cultivable fungi and 18S gene copies in AS soils compared to S soils (Darriaut et al., 2021). These results suggested that the symptomatic soils were either subject to microbial dysbiosis involving some keystone taxa in the soil ecosystemic processes, or to a dysregulation of the richness and diversity of bacterial and fungal communities. The aim of this study was to compare the structure and diversity of microbial communities between the soils with asymptomatic and symptomatic (declining) vines. To this end, following the preliminary analysis performed in Darriaut et al. (2021) on vineyard bulk soils, the bacterial and fungal communities structures were investigated using a metabarcoding approach, with a focus was on the *Glomeromycota* division.

2. Material & methods

2.1. Study sites and sampling

The samples were collected in four vineyards from two Bordeaux region terroirs, namely Graves and Haut-Médoc (Fig. S1), in autumn and spring, as described in Darriaut et al. (2021). The detail of the characteristics of the studied vineyards, such as the physicochemical parameters of the soils, the different rootstock/scion combinations, or the vigour of the plants is described in Darriaut et al. (2021). Briefly, the vineyards, ranging from 11 to 59 years old (Fig. S1), were located on sand soils according to USDA soil taxonomy (i.e., ranging from 82 % to 95 % of sand; 2 % to 0 % of silt; 3 % to 8 % of clay) under a sub-humid temperate climate with cool nights and a low risk of extreme temperatures, subtyped as Cfb in Köppen climate classification. Organic matter was ranging from 0.39 % to 0.97 % with the exception of one Haut-Médoc asymptomatic area with 2.40 %. In each of these vineyards, an area displaying grapevine decline has been identified by the winegrowers and has been named symptomatic area (S), compared to an asymptomatic (healthy) area in the same plot (called AS). In the S areas, the higher mortality (38–65 % and 1–19 % of dead or one-year old plants in S and AS areas, respectively) and lower vigour of the living plants (1.8 to 2.7 times less pruning weight per vine in S areas compared to AS areas) was not related to disease symptoms or the presence of the main viruses (GFLV or ArMV), and could not be explained by some soil mineral deficiencies (Darriaut et al., 2021).

The samples were taken in the middle of the inter-rows, about 50 cm from the plants from the 10 cm below the upper soil surface to a depth of approximately 30 cm using an auger (10 cm × 25 cm). The soil samples were then sieved (<2 mm), lyophilized for 48 h using Christ Alpha® 1–4 (Bioblock Scientific), and stored at −80 °C prior to DNA extraction. Forty-eight samples were then collected and analyzed: 6 biological replicates × 2 terroirs × 2 soils × 2 seasons.

2.2. DNA extraction, library preparation, and sequencing

Total DNA was extracted from 250 mg of the lyophilized soils using the DNeasy PowerSoil Pro kit (Qiagen) according to the manufacturer recommendations with an additional C5 washing step. The extracted DNA samples were quantified on a Qubit® 3.0 fluorometer (Thermo Fisher Scientific) using the Qubit™ dsDNA HS Assay Kit and checked for quality using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). The DNA was then stored at −20 °C for further use.

The DNA samples were randomized across plates and amplified using the universal primers listed in Table 1, specific to either the bacterial and archaeal 16S rRNA gene, the fungal ITS1 region, or the fungal SSU, which was used for *Glomeromycota* identification. All the primers included the specific overhang Illumina adapters used for the amplicon library construction. Each 25 µl reaction contained 12.5 µl of 5× GoTaq® reaction buffers (Promega, France), 8 µl of nuclease-free water, 1 µl of each primer (10 µM), 2.5 µl of DNA template (5 ng/µl), and 0.625 u of GoTaq® G2 DNA polymerase (Promega, France).

PCR amplifications were performed in triplicate for each condition. The cycling conditions are listed in **Table S1**. Further steps were processed at the PGTB sequencing facility (Genome Transcriptome Facility of Bordeaux, Pierroton, France) using first a Nano V2 with 2 × 250 nucleotides paired reads to calibrate the homogeneity of the 3 gene targets, followed by a V2 with 2 × 250 nucleotide paired reads protocol. The PCR products were purified with platform specific SPRI magnetic

Table 1
Primers used for 16S rRNA, ITS, and 18S rRNA amplifications. Specific overhang Illumina adapters are in italics and underlined.

Primer	Primer sequence (5′ to 3′)	Target and size of the amplicon	Reference
341F	*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*CCTACGGGNGGCWGCAG	Bacterial 16SrRNA gene V3-V4 regions (464 bp)	(Klindworth et al., 2013)
785R	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GACTACHVGGGTATCTAATCC		
ITS1F	*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*CTTGGTCATTTAGAGGAAGTAA	Fungal ITS1 region (highly variable)	(Gardes and Bruns, 1993)
ITS2	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GCTGCGTTCTTCATCGATGC		(White et al., 1990)
AMV4.5Nf	*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*AAGCTCGTAGTTGAATTTCG	Fungal SSU 18SrRNA gene (350 bp)	(Suzuki et al., 2020)
AMDGr	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*CCCAACTATCCCTATTAATCAT		

beads (1× ratio) and quantified using Quant-iT™ dsDNA Assay kit (ThermoFisher, France). MID and Illumina sequencing adapters were added. Libraries were pooled in equimolar amounts using a Hamilton Microlab STAR robot and sequenced on an Illumina MiSeq platform using the MiSeq Reagent Kit v2 (2 × 250 bp). The obtained sequences were demultiplexed with index searches at the PGTB facility.

2.3. Bioinformatic methods

Sequence quality was preliminarily checked with FastQC v.0.11.8 (Andrews, 2010). Sequences were quality filtered, trimmed, denoised, and clustered into Operational Taxonomy Units (OTUs) using FROGS pipeline from Galaxy instance (Escudié et al., 2018). Raw forward and reverse reads for each sample were assembled into paired-ended reads with a minimum overlapping of 50 nucleotides and 0.1 mismatch using the VSEARCH tool (Rognes et al., 2016). Primers were removed using Cutadapt (Martin, 2011), and chimeras were detected and removed with UCHIME (Edgar et al., 2011). Clustering was performed using SWARM (Mahé et al., 2014) in the FROGS pipeline. The minimum proportional abundance of OTUs was set at 5e-05, and singletons were removed using a phiX contaminant databank. Taxonomic assignments of 16S rRNA, 18S rRNA gene OTUs and ITS-based OTUs were performed against silva138.1 (16S pintail100) (Quast et al., 2012), MaarJAM (2019) (Öpik et al., 2010), and Unite8.2 (Nilsson et al., 2019), respectively, using RDPClassifier from Galaxy.

All analysis and graphs were performed on R (4.1.2) using RStudio (2021.9.1.372). Figures were generated with *ggplot2* (3.3.5) and *ggthemes* (4.2.4) packages and arranged using *ggpubr* (0.4.0) (Wickham and Chang, 2008; Kassambara, 2020; Arnold, 2021). Datasets were gathered and analyzed with *phyloseq* package (1.38.0) (McMurdie and Holmes, 2013). Taxa related to mitochondrial and chloroplast OTUs were removed. Venn diagrams were generated to demonstrate the distinct and shared OTUs within soils in relation to soil status, season, and terroir factors. Overlaps of the Venn diagrams were tested for their significance based on hyper-geometric distribution using "phyper" function from R stats (4.1.2). Bacterial and fungal OTUs shared between Soil × Terroir × Season conditions were visualized using *UpSetR* (1.4.0) (Conway et al., 2017). Richness and diversity metrics, represented by Chao1, Shannon's diversity, and Bray-Curtis dissimilarity, were calculated through *phyloseq* using "estimate_richness" function. Pairwise comparisons were used to test for significant differences between the mean α-diversity metrics by conditions based on either *t*-tests or Wilcoxon tests, following homogeneity and normalization checks using Levene and Shapiro tests. Nonmetric multidimensional scaling (NMDS) was used to plot samples on a two-dimensional space using Bray-Curtis distances using the *phyloseq* ordinate function with the "NMDS" method. Linear models and permutational analysis of variance (PERMANOVA), for richness and diversity metrics, were demonstrated using the following formula: variable ~ Soil status × Season + Terroir. Type-II ANOVAs were performed using *car* (3.0–12) on Chao1 and Shannon's diversity metrics while PERMANOVAs were assessed on Bray-Curtis dissimilarity using the *vegan* package (2.5–7) with 999 permutations, and tests of multivariate homogeneity of group dispersions were checked using the "betadisper" function in the same package. Vector fitting to ordinations using "envfit" function from *vegan* was used to identify the environmental factors that best predicted bacterial and fungal community structures. The "ggeffectsize", "ggdiffbox", and "ggdiffclade" functions from *MicrobiotaProcess* (1.2.2) were used to reveal significantly different taxa across conditions (Xu and Yu, 2021). This process was set with a Kruskal ($\alpha = 0.05$) test based on linear discriminant analysis (LDA) effect size (LEfSe) and Wilcox ($\alpha = 0.05$), and corrected for the False Discovery Rate (FDR).

3. Results

3.1. Taxonomic distribution across the different conditions

Samples were taken from inter-row bulk soils in symptomatic (S) and asymptomatic (AS) areas across four different vineyards in the autumn and spring, accounting for 48 samples. A total of 4,649,863 16S, 4,191,712 ITS, and 3,844,836 18S raw sequences were generated from the libraries run. Subsequent to chimera removal, paired-end sequences were clustered into 2684, 810, and 244 operational taxonomic units (OTUs). The OTU accumulation curves tended to saturate as the number of samples increased, indicating that the sequencing depth was sufficient to provide an overview of the taxonomic distribution in each microbial community in the samples (Fig. S2).

The percentage of shared and specific OTUs across soil status, season, and geographical location factors were shown in Fig. 1**.A**. From 52.05 % to 96.38 % of OTUs were found in common depending on the comparison. The overlaps were not significant ($P > 0.05$) according to hypergeometric test, which indicates that the groups are significantly different for all the comparisons. The co-occurrence of OTUs revealed 1423 bacterial OTUs, 206 fungal OTUs, and 14 OTUs associated with *Glomeromycota*, shared across the eight Soil × Terroir × Season conditions (Fig. S3).

Regardless of soil status, terroir, and season, *Actinobacteriota* (33 %), *Proteobacteria* (15 %), *Chloforexi* (14 %), *Acidobacteria* (13 %), *Firmicutes* (14 %), *Verrucomicrobiota* (3 %) were the most abundant bacterial phyla, accounting for 92 % of total bacterial communities (Fig. 1**.B**), while the "Others" group was composed of *Gemmatimonadota*, *Myxococcota*, *Methylomirabilota*, *Nitrospirota*, *Bacteroidota*, *Desulfobacterota*, *Latescibacterota*, *RCP2-54*, *MBNT15*, *Entotheonellaeota*, GAL15, *Halobacterota*, *Cyanobacteria*, *Patescibacteria*, *Fibrobacterota*, and *Bdellovibrionota*. *Ascomycota* (58 %), *Basidomycota* (16 %), *Mortierellomycota* (8 %), and *Rozellomycota* (6 %) were the predominant fungal phyla, while unaffiliated fungal OTUs accounted for 9 % and the "Others" group was constituted of *Calcarisporiellomycota*, *Chytridiomycota*, *Glomeromycota*, *Basidiobolomycota*, *Kickxellomycota*, *Monoblepharomycota*, *Blastocladiomycota*, *Zoopagomycota*, *Olpidiomycota*, and *Entorrhizomycota*. Regarding the 18S rRNA sequencing data, 31 % of OTUs were unaffiliated. *Glomus* (55 %), *Paraglomus* (24 %), *Claroideoglomus* (16 %), and *Acaulospora* (3 %) were the predominant genera from the 49 % identified, while *Scutellospora* (0.53 %), *Diversispora* (0.41 %), *Archaeospora* (0.35 %), *Gigaspora* (0.16 %), *Pacispora* (0.08 %), and *Ambispora* (0.01 %) were grouped in "others" (Fig. 1**.B**).

Several fungi affiliated to grapevine diseases (i.e., grey mold, Petri disease, black foot, grapevine canker) (**Table S2**) were detected across the conditions. Among the 196 genera found in the samples, *Phaeoacremonium*, *Ilyonectria*, *Neonectria*, *Cadophora*, *Botrytis*, and *Curvularia*,

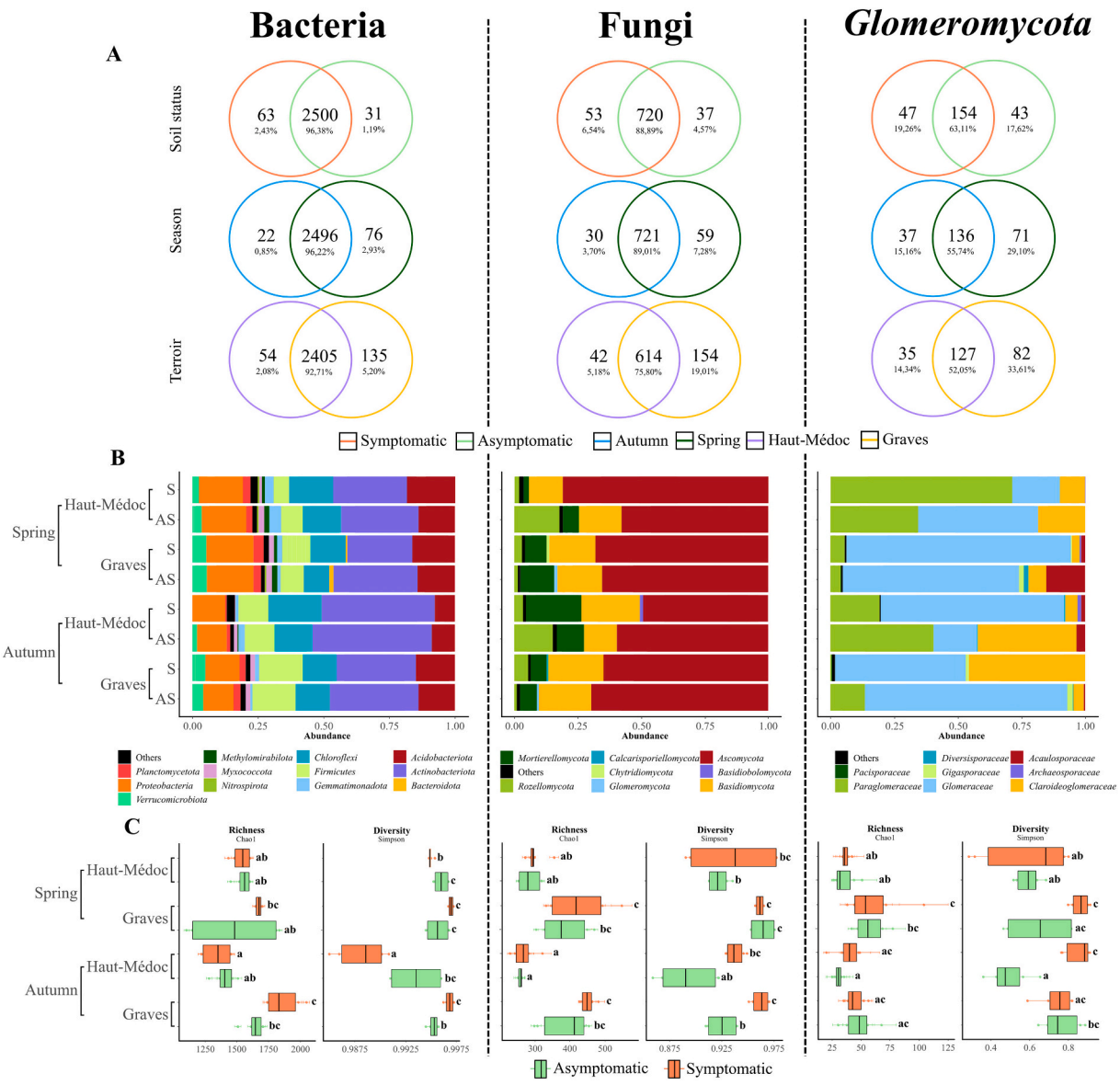

Fig. 1. Taxonomic distribution of the bacterial, fungal, and *Glomeromycota* communities across season × terroir × soil status (S: Symptomatic; AS: Asymptomatic) conditions. (A) Venn diagram presenting the number of common and specific OTUs between conditions. (B) Relative abundances of the bacterial and fungal phyla and *Glomeromycota* families identified. The phyla individually representing <1 % of the total communities were grouped in the "Others" phylum. (C) Richness and diversity, represented by Chao1 and Simpson's indexes, respectively. Different letters represent significantly different groups ($P < 0.05$).

all belonging to *Ascomycota* phylum, were identified in both symptomatic and asymptomatic soils (Fig. 2), accounting for 1.24 % of the total OTUs. A significantly higher percentage of sequences affiliated with these six pathogenic fungal genera, relative to the total number of sequences, was found in symptomatic compared to asymptomatic conditions in Graves for both seasons ($P = 0.0007$ and $P = 1.78E\text{-}05$ for spring and autumn, respectively) and in Haut-Médoc in spring ($P = 0.0375$). *Cadophora* and *Curvularia* were specifically and significantly more abundant in these symptomatic conditions compared to the asymptomatic ones (**Table S3**).

3.2. Richness differed between terroir and season, whereas diversity was affected by soil status

Similar bacterial richness, represented by the Chao1 metric, was observed in the spring for both Graves and Haut-Médoc terroirs between symptomatic and asymptomatic conditions, whereas significant differences were detected in the autumn due to geographical location, accounting for 20.6 % of the total variance (Fig. 1**.C;** Table 2). The bacterial Simpson's index was globally affected by terroir and season parameters, with symptomatic soils in Haut-Médoc presenting significantly lower diversity than asymptomatic ones.

In regard to ITS-sequenced fungal community, richness was significantly influenced by soil status and terroir, accounting for 4.5 % and 79.5 % of the observed variance, respectively. The fungal Simpson's index was affected by both soil status, season, and terroir factors, accounting for 11.3 %, 5.6 %, 31.3 % of the observed variance, respectively, with greater diversity in symptomatic conditions compared to asymptomatic ones, and higher diversity in Graves compared to Haut-Médoc.

Concerning the *Glomeromycota* division, the Chao1 metric was influenced by both season and terroir, accounting for 4.4 % and 22.7 % of the total variance, respectively, with lower richness in Haut-Médoc and in autumn compared to the Graves terroir and the spring. The Simpson's index diversity of *Glomeromycota* communities was influenced by both terroir and soil status, accounting for 10.9 % and 5.6 % of total

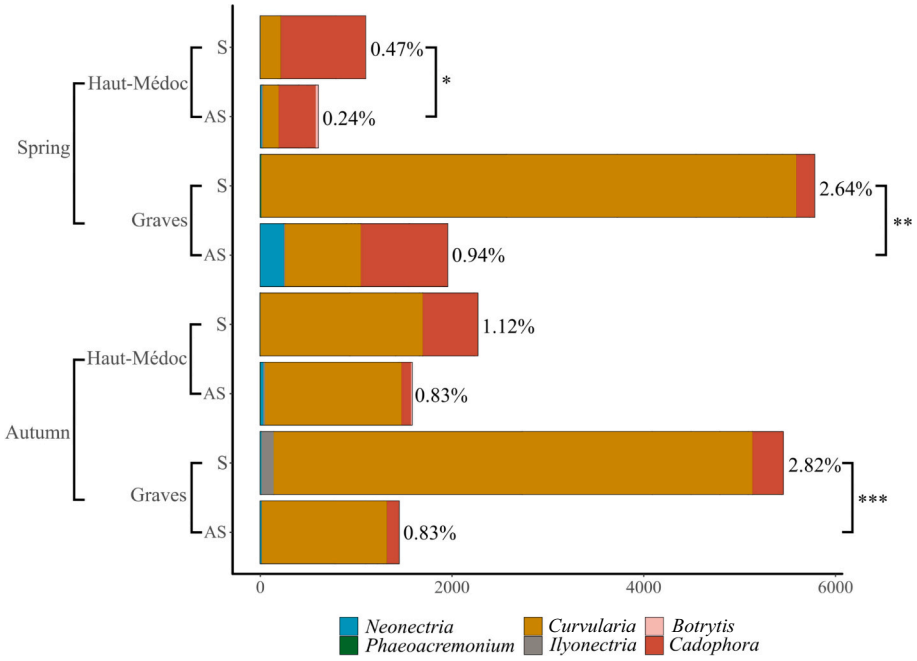

Fig. 2. Abundances of fungal OTUs potentially associated with grapevine diseases across season × terroir × soil status (S: Symptomatic; AS: Asymptomatic) conditions. Percentages indicate proportions of sequences affiliated with pathogenic fungi relative to total sequences. Significant differences between S and AS conditions (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) were detected using student *t*-tests or Wilcoxon-tests, depending on the normality and variance ($n = 6$).

Table 2
Soil composition (S, AS), season (autumn, spring), and terroir (Graves and Haut-Médoc), effects on richness (Chao1), α-diversity (Shannon), and β-diversity (Bray-Curtis) related to bacterial, fungal, and *Glomeromycota* communities in the bulk soils. Significances were assessed by Type II ANOVA for richness and α-diversity, while PERMANOVA was used for β-diversity. *P* values below 0.05 are highlighted in bold.

		Richness (Chao1)		α-diversity (Shannon)			β-diversity (Bray)	
		F(1,43)	*P*	*F*(1,43)	*P*	*F*(1,43)	R^2	*P*
Bacteria	Soil	3.467	0.070	0.538	0.468	5.092	0.075	**0.001**
	Season	0.001	0.993	28.631	**<0.001**	5.111	0.074	**0.001**
	Terroir	20.608	**<0.001**	38.710	**<0.001**	9.217	0.134	**0.001**
	Soil × Season	0.003	0.959	0.754	0.390	1.847	0.027	**0.048**
	Soil × Terroir	7.432	**0.009**	10.193	**0.003**	3.237	0.047	**0.006**
	Season × Terroir	14.336	**<0.001**	22.073	**<0.001**	2.956	0.043	**0.004**
	Soil × Season × Terroir	0.172	0.681	1.270	0.266	1.292	0.188	0.205
Fungi	Soil	4.474	**0.041**	11.318	**0.001**	4.517	0.063	**0.001**
	Season	0.265	0.609	5.583	**0.023**	4.094	0.057	**0.001**
	Terroir	79.525	**<0.001**	31.286	**<0.001**	11.812	0.164	**0.001**
	Soil × Season	0.098	0.756	6.584	**0.014**	1.902	0.026	**0.033**
	Soil × Terroir	1.597	0.214	2.465	0.124	4.059	0.056	**0.001**
	Season × Terroir	1.916	0.174	0.001	0.982	3.833	0.053	**0.001**
	Soil × Season × Terroir	0.191	0.664	0.022	0.882	1.790	0.025	0.060
Glomeromycota	Soil	0.285	0.596	9.317	**0.004**	5.655	0.073	**0.001**
	Season	4.431	**0.041**	0.319	0.575	4.336	0.056	**0.001**
	Terroir	22.73	**<0.001**	11.320	**0.001**	10.878	0.141	**0.001**
	Soil × Season	0.004	0.949	1.399	0.244	4.358	0.056	**0.001**
	Soil × Terroir	0.536	0.468	2.157	0.150	4.155	0.054	**0.001**
	Season × Terroir	2.683	0.109	2.119	0.153	3.623	0.047	**0.001**
	Soil × Season × Terroir	0.888	0.352	11.702	**0.001**	4.075	0.053	**0.001**

variance, respectively, with greater diversity in Graves terroir and symptomatic conditions.

3.3. Microbial community structure is different in symptomatic and asymptomatic soils

Sampling time, vineyard location, and the β-diversity using Bray-Curtis distance were computed in order to determine whether differences in microbiome structure and composition were correlated with decline features. NMDS analysis identified groups of microbial communities with similar soil status based on the season or terroir (Fig. 3). Each of the three factors (i.e., soil status, season, terroir) displayed significant correlations with the first two dimensions of the NMDS analysis. Although clustering was more pronounced for the terroir factor compared to the season, statistical differences were confirmed through PERMANOVA and revealed that across both bacterial and fungal samples, the overall microbial community differed in relation to decline features, sampling times, and vineyard location (Table 2). The beta-dispersion analysis showed non-significant comparisons for the soil status factor across the microbial communities (**Table S4**). These results suggest that the significant effects of the symptomatic and asymptomatic features observed above are not an artifact of dispersion, but rather

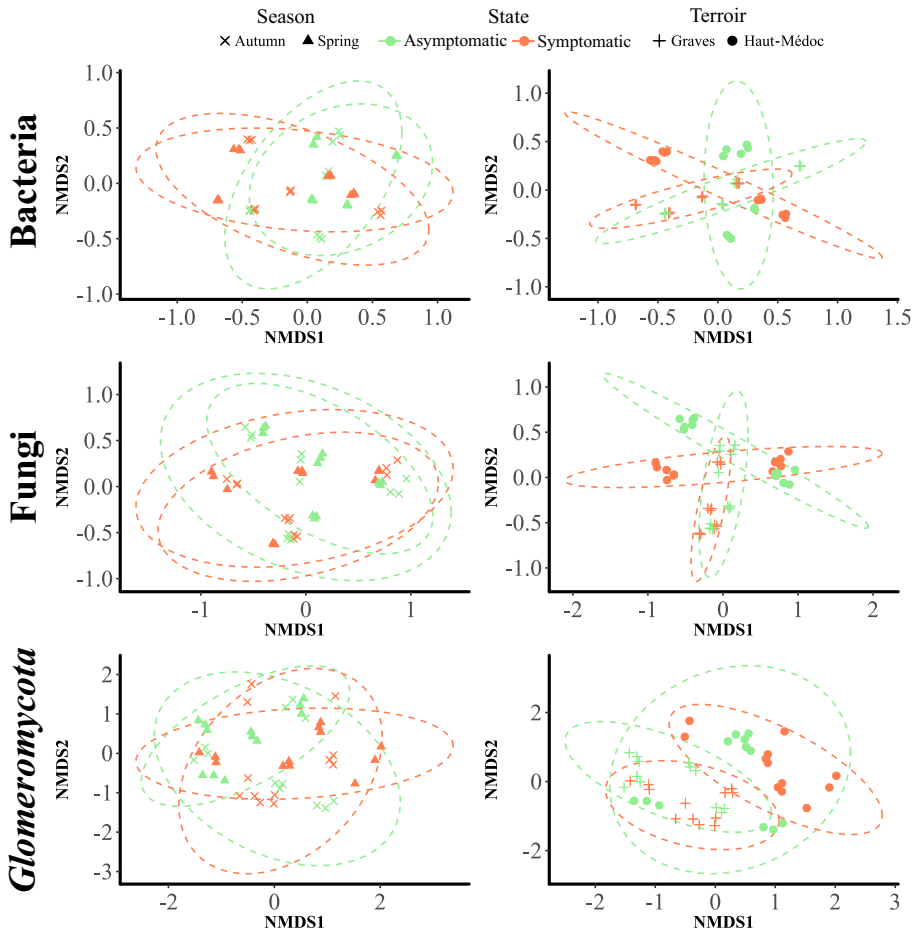

Fig. 3. Non-metric multidimensional scaling (NMDS) plot ordination of bacterial, fungal, and *Glomeromycota* communities among the Graves (cross) and Haut-Médoc (circle) terroirs during spring (triangle) and autumn (cross) seasons, depending on symptomatic S (orange) and asymptomatic AS (light green) conditions. Ordinations were based on Bray-Curtis distance method, and dashed lines represent 95 % confidence ellipses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reflect biological differences. On the other hand, the season and terroir factors significantly affected bacteria in fungal and *Glomeromycota* communities (**Table S4**). At the same time, the strongest relationship between the factors and bacterial community was observed in soil status ($R^2 = 0.087$), whereas ITS-fungal and *Glomeromycota* communities were more affected by the terroir component ($R^2 = 0.125$ and $R^2 = 0.246$, respectively).

3.4. Taxa enrichment is influenced by soil status

The LEfSe was conducted to report significantly enriched taxa and to get an overview of microbial community changes between soil, sampling time and vineyard location. The results presented in Fig. 4 showed enrichment related to each of the three environmental factors. LDA scores and the associated *P*-values with their FDR corrections were reported in **Fig. S4** and Table S5, respectively. The number of bacterial taxa showing significant differential enrichments was more extensive when comparing season and terroir than soil status factor, while fungi enrichments were mainly influenced by terroir and soil factors.

Asymptomatic soils were, regardless of season or vineyard location, significantly enriched (FDR-adjusted *P*-value < 0.05; Table S5) in 18 bacterial taxa, mainly from *Actinobacteriota* (i.e., *Acidimicrobiia*, *MB-A2-108*, *Thermoleophilia* classes) and *Myxococcota* (i.e., *bacteriap25* class) phyla, whereas symptomatic soils were enriched in 16 taxa belonging partly to *Ktedonobacteria* and *Acidobacteriae* classes, as well as *Blastococcus*, *Terrabacter*, *Gemmatimonas*, *Sphingomonas*, and *Fonticella* genera. When comparing the seasons, 30 bacterial taxa were enriched in autumn compared to spring, primarily belonging to *Actinobacteriota* and *Firmicutes* phyla, whereas 56 taxa were enriched during spring, belonging to *Proteobacteriota*, *Acidobacteriota*, *Planctomycetota*, *Methylomirabiota*, *Bacteroidota*, *Nitrospirota*, *Myxoccocota*, and *Desulfobacterota*. In regard to terroir, 22 taxa largely belonging to *Chloroflexi* and *Gemmatimonadota* were enriched in Haut-Médoc while 58 taxa were more abundant in Graves from *Firmicutes*, *Acidobacteriota*, *Verrucomicrobiota*, *Planctomycetota*, and *Myxococcota* phyla.

Regarding fungi sequenced-based on ITS, 21 taxa were significantly enriched (FDR-adjusted *P*-value < 0.05) in symptomatic soils, with most fungal taxa belonging to *Trichocomaceae*, *Filobasidiaceae*, *Pleosporaceae* (i.e., affiliated to *Curvularia* genus), *Hyaloscyphaceae*, and *Dermateaceae* families, whereas asymptomatic soil was enriched in 17 taxa primarily belonging to *Pyronemataceae*, *Russalaceae*, *Lyophyllaceae*, and *Minutisphaeraceae* families. Few seasonal taxonomic enrichments were found in fungal communities, with 9 taxa enriched during autumn (e.g., *Sporidiobolales* family) and 9 during spring (e.g., *Helotiales*, *Sebacinales*, and *Auriculariale* orders). On the other hand, the most important effect on a microbial community was the terroir factor on the ITS-sequenced fungal community, with 59 more abundant taxa in the Graves terroir (e.g., *Tremellales*, *Thelebolales*, *Eurotiales*, *Pleosporales*, and *Glomerales* orders) and 34 enriched taxa in Haut-Médoc (e.g., *Holtermanniales* and *Cystofilobasidiales* orders).

Terroir had a great impact on taxa enrichment in the *Glomeromycota* division, with 17 enriched taxa (e.g., species from *Glomerales*, and *Diversiporales* orders) in Graves and 5 in Haut-Médoc (exclusively from *Paraglomus* genus). When comparing soil status, 7 taxa were enriched in symptomatic soils and 6 in asymptomatic soils. Sampling time seems to have a low impact on *Glomeromycota* division, with 3 significantly enriched taxa in autumn and 5 during spring.

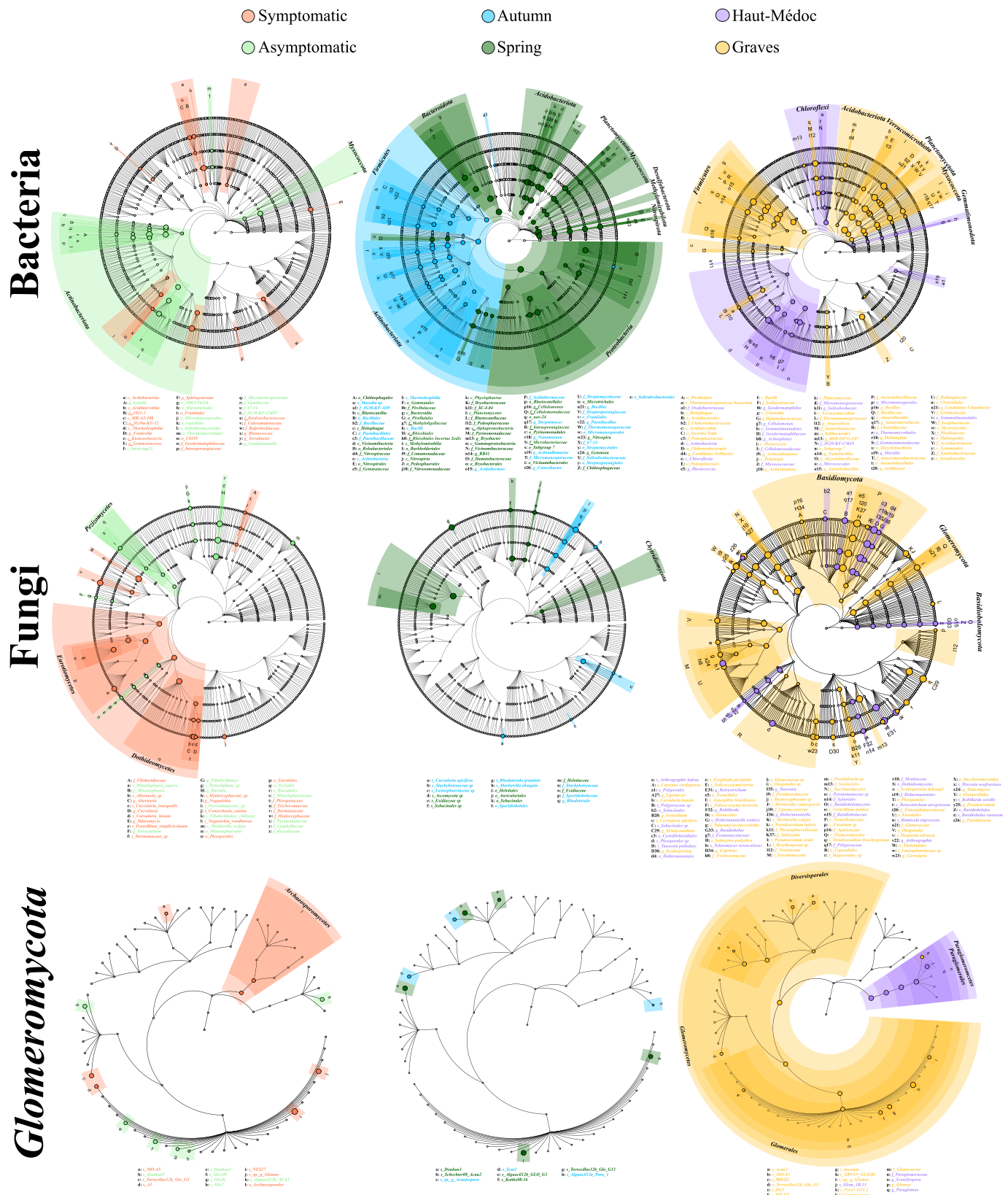

Fig. 4. Circular cladograms reporting LEfSe analysis related to identified OTUs from bacterial, fungal, and *Glomeromycota* communities according to phylogenetic features around the circle. The center of the circle represents the kingdom while the outer circle presents the OTUs at the species level. The color of the points and sectors indicate the factor in which the related OTUs are significantly enriched (FDR-adjusted *P*-value < 0.05). Letters indicate the enriched taxa that are reported below the figures.

4. Discussion

One of the main goals of microbial ecology is to understand how specific factors modulate microbial communities. The bulk soil is the microbial reservoir where the plant uptakes its associated microbiome through the chemoattraction of root exudates. Since a balanced grapevine-associated microbiome is essential to vine development and berry quality, the surrounding soil should provide a high diversity of microorganisms with the best activities for the plant. This work aimed to investigate the bacterial, fungal, and *Glomeromycota* communities associated with the bulk soil from the inter-rows of asymptomatic and symptomatic areas at four vineyards, presenting vines affected by decline unrelated to mineral deficiencies or pathogen infection. Since microbial communities are highly sensitive to environmental changes, specific attention was paid to the microbial richness and diversity linked to the community structures influenced by soil status, season, and terroir.

4.1. Do microbial diversity metrics in bulk soil offer a biological indicator of grapevine decline?

Vineyard decline is related to a wide range of environmental factors and can be alleviated by human practices if managed properly. For instance, cover crops can be used to counteract vine decline by conferring increased microbial diversity that forms a line of defense against soil pathogens (Richards et al., 2020). In the studied vineyards, cover cropping was used in Graves for both symptomatic and asymptomatic soils, suggesting that the observed differences in diversity were not dependent on the use of cover crops. The results obtained indicate that microbial richness was more influenced by vineyard location and season than by soil condition. This may not be surprising, since the main drivers of microbial communities in vineyard soils are edaphic (e.g., soil physicochemical parameters) and environmental factors (e.g., temperature or moisture) (Burns et al., 2015; Coller et al., 2019). Two terroirs were explored in this study, Graves (vineyards 1 and 2) and Haut-Médoc (vineyards 3 and 4). Both were located in the Bordeaux region and managed with conventional practices, with different physicochemical parameters depicted in Darriaut et al. (2021).

Notwithstanding seasonal and terroir-related impacts on microbial communities, no significant differences in bacterial richness and few significant differences in diversity were observed between symptomatic and asymptomatic soils. A previous study on vineyards affected by esca complex did not show any significant differences in fungal and bacterial richness and diversity between symptomatic and asymptomatic bulk soils (Nerva et al., 2019). In addition, similar bacterial diversity was found in the rhizosphere of symptomatic and asymptomatic grapevine affected by esca complex (Saccà et al., 2019). In our study, symptomatic soils presented significantly higher fungal richness and diversity compared to asymptomatic ones, which did not corroborate to Nerva et al. (2019) but were consistent with previous results related to higher level of cultivable fungi in symptomatic soils (Darriaut et al., 2021).

Moreover, the *Glomeromycota* division, affiliated to AMF, also presented significantly higher diversity in symptomatic soils compared to asymptomatic ones (Table S6). To our knowledge, only Bezerra et al. (2021) investigated the richness of AMF in soil affected by decline and dieback features by comparing them to asymptomatic soils within the same vineyards. It appeared that the location of the vineyard had a greater influence on the AMF diversity and richness than the soil status.

Degraded soil generally presents less fungal diversity or richness than normal soil since biodiversity correlates positively with soil ecosystemic processes, whereas a loss of fungal diversity impairs several functions, including nutrient cycling, organic matter decomposition, and plant defense systems (Wagg et al., 2014, 2019). It has been proposed that the higher the microbial diversity, the better the soil ecosystemic services are (Maron et al., 2018). In our case, the soils showing grapevine decline presented no changes in bacterial richness and sometimes increased bacterial diversities, whereas the fungal communities displayed higher diversity metrics when compared to soils with normal-growing vines, which can be considered as controversial indicator of soil health (Fierer et al., 2021).

4.2. Fungal pathogens were significantly more abundant in symptomatic than asymptomatic soils

Among these genera, *Cadophora luteo-olivacea*, *Curvularia inaequalis*, *Curvularia lunata*, *Curvularia portulacae*, *Curvularia spicifera*, *Ilyonectria destructans*, and *Neonectria lugdunensis* species were detected. Some of them were clearly identified as grapevine pathogens (Gramaje et al., 2018, 2022; Bahmani et al., *2021;* Lade et al., 2022).

Interestingly, the abundances affiliated to these fungal genera were significantly more affected by soil ($F(1,284) = 7.21$, $P = 0.008$) and terroir ($F(1,284) = 7.31$, $P = 0.007$) than by season ($F(1,284) = 0.15$, $P = 0.694$) and were significantly higher in symptomatic compared to asymptomatic soils. Nerva et al. (2019) found a higher abundance of the *Phaeoacremonium* and *Phaeomoniella* genera in soils associated with symptomatic vines affected by esca, compared to asymptomatic ones . In our study, the *Phaeoacremonium* genus was identified as having a similar abundance between both bulk soils. These findings were not correlated with the listed grapevine diseases, supporting the idea that the declines observed were not related to the invasion of pathogens from the bulk soil. However, it supports the postulate that soil is one of the inoculum sources of grapevine fungal pathogens grapevine that are present even in healthy and asymptomatic bulk soil (Giménez-Jaime et al., 2006; Gramaje and Armengol, 2011; Nerva et al., 2019).

The changes in microbial richness and diversity previously discussed could be related to several mechanisms to which microbial communities are sensitive. With the higher abundance of some fungal pathogens in symptomatic soils (i.e., *Cadophora*, *Curvularia*, and *Ilyonectria*), the potential effects of some secondary metabolites secreted by these microorganisms could affect the bacterial and fungal communities in the bulk soil. For instance, soil-borne *Curvularia* microbes are known to produce large amount of extracellular proteins, and secrete biologically active metabolites having antibacterial and antifungal properties (Bengyella et al., 2019). Similar antimicrobial properties caused by different secondary metabolites were found for *Cadophora* (Rusman et al., 2015; Yakti et al., 2019) and by several toxins and other extracellular enzymes for *Ilyonectria* (Manici et al., 2018).

4.3. Symptomatic bulk soil contains a high abundance of potentially beneficial bacteria and high diversity of Glomeromycota

The greater abundance of potential fungal pathogens in symptomatic soils was accompanied by an enrichment of several bacterial taxa compared to asymptomatic conditions, regardless of season or vineyard location. These enriched taxa included the bacterial genera *Blastococcus*, *Terrabacter*, *Sphingomonas*, *Gemmatimonas*, and *Fonticella*. None of these genera were affiliated to known plant diseases. On the contrary, they were related to pathogen control or nutrient regulation. For instance, *Blastococcus* is involved in both nitrogen metabolism (Cobo-Díaz et al., 2015) and pathogen inhibition, as observed in *Fusarium oxysporum* (Zhao et al., 2019). *Terrabacter* genus is also a potential keystone taxa involved in pathogen suppression (Wei et al., 2019), responsible for wheat decline (Chng et al., 2015). *Terrabacter*, on the other hand, has been negatively correlated with bacterial wilt disease index, and was linked to the restauration of declining soil properties by increasing soil nutrients (Qi et al., 2020). *Sphingomonas* genus displayed high nitrogenase activity and a plant-promoting growth capacity in maize and wheat plants (Xu et al., 2018). It is also known to display biocontrol properties on powdery mildew and *Fusarium* blight in Arabidopsis and wheat (Innerebner et al., 2011; Wachowska et al., 2013). These results are based on the assumption that these bacterial genera are biologically active and actually improve soil health in various processes involving

nutrient cycling and pathogen inhibition. Further microbial screening of the isolates should be conducted to confirm or refute this postulate by unraveling the functional diversity of the symptomatic and asymptomatic bulk soils.

In addition to this bacterial enrichment, higher *Glomeromycota* diversity was found in symptomatic vineyard bulk soils compared to asymptomatic ones. Although Landi et al. (2021) did not investigate diversity, the authors found greater mycorrhizal fungi colonization, as well as greater abundance of *Glomeromycota*, in the rhizosphere and roots of esca-affected grapevine compared to asymptomatic ones.

The "cry-for-help" phenomenon is a plant's adaptative response to biotic (Rolfe et al., 2019) or abiotic stresses (Rolli et al., 2021), and could make sense in this context of decline, since there was no evidence of edaphic factors or mineral deficiencies explaining the growth problem of grapevines at the four vineyards (Darriaut et al., 2021). These data might be the results of the decline and may not reflect an initial dysbiosis in the soil. Wei et al. (2019) demonstrated the determinant function of soil microbiome composition in plant health through the presence of rare taxa and pathogen-suppressing genus. A study of the potentially beneficial effects of isolates from areas of decline would be of great value. Stressful environments may indeed provide microorganisms that can alleviate diverse stresses and provide plants with valuable growth-promoting traits or relevant metabolic activities for soil nutrient cycling (Ashry et al., 2021). As suggested previously, the higher presence of fungal latent pathogens in symptomatic bulk soils might be related to changes in their microbial communities. Although dysbiosis of the soil microbiome, represented here by differences in microbial composition and diversity metrics, may not be the main cause of this decline, it is questionable whether microbiome engineering would be a relevant solution to overcome this soil dysfunction. Some plant-growth promoting microorganisms have been proposed to stimulate the grapevine growth through nutrient uptake or pathogen control by dipping grapevine roots or by drenching microbes directly into soil (Darriaut et al., 2022). Nonetheless the use of microbial inoculation may not enhance the microbial diversity or soil functional resilience (Ambrosini et al., 2016), while vineyard practices such as the use of organic fertilizers or the reasoned soil management should restore this loss in diversity.

5. Conclusions

For the first time, this study examined microbiome from the bulk soil of vineyards in two different terroirs affected by unexplained decline in the spring and autumn. The study highlighted differences between symptomatic and asymptomatic soils surrounding grapevine in terms of both bacterial and fungal divisions. Despite the presence of fungal genera associated with grapevine diseases, soils in symptomatic areas were enriched in potentially beneficial bacterial genera, with also higher diversity in fungal communities, including the *Glomeromycota* phylum. These findings suggest that soil is a source of both fungal pathogens and of beneficial microorganisms for grapevines, which generates interest in the isolation of bacteria in stressful environments. This conclusion relies on the speculative functionality of the enriched genera, further analysis based on microbial screening of isolates present in the asymptomatic and symptomatic bulk soils should be performed. Omics technologies represent a pivotal step towards understanding biological events related to plant health, particularly in describing the taxonomic composition of microbial communities in the soil of healthy or declining vines. The study of microbiota in a context of decline offers a breakthrough in the reduction of pesticides and chemical fertilizers through biocontrol, biostimulation, and biofertilization, which are key drivers for sustainable viticulture.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw sequences have been deposited in Sequence Read Archive (NCBI), Bioproject PRJNA826282. The data have been publicly released. https://www.ncbi.nlm.nih.gov/bioproject/PRJNA824657.

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