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Endophytic microbiota diversity in the phyllosphere of Sicilian olive trees across growth phases and farming systems

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A R T I C L E I N F O

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Olea europaea subsp. *europaea* var. *sylvestris*
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Olive management system

A B S T R A C T

This study investigates the diversity and interactions of endophytic microbial communities in the phyllosphere of Sicilian olive trees, focusing on both cultivated varieties (cultivars) and wild accessions. The research aims to explore the influence of anthropogenic factors, phenological stages, and farming practices on endophytic diversity. Samples were collected from three Sicilian olive cultivars subjected to two different olive cultivation management (organic and conventional) and six wild olive accessions (natural environments), across four key phenological phases. Using culture-independent methods, bacterial and fungal communities have been characterized through high-throughput sequencing. The results indicate that phenological stages and agricultural practices significantly affect microbial communities, while the type of olive host mainly affects the fungal diversity. Winter season emerged as a key period for microbial diversity, especially for bacteria, whereas fungal diversity varied less across growth phases. Organic farming management reduced bacterial diversity compared to conventional management and wild habitats. Furthermore, interactions between bacterial and fungal communities revealed positive correlations, highlighting potential synergy among endophytes. These findings underscore the dynamic nature of olive tree microbiota and suggest that both plant–microbe and microbe–microbe interactions play vital roles in structuring endophytic communities. This study is innovative as it compares, for the first time, the complete phenological cycle of local olive cultivars and wild accessions. It also analyzes the endophytic microbial community and its relationship with organic and conventional management.

1. Introduction

Plants are now recognized as "holobionts," forming mutualistic or pathogenic associations with their associated microorganisms [1] which can enhance nutrient bioavailability and acquisition, counteract pathogen presence, and protect plants from both biotic and abiotic stressors [2–4]. Several factors, such as host species, soil composition, climate and farming practices, can affect the microbial communities associated with different plant tissues [5]. The Mediterranean olive tree is a resilient, evergreen crop that encompasses two closely related entities, the spontaneous wild olive or oleaster [*Olea europaea* L. subsp. *europaea* var. *sylvestris* (Mill.) Lehr.], and the cultivated olive (*Olea europaea* subsp. *europaea* var. *europaea*) [6]. Olives and olive-derived products are an important source of income in traditional Mediterranean regions [7]. Sicily is particularly rich in olive germplasm and without taking into account ancient varieties not yet identified, 25 recognized cultivars [8] are used for both oil and table olives, covering 97.3 % of the cultivated area [9].

Previous studies on olive tree microbiota have explored potential relationships between microbial communities and host species or cultivars [10–14]. These studies also examined different olive compartments, such as the rhizosphere, phyllosphere, carposphere, and xylem sap [10,15–23]. Furthermore, factors such as altitude, geographic location, seasonality [15,22,24–26], agricultural practices [27,28], and abiotic stresses like salinity and drought [29,30] have been shown to affect olive microbiota. Advancement of high-throughput sequencing

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technologies has significantly enhanced our understanding of plant-associated microbiota, allowing the identification of many previously undetectable microorganisms [31]. Early research of culture-independent plant microbiomes primarily focused on microbial diversity and community composition using amplicon-based profiling techniques. These studies provide insights into the environmental and biological factors that could affect plant-associated microbial populations [19].

Recently, de Oliveira *et al.* [24] used metabarcoding amplicon sequencing to assess the influence of host age and geographic location on the bacterial and fungal communities of Brazilian olive trees. Kakagianni *et al.* [23] investigated prokaryotic, fungal, and arbuscular mycorrhizal fungal communities in various above-and below-ground tree parts of two Greek olive cultivars, during their annual growth cycles. Wentzien *et al.* [28] explored the impact of two agricultural management practices on the root microbiota of a Spanish olive cultivar. To date, only Ferraro *et al.* [32] and Crucitti *et al.* [33] have conducted in-depth studies on the culturable microbiota of different Sicilian olive cultivars, focusing on factors affecting the phyllosphere. However, no studies using metabarcoding sequencing have yet documented, with high resolution, the microbial composition, diversity, and interactions in both cultivated and wild olive samples. Moreover, no investigations have examined how host and environmental factors affect the composition and structure of the "core microbiota" of Sicilian olive trees.

This study aims to characterize the endophytic microbiota that has adapted to Sicilian olive biodiversity to better understand the biotic factors contributing to the success of olive tree health in the island. This research differs from previous studies on Mediterranean olive trees, taking into consideration the entire phenological cycle of local cultivars and wild accessions that have never been compared until now. In addition, the composition and correlations of the entire community of the endophytic microbiota (fungi and bacteria) are described, also considering the effect of different crop treatment (organic versus conventional).

The research assessed the presence of distinct microbial communities among different olive genotypes, throughout the olive annual growth cycle, and in various management systems. Using culture-independent methods, we characterized the prokaryotic and fungal endophytic communities in three dual-purpose (olive oil extraction and table olive) Sicilian olive cultivars namely ’Nocellara del Belice’, ’Nocellara Etnea’ and ’Nocellara Messinese’, and wild accessions. Particularly the following aspects were studied: (a) the effects of cultivation practice impact, annual cycle phenological phases, and farming management on the abundance and diversity of these communities; (b) the correlations between endophytic bacteria and fungi; and (c) the trophic modes of the fungal communities. These insights may contribute to a better understanding of the ecological dynamics and functional roles of microbial communities in olive trees.

2. Material and methods

2.1. Orchard site, sampling and metagenomic DNA extraction

Surveys were conducted in five olive orchards located in different olive Protected Denomination Origin (PDO) districts of Sicily, from December 2021 to October 2022 (Table 1). To study the effects of host variety and cultivation practices (wild vs cultivated conditions), the apical part of current season shoots was collected from three cultivars: ’Nocellara del Belice’ (NB), ’Nocellara Etnea’ (NE), and ’Nocellara Messinese’ (NM)—as well as from six wild Sicilian olive accessions (*Olea europaea* subsp. *europaea* var. *sylvestris*) from the Pisano forestry population. To compare the role of management systems on the endophytic microbiota, for each olive cultivar, three healthy trees were randomly selected from orchards conducted with differing farming practices: organic management (ORG), conventional management (CONV), and no agricultural intervention (NONE). To evaluate the effects of olive development stages on endophytic composition and diversity, 24 cultivated and wild olive trees were sampled at four different times, corresponding to key phenological phases: winter season (December 2021 to February 2022), full bloom (May 2022), fruit set (June to July 2022), and fruit ripening (October 2022). From each olive tree, a branch from each of the four cardinal directions was collected and stored at 4 °C for 48 h. From each of the above-mentioned branches, six shoot tips (7–8 cm) were randomly selected. The shoot tips were rinsed with tap water, then sterilized under aseptic conditions by immersion in 70 % (v/v) ethanol for 2 min, 3 % (w/v) sodium hypochlorite for 3 min, 70 % (v/v) ethanol for 1 min, followed by three 1-min rinses in sterile distilled water (SDW). The ninety-six sterilized shoot samples were first placed in BIOREBA extraction bags containing 5 ml of CTAB and subsequently crushed with the aid of a manual press equipped with opposing steel cylinders (flat rolling). An equal volume of CTAB/β-mercaptoethanol was added to a volume of the liquid fraction and the obtained suspension

Table 1
Sicilian olive cultivars and wild accessions sampled, sampling sites and farming systems.

Tree Host	Farming System	Accession Code	Olive District	GPS Coordinates
’Nocellara del Belice’	Organic	GIAL01	Castelvetrano (Trapani, South-West Sicily)	N 37°41’56.1"
		GIAL02		E 12°47’33.1"
		GIAL03		
	Conventional	GIAL04	Castelvetrano (Trapani, South-West Sicily)	N 37°41’49.2"
		GIAL05		E 12°48’21.9"
		GIAL06		
’Nocellara Etnea’	Organic	NEB01	Motta Sant’Anastasia (Catania, North-East Sicily)	N 37°31’15.8"
		NEB02		E 14°56’21.1"
		NEB03		
	Conventional	NEC04	Adrano (Catania, North-East Sicily)	N 37°41’21.6"
		NEC05		E 14°50’25.1"
		NEC06		
’Nocellara Messinese’	Organic	NMB01	Motta Sant’Anastasia (Catania, North-East Sicily)	N 37°31’15.8"
		NMB02		E 14°56’21.1"
		NMB03		
	Conventional	NMC01	Modica (South-East Sicily)	N 36°50’04.4"
		NMC02		E 14°46’57.4"
		NMC03		
Wild olive	Natural environments	SYLV01	Pisano forestry (Buccheri, Eas.t Sicily)	N 37°10’58.9"
		SYLV02		E 14°52’28.4"
		SYLV03		
		SYLV04		
		SYLV05		
		SYLV06		

processed according to the Doyle & Doyle [34] protocol. All DNA samples were quantified by fluorometer (Synergy H4 Microplate Reader, Bio Tek), to measure purity (Absorbance 260/280) and concentration (ng/μL). To assess the extracted DNA integrity, DNA samples were separated by 1.0 % agarose gel electrophoresis in TBE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) and 30 ng/μL (final concentration) of sample was used for sequencing with the Illumina MiSeq platform at Bio-Fab Research (Roma, Italy). To characterize the main soil physical-chemical properties, in each olive district for both orchard managements, pooled soil samples were collected at a 0–30 cm depth after removing surface residues (Supplementary Table S1). Each sample was placed in a sterile plastic bag, labelled, and stored in a cold chamber at 4 °C until analyses performed according to official methods in accredited reference laboratories courtesy of the research partner owners of each olive orchard.

2.2. Illumina sequencing

A prokaryotic library was constructed amplifying the hyper-variable regions V3-V4 (~ 300–350 bp) of the 16S rRNA gene using the primer pair 515 F (5'-GTGYCAGCMGCCGCGGTAA-3') [35] and 806 R (5'-GGACTACNVGGGTWTCTAAT-3') [36], together with two peptide nucleic acid (PNA) PCR clamps to reduce plastid and mitochondrial DNA amplification. In PCR reactions, 2.5 μL of microbial genomic DNA (5 ng/μL in 10 mM Tris pH 8.5), 5 μL of each Forward and Reverse primer, 0.75 μM of each PNA clamp (pPNA: 5'-GGACTACNVGGGTWTCTAAT-3'; mPNA: 5'-GGCAAGTGTTCTTCGGA-3') [37], DMSO (final concentration 3 %) and 12.5 μL of 2 × KAPA HiFi HotStart ReadyMix to a final volume of 25 μL were used. Thermocycler conditions were set as follows: initial denaturation at 98 °C for 1 min, 35 cycles of 98 °C (10 s), 78 °C (10 s), 50 °C (45 s), 72 °C (30 s), final extension at 72 °C for 10 min.

A eukaryotic library was obtained amplifying the ITS2 region (~ 270–450 bp) using the primer pair ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR reaction mixture (25 μL) contained 1x reaction buffer, 100 μM of each dNTP, 200 nM of each primer, 2 mM MgCl2, 0.05 U Taq DNA Polymerase (PCRBIO HiFi Polymerase PCRBIOSYSTEMS) and 1 μL of DNA template (30 ng/μL). Amplifications were performed using the following protocol: initial denaturation at 98 °C for 1 min; 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C; final elongation at 72 °C for 10 min.

All PCR products were run on a 2 % (w/v) agarose gel for 40 min at 90 V, stained with Gel Red (GelRed millipore Nucleic Acid Stain 10000X, Water). Amplified samples were purified using the AMPure XP beads (Beckman Coulter) kit and indexing/multiplex sequencing were performed following the manufacturer's protocols (NEXTERA XT, Illumina). Libraries were further cleaned-up, normalized, pooled by denoising processes, and sequenced on Illumina MiSeq Platform with 2 × 300 bp paired-end reads.

2.3. Data analyses

Raw DNA sequence data were processed by demultiplexing, denoising, quality filtering and chimera removing using DADA2 plugin in QIIME 2 (Quantitative Insights Into Microbial Ecology) qiime2–2023.5 version, to infer Amplicon Sequence Variants (ASVs). Bacterial taxonomic assignment of ASVs was achieved using "homemade" Naive Bayesian Classifier trained on V3-V4 16S sequences of SILVA database release 138.1. The classifier was set to include V4 regions of 16S rRNA genes at 99 % sequence similarity. The Amplicon Sequence Variants (ASVs) corresponding to chloroplasts and mitochondria were removed from the final set. For fungal communities, taxonomic assignment was conducted with the UNITE v9.0 Fungi reference datasets (2023–07–18) trained for use with qiime2–2023.5 version and eukaryotic ASVs not classified as fungi at the kingdom level were removed. Taxonomy BarPlots were generated through a R version 4.3.3 (2024–02–29) [38] using ggplot2 v3.5.1 [39].

Species diversity, rarefaction curves and evenness of the communities within samples were estimated by α-diversity indices (Observed_ASVs, qualitative Chao1 index, quantitative Shannon, Simpson and Faith's PD indices, Pielou index) at two taxa levels (Level 6 = Genus, Level 7 = Species). Beta-diversity between samples and Principal Coordinate Analysis (PCoA) were also carried out by analysis based on Bray-Curtis, Jaccard, Unweighted Unifrac and Weighted Unifrac dissimilarities matrices. To assess the differences in alpha and beta diversity, in correspondence with anthropogenic and environmental effects, three explanatory factors were defined: host (cultivated trees and wild accessions), phenological phase (winter dormancy, flowering, fruit set and fruit maturation), agricultural intervention (organic, conventional, none) Significant differences were determined by statistical analyses, including Kruskal-Wallis test for alpha-diversity indices, and Permutational Multivariate Analysis of Variance (PERMANOVA) with 1000 permutations for beta-diversity analysis. Moreover, differences in taxonomical abundances among sample groups were evaluated by the statistical Analysis of Compositions of Microbiomes (ANCOM) implemented in QIIME2.

From the normalized biom-table of 16S and ITS, respectively, ASVs representing at least 1 % of the amplicons in at least one sample were taken (55 taxa and 76 samples for the 16S biom-table; 38 taxa and 76 samples for the ITS biom-table). The regularized extension of canonical correlation analysis (rCCA) was used to search for correlations between the filtered biom-table matrices. After adjusting the lambda parameters ($\lambda 1 = 0.010$, $\lambda 2 = 0.231$) via a cross-validation approach, rCCA was performed including a ridge regularization step with four pairs of calculated canonical variables. Raw sequence reads are available on the NCBI BioProject under the accession number PRJNA1212469.

2.4. Differential abundance analysis of bacterial and fungal endophytes

To identify key ASVs driving the observed patterns of alpha and beta diversity, the differential abundance (DA) analysis of microbiome count data was performed using an Analysis of Compositions of Microbiomes with Bias Correction 2 (ANCOM-BC2) with sensitivity analysis. Sensitivity analysis was also incorporate into final assessment of taxon significance as aquamarine-colour in the plots. The primary analysis identified taxa with differential abundance based on the covariates phenological phase and type of management, using "blooming" and "organic" levels as baseline, respectively. As part of ANCOM-BC2, the Holm-Bonferroni method was employed to correct P values, using a significance cutoff of $p_{adj} \leq 0.05$.

2.5. Prediction of functional capabilities of bacterial communities

Prokaryotic metagenome profiles were functional annotated by mapping the 16S rRNA gene sequences to SILVA database. The tool Tax4Fun v1.1.5 [40] with Reference Data v2 of the database Ref99NR was used to identify significant functional categories of bacterial communities. Differential analysis on the KEGG functions was performed in R environment by microeco package v0.7.1. Significance functional abundance with 0.05 threshold was shown in Lefse plots performing a Linear Discriminant Analysis (LDA) Effect size (LEfSe) among the three explanatory factors. Based on gene content, bacterial reads were linked to metabolic function using different KEGG (Kyoto Encyclopedia of Genes and Genomes) levels to predict the functional community profiling. Putative metabolic phenotypes and relevant ecological functions based on the literature of cultured representatives were also assigned with FAPROTAX v1.2.10 database [41].

Collinearity was observed between the two explanatory factors, host and agricultural intervention, and the latter was used to predict the value of the relative abundance for each ecological function. Differences of metabolic and ecological categories among phenological phases and among farming practices were statistically assessed by a multiple hypothesis testing using the analysis of variance (ANOVA) implemented in R environment.

2.6. Prediction of fungal trophic modes and guilds

To obtain information about the functional diversity of fungal communities in shoot habitat, FUNGuild database was used to estimate the trophic modes and guilds of fungi [42]. This tool allows to predict the primary fungal lifestyle such as Pathotroph: receiving nutrients at the expense of the host cells and causing disease; Saprotroph: receiving nutrients by breaking down dead host cells; Symbiotroph: receiving nutrients by exchanging resources with host cells. The identified ASVs at genus level were classified with the FUNGuild tool and differences into ecological guilds among phenological phases and between farming systems were assessed. Only those ASVs identified with a confidence ranking "Highly Probable" (absolutely certain), and "Probable" (fairly certain) were considered in this analysis. Statistically significant differences of fungal guild descriptors by phenological phases and farming systems were performed by a multiple hypothesis testing using the analysis of variance (ANOVA) implemented in R environment.

3. Results

3.1. Bacterial community analysis through metabarcoding amplicon sequencing

Starting from 96 amplified samples, a total of 8869,170 raw reads were obtained ranging from 15,870 to 127,554 per sample. About 96 % reads were maintained after sequence quality control (denoising), generating a total of 2262,331 non-chimeric amplicons. After removing low-quality sequences, mitochondrial, and chloroplast sequences, 3449 taxa or ASVs (Amplicon Sequence Variants) were recovered. The bacterial community composition across all samples and phenological phases was predominantly represented by the classes Gammaproteobacteria (relative abundance [RA] ranging from 12.8 % to 96 %), Bacteroidia (RA ranging from 3.5 % to 60.8 %), Campylobacteria (RA up to 17.3 %), Alphaproteobacteria (RA up to 78.2 %), and Actinobacteria (RA up to 53.4 %). Twenty-seven ASVs were shared by at least 95 olive twig samples. Among the most commonly recovered bacterial genera were *Neptuniibacter*, *Neptunomonas*, *Malaciobacter*, *Winogradskyella*, *Maribacter*, *Desulforhopalus*, *Alteromonas* and *Saccharospirillum* (Fig. 1). When comparing different olive cultivars and wild olive accessions, the microbial composition generally showed the same dominant classes. However, in some samples of 'Nocellara Etnea' and 'Nocellara Messinese', the genus *Wolbachia* (Alphaproteobacteria) showed higher levels of relative frequency (Fig. 1). Considering the bacterial taxonomic genera with a good level of coverage among olive hosts, the genus *Candidatus* Cardinium was exclusively detected in olive cultivar samples (Fig. 2), whereas the genera *Robbsia*, *Kineosporia*, *Bryocella*, and *Amnibacterium*, although less abundant, were found in wild olive trees but not in cultivars.

Considering the phenological phases, bacteria from the classes Gammaproteobacteria, Bacteroidia, and Campylobacteria had an almost uniform distribution across all phases. However, the presence of Alphaproteobacteria increased during blooming (RA up to 58.3 %), with higher values also observed during fruit set (RA up to 39.8 %) and fruit ripening (RA up to 78.2 %). This increase was largely due to the presence of the genus *Wolbachia* (Fig. 1). This trend became clearer when comparing the microbial composition across different farming systems. Olive cultivars organically managed showed higher relative abundance of the class Alphaproteobacteria, at the expense of Bacteroidia and Campylobacteria, when compared to conventionally managed cultivars and Sicilian wild olive trees (Fig. 1). The most frequently identified Alphaproteobacteria were from the genus *Wolbachia*, observed in 41 samples, and the genus *Sulfitobacter*, observed in 95 samples.

3.2. Fungal community analysis through metabarcoding amplicon sequencing

Considering the samples that did not show an amplification signal, metabarcoding analyses were performed on 76 samples. A total of 8077,149 raw reads were obtained ranging from 53,963 to 264,156 per sample. About 65 % reads were maintained after sequence quality control (denoising), generating a total of 1945,823 non-chimeric amplicons. After removing low-quality, and chimeric sequences, were identified 1796 taxa of fungi associated with olive tree twigs. The primary taxa revealed that *Dothideomycetes* was the predominant fungal class (relative abundance [RA] ranging from 1.8 % to 98 %), followed by *Fungi_cls_Incertae_sedis* (RA up to 96.9 %), *Sordariomycetes* (RA up to 57.04 %), *Eurotiomycetes* (RA up to 83.3 %), and other less abundant classes. All analyzed samples frequently shared ASVs identified as *Fungi_gen_Incertae_sedis* and the genus *Ochrocladosporium*, while *Alternaria* was the most frequently observed fungal genus, recorded in 72 samples (Fig. 3A). Across all phenological phases, the genera *Ochrocladosporium* (RA up to 80.9 %) and *Furfurella* (RA up to 56.9 %) were well represented in the fungal communities of Sicilian wild olive trees, while the genus *Foliophoma* (RA up to 49.7 %) was primarily found in twig samples from wild olive accessions and 'Nocellara Messinese' in conventional orchards (Fig. 3). In addition, among the most represented fungal genera, the Sicilian wild olive trees hosted endophytes of genus *Polyporales_gen_incertae_sedis* (Fig. 4), and to a lesser extent the genera *Phallus*, *Orbilia*, *Neopyrenopeziza*, *Bellamyces*, *Stagonospora*, *Hysteriaceae_gen_incertae_sedis* and *Muriphaeosphaeria* not present in the samples of olive cultivars. Regarding the fungal composition across different phenological phases, the genus *Fungi_gen_Incertae_sedis* was clearly predominant during the fruit set and ripening stages (Fig. 3). When comparing managing systems, wild olive trees, which were not subjected to any agricultural practices, showed the highest abundance of the genera *Ochrocladosporium* and *Furfurella* (Fig. 3). The genus *Aureobasidium* (RA up to 37.7 %) was better represented in organic orchards, while the genus *Quambalaria* (RA up to 79.3 %), one of the most frequently identified genera, was more abundant in samples from both organic and conventional orchards (Fig. 3 and Supplementary Figure S1).

3.3. Alpha diversity of the endophytic communities in olive tree twigs

The statistical analyses were carried out to determine the influence of the explanatory factors (host cultivars/wild accessions, phenological phases and farming systems) on the response variable (Observed_ASV). Alpha rarefaction curves on the observed features and the three diversity indices (Chao1, Shannon and Simpson) suggested that sample diversity was well covered for both target regions (16S rDNA and ITS2), reaching an asymptote for all considered factors, suggesting that collection efforts were enough to represent the diversity of microbial species (Supplementary Figure S2).

Analysing the bacterial species diversity among the three cultivars of Nocellara and the wild olive trees, no significant differences were found (Kruskal-Wallis $H = 29.06$, p-value $= 0.178$). However, variation among each olive cultivar and wild olive trees was assessed showing significant differences ($p \leq 0.05$), specifically between some wild trees (SYLV02, SYLV03, SYLV04 and SYLV06) and some cv. 'Nocellara Etnea' (NEB02, NEB03, NEC04, NEC05, NEC06) and cv. 'Nocellara Messinese' (NMB01, NMB02, NMB03) trees. Similarly, the bacterial species evenness showed no significant differences among cultivated and wild olive trees but two oleaster accessions (SYLV 03 and SYLV04) had a significantly difference in endophytic taxon uniformity compared to one sample of cv. 'Nocellara Etnea' (NEB02 – SYLV04, $p = 0.043$) and two samples of cv. 'Nocellara del Belice' (GIAL01 – SYLV04, $p = 0.034$; GIAL04 – SYLV03, $p = 0.050$; GIAL04 - SYLV04, $p = 0.021$). Considering the impact of phenological phases on bacterial alpha diversity (Supplementary Figure S3), both species diversity (Shannon entropy, Kruskal-Wallis H =

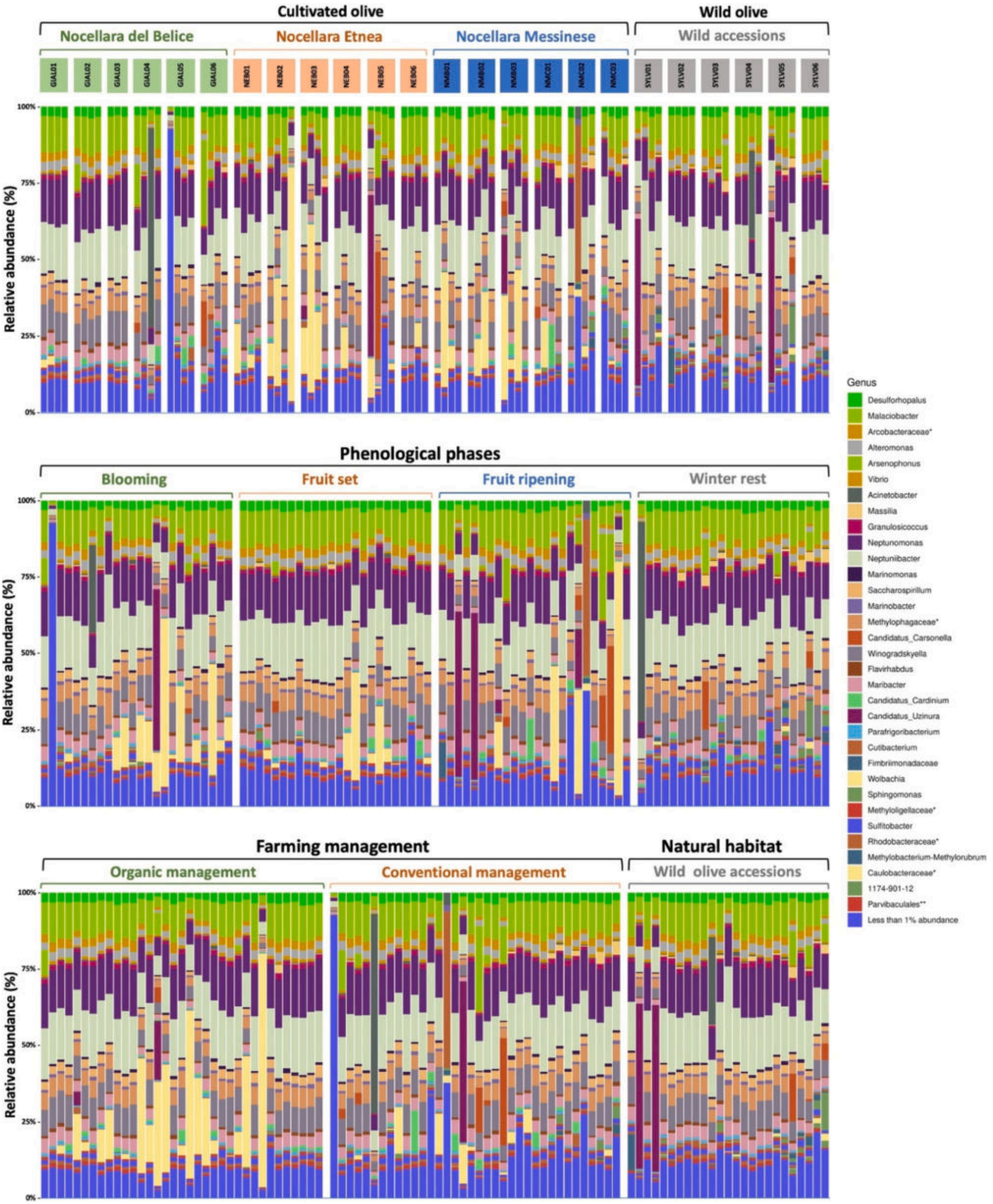

Fig. 1. Relative abundance of main bacterial ASVs recovered from olive twig samples grouped for olive hosts, phenological phases and farming systems. Different colours in the bar plot represent the main bacterial taxonomic genera, as reported in legend.

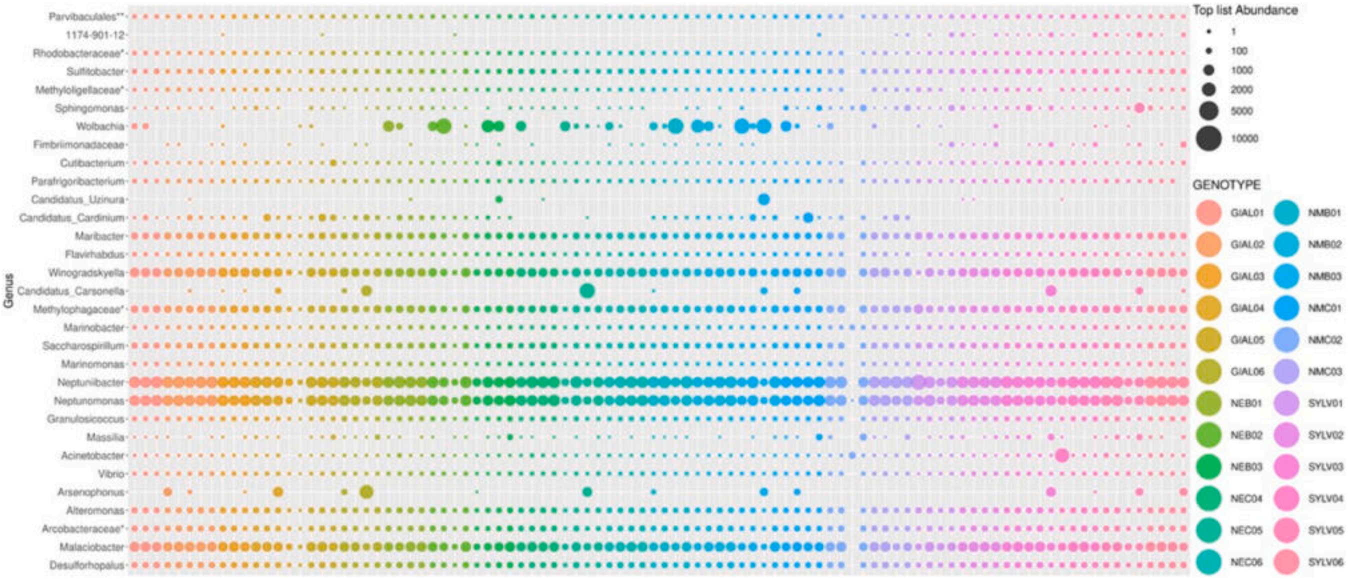

Fig. 2. Bubble plot showing the distribution of bacterial ASVs by genus across olive twig samples. Different colours represent the olive twig samples as indicated in the legend, while bubble size corresponds to the relative abundance of each genus.

9.89, $p = 0.019$) (Supplementary Figure S3A) and evenness (Pielou's index, Kruskal-Wallis $H = 28.5$, $p < 0.0001$) (Supplementary Figure S3B) showed significant differences across the sampling points, with the highest values recorded during the winter dormancy period. In contrast, phylogenetic diversity (Faith's PD index) did not differ significantly among the four phenological phases. When analysing the interaction between orchard management (organic vs. conventional) and alpha diversity, the Kruskal-Wallis's statistic revealed a significant effect on both species diversity ($p = 0.005$) and phylogenetic diversity ($p = 0.024$). Specifically, the endophytic bacterial communities of cultivars in organic orchards exhibited significantly lower species diversity compared to those managed conventionally ($p = 0.032$) and to wild olive accessions ($p = 0.002$) (Supplementary Figure S4A). Additionally, a significant reduction in phylogenetic diversity ($p = 0.006$) — based on the phylogenetic distances of detected taxa — was observed in cultivars under organic management compared to wild olive trees. A less pronounced, though non-significant, decrease was also found between cultivars in conventional orchards and wild olive accessions (Supplementary Figure S4B).

In term of Shannon entropy index, mycobiota alpha diversity did not respond to host type (Kruskal-Wallis $H = 30.32$, p-value $= 0.140$), phenological phase (Kruskal-Wallis $H = 7.61$, p-value $= 0.055$) and agricultural management effect (Kruskal-Wallis $H = 2.24$, p-value $= 0.326$) among all olive trees. At single sample level, the most frequent significant differences ($p < 0.05$) were found between trees of cv. 'Nocellara del Belice' in conventional management and some trees of cv. 'Nocellara Etnea', cv. 'Nocellara Messinese' and some samples of wild olive trees. A significant effect of phenological phases on fungal species diversity was observed between blooming and fruit maturation ($p < 0.01$) (Supplementary Figure S5A), and between winter and fruit ripening ($p = 0.030$) (Supplementary Figure S5A). Also, the blooming phase recorded a significantly different Faith's PD (p-value < 0.01) in pairwise comparisons with the other considered phenological phases (Supplementary Figure S5B). Mycobiota species evenness not showed significant differences (Pielou index, p-value > 0.05) among all analysed factors, demonstrating that all identified fungal species are uniformly represented among the samples.

3.4. Beta diversity of the endophytic communities in olive tree twigs

The analyses of the variation of species composition among the studied factors revealed that phenological phases (PERMANOVA Unweighted UniFrac: all groups Pseudo-F $= 1.26$, p-value $= 0.013$) and type/absence of management (PERMANOVA Unweighted UniFrac: all groups Pseudo-F $= 1.85$, p-value $= 0.001$) affect the bacterial community in olive tree shoots. Each phenological phase was also compared in pairs, and during the winter showed significant results compared to blooming (PERMANOVA Unweighted UniFrac: pairwise Pseudo-F $= 1.35$, p-value $= 0.029$), to fruit set (PERMANOVA Unweighted UniFrac: pairwise Pseudo-F $= 1.59$, p-value $= 0.001$) and to fruit ripening (PERMANOVA Unweighted UniFrac: pairwise Pseudo-F $= 1.33$, p-value $= 0.035$). The ANCOM analyses showed that the family Methyloligellaceae ($W = 1226$), and the genera *Parafrigoribacterium* ($W = 1152$) and *Reinekea* ($W = 1124$) were differentially abundant among phenological phases. Regarding the pairwise comparison among farming managements (organic or conventional) and the absence of any type of agricultural intervention, beta diversity differences were observed between the groups of wild olive accessions and olive cultivars managed both in organic (PERMANOVA Unweighted UniFrac: pairwise Pseudo-F $= 2.67$, p-value $= 0.001$) and conventional system (PERMANOVA Unweighted UniFrac: pairwise Pseudo-F $= 1.91$, p-value $= 0.001$). According to ANCOM analyses, Sicilian wild olive trees hosted differentially more abundant the bacterial genera *1174–901–12* (family Beijerinckiaceae, Rhizobiales) ($W = 949$) and *Robbsia* ($W = 908$) compared to cultivars under management conditions that shared the genera *Candidatus* Cardinium and *Wolbachia* ($W = 948$ for both). Although the principal coordinates analyses (PCoA) revealed the majority of samples cluster together, the PCoA with the Bray-Curtis distance matrix (Fig. 5) showed that the bacterial communities of wild olive accessions were separated from those of cv. 'Nocellara Etnea' and cv. 'Nocellara Messinese' samples (Fig. 5A). As well as two clusters partly distinct were observed between the bacterial endophytes of the winter phase and blooming /fruit set phases (Fig. 5B). Also, the organic management system contributed to the spatial differences of bacterial communities distinguishing a main cluster compared to the mixed one of conventional and no agricultural intervention (Fig. 5C).

Endosphere olive mycobiota beta diversity was significantly affected by all factors examined. Fungal community composition was shaped by the olive host (PERMANOVA Unweighted UniFrac: all groups Pseudo-F $= 1.45$, p-value < 0.001), particularly for the wild olive group; by phenological phases (PERMANOVA Unweighted UniFrac: all groups Pseudo-F $= 2.07$, p-value < 0.001) with the highest degree of

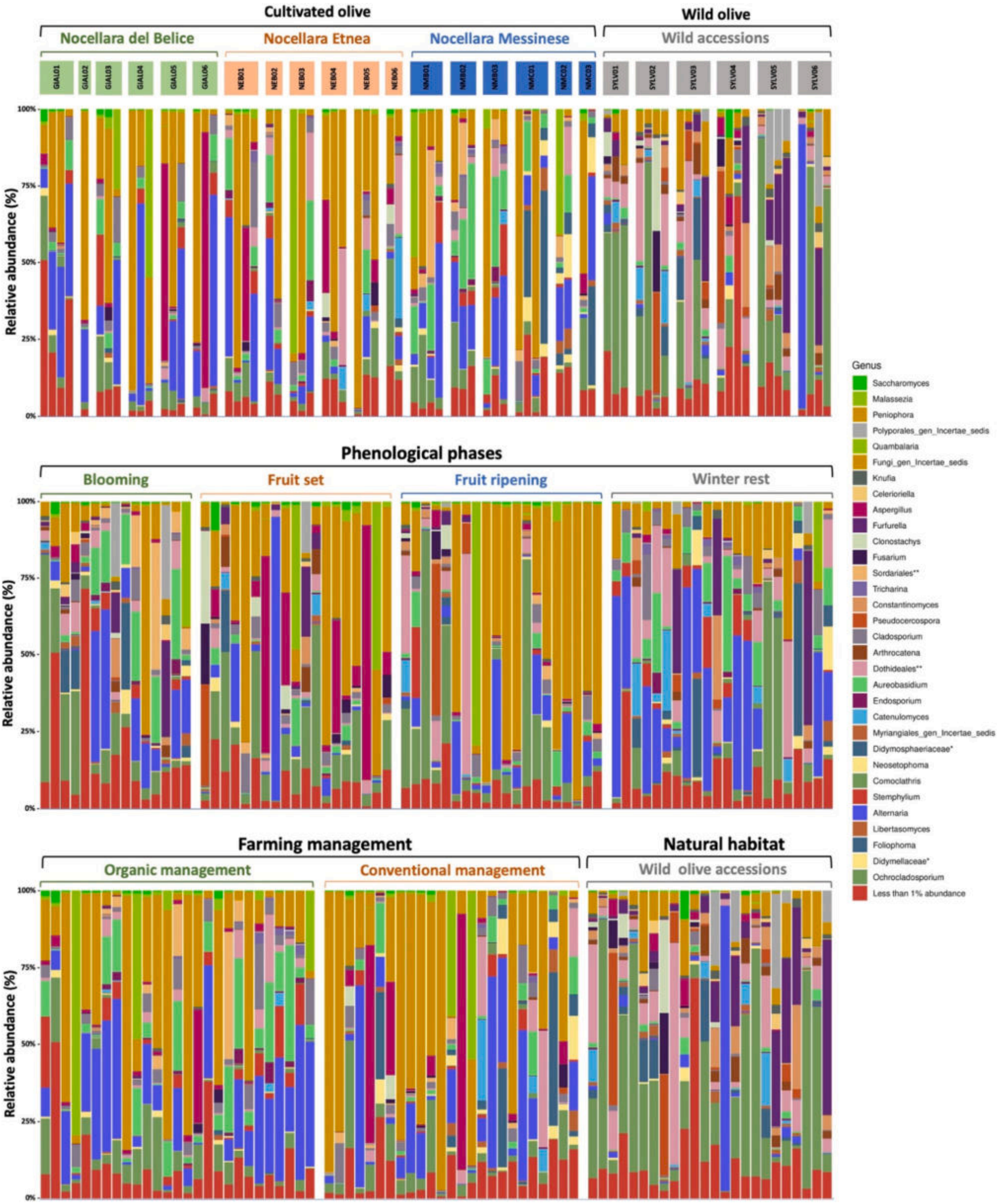

Fig. 3. Relative abundance of main fungal ASVs recovered from olive twig samples grouped for olive hosts, phenological phases and farming systems. Different colours in the bar plot represent the main fungal taxonomic genera, as reported in legend.

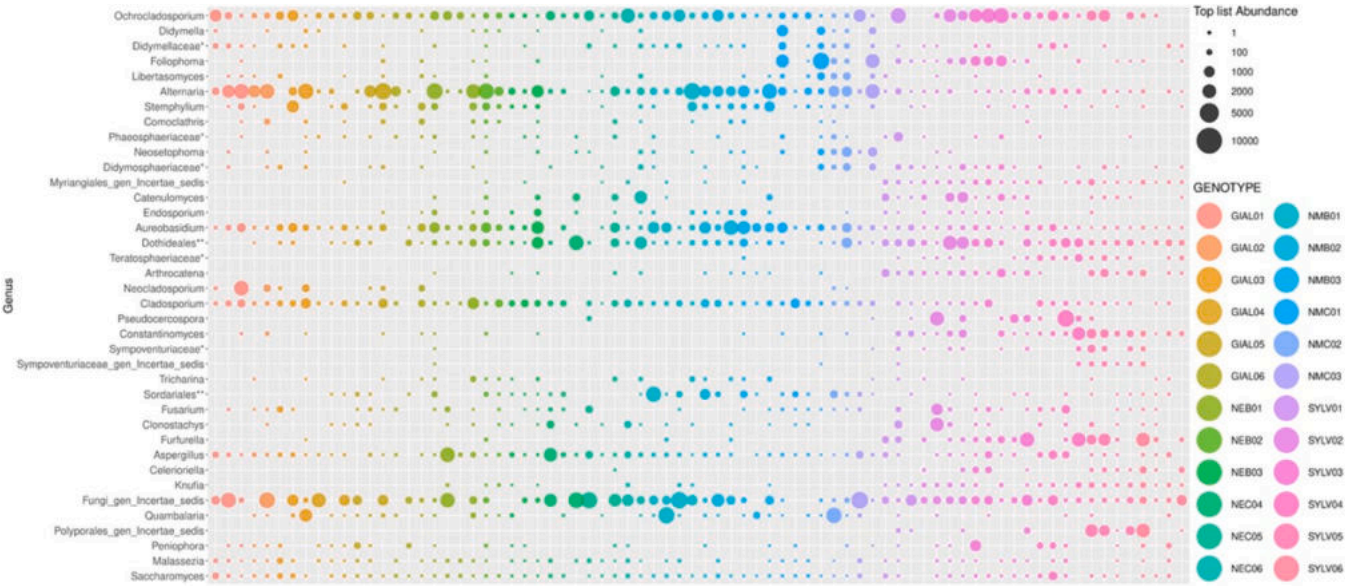

Fig. 4. Bubble plot showing the distribution of the fungal ASVs dataset for taxonomic genus and among olive twig samples. Different colours represent the olive twig samples and the size of the bubbles the respective abundance, as reported in legend.

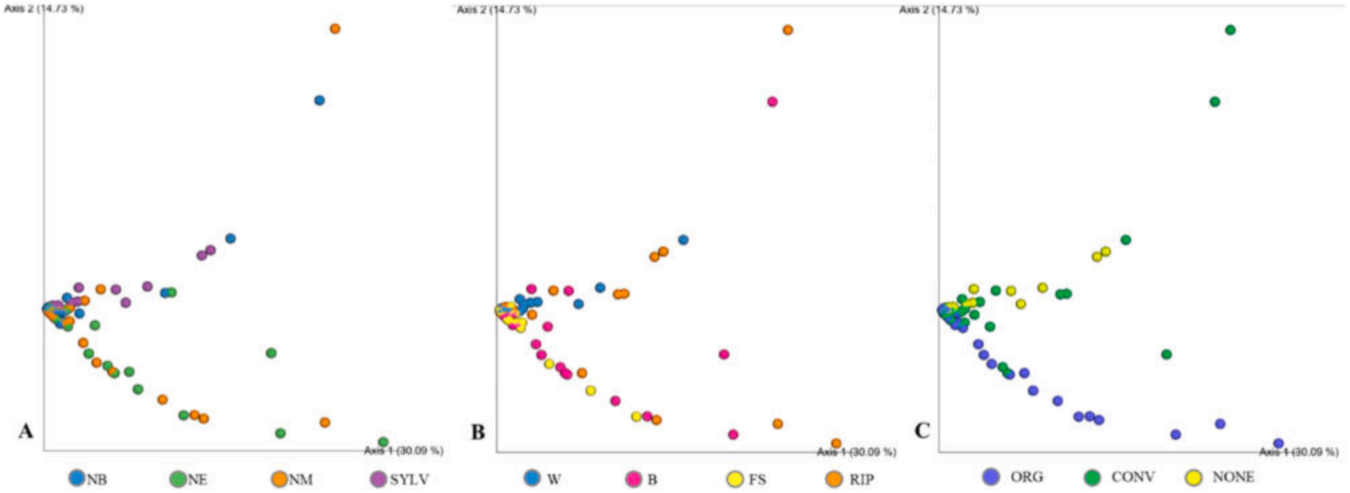

Fig. 5. Beta diversity results with Principal coordinates analysis (PCoA) with Bray-Curtis distance illustrating bacterial community structure among shoots of the Sicilian olive trees. A) PCoA for olive hosts, each colour represents a different host group. B) PCoA for phenological phases (B: Blooming; FS: fruit set; RIP: fruit ripening; W: winter), each colour represents a different phenological phase. C) PCoA for farming systems, each colour represents a different management (ORG: organic; CONV: conventional; NONE: no agricultural interventions).

dissimilarity between the group of winter phase and the other phenological stages. Moreover, fungal communities were under the control of the agricultural practices (PERMANOVA Unweighted UniFrac: all groups Pseudo-F = 5.64, p-value < 0.001) indicating a greater diversity between the group of oleasters in absence of horticultural management and olive trees in organic (PERMANOVA Unweighted UniFrac: pairwise Pseudo-F = 8.89, p-value < 0.001) and conventional (PERMANOVA Unweighted UniFrac: pairwise Pseudo-F = 6.48, p-value < 0.001) management, the latter less distant from the mycobiota composition of the olive cultivars in organic farming (PERMANOVA Unweighted UniFrac: pairwise Pseudo-F = 1.62, p-value = 0.019). The ANCOM analyses showed that fungal genera *Arthrocatena* (W = 433), *Saccharomyces* (W = 365) and *Furfurella* (W = 441) were differentially abundant among olive hosts, phenological phases and management systems, respectively. The PCoA using the Jaccard distance matrix (Fig. 6) explained, better than the others, the differences obtained from the beta diversity analyses: when compared to the other relative groups, clustering was clear in case of wild olive trees (Fig. 6A), and no farming system (Fig. 6C), while it was less evident although significant for wintertime (Fig. 6B).

3.5. Taxa driving alpha and beta diversity

Thirty-one bacterial ASVs and one fungal ASV differentially abundant were identified and associated with phenological phases. Among assigned taxa, two ASVs (*Wolbachia* and Sordariales) displayed a considerable log-fold decrease (up to −4) in winter compared to blooming (Fig. 7A) in which other 29 bacterial genera were more abundant (log fold up to −1), including the most significant ones were *Parafrigoribacterium*, *Cohaesibacter*, *Rhodopirellula*, *Reinekea*, *Maribacter*, *Marinobacterium*, *Lentimonas*, *Pseudoalteromonas*, *Neptunomonas*, *Marinobacter* and the bacterial families Saprospiraceae, Methyloligellaceae and Microtrichaceae (Fig. 7A). On the other hand, the *Candidatus* Cardinium genus demonstrated a log-fold increase (greater than 2) in fruit set and fruit ripening respect to blooming (Supplementary Figure S6). A

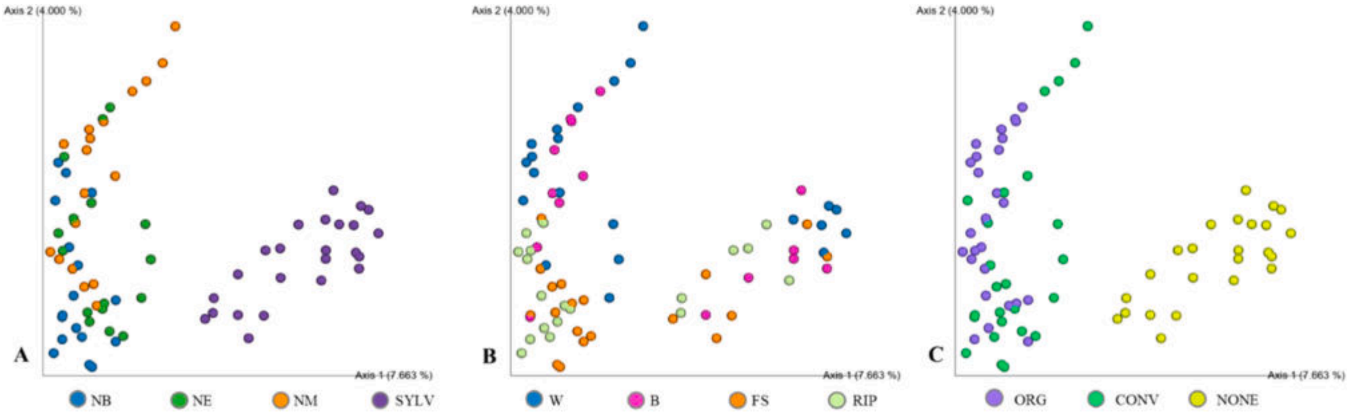

Fig. 6. Beta diversity results with Principal coordinates analysis (PCoA) with Jaccard distance illustrating fungal community structure among twigs of the Sicilian olive trees. A) PCoA for olive hosts, each colour represents a different host group. B) PCoA for phenological phases (B: blooming; FS: fruit set; RIP: fruit ripening; W: winter), each colour represents a different phenological phase. C) PCoA for managing systems, each colour represents a different management (ORG: organic; CONV: conventional; NONE: no agricultural interventions).

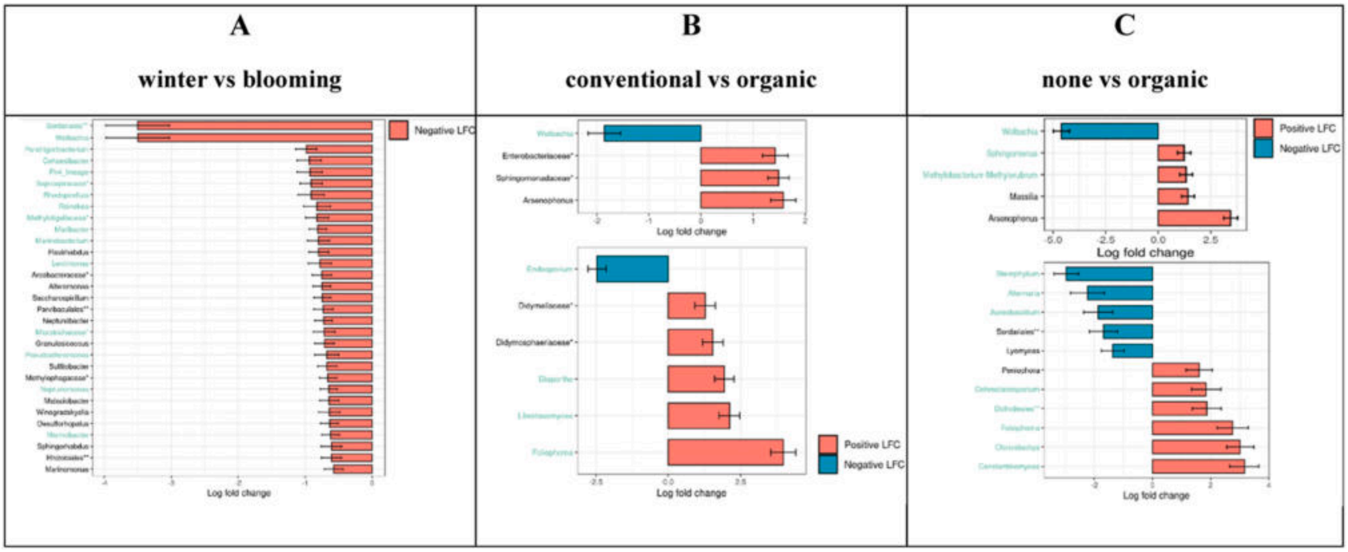

Fig. 7. Differential abundance analysis of microbial genera using ANCOM-BC2: A) phenological phases: winter versus blooming; B) type of management: conventional versus organic; C) type of management: none versus organic.

higher degree of differential abundances of endophytic taxa was observed in the groups of conventional management and absence of agricultural practices (Fig. 7B and Fig. 7C). The Enterobacteriaceae, Sphyngomonadaceae, Didymellaceae and Didymosphaeriaceae together with the genera *Arsenophonus*, *Diaporthe*, *Libertasomyces* and *Foliophoma* were significantly more abundant in olive trees in conventional farming compared to organic farming (Fig. 7B). Within the group of 'no agricultural interventions', corresponding to wild olive trees, the taxa *Sphingomonas*, *Methylobacterium*, *Methylorubrum*, *Massilia*, *Arsenophonus*, *Peniophora*, *Ochrocladosporium*, Dothideales, *Foliophoma*, *Clonostachis* and *Constantinomyces* increased significantly in relative abundance compared to organic cultivated varieties (Fig. 7C). In contrast, fewer taxa (six genera and one order) were significantly different for the organic management group, including *Wolbachia* which showed a higher differential abundance than both conventional and no management (Fig. 7B and Fig. 7C).

3.6. Correlation analysis of bacterial and fungal communities

The total of 31 bacterial genera and 38 fungal genera was found to be significantly different among samples. The bacterial genera that contributed most to bacterial communities' distinction in shoot samples (*Neptuniibacter*, *Neptunomonas*, *Malaciobacter*, *Winogradskyella*, *Maribacter*, *Desulforhopalus*, *Alteromonas* and *Saccharospirillum)* presented positive relationships among them and with other five taxa, *i.e.* Arcobacteraceae, *Desulforhopalus*, *Granulosicoccus*, Methylophagaceae and *Parvibaculales*. The genus *Arsenophus* correlated positively only with the candidatus genus *Carsonella*, while the two genera *Wolbachia* and *Candidatus* Uzinura negatively related especially to all the most recovered genera (Supplementary Figure S7A).

Differently, the two most represented fungal genera, such as *Fungi_gen_Incertae_sedis* and *Ochrocladosporium*, showed a low positive correlation between themselves and respectively a negative or slightly positive correlation with the other more abundant genera. The main positive correlations were found among the genera *Alternaria*, *Aureobasidium* and *Comoclathris*, among *Aspergillus*, *Saccharomyces* and *Malassezia*, between *Endosporium* and *Tricharina* and among *Arthrocatena* and *Catenulomyces*, *Celerioriella*, *Furfurella* and *Myriangiales_gen_Incertae_sedis*. Moreover, the strongest negative interactions were found between the most frequently genera *Fungi_gen_Incertae_sedis* and *Ochrocladosporium* with *Constantinomyces* and *Alternaria*, respectively (Supplementary Figure S7B).

3.7. Interactions between bacterial and fungal endophytes communities

To elucidate the relationship between bacteria and fungi, a network analysis was implemented using only statistically significant correlations. Bacterial and fungal communities showed positive correlations more frequently than negative ones (Fig. 8 and Supplementary Table S2).

Correlations were identified between 36 ASVs, 17 from the 16S rRNA dataset and 19 from the ITS dataset. The strongest positive correlations were observed between fungal ASV4442, assigned to *Neosetophoma rosarum*, and bacterial ASVs2274 and ASVs3077, assigned to *Pseudomonas* sp. and *Kineococcus mangrovi*, respectively. *Neosetophoma rosarum* also showed positive associations with *Comamonadaceae* and *Massilia*, as well as the bacteria *Pseudomonas* sp. and *Kineococcus mangrove* with the fungal genus *Endoconidioma* (ASVs4607). In the case of the four major negative correlations, five bacterial and five fungal ASVs were involved. Bacterial ASV1474, corresponding to *Sphingomonas* sp., showed negative correlations with two fungal ASVs assigned to *Pseudocercospora* (ASV4502) and *Biscogniauxia rosacearum* (ASV4406). The latter also had a negative correlation with the prokaryote *Massilia* (ASV0449). Additionally, the genus *Rickettsiella* (ASV2357) and the family *Rhodobacteraceae* (ASV3242) were negatively correlated with the fungi *Furfurella luteostiolata* (ASV3873) and *Neosetophoma rosarum* (ASV4442), respectively.

3.8. Putative functional categories of endophytic bacteria

The most significant functional categories at the minimal LDA score varied among olive hosts, phenological phases and managements (Supplementary Figure S8). Among specific functional categories of olive hosts, cellular community-prokaryotes, translation, signal transduction, replication and repair, nucleotide, carbohydrate and lipid metabolism had the highest LDA scores (Supplementary Figure S8A). The cellular community-prokaryotes and signal transduction categories were associated with winter, showing greater scores, followed by xenobiotics biodegradation and metabolism (Supplementary Figure S8B); while translation, replication and repair were the most representative functionalities of fruit ripening (Supplementary Figure S8C). Translation, membrane transport and signal transduction had also high LDA scores in organic, conventional and no management, respectively. The relative abundance of the putative functional categories varied slightly with the experimental factors (Supplementary Figure S9). The most abundant putative functional categories belonged to the "metabolism" module (global and overview maps, carbohydrate metabolism, amino acid metabolism, energy metabolism, metabolism of cofactors and vitamins, lipid metabolism, nucleotide metabolism), which accounted for at least 75 % in each host, phase and management. Phenological phases and types of management exhibited positive correlations with methylotrophy, methanol oxidation, sulfate and sulfite respiration, dark sulfite oxidation, methanotrophy, nitrification and aerobic ammonia oxidation (Supplementary Figure S10). Among ecological functions, sulfate respiration showed a greater relative abundance in the endosphere microbiota of olive twigs during the winter with significative differences respect to blooming, as well as for dark sulfite oxidation and sulfite respiration functions compared to all other phenological phases (Supplementary Figure S10). Sulfate respiration was also detected as biochemical function of wild olive trees endophytes, grew up without any form of agriculture, differing significantly from olive trees farmed under conventional management (Supplementary Figure S10).

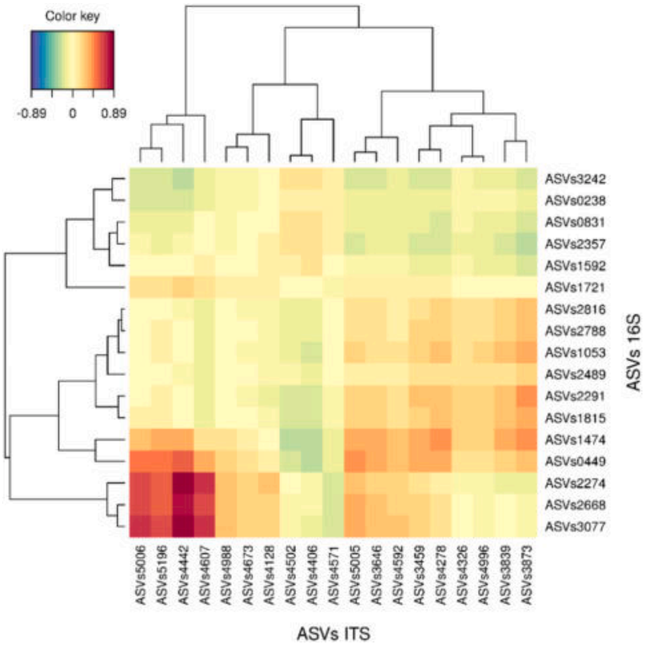

Fig. 8. Analysis of correlations between bacterial ASVs and fungal communities (green ovals) obtained from 16S rRNA gene and ITS metabarcoding analyses, respectively. Negative correlations are indicated in light blue, while positive correlations are represented by orange and red shades. A detailed list of ASVs, along with their corresponding bacterial and fungal taxa, is provided in Supplementary Table S2.

3.9. Trophic mode of endophytic fungi

Fungal taxonomic functional analyses by FUNGuild classified the fungal sequences into different trophic modes. The most common trophic mode was saprotroph (50 % of identified taxa), followed by pathotroph (29 %) and symbiotroph (15 %). Among olive hosts, the saprotroph group showed a greater abundance in wild olive compared to olive cultivars (Supplementary Figure S11) with significative differences during the winter respect to fruit ripening phase, as well as for the pathotroph and symbiotroph groups (Supplementary Figure S12A). The main saprophytes were represented by wood rotting fungi, while plant and animal pathogens and endophytes composed the groups of pathotrophs and symbiotrophs, respectively. Among management systems, the highest relative abundance was recorded under organic management for all the three trophic categories (Supplementary Figure S12B). Most trophic modes did not differ between organic and conventional management except for the plant pathogen and epiphyte groups, with greater abundance in organic farming system (Supplementary Figure S12B). However, wood saprotroph, animal pathogen and symbiotroph endophytes were significantly influenced by the adoption of horticultural practices, showing less abundance under no horticultural management (Supplementary Figure S12B).

4. Discussion

4.1. Bacterial and fungal community analyses

This is the first study analyzing both endophytic bacterial and fungal communities in Sicilian olive cultivars and wild accessions. In our study we investigated the microbiota of olive twigs that was characterized by a lower presence of microbial targets. Our results are in line with other studies focused on hyper-arid soils, sediments, dry permafrost or filtered waters [43–45]. In this regard, bioinformatic tools for chimera detection, filtering and removal have been adopted in order to obtain accurate metabarcoding results. Phenological phase and horticultural management affected both microbial groups, while host type specifically impacted fungi. The dominant bacterial and fungal classes were Gamma-proteobacteria and Dothideomycetes, consistent with previous studies on olive phyllosphere endophytes in both cultivated [10,11,15,24] and wild plants [14].

4.2. Cultivars did not influence Sicilian olive tree endophytic communities

Previous studies on Iberian olive cultivars, which focused exclusively on either bacterial [13] or fungal [10] endophytic communities in the phyllosphere, reported that host genotype played a significant role in structuring microbiota composition. However, our study found no significant diversity among three Nocellara varieties, though clear differences were observed between cultivated and wild olives. This suggests that anthropogenic practices had a greater impact on shoot microbiota structure, similarly to results showed by Ali *et al.* [46] regarding the soil-borne parasite diversity. According to de Oliveira *et al.* [24], Sicilian olive varieties displayed similar microbial communities, supporting the concept of a putative core microbiota with regional specificity. The microbiota of wild olives lacked *Wolbachia* spp. and showed a reduced abundance of *Alternaria*, accompanied by an increase in *Ochrocladosporium*, a known antagonist of *Alternaria* [47]. This finding reinforces the idea that wild plants, which have not been subjected to selection for cultivation, are more resilient to biotic stresses and represent valuable reservoirs of potential biocontrol agents. To date, only two studies [14, 48] have compared bacterial communities in the roots and leaves of wild olive trees in Mediterranean countries (Spain, Portugal, and Greece) with those of cultivated olives from the same regions. While Aranda *et al.* [48] suggested that wild olives serve as reservoirs of unique bacterial diversity, Müller *et al.* [14] reported that bacterial communities in wild and cultivated olives were closely related. Our beta-diversity analysis aligns with these observations, showing that Sicilian wild olive accessions formed distinct clusters, while still sharing certain taxonomic groups with cultivated varieties.

4.3. Phenological phases influenced Sicilian olive tree endophytic communities

Seasonality is known to influence the structure of bacterial and fungal communities in various Mediterranean ecosystems [5], tropical regions [49,50] and forest/woody plants [18,22,23,25,26,51,52]. This study is the first to explore the impact of winter on olive microbiota, revealing that bacterial diversity peaks during colder months, although in earlier studies bacterial alpha diversity increased during warmer months [25]. Fungal alpha diversity tended to decrease from spring (blooming) to autumn (fruit ripening), as previously reported [22,26], confirming that humid and rainy winters and springs favour fungal cycle. In line with previous studies, Gamma-proteobacteria dominated bacterial communities [13], while Dothideomycetes were the main fungal class [12,15,22]. Notably, 'Nocellara Etnea' and 'Nocellara Messinese' exhibited a high abundance of the genus *Wolbachia* (Alphaproteobacteria class) from May to October. The presence of bacteria *Wolbachia*, *Candidatus* Uzinura and *Candidatus* Cardinium, known arthropod endosymbionts [53,54], has been observed in olive trees for the first time, whereas the presence in plants as endophytes is less documented [55–57]. Previous studies have focused on *Wolbachia* in sap-feeding insects which may act as vectors for plant pathogens. Li *et al.* [58] demonstrated horizontal transmission of *Wolbachia* in the phloem-feeding whitefly, *Bemisia tabaci*, across different herbaceous plant species. *Candidatus* Uzinura occurred dominantly together with *Wolbachia* in the phyllosphere of *T. landbeckii* [56]. Our study confirms that *Wolbachia* and *Candidatus* Uzinura can reside in the plant as endophytes and suggests that their presence in olive trees could be linked to the associated insect community. A more in-depth analysis of the olive tree microbiome and its associated insects could further clarify the dynamics of these bacteria and their potential impact on plant physiology and pathogen transmission.

Across the phenological phases, the fungal genera *Alternaria*, *Aureobasidium*, *Quambalaria*, *Aspergillus* and *Cladosporium* prevailed, in accordance with previous findings on Mediterranean olive aboveground endosphere [12,15].

4.4. Horticultural management influenced Sicilian olive tree endophytic communities

Phyllosphere microbiota is shaped by environmental factors, including soil and surrounding vegetation [50]. Soil microbes that colonize root tissues can move to the phyllosphere via xylem [59]. Previous studies highlighted the impact of orchard management on microbial communities, showing that the use of organic fertilizers improved microbial biomass, richness, and diversity in both soil and olive rhizosphere [28,60]. Our findings align with bacterial and fungal diversity patterns observed in phyllosphere communities of other woody crops under organic and conventional agriculture [61,62]. In this case, there is a loss of biodiversity of the indigenous bacterial communities under organic management, and although the indigenous fungal communities are less influenced by the horticultural practices, what stands out is that both microbial compositions (bacterial and fungal) separate from the corresponding microbial communities of the wild olive. However, this study is the first to investigate how olive phyllosphere microbiota responds to horticultural management systems as well as in native forest. To compare the effects of organic and conventional agricultural management on the three studied Sicilian olive cultivars, fields with similar soil texture (clay loam and sandy loam) and pH categories were selected, as these factors are known to influence soil microbiota diversity and composition, particularly the fungal fraction [63]. According to the literature [64], higher concentrations of cations in soil solution are linked to greater fertility and increased nutrient availability for both plants and soil microorganisms. An analysis of the key physicochemical properties of soils across all olive orchards revealed that cation exchange capacity was high or moderately low, while electrical conductivity fell into three categories: negligible, strong, and very strong. These differences primarily reflect the geographical origin of the soils, rather than the type of agricultural management or cultivated olive variety. Further research is needed to explore the variations in alpha diversity observed between the two agricultural systems and to determine which environmental factors may have indirectly shaped the structure of the olive phyllosphere microbiota in Sicily.

4.5. Correlations and interactions among endophytes

The dominant bacterial communities included several marine-associated microorganisms, which, to date, have not been reported in Mediterranean or Brazilian olive cultivars [11,14,23,24,28]. The detection of halophilic genera such as *Neptuniibacter*, *Neptunomonas*, *Malaciobacter*, *Winogradskyella*, *Maribacter*, and *Granulosicoccus*—all showing positive correlations—suggests an adaptation to the naturally saline soils of Sicily [65–68]. Some of these genera are known for their PGP roles in algae and higher plants colonizing marine [69–74] or saline environments [75].

The detection of key fungal genera displaying positive correlations aligned with previous studies on the endophytic mycobiota of olive trees [76–79], xerophytic plants [80], and other plant species [42,81–83]. Notably, a negative correlation between *Ochrocladosporium* and *Alternaria* was observed, with the former increasing and the latter decreasing, particularly in wild olive samples. This supports earlier findings by Gomes *et al.* [84], who highlighted the potential beneficial role of *Ochrocladosporium*, a relatively understudied genus isolated from asymptomatic shoots of an olive knot-tolerant cultivar.

Metabarcoding analyses also uncovered rare or unidentified taxa resolved only to the genus level, limiting inferences about their ecological roles. This is particularly important, as species within the same genus, such as *Alternaria*, can exhibit different behaviors (saprophytic, opportunistic, or pathogenic). Tao *et al.* [47] demonstrated that pathogen invasion in plants can alter and reconstruct the existing fungal community structure, assigning suppressive activities to *Ochrocladosporium* against pathogenic *Alternaria* and confirming a negative interaction between these two genera. According to da Rocha *et al.* [85], *Ochrocladosporium* also possesses antibacterial and antioxidant properties, warranting further research to explore its antimicrobial and antagonistic potential.

In terms of community biodiversity and microbial interaction networks, co-occurring taxa were often linked by their shared capacity to tolerate harsh environments, while taxa exhibiting mutual exclusion typically included fungal pathogens and antimicrobial-producing bacteria. Microorganisms showing positive associations were predominantly isolated from arid, semi-arid, or aquatic ecosystems. For example, *Kineococcus* has been recovered from desert sands, radioactive sites, saline sediments, and plant roots, demonstrating growth at temperatures up to 32 °C and in NaCl concentrations of up to 10 %, with proven plant growth-promoting effects under drought conditions [86–88]. Likewise, *Massilia* species have been isolated from Antarctic and subtropical streams, oceans, and saline soils, where they promote plant growth, often in association with arbuscular mycorrhizal fungi [89,90]. Some *Massilia* strains also produce violacein, a secondary metabolite with antibacterial, antifungal, antiprotozoal, and anticancer properties [91,92] potentially explaining the negative interaction observed between *Massilia* and the phytopathogenic fungus *Biscogniauxia rosacearum* in this study.

Psychrophilic and psychrotolerant strains of *Pseudomonas* thrive in a wide range of habitats due to their physiological flexibility and trehalose synthesis able to protect them from salt stress and other abiotic stressors [93,94]. Also, the fungus *Neosetophoma* was isolated from phyllosphere of *Citrus reticulata* in southern Iran, an arid region with limited and saline groundwater resources [95,96]. Additionally, fungal taxa such as *Didymosphaeriaceae* and *Foliophoma*, which can act as saprobes or necrotizing pathogens, were identified in salt marsh ecosystems [97]. The presence of *Pseudocercospora* and *Biscogniauxia rosacearum* could be linked to the pathogenic characteristics of these taxa, potentially counteracted by the bacterial genus *Sphingomonas*, which is antagonistic to phytopathogenic fungi.

Pseudocercospora species occur across a wide climatic range, from desert to humid regions, causing leaf and fruit spots and blights on various host plants, including olive [77,98,99]. A recent survey in South African olive orchards identified *B. rosacearum* as a key pathogen associated with olive trunk disease, due to its high incidence and aggressiveness [100]. *Sphingomonas*, a widely distributed genus found in oligotrophic environments such as agricultural soils, polar regions, marine sediments, and plant tissues, has demonstrated antagonism against several phytopathogens, including *Verticillium dahliae*, the causal agent of Verticillium wilt in olives and other crops [101,102]. More recently, strong antagonistic effects have also been reported against *Alternaria alternata*, *Sclerotium rolfsii*, *Botrytis cinerea*, and various *Fusarium* species [101,103].

4.6. Putative functional categories of endophytic bacteria

This is the first study to explore the functional role of bacterial communities in the olive phyllosphere. Our findings could enhance the KEGG database, which currently lacks specific data on plant endophytes. Among olive twig endophytic communities, putative functional categories related to metabolism were the most represented. Notably, sulfate respiration emerged as a significant ecological function. Bacteria involved in natural sulfur cycle colonize various environments, including seawater, freshwater, industrial wastewater, and soils [104,105], in this study they are represented by genera such as *Desulforhopalus* and *Sulfitobacter*. These bacteria play a crucial role in the sulfur cycle, thereby influencing soil nutrient cycling and plant health. Their presence as olive endophytes may be linked to both agricultural treatments and natural soil conditions, particularly in volcanic soils and irrigation water. Additionally, sulfate respiration has been identified as a key biochemical function among the endophytes of wild olive trees, indicating that bacteria of the sulphur cycle contribute to sulfur metabolism even in unmanaged environments. Our results further reveal that sulfur bacteria activity is influenced by seasonal variations, with increased sulfate respiration observed in winter. This trend may reflect shifts in microbial community composition and metabolic processes occurring before the complete vegetative recovery of the olive trees.

4.7. Trophic mode of endophytic fungi

In recent years, interest in the functional roles of fungi and their ecological classifications has grown. However, only de Oliveira *et al.* [24] have explored the putative functional ecology of fungal communities in different *Olea europaea* cultivars. Their study, conducted in Brazilian olive orchards, found that the most common trophic mode among fungal endophytes in olive leaves was unclassified, followed by symbiotrophs, pathotrophs, and saprotrophs. In contrast, our study identified saprotrophs as the dominant group, likely due to the sampling of shoot endophytes rather than leaf tissues.

Wentzien *et al.* [28] assessed the effects of manure application on olive rhizosphere and root endosphere microbiota, reporting saprotrophic and plant-parasitic fungi as predominant in organic orchards. These findings are consistent with ours, which also revealed higher abundances of wood saprotrophs and pathogens in organic farming systems, including wood decomposers, and soil saprobes and soil saprophytes, possibly driven by degradation of the abundant organic matter in these systems. Further research is needed to deepen our understanding of the functional roles and classifications of endophytic fungi in olive trees.

5. Conclusion

The structure of Sicilian olive endophytic communities is primarily driven by phenological phases and farming systems. Bacterial diversity was influenced by the winter season, which was characterized by a more uniform distribution of the dominant microbial classes forming the phyllosphere microbiota. Organic management resulted in a lower bacterial species and phylogenetic diversity compared to both conventional system and wild habitat. Although the overall mycobiota structure appeared less influenced by host, phenological phase and farming system, a clearer pattern emerged when beta diversity was considered. Wild olive trees displayed a distinct fungal composition compared to cultivated groups, reflecting differences associated with both host type and cultivation practices. Additionally, beta diversity of olive mycobiota was shaped by the winter season, contributing to the structuring of fungal communities.

Analyses of potential interactions revealed that positive correlations among microbial taxa were more frequent than negative ones. Fungal communities were classified into functional guilds, with saprotrophs (mainly wood-rotting fungi) predominating, particularly in wild accessions, during winter and under organic management. Overall, our findings confirm that plant microbiota is dynamic and that the composition and plasticity of the microbiota are determined by complex plant–microbe and microbe-microbe interactions.

Author statement

Dalila Crucitti: Conceptualization, Methodology; Validation; Formal analysis; Investigation; Resources; Data Curation; Writing - Original Draft; Writing - Review & Editing; Supervision; Project administration, **Michele Sonnessa:** Methodology; Software; Validation; Formal analysis; Investigation; Data Curation; Writing - Review & Editing, **Francesco Carimi:** Conceptualization; Resources; Writing - Review & Editing, **Tiziano Caruso:** Conceptualization; Writing - Review & Editing, **Davide Pacifico:** Conceptualization; Methodology; Investigation; Resources; Data Curation; Writing - Review & Editing; Supervision; Project administration

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2025.100510.

Data availability

Project information is accessible with the following link http://www.ncbi.nlm.nih.gov/bioproject/1212469

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