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CHARACTERIZATION OF THE BIODIVERSITY OF SICILIAN OLIVE TREE MICROBIOTA AND EVALUATION OF ENDOPHYTES AS PLANT GROWTH PROMOTING AND POTENTIAL BIOCONTROL AGENTS

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Abstract

Plants are the primary producers in terrestrial ecosystems and coexist with a wide community of microorganisms that inhabit almost all plant organs, both above and below ground, both inside and outside their tissues. These microorganisms are involved in intricate networks of interactions that influence plant health and have a significant impact on ecosystem functions. While our understanding of plant microbiota continues to grow, we still lack the ability to fully control these communities, determine their complete ecological roles, or predict their responses to environmental changes. Despite the important cultural and economic role of the olive tree, the bacterial and fungal communities associated with olive trees have received less attention compared to the microbiota of other crops, especially in Sicily, a region renowned for its biodiversity and natural vulnerability to climate change. This thesis, conducted as part of a three-year PhD program, explores the endophytic communities hosted in Sicilian olive trees, emphasizing the crucial role of plant-associated microorganisms in both plant relationships and in maintaining olive tree health and disease control through a holistic and sustainable approach.

The experimental studies are organised into chapters as follows.

Chapter 1 highlights the significance of olive cultivation in Sicily, focusing on the adaptive strategies that olive trees have developed to cope with various abiotic stressors. Particular attention is given to the resilience of three traditional Sicilian cultivars—'Nocellara del Belice', 'Nocellara Etnea', and 'Nocellara Messinese'—with an emphasis on microbiota mediation and its mechanisms for enhancing plant growth and health. The first chapter also introduces the various pests and diseases that threaten olive trees, with a focus on pathogens affecting the vascular system, their impact on olive production, and potential integrated control methods.

Chapter 2 presents a culture-independent metabarcoding method for studying bacterial and fungal endophytes, offering an effective protocol for DNA extraction and purification—typically a limiting factor in studying wood-associated microbiota. Metabarcoding of plant endophytic communities is often hampered by the co-amplification of host DNA during polymerase chain reaction (PCR) of phylogenetic marker genes. This chapter introduces an innovative method involving the use of peptide nucleic acid clamps to prevent host DNA amplification, facilitating the efficient metabarcoding of endophytic bacterial communities in olive trees. The chapter also investigates phyllosphere microbial communities to provide

a comprehensive picture of Sicilian olive microbiota biodiversity and offers insights into endophyte assemblages based on statistical analyses of various factors influencing microbiota diversity and microbe-microbe interactions.

Olive microbiota hosted by twigs of the three different Sicilian olive cultivars and wild olive accessions were affected by phenological phases and farming systems both in diversity and composition. Bacterial and fungal communities showed positive correlations more frequently than negative ones. The strongest positive correlations were observed between fungus *Neosetophoma rosarum*, and bacteria *Pseudomonas* sp. and *Kineococcus mangrovi*. While *Sphingomonas* sp. Was involved in negative correlations with two fungi *Pseudocercospora* and *Biscogniauxia rosacearum*. Fungal communities were mainly categorized as saprotroph represented by wood rotting fungi, more abundantly found in wild accessions, during the winter and under organic management.

Chapter 3 outlines the sampling, isolation, and identification methods used to assess the endophytic microbiota of Sicilian olive cultivars and wild accessions from different phyllosphere tissues, employing a culturomic approach. This approach was used to identify the optimal conditions for establishing a functional microbial collection from leaves and twigs of three Nocellara cultivars and a wild olive population across four phenological stages (winter dormancy, flowering, fruit set, and fruit maturation) and two farming systems (organic and conventional). Additionally, alpha and beta diversity measures were inferred using statistical analyses to describe the structure, composition, and potential ecological roles of endophytic communities. The results revealed that a more extensive fungal consortium, compared to bacterial communities, characterizes the olive microbiota, with twigs hosting a greater diversity of microorganisms than leaves. Furthermore, during winter dormancy, a higher diversity in taxa and microbial composition was observed. Wild olives displayed greater species richness and distinct endophytic compositions. Differences in endophytic community structure between the two agricultural management systems were less pronounced. Culturable bacterial endophytes were characterized to identify strains with plant growth-promoting traits. This phase of the research was conducted at the Department of Microbiology and Parasitology at the University of Seville, under the supervision of Prof. Salvadora Navarro de la Torre. Functional characterization revealed that most of bacterial isolates produced siderophores, followed by nitrogen-fixing abilities, and indole-3-acetic acid (IAA) synthesis. *Bacillus* spp. dominated the enzymatic activities, including amylase,

protease, and lipase production. Additionally, top-performing endophytes along with a soil-borne fungus (*Trichoderma harzianum*), were tested *in vitro* for antifungal activity against major olive vascular pathogens, including *Verticillium dahliae*, *Neofusicoccum parvum*, and *Neofusicoccum vitifusiforme*. *Bacillus licheniformis* displayed a relevant antifungal activity against the olive fungal pathogen *Neofusicoccum vitifusiforme.*

Chapter 4 consolidates current insights into the diversity, composition, and ecological determinants of phyllosphere-associated endophytes in olives, with particular attention to bacterial and fungal taxa. Emphasis was placed on host-related factors—such as genotype, organ specificity, developmental stage, and plant age—that modulate microbiota composition as well as geographic location, environmental conditions, and seasonal dynamics that exert significant influence on endophyte diversity and distribution. A further overview was given on the influence of agricultural management practices, as well as biotic and abiotic stressors, on the stability and functionality of the endophytic microbiota. Of particular interest were several cultivable microbial taxa—including *Bacillus*, *Paenibacillus*, *Pantoea*, *Aureobasidium*, and *Penicillium*—which showed antagonistic properties against key olive pathogens, highlighting their promise as biological control agents.

Finally, concluding reflections and future perspectives were provided, acknowledging that the findings of this thesis contribute to a better understanding of olive microbiota biodiversity, distribution, and function in relation to crop health. This research paves the way for developing targeted integrated approaches that, when combined with other sustainable cultivation systems, can be transferred to Sicilian olive farming.

1. CHAPTER 1

1.1 The resilience of olive cultivation

The olive tree (*Olea europaea* L.), one of the most significant crops in Mediterranean agriculture, has become a cultural and economic symbol of the Mediterranean basin. Among European countries, Italy ranks second only to Spain in terms of hectares cultivated with olive trees and annual olive production (*Crops and Livestock Products Rome*, 2023). *Olea europaea* subspecies *europaea* includes both the wild type (*Olea europaea* subsp. *europaea* var. *sylvestris*) and the cultivated type (*Olea europaea* subsp. *europaea* var. *europaea*). As a xerophyte, the olive tree is well-adapted to diverse and fluctuating environments, earning a reputation as a resilient species capable of withstanding various environmental stresses such as heat, drought, salinity, and high levels of UV-B radiation (Brito *et al.*, 2019; Regni *et al.*, 2019; Silva *et al.*, 2018). Olive trees employ several strategies to cope with these stressors, including morphological changes and the activation of physiological and biochemical mechanisms (Dias *et al.*, 2022). Exposure to high temperatures and UV-B radiation, which leads to a reduction in leaf relative water content and a significant increase in cell membrane permeability, is positively correlated with elevated levels of proline and total anthocyanins. These compounds help maintain cell turgor and control membrane damage (Silva *et al.*, 2018). Water deficits in plants occur either due to insufficient water uptake to compensate for evapotranspiration losses or due to conditions that hinder water absorption, such as saline soils, low temperatures, or flooding (Brito *et al.*, 2019). To manage drought conditions and minimize water loss, olive trees possess well-adapted organs, including roots with plastic behavior suited to various soil conditions, cork-coated stems, and adaxial leaf epidermis covered with a waxy cuticle (Fernández, 2014). They also tightly regulate stomatal aperture (Nteve *et al.*, 2024), maintaining leaf water potential within physiological limits to prevent xylem cavitation and preserve xylem function. Root and leaf hydraulic efficiency, along with biochemical signals like abscisic acid (ABA) affecting guard cells, play a crucial role in controlling stomatal closure, ensuring both direct and indirect regulation of stomatal activity (Torres-Ruiz *et al.*, 2015). Olive trees subjected to salinity stress undergo physiological and biochemical changes. A marked reduction in leaf net photosynthesis and CO2 assimilation rates, coupled with increased catalytic activity, results in an altered redox state. This is accompanied by an increase in reactive oxygen species (ROS)-scavenging enzymes, such as

catalase and glutathione reductase, as part of the tree's physiological response (Regni *et al.*, 2019). Additionally, the regulation of salt translocation from roots to shoots further supports the tree's ability to manage salinity stress (Chartzoulakis, 2005).

Crossroads of peoples and cultures, Sicily boasts a rich heritage of olive germplasm, with 25 recognized cultivars (Marchese *et al.*, 2023). Three native cultivars used for both oil and table olive production in Sicily are 'Nocellara del Belice', primarily from the provinces of Trapani and Agrigento; 'Nocellara Etnea', from the central and eastern regions of Sicily (provinces of Enna, Catania, Messina, Syracuse, and Ragusa); and 'Nocellara Messinese', which is less well-known and less widely cultivated, found mainly in the olive orchards of the Messina, Catania, and Syracuse districts. Although 'Nocellara del Belice' trees are not particularly vigorous, they have a notable ability to adapt to a variety of environmental conditions. However, they are more sensitive to drought compared to other cultivars—an issue linked to the dense foliage and the thick mesocarp, which requires large amounts of water to maintain cell turgidity (Scollo *et al.*, 2018). 'Nocellara Etnea', a native olive cultivar that grows in volcanic soils, is highly resistant to wind, cold, and drought, and thrives even at high altitudes, up to 1,000 meters above sea level (https://cutrera.com/fr/olio/monocultivar-nocellara-etnea-igp-olio-extravergine-di-oliva-18.html; https://www.oliodellasicilia.com/cultivar/nocellara-etnea). Less well-known than the previous two, 'Nocellara Messinese' is also less widespread, likely due to its poor resistance to low temperatures (which discourages its cultivation in hilly or mountainous areas) and its sensitivity to water scarcity (https://tholos-alcantara.com/la-nocellara-dell-etna-e-le-sue-cugine/; https://www.polliceverdesas.com/quel-che-devi-sapere-sull-ulivo-lalbero-sacro-del-mediterraneo/).

1.2 Plant microbiota and influencing factors

Plants are considered holobiont systems that coexist with associated microbes (Sandrini *et al.*, 2022), which can influence plant growth and health through beneficial, neutral, or detrimental interactions (Cesaro *et al.*, 2021; Müller *et al.*, 2016). Microbes can colonize either the external (epiphytes) or internal (endophytes) tissues of plants, in both above-ground (phyllosphere) and below-ground (rhizosphere) compartments (Gross, 2022). The host-associated microbial community, known as the microbiota, includes bacteria, fungi, oomycetes, algae, protozoa, nematodes, and viruses. Plant microbiota likely coevolves with

their hosts (Mesny *et al.*, 2023); some microbes are transmitted through seeds or clonal propagation, others are recruited from the environment, while some establish stable associations with their hosts regardless of external conditions (Compant *et al.*, 2019). Several biotic and abiotic factors, including soil type, climate, farming practices, and plant domestication, can shape the composition of the microbiota (Dastogeer *et al.*, 2020; Müller *et al.*, 2016; Pacifico *et al.*, 2019), and host selection can significantly influence microbial communities (Mesny *et al.*, 2023) (Figure 1). Research has shown that variations in soil types and composition correlate with differences in rhizosphere microbial communities, and that soil pH affects the taxonomic abundance of root microbiota (Chialva *et al.*, 2022; Lauber *et al.*, 2009; Peiffer *et al.*, 2013; Zarraonaindia *et al.*, 2015). Recent studies have also demonstrated the impact of climatic variables on plant and soil microbiomes, showing that the abundance of bacteria and fungi increases with higher annual precipitation levels (de Vries *et al.*, 2012). Agricultural activities can either reduce the interaction between soil microorganisms and below-ground plant parts, or, in some cases, have no impact on rhizosphere community composition, which may instead be influenced by plant species (Estendorfer *et al.*, 2017; Gottel *et al.*, 2011). The identity of a plant's microbiota is closely linked to the identity of the host plant. Plants of different species growing in the same location harbour distinct microorganisms (Aleklett *et al.*, 2015; Samad *et al.*, 2017), and the variation in microbiota composition is more pronounced in plants that are not closely related phylogenetically (Bouffaud *et al.*, 2014). Additionally, host plants specifically select endophytes based on host identity, rather than the plant organ or location (Dastogeer *et al.*, 2018; Manter *et al.*, 2010). There is substantial evidence showing that plant genotypes and cultivars play a key role in shaping microbiota structure (Bulgarelli *et al.*, 2015; Fitzpatrick *et al.*, 2017; Inceoğlu *et al.*, 2011). More specifically, domestication (Bulgarelli *et al.*, 2015) and genetic variability between and within species contribute to the microbial composition of plant organs (Horton *et al.*, 2014; Mina *et al.*, 2020; Wagner *et al.*, 2016). The ability of each microorganism to colonize plant tissues, as well as its adaptation to external conditions in outer tissues and variations in internal tissue states, determines plant microbiota diversity. Furthermore, factors such as plant age, developmental stage, health, fitness, and plant-derived antimicrobial compounds, along with microbial factors like microbe-microbe interactions, can influence microbiota assembly, structure, function, and performance (Santoyo, 2022).

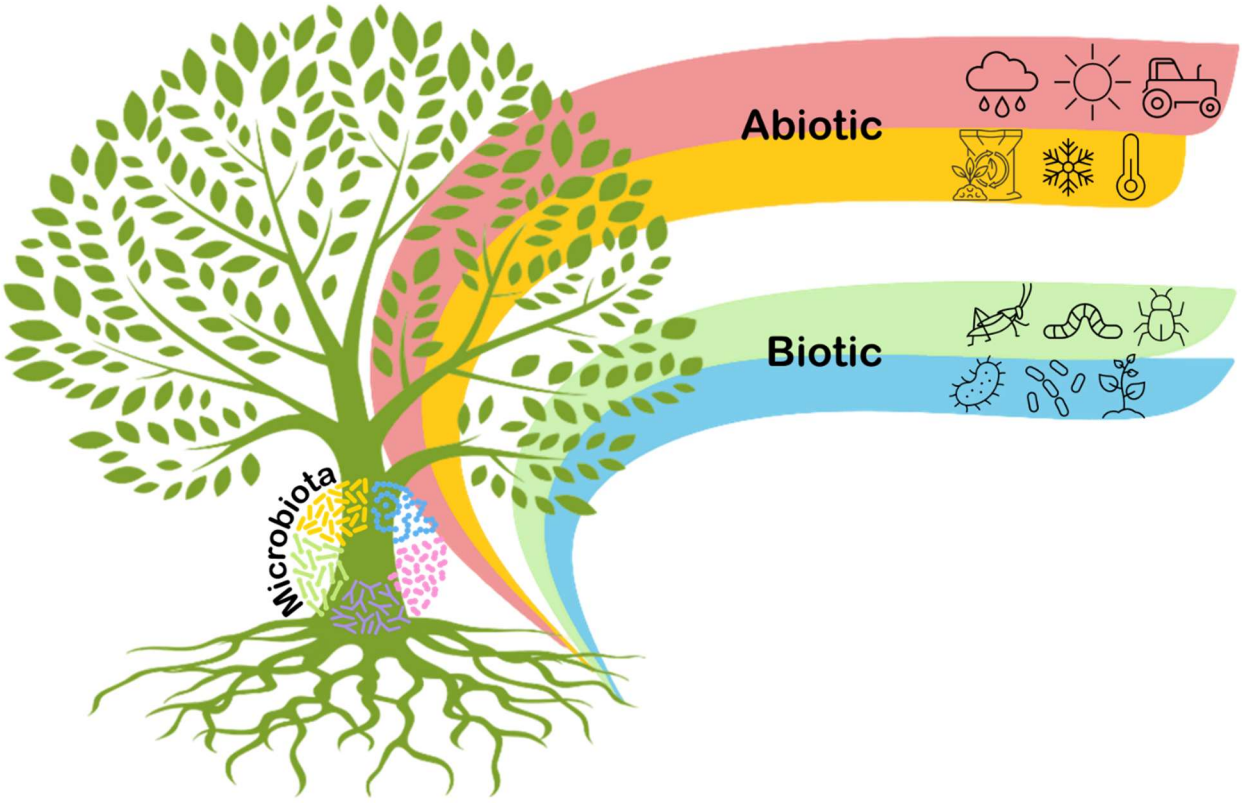

Figure 1 Simple illustration of the main driver factors that influence the whole plant microbiota. The host, microbial species, and environmental factors are all interrelated and together shape the microbiota community structure.

1.2.1 Crop endosphere: an account

The plant endospheric microbiota comprises specific microbial communities, including plant root-arbuscular mycorrhizal fungi and other endophytic fungi and bacteria that coexist, interact with their host, and possibly promote its growth. The presence of stable environmental conditions and nutrient-rich plant compartments affects the diversity and identity of the endophytic community as compared to the outside microbial communities and among above-and below-ground inner tissues (Harrison & Griffin, 2020; Kandasamy & Kathirvel, 2023). Previous studies explored the relationship between host species or cultivars and their bacterial endophyte communities. Manter *et al.* (Manter *et al.*, 2010) analysed the composition of endophytic bacteria across twenty potato cultivars/clones, revealing variations in both genera and abundance. Similarly, research on the transgenic *Bacillus thuringiensis* (Bt) maize indicated that its bacterial community composition was similar to that of non-transgenic maize, although microbial density varied between the two maize

genotypes during the reproductive stage, likely due to plant age and soil type (Lopes *et al.*, 2018; Mashiane *et al.*, 2018; Saxena *et al.*, 2002). Moreover, Mina *et al.* (Mina *et al.*, 2020) compared endophytic bacteria isolated from leaves and twigs of two Portuguese olive tree cultivars and confirmed that host genotype at the cultivar level affects bacterial community structure.

Studies have also examined how environmental and plant factors shape fungal communities. Significant variation in the endophytic mycobiota of wild Nicotiana plants was observed, with higher α-diversity in root tissues compared to stems and leaves, depending on the genotype and tissue origin (Dastogeer *et al.*, 2018). Research on endophytic phyllosphere fungal communities in Iberian *Olea europaea* L. showed that abiotic factors, particularly the spring season and rainfall, primarily drive fungal spore dispersion and olive tree endophyte colonization (Gomes *et al.*, 2018). Similarly, the colonization frequency, diversity, and composition of fungal endophytes in *Citrus reticulata* were affected by season, location, and tissue type (Sadeghi *et al.*, 2019). According to Chen *et al.* (Chen *et al.*, 2020), cultured endophytic fungi colonization of stems, roots and leaves of three cruciferous species, changes depending on season, plant identity and plant tissue, while species richness is influenced by season and tissue. While the beneficial interactions between plants and phyllosphere endophytes are well accepted by the scientific community, there remains a need for more studies to evaluate the factors that influence the composition of endophytic communities, both bacterial and fungal, in Mediterranean woody crop plants (de Oliveira *et al.*, 2022; Kakagianni *et al.*, 2023; Wentzien *et al.*, 2023).

1.3 Culture-dependent and Culture-independent techniques

Microbes are ubiquitous and colonize varied natural environments enough to dominate the biosphere. The microbial community composition and its changes in the space along the time has always been the subject of ecological analyses. In general, the study of microbial diversity means to measure the number of different species (richness) and their relative abundance (evenness) in a particular habitat, determining the alpha-diversity, and to analyse the similarity or dissimilarity among several ecosystems by beta-diversity (Kers & Saccenti, 2022). Understanding the diversity of microorganisms that inhabit different environments and carry out important ecological processes is important both to know the ecosystem functioning and from the prospect of ecological role of microbes involved in the ecosystem

stability and resilience. Culturing of microorganisms is indispensable to increase knowledge on physical, biochemical and functional characteristics of an organism. Culture-dependent method mainly comprises plate count techniques, visualization of colony features in cultured petri plate (shape, pigment production, colony form, surface and opacity, smell), microscopic and staining techniques and community-level physiological profiles (CLPP) (for example, BIOLOG approach, enzymatic activities, biochemical properties, etc.). Identification of microorganisms involves the amplification of target genes using extracted genomic DNA as template followed by sequencing and comparison of obtained sequences with reference sequences in public database to assign the most relative taxonomic group (Figure 2).

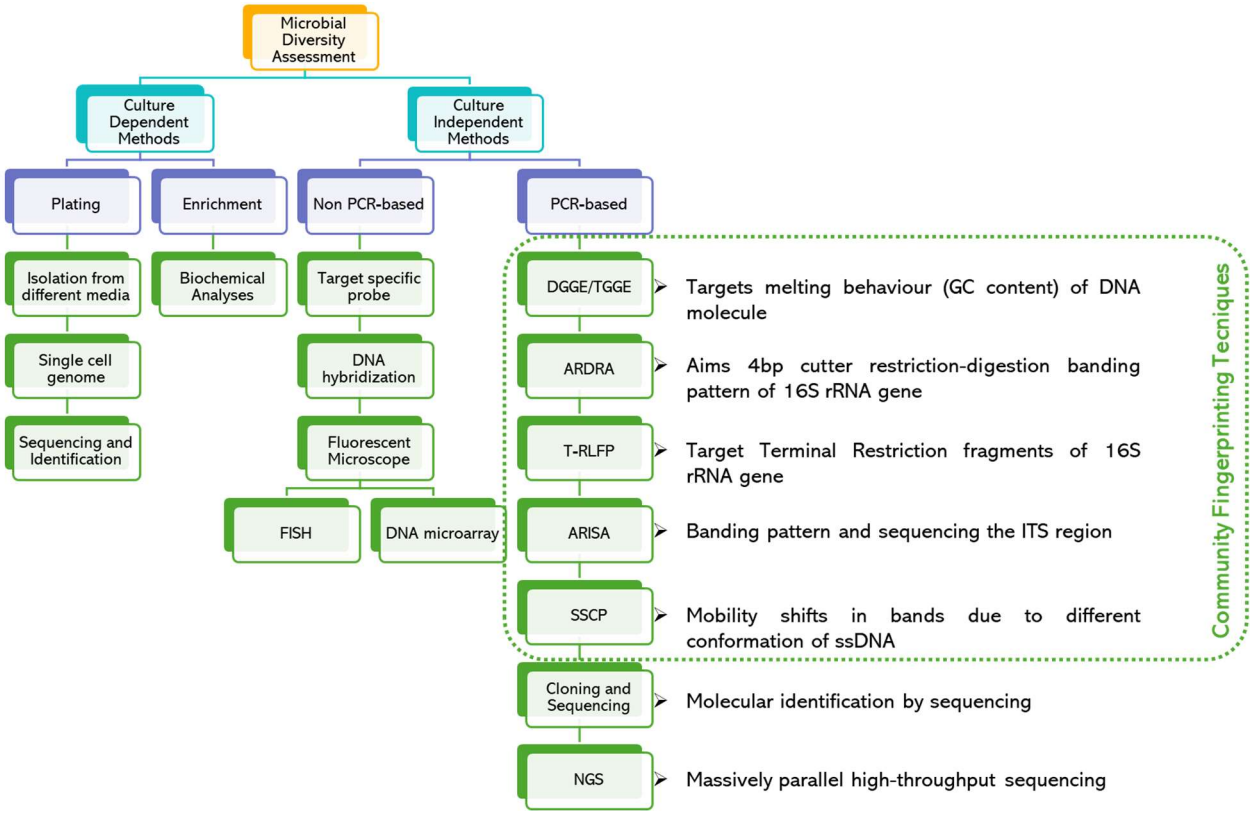

Figure 2 Overview of culture-dependent and culture-independent methods used to characterize microbial diversity in the environment.

By culturing, collections of microorganisms are obtained and information on microbial genetics, physiology and evolution can be provided. However, according to enigma of "great plate count anomaly" (Figure 3), agar plate and total cell count do not allow to analyse whole microbial communities (Rajeev *et al.*, 2021).

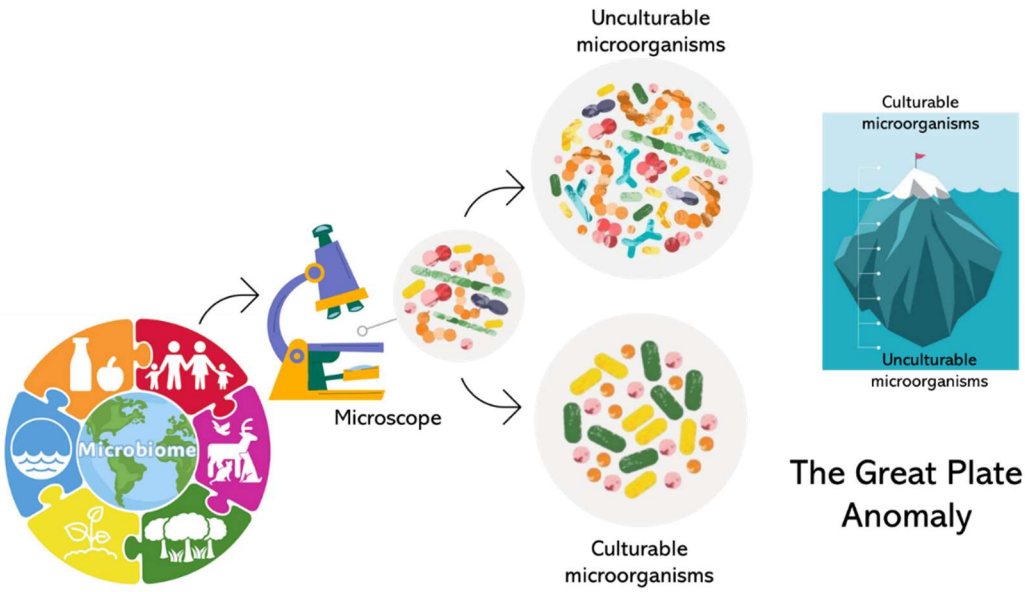

Figure 3 Great Plate Count Anomaly: observed discrepancy between technique of microscopic visualization, plating methods and culture-independent approaches of microbial diversity detection in samples of various habitats. Metagenomic analyses provide the highest diversity followed by microscopy and plate isolation.

Considering that in any environment, only 1% of the total microbial community is cultivable, the importance of culture-independent methods is increased precisely to bridge the discrepancy between cultivated and real diversity. The use of advanced molecular approaches, such as TGGE (thermal gradient gel electrophoresis), DGGE (denaturing gradient gel electrophoresis), SSCP (single-strand conformation polymorphism), RFLP (restriction fragment length polymorphism), TRFLP (terminal restriction fragment length polymorphism), ARISA (automated ribosomal intergenic spacer analysis), ARDRA (amplified ribosomal DNA restriction analysis), pyrosequencing, and Illumina MiSeq sequencing (Figure 2), promotes the culture-independent techniques to more performing methods capable of discovering the hidden diversity by analysing the metagenome (genome of total microbiota) directly from the environmental source (Rungjindamai & Jones, 2024). The combination of advanced molecular instruments and computational software led to next-generation studies (NGS) that detect more diverse groups of taxa of both culturable and unculturable microflora within environmental samples without requiring axenic cultures. To explore microbiomes and predict phylogenetic relationships, molecular markers such as

prokaryotic 16S rDNA, eukaryotic 18S rDNA and internal transcribed spacer (ITS) region are investigated. When investigating microbial communities associated with a eukaryotic host, universal primers in metabarcoding analysis amplify sequences that can originate from the host's genome. To overcome this "contamination", peptide nucleic acid (PNA) PCR clamps can be introduced during the library preparation. The PNA PCR clamps are synthetic nucleic acids linked to a peptide backbone that bind specifically to a complementary contaminant sequence and physically block its amplification (Fitzpatrick *et al.*, 2017; Lundberg *et al.*, 2013). In this way, by using PNAs or a combination of PNAs that block more types of host contaminant sequences in the same reaction, the yield of sequences of interest will be increased. Despite this, metagenomic analyses allow to identify most of microbial isolates less specifically by assigning the obtained Amplicon Sequence Variants (ASVs) only to order, family or genus level.

However, if on the one hand the culture-independent approaches identify the predominant unculturable microflora, on the other the lack of pure cultures of microorganisms does not allow their use in industrial applications nor the study of their ecological functions. Therefore, both methods are complementary to provide comprehensive knowledge of microbial diversity.

1.4 Plant growth promoting microorganisms (PGPMs)

In recent decades, extensive research has explored the intricate relationships between organisms and their associated microbial communities. Plants are considered holobionts due to their interactions with a diverse range of collaborating and competing microorganisms (Naamala & Smith, 2020). These interactions can occur in various plant compartments, including the phyllosphere, endosphere, and rhizosphere, where microbes can be pathogenic, neutral, or beneficial (Sánchez-Cañizares *et al.*, 2017).

Microbes that are essential to plant health are known as plant growth-promoting microorganisms (PGPMs). These microbes have been shown to enhance plant growth under both stressed and non-stressed conditions. PGPMs promote plant growth through both direct and indirect mechanisms (Elnahal *et al.*, 2022) (Figure 4). Direct mechanisms include nitrogen fixation, production of phytohormones (such as indole acetic acid, ethylene, cytokinin, and gibberellin), phosphate solubilization, and iron sequestration. Indirectly, PGPMs can act as biological control agents by reducing biotic stresses through mechanisms

such as the synthesis of lytic enzymes (e.g., chitinases, cellulases, 1,3-glucanases, proteases, and lipases), production of antibiotic molecules, siderophores, and cyanide, competition for nutrients, and stimulation of stress-related gene expression (El-Saadony *et al.*, 2022).

Main Direct Mechanisms Mediated by PGPMs with Beneficial Effects on Host Plants

In nature, nitrogen (N_2) is typically inert, but it can be fixed into usable forms such as ammonia (NH_3) through lightning or UV rays. Plants use nitrogen compounds like ammonia, nitrates (NO_3^-), and nitrites (NO_2^-) for growth, including root promotion, shoot elongation, and biomass production (Marques *et al.*, 2010). Plants assimilate inorganic nitrogen forms through processes such as anoxic nitrogen fixation (conversion of N_2 into NH_3) and ammonia/nitrite oxidation (NH_3 is oxidized into NO_2^- and then into NO_3^-) by nitrogen-fixing and nitrifying bacteria. Over 90% of nitrogenous compounds are synthesized by soil microorganisms. Two types of nitrogen-fixing microorganisms are recognized: free-living (non-symbiotic) bacteria, such as cyanobacteria (*Anabaena*, *Nostoc*) and genera like *Azotobacter*, *Beijerinckia*, and *Clostridium*; and mutualistic (symbiotic) bacteria, such as *Rhizobium* (associated with leguminous plants) and various *Azospirillum* species (associated with cereal grasses) (Britannica, https://www.britannica.com/science/nitrogen-fixation).

Inorganic phosphorus (HPO_4^{2-} and $H_2PO_4^-$) is a crucial macronutrient for plant growth and development. Phosphorus in soil exists in two forms: organic (about 30-65%) and inorganic, 35-70% (Ducousso-Détrez *et al.*, 2022). The inorganic fraction that is "plant-available" is made accessible through solubilization by phosphate-solubilizing bacteria (e.g., *Pseudomonas*, *Bacillus*, *Micrococcus*) and fungi (e.g., *Aspergillus*, *Fusarium*, *Penicillium*). Plants and microbes solubilize phosphate through two strategies: releasing organic acids to improve the mobility of inorganic phosphorus ions and secreting hydrolytic enzymes to unbind phosphate groups from organic matter (X. Liu *et al.*, 2022; Suleimanova *et al.*, 2023).

Sulphur (S) is vital for plant growth and crop production. In soil, sulphur exists in various inorganic and organic forms. Plants absorb sulphur after its conversion to sulphate (SO_4^{2-}) by sulphur-oxidizing bacteria (SOB), fungi, and actinomycetes (Joshi *et al.*, 2021). SOB are also crucial for oxidizing hydrogen sulphide (H_2S) into sulphate and hydrogen ions, as H_2S is toxic to both plants and humans (Yousef *et al.*, 2019).

Iron (Fe) is essential for plant growth, involved in processes such as DNA synthesis, energy transfer, respiration, and photosynthesis (Ahmed & Holmström, 2014; Ning *et al.*, 2023).

Plants absorb iron in its ferrous form (Fe^{2+}) through proton release mechanisms and the ferric form (Fe^{3+}) by reducing Fe^{3+} and binding it to cellular transport systems. In iron-deficient soils, plants secrete phytosiderophores to chelate and solubilize iron for uptake (Ahmed & Holmström, 2014). Certain microorganisms also produce siderophores to chelate ferric iron from the environment and transport it into the cell (Miethke & Marahiel, 2007). Over 500 siderophores have been identified, categorized into three groups based on their chemical structure and affinity for iron: catecholates, hydroxamates, and carboxylates (Himpsl & Mobley, 2019).

Potassium is crucial for plant protein and starch synthesis, enzyme activation, osmoregulation, and stomatal regulation (Almeida *et al.*, 2017). About 2% of potassium (K) is soluble, while the remaining 98% is in insoluble forms. Potassium-bearing minerals can be solubilized by soil potassium-solubilizing microorganisms (KSMs), which enhance crop productivity and nutrient availability. KSMs include *Aspergillus* spp., *Flavobacterium* spp., *B. pumilus*, *B. mucilaginosus*, *Rhizobium* spp., *B. edaphicus*, *Agrobacterium tumefaciens, B. circulans, B. subtilis*, and plant-growth-promoting fungi like *Trichoderma* spp. (El-Egami *et al.*, 2024; Kumar Meena *et al.*, 2015).

The auxin indole-3-acetic acid (IAA) is a key phytohormone involved in various plant physiological processes, including cell division, elongation, vascular differentiation, gravitropism, and phototropism. Numerous bacteria (e.g., *Bacillus, Enterobacter, Pseudomonas, Agrobacterium,* and *Rhizobium*) produce IAA, which promotes plant growth (Zhang *et al.*, 2021).

Main Indirect Mechanisms Mediated by PGPMs with Beneficial Effects on Host Plants

Plants produce ethylene, a volatile compound that regulates cellular processes and confers tolerance to abiotic stressors (Schaller, 2012). Under stress conditions, plants increase the synthesis of ethylene by upregulating ACC synthase and ACC oxidase. While ethylene is important for plant growth, excessive amounts can inhibit root growth and induce senescence (Saravanakumar & Samiyappan, 2007). Phytoremediation efficiency is therefore enhanced by PGPMs that suppress ethylene production through 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCD) activity (Orozco-Mosqueda *et al.*, 2020).

Cyanide is a volatile and toxic molecule that inhibits cellular respiration by blocking cytochrome oxidase. Many plants, including agriculturally important ones, release cyanide

into the soil (Martinez & Diaz, 2024). Several fungi (e.g., *Aspergillus* and *Fusarium*) and bacteria (e.g., *Bacillus*, *Pseudomonas*, *Klebsiella*, *Citrobacter*) produce hydrogen cyanide (HCN), which has antibiotic activity and promotes plant growth by antagonizing phytopathogens (Knowles, 1976; Ripa *et al.*, 2019).

Exocellular polysaccharides (EPSs) are long-chain polysaccharides secreted by bacteria and fungi. EPSs play roles in protection, surface attachment, hydrophilic biofilm formation, microbial aggregation, plant-microbe interactions, and bioremediation (Ajijah *et al.*, 2023).

Amylases are enzymes found in various organisms, including fungi, oomycetes, and bacteria. They catalyse the hydrolysis of starch into glucose and maltose sugars and are generally produced by pathogens to degrade plant tissues. Recent studies on plant growth-promoting microorganisms have shown that amylase production aids in seed germination by hydrolysing the endosperm, thus supporting root and shoot development. Amylase production is also linked to phytohormone production, such as gibberellins, which further enhances plant growth and mitigates pathogen effects (Carro & Menéndez, 2020).

Cellulases, enzymes that break down cellulose into monosaccharides or shorter polysaccharides and oligosaccharides, play a crucial role in the lifestyle of endophytic bacteria. They aid in colonizing plants by allowing bacteria to penetrate plant cell walls and spread throughout host tissues (Afzal *et al.*, 2017; Ma, 2017). These enzymes also facilitate the translocation of various compounds from outside into the plant apoplast and contribute to biocontrol activity through direct interactions with microbial pathogens (Carro & Menéndez, 2020). Chitinases are enzymes that hydrolyse the β-1,4-glycosidic bonds of chitin and other N-acetylglucosamine compounds. Produced by plants, viruses, bacteria, and fungi, chitinases are involved in nutrition, morphogenesis, pathogenicity, and symbiosis (Vaghela *et al.*, 2022). In plants, these enzymes are important for growth, development, systemic defence mechanisms, and tolerance to biotic and abiotic stresses. Microbial chitinases from plant growth-promoting microorganisms (PGPMs) induce beneficial responses in plants, acting both as an energy source and as biocontrol agents to inhibit attacks from phytopathogens (Carro & Menéndez, 2020).

Lipases, enzymes that catalyse the hydrolysis of various lipid substrates, play a wide range of roles in plants, including germination, signalling processes, growth and development, lipid turnover, and stress responses (Matos & Pham-Thi, 2009). Many bacteria, yeasts, and fungi produce extracellular lipases. A specific type of microbial lipase, known as cutinase,

helps degrade cuticular polyesters in plants, allowing pathogens to penetrate the host (Carro & Menéndez, 2020). Studies have shown that endophytic bacteria strains with broad-spectrum antimicrobial activity produce lipases, which are closely linked to their plant-protective capabilities. Lipases contribute to plant defence by degrading fungal cell walls or membranes, as well as by stimulating the plant's immune system (Mohamad *et al.*, 2018).

Proteases, also known as peptidases or proteinases, are enzymes that hydrolyse peptide bonds in protein chains. In plants, proteases trigger programmed cell death in response to microbial invasion, activating defences in distant tissues (Carro & Menéndez, 2020). Biocontrol microorganisms in plants employ antagonistic mechanisms to produce pathogen-repelling compounds. Metabolites such as chitinases, β-1,3-glucanases, and proteases can lyse and parasitize antagonistic pathogens. Extracellular proteases control plant infections by digesting the protein components of pathogen cell walls (Illakkiam *et al.*, 2013).

Some bacteria and fungi closely associated with plant roots have been shown to exhibit extracellular DNase activity. Microbial DNases play important roles in soil functions, nutrient breakdown, and microbial survival, improving growth conditions for microbes (Kamino & Gulden, 2021; Uesugi *et al.*, 2024). In addition to their role in competitive antagonism, biofilm development, toxin production, and plant-microbe interactions, extracellular DNases are selectively influenced by crop plants (Ogawa *et al.*, 2022). DNase production has been reported in bacterial isolates from genera such as *Bacillus*, *Brevibacillus*, *Flavobacterium*, *Microbacterium,* *Pseudomonas*, *Serratia*, *Stenotrophomonas*, and *Streptomyces*.

PGPMs have developed several strategies to enhance plant disease resistance, including antibiosis (the production of inhibitory metabolites), competition for micronutrients like iron, mycoparasitism, and the production of hydrolytic enzymes. Additionally, they can produce volatile organic compounds (VOCs) that play a significant role in plant growth and trigger induced systemic resistance (ISR) and acquired systemic resistance (ASR) to pathogens in host plants (El-Saadony *et al.*, 2022).

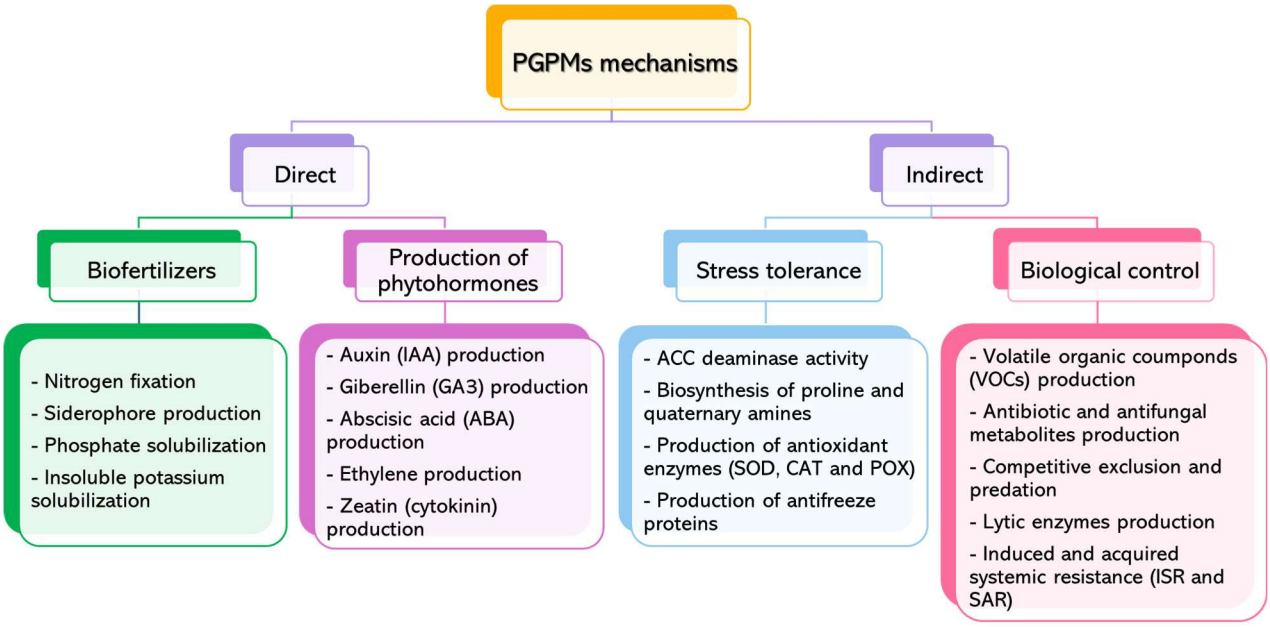

Figure 4 Direct and indirect mechanisms mediated by plant growth-promoting microorganisms (PGPMs) with beneficial effects on host plants (ACC, 1-aminocyclopropane-1-carboxylic acid; SOD, superoxide dismutase; CAT, catalase; and POX, peroxidase).

1.5 Plant diseases and main olive tree pathogens

Plant diseases can cause significant losses in horticulture, forestry, and agriculture by severely impacting crop productivity. These diseases occur when pathogens or adverse environmental factors disrupt the normal physiological processes of plants. The concept of the "plant disease triangle," introduced by Stevens (1960), is fundamental for understanding plant disease development. It requires three key conditions: a susceptible host plant, a virulent pathogen, and favorable environmental conditions. Changes in global climate patterns influence the distribution and impact of plant pathogens, often creating microenvironments that are optimal for pathogen proliferation (Roussin-Léveillée *et al.*, 2024). Increased trade, climate change, and evolving farming practices have facilitated the introduction, reemergence, spread, and establishment of pests and diseases in various crops. Olive trees are particularly susceptible to a variety of pests, insects, nematodes, and weeds, whose prevalence has increased in recent years, significantly affecting olive production, yields, and the profitability of olive cultivation in certain European countries. The primary causes of olive tree diseases fall into three major categories: bacterial, fungal, and viral

pathogens; parasites (mainly phytophagous insects and vector animals); and nutrient deficiencies in the plant.

Among bacterial diseases, two are particularly notable: olive tree canker, caused by *Pseudomonas savastanoi* pv. *savastanoi*, which induces the formation of hyperplastic growths (tumorous galls or knots) on stems and branches (Košćak *et al.*, 2023); and Olive Quick Decline Syndrome (OQDS), caused by a systemic infection of *Xylella fastidiosa* (Beretta *et al.*, 2022). The primary fungal pathogens affecting olive trees include *Venturia oleaginea*, *Colletotrichum* spp., and *Verticillium dahliae*, which cause scab or peacock spot on leaves (Buonaurio *et al.*, 2023), anthracnose with drupe mummification (Moral *et al.*, 2021), and verticillium wilt of the foliage (Montes-Osuna & Mercado-Blanco, 2020), respectively.

Olive trees can also be affected by systemic pathogens such as viruses, which cause leaf and fruit deformations, leaf discoloration, chlorosis, and yellowing (Martelli, 2013). In some cases, viral infections lead to deformities in the woody cylinder, such as stem pitting and woody gall disease. Among the recognized seventeen olive viruses (ArMV, *nepovirus arabis*; CLRV, *nepovirus avii*; OLRSV, *nepovirus oleae*; SLRSV, *stralarivirus fragariae*; OVYaV, *olive vein yellowing associated virus*; OSLV, *olive semilatent virus*; OYMDaV, *olive yellow mottling and decline associated virus*; OLYaV, *olive leaf yellowing associated virus*; OLV-1, *alphanecrovirus oleae*; OLV-2, *olive latent virus 2*; OLV-3, *olive latent virus 3*; OMMV, *alphanecrovirus tessellati*; CMV, *cucumber mosaic virus*; TMV, *tobacco mosaic virus*; TNV-D, *betanecrovirus nicotianae*; OlVT, *olive virus T*; OEGC, *olea europaea geminivirus*), some are soil-borne (SLRSV, ArMV, TNV), others can be transmitted mechanically (TMV), by seed (CLRV, OLV-1), by aphids (CMV), nematodes (ArMV, SLRSV), or through mechanical inoculation and grafting (OLV-2, OLRSV, OlVT, OLYaV) (Çağlayan & Faggioli, 2024). All of these viruses can be easily spread through infected propagation material (Martelli, 2013; Mathioudakis *et al.*, 2020; Mounir & Afechtal, 2020).

The olive fruit fly (*Bactrocera oleae* or *Dacus oleae*) is a widespread parasite whose larvae feed exclusively on wild and cultivated olives, destroying the pulp and facilitating secondary infections by bacteria and fungi, which significantly reduce oil quality (Lantero *et al.*, 2023; Malheiro *et al.*, 2015). Another major pest in Europe is the olive moth (*Prays oleae*), with each of its three annual generations specializing in damaging different parts of the plant, including leaves, flowers, and drupes (Lantero *et al.*, 2023; Pascual *et al.*, 2022). Alongside

the fly and the moth, the black scale (*Saissetia oleae*) is considered one of the three main pests of olive trees. These sap-sucking insects attack leaves and twigs, causing reduced tree vigor and promoting secondary fungal infections through honeydew secretion, which leads to sooty mold formation, leaf fall, and twig dieback (Camacho & Chong, 2015; Tena *et al.*, 2007). The grey oval fruit scale insect (*Parlatoria oleae*) also parasitizes olive trees, infesting leaves, branches, and sometimes fruits, where visible markings or pitting can appear (Bubola *et al.*, 2014). Like all plants, olive trees require a variety of nutrients (macro and micronutrients), and deficiencies can negatively affect their growth and productivity. For instance, shortages of nitrogen, potassium, boron, calcium, phosphorus, and iron are often indicated by specific discolorations such as yellowing, browning, and chlorosis (de Souza *et al.*, 2019).

1.5.1 Plant Vascular Pathogens

Plant pests and pathogens can infect both above-ground parts of plants, such as leaves, stems, flowers, and fruits, as well as below-ground organs, like roots and tubers (Belli, 2011). Others have evolved to infect the plant's vascular system, which includes the xylem vessels (dead tracheary components that transport water and nutrients absorbed by the roots to the photosynthetic organs) and the phloem (living tissue responsible for moving organic products of photosynthesis). Most vascular pathogens preferentially colonize the xylem vessels. Although the xylem is nutrient-poor compared to the phloem, its low osmotic pressure facilitates easier access for invading organisms (Yadeta & Thomma, 2013). Several bacteria, fungi, and oomycetes are known to invade the xylem, causing vascular wilt diseases that can devastate entire crops. These diseases typically present acropetal symptoms, such as foliar epinasty, wilting, chlorosis, vascular browning, and tissue necrosis (Agrios, 2005). Depending on the pathogen species and host plant, symptoms may range from stunting to complete wilting and dieback. Most vascular wilt pathogens are soil-dwelling and reach the vascular system through the roots or root wounds; others exploit natural openings on leaves, while some use insect vectors or chewing insects to gain access to the plant's vascular system and proliferate (Kashyap *et al.*, 2021). Controlling vascular wilt diseases is notoriously difficult due to the lack of effective treatments, the persistence of quiescent pathogen structures in the soil, and the wide host range of these pathogens. Promising approaches to managing these diseases include biological control agents (Eljounaidi *et al.*, 2016), organic soil amendments, and the use of resistant plant genotypes (Díaz-Rueda *et al.*, 2021; Yadeta

& Thomma, 2013). However, further research is needed to better understand the molecular mechanisms underlying vascular wilt diseases and to develop new, more effective control strategies.

1.5.2 Verticillium Wilt of Olive (VWO)

Verticillium wilt of olive (VWO) is currently regarded as one of the most devastating diseases affecting olive trees, severely impacting both olive oil production and its commercial value, in addition to incurring economic costs due to tree mortality and reduced fruit yield (Montes-Osuna & Mercado-Blanco, 2020). Widely distributed around the world, VWO was first reported in Italy in 1946 (Ruggieri, 1946). According to Lo Giudice *et al.* (2010), typical symptoms associated with VWO were observed on olive trees in irrigated orchards in Sicily for the first time in 2008. The advent of modern management practices and mechanization in crop fields, driven by the need to meet growing consumer demand, has revolutionized olive cultivation systems, altering the traditional olive landscapes commonly found in Mediterranean regions. Low-density olive orchards with irregular rainfall have been replaced by high-density and super high-density systems with drip irrigation (Connor *et al.*, 2014). The limited number of cultivated olive varieties, the use of infected plant material, susceptible olive cultivars, and practices such as the application of fertilizers, fungicides, and irrigation with contaminated water can disrupt soil microbiota, increasing the incidence and severity of specific pests and soil-borne diseases (Fernández-González *et al.*, 2019; Mercado-Blanco *et al.*, 2018). Additionally, inadequate agronomic practices, the lack of measures to control the pathogen, and its persistence in the soil and on various hosts can exacerbate the spread of VWO, severely compromising olive production in many growing regions.

Causal agent

Verticillium wilt of olive (VWO) is caused by the hemibiotrophic soil-borne fungus *Verticillium dahliae* Kleb. (Klebahn, 1913). Belonging to the Ascomycota phylum, Pezizomycotina subdivision, Sordariomycetes class, and Plectosphaerellaceae family, *V. dahliae* is generally considered an asexually reproducing fungus, as the sexual cycle has not been reported (Liu *et al.*, 2021). This pathogen has a broad host range, affecting both annual and perennial dicotyledonous plants and causing significant economic losses in crops throughout tropical and temperate regions worldwide. The saprophytic *V. dahliae* can

survive in the soil for extended periods due to its ability to form microsclerotia, dark and condensed masses of mycelium that act as propagules, germinating under favorable conditions. Two virulence groups or pathotypes have been identified: highly virulent defoliating (D) and moderately virulent non-defoliating (ND) (Jiménez-Díaz *et al.*, 2012b; Rodríguez-Jurado, 1993). The D pathotype causes the death of both young and old olive trees, while the ND pathotype is less virulent, associated with wilting and sometimes leading to host death (Jiménez-Díaz *et al.*, 2012a; López-Escudero & Mercado-Blanco, 2011; Mercado-Blanco & López-Escudero, 2012).

Disease Cycle

The life cycle of *V. dahliae* consists of three main phases: the "dormant" phase, during which black, thick-walled resting structures known as microsclerotia form; the "parasitic" phase, when the pathogen colonizes the host vascular system; and the "saprophytic" phase, occurring in necrotic tissue or during plant senescence. Microsclerotia remain viable for a long time, more than 10-15 years in soil (Wilhelm, 1955) and germinate under favorable environmental conditions such as root exudates and the presence of soil nutrients, forming mycelium that invades plants through root tips or wounds. Once the root cortex is penetrated, the mycelium moves into the xylem vessels, where it forms hyaline conidiophores with phialides arranged in a verticillate pattern (from which the fungus gets its name). These conidia are transported to the aerial parts of the plant by transpiration (Tsror, 2011). After vessel colonization, sporulation occurs, initiating another infection cycle, fungal elimination, and dissemination (Fradin & Thomma, 2006). The pathogen produces pectolytic enzymes, which, along with plant-produced tyloses, occlude the vessels and block xylem flow, contributing to symptoms such as loss of turgidity, wilting, branch dieback, shortened internodes, defoliation, chlorosis, and leaf necrosis (López-Escudero & Mercado-Blanco, 2011).

Symptomatology

Olive trees can exhibit two distinct forms of VWO: the acute form, known as "apoplexy," and the chronic form, referred to as "slow decline." The acute form appears from late winter to early spring, with chlorotic leaves that turn light brown and curl inward. Rapid dieback of shoots and branches can lead to the death of the entire tree within a short period. A characteristic symptom in cross-sections of branches is punctate vascular browning. The

slow decline form is observed in spring and progresses slowly through early summer. Initial symptoms include mummified flowers, followed by dull green leaves, massive defoliation, and reddish-brown coloration with necrosis of affected branches and shoots (Tsror, 2011). Both symptom complexes can occur simultaneously on the same tree and affect it either partially or entirely.

Integrated Control Strategy for Verticillium Wilt of Olive

Modern olive cultivation systems and the inability of fungicides to act systemically in plants make controlling VWO a significant challenge. An integrated control strategy should combine pre-planting and post-planting measures, along with alternative methods such as using biological control agents (BCAs), organic amendments, and essential oils. Pre-planting measures include using certified, healthy plant material, resistant cultivars, and establishing new olive orchards in *V. dahliae*-free soils through solarization. Post-planting measures aim to prevent or reduce pathogen spread and disease progression. This includes using clean, disinfected tools and vehicles to avoid spreading the inoculum, implementing cultural practices such as burning host weeds and using antagonists to reduce inoculum density, and reducing irrigation and fertilization to lower infective inoculum levels (Jiménez-Díaz *et al.*, 2012a; López-Escudero & Mercado-Blanco, 2011; Tsror, 2011). Promising microorganisms can interact with olive trees, either directly or indirectly, by competing for nutrients, space, or by activating systemic resistance (Varo *et al.*, 2017). Several strains of *Pseudomonas* spp. (Triki *et al.*, 2011; Varo *et al.*, 2017), *Trichoderma* spp. (Carrero-Carrón *et al.*, 2016), and non-pathogenic isolates of *Fusarium oxysporum* (Mulero-Aparicio *et al.*, 2020; Varo *et al.*, 2017) have been identified as promising BCAs against Verticillium wilt in various crops, including olive trees. Composting produces stable organic amendments free of pathogens and capable of hosting antagonistic microorganisms, which protect plants either directly through chemical or biological toxicity or by improving nutritional conditions. Secondary plant metabolites (e.g., carvacrol and thymol) and essential oils, though less studied, offer sustainable alternatives to chemical products due to their mechanisms of action against plant pathogens: morphological alteration, cell wall lysis, germination inhibition (Antón-Domínguez *et al.*, 2024; Varo *et al.*, 2017).

1.5.3 Emerging complex olive wilt syndrome

In olive-producing countries, pests and diseases are major limiting factors. Beyond the well-known fungal and bacterial olive diseases, a complex wilting syndrome has recently affected several olive orchards. In various countries and continents, including Australia (Sergeeva *et al.*, 2009), California (Moral *et al.*, 2010; Úrbez-Torres *et al.*, 2013), Croatia (Ivić *et al.*, 2023), Spain (Moral *et al.*, 2010, 2017), and Uruguay (Hernández-Rodríguez *et al.*, 2022), decline syndromes have emerged, and a complex mycobiota has been identified as the causal agent of what is referred to as Branch and Twig Dieback (BTD) of olive trees (Moral *et al.*, 2010, 2017; Úrbez-Torres *et al.*, 2013). Fungal species from the Botryosphaeriaceae family, including *Neofusicoccum* spp., *Pleurostomophora richardsiae*, *Phaeoacremonium* spp., and *Hymenomycetes*, have been identified as emerging pathogens. Since 2014, following the OQDS outbreak in Veneto and Lombardy, and later in Apulia, Calabria, and Sardinia, a new wilting syndrome has been reported, characterized by general dieback, cankers, and twig wilting. By assessing the occurrence of potential pathogens, fungal isolates of *Botryosphaeria dothidea*, *Diplodia olivarum*, *Neofusicoccum mediterraneum*, *Neofusicoccum parvum*, *Neofusicoccum vitifusiforme*, *Phaeoacremonium aleophilum*, *Pleurostomophora richardsiae* (Brunetti *et al.*, 2022; Carlucci *et al.*, 2013; Lazzizera *et al.*, 2008; Linaldeddu *et al.*, 2023; Manca *et al.*, 2020; Manetti *et al.*, 2023) were more frequently identified. This syndrome strongly reduces olive tree productivity and vitality, oil quality and causes extensive losses over large areas (Slippers & Wingfield, 2007). Currently, few effective chemical products are available to reduce the crop damage, moreover the complexity of aetiology and the overlap of symptoms caused by different pathogens emphasize the need to develop new management strategies based on holistic approaches to protect one of the most iconic crops in Italy against this new emerging threat.

Causal agent

Most fungal pathogens responsible for the complex olive wilt syndrome belong to the *Botryosphaeriaceae* family, which includes filamentous fungi of the *Ascomycota* phylum. Currently, this family encompasses 22 genera and approximately 300 species, including major plant pathogens such as *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, *Neoscytalidium*, and *Macrophomina*. Some species also function as endophytes in healthy plants or as saprophytes on dead tissues (Aiello *et al.*, 2023).

Generally, *Botryosphaeriaceae* species are non-obligate parasites capable of infecting numerous angiosperms and gymnosperms, with a global distribution (Moral *et al.*, 2019). These fungal species exhibit variation in their conidia, which may be hyaline, narrow, wide, or colored, and their morphological traits, combined with phylogenetic analyses, are key to identifying *Botryosphaeriaceae* species affecting woody crops. Studies on *Botryosphaeria* dieback in grapevines have shown that these pathogens reside in the vascular tissues (phloem and xylem) of basal internodes of annual stems, blocking the xylem (Moret *et al.*, 2024). The pathogens are consistently found in the wood of infected plants but never in symptomatic leaves, which are nonetheless damaged by fungal exopolysaccharides, secreted proteins, and phytotoxins translocated from the woody tissues of the trunk to the leaves via the transpiration stream (Belair *et al.*, 2022; Bénard-Gellon *et al.*, 2015; Martos, 2008).

Disease cycle

Botryosphaeriaceae species exhibit two distinct phases: a dormant period during which they exist as endophytes within the plant, and a pathogenic phase when they become virulent and actively colonize the wood (Slippers & Wingfield, 2007). In the absence of stress, these fungi can enter healthy plants through lenticels, stomata, or other openings (e.g., wounds on leaves, branches, or stems), establishing endophytic infections. In some hosts, *Botryosphaeriaceae* fungi have been linked to seeds or propagation materials from nurseries, suggesting a potential role in the global spread of these pathogens (Aiello *et al.*, 2023). In woody hosts, endophytic infections are typically transmitted horizontally via rain-splashed conidia or water dispersal (e.g., sprinkler irrigation), with aerial dispersal of ascospores being less common. *Botryosphaeriaceae* fungi may also interact with insects and can infect plants through contaminated pruning tools and equipment (Moral *et al.*, 2019). Various stress conditions, including drought, physical damage (e.g., hail), biotic factors (pathogens, insects, snow), and abiotic stressors (elevation, soil type, temperature), are strongly linked to the pathogenic phase, which manifests in various disease symptoms. Upon reaching the host, the pathogens initiate pre-infection processes such as adhesion, spore germination, and host recognition (Sammonds *et al.*, 2016). They then penetrate the host tissues either mechanically via an appressorium or through the production of enzymes that degrade the cuticle and cell walls, facilitating colonization (Esteves *et al.*, 2014; Moral *et al.*, 2019).

Symptomatology

Initially attributed to *Verticillium* species, symptoms of olive tree decline include browning and shedding of leaves, wilting of apical shoots (giving the canopy a scorched appearance), dieback of twigs and branches, and brown streaks beneath the bark of the trunk, branches, and twigs. As the disease progresses, necrosis and cankers may develop. According to Brunetti *et al.* (2022), symptoms begin with red-bronze necrosis on the leaf blade, edge, or vein, sometimes accompanied by a chlorotic halo. As necrotic patches merge, leaves fold downwards and wilt. In affected branches, the vascular tissue is discolored, suggesting impaired water transport. Externally, the bark may appear reddish, with sunken and necrotic areas. Linaldeddu *et al.* (Linaldeddu *et al.*, 2023) reported that unripe olives drop during summer, with infected drupes displaying small sunken necrotic lesions that expand until causing fruit drop (carpoptosis). Depending on the fungal species, ripe olives may exhibit light brown soft rot, roughness, mummification, or reddish-brown rot, often remaining attached to the tree.

Integrated control strategy of the emerging complex olive wilt syndrome

The management of the complex olive wilt syndrome is challenging once introduced to a new area due to its potential to infect all plants of a given species. Large-scale chemical control (e.g., in forests or plantations) is extremely difficult, if not impossible, and may be impractical due to environmental concerns. In intensively managed orchards, removal or treatment of infected tree parts, combined with sanitation measures to reduce spore loads, can help mitigate the disease (Brown-Rytlewski & Mcmanus, 2000; Flowers *et al.*, 2001; Slippers & Wingfield, 2007). Effective disease control requires an integrated approach. Reducing tree density, improving canopy ventilation and sunlight exposure, pruning dead or infected tissues, and adopting irrigation practices that minimize wetting of the trunk or canopy can lower inoculum levels, infection risk, and symptom severity (Moral *et al.*, 2019). Additional research is needed to identify cultivars that are fully resistant or less attractive to olive insects, which play a key role in fungal dissemination and drupe infection. The use of synthetic fungicides (strobilurin and carboxamide premixes) must be complemented with biological control measures (yeasts, bacteria, plant-based substances) or chemo-physical approaches (Aiello *et al.*, 2023).

The emergence of complex olive wilt syndrome underscores the difficulties of controlling olive diseases in modern cultivation systems. A thorough understanding of soil health, pathogen biology, and cultivation practices is essential for developing effective management strategies. Integrated approaches that combine cultural, biological, and chemical methods, alongside continuous monitoring and research, are vital to mitigating the impact of Verticillium wilt of olive (VWO) and other emerging threats to olive production.

1.5.4 Olive Quick Decline Syndrome (OQDS)

The European olive industry has been severely impacted by the ongoing Olive Quick Decline Syndrome (OQDS) outbreak, caused by the bacterium *Xylella fastidiosa*, resulting in significant socio-economic and environmental damage (Beretta *et al.*, 2022). Currently, in the absence of effective control strategies, farmers are forced to uproot infected and neighboring olive trees and replant with more resistant cultivars to limit the spread of the pathogen and mitigate the damage. However, this approach has dramatically altered the olive-growing landscape, with centuries-old trees being removed, and has resulted in economic losses due to the cost of replanting, several years of lost production, and uncertainty about achieving previous high production levels. Until recently, *Xylella fastidiosa* was considered endemic to the Americas, where it is associated with economically significant diseases such as Pierce's disease in grapevines, citrus variegated chlorosis, and various other diseases affecting perennial crops and ornamental plants (Saponari *et al.*, 2017). Global trade networks and transportation infrastructure have contributed to the spread of invasive species, and the OQDS outbreak exemplifies how the unintentional introduction of a pathogen into a new, non-competitive environment can cause an ecological and environmental disaster (Bajocco *et al.*, 2023). The first cases of olive decline in the Ionian coastal region of the Salento peninsula, southeast Italy, were observed between 2008 and 2009 by farmers and regional technicians, with the disease officially identified as Olive Quick Decline Syndrome in autumn 2013 (Martelli *et al.*, 2016). According to the latest EPPO report (EPPO RS 2022/05, https://gd.eppo.int/reporting/article-7342), *Xylella fastidiosa* is currently recorded in Italy in the regions of Apulia, Tuscany, and Lazio. The suitable climatic conditions of southern Europe, the presence of other susceptible host plants near olive orchards, and the existence of hidden reservoirs of asymptomatic but infectious

plants facilitate the pathogen's spread, making attempts at control and eradication challenging (Strona *et al.*, 2017).

Although this olive vascular pathogen has not been studied in this doctoral thesis, the main characteristics of the bacterium and the associated disease are reported for completeness of the discussion.

Causal agent

The pathogen responsible for OQDS is *Xylella fastidiosa*, a Gram-negative, xylem-limited, slow-growing bacterium from the Gammaproteobacteria class (order Lysobacterales; family Lysobacteraceae). This strictly aerobic, non-flagellate bacterium colonizes the xylem tissue of over 560 plant species across 80 plant families and is listed as an EPPO A2 quarantine pathogen (Scortichini, 2022). It is transmitted by various xylem-feeding insect vectors from the *Aphrophoridae*, *Cicadellidae*, and *Cercopidae* families (Cornara *et al.*, 2019). *Xylella fastidiosa* exhibits a remarkably minimalist metabolism, enabling it to survive and thrive in the nutrient-poor, oxygen-deficient xylem environment, which also experiences fluctuating negative pressure (Landa *et al.*, 2022). Lacking motility, the bacterium relies on producing surface adhesins to promote attachment, cell aggregation, and biofilm formation, facilitating movement within the xylem, bacterial multiplication, and transmission by insect vectors (Ionescu *et al.*, 2014). Genetic analyses comparing strains of *Xylella fastidiosa* from Central and South America with the strain isolated in Italy from symptomatic olive trees revealed a close genetic relationship and sequence type (ST53) similarity with *Xylella fastidiosa* subspecies *pauca*, suggesting the pathogen was introduced into Apulia via imported plant material (Martelli *et al.*, 2016; Scortichini, 2022).

Disease cycle

Xylella fastidiosa has never been detected outside of its plant hosts or insect vectors. To colonize the xylem or the cuticular lining of an insect's foregut, the bacterium forms biofilms. After xylem colonization, the pathogen transitions from an adhesive to an exploratory state by producing outer membrane vesicles (OMVs) that reduce adhesion to xylem surfaces and secrete endo-polygalacturonase (endo-PG) to enzymatically degrade the pit membrane pores, facilitating systemic plant colonization (Rapicavoli *et al.*, 2018). *Xylella fastidiosa* is efficiently transmitted by insect vectors in a persistent manner, with no latent period between acquisition and transmission. While feeding on infected plants, the insect

vectors acquire the bacterium, which adheres to their foregut and multiplies before being transmitted to a new host during subsequent feeding (Martelli *et al.*, 2016; Rapicavoli *et al.*, 2018).

Symptomatology

The Olive Quick Decline Syndrome is characterized by extensive lesions on leaf tips and margins, followed by desiccation of twigs and small branches. These symptoms initially appear in the upper parts of the canopy and gradually spread throughout, giving the tree a "burned" appearance. Severely affected trees are often pruned to stimulate new growth, but this new growth is sparse and desiccates quickly. Although the skeletal-looking trees may not immediately die, as evidenced by the generation of suckers at their base, they ultimately succumb to the disease. The most severe symptoms are observed in centuries-old olive trees, which suffer from xylem occlusion caused by *Xylella fastidiosa* and the concurrent presence of other pathogens, such as fungi, and tissue damage from insect galleries (Saponari *et al.*, 2013). Within a few years of symptom onset, OQDS causes tree death (Nuti *et al.*, 2021; Saponari *et al.*, 2017).

Integrated control strategy of Olive Quick Decline Syndrome

To prevent the spread of *Xylella fastidiosa*, European authorities have established containment and eradication measures based on demarcated zones (infected and buffer zones) alongside direct pathogen and vector control, plant breeding, and integrated pest management (Morelli *et al.*, 2021). Research has shifted towards plant-derived minerals and compounds that affect biofilm formation, bacterial growth, strain virulence, and vector acquisition, though these approaches have yet to be validated for in planta use (D'attoma *et al.*, 2019). Natural products, including plant oils, extracts, and microorganisms, are being tested for their ability to inhibit bacterial growth and promote plant defense (Bleve *et al.*, 2018). Given the lack of a cure for infected plants, vector control remains the most effective strategy. Efforts have focused on reducing vector populations through chemical treatments during critical developmental stages (Bodino *et al.*, 2020) and mechanical methods such as pyroweeding and tillage to reduce juvenile populations (Dongiovanni *et al.*, 2018). Various insecticides and natural compounds (e.g., neonicotinoids, pyrethroids, essential oils, kaolin, and zeolite) have shown promise in reducing vector numbers. Investigations into vector feeding and mating behaviors using volatile compounds and pheromones may lead to

innovative, environmentally friendly control methods in the future (Ganassi *et al.*, 2020; Germinara *et al.*, 2017). The integrated pest management strategy for *Xylella fastidiosa* includes early detection, rapid phytosanitary measures, vector control, removal of infected plants in buffer zones, and the use of resistant or tolerant cultivars (Bragard *et al.*, 2019; Morelli *et al.*, 2021).

1.6 Aim of the Research

The olive tree (*Olea europaea* L.) is one of the most ancient crops in the Mediterranean basin, and its cultivation in Sicily holds significant economic, environmental, historical, and cultural value. The widespread cultivation of olives in Mediterranean countries is largely due to the species' ability to adapt to extreme ecological conditions, often characterized by prolonged water shortages, high temperatures, and high soil salinity. However, several factors—such as climate change, improper olive grove management, transplantation of infected plants, the use of agricultural tools shared between farms, poor-quality irrigation water, misuse of fungicides, insecticides, or herbicides, and the spread of pollutants—can negatively affect the natural resilience of olive trees, making them more susceptible to biotic and abiotic stress, and leading to significant reductions in both yield and quality. In this context, the development of alternative, eco-friendly practices are essential to reduce the reliance on synthetic products (fertilizers and pesticides), control biotic threats, and enhance olive tree health and productivity. Plants have co-evolved with specific microbial communities (the plant microbiota), which play a crucial role in supporting plant growth and health. Interactions between plants and their microbiota can also offer protection against biotic stressors through the production of bioactive compounds and/or stimulation of the plant immune system.

Thus, the objectives of this research are:

* To provide a detailed description of the biodiversity of microbial communities hosted by native Sicilian olive cultivars and oleasters in different pedoclimatic conditions, analyzing both culturable and unculturable endophytic microorganisms through a combined approach of microbiology, molecular biology, and metabarcoding techniques.
* To create a culture collection of olive endophytes representative of the Sicilian olive germplasm, to be used in further studies on their ecological roles and interactions with the plant host.
* To characterize endophytes with plant growth-promoting properties and antagonistic activity against olive pathogens, particularly fungal vascular pathogens, through various *in vitro* assays, with the goal of applying native microbial consortia in sustainable cultivation practices.

1.7 References

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2. CHAPTER 2

Experiment 1: Endophytic Microbiota Diversity in the Phyllosphere of Sicilian Olive Trees Across Growth Phases and Farming Systems

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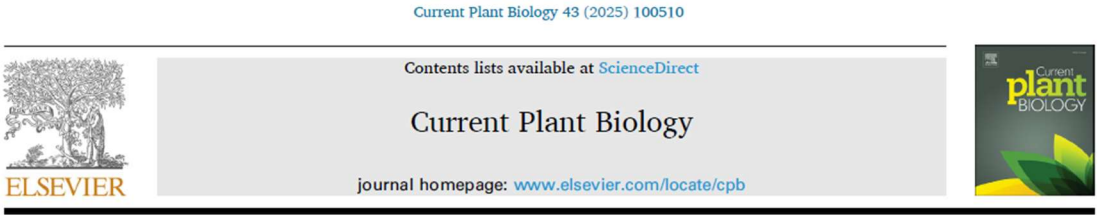

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Endophytic microbiota diversity in the phyllosphere of Sicilian olive trees across growth phases and farming systems

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2.1 Abstract

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.

2.2 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 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.

2.3 Materials and Methods

2.3.1 Orchard site and sampling, 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 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.

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

Tree Host	Farming System	Agrochemical treatments	Accession Code	Olive District	GPS Coordinates
'Nocellara del Belice'	Organic	Fertilization with compost; Copper-based treatments and mass trapping traps	GIAL01 GIAL02 GIAL03	Castelvetrano (Trapani, South-West Sicily)	N 37°41'56.1" E 12°47'33.1"
	Conventional	Fertilization with NPK (Nitrogen-Phosphorus-Potassium) formulations; Copper-based treatments and systemic insecticides	GIAL04 GIAL05 GIAL06	Castelvetrano (Trapani, South-West Sicily)	N 37°41'49.2" E 12°48'21.9"
'Nocellara Etnea'	Organic	Fertilization with compost and manure; Copper- and Kaolin-based treatments; Use of bio-stimulants	NEB01 NEB02 NEB03	Motta Sant'Anastasia (Catania, North-East Sicily)	N 37°31'15.8" E 14°56'21.1"
	Conventional	Fertilization with NPK (Nitrogen-Phosphorus-Potassium) formulations; Copper-based treatments and systemic insecticides	NEC04 NEC05 NEC06	Adrano (Catania, North-East Sicily)	N 37°41'21.6" E 14°50'25.1"
'Nocellara Messinese'	Organic	Fertilization with compost and manure; Copper- and Kaolin-based treatments; Use of bio-stimulants	NMB01 NMB02 NMB03	Motta Sant'Anastasia (Catania, North-East Sicily)	N 37°31'15.8" E 14°56'21.1"
	Conventional	Fertilization with NPK (Nitrogen-Phosphorus-Potassium) formulations; Copper- and Zinc-based treatments; systemic insecticides	NMC01 NMC02 NMC03	Modica (South-East Sicily)	N 36°50'04.4" E 14°46'57.4"
Wild olive	Natural environments	None	SYLV01 SYLV02 SYLV03 SYLV04 SYLV05 SYLV06	Pisano forestry (Buccheri, East Sicily)	N 37°10'58.9" E 14°52'28.4"

2.3.2 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.

2.3.3 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 "home-made" 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.

2.3.4 *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 $p_{adj} \leq 0.05$.

2.3.5 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.

2.3.6 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.

2.4 Results

2.4.1 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.

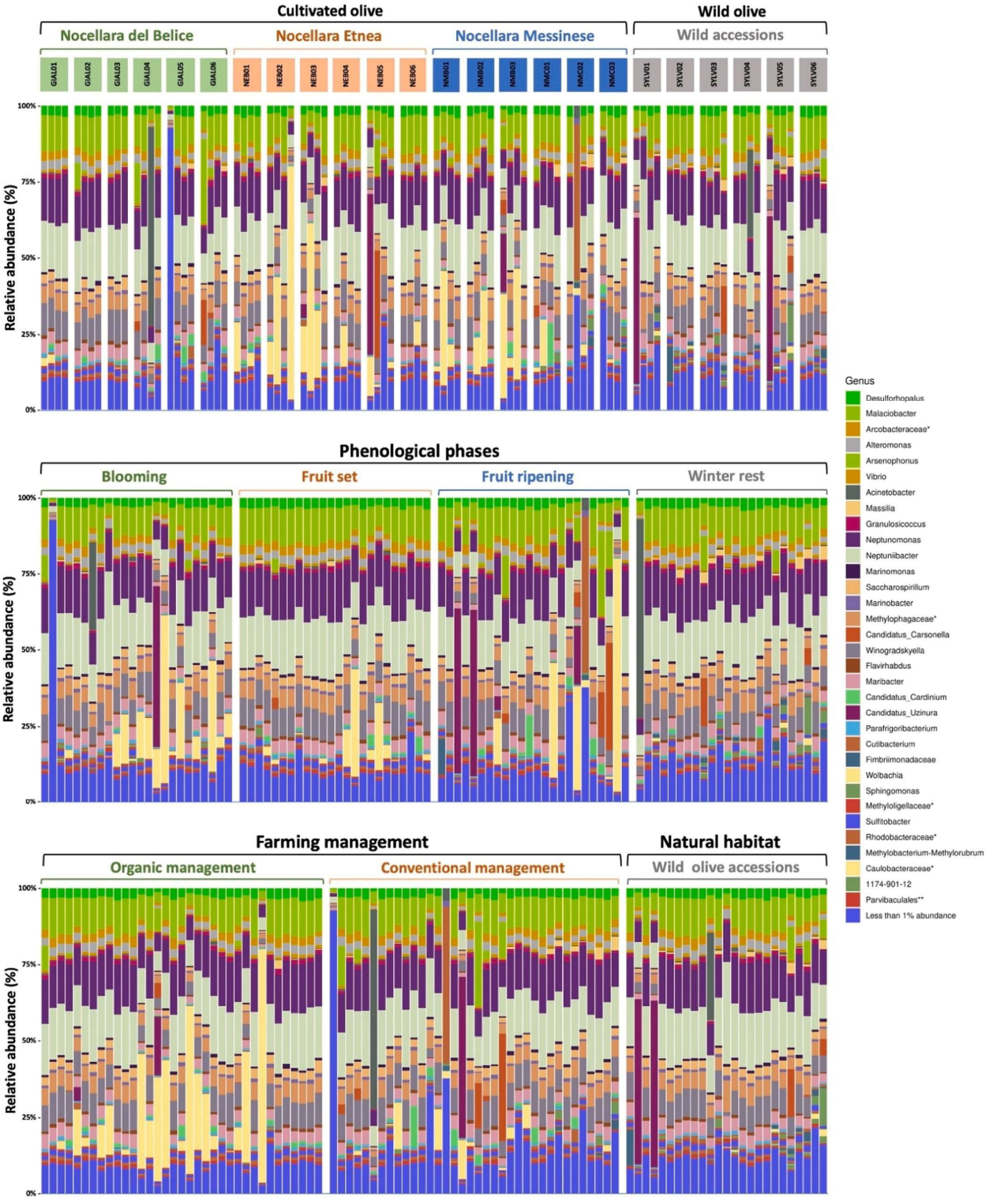

Fig. 1. Relative abundance of main bacterial ASVs recovered from olive twig samples grouped for olive hosts, phenological phases and farming systems. Different colours in the bar plot represent the main bacterial taxonomic genera, as reported in legend.

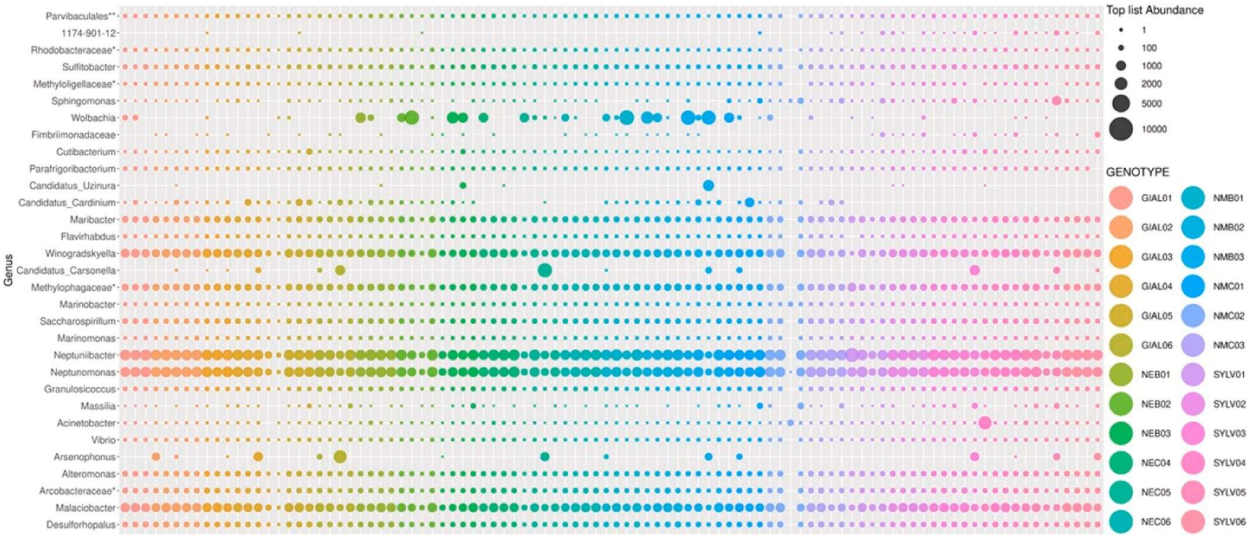

Fig. 2. Bubble plot showing the distribution of bacterial ASVs by genus across olive twig samples. Different colours represent the olive twig samples as indicated in the legend, while bubble size corresponds to the relative abundance of each genus.

2.4.2 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).

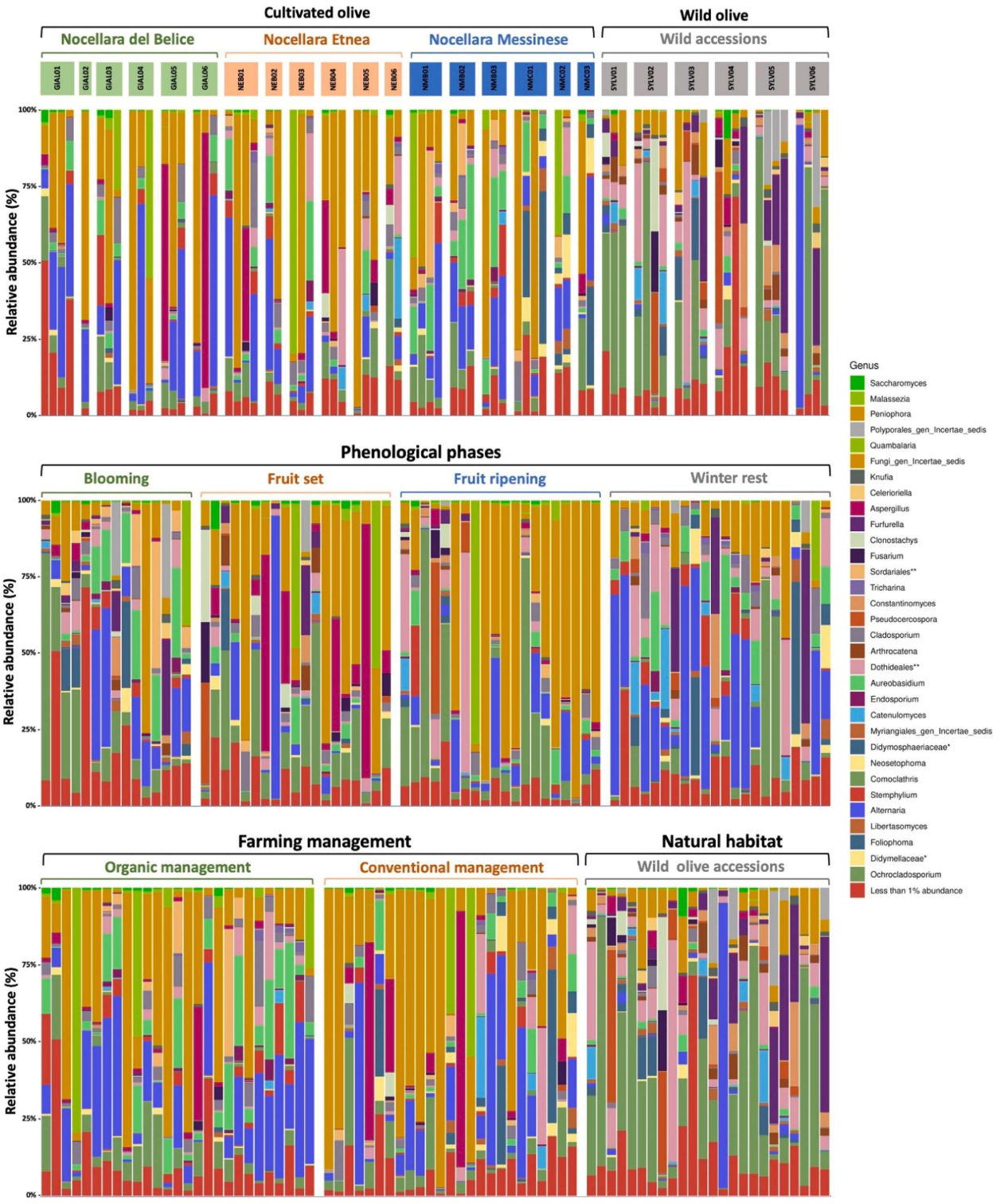

Fig. 3. Relative abundance of main fungal ASVs recovered from olive twig samples grouped for olive hosts, phenological phases and farming systems. Different colours in the bar plot represent the main fungal taxonomic genera, as reported in legend.

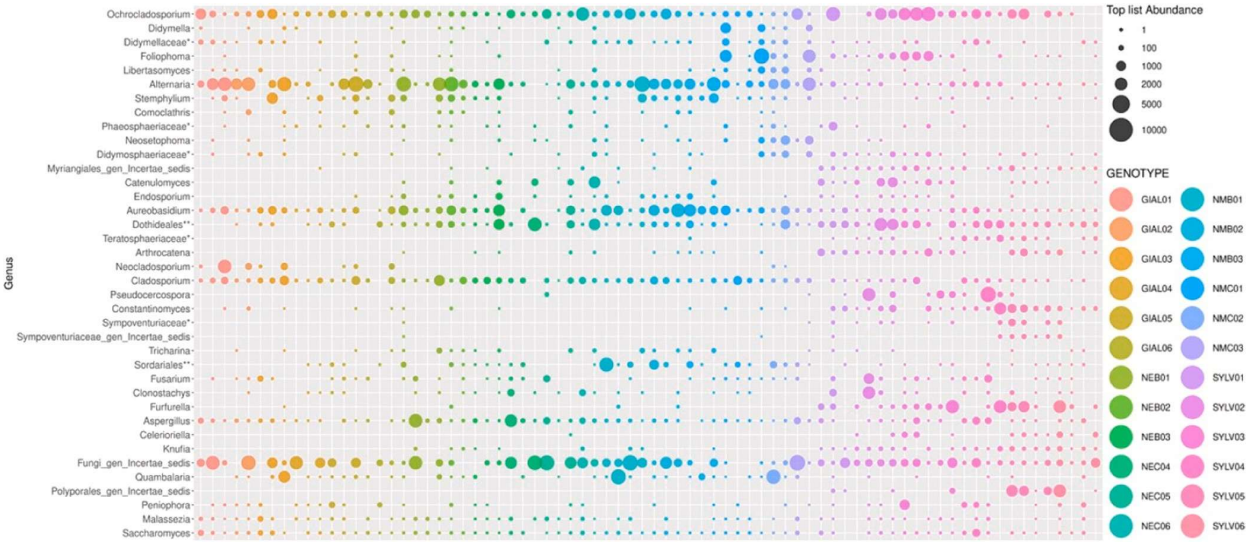

Fig. 4. Bubble plot showing the distribution of the fungal ASVs dataset for taxonomic genus and among olive twig samples. Different colours represent the olive twig samples and the size of the bubbles the respective abundance, as reported in legend.

2.4.3 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 =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.

2.4.4 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 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).

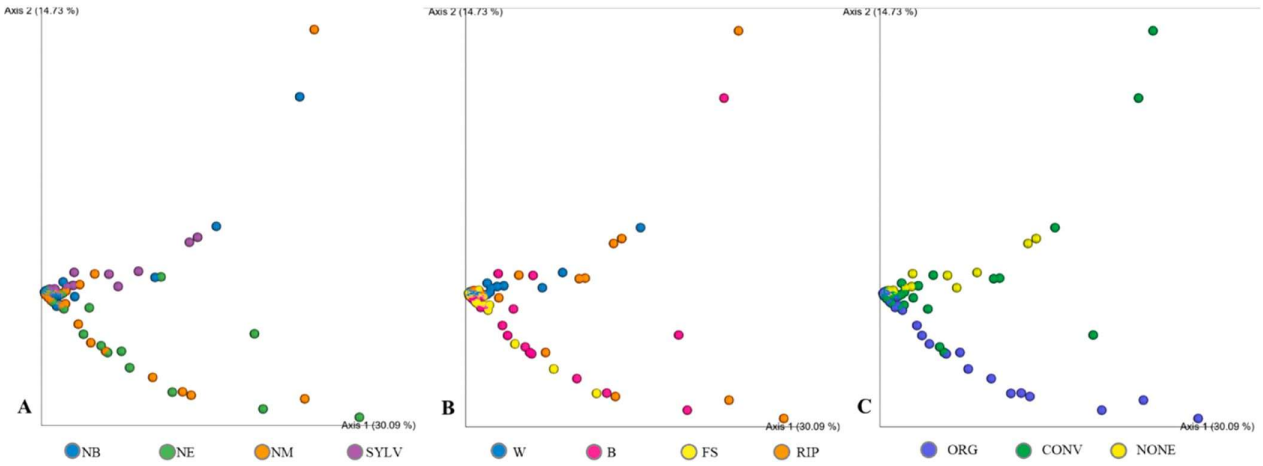

Fig. 5. Beta diversity results with Principal coordinates analysis (PCoA) with Bray-Curtis distance illustrating bacterial community structure among shoots of the Sicilian olive trees. A) PCoA for olive hosts, each colour represents a different host group. B) PCoA for phenological phases (B: Blooming; 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).

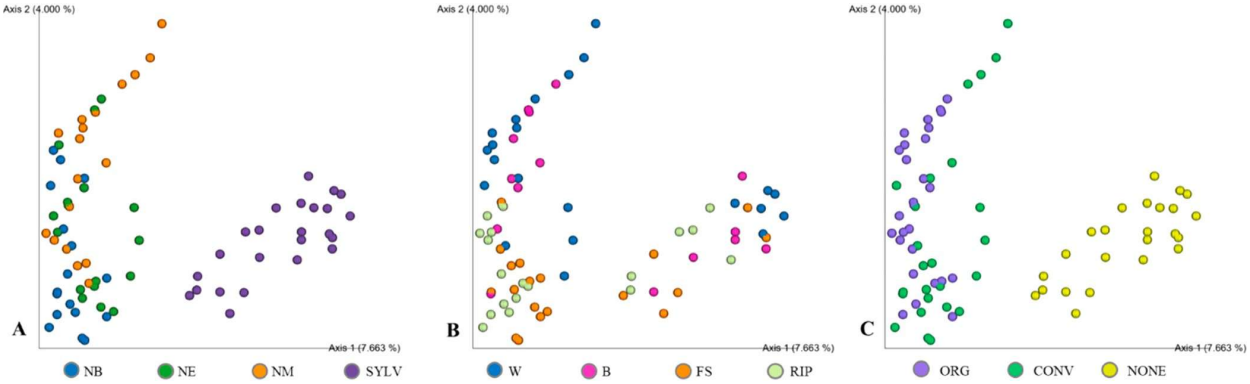

Fig. 6. Beta diversity results with Principal coordinates analysis (PCoA) with Jaccard distance illustrating fungal community structure among twigs of the Sicilian olive trees. A) PCoA for olive hosts, each colour represents a different host group. B) PCoA for phenological phases (B: blooming; FS: fruit 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).

2.4.5 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 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).

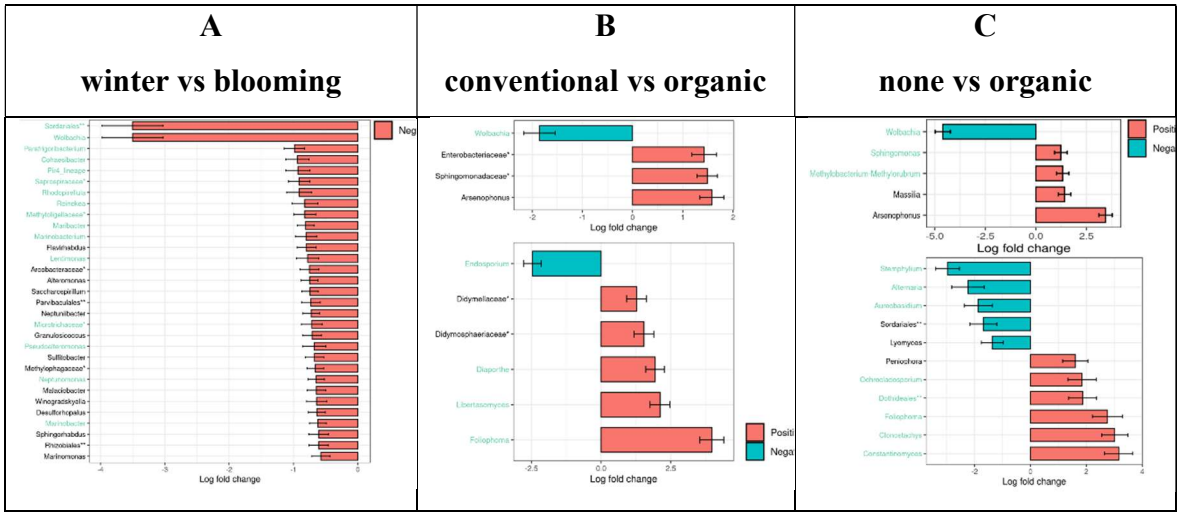

Fig. 7. Differential abundance analysis of microbial genera using ANCOM-BC2: A) phenological phases: winter versus blooming; B) type of management: conventional versus organic; C) type of management: none versus organic.

2.4.6 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).

2.4.7 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.

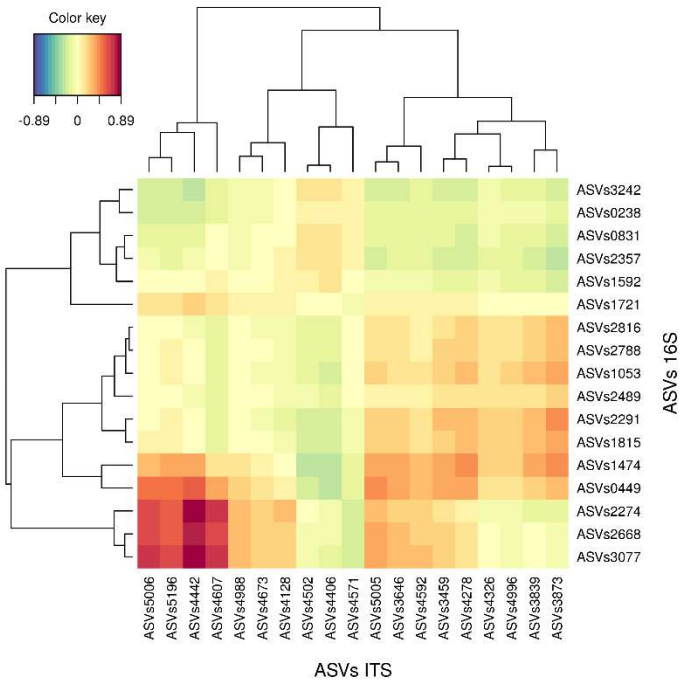

Fig. 8. Analysis of correlations between bacterial ASVs and fungal communities (green ovals) obtained from 16S rRNA gene and ITS metabarcoding analyses, respectively. Negative correlations are indicated in light blue, while positive correlations are represented by orange and red shades. A detailed list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

2.4.8 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).

2.4.9 *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).

2.5 Discussion

2.5.1 Bacterial and fungal community analyses

This is the first study analysing 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].

2.5.2 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.

2.5.3 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 above- ground endosphere [12,15].

2.5.4 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.

2.5.5 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].

2.5.6 *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.

2.5.7 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.

2.6 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.

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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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3. CHAPTER 3

Experiment 2: Endophytic Diversity in Sicilian Olive Trees: Identifying Optimal Conditions for a Functional Microbial Collection

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Endophytic Diversity in Sicilian Olive Trees: Identifying Optimal Conditions for a Functional Microbial Collection

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3.1 Abstract

This study aims to identify the optimal conditions—host, plant material, seasonality, and agricultural practices—for isolating and developing a collection of culturable endophytic microorganisms to support sustainable *Olea europaea* L. cultivation. Samples were collected from three Sicilian olive cultivars ('Nocellara del Belice', 'Nocellara Etnea', and 'Nocellara Messinese') and six wild olive accessions across different phenological phases and under organic and conventional agronomic management. Endophytes were isolated from leaves and twigs using a culture-dependent approach, and their taxonomic diversity and plant-growth- promoting (PGP) traits were analyzed. A total of 133 endophytic isolates were identified, spanning bacterial (*Proteobacteria*, *Firmicutes*, and *Actinobacteria*) and fungal (*Ascomycota* and *Basidiomycota*) phyla. Wild olive trees contributed more than cultivated

varieties to enriching the diversity and composition of culturable endophyte collection as well as twigs instead of leaves. Winter sampling allowed to implement the taxonomic genera of olive endophyte collection. Both farming systems favored an increase in the composition of microbial collection, though organic farming systems supported greater microbial richness. Functional analysis highlighted key PGP traits in a selection of bacterial isolates, including indole-3-acetic acid and siderophore production, nitrogen fixation, and antifungal activity. *Bacillus* spp. dominated enzymatic activities, such as amylase, protease, and lipase production, as well as antifungal activity against the olive fungal pathogen *Neofusicoccum vitifusiforme*. This research highlights the significant diversity and functional potential of Mediterranean olive endophytes. Our findings emphasize the role of native microbial communities as bio-inoculants, promoting plant growth, nutrient uptake, and disease resistance. These insights lay the groundwork for developing targeted olive-microbial consortia for biocontrol and stress tolerance applications.

3.2 Introduction

The plant microbiota—comprising bacteria, fungi, oomycetes, algae, protozoa, nematodes, and viruses—continuously co-evolves with its host, playing a crucial role in plant growth and health [1]. Among these microorganisms, plant growth-promoting endophytes (PGPEs) are particularly valuable, as they colonize internal plant tissues, enhancing biomass accumulation, nutrient acquisition (such as nitrogen, phosphorus, iron, etc.), and the production of beneficial secondary metabolites. PGPEs also contribute to seed germination, root and shoot development, and overall plant resilience by modulating the host immune system, improving tolerance to abiotic stressors and pathogens [2,3].

These beneficial effects are mediated by a range of bioactive compounds and enzymatic activities, including (i) phytohormones such as indole-3-acetic acid (IAA) and gibberellins, which regulate plant development; (ii) siderophores, which enhance iron bioavailability; (iii) phosphate-solubilizing molecules; (iv) trehalose, which improves drought and salt-stress tolerance; (v) hydrolytic enzymes that facilitate bacterial entry into plant tissues; (vi) 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which reduces ethylene levels and mitigates abiotic stress; (vii) nitrogen-fixing enzymes; (viii) enzymes capable of degrading complex organic compounds; and (ix) antimicrobial compounds that suppress phytopathogens [4,5]. However, the effectiveness of endophytes depends on microbial

strain, plant genotype, physiological state, environmental conditions, and external stressors, making plant-microbiota interactions highly dynamic and complex [6–8].

The olive tree (*Olea europaea* L.), a key species in Mediterranean agriculture, is increasingly vulnerable to biotic and abiotic stressors due to shifting climatic conditions and evolving cultivation practices [9]. Italy, the second largest olive producer in Europe [10], possesses a rich genetic heritage, with 25 officially recognized cultivars in Sicily alone [11]. Despite the economic and ecological importance of olive cultivation, studies on the culturable microbial diversity of the olive phyllosphere remain limited [12–15]. Most research has focused on how climate, host genotype, plant organ specificity, and seasonal variation influence microbial communities, while investigations into beneficial microorganisms in Italian olive groves have largely concentrated on soil-dwelling microbiota [16]. To bridge these gaps, we investigated the culturable fraction of olive phyllosphere microbiota by maximizing the diversity of both plant hosts and environments, *i.e.*, including wild and cultivated accessions of different varieties collected in Sicilian traditional olive districts.

Considering the loss of microbial diversity associated with domestication [17] and the positive influence of manure amendments on the plant-associated microbial community [18], we hypothesized that (a) wild olives harbor a higher endophytic diversity than cultivars, and (b) organic farming practices enhance microbial richness.

This study aims to identify the optimal conditions for establishing a functional microbial collection from the phyllosphere of Sicilian olive cultivars and wild olive trees. Using a culture-dependent approach, we assessed microbial diversity across different host types, plant organs, phenological stages, and farming systems. Selected bacterial isolates were characterized in vitro for their plant growth-promoting properties, enzymatic activity, and antagonistic potential against major olive pathogens, including *Verticillium dahliae* and *Neofusicoccum* spp. By linking microbial functionality to environmental and host-related factors, this research provides critical insights for the development of an optimized microbial collection, enabling targeted applications in sustainable olive production and biological control strategies.

3.3 Materials and Methods

3.3.1 Orchard site and sampling

Plant material was collected from five olive orchards (Table 1 and Supplementary Figure S1) under different pedo-climatic conditions, including mild winters and dry summers in southern Sicily, as well as rainfall and strong thermal fluctuations in suburban areas around Mount Etna in eastern Sicily. Three dual-purpose (table and olive oil use) cultivars (*Olea europaea* L. subsp. *europaea* var. *europaea*), namely 'Nocellara del Belice' (NB), 'Nocellara Etnea' (NE), and 'Nocellara Messinese' (NM), were selected from different sites listed in Table 1. Additionally, wild olive accessions (SYLV) were chosen from two sites within the Pisano Forest (SAC—Special Conservation Area, ITA090022), characterized by a lower humid meso-Mediterranean bioclimate and hosting a rich population of *Olea europaea* L. subsp. *europaea* var. *sylvestris* (Table 1). At each sampling site, leaves and twigs were collected from three randomly selected olive trees during four different periods corresponding to phenological phases: winter rest (WD, December 2021–February 2022), flowering (FL, May 2022), fruit set (FS, June–July 2022), and fruit maturation (MAT, October 2022).

Table 1. List of olive trees, cultivars, and wild accessions with the corresponding sampling sites and farming systems; NB: 'Nocellara del Belice'; NE: 'Nocellara Etnea'; NM: 'Nocellara Messinese'; SYLV: wild olive trees. A map of sampling sites is also available in Supplementary Figure S1.

Olive Tree ID	Olive Host	Olive Yards	GPS Coordinates	Farming System
GIAL01 GIAL02 GIAL03	NB	Castelvetrano (Trapani, South-West Sicily)	N 37°41′56.1″; E 12°47′33.1″	Organic
GIAL04 GIAL05 GIAL06	NB	Castelvetrano (Trapani, South-West Sicily)	N 37°41′49.2″; E 12°48′21.9″	Conventional
NEB01 NEB02 NEB03	NE	Motta Sant'Anastasia (Catania, North-East Sicily)	N 37°31′15.8″; E 14°56′21.1″	Organic
NEC04 NEC05 NEC06	NE	Adrano (Catania, North-East Sicily)	N 37°41′21.6″; E 14°50′25.1″	Conventional

NMB01 NMB02 NMB03	NM	Motta Sant'Anastasia (Catania, North-East Sicily)	N 37°31′15.8″; E 14°56′21.1″	Organic
NMC01 NMC02 NMC03	NM	Modica (South-East Sicily)	N 36°50′04.4″; E 14°46′57.4″	Conventional
SYLV01 SYLV02 SYLV03	SYLV	Pisano forest (Buccheri, East Sicily)	N 37°10′58.9″; E 14°52′28.4″	None
SYLV04 SYLV05 SYLV06	SYLV	Pisano forest (Buccheri, East Sicily)	N 37°10′76.2″; E 14°52′28.2″	None

3.3.2 Endophyte Isolation

Samples were collected from four distinct branches distributed across the four cardinal points of the canopy of each olive tree, handled with sterile tools, placed in sterile plastic bags, maintained at 4 °C, and processed within 48 h. Leaves and twig tips (7–8 cm) from each sample were separated and randomly divided into two distinct pools of 10 leaves and 6 twigs. Each pool was weighed, washed with tap water, and surface-disinfected through immersion in 70% (*v/v*) ethanol for 2 min, 3% (*w/v*) sodium hypochlorite for 3 min, and 70% (*v/v*) ethanol for 1 min, followed by three rinses of 1 min each in sterile distilled water (SDW). The disinfected samples were then ground in sterile mortars with 5 mL of SDW. Plant extracts (100 µL) were serially diluted and plated on Nutrient Agar (NA, Condalab) and Potato Dextrose Agar (PDA, Condalab) plates. A negative control was prepared by plating an equal volume of the final wash water onto the same media. Plates were incubated at 26 °C in the dark and observed daily. Total colonies were counted and the number of colony-forming units (CFUs) converted to log10 CFUs/g of fresh plant material. Bacterial and fungal colonies were initially grouped according to colony morphological features (shape, color, elevation, surface, margin, aerial mycelium, presence of exudate, and growth rate) and microscopic similarity, and at least two representative colonies of each morphotype for each organ and host sample were selected, purified, and maintained in pure cultures at −80 in glycerol (10–50% *v/v*).

3.3.3 Identification of Bacterial and Fungal Endophytes

Molecular identification was performed through sequence analysis of PCR-amplified bacterial 16S rDNA genes and fungal internal transcribed spacer (ITS1, 5.8S, ITS2) rDNA regions. Bacterial isolates were cultivated in Nutrient Broth (NB) medium and incubated at 28 ± 2 °C for 24 h. Bacterial genomic DNA was extracted from cell pellets resuspended in 500 µL of TE buffer, enzymatically lysed with Proteinase K (20 mg/mL), and purified using the phenol/chloroform/isoamyl alcohol (25:24:1) method [19]. The extracted DNA was amplified using universal primers (27f and 1492r) following the original PCR protocol [20]. Pure fungal cultures were obtained on PDA plates incubated for seven days at 28 ± 2 °C. Fungal genomic DNA was extracted from 200 µg of scraped aerial mycelium, disrupted with tungsten carbide beads in a Tissue Lyser II (Qiagen, Hilden, Germany) set at 30 Hz for 2 min, following a Cetyl Trimethyl Ammonium Bromide (CTAB) protocol [21]. DNA amplification was performed using the ITS5/ITS4 primer set [22], following the original PCR protocol with an annealing temperature of 56 °C. The amplified products were purified and sequenced by Eurofins Genomics (Ebersberg, Bayern, Germany).

Bacterial and fungal identification was performed using the highest identity score from BLAST 2.15.0 software of the National Center for Biotechnology Information (NCBI) against the NR database. Sequences were deposited in the GenBank database under accession numbers PP506680-PP506717, PV240332-PV240334, PP513240-PP513283, and PP513285-PP513302 (Supplementary Table S1). A percentage identity > 98% with NCBI sequences was accepted for species-level identification, while identities between 95% and 97% were classified at the genus level. Isolates with identity < 95% were designated as "unknown" [23].

3.3.4 Determination of Plant Growth Promotion Properties and Enzymatic Activities of Bacterial Isolates

The production of IAA was evaluated using a colorimetric technique by incubating 3 mL of a liquid culture in NB with L-tryptophan (100 mg/L) and incubated at 28 °C for 72 h with shaking. Cultures were centrifuged for 5 min at 15,000 × g and supernatants transferred to glass tubes. Color changing to pink (positive reaction) was verified following the addition of 4 mL of Salkowski's reagent [24]. The amount of IAA was calculated by measuring the

absorbance at 535 nm in a spectrophotometer (ASYS UVM-340 Microplate Reader, Biochrom, Ltd., Cambridge, UK), accordingly to a standard curve appropriately realized.

Siderophores production was evaluated in Chrome Azurol S (CAS) agar plates [25]. Aliquots (100 µL) of bacterial cultures were inoculated in the wells of the CAS plates and incubated for 7 days at 28 °C in darkness. Bacteria that produced siderophores showed an orange halo.

Phosphate solubilization was determined by the presence of a transparent halo around the bacterial cultures (100 µL) inoculated in wells of NBRIP plates (phosphate growth medium from the National Institute of Botanical Research) after 7 days at 28 °C [26].

Nitrogen fixation was assessed by plating strains in a nitrogen-free minimum medium (NFB; [27]) and incubating for 5 days at 28 °C. Bacterial growth indicated the possible ability of the bacteria to fix atmospheric nitrogen.

The formation of biofilms was determined by checking the adhesion capacity of the bacteria in microplates with 12 wells in NB at 28 °C, incubating for 4 days. After incubation, the biofilm formation was observed in the surface and/or bottom of the wells. Then, each well was stained for 20 min with 0.01% crystal violet [28] to evaluate the presence of a ring of biofilm around the well walls.

The ACC deaminase activity was performed as described by Penrose and Glick (2003) [29]. Briefly, bacterial cultures were incubated in a salts minimal broth added with ACC (5 mM) for 24 h at 28 °C by shaking. The α-ketobutyric acid produced was quantified using a standard curve with known concentrations at 540 nm in a spectrophotometer (ASYS UVM-340 Microplate Reader, Biochrom, Ltd., Cambridge, UK), and ACC deaminase activity was expressed in µmoles of α-ketobutyrate per mg of protein per hour.

Enzymatic activities were determined on plates incubated at 28 ◦C for 7 days, observing the formation of halo around the bacterial biomass. DNAse activity was determined by streaking the isolates on DNA agar plates revealed with 1 M HCl. Amylase activity was performed on starch agar plates (Scharlab, Barcelona, Spain) and revealed with 10 mL Lugol. Protease and lipase activities were assessed in casein agar and Tween 80 media, respectively, as described by Harley and Prescott (2002) [30]. Pectinase and cellulase activities were examined as described by Elbeltagy *et al.* [31]. For pectinase activity, strains plated on ammonium mineral agar plates were revealed with 2% CTAB, and positive bacteria showed a halo. For cellulase activity, strains were plated on solid minimal medium supplemented with yeast extract (0.2%) and carboxymethylcellulose (1%). Plates were developed by covering the

plate with 1 mg/mL Congo Red solution for 15 min and decolorizing with 1 M NaCl for 20 min. Finally, chitinase activity was performed as described by Mesa *et al.* [32] on agar plates containing minimal medium supplemented with colloidal chitin.

3.3.5 *Dual Culture Plate Screening for Antagonistic Activity of Endophytes*

Five top-performing bacterial isolates that exhibited a higher number of plant growth promoting (PGP) properties (PGP properties ≥ 3 and enzymatic activities ≥ 2), along with the only isolate presenting chitinase activity, were selected. The six isolates were tested in vitro for their antifungal activity against olive fungal pathogens: two no-defoliating strains of *Verticillium dahliae* (Vd-LT and Vd-CS), one strain of *Neofusicoccum parvum* (Np- P4), and one strain of *Neofusicoccum vitifusiforme* (Nv-P3). Three replicate plates were prepared for each bacterial isolate. For screening, round agar plugs of 5 mm diameter of the filamentous fungi were obtained from the margins (active growth zone) of young mycelia grown on PDA and placed at a distance of 21 mm away from the Petri dish edge (standard 90 mm Petri dishes) with fresh nutrient agar. One hundred microliters of bacterial suspension (~106 cells/mL) were inoculated 45 mm away from the fungal plug on the opposite side of the plate. Negative control plates were inoculated only with the filamentous fungi, while a strain of *Trichoderma harzianum* S3, a fungus commonly used as biocontrol agents [33], was inoculated instead of bacteria as positive control. The plates were incubated for 42 days at 25 °C in the dark. Mycelial growth was observed weekly, and images were captured to record the growth. The inhibition percentage was calculated as $[(R_c\text{-}R_{exp})/R_c] \times 100\%$, where R_c represents the longest distance of fungal mycelium from the inoculated fungal plug, and R_{exp} is the horizontal distance from the inoculated fungal plug towards the bacterial colony, which shows the inhibitory effect [34,35].

3.3.6 *Data Analysis*

A non-parametric approach was adopted to analyze the culturable endophyte occurrence in leaves and twigs of different olive varieties and wild accessions during the four phenological phases. To investigate the differences in CFU/g, the Kruskal–Wallis statistic with adjustment for ties [36] was adopted and implemented on Minitab statistical software version 22.1.0 [37].

Richness and evenness were calculated to determine the α-diversity of the culturable fungal and bacterial endophytes identified at genus level in different hosts under several

environmental factors. To carry out diversity analyses, the PAST 4.17 software tool [38], version 4.17, was used, estimating the diversity indices and the species rarefaction curves in leaves and twigs for each phenological phase based on the Simpson index (1/D). For diversity analyses, only the identified taxonomic genera were considered, excluding the "unknown".

β-diversity was examined by means of non-metric multidimensional scaling (NMDS), using the Bray–Curtis coefficient and the Jaccard indexes as similarity measures. Analyses were performed using the PAST 4.17 software, and the quality of the results was visualized in the Shepard plots, accepting stress values ≤ 0.2 as goodness of fit. To assess significant differences among endophyte community groups obtained by NMDS plots, p-values (significance assumed for $p \leq 0.05$) were generated by one-way analysis of similarity (ANOSIM) from Bray–Curtis matrices automatically computed in PAST 4.17 software. To assess which taxa are primarily responsible for a difference between groups of samples, the similarity percentage (SIMPER) analysis was implemented on PAST 4.17 software.

Venn diagrams were made using the opensource component for web environment Jvenn (available online: http://jvenn.toulouse.inra.fr, accessed on 30 April 2025).

To obtain information about the functional diversity of fungal strains, the FUNGuild database was used to estimate the trophic modes and guilds of fungi [39]. This tool allows to predict the following primary fungal lifestyles: 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.

3.4 Results

3.4.1 Culturable Endophyte Occurrence in Sicilian Olive Cultivars and Wild Accessions

The presence of bacterial and fungal colonies on NA and PDA media increased during the incubation period, and most of them appeared within 10 days. No colonies were obtained from any dilutions. The absence of colonies was observed on some NA and PDA plates regardless of olive samples, organs and phenological phases, probably due to the low concentration and erratic distribution typical of plant endophytes. Considering the plant material, most of colonies were recovered from twigs compared to occasional microbial

isolation from leaves. Twigs enabled the isolation of endophytes from more olive trees along all phenological phases (Figure 1A, B) as well as farming systems (Figure 1C, D).

Within cultivars, the occurrence of culturable endophytes was significantly influenced by twig samples in ‘Nocellara del Belice’ ($p = 0.036$), ‘Nocellara Messinese’ ($p = 0.007$), and wild olive accessions ($p = 0.038$) compared to leaves. The highest value of total microbial count in twigs was observed during fruit maturation in ‘Nocellara del Belice’, ‘Nocellara Etnea’, as well as wild olive trees and during flowering in ‘Nocellara Messinese’ (Figure 1B). Regarding farming systems, samples from olive trees under organic management showed a higher microbial occurrence (Figure 1C, D), which varied significantly in samples from ‘Nocellara Etnea’ ($p = 0.001$) compared to those under conventional management. Among phenological phases, no significant differences were observed in olive cultivar and wild olive tree total microbial count.

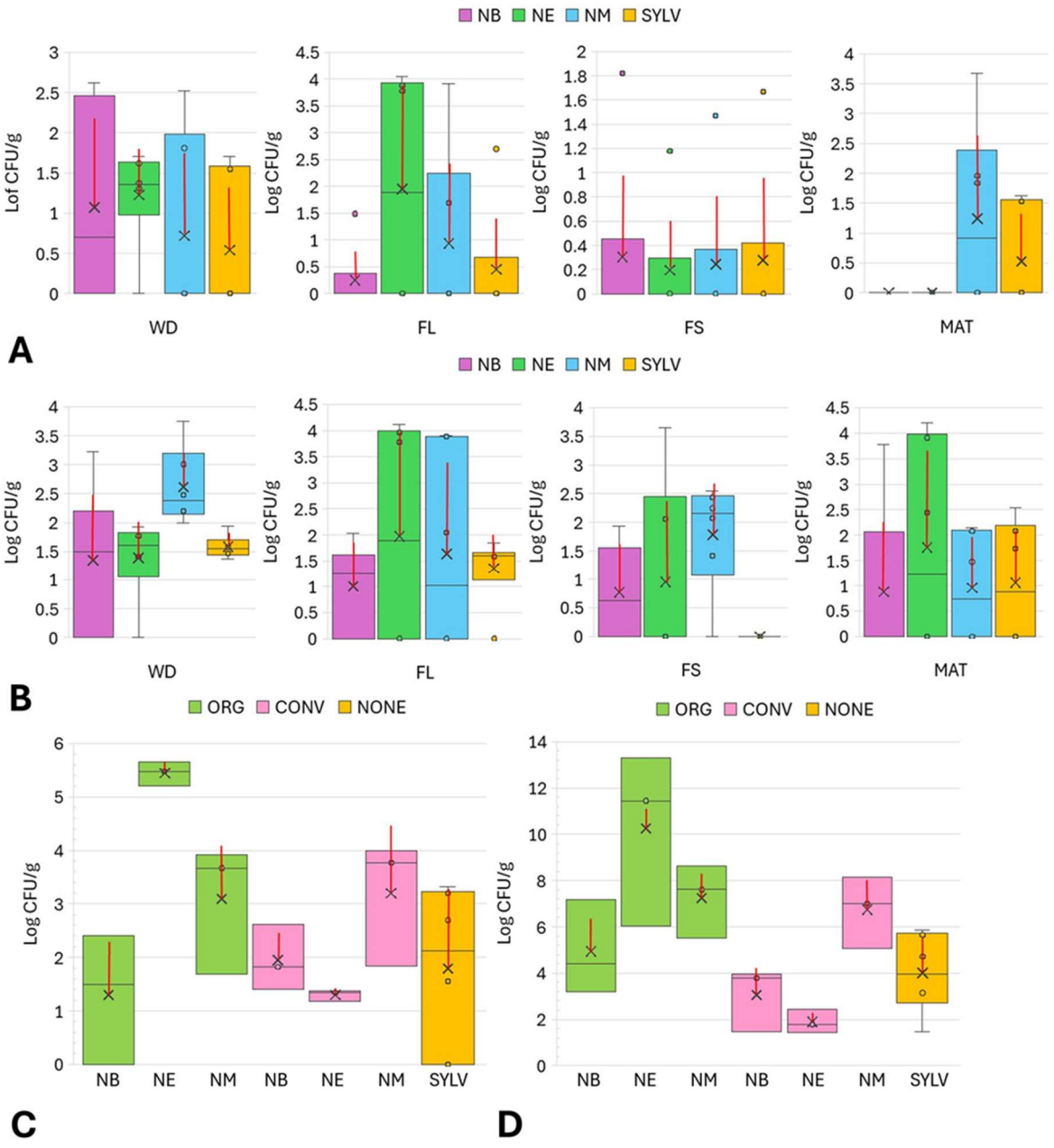

Figure 1. Box plots showing total microbial counts (log10 CFU/g) of leaf (A, C) and twig (B, D) endophytes isolated from the different cvs. 'Nocellara del Belice' (NB), 'Nocellara Etnea' (NE), and 'Nocellara Messinese' (NM) and Sicilian wild olive trees (SYLV) during the four phenological phases (WD, winter rest; FL, flowering; FS, fruit set; MAT, fruit maturation) (A,B) and farmed under organic (ORG) and conventional (CONV) management and no agricultural practices (NONE) (C,D). The central horizontal bars are the medians. The central symbol X corresponds to average values, and the red vertical lines are the standard deviations. Points above and below the whiskers' upper and lower bounds are outliers.

3.4.2 *Diversity of Culturable Endophytes*

From olive leaves and twigs collected during all phenological phases, a total of 133 phenotypically different endophytic bacterial and fungal isolates were selected and identified by 16S rRNA gene and ITS sequencing analysis, respectively. Sequence lengths varied from 474 to 1425 bp for 16S rRNA region and from 417 to 651 bp for the ITS region (Supplementary Table S1).

The 60 bacterial isolates identified were affiliated to three phyla (Actinobacteria, Firmicutes, and Proteobacteria) and mainly associated most with the genera *Bacillus* (43.3%), *Staphylococcus* (18.3%), and *Sphingomonas* (10.0%) (Figure 2). The 68 fungal isolates identified were assigned to two phyla (Ascomycota and Basidiomycota) and affiliated to the genera *Quambalaria* (17.4%), *Alternaria* (10.1%), and *Biscogniauxia* (8.7%) (Figure 3). The five isolates that showed a sequence identity ≤ 94.7% were excluded from analyses (Supplementary Table S1). The microbial diversity (high or low) found in each sample was independent of the overall number of microorganisms present, regardless of host, organ, phenological phase, or farming system.

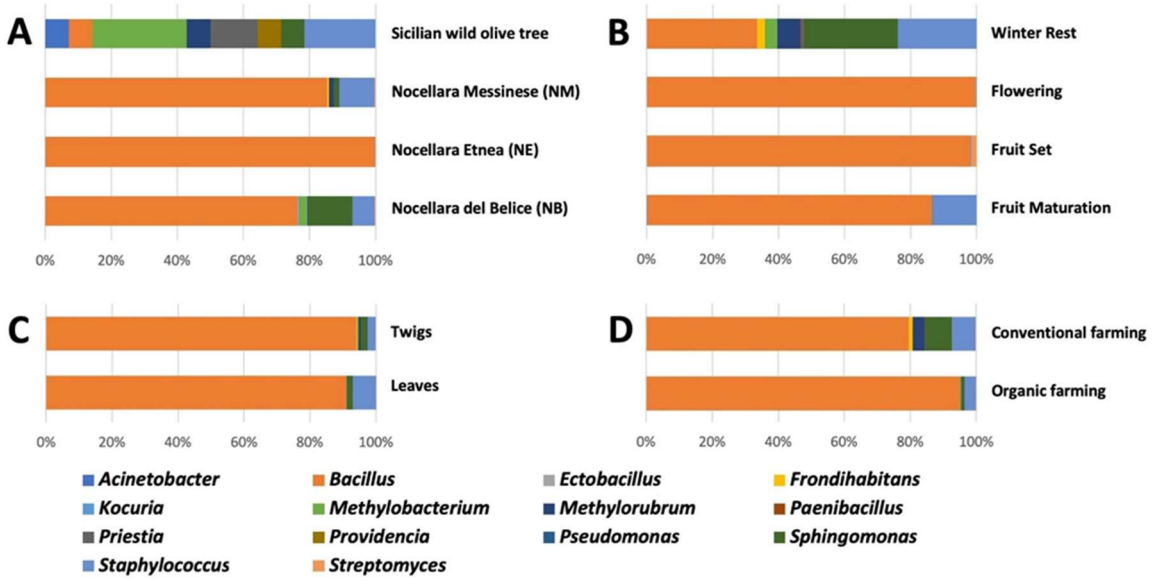

Figure 2. Relative abundance of endophytic bacterial genera in hosts (A), phenological stages (B), plant organs (C), and farming systems (D) of olive trees cvs. 'Nocellara del Belice' (NB), 'Nocellara Etnea' (NE), and 'Nocellara Messinese' (NM) and Sicilian wild olive trees (SYLV).

Considering olive hosts, Sicilian wild olive trees contributed most to the diversity of bacterial and fungal endophyte collection (Figures 2A and 3A) and significantly to microbial composition ($R = 0.562$; $p = 0.008$) compared to cultivated varieties that showed a more homogeneous endophytic composition (few different dominant genera with individuals uniformly distributed), with minor differences (Table 2; Supplementary Figure S2). Rarefaction curves showed that the probability to enrich the microbial collection of endophytes can be further increased by analyzing a larger number of wild olive samples, while for cultivated olive trees, the plateau seems to be reached (Supplementary Figure S3). Nine microbial genera (*Acinetobacter, Chaetomium, Eurotiomycetes, Peniophora, Pleosporineae, Preussia, Priestia, Providencia,* and *Tritirachium*) derived exclusively from wild plants (Figure 4A). Endophytes belonging to ten genera, *i.e.*, *Bacillus, Methylobacterium, Methylorubrum, Sphingomonas, Staphylococcus, Biscogniauxia, Cladosporium, Diaporthe, Penicillium*, and *Quambalaria*, were isolated both from wild olive accessions and at least one of the three cultivars of 'Nocellara', representing ~25% of the endophytic communities (Supplementary Table S1). At species level, the bacterial endophytes *Ectobacillus funiculus*, *Pseudomonas savastanoi*, and *Streptomyces bryophytorum* and the fungal ones, namely *Aspergillus stella-maris* and *Cladosporium sphaerospermum*, were isolated only from leaf samples of the cultivar 'Nocellara del Belice', which clustered separately from the samples of other cultivars (Supplementary Figure S2A, B). Although differences in pairwise comparisons of microbial communities from different hosts were not significant ($p > 0.05$), the SIMPER analysis showed that the species of the genus *Bacillus* and secondarily those of the genus *Pyronema* and the species *Sphingomonas paucimobilis* contributed greatly to determining the differences among hosts, influencing more the diversification of isolation of culturable olive endophytes. Despite this diversity, a putative microbial core, defined as the community systematically associated with a given host plant [40], of culturable endosphere-associated microorganisms from Sicilian olive trees can be attributed to the presence of the genera *Bacillus*, *Staphylococcus*, and *Quambalaria*.

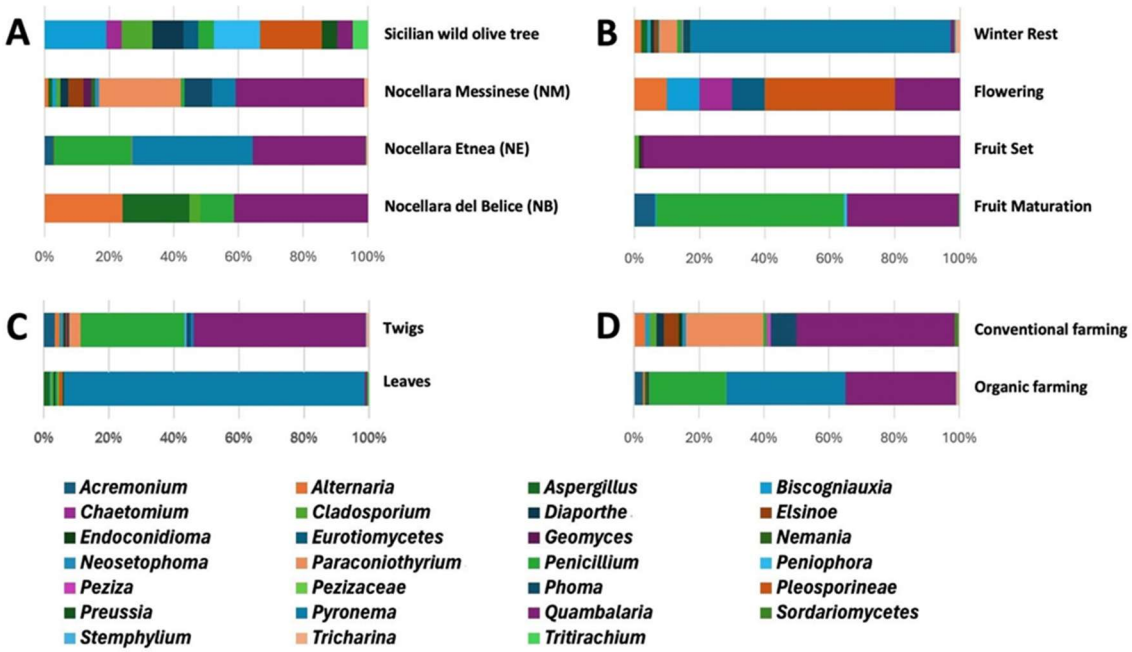

Figure 3. Relative abundance of endophytic fungal genera in hosts (A), phenological stages (B), plant organs (C), and farming systems (D) of olive trees cvs. 'Nocellara del Belice' (NB), 'Nocellara Etnea' (NE), and 'Nocellara Messinese' (NM) and Sicilian wild olive trees (SYLV).

Table 2. Diversity indices of endophytes associated with leaves (L) and twigs (T) of different olive hosts (NB, 'Nocellara del Belice'; NE, 'Nocellara Etnea'; NM, 'Nocellara Messinese'; SYLV, wild olive accessions).

Diversity Indexes	NB-L	NB-T	NE-L	NE-T	NM-L	NM-T	SYLV-L	SYLV-T
Taxa (genera)	7	10	6	10	5	21	9	15
Individuals	33	398	1204	2002	397	853	16	19
Dominance	0.428	0.592	0.621	0.590	0.633	0.662	0.108	0.029
Simpson (1-D)	0.572	0.408	0.380	0.410	0.367	0.338	0.892	0.971
Shannon	1.282	0.963	0.589	0.796	0.605	0.935	2.243	2.993
Equitability	0.659	0.418	0.329	0.346	0.376	0.307	1.021	1.105
Chao-1	9.91	10.50	12	20	6.99	36.98	16.03	35.84

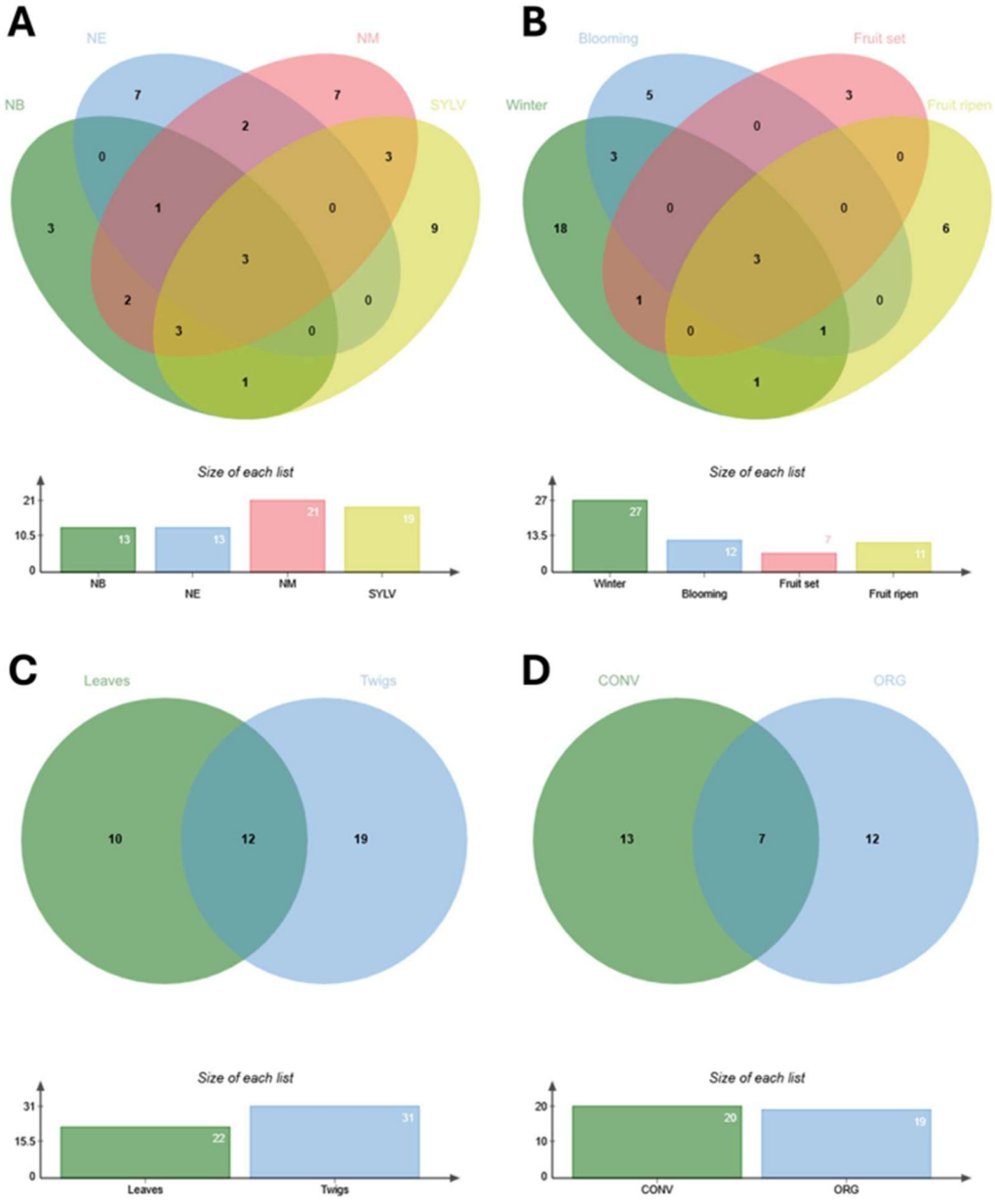

Figure 4. Venn diagram of shared and specific microbial genera from different cvs. 'Nocellara del Belice' (NB), 'Nocellara Etnea' (NE), and 'Nocellara Messinese' (NM) and Sicilian wild olive trees (SYLV); (A) phenological phases (B), plant organs (C), and farming systems (CONV, conventional; ORG, organic) (D).

In almost all phenological phases, microbial isolates from wild olive trees enriched culture collection diversity and composition more than those from cultivated varieties (Figures 2B and 3B, Supplementary Table S2). Among all samples, the highest number of microbial genera was isolated during the winter rest (Figure 4B), with a prevalence of fungal

endophytes. The endophytic genera *Bacillus*, *Quambalaria*, and *Staphylococcus* were isolated during all sampling periods.

A wider diversity of endophytic isolates was reached in twigs rather than leaves both along all phenological phases and different olive hosts (Table 2 and Supplementary Table S2). Twig samples were richer on genera (Figures 2C, 3C, and 4C); about 35.7% of bacterial (*Acinetobacter, Frondihabitans, Kocuria, Priestia,* and *Providencia*) and 55.5% of fungal (*Alternaria, Acremonium, Chaetomium, Diaporthe, Dydimella, Elsinoe, Geomyces, Nemania, Neosetophoma, Paraconiothyrium, Peniophora, Peziza, Phoma, Stemphylium,* and *Tricharina*) genera were isolated only from that plant material, mainly from cv. 'Nocellara Messinese'. More than 20% of both bacterial and fungal genera were isolated from only leaves (*Ectobacillus, Pseudomonas, Paenibacillus, Endoconidioma, Lybertasomyces, Preussia, Pleosporinae, Sordariomycetes, Pezizaceae,* and *Tritirachium*). Almost 30% of microbial genera (*Bacillus, Methylobacterium, Methylorubrum, Sphingomonas, Staphylococcus, Streptomyces, Aspergillus, Biscogniauxia, Cladosporium, Penicillium,* and *Quambalaria*) were recovered from both plant organs.

The microbial diversity of the culturable endophytic collection was considerably increased by the contribution of microbial communities from samples of the organically farmed cv. 'Nocellara del Belice' as well as cultivars 'Nocellara Etnea' and 'Nocellara Messinese', farmed under conventional practices (Supplementary Table S3). Regarding taxonomical genera, the endophyte collection was enriched both from samples collected under organic (12) and conventional (13) practices (Figure 4D). The bacterial genera (*Ectobacillus, Kocuria, Methylobacterium,* and *Pseudomonas*) and fungal genera (*Aspergillus, Geomyces, Nemania, Pezizaceae* sp., *Pyronema, Stemphylium,* and *Tricharina*) associated with organic farming systems were different from the genera (bacterial: *Frondihabitans, Methylorubrum,* and *Paenibacillus*; fungal: *Biscogniauxia, Cladosporium, Diaporthe, Elsinoe, Endoconidioma, Neosetophoma, Paraconiothyrium, Peziza,* and *Sordariomycetes* sp.) associated with conventional managements (Figures 2D and 3D). Only seven microbial genera (*Alternaria, Bacillus, Penicillium, Quambalaria, Sphingomonas, Staphylococcus,* and *Streptomyces*) were found to be widespread in cultivars farmed under both agricultural practices. Endophyte isolates from samples of each cultivar under organic management were taxonomically different from those isolated from conventional management, but no clear differentiation and no significant dissimilarities of two groups (ORG and CONV) was

observed by NMDS ordination (Supplementary Figure S2C, D) and ANOSIM analysis. *Bacillus, Sphingomonas, Alternaria,* and *Quambalaria* from cv. 'Nocellara del Belice'; *Staphylococcus* and *Quambalaria* from cv. 'Nocellara Etnea'; and *Bacillus* and *Staphylococcus* from cv. 'Nocellara Messinese' were the microbial genera found in common between the two farming systems.

3.4.3 Characterization of Endophytic Bacteria from Olive Leaves and Twigs

3.4.3.1 Plant Growth-Promotion Properties and Enzymatic Activities

A total of 45 bacterial isolates were characterized to evaluate the presence of plant growth-promotion traits, and about 90% of them, representing those with greater growth capacity in vitro, showed PGP properties (Supplementary Table S4) and enzymatic activities (Supplementary Table S5). All isolates showed at least one of the screened characteristics, and 65% of them exhibited from five to ten PGP traits concurrently. The most common PGP properties were siderophore production (80%), biofilm formation (77.5%), and nitrogen fixation (65%). Bsp_GIAL05R strain (*Bacillus* sp.) showed the highest production of siderophores according to the diameter of the halo in CAS medium (Supplementary Table S4), and Bm_GIAL02R (*Bacillus megaterium*), Msp_SYLV02F (*Methylobacterium* sp.), and Pv_SYLV05R (*Providencia vermicola*) were the best phosphate solubilizers (Supplementary Table S4). A high number of isolates (26) were able to grow in N-free medium. The strain Pv_SYLV05R (*Providencia vermicola*) produced the highest concentration of IAA, more than 9 mg/L (Supplementary Table S4). Bacterial isolates did not show biological ACC deaminase activity in vitro. Among the seven enzymatic activities studied, amylase (57.5%), lipase (52.5%), and DNase (52.5%) were the most represented (Supplementary Table S5). Protease synthesis was found in 15 endophytes, and four strains of *Bacillus* genus (Bsp_NMC03R, Bsp_NMC03F, Bm_GIAL02Rb, and Bsp_NEB03RIII) showed a strong activity (Supplementary Table S5). Cellulase, pectinase, and chitinase activities were detected in a low number of isolates (three to seven).

3.4.3.2 Antagonistic Activities

Among the screened bacterial isolates, *Bacillus licheniformis* Bl_SYLV02R displayed inhibitory activity against the fungal pathogen *Neofusicoccum vitifusiforme* (Nv-P3) in dual-plate assay (Figure 5). A mycelial inhibition percentage equal to 40.3% was recorded compared to that of the positive control, explained by the 60% inhibition by *Trichoderma*

harzianum S3 against the same pathogenic fungus (Supplementary Table S6). It is noteworthy that the other different tested species within the *Bacillus* genus (*Bacillus* sp. and *Bacillus marisflavi*) did not exhibit the same inhibition capability. No inhibitory activity was observed against the two no-defoliating strains of *Verticillium dahliae* (Vd-LT and Vd-CS) and the fungus *Neofusicoccum parvum* (Np-P4) by *Bacillus licheniformis* Bl_SYLV02R or by the other bacterial isolates against all the pathogenic fungi considered.

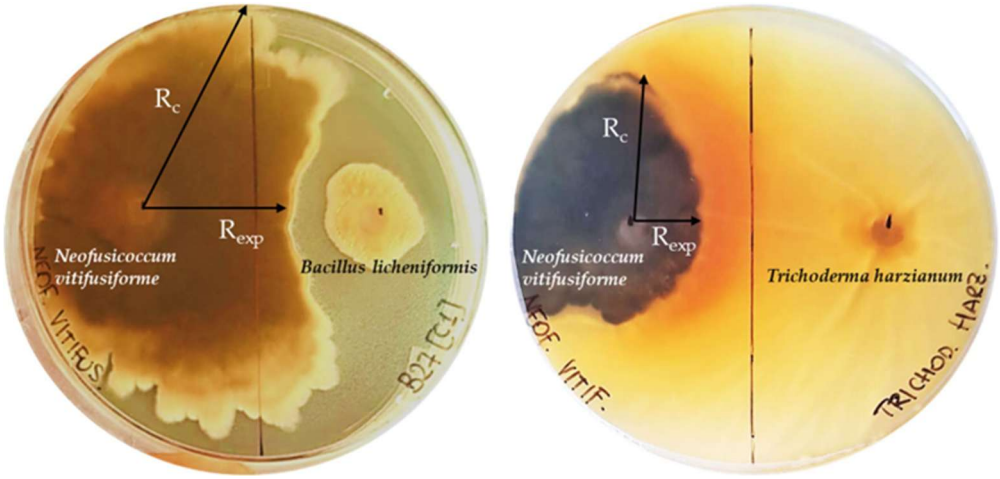

Figure 5. Representation of the dual culture assay showing antifungal activity of *Bacillus licheniformis* Bl_SYLV02R and *Trichoderma harzianum* S3 against the fungal pathogen *Neofusicoccum vitifusiforme* (Nv-P3) and how the inhibition was measured. R_c represents the longest distance of fungal mycelium growth from the inoculated fungal plug, and R_{exp} is the horizontal distance from the inoculated fungal plug towards the screened isolates.

3.4.4 Trophic Mode of Endophytic Fungi

Fungal taxonomic functional analyses by FUNGuild classified the fungal strains into different trophic modes (Supplementary Table S7). The most common trophic mode was pathotroph–saprotroph–symbiotroph (41.2% of identified isolates), followed by pathotroph (13.2%) and saprotroph (11.7%) (Supplementary Table S7). The main trophic mode was mainly represented by endophytes, animal and plant pathogens, and wood saprotrophs (Supplementary Table S7). The pathotroph group was exclusively represented by the plant pathogen *Quambalaria cyanescens*, most frequently isolated under conventional farming. The species *Endoconidioma populi*, *Geomyces* sp., *Libertasomyces platani*, *Nemania serpens*, *Paraconiothyrium brasiliense*, and *Preussia minima* were classified as wood and plant saprotroph. The endophytes *Neosetophoma* sp., *Pyronema* sp., and *Tricharina* sp. and

the dung saprotrophs *Peziza* sp. and *Pezizaceae* sp. belonged to the saprotroph–symbiotroph group and were isolated only during the winter rest and mostly under organic farming systems.

3.5 Discussion

The beneficial interaction between plants and their phyllosphere endophytes is well recognized, yet relatively few studies have explored the functional potential of endophytic communities associated with olive trees in Mediterranean environments. This study aimed to establish a collection of beneficial microorganisms for potential application in olive cultivation to enhance plant tolerance to abiotic and biotic stresses. Focusing on the culturable fraction of the olive endophytic microbiota, we leveraged the plant's natural selection of symbiotic partners to isolate microorganisms with multiple PGP traits, ultimately identifying candidates for future bioinoculant development. To maximize microbial diversity, we sampled different olive cultivars representative of the local germplasm, cultivated under varying pedoclimatic conditions and either organic and conventional farming systems, and including wild olive accessions, across different phenological stages. A sampling protocol including the minimal standard number of biological replicates (three olive trees) was adopted, as applied in similar studies [41]. To recover the culturable fraction of microbial communities, we prepared leaf and twig pools prior to surface disinfection. This procedure is a standard method for isolating endophytes from woody crops [42], ensuring efficient sample processing within 24 h. It is important to note that this work does not seek to provide a comprehensive overview of the entire olive endophytic microbiota but rather to define optimal conditions for exploring the culturable fraction associated with some of the most widespread Sicilian varieties, identifying potential key taxa under different agroecological conditions.

For microbial isolation and maintenance, two general-purpose media (NA and PDA) were selected as widely used to isolate endophytes from plants [13,43–48]. As highlighted by Eevers *et al.* [49], complex media containing glucose and yeast extract foster higher endophyte cultivability than minimal media. Despite this, colony counts remained low across both media, irrespective of sample origin. Isolation efficiency likely depends not only on culture conditions and sterilization protocols but also on the intrinsic rarity, uneven distribution, and varied growth capacities of plant endophytes under aseptic conditions [50].

Moreover, fewer colonies are generally expected when surface disinfection is applied on plant tissues prior to strain isolation compared to soil samples. The use of additional selective, enriched, as well as oligotrophic media could improve future recovery rates as well as allowing the isolation of slow-growing taxa.

Considering that a species-level identification needs to combine morphological and muti-loci phylogenetic analyses [51], particularly for bacterial and fungal isolates, identification was limited at genus level for diversity analyses. The single target molecular identification used in this work did not allow us to clarify the taxonomic position of "unknown" isolates, which could represent novel taxa beneficial to the olive host. Future phylogenetic studies could better define their taxonomic position. Lacking sufficient genetic information and experimental evidence for pathotroph isolates, it was not possible to evaluate their real pathogenicity.

Many isolates recovered in this study have been previously reported as olive endophytes with plant-beneficial properties [14,46,52–55]. Notably, genera such as *Ectobacillus*, *Geomyces*, and *Tritirachium* were identified in olive trees for the first time, though they have been described as endophytes in other plant species [52,56]. According to the literature and based on our results, the isolates Ef_GIAL02F, Geosp_NMB03R, and Tritb_SYLV06F could play a role in thermal regulation, adaptation, and plant protection through enzyme and antibiotic production. The microbiota of Sicilian olive cultivars was predominantly composed of *Proteobacteria*, *Firmicutes*, and *Actinobacteria*, alongside fungal members of the *Ascomycota* phylum. Similar profiles have been reported in previous studies on Mediterranean olive trees [14,57,58], with *Proteobacteria*—particularly Pseudomonadales—frequently dominating, along with *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and other *Proteobacteria* isolated from cultivated and wild olive xylem sap [57]. Similarly, a higher prevalence of *Proteobacteria* was observed in Spanish cultivars and Portuguese wild olive trees [58]. The recovered fungal community was rich and largely composed of Dothideomycetes and Sordariomycetes, in agreement with earlier findings on Portuguese and Spanish olive orchards [13,59].

By accounting for host type (cultivated vs. wild), plant organs, phenological stages, and farming systems, this study sought to identify optimal conditions to build a representative collection reflecting the natural diversity of olive endophytes. Previous research by Müller *et al.* [58] compared the microbiota of cultivated and wild olive trees across Mediterranean

regions, revealing that both genotype and geographical origin shape bacterial endophyte communities. Their analysis showed that wild olives clustered with cultivars from the same region, yet distinct taxonomic groups differentiated cultivated from wild trees. Our findings align with this pattern: Sicilian wild olive accessions formed distinct groupings compared to domesticated varieties while maintaining a closely related microbiota characterized by the presence of the class Eurotiomycetes, the family Pleosporineae, and the genera *Acinetobacter*, *Chaetomium*, *Peniophora*, *Preussia*, *Priestia*, *Providencia*, and *Tritirachium*. This result supports the hypothesis that domestication has progressively reduced microbial biodiversity. Historically, the selection of cultivated plants has prioritized agronomic and productive traits, often overlooking their capacity to establish beneficial relationships with soil and plant-associated microorganisms. Modern varieties, bred for high yields under intensive management, may have lost traits involved in hosting growth-promoting and pathogen-antagonistic endophytes. In contrast, wild relatives typically maintain richer and more diverse microbial communities [17]. Despite this, a conserved "core" microbiota persists across both wild and cultivated olives, which is functionally essential for plant fitness through genes acquired by evolutionary selection [60]. Within this core, the keystone taxa, *i.e.*, highly connected taxa that influence community structure and function irrespective of their abundance across space and time [61], play a crucial role in ecosystem stability, nutrient cycling, pathogen suppression, and plant health [40]. Meanwhile, less abundant and transient satellite taxa contribute to disease resistance, resilience, and functional redundancy [62]. In our study, while 'Nocellara' and wild olive trees shared a core microbiota represented by *Bacillus*, *Staphylococcus*, and *Quambalaria*, wild accessions harbored greater microbial diversity, likely reflecting their adaptation to more heterogeneous and unmanaged environments.

Regarding plant organs, twigs hosted a higher number of taxa than leaves, a trend well documented across plant species. A meta-analysis by Harrison and Griffin [63] confirmed the predominance of endophytic fungi and bacteria in woody stems over other tissues. This was also evident in our study, with most culturable fungal species isolated from twigs, likely due to the richer presence of structural carbohydrates that promote fungal colonization [64]. Seasonal variation in olive endophytic communities remains poorly explored [12,46,65]. In line with Abdelfattah *et al.* [65], we recorded peak species richness and diversity during the winter rest period, dominated by fungal isolates—a pattern also reported for other

Mediterranean woody crops [44]. According to results reported by Gomes *et al.* [13], climatic conditions typical of Sicilian winters, including higher winds, rainfall, and marked temperature–humidity fluctuations, likely enhance microbial dispersal and colonization. Notably, microbial composition shifted during sampling, favoring *Bacillus* and pigmented taxa, groups widely acknowledged for their stress tolerance and ecological versatility (mesophilic nature and endospore formation), making them valuable biofertilizers [66–68]. Most studies on farming practices and plant microbiota have focused on soil communities, but relatively few have addressed endophytes under different cultivation systems. Rhizosphere diversity is known to depend on soil properties, plant species, and management system, often increasing under organic practices [69]. Supporting this, Sofo *et al.* [70] and Wentzien *et al.* [18] found higher microbial abundance and diversity in organic olive orchards. Although focused on phyllosphere endophytes, our work also revealed greater microbial richness and abundance among plants under organic management.

While studies have begun to describe the olive root microbiome and its role in enhancing plant physiology [53,71–73], knowledge of the functional traits of olive phyllosphere endophytes remains limited. In this study, bacterial endophytes exhibited multiple PGP traits, including siderophore production, biofilm formation, auxin (IAA) synthesis, nitrogen fixation, and hydrolytic enzyme activity. Iron is a key nutrient for plant metabolism [74,75]. Microorganisms assist in its acquisition by producing siderophores that chelate ferric iron [76]. In our collection, 76.2% of bacterial isolates produced siderophores, with *Bacillus*, *Sphingomonas*, and *Staphylococcus*, mainly isolated from organic management, being predominant. While siderophore production by *Bacillus* and *Staphylococcus* has been documented in the olive rhizosphere [73], this is the first report of siderophore-producing *Sphingomonas* endophytes in olives, expanding their documented plant-beneficial roles [77]. Over 80% of isolates formed biofilms, with *Sphingomonas*, *Bacillus*, *Providencia*, and *Kocuria* showing the strongest capacity, in agreement with their known contribution to stress resilience and crop productivity [78,79]. Auxin IAA production was observed in 38% of isolates mostly collected in organic olive orchards and wild accessions, with *Priestia*, *Bacillus*, and *Providencia* being the most active producers. These findings are consistent with reports associating these genera with plant growth promotion, nitrogen fixation, and biocontrol activities against phytopathogenic nematodes and fungi [80–85]. Nitrogen fixation capacity was demonstrated by over 60% of isolates, including *Acinetobacter*,

Bacillus, *Paenibacillus*, *Priestia*, *Pseudomonas*, *Sphingomonas*, and *Staphylococcus*, which are generally found regardless of management practices. These genera have previously been recognized as nitrogen fixers and potential biofertilizers [77,86–89]. Hydrolytic enzymes such as DNase, amylase, cellulase, chitinase, lipase, protease, and pectinase, involved in microbial colonization and plant protection, were widely produced among isolates. *Bacillus* showed the greatest enzymatic activity, followed by *Frondihabitans*, *Paenibacillus*, *Pseudomonas*, and *Sphingomonas*. *Bacillus* and *Pseudomonas* are the most prevalent PGP bacteria for enzymatic activities, with *Bacillus*-based biostimulants being widely commercialized due to their efficient metabolite synthesis [90]. The ecological role of *Bacillus* endophytes can include mechanisms of competitive exclusion such as resource competition, competition for attachment sites, and the production of antimicrobial compounds. The dominant presence of the genus *Bacillus* could be attributed to its ecological trade-offs as well as to selective recruitment by the host. *Bacillus* protein toxins and metabolites enhance colonization while exerting antagonistic effects on plant pathogens and insect pests [91,92]. Antifungal, antibacterial, and algicidal activities of siderophores are well documented [93–97]. *Bacillus licheniformis* lipopeptides, cell lytic enzymes, and siderophores contribute to the inhibition of pathogenic fungi [98,99]. In this study, *Bacillus* Bl_SYLV02R, characterized by siderophore and biofilm production as well as multiple enzymatic activities, showed significant antifungal activity against the olive pathogen *Neofusicoccum vitifusiforme*. Further studies are warranted to elucidate the mechanisms underlying this antifungal capacity and to assess the performance of these PGP isolates in planta, both individually and as microbial consortia.

3.6 Conclusions

In conclusion, wild olive trees, twig samples, and winter sampling were the optimal conditions to build a variegated collection of Sicilian olive culturable endophytes. Twigs hosted significantly higher endophyte abundance than leaves, and uncultivated olive trees could be considered as custodians of higher biodiversity. Organic management of olive yards seems promising for a richer functional endophyte collection, although further studies should be performed. Potential plant growth-promoting features for nutrient uptake, plant growth promotion, and protection against fungal diseases were demonstrated by bacterial endophytes, which would improve the overall fitness of the host plants.

The potential role of these Sicilian olive endophyte communities needs to be further investigated, evaluating their possible use as native microbial consortia in sustainable cultivation practices. Future applications for sustainable agriculture could be enhanced by integrating the native microbial communities of both cultivated and wild olives, selecting microbial consortia for plant growth promotion, biocontrol, and tolerance/resistance to biotic stresses.

Supplementary Materials: The following supporting information can be downloaded at https:// www.mdpi.com/article/10.3390/microorganisms13071502/s1. Figure S1: Sampling sites; Figure S2: NMDS plots; Figure S3: Rarefaction curves; Table S1: Endophyte molecular identification; Table S2: Diversity indices during phenological phases; Table S3: Diversity indices in farming systems; Table S4: PGP properties; Table S5: Enzymatic activities; Table S6: Antagonistic abilities; Table S7: Functional diversity of fungal endophytes isolated from Sicilian olive tree, according to FUNGuild database.

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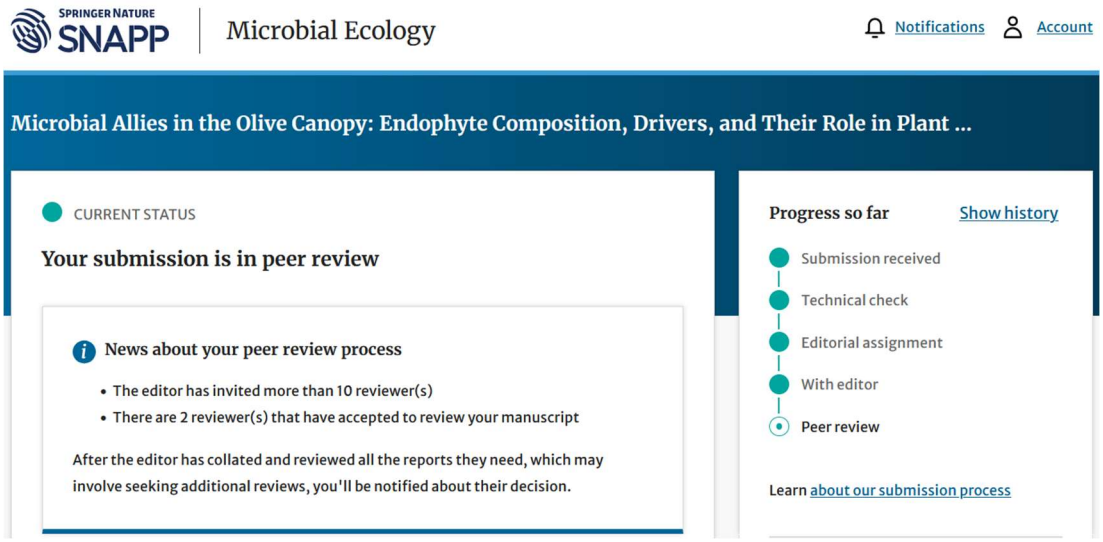
4. CHAPTER 4

Review: Microbial Allies in the Olive Canopy: Endophyte Composition, Drivers, and Their Role in Plant Protection

This review study was submitted to Microbial Ecology (Springer) on July 24, 2025, and has been accepted for peer review.



4.1 Abstract

The olive tree (*Olea europaea* L.) hosts diverse endophytic microbial communities that contribute to its resilience, productivity, and adaptation to environmental stressors. Since the temperature increases caused by global climate change primarily affects the aerial part of the plant, this review synthesizes current knowledge on the diversity, composition, and ecological drivers of olive phyllosphere endophytes, with a focus on bacterial and fungal communities. We highlight the role of host-related factors—including plant genotype, organ specificity, age, and phenological stage—in shaping microbiota structure across spatial and temporal scales. Genotype consistently emerges as a major determinant of microbial composition, while leaves and twigs harbor distinct yet overlapping communities. Geographic location, environmental variables, and seasonal shifts significantly influence microbial assemblages, with closer sites often supporting more similar communities. We also discuss the impact of agricultural practices and biotic and abiotic stressors on microbiota stability and function. Notably, several cultivable taxa—including *Bacillus*, *Paenibacillus*, *Pantoea*, *Aureobasidium*, and *Penicillium*—exhibit antagonistic activity against key olive

pathogens, underscoring their potential as biological control agents. We conclude by emphasizing the need for functional studies to elucidate the roles of keystone endophytes and to inform microbiome-based strategies for sustainable olive cultivation.

4.2 Introduction

The olive tree (*Olea europaea* subsp. *europaea* var. *europaea*) has been cultivated for millennia across the Mediterranean basin, where it holds a central role not only in agriculture, but also in the economic and cultural heritage of the region [1]. Renowned for its adaptability, the olive tree thrives under a wide range of environmental conditions, including those characterized by abiotic stresses such as prolonged drought [2], high soil salinity [3, 4], and nutrient-poor substrates [5]. To cope with this challenging environment, olive trees have evolved intricate relationships with their associated microbiota, particularly with the microbial communities inhabiting the phyllosphere.

The phyllosphere—the aerial habitat encompassing leaves, stems, flowers, and fruits—constitutes a dynamic ecological niche where plant tissues interface directly with surrounding microbial communities. This compartment supports a complex network of interactions, many of which are mediated by endophytic microorganisms that colonize internal plant tissues without eliciting disease symptoms. These endophytes contribute significantly to plant fitness by enhancing nutrient acquisition, conferring tolerance to abiotic stresses, and suppressing pathogens [6]. Unlike root-associated microbiota, which are predominantly shaped by edaphic properties such as soil composition and nutrient availability [7, 8], phyllosphere communities are primarily shaped by host genotype, environmental exposure, seasonality and agrochemical treatments.

Deciphering the composition and function of the olive phyllosphere microbiota is essential for harnessing its full potential in sustainable crop production. The increasing global interest in microbiome-informed strategies for enhancing plant resilience and productivity highlights the need for a deeper understanding of these microbial partners and their ecological roles. Recent developments in high-throughput sequencing and omics-based technologies have provided unprecedented insights into the taxonomic and functional diversity of bacterial and fungal communities in the olive phyllosphere [9–14], revealing their potential in promoting plant health and mitigating disease impact.

In this review, we focus specifically on the phyllosphere microbiota of olive trees, while fully recognizing the critical importance of root-associated microbial communities. Our intent is not to diminish the role of belowground microbiota, but rather to underscore the unique ecological features and functional roles of microbial communities inhabiting the aerial plant environment which are gaining increasing interest because they are more sensitive to climate change. Although the rhizosphere and phyllosphere are interconnected via plant physiological processes, they are shaped by distinct environmental filters and selective pressures, resulting in functionally and compositionally divergent microbiomes.

Root-associated endophytes are strongly influenced by soil texture, organic matter content, and microbial interactions in the rhizosphere [15], whereas phyllosphere microbes are primarily affected by environmental exposure, atmospheric dynamics, UV radiation, humidity, and temperature fluctuations, as well as host-derived factors such as surface exudates and cuticle characteristics. Given these fundamental differences, we argue that a dedicated analysis of phyllosphere microbiota is necessary to accurately assess their contributions to olive tree health. Future research should aim to integrate both below- and above-ground microbial compartments in order to achieve a comprehensive understanding of olive-microbiome interactions. However, within the scope of this review, we offer a detailed examination of the phyllosphere endophytic microbiota and its ecological relevance, with a particular emphasis on its potential application in biological control and climate-resilient olive cultivation.

4.3 Drivers of olive microbiota communities

The structure of the olive-associated microbiota results from intricate interactions among the host plant, microbial communities and a range of environmental drivers, including climatic conditions, agronomic practices, particularly agrochemicals treatments to the canopy and both biotic and abiotic stressors. This section provides an overview of current scientific understanding concerning the main host and environmental factors that influence the composition and diversity of the endophytic microbiota in the olive phyllosphere (Figure 1). The synthesis includes evidence from both classical plant–microbe interaction studies based on culturable microorganisms, and more recent meta-omics approaches, which have significantly expanded our capacity to characterize microbial diversity, structure, and function under varying ecological and genetic contexts.

4.3.1 Host factors influencing endophytic microbiota of olive trees

4.3.1.1 Plant genetics

Studies on the olive phyllosphere microbiota have been primarily conducted in Mediterranean countries with a well-established history of olive cultivation (such as Spain, Italy, Portugal, and Greece) and more recently in Brazil. These investigations, often adopting comparable sampling strategies and analytical pipelines, have produced a comprehensive depiction of the microbial assemblages associated with different olive genotypes. Olive orchards are recognized as rich microbial reservoirs, hosting a variety of bacterial and fungal communities across different cultivars and plant genotypes. According to several studies [9–14, 16–23], the olive phyllo-endosphere is predominantly inhabited by bacterial phyla such as Proteobacteria, Actinobacteria and Firmicutes, and fungal communities mainly from the phylum Ascomycota.

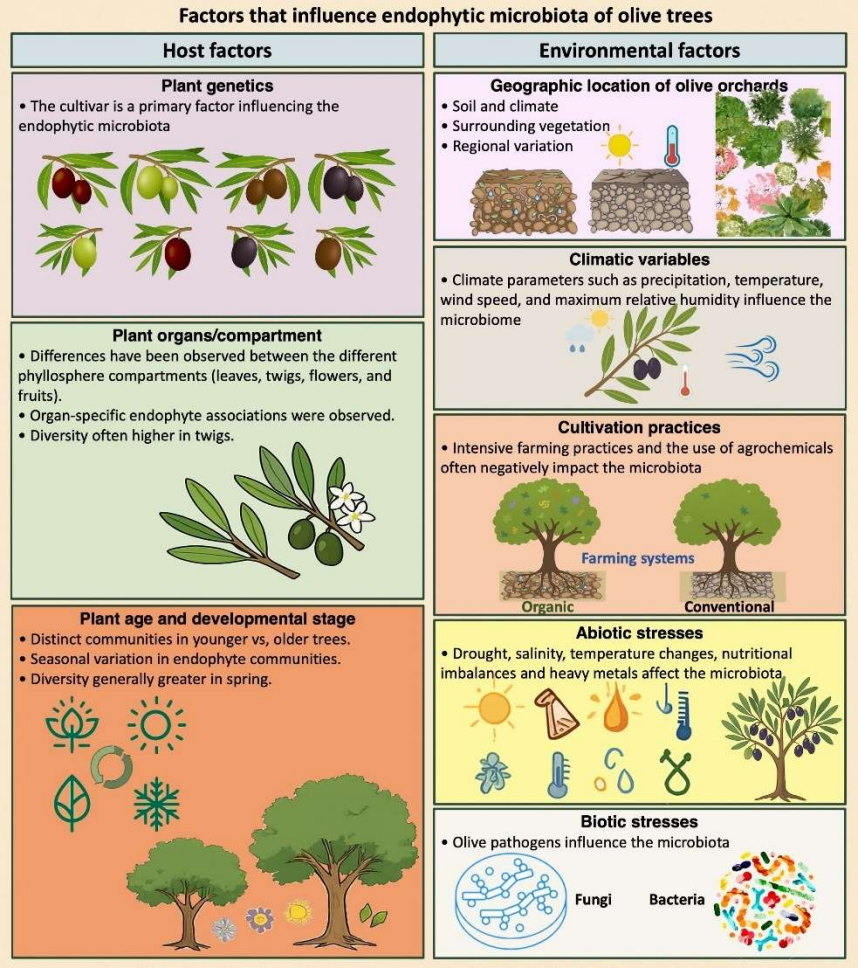


Fig. 2 Host and environmental factors that influence the composition and diversity of the endophytic microbiota in the olive phyllosphere

The role of host genotype and geographic origin in shaping the structure and function of endophytic communities was first highlighted by Müller *et al.* [10] through a study conducted in a Spanish olive orchard. The authors analyzed microbial communities from ten cultivated olive varieties of Mediterranean origin and nine wild olive accessions from Cyprus, Greece, and Madeira Island. Using 16S rRNA gene amplicon sequencing, the study demonstrated a strong correlation between the microbial composition and the geographical origin of the olive genotypes, which reflected a broader differentiation between "Eastern" and "Western" Mediterranean regions. Notably, wild olive trees from the same regions as their cultivated counterparts harbored similar endophytic communities, suggesting the presence of a regional microbial signature conserved across domestication boundaries. The bacterial taxa were dominated by Alpha-, Beta-, and Gammaproteobacteria, followed by Firmicutes, Actinobacteria, and Bacteroidetes. Bacteria *Pelomonas* sp., *Ralstonia* sp., *Pseudomonas* sp., and *Actinobacter* sp. were considered the main representatives of the putative core microbiota.

In Portugal, Mina *et al.* [19] examined the impact of plant genotype at cultivar level on bacterial communities within the phyllosphere. The study, based on culture-dependent techniques, was carried out in three orchards located in the Mirandela region, comparing two widely cultivated olive varieties: ‘Cobrançosa’ and ‘Verdeal Transmontana’. Results revealed that host genotype significantly influenced both the diversity and composition of bacterial communities. Specifically, ‘Verdeal Transmontana’ hosted a greater abundance and richness of endophytes, along with higher alpha diversity, compared to ‘Cobrançosa’. Distinct microbial signatures were associated with each cultivar: *Pseudomonas aeruginosa*, *Pseudomonas graminis*, and *Brevundimonas* sp. were more frequently recovered from ‘Cobrançosa’, whereas *Pantoea vagans*, *Pantoea brenneri*, and other *Pseudomonas* species predominated in ‘Verdeal Transmontana’. These findings support the hypothesis that host genotype acts as a selective filter in shaping the phyllosphere microbiome.

Anguita-Maeso *et al.* [20] analyzed the xylem sap microbiota of two widely cultivated Spanish olive varieties, ‘Arbequina’ and ‘Picual’, and reported significant differences in bacterial composition. Notably, a higher number of unique bacterial genera were detected in

‘Picual’, reinforcing the hypothesis that plant genotype exerts a selective influence on the structure of endosphere microbiota.

To further investigate genotype-driven variation across different plant compartments, Malacrinò *et al.* [13] examined bacterial communities in fruits, leaves and surrounding soil of two olive genotypes (cv. ‘Sinopolese’ and cv. ‘Ottobratica’), revealing that plant genotype was a key determinant in shaping microbiota composition. Each compartment harbored distinct microbial assemblages, and a significant genotype × compartment interaction was observed. The influence of genotype was particularly pronounced in fruit tissues, with *Pseudomonas* and *Escherichia-Shigella* more abundant in ‘Sinopolese’, and Raoultella and Klebsiella in ‘Ottobratica’.

In one of the earliest studies on fungal endophytes of olive fruits, Preto *et al.* [24] investigated two Portuguese cultivars with differing susceptibilities to olive anthracnose: the highly susceptible ‘Madural’ and the moderately tolerant ‘Verdeal Transmontana’. The fungal community composition and abundance varied significantly between cultivars. Cultivar ‘Madural’ had lower colonization rates and species evenness, with Gibberella dominating. In contrast, *Neofabraea vagabunda* was exclusive to ‘Verdeal Transmontana’, suggesting a genotype-driven selection of endophytic fungal communities. Further evidence for genotype effects on fungal diversity was provided by Materatski *et al.* [18], who isolated endophytes from olive leaves of three cultivars (‘Galega vulgar’, ‘Cobrançosa’, and ‘Azeiteira’) collected from different sites in the Alentejo region of southern Portugal. The results showed that both endophytic richness and diversity varied significantly by cultivar and location, with ‘Galega vulgar’ exhibiting particularly low evenness, indicating that host genotype interacts with local environmental conditions to shape fungal community structure.

Costa *et al.* [11] employed ITS1 amplicon sequencing to analyze the fungal communities of the phyllosphere (leaves and twigs) in five olive cultivars (‘Cobrançosa’, ‘Galega vulgar’, ‘Madural’, ‘Picual’, and ‘Verdeal Transmontana’). Their results confirmed that host cultivar was the most significant driver of fungal community assembly. Although taxa from the phyla Ascomycota and class Dothideomycetes were predominant across all cultivars, distinct fungal profiles emerged for each genotype, suggesting the coexistence of a conserved core microbiota and cultivar-specific components.

In a biogeographically distinct context, Ngubane *et al.* [25] conducted the first comparative study of fungal endophytes in two subspecies of *Olea europaea* from South Africa, namely the native African olive (*O. europaea* subsp. *cuspidata*) and the introduced cultivated European olive (*Olea europaea* subsp. *europaea* var. *europaea*). Twigs were sampled across six locations in the Western Cape Province. Native trees harbored more diverse and species-rich fungal communities. Moreover, distinct clustering patterns were observed between the two subspecies, with European cultivars forming more homogeneous groups. These findings highlight that host identity shapes endophytic assemblages and that cultivated varieties may lose or acquire endophytes during geographical relocation.

A genotype effect was also documented by Hanani *et al.* [22] in Apulia, southern Italy. The authors investigated culturable endophytes in the sapwood of three cultivars with varying susceptibility to *Xylella fastidiosa*: the resistant 'Leccino' and the susceptible 'Ogliarola salentina' and 'Oliva rossa'. 'Leccino' exhibited significantly higher bacterial richness and a distinct profile of dominant genera such as *Bacillus*, *Methylobacterium*, and *Paenibacillus*. Fungal colonization and endophyte density also varied among cultivars. 'Leccino' hosted larger populations of commonly found genera including *Aspergillus*, *Cladosporium*, *Fusarium*, and *Pithomyces chartarum*. In contrast, de Oliveira *et al.* [12] found that the phyllosphere microbiota of five Brazilian olive cultivars ('Arbequina', 'Arbosana', 'Ascolana', 'Koroneiki', and 'Grappolo') was not significantly influenced by genotype in terms of abundance or composition. The authors attributed this to the wide geographic distances between sampling sites, which were located in different Brazilian states. Supporting the role of regional specificity, Crucitti *et al.* [14] employed high-throughput sequencing to assess endophyte diversity in twigs from three Sicilian cultivars ('Nocellara del Belice', 'Nocellara Etnea', 'Nocellara Messinese') as well as from wild olive trees (*O. europaea* var. *sylvestris*). While alpha diversity was not significantly influenced by host type, fungal community composition clearly diverged between wild and cultivated olives. Wild olives clustered separately and hosted unique taxa such as *Robbsia, Kineosporia, Bryocella, Phallus, Orbilia, Neopyrenopeziza, Bellamyces,* and *Stagonospora*. In a related culture-dependent study, Crucitti *et al.* [23] confirmed these findings: wild olives exhibited higher bacterial and fungal diversity than cultivated ones. Genera such as *Bacillus*, *Staphylococcus*, and *Quambalaria* were identified as part of the core culturable microbiota of Sicilian olives.

Overall, olive cultivar plays a pivotal role in structuring both fungal and bacterial endophytic communities in the phyllosphere. The evidence suggests that cultivar-specific features—such as tissue chemistry, morphological traits, and defense mechanisms—act as selective filters, shaping the composition and ecological function of resident microbial assemblages.

4.3.1.2 Plant organs/compartments

An important dimension in understanding endophytic diversity within the olive phyllosphere is the specific plant organ or tissue from which microbial communities are recovered. Different organs provide distinct microenvironments, potentially selecting for organ-specific microbial assemblages. Mina *et al.* [19] investigated the influence of leaf versus twig tissues on culturable bacterial communities in the Portuguese cultivars 'Cobrançosa' and 'Verdeal Transmontana'. They found that the effect of plant organ on bacterial composition was less pronounced than that of host genotype or the structure of the epiphytic microbiota. Nonetheless, some degree of organ-level differentiation was evident, with differences more evident in leaves than in twigs: *Ochrobactrum* was predominantly associated with leaf tissues, while *Brevundimonas* characterized twig-associated communities. The limited distinction between leaf and twig endospheres may be due to their shared physiological roles and similar endospheric conditions. Using a metabarcoding approach, Abdelfattah *et al.* [9] found that the endophytic fungal consortia associated with leaves, flowers, and fruits of *Olea europaea* subsp. *europaea* var. *europaea* were most distinct in the leaf samples, which clearly segregated from all other organs. The higher fungal colonization in leaves may be attributed to their constant presence on the tree, their large surface area relative to volume compared to fruits, and their longer lifespan compared to both flowers and fruits. Martins *et al.* [16], in a study across nine olive groves of cv. 'Cobrançosa' in the Bragança District (Northeast Portugal), and Costa *et al.* [11], analyzing several cultivars ('Cobrançosa', 'Galega vulgar', 'Madural', 'Picual', and 'Verdeal Transmontana') in the Iberian Peninsula, found that fungal colonization rates were higher in leaves than in twigs. However, the number of fungal taxa was greater in twigs. Although fungal communities from leaves and twigs formed a tightly clustered and compositionally similar group, some taxa were exclusively isolated from one organ: *Trichoderma gamsii*, *Trichoderma* sp. 1, *Epicoccum nigrum*, and *Penicillium canescens* were found only in twigs, while *Paraphoma chrysanthemicola*, *Alternaria arborescens*, *Alternaria alternata*, and *Penicillium restrictum*

were exclusive to leaves. One possible explanation for the observed overlap between leaf and twig microbiota is the vegetative propagation of olive trees and their common exposure to aerial inocula, facilitating horizontal transmission of microbial taxa across organs. Despite this overlap, the structural and biochemical differences between tissues can still shape organ-specific microbial consortia.

Gomes *et al.* [17] also reported high compositional similarity between leaf and twig fungal endophytes in Portuguese olives but found significantly greater abundance, richness, and diversity in twig samples. This was hypothesized to result from the structural robustness of twig tissues, which may offer a more stable and protected niche, less affected by external environmental stressors compared to the more delicate and exposed leaf surfaces.

The greater endophytic richness associated with twigs was further confirmed by Crucitti *et al.* [23], who evaluated culturable endophytes in cultivated and wild Sicilian olive trees. The study showed that 35.7% of bacterial genera—including *Acinetobacter, Frondihabitans, Kocuria, Priestia,* and *Providencia*—and 55.5% of fungal genera—including *Alternaria, Acremonium, Chaetomium, Diaporthe, Dydimella, Elsinoe, Geomyces, Nemania, Neosetophoma, Paraconiothyrium, Peniophora, Peziza, Phoma, Stemphylium,* and *Tricharina*—were isolated exclusively from twig tissues. These findings underscore the importance of twigs as a reservoir of endophytic diversity in the olive phyllosphere.

Beyond vegetative tissues, reproductive organs have also been shown to harbor unique microbial assemblages. Martins *et al.* [21] analyzed flower buds, flowers, and fruits of cv. 'Madural' and found clear differences in fungal community composition and richness across developmental stages and tissue types. *Biscogniauxia mediterranea* and *Cladosporium cladosporioides* were predominant in flower buds, while flowers were characterized by *Pezizomycetes* sp. and *Diaporthe rudis*. Fruits hosted a distinct community, dominated by *Epicoccum nigrum*, *Trametes* sp., and *Neofabraea vagabunda*. These results reinforce the idea that plant organs impose selective pressures that shape the composition and ecological roles of associated endophytic microbiota.

4.3.1.3 Plant age and developmental stage

The influence of olive tree age on the composition and diversity of associated microbial communities has been, to date, only marginally investigated. One of the first studies

addressing this factor was conducted by Anguita-Maeso *et al.* [20], who compared the xylem sap microbiota of adult olive trees (10 years old) and young plantlets (1 year old) of the cultivars 'Picual' and 'Arbequina'. Their results revealed significant age-related differences in microbial assemblages. Specifically, adult trees exhibited higher bacterial diversity, as measured by Shannon alpha-diversity, and a greater number of unique bacterial genera not shared with their younger counterparts. To further evaluate the influence of age, de Oliveira *et al.* [12] analyzed microbial diversity and composition in Brazilian olive orchards. Leaf-associated bacterial communities from eight-year-old trees differed significantly from those of four-, five-, and seven-year-old trees. Both alpha and beta diversity of associated fungal communities also varied with plant age. The main microbial taxa contributing to these differences were *Stenotrophomonas* and *Achromobacter* (bacteria), and *Pseudocercospora*, *Hyphozyma*, and *Symmetrospora* (fungi). These findings underscore the importance of further research on age-related microbiota shifts in olive trees, ideally involving a broader range of genotypes and agronomic conditions.

These findings suggest that microbial community structure evolves over time, likely influenced by cumulative environmental exposures and physiological changes in the host. Age-related differences in phyllosphere endophytes may be attributed to changes in leaf and cuticle structure, trichome distribution, volatile compound profiles, hormone levels, and other age-dependent physiological factors.

Microbial community dynamics have also been explored across different developmental (phenological) stages of the olive tree. Seasonal variation in phyllosphere microbiota has been documented in a limited number of studies, with a focus on both culturable and unculturable fractions. Hanani *et al.* [22] assessed culturable endophytes in the sapwood of three cultivars sampled in fall (November), winter (February), and summer (July). They observed pronounced seasonal shifts in bacterial communities, with peak richness in summer. Interestingly, the microbial profiles from fall were more similar to those of winter, suggesting a possible dormancy or stabilization effect during colder months. Fungal communities also varied with season: while winter and fall supported comparable fungal abundance, summer was associated with a marked reduction in fungal isolates. Similar trends were reported by Crucitti *et al.* [14], who analyzed alpha diversity in three Sicilian cultivars ('Nocellara del Belice', 'Nocellara Etnea', and 'Nocellara Messinese') and wild

olives during winter dormancy, full bloom, fruit set, and fruit ripening. Bacterial species diversity and evenness varied significantly across growth stages, with the highest values recorded during winter dormancy. In a complementary culture-dependent study, Crucitti *et al.* [23] isolated bacteria and fungi from leaves and twigs of the same cultivars and wild genotypes, confirming winter as the most productive season in terms of microbial genera recovery, with a predominance of fungal taxa such as *Pyronema*.

The influence of the development stage of the olive host on fungal microbiota has been previously investigated. In the first metabarcoding study on this topic, Abdelfattah *et al.* [9] monitored the fungal diversity of the phyllosphere and carposphere of cv. 'Ottobratica' at four phenological stages (May, June, October, and December). In the phyllosphere, dominance values increased progressively from May to December, while Shannon diversity showed notable variation in flower samples during May, likely due to the transition from flower to fruit. *Devriesia* and *Pseudocercospora* were dominant in October and December, while *Aureobasidium* and *Cladosporium* were more prevalent during the summer. In Portugal, Martins *et al.* [16] studied seasonal variation in culturable fungal endophytes from cv. 'Cobrançosa' across several groves. Sampling occurred once per site from late spring (June) to autumn (November). Colonization frequency and abundance increased from June to November, while fungal richness and diversity decreased. Additionally, the community structure changed: in June, *Phomopsis columnaris* represented 47% of isolates, but dropped to 36% in November, while *Fusarium oxysporum* increased to 35%. *Trichoderma* sp. 1, absent in June, became the third most abundant isolate in November (20%). These findings suggest that some fungal endophytes are progressively established over time, while others may decline or disappear, possibly due to interspecific competition or changes in plant tissue chemistry during phenological transitions.

In a study conducted in Mirandela [17], three olive orchards of different cultivars were sampled during two seasons: autumn (October–November) and spring (March–May). In line with earlier culture-based findings, fungal species richness, diversity (Simpson index), and community composition were significantly greater in spring than in autumn. Specific endophyte families were more strongly associated with spring (Pyronemataceae, Pleosporaceae, Pezizaceae) or autumn (Leptosphaeriaceae, Trichocomaceae).

In southern Portugal, Materatski *et al.* [18] examined seasonal variation in fungal endophytes of three cultivars (‘Galega vulgar’, ‘Cobrançosa’, and ‘Azeiteira’) sampled during spring, summer, and autumn. Unlike previous studies, they observed an increase in the number of isolated fungal operational taxonomic units (OTUs) from spring to autumn, with a significant rise in diversity and dominance in the latter season. These findings suggest that autumn had the strongest influence on fungal endophyte community structure and richness.

This discrepancy with earlier Portuguese studies (*e.g.*, Martins *et al.*, [16]; Gomes *et al.*, [17]), which found higher diversity in spring, may be attributed to differences in sample size and geographic location. Materatski *et al.* [18] sampled 270 trees over three seasons, compared to 63 trees in one-time-point sampling by Martins *et al.* [16], and two seasonal time points by Gomes *et al.* [17]. Furthermore, Materatski's study was conducted in southern Portugal, a region with different climatic conditions—particularly in terms of rainfall and humidity—than the mountainous Bragança and Mirandela areas in the northeast. As reported in the mentioned literature, higher rainfall and relative humidity positively influence fungal endophyte colonization and dispersal.

The dynamics and the underlying processes regulating the endophyte variation in olive trees remain largely unexplored, highlighting the need for further in-depth and geographically diverse investigations.

4.3.2 Environmental factors as drivers of endophytic microbiota assembly of olive tree phyllosphere

Geographic location and local environmental conditions are key determinants of endophytic microbiota structure and diversity in olive trees. These factors often exert a greater influence than topographic variables such as altitude, particularly when sites differ in land use, vegetation cover, or microclimatic conditions.

4.3.2.1 Geographic location of olive orchards

Endophytic bacteria associated with olive leaves in the southeastern region of South America were recently identified by de Oliveira *et al.* [12]. Their study revealed that the diversity of microbial communities in five different cultivars was affected more by geographic location than by altitude, specifically based on differences of position among the six and three farms in the states of São Paulo and Minas Gerais, respectively. Regarding microbial composition,

counties could be distinguished based on the presence of *Stenotrophomonas* and *Achromobacter*. Similarly, both geographic location and altitude affected the diversity, abundance, and composition of fungal communities, with *Hyphozyma* and *Pseudocercospora* identified as the main contributors to inter-county differences for both factors.

Comparable patterns have been observed in Portugal. Martins *et al.* [16] assessed fungal endophyte diversity in leaves of cv. 'Cobrançosa' across three locations in the Trás-os-Montes region: Mirandela, Bragança, and Carrazeda de Ansiães. Fungal richness was highest in Mirandela, and community composition clustered distinctly by location. The distance between sites was positively correlated with dissimilarity in microbial profiles, with *Phomopsis columnaris* and *Fusarium oxysporum* identified as key taxa differentiating the three fungal communities. In southern Portugal, fungal endophyte diversity also varied considerably among olive cultivars across three sites: Vidigueira, Monforte, and Elvas [18]. The lowest fungal richness was recorded in Elvas, indicating a distinct endophyte profile compared to the other two locations and confirming high spatial variability in endophyte diversity.

At broader geographic scales, endophytic microbiota tends to display non-random regional distributions. Factors such as biogeography and soil physicochemical properties likely play important roles in shaping microbial communities, especially when production sites differ significantly in environmental conditions. In general, closely located sites with similar vegetation types often support more similar microbial communities. Ngubane *et al.* [25] found that fungal abundance and community composition in cultivated European olives and surrounding native African olives differed among South African sites, with less variation among nearby locations and those sharing similar vegetation. Since the environment is the primary source of many plant-associated endophytes, local factors such as geographic location and surrounding flora can have a major influence on endophyte community composition [26].

4.3.2.2 Climatic variables

The influence of seasonal changes on olive phyllosphere endophytes has already been discussed, highlighting the role of climate in shaping microbial communities. Among the limited studies investigating this aspect in detail, Gomes *et al.* [17] assessed the effects of

various microclimatic parameters—including mean temperature, maximum and minimum relative humidity, cumulative rainfall, and mean wind speed—on culturable fungal assemblages in olive cultivars in Mirandela. The most influential variable was rainfall, followed by mean temperature, wind speed, and maximum relative humidity, all of which contributed to variation in the endophytic community.

4.3.2.3 Cultivation practices

The expansion of olive cultivation and the growing global demand for olives and olive oil have driven the adoption of high-density orchard systems to increase yield. Most olive groves are managed conventionally, often involving intensive use of agrochemicals and practices that negatively impact soil health and biodiversity.

Pascazio *et al.* [27] investigated the influence of farming systems on the carposphere bacterial endophytes of cv. 'Maiatica'. Using 16S rRNA gene analysis and DGGE, they compared olive pulp (mesocarp) microbiota after 13 years of sustainable versus conventional management in Ferrandina (Basilicata, Italy). Their results showed that sustainable farming supported a greater number of bacterial species, with *Rahnella* spp., *Kluyvera intermedia*, *Averyella dalhousiensis*, *Pantoea* sp., and *Serratia* sp. (Enterobacteriaceae) being the most abundant.

Crucitti *et al.* [14] studied endophytic microbiota in the phyllosphere of Sicilian olive cultivars under organic and conventional farming systems. Bacterial species richness and phylogenetic diversity varied significantly between management types. In organic orchards, where *Wolbachia* and *Sulfitobacter* (Alphaproteobacteria) dominated, bacterial diversity was significantly lower than in conventionally managed orchards. In contrast, fungal community diversity showed no significant differences between the two systems.

Further analysis by Crucitti *et al.* [23] showed that the diversity of culturable endophytes also varied depending on cultivar and farming method. Organically farmed cv. 'Nocellara del Belice' and conventionally farmed cvs. 'Nocellara Etnea' and 'Nocellara Messinese' contributed most to overall endophytic isolate diversity, suggesting cultivar-specific responses to management practices.

4.3.2.4 Abiotic stresses

Environmental changes cause phenotypic shifts in plants, which in turn influence the structure of their associated microbiota. Endophytic microorganisms can support plant adaptation to various abiotic stresses, such as drought, salinity, temperature extremes, nutrient imbalances, and heavy metal contamination [28].

Using a metabarcoding approach, Vita *et al.* [29] documented a significant shift in the leaf endophytic bacterial community of olive trees exposed to moderate salt stress, but not under extreme salt stress conditions. Salt stress notably altered the abundance of bacterial endophytes in the four tested cultivars ('Frantoio', 'Leccino', 'Oliana', and 'Lecciana'), with Burkholderiales and Pseudomonadales showing contrasting trends. 'Oliana' and 'Lecciana' under moderate salt stress also exhibited reduced community diversity. The osmotic changes induced by salinity likely selected for genera such as *Burkholderia* and *Ralstonia*, which are capable of thriving under such conditions.

Interestingly, while bacterial endophytes were negatively affected by high salinity, fungal communities appeared to respond more positively, often replacing bacterial populations under extreme stress.

To date, salinity is the only abiotic factor investigated for its effect on both the phyllosphere and carposphere endophytes in olive trees. More research is needed to assess the impact of other abiotic stresses and to fully understand the role of microbial communities as key allies for plant health and sustainable agriculture.

4.3.2.5 Biotic stresses

Knowledge regarding the diversity and composition of olive-associated endophytes—and how microbial community shifts might select for keystone taxa in response to pathogen or pest invasion—remains incomplete. In this context, the first study to explore fungal communities in olives from cultivars with differing susceptibility to anthracnose, though asymptomatic, was conducted by Preto *et al.* [24]. Anthracnose, caused by several *Colletotrichum* species, primarily affects fruits, ranging from latent infections during flowering to a necrotrophic phase upon ripening [30]. Prior to fruit ripening, these pathogens may exist in the host as endophytes or hemi-biotrophs. In cv. 'Madural', the olive fruit endosphere contained *Colletotrichum* spp., confirming the cultivar's susceptibility to anthracnose. Conversely, in the tolerant cv. 'Verdeal Transmontana', *Colletotrichum* was not

isolated, though another phytopathogen, *Neofabraea vagabunda*—the causal agent of olive leprosy—was found in high abundance. This contributed to the differing microbial composition of endophytes among cultivars. Although no direct correlation was found between cultivar resistance and endophytic fungal community structure, each cultivar shaped its own distinct fungal microbiota, both in terms of phytopathogenic and total fungal communities.

Similarly, the culturable endophytic fungal community of cv. 'Madural' was assessed in two orchards with contrasting levels of anthracnose incidence and severity [21]. Asymptomatic flower buds, flowers, and fruits were collected across the flowering-to-fruit set period. Fungal endophyte abundance and richness were significantly greater in the orchard with low anthracnose incidence, particularly in flowers (both abundance and richness) and fruits (abundance). In high-disease orchards, beneficial fungi were notably reduced in fruit tissues, while commensal fungi were significantly more abundant in flower buds and flowers of low-incidence orchards. Mycobiota composition also differed between orchards, with *Biscogniauxia*, *Cladosporium*, and *Colletotrichum* dominating in high-incidence orchards, whereas *Alternaria* and *Biscogniauxia* prevailed in low-incidence ones. Furthermore, the endophytes *Pseudophaeomoniella oleae*, *Neofabraea vagabunda*, and *Parastagonospora avenae* were positively correlated with *Colletotrichum* spp. in high-incidence sites. The decreased abundance of beneficial fungi may result from antagonistic interactions with the pathogen or be influenced by the presence of other plant pathogens.

Other major fungal pathogens affecting olive canopy include *Venturia oleaginea* (causal agent of peacock spot) and *Pseudocercospora cladosporioides* (responsible for cercosporiosis). Varanda *et al.* [31] used culture-dependent and -independent methods to investigate leaf mycobiota in three cultivars with differing susceptibilities to these diseases, sampling both symptomatic and asymptomatic trees. In southern Portugal (Alentejo region), the most widely grown cultivars—'Galega vulgar', 'Cobrançosa', and 'Picual'—are considered resistant, moderately susceptible, and highly susceptible to *V. oleaginea*, respectively. Excluding the pathogens themselves, fungal richness generally declined in symptomatic trees of 'Cobrançosa' and 'Picual', whereas it increased significantly in 'Galega vulgar'. The resistant cultivar exhibited significantly higher fungal richness and diversity, with five exclusive fungal genera (*Bullera, Cryptococcus, Fusicladium,*

Saccharata, and *Sporobolomyces*), along with *Botrytis*. These taxa contributed to higher fungal richness in symptomatic trees of 'Galega vulgar', suggesting better adaptation and potential antagonistic activity in this cultivar.

Similar observations were made by Vergine *et al.* [32], who characterized the bacterial endophytic microbiota of the resistant cv. 'Leccino' and the susceptible cv. 'Cellina di Nardò', both infected and uninfected with *Xylella fastidiosa*—a xylem-limited bacterium causing Olive Quick Decline Syndrome in southern Italy. Endophytic bacterial diversity in leaves was significantly higher in 'Leccino' than in 'Cellina di Nardò', with fungal differences being less pronounced. The endophytic microbiota of 'Leccino' remained stable regardless of infection, in contrast to 'Cellina di Nardò', where significant shifts occurred in infected trees. Infected 'Leccino' samples hosted bacterial taxa (*Silanimonas*, *Xanthomonadaceae*) absent in healthy samples. Two fungal genera, *Neodevriesia* and *Sphaceloma*, were negatively correlated with *X. fastidiosa*, suggesting potential inhibitory roles.

A comprehensive study by Giampetruzzi *et al.* [33] employed whole metagenome shotgun sequencing (WMSS) and metataxonomics to analyze xylem microbiomes in infected twigs of the susceptible cv. 'Kalamata' and resistant cv. 'FS17' during spring and autumn. While *X. fastidiosa* levels remained low in 'FS17', they increased sharply in 'Kalamata', comprising over 50% of total microbial reads in autumn. This increase in pathogen abundance was linearly correlated with overall bacterial population growth, suggesting pathogen dominance of the bacterial niche in susceptible cultivars. Fungal communities were shaped more by seasonal and environmental factors than by cultivar susceptibility or pathogen load. Overall microbial communities differed more in 'FS17' than in 'Kalamata', especially in spring and in plants with low *Xylella* levels, indicating that the pathogen influences microbiome dynamics more strongly in susceptible hosts.

Hanani *et al.* [22] corroborated the stability of sapwood endophytic communities in resistant cultivars by comparing culturable bacteria and fungi in one resistant ('Leccino') and two susceptible ('Ogliarola salentina', 'Oliva rossa') cultivars. Bacterial richness declined significantly in infected 'Ogliarola salentina', whereas it remained stable in 'Leccino'. Fungal endophyte dynamics were not influenced by cultivar susceptibility or symptom severity. In 'Leccino', *Curtobacterium*, *Bacillus*, *Pantoea* (bacteria), and *Paraconiothyrium*,

Pithomyces, *Cladosporium* (fungi) were abundant regardless of health status, making them promising candidates for biocontrol against *Xylella fastidiosa*—a role already supported by studies in citrus and wheat [34, 35].

Vergine *et al.* [36] further analyzed the phyllosphere microbiota of genotypes with contrasting responses to *X. fastidiosa* in Salento. Asymptomatic or paucisymptomatic plants were compared to symptomatic 'Cellina di Nardò'. Metabarcoding revealed that endophytic structure and diversity were shaped by the presence of *X. fastidiosa* subsp. *pauca*, especially among fungi. Asymptomatic genotypes hosted overrepresented fungal genera (*Pseudocercospora*, *Xenosonderhenioides*) and underrepresented ones (*Venturia*, *Fusicladium*, *Hormodochis*, *Paracucurbitaria*). Susceptible plants exhibited marked reductions in dominant xylem-associated bacterial genera (*Burkholderia, Dolichospermum, Bacillus, Hymenobacter, Sphingomonas*) and changes were also observed for several fungal taxa (such as *Lembosiella, Myriospora, Recurvomyces, Quambalaria, Phaffia,* and *Phaephleospora*). The persistent presence of beneficial endophytes such as *Burkholderia*, *Quambalaria*, *Phaffia*, and *Rhodotorula* in asymptomatic plants highlights the potential role of endophytes in disease tolerance.

Recently, Vergine *et al.* [37] demonstrated that infection by *Xylella fastidiosa* subsp. *pauca* (*Xfp*) modulates the endosphere microbiota of olive trees. The authors investigated the diversity and composition of endophytic communities in the canopy of both resistant ('Leccino') and susceptible ('Cellina di Nardò') olive cultivars, grouped according to different *Xfp* titers (control, low, and high). Proteobacteria and Ascomycota were the predominant groups in control plants, although microbial composition varied depending on the cultivar and infection level. At the genus level, *Pseudomonas*, *Acremonium*, and *Aureobasidium* dominated in healthy plants, regardless of cultivar. In infected plants, the bacterial microbiota was reshaped, with *Enterobacter* emerging as the dominant genus. The presence of the fungal genus *Acremonium* exhibited cultivar-dependent behavior: its abundance increased in the resistant cultivar and decreased in the susceptible one following *Xfp* infection. Similarly, *Aureobasidium* content increased in 'Leccino' in response to infection, while in 'Cellina di Nardò' its abundance varied with plant health status. Overall, microbial biodiversity was more uniform in the resistant cultivar, showing only minor fluctuations across health conditions. In contrast, high *Xfp* infection caused significant

changes in the susceptible cultivar, increasing bacterial diversity while reducing fungal diversity. Moreover, ‘Leccino’ exhibited a higher abundance and diversity of beneficial endophytes, which helped mitigate some of the pathogen's effects, highlighting their potential role in sustainable agriculture.

Olive knot disease (OKD), caused by *Pseudomonas savastanoi* pv. s*avastanoi* (*Psv*), is another major bacterial threat in the Mediterranean region. Gomes *et al.* [38] examined fungal endophytes isolated from three cultivars with different OKD susceptibilities. Fungal diversity was highest in the moderately tolerant cv. ‘Cobrançosa’ and lowest in the highly susceptible cv. ‘Verdeal Transmontana’. The presence of knots (infected twigs) decreased fungal diversity in all cultivars, with the most notable drop in the tolerant cultivar. Host-pathogen interactions and cultivar-specific endophyte communities—likely shaped by resident microbiota, tissue chemistry, or competitive interactions—appear to influence fungal community structure.

Licciardello *et al.* [39] investigated whole endophytic communities in twigs of the highly susceptible cv. ‘Giarraffa’ and resistant cv. ‘Zaituna’ to OKD. Metataxonomic analyses showed higher bacterial richness but lower diversity in ‘Giarraffa’, and vice versa for fungi in ‘Zaituna’. Distinct endophytic profiles emerged, with *Amnibacterium*, *Methylobacterium*, *Sphingomonas*, and *Pithomyces chartarum* dominating in ‘Zaituna’, and *Alternaria*, *Neofusicoccum*, *Ascochyta*, *Elsinoë*, *Devriesia*, *Pseudocercospora*, and *Epicoccum nigrum* in ‘Giarraffa’. These findings suggest that host susceptibility influences pathobiome structure, likely through differential interactions between resident endophytes and the pathogen.

4.4 Phyllosphere endophytes as antagonists of olive pathogens

The olive crop is affected by numerous diseases that often lead to severe yield losses [40]. Some of these diseases are considered highly destructive and devastating. One of the most recent and threatening is Olive Quick Decline Syndrome (OQDS), caused by *Xylella fastidiosa* subspecies *pauca*, a xylem-limited phytopathogenic bacterium. In the plant, *X. fastidiosa* behaves similarly to other vascular pathogens, with symptoms progressing from leaf scorch to complete tree collapse [41, 42]. Olive anthracnose, mainly associated with fungal species *Colletotrichum acutatum* and *C. gloeosporioides*, affects fruits negatively impacting the sensory qualities of olive oil and causing considerable production losses [43].

Another widespread and damaging disease is Verticillium wilt of olive (VWO), caused by the soil-borne fungus *Verticillium dahliae*. This pathogen is particularly difficult to manage due to its ability to produce resting structures (microsclerotia) and the existence of two virulent pathotypes (defoliating and non-defoliating), which enhance its dissemination and epidemiology [44].

Currently, disease management relies heavily on copper-based pesticides, which have limited effectiveness and are not compatible with sustainable agricultural practices [18, 45, 46]. The lack of specific, effective treatments underscores the urgent need for alternative, eco-friendly strategies. One promising approach is the use of biological control agents (BCAs), including antagonistic microorganisms that protect plants through direct inhibition of pathogens (Table 1) or by inducing plant defence mechanisms.

Bacterial and fungal endophytes isolated from resistant Apulian olive cultivars have demonstrated in vitro antagonistic activity against *X. fastidiosa* subsp. *pauca* ST53 [22]. Strains such as *Bacillus subtilis*, *B. pumilus*, *Pantoea agglomerans*, and *Paraconiothyrium brasiliense* significantly inhibited the growth of the pathogen in both dual culture and disc diffusion assays. Notably, *B. subtilis* and *P. brasiliense* successfully colonized the internal tissues of inoculated plants and triggered upregulation of defense-related genes in cultivars 'Leccino' and 'Cima di Mola' [22]. Similarly, Mourou *et al.* [47] screened bacterial endophytes isolated from symptomatic and asymptomatic olive trees of cv. 'Leccino' and cv. 'Ogliarola Salentina', identifying *Paenibacillus naphthalenovorans* as the most effective antagonist, followed by *P. rigui*, *B. subtilis*, *Pseudomonas hibiscicola*, and *B. pumilus*. Cell-free supernatants from *B. subtilis*, *B. pumilus*, and *P. rigui* also showed potent antimicrobial effects, confirming their potential as biocontrol agents.

Several fungal endophytes have also exhibited strong antagonistic effects against *C. acutatum* and *V. dahliae*. Martins *et al.* [48] identified *Hypocrea lixii* and *Paecilomyces lilacinus* as the most effective in inhibiting mycelial growth of these pathogens, while Penicillium commune was able to significantly reduce *C. acutatum* sporulation. In *in planta* assays, *P. commune* (strain CIMO 14FM009) inoculated onto olive branches led to reduced pathogen growth and sporulation, as well as increased release of volatile organic compounds (VOCs) with protective functions [49].

Landum *et al.* [50] evaluated six fungal endophytes from asymptomatic cv. 'Galega vulgar' trees and found that *Nigrospora oryzae* showed strong mutual inhibition with *C. acutatum*. Other isolates such as *Alternaria* sp., *Epicoccum nigrum*, and *Fusarium* sp. also demonstrated inhibitory effects via direct antagonism and VOC emission. Additional promising isolates included *Chaetomium* sp. and *Diaporthe* sp., with the latter producing substances that reduced the radial growth of the pathogen. Preto *et al.* [24] selected *Chondrostereum purpureum* from olives of susceptible cv. 'Madural', which inhibited pathogen growth by 30.9% and, although it did not reduce sporulation significantly, it showed potential for future applications. *Aureobasidium pullulans* (strain CIMO 19DM275) also effectively reduced *C. acutatum* growth, sporulation, and infection severity on detached olive fruits [51].

Further evidence of *A. pullulans* efficacy came from Varo *et al.* [52], who tested four strains from cv. 'Picual' and 'Arbequina'. These strains inhibited mycelial growth of the virulent defoliating pathotype *V. dahliae* V024 by more than 56%. Application of strain AP06 (via foliar spray or irrigation) to olive plants in pathogen-infested soil significantly reduced disease-related mortality. Similarly, *Bacillus amyloliquefaciens*, isolated from European and African olive trees, showed high antagonistic activity against *V. dahliae* V25 in dual culture assays [10]. Finally, *Bacillus licheniformis* Bl_SYLV02R, isolated from wild Sicilian olive twigs and selected for its plant-growth-promoting properties, inhibited more than 40% of the emerging fungal pathogen *Neofusicoccum vitifusiforme* in *in vitro* tests [23].

Pathogen	Endophytes	Origin of endophyte isolation	Olive host	*In vitro* inhibition zone (mm) or growth inhibition (%)	Reference
Xylella fastidiosa	*Bacillus subtilis*	Sapwood	cv. 'Leccino', cv. 'Ogliarola salentina', cv. 'Oliva rossa'	15.2 ± 0.69 (mm)	[22]
	Bacillus pumilus	Sapwood	cv. 'Leccino', cv. 'Ogliarola salentina', cv. 'Oliva rossa'	8.7 ± 0.47 (mm)	[22]
	Pantoea agglomerans	Sapwood	cv. 'Leccino', cv. 'Ogliarola salentina', cv. 'Oliva rossa'	11.82 ± 0.42 (mm)	[22]
	Paraconiothyrium brasiliense	Sapwood	cv. 'Leccino', cv. 'Ogliarola salentina', cv. 'Oliva rossa'	19.28 ± 0.86 (mm)	[22]
	Paraconiothyrium brasiliense	Sapwood	cv. 'Leccino', cv. 'Ogliarola salentina', cv. 'Oliva rossa'	17.3 ± 0.52 (mm)	[22]
	Paenibacillus naphthalenovorans	Twigs	cv. 'Leccino'	38.6 ± 0.94 (mm)	[47]

	Paenibacillus rigui	Twigs	cv. 'Ogliarola salentina'	21.6 ± 0.47 (mm)	[47]
	Bacillus subtilis	Twigs	cv. 'Leccino'	19 ± 0.4 (mm)	[47]
	Pseudomonas hibiscicola	Twigs	cv. 'Leccino'	15 ± 0.4 (mm)	[47]
	Bacillus pumilus	Twigs	cv. 'Leccino'	9.8 ± 0.23 (mm)	[47]
Colletotrichum acutatum	*Hypocrea lixii*	Leaves, branches and roots	cv. 'Cobrançosa'	50%	[48]
	Paecilomyces lilacinus	Leaves, branches and roots	cv. 'Cobrançosa'	38%	[48]
	Penicillium commune	Leaves, branches and roots	cv. 'Cobrançosa'	n.a.	[48]
	Penicillium commune	Twigs	cv. 'Cobrançosa'	*In plant assay	[49]
	Nigrospora oryzae	Leaves of	cv. 'Galega vulgar'	68%	[50]
	Alternaria sp.	Leaves	cv. 'Galega vulgar'	28%	[50]
	Epicoccum nigrum	Leaves	cv. 'Galega vulgar'	25%	[50]
	Chaetomium sp.	Leaves	cv. 'Galega vulgar'	21%	[50]
	Fusarium sp.	Leaves	cv. 'Galega vulgar'	14%	[50]
	Diaporthe sp.	Leaves	cv. 'Galega vulgar'	12%	[50]
	Chondrostereum purpureum	Olive fruits	cv. 'Madural'	30.9%	[24]
	Aureobasidium pullulans	Leaves	cv. 'Cobrançosa' and cv. 'Madural'	39%	[51]
Verticillium dahliae	*Hypocrea lixii*	Leaves, branches and roots	cv. 'Cobrançosa'	55%	[48]
	Paecilomyces lilacinus	Leaves, branches and roots	cv. 'Cobrançosa'	32%	[48]
	Aureobasidium pullulans AP06	Leaves	cv. 'Picual'	57.8%	[52]
	Aureobasidium pullulans AP07	Leaves	cv. 'Arbequina'	56%	[52]
	Aureobasidium pullulans AP08	Leaves	cv. 'Picual'	55.9%	[52]
	Aureobasidium pullulans AP09	Leaves	cv. 'Picual'	58.3%	[52]
	Bacillus amyloliquefaciens	Branches and leaves	Different olive cultivars and wild olive trees	n.a.	[10]
Neofusicoccum vitifusiforme	*Bacillus licheniformis*	Twigs	Wild olive trees	40.3%	[23]

Table 1 Main endophytes from olive phyllosphere involved in biotic stress tolerance

4.5 Conclusions

Current research on the olive tree microbiota highlights the complexity and ecological significance of associated-endophytic communities. Host-related factors, particularly plant genotype, organ specificity, age, and developmental stage, are key drivers in shaping the diversity and composition of endophytic microbial communities in olive trees. Among these, plant genotype consistently emerges as a major determinant, with both bacterial and fungal communities specific to multiple compartments and geographic regions. Endophyte richness and community composition often differ between leaves and twigs, with some genera uniquely associated with one organ. phyllosphere and carposphere harbor distinct microbiota shaped by their temporal and physiological transitions. Older trees consistently harbor more diverse and established bacterial communities, while seasonal changes influence both bacterial and fungal community dynamics. Geographic location plays an important role in microbial community assembly. Regional differences in soil, climate, and surrounding vegetation strongly influence both bacterial and fungal endophyte profiles, with closer sites typically exhibiting more similar communities. Changes across seasons and local microclimates modulate microbial abundance and diversity, highlighting the dynamic and responsive nature of endophyte assemblages to external environmental cues. Responses to agricultural management appear to be taxa- and cultivar-dependent, indicating complex interactions between host genotype, microbial ecology, and farming inputs. Abiotic and biotic stresses have been shown to alter the balance and structure of endophytic communities, reflecting differential resilience across microbial kingdoms. Certain bacterial and fungal taxa potentially act as keystone microorganisms in suppressing or mitigating infection by olive pathogens. In this context, endophytic microorganisms—particularly cultivable strains of *Bacillus*, *Pantoea*, *Paenibacillus*, *Aureobasidium*, and *Penicillium*—have demonstrated antagonistic activity against major olive pathogens such as *Xylella fastidiosa*, *Colletotrichum* spp., *Verticillium dahliae*, and *Neofusicoccum* species. These microbial antagonists act via direct competition, inhibition of pathogen growth, or induction of host defense responses, offering promising prospects for their application as biological control agents (BCAs) in integrated and sustainable disease management strategies. Altogether, these findings highlight the need for deeper functional studies to clarify the roles of keystone endophytes in plant–microbe–pathogen interactions and to explore the use of endophytic consortia in integrated management. Future research should aim to unravel the mechanisms by which microbial communities contribute to host health and defence,

focusing on their metabolic traits, colonization patterns, and synergistic interactions with the host plant, advancing the development of more resilient, eco-friendly, and productive olive crops.

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5. CHAPTER 5

Conclusive Remarks and Future Perspectives

This doctoral research contributes to the knowledge of the endophytic microbiota inhabiting Sicilian olive trees, integrating culture-dependent and culture-independent approaches. The results significantly expand current knowledge on the diversity, composition, and ecological roles of bacterial and fungal endophytes in both cultivated and wild olive germplasm under varying environmental and management conditions.

Metabarcoding analyses provided the most comprehensive characterization to date of the endophytic bacterial and fungal communities inhabiting Sicilian olive tree twigs, highlighting how host identity, phenological stage, and farming practices interact to shape microbiota structure and dynamics. Amplicon-based sequencing revealed highly diverse and complex communities, dominated by Proteobacteria, Bacteroidia, Actinobacteria and Dothideomycetes, alongside a wide range of less abundant but ecologically relevant taxa. A core microbiota of both bacterial and fungal genera was consistently recovered across most samples, yet strong signatures of differentiation emerged depending on biological and environmental contexts.

Alpha and beta diversity analyses demonstrated that bacterial assemblages are significantly modulated by phenological phases and orchard management, with higher species diversity observed in winter and distinct community structures in organically managed orchards compared to conventionally managed or unmanaged wild olives. In contrast, fungal diversity responded more strongly to host type and phenological stages, with wild olives harboring unique genera absent from cultivated varieties. The winter phase consistently represented a turning point for both bacterial and fungal communities, suggesting that dormancy exerts a selective pressure favoring taxa adapted to lower metabolic activity and environmental stress.

Differential abundance and correlation analyses further indicated that endophytic networks are shaped by both cooperative and antagonistic interactions among bacterial and fungal taxa. Positive interactions between bacterial and fungal taxa were more frequent than antagonistic ones, suggesting the existence of cooperative microbial networks within the olive phyllosphere. Notably, certain fungal and bacterial taxa, such as *Neosetophoma rosarum*, *Pseudomonas* spp., and *Kineococcus mangrovi*, exhibited strong positive

associations, while *Sphingomonas* spp. showed negative correlations with known pathogenic fungi. Moreover, *Wolbachia* and *Candidatus* Cardinium were linked to olive cultivars, while several genera with putative beneficial roles (*e.g.*, *Ochrocladosporium*, *Furfurella*, *Robbsia*) were associated with wild olives. Management practices influenced not only the taxonomic composition but also the ecological and functional traits of endophytes. Organic systems were enriched in taxa with symbiotrophic and saprotrophic potential, whereas conventional orchards and unmanaged trees displayed higher abundances of putative pathogens and stress-associated taxa.

Functional predictions supported these patterns, revealing shifts in metabolic pathways across hosts, phenological stages, and farming systems. In particular, processes related to sulfur cycling, methanotrophy, and nitrification were enriched in wild olives and during winter dormancy, indicating a potential role of these taxa in sustaining tree physiology under nutrient-limited or stressful conditions. Fungal trophic mode analysis confirmed a predominance of saprotrophic taxa, but with a significant representation of pathotrophs and symbiotrophs that may influence host health in contrasting ways depending on ecological context.

Overall, these findings underscore the dynamic and multifactorial nature of olive endosphere microbiota. The differentiation observed between cultivars and wild olives, together with the clear imprint of phenological transitions and management regimes, suggests that both evolutionary history and agricultural practices leave lasting signatures on microbial community assembly. By disentangling the ecological forces shaping olive-associated microbiota, this work lays the foundation for exploiting beneficial endophytes as natural allies in sustainable olive cultivation, while also highlighting the need to preserve the unique microbial diversity hosted by wild olive populations.

The culturomic approach enabled the creation of a representative microbial collection from olive twigs and leaves, including strains with promising plant growth-promoting (PGP) traits and antagonistic activity against major olive pathogens, highlighting how host identity, plant organ, and farming practices collectively influence microbial occurrence and diversity.

Overall, twigs represented the most reliable source of endophytes compared to leaves, yielding a higher number of isolates across all olive hosts, growth stages, and management regimes. Organic orchards generally supported higher microbial occurrence than conventional systems, with wild olive trees consistently enriching the endophytic collection.

More than one hundred phenotypically distinct isolates were recovered, with bacteria mainly affiliated to the genera *Bacillus*, *Staphylococcus*, and *Sphingomonas*, and fungi primarily represented by *Quambalaria*, *Alternaria*, and *Biscogniauxia*. Wild olive accessions were a key reservoir of unique taxa, contributing several bacterial and fungal genera absent from cultivated varieties. This finding underscores the evolutionary and ecological importance of wild germplasm in preserving microbial diversity and suggests that wild populations may represent a valuable source of beneficial endophytes for olive health and resilience. Despite such diversity, a putative microbial core composed of *Bacillus*, *Staphylococcus*, and *Quambalaria* was consistently recovered across most hosts, suggesting stable associations within the olive endosphere.

Functional characterization of bacterial isolates revealed that nearly 90% expressed plant growth–promoting (PGP) traits, with many strains displaying multiple properties such as siderophore production, phosphate solubilization, nitrogen fixation, and biofilm formation. These activities highlight the biotechnological potential of olive endophytes in supporting plant nutrition and stress adaptation. Enzymatic assays demonstrated frequent occurrence of hydrolytic activities, particularly amylase, lipase, and DNase, while a subset of *Bacillus* strains exhibited strong protease production. Antagonism tests revealed that *Bacillus licheniformis* Bl_SYLV02R significantly inhibited *Neofusicoccum vitifusiforme*, one of the causal agents of olive branch and twig dieback, pointing to possible applications in biocontrol strategies. These findings support the potential of native olive endophytes as sustainable biocontrol agents against other emerging vascular bacterial (*i.e.*, *Xylella fastidiosa*) and fungal (*i.e.*, *Verticillium albo-atrum*, *Neofusicoccum parvum*) pathogens.

Fungal trophic mode analysis indicated a dominance of mixed pathotroph–saprotroph–symbiotroph lifestyles, reflecting the ecological versatility of olive-associated fungi. While potential pathogens such as *Quambalaria cyanescens* were more frequent in conventional orchards, saprotrophic and symbiotrophic fungi were largely associated with wild olives and organic farming systems, particularly during winter dormancy.

Taken together, these results emphasize the pivotal role of olive endophytes as reservoirs of functional diversity with implications for plant health, adaptation, and sustainable management. The unique microbial taxa recovered from wild olive trees highlight the value of conserving natural populations as sources of beneficial strains. Moreover, the consistent recovery of endophytes with PGP and antifungal properties suggests that culture-dependent

collections represent an important resource for future applications in olive breeding, disease management, and biofertilizer development.

The review component of this thesis further contextualized the experimental results within the broader framework of plant–microbe–environment interactions, emphasizing the role of environmental drivers, agricultural management, and plant compartment specificity in shaping olive microbiota. The synthesis of literature and experimental evidence underscores the strategic value of exploiting beneficial microbial consortia to enhance olive resilience to biotic and abiotic stresses.

The outcomes of this work open new opportunities for the application of endophytic microorganisms in sustainable olive cultivation. Future research should address the following priorities:

- *In planta validation of beneficial strains* – Field trials are necessary to confirm the efficacy of selected endophytes under natural conditions, considering environmental variability and long-term stability of microbial inoculation.
- *Consortium-based biocontrol strategies* – Given the cooperative interactions detected within endophytic communities, the design of microbial consortia rather than single-strain inoculants may provide broader-spectrum and more resilient plant protection.
- *Integration with precision agriculture* – Linking microbiome management with precision farming tools could enable targeted applications of beneficial microbes, optimized for specific phenological stages, climatic conditions, or pathogen pressures.
- *Mechanistic studies on plant–microbe interactions* – Multi-omics approaches, including transcriptomics, metabolomics, and proteomics, should be employed to unravel the molecular basis of endophyte-mediated plant growth promotion and disease suppression.
- *Adaptation to climate change* – Given the increasing impact of drought, heat, and emerging pathogens on Mediterranean olive cultivation, microbial solutions should be tested for their ability to enhance stress tolerance in both traditional and modern high-density orchards.

- *Conservation of microbial diversity* – The microbial collection developed in this study represents an important genetic resource. Its preservation, expansion, and functional characterization will be crucial for future biotechnological applications in olive and other perennial crops.

In conclusion, this thesis demonstrates that the native microbiota of Sicilian olive trees constitutes a valuable reservoir of functional biodiversity that can be harnessed to support plant health, productivity, and resilience. The integration of microbial-based solutions into sustainable olive farming has the potential to reduce reliance on synthetic inputs, mitigate the effects of climate change, and preserve the cultural and economic heritage of olive cultivation in Sicily and beyond.

List of Publications

1. **Crucitti D**, Chiapello M, Oliva D, Forgia M, Turina M, Carimi F, La Bella F, Pacifico D (2022) Identification and Molecular Characterization of Novel Mycoviruses in *Saccharomyces* and Non-*Saccharomyces* Yeasts of Oenological Interest. Viruses, 14(1), 52. https://doi.org/10.3390/v14010052
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   *Fiorella Neri and Dalila Crucitti contributed equally to the paper.
4. **Crucitti D.**, Sutera A., De Michele R., Carimi F., Pacifico D. (2023) Mining the seagrass microbiota: the endophyte community of *Posidonia oceanica* seeds. In: Proceedings of *International Plant Biology Europe meeting* (PBE 2023), 3-6 July 2023, Marseille, France.
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14. **Crucitti D.**, Barone S., Navarro-Torre S., Quatrini P., Carimi F., Caruso T., Pacifico D. (2025) Endophytic Diversity in Sicilian Olive Trees: Identifying Optimal Conditions for a Functional Microbial Collection. Microorganisms, 13, 1502. https://doi.org/10.3390/microorganisms13071502

Oral Presentation in Conference or Congress

1. Varietal identification of mango genotypes using molecular markers. In: Conference of Frutticoltura Tropicale Mediterranea: Ricerca, Innovazione e Trasferimento alle aziende, 6 December 2022, Palermo, Italy.
2. Culturable endophyte isolation and propagation: seasonal dynamics. In: International Conference of Plant microbiomes in sustainable viticulture PROSIT, 22 June 2023, Padova, Italy.
3. Host and Environmental Factors shape the Endophytic diversity and Composition of Sicilian Olive Tree Phyllosphere. In: V Conference AISSA #under 40 – Le scienze agrarie nell'antropocene: dalla produttività alla tutela del patrimonio materiale e culturale, 26-27 June 2024, Florence, Italy.