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Biotechnological strategies for the adaptation of olive trees (*Olea europaea* L.) to climate change

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SUMMARY

The focus of this doctoral thesis is on olive trees (*Olea europaea* L. subsp. *europaea*), the dominant tree crop over large areas of the Mediterranean Basin.

The drought resistance and nutritional efficiency of the olive tree allow it to survive in arid and semi-arid areas where other crops fail (Martins et al., 2024), making it a key species for the economic livelihood of local communities. From an ecological point of view, it provides habitat and food for a variety of species (such as birds, insects and mammals), and its extensive root system stabilises the soil, preventing erosion (Grego, 2022).

Like most crops, the olive tree is facing important problems and challenges related to adverse environmental conditions, either biotic or abiotic ones, a situation that can be worsened in a climate change scenario. In this regard, the most threatened olive crops will be those with sensitive cultivars and management methods that do not maximise their resilience.

Terrestrial agroecosystems and their management can play a key role in mitigating climate change: maintaining the carbon sink role of ecosystems and productive low-emission agriculture is an ambitious challenge given the continuous increase in resource use, on which a constantly growing population depends. The use of sustainable management systems such as organic fertilisation, pruning residue recycling, livestock integration and controlled deficit irrigation has been encouraged in olive agroecosystems to improve ecosystem services and ensure the multifunctionality of olive groves (Palese et al., 2013). Agronomic management can also shape the microbiome associated with olive trees, which in turn influences the dynamics that enable the plant to cope with environmental stress.

The olive tree microbiome, i.e. the heterogeneous population of bacteria, fungi and archaea, that live associated with the host plant, essential for environmental sustainability, is valuable for the growth, health and adaptation of the olive tree to environmental stresses (Avramidou et al., 2024). In fact, some plant growth-promoting microorganisms (PGPMs) can exert direct effects through nitrogen fixation or phosphorus solubilisation or even by modulating hormones. Some microbial groups can act indirectly on the host through mechanisms that lead to induced systemic resistance, osmotic adjustment, siderophore production, antibiotic production, and cell wall-degrading enzymes (Dias et al., 2024). Olive trees have the capacity to undergo “stress imprinting” due to previous exposure to abiotic stress. The responses resulting from this

mechanism depend not only on the plant's intrinsic ability to cope with a specific stress, but also on habitat conditions and the availability of symbionts. Specific plant growth-promoting rhizobacteria (PGPR) in the soil can induce a preconditioned state of high alert, enabling rapid and robust defence responses in the event of stress (Singh et al., 2024).

The literature shows that beneficial microorganisms significantly affect both soil health and crop productivity, by solving several problems related to soil stress, soil fertility, soil degradation and plant growth. The interactions between genotype and the microbiome have only recently been analyzed, and few studies have focused on the particular priming capacity of the olive holobiont, which has nevertheless proven to be an excellent ally in coping with drought stress.

Furthermore, especially in contexts characterised by arid environments, there are few studies that have analysed the contribution of genotype, climatic conditions and management practices at the same time, making it difficult to find a universal model that explains how all these variables influence the composition of the microbiome associated with olive trees.

Therefore, to test the hypothesis that sustainable management systems reshape the taxonomic profiles of the olive tree microbiome and that inoculated microbial consortia (PGPR) induce priming for drought tolerance, this thesis has been organised into six chapters in order to clarify the interactions between the olive microbiome, agronomic practices and resilience/priming capacity in response to water stress.

The first chapter (INTRODUCTION) provides a general overview of olive groves in the Mediterranean, starting with their botanical characteristics, through their history and their current economic and ecological role. Attention is paid to the problem of drought in agriculture and the concept of holobionts is integrated and priming and stress memory strategies in the plant world are defined. The second chapter (STATE OF THE ART) summarises current knowledge, starting with the definition of bacterial and fungal communities in the above- and below-ground parts of the olive tree, and then illustrates how genotype, soil and environmental pressures guide microbial assembly. It then analyses the main olive grove management techniques and their specific impacts on the microbiome, culminating in an examination of the physiological, biochemical and molecular responses activated by the olive tree under water stress, including priming mechanisms against drought. Follow the third chapter (OBJECTIVES) that defines the hypotheses, general and specific objectives of the thesis.

Chapter 4 (RESULTS) contains two research articles. In particular, section 4.1 refers to the paper “*Management affects the diversity and functions of root- and leaf-associated microbiomes: implications for olive resilience*”, published in the journal *Frontiers in Plant Science*. This study reports on the differences in composition and function of the microbial communities of the *Olea europaea* L. subsp. *europaea* 'Ortice' when subjected to different agronomic management practices. While section 4.2 reports the article “*Priming and memory in ancient olive cultivars: Enhancing drought resilience through rhizosphere microbial consortia under repeated stress cycles*” published in the journal *Environmental and Experimental Botany*. This study examined the physiological, biochemical and molecular responses of three olive cultivars (*Olea europaea* L. subsp. *europaea* 'Ortice', *Olea europaea* L. subsp. *europaea* 'Ortolana', *Olea europaea* L. subsp. *europaea* 'Racioppella') when subjected to repeated water deficits. In addition, the resilience effects of a PGPR consortium (*Bacillus subtilis*, *Pseudomonas fluorescens*) were evaluated. The fifth chapter (INTEGRATED DISCUSSION), aims to highlight how agricultural management practices directly influence the assembly of olive tree microbial communities, their ability to cope with environmental stresses (in terms of stress memory) and, the limitations of the studies and possible solutions. The sixth final chapter (CONCLUSION) concludes the manuscript by summarising the main results obtained, and finally the implications of a biotechnological solution for sustainable agriculture.

In this thesis, we observed how conservation-focused management (organic and traditional) increases the microbiome associated with the rhizosphere (enrichment of fungal biomarkers) and improves its functionality (abundant presence of bacteria of the *Actinomycetales* order). The different resilience capacities of three olive tree genotypes under drought conditions were explored ('Ortolana' maintained a certain degree of water stability, 'Ortice' proved to be plastic and adaptive, while 'Racioppella' showed a certain sensitivity). The effect of the PGPR consortium increased the accumulation of osmolytes and antioxidants, and the gene expression of aquaporins was reduced during drought events.

In summary, the results show that the plant microbiome is not just a passive recipient of environmental influences, but a dynamic actor that adapts and, in turn, influences the plant's ability to tolerate stress. This bidirectional interaction, in which microbial communities are shaped by both agronomic management and extreme environmental conditions, is key to understanding the resilience of the plant holobiont (Singh et al., 2024).

Chapter 1 INTRODUCTION

1.1 Olive groves in the Mediterranean basin

The olive tree (*Olea europaea* L.) is one of the oldest and most important crops in the Mediterranean basin, cultivated by the ancient civilizations (Fraga et al., 2021), this crop represents a pivotal point in the traditions, diet, landscape and economy of the countries of the Mediterranean basin.

1.1.1 Botanical characteristics and life cycle

The olive, belonging to the *Oleaceae* family, is an evergreen tree, particularly long-lived some can live for more than 1000 years (Seifi et al., 2015) and can reach a height of between 8 and 15 metres (Guerrero et al., 2016). The root system is shallow and does not exceed 90-120 cm in depth, while it can develop laterally for at least 15 m in more mature individuals (Seifi et al., 2015).

It has thick, silver-green leaves that can live for 2-3 years (Guerrero et al., 2016), with an elliptical or lanceolate oblong outline and sizes ranging from small to very large depending on the cultivar, the vigour of the branch on which they grow and environmental characteristics (Franchini et al., 2006). The upper side of the leaf is dark green, while the underside is lighter in colour, being covered with dense stellate hairs or trichomes to prevent stomatal evapotranspiration.

The flower buds are grouped in inflorescences called panicles that develop in the axils of the leaves. The flowers are small, white and hermaphroditic, pollination is anemophilous and the fruit is a 2-2.5 cm long drupe, which develops in summer and ripens during autumn until it turns black (Guerrero et al., 2016).

The drupes consist of three layers: epicarp, mesocarp and endocarp. The outermost layer is the epicarp (skin), formed by a thin layer of globular cells cemented by cutin, which serve to protect the fruit and the colour changes from green to yellow to purple to purplish black during ripening (Longo, 2019). Next is the mesocarp, which contains 90% of the total oil content; the cells that form this tissue are globular in shape and become smaller and more fusiform as they approach the stone. Finally, the endocarp (stone) consists of cells which, after the early stages of fruit

development, harden the membranes to give the shell a woody consistency to protect the seed (Franchini et al., 2006; Sánchez Lucas, 2019) (Fig.1).

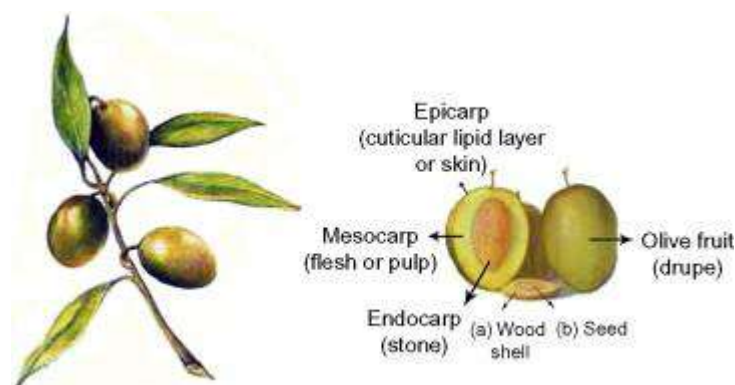


Fig.1 Cross-section of an olive fruit (From Juan et al., 2010)

The vegetative growth of the olive tree follows the Mediterranean climate cycle: shoots develop rapidly in spring, slow down in summer and then develop again with the rains at the end of August-September; then, as temperatures drop, growth slows down again (Franchini et al., 2006). The reproductive cycle takes two years, and the processes that lead to fruit formation begin the year before harvest. In fact, the shoots that give rise to flower buds and then fruits develop on the young branches of one year (Sánchez Lucas, 2019). Flowering occurs from April to June, simultaneously with the development of new shoots from the apical buds of the same fruiting branches, which will give fruit the following year (Longo, 2019). Since the fruits are centres of preferential translocation of metabolites compared to shoots, the olive tree alternates between years of high and low fruit production. During years of low production, the new shoots will be short with brief internodes and therefore unlikely to produce many fruits (Longo, 2019).

1.1.2 Historical spread and economic importance

The history of the olive tree began around 6000 years ago with the domestication of the wild olive tree *Olea europaea* subsp. *europaea* variety *oleaster* (or *sylvestris*), known as oleaster in the Middle East. These ancient populations managed to obtain a variety of olive tree with oil-rich drupes and no thorns, which made the oleaster less exploitable (Calabrese et al., 2012). In fact, the oleaster, being the ancestor of the current cultivated olive tree (*Olea europaea* L. subsp. *europaea* var. *europaea*), is widespread throughout the Mediterranean basin and shows a distribution that overlaps with that of cultivated olive trees (Fanelli et al., 2022). The origin of

Olea europaea in the Mediterranean is currently debated due to the complex, multi-localised domestication of the first cultivated entities (Loumou & Giourga, 2003).

From the 16th century BC, the Phoenicians and Greeks spread the olive tree throughout Greece and the Aegean archipelagos, eventually reaching southern Italy (Calabrese et al., 2012; IOC, 2026). Subsequently, the Romans continued to spread the olive tree throughout the Mediterranean countries, where it reached its maximum distribution, favoured by the soil and climate conditions of the basin. Around 600 BC, it was introduced to Marseille and then spread throughout Gaul. During the period of Phoenician maritime domination (1050 BC), the olive tree was also introduced to Spain, where it only really developed in 45 BC under Roman rule (Fig.2). Finally, during the modern age, the olive tree spread beyond the Mediterranean, exported from Seville first to the West Indies and then to Peru, California, Chile and Argentina (IOC, 2026).

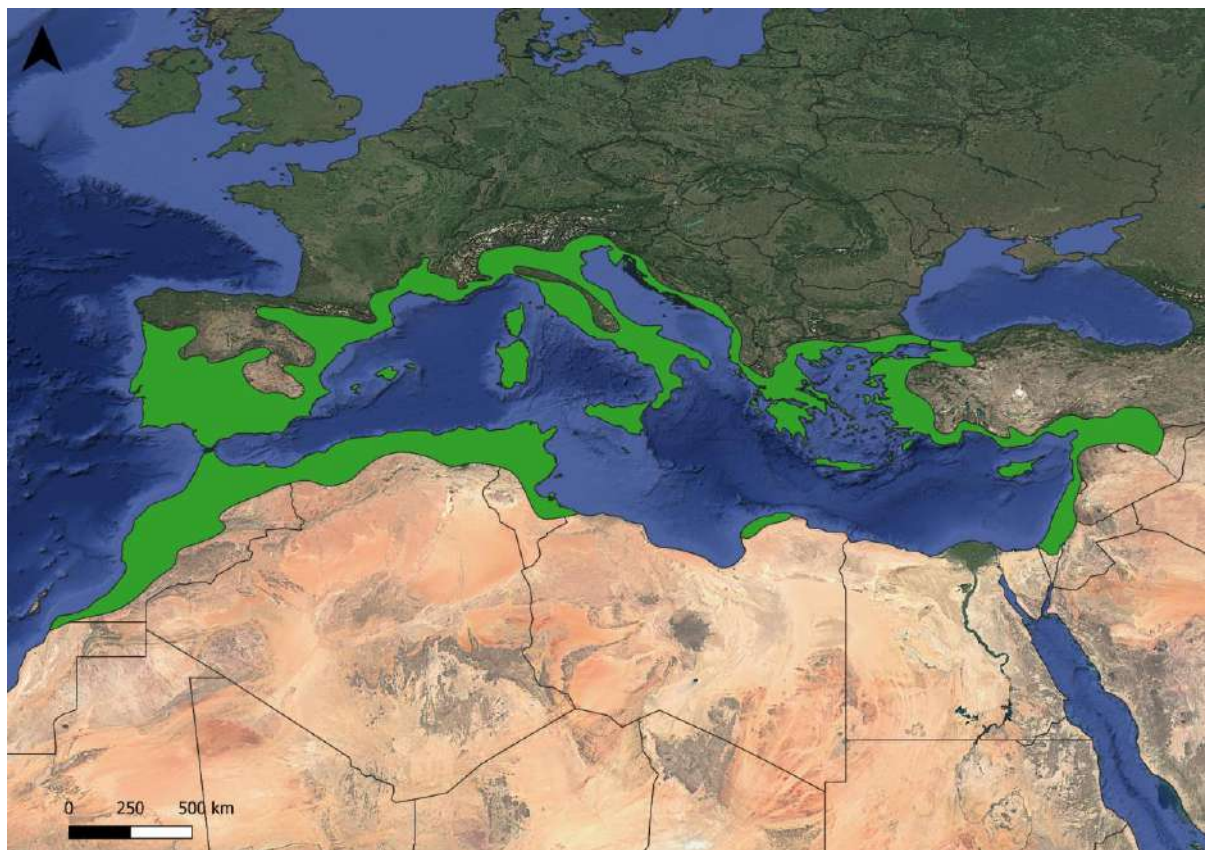


Fig. 2. Distribution of the olive orchards in the Mediterranean region (Caudullo et al., 2017).

Over the centuries, olive groves have boosted rural development, becoming one of the main sources of income and employment in relatively poor countries with limited water resources (Loumou & Giourga, 2003). The main product of *Olea europaea* is olive oil, whose renowned

nutritional properties make it a staple food in the Mediterranean diet and, at the same time, particularly important in the economies of the countries that produce it. In fact, 90% of olives are used for oil production and only 10% are used for table olives (Fraga et al., 2021; Sánchez Lucas, 2019). To meet the demand for olive oil, new olive groves were created by irrigating and fertilising large agricultural areas, and crops were adapted to pruning and mechanical harvesting in high-density plots (Brito et al., 2019). Currently, there are 41 olive oil-producing countries worldwide, with a total cultivated area of 10.58 million hectares (Kaniewski et al., 2025) and over 750 million olive trees, of which approximately 95% are located in the Mediterranean region (Muzzalupo et al., 2014). Based on official country data and estimates by the IOC Executive Secretariat, world olive oil production in the 2025/26 season is expected to be around 3,440,000 tonnes, down 4% from the 2024/2025 season. According to IOC estimates, the main olive oil producing countries remain Spain (65.4%), Greece (12.7%) and Italy (11.3%) in the 2024/2025 season. The main olive oil importing countries are the United States with 35%, the European Union with 17%, Brazil with 8%, Japan with 6%, Canada with 5%, China with 4% and Australia with 3%, with Spain (27.9%), Tunisia (24.3%) and Italy (19.6%) as the main exporters (IOC, 2026). From 1990 to 2019, olive oil consumption increased by 94%, along with growth in exports (5,794 million dollars in 2019), but the effects of climate change (increased temperatures and reduced rainfall) negatively affect crop fitness and the resulting oil production (Kaniewski et al., 2025).

1.2 The olive agroecosystem

Today, olive groves reflect an important part of the Mediterranean agricultural landscape, along with other important crops such as vines and cereals. This crop, in addition to being an integral part of the culture and economy of rural populations, is also perfectly integrated into the natural system with the Oleo-Ceratonion plant community, which, according to phytogeographers, is one of the most extensive communities in the Mediterranean area. Olive groves can grow easily on sloping, shallow and infertile land, and on hand-built stone terraces, thanks to their limited water requirements (Loumou & Giourga, 2003).

Despite their integration into the Mediterranean environment, the ecological importance of olive groves has only recently been recognised. Olive growing is often confined to hillsides or rather rugged terrain and occupies significant portions of mountainous and hilly areas, as its cultivation reduces soil erosion and also promotes the conservation of both aboveground and belowground biodiversity (Calabrese et al., 2012).

Many centuries-old olive groves are managed in a traditional way and are immersed in a mosaic of semi-natural areas interspersed with small-scale structural elements such as copses, shrubby hedges and dry stone walls. The presence of these natural corridors, together with the complex conformation of the trunks with large cavities and the production of high-energy fruits, make olive groves an ideal refuge for many species of wintering and migratory birds and small mammals (Calabrese et al., 2012; Loumou & Giourga, 2003).

The wild vegetation that grows naturally within or on the edges of an olive grove is extremely similar to the vegetation found in spontaneous Mediterranean ecosystems, making the entire olive-growing agroecosystem more natural than others. If olive groves are completely abandoned for a long time, through the process of ecological succession, the cistets that first colonise the olive groves will evolve into true Mediterranean-type natural forests, depending on the climatic and territorial conditions of each region (Loumou & Giourga, 2003).

However, the change in land use of an olive grove is towards grazing for sheep and goats. In this case, the impact on the environment is negative. In fact, animals attracted by plant biomass begin to cause erosion, degrading the soil and hindering the natural regeneration of spontaneous vegetation. The change in land use also negatively affects soil depth, which, deprived of the root systems of shrubs and vegetation cover, remains more exposed to erosion. Maintaining traditional olive groves is beneficial for the environment, as these systems have low soil erosion rates and high biodiversity. Olive growing becomes even more important when carried out on sloping land with marginal productivity, which is at high risk of irreversible deterioration, in fact they are able to reduce runoff and prolong the time that rainfall remains on the soil surface (Loumou & Giourga, 2003).

1.3 Climate change challenges: drought and sustainability

The IPCC Sixth Assessment Report (AR6) states that global surface temperature was 1.09 °C higher in the period 2011-2020 than in the period 1850-1900 and that human influence has likely contributed to the pattern of precipitation change since the mid-20th° century. Furthermore, the climatic zones of both hemispheres have shifted towards their respective poles, and that in the mid-latitudes, the growing season has lengthened on average up to two days per decade. In practice, mankind has triggered unprecedented global warming (Calvin et al., 2023). Rising global temperatures, projected to increase by 1.5–2 °C during the 21st century (IPCC, 2013), are expected to substantially alter regional climate patterns, particularly in the

Mediterranean basin. Most climate scenarios predict higher temperatures, reduced precipitation, and an increased frequency of extreme events such as heatwaves and droughts (Giorgi & Lionello, 2008; Gualdi et al., 2013; Hertig et al., 2014).

One of the main manifestations of climate change is drought, which can be defined as "an abnormally dry period of time, long enough to cause a severe hydrological imbalance" (IPCC, 2012). Drought can be caused by a lack of rainfall during a certain period or by inadequate distribution and timing of rainfall. Excessive water evaporation caused by high temperatures and strong winds can create a feedback loop in which increased heat waves cause the soil to dry out, with a consequent negative impact on crops (Erian et al., 2021).

The Mediterranean basin is characterised by long, hot, dry summers and mild, wet winters, and olive trees are one of the species best adapted to these conditions. In fact, as an xerophyte, the olive tree has morphological and biochemical adaptations that help it cope with water shortages, some of which are: low leaf conductance, long and deep roots, good osmotic regulation of cell content and variations in cell wall elasticity (Sanzani et al., 2012). However, rising temperatures, decreasing rainfall and more frequent extreme weather events are putting this crop at risk (Fraga et al., 2021). In particular, successive unusually warm winters can delay or prevent the fulfillment of chilling requirements, thereby inhibiting flowering, while heat waves and reduced water availability during critical growth stages may damage floral organs, reduce leaf area, and limit photosynthetic activity (Fraga et al., 2021; Kaniewski et al., 2025). These physiological stresses ultimately translate into reduced olive and oil yields, with significant economic consequences.

The centre of oil production has shifted towards the eastern Mediterranean, but already in the 2023-2024 season, Turkey and the Levant recorded an average decline of $44 \pm 18\%$ in olive oil production compared to the previous year (Kaniewski et al., 2025).

Approximately 90% of olive trees cultivated in the Mediterranean basin are rain-fed, and their distribution in arid areas is limited by annual rainfall of less than 350 mm. This highlights the importance of rainfall for this crop, to the extent that olive growers use management practices such as sparse planting and heavy pruning to maximise water resources and avoid severe water stress (Fraga et al., 2021).

Fraga et al., (2021) identifies a series of climate change adaptation actions that the olive sector can apply at regional/local level. The author defines two types of strategies: short-term

strategies, which can be applied to orchards over 1 or 2 seasons, and long-term strategies, which can be implemented over 3 or more seasons but involve significant socio-economic costs for growers, stakeholders and policy makers.

Short-term actions include low tillage (which limits CO₂ emissions), the use of cover crops with seed mixtures, and the application of mulch on bare soil, which can reduce water evaporation from the soil and erosion. Pruning measures aimed at improving light distribution within the canopy and facilitating aeration are also included. These measures should limit the years of alternating high and low yields and thus stabilise annual production and, consequently, labour demand. The use of PGPR-based microbial inoculants can be included in these types of action. Several studies have shown that PGPR improve the resilience and adaptability of olive trees to drought conditions (Crespo-Barreiro et al., 2025; Sbbar et al., 2025).

Long-term adaptation strategies include measures such as planting cultivars that are resistant to high temperatures and drought and have a high tolerance to diseases and harmful insects. There are also plans to move olive cultivation northwards and to higher latitudes, which, due to milder winters and hotter, drier summers, will become viable areas for this crop (Sparks & Menzel, 2002; Tanasijevic et al., 2014). On the other hand, however, this would lead to the abandonment of Mediterranean olive groves and thus to the extinction of the bioclimatic niche (Fraga et al., 2021).

Understanding how olive trees respond to drought stress is the first step towards improving their profitability (Brito et al., 2019). The use of resistant cultivars, sustainable olive grove management techniques and new biotechnological applications (such as microbial inoculants) are just some of the strategies that can be employed to counteract the effects of climate change (Mairech et al., 2021). It is therefore necessary that these techniques are always able to adapt to the new climatic conditions we are experiencing.

1.4 The holobiont concept

Plants live in close association with complex microbial communities (microbiome) involved in vital functions such as resistance to biotic and abiotic stresses (Vandenkoornhuyse et al., 2015). The co-evolution of fungi and bacteria and the plant itself results in intimate relationships that create highly specific and stable microbiota, to the point that the entire organism can be defined as a plant holobiont (Cardoni & Mercado-Blanco, 2023; Vandenkoornhuyse et al., 2015).

The microbiota associated with plants can be found outside plants (in the rhizosphere or phyllosphere) or inside plants (the endosphere) and can consist of both pathogens and non-pathogenic endophytes, which spend at least part of their life cycle inside host plants (Giordani, 2021). Endophytes in the root endosphere show less diversity than those in the rhizosphere, but they are quite specific and contribute significantly to nutrient acquisition, hormonal regulation and systemic pathogen resistance (Kacem, 2023). The rhizosphere (defined as the soil near the root surface) is considered a hotspot of microbial diversity: it can contain up to 10^{11} microbial cells per gram of root (Egamberdieva et al., 2008) and more than 30,000 prokaryotic species (Mendes et al., 2011).

In plant ecology, the microbiota is considered the “facilitator” of the holobiont as it maintains or strengthens the host's homeostasis (Sánchez-Cañizares et al., 2017). Some aspects of the host's life that the soil microbial community can influence relate to plant growth and development, survival and tolerance to biotic and abiotic stresses, and inducing systemic resistance against pests and pathogens in both leaves and roots (Giordani, 2021). A concrete example is provided by beneficial rhizosphere bacteria (PGPR), which can promote plant growth through both direct and indirect mechanisms, such as: the synthesis of phytohormones, increased availability of nutrients in the soil; acting as biocontrol agents or biopesticides through the inhibition or suppression of phytopathogenic organisms thanks to antibiosis and induced systemic resistance (Bashan & Holguin, 1998; Gamalero & Glick, 2015; Hayat et al., 2010).

The concept of holobiont involves bidirectional dynamics. Depending on its age, stage of development or other physiological condition, plants can also select the microbial community to host from the soil, releasing root exudates into the soil through the process of rhizodeposition. Through the process of rhizodeposition, roots release low molecular weight compounds (sugars, amino acids and organic acids), polymeric sugars (mucilage) and dead root cap cells that are used by bulk soil bacteria as a carbon source. Rhizodepositions may also contain secondary metabolites, such as antimicrobial compounds, nematocides and flavonoids involved in the activation phases of associative biocenoses and symbiosis or in repelling pathogens and parasites (Giordani, 2021).

The host's ability to recruit bacteria and fungi with biofertilisation, biopromotion and bioprotection capabilities allows us to define it as a “central stress microbiome” (Marasco et al., 2021). The genotype also influences the selection of the microbiota: different olive cultivars,

despite growing in similar conditions, attract different microbial assemblages (Ahmad et al., 2026).

The importance of this interaction is highlighted by the concept of microbe-mediated adaptation in plants, which attributes the host's ability to adapt to the environment (totally or in part) to the microbiota (Petipas et al., 2021). In other words, the microorganisms associated with the plant act as an extension of the host's genotype, capable of responding rapidly to environmental changes, including drought stress in the olive holobiont (Marasco et al., 2021). But, at the same time, the structure and function of the microbiota are influenced by biotic and abiotic factors such as climate change, pesticide use, soil type and structure, overall plant health, and the presence of pathogens and pests (Cardoni & Mercado-Blanco, 2023). In particular, it is edaphoclimatic factors, represented by the combined effect of soil properties and climatic conditions, that drive the microbial assembly of olive trees. Elements such as soil texture, pH, organic matter, nutrient availability and salinity in moisture act in synergy with temperature, irradiation and precipitation patterns to determine the microbiota assemblage (Ahmad et al., 2026).

In a scenario where climate change is affecting Mediterranean agroecosystems, it is essential to consider olive trees from a holistic perspective. Therefore, any action aimed at strengthening crop resilience to biotic or abiotic stress should take into account the host and the associated microbiota.

A wide range of factors influence the composition and structure of the plant-associated microbiome over time and space. In addition to natural fluctuations in temperature, rainfall, exposure to UV rays, soil pH, etc., anthropogenic factors such as pollution and practices can also alter the microbiome of tree crops. The latter have a strong impact on the microbiome, stimulating or inhibiting its activity based on the nutritional preferences of microorganisms, or influencing the way plants “recruit” their microorganisms (Cardoni & Mercado-Blanco, 2023).

In summary, the olive tree's microbiota acts as an interface between the plant and the external environment, promoting adaptation to adverse climatic conditions and more sustainable olive production practices (Cardoni & Mercado-Blanco, 2023).

1.5 Priming and stress memory in plants

As sessile organisms, plants are subject to adverse environmental stimuli that affect their germination, growth and productivity. These stimuli can be biotic in nature, such as pathogens and herbivores, or abiotic, such as salinity, drought, cold, heat and UV rays (Turgut-Kara et al., 2020).

Under conditions of water scarcity, plants have developed strategies for adaptation and acclimatisation at various levels, including morphological and anatomical variations and physiological and biochemical reactions. The duration and intensity of plant responses depend on the duration of water shortage, species and cultivar (plant genotype), age and stage of development .

It is recognised that plants can implement mechanisms that allow them to identify a stress event that has already occurred and respond in a timely and efficient manner. The ability to remember the stressor and apply protective actions is called stress memory (Nteve et al., 2024). Past experiences can shape or “prime” the response of plants to future environmental stimuli, resulting in phenotypic plasticity adapted to the characteristics of the stressful event (Hilker et al., 2016).

This mechanism begins with an initial stress event that stimulates physiological and molecular mechanisms (e.g., the accumulation of stress-sensitive osmoregulatory metabolites or the synthesis of protective proteins) leads to the accumulation of signalling proteins or transcription factors and epigenetic mechanisms in plants (DNA methylation or acetylation, chromatin remodelling or histone alteration) (Ben Abdallah et al., 2017). This is followed by a post-stress period during which this information is stored and will enable faster responses to a further stressful event (Jacques et al., 2021).

In plant biology, priming is represented by the occurrence of an event whose triggering process will positively influence the future performance (responses) of the plant if it finds itself in conditions of particular stress (Hilker et al., 2016).

Priming in plants can also be mediated by external factors such as the colonisation of beneficial fungi or bacteria in the roots (PGPR), which can give rise to induced systemic resistance (ISR), which improves the response against plant pathogens that infect plants (Hilker et al., 2016; Hilker & Schmülling, 2019).

To understand the concept of stress memory in plants, according to Hilker et al. (2016), we define a primable organism as one capable of perceiving a stress event and storing information about it. The priming process develops through different physiological stages of an organism. It starts from the naïve state (control, C), a condition without prior exposure to stress, which evolves into the primed state (P), a latency phase in which the organism, alerted, remains in a “state of readiness”; in this phase, the organism prepares itself for a better response to future stress. In the primed-and-triggered state (P+T), previously acquired information is retrieved to shape the response to a stressful event, i.e. the triggering stimulus.

The purpose of priming is to improve an organism's fitness during a stressful event, with minimal waste of energy resources. However, even the initial response to the priming event itself involves costs associated with maintaining a state of alertness; this reversible “standby” condition will remain in place until other mechanisms are activated in response to the triggering event. Since priming involves the performance of the entire organism, from the point of view of fitness and efficiency, better performance is expected in a primed-and-triggered (P+T) organism than in a stressed but not primed organism (Hilker et al., 2016).

In other words, priming provides broad-spectrum protection in harsh environments, increasing crop productivity with minimal costs in terms of fitness (Singh et al., 2024).

Chapter 2 STATE OF THE ART

2.1 The olive holobiont: an integrated perspective

The olive holobiont concept views the olive tree (*Olea europaea* L.) and its associated microbial communities (such as bacteria, fungi, and other microbes in roots, leaves, and soil) as a single functional unit (Cardoni & Mercado-Blanco, 2023). This holistic perspective highlights how the microbiome influences the tree's growth, stress tolerance, productivity, and resilience, especially amid modern intensive farming changes in the Mediterranean basin. The plant-microbes interplay forms a feedback loop: plants select for functional microbes that, in turn, modulate root growth and holobiont health amid stresses, as seen in shared core microbiomes across olive genotypes and management (Fernández-González et al., 2025).

2.2 Olive-associated microbiomes

Given the above, the number of studies on the olive microbiome is constantly growing. To date, metagenomic analyses are proving to be fundamental in detecting the vast microbial biodiversity of different environments that was previously unknown (Abdelfattah et al., 2015). The bacterial and fungal communities of olive tree organs/compartments have been described, such as: root endosphere, rhizosphere, xylem, carposphere and phyllosphere. Other factors have also been taken into consideration, such as olive tree genotype, cultivation system, tree phenology, and tolerance/susceptibility to disease (Cardoni & Mercado-Blanco, 2023; Dias et al., 2024).

However, Kakagianni et al., (2023) points out that much research does not consider the interconnection between the microbial communities that inhabit the different compartments of the plant, and we agree that, in order to provide a complete picture of the interactions that take place in the olive holobiont, it is necessary to study all compartments of the plant.

2.2.1 The role of rhizosphere-associated microbial communities

Much of the research related to hypogean microbiota has been conducted in Mediterranean countries dominated by olive cultivation (Spain, Italy, Portugal, Greece and Tunisia) using methods and analyses that allow for a detailed description of microbial communities (Dias et al., 2024).

These studies show that the rhizosphere and root endosphere of olive trees are colonised by the bacterial phyla *Actinobacteria*, *Proteobacteria*, *Acidobacteria*, *Firmicutes*, *Bacteroidetes* and *Verrucomicrobia*. Some of these are defined as BCAs (biological control agents) capable of suppressing pathogens through various mechanisms (e.g. antibiosis, competition, parasitism, etc.).

For example, some PGPMs can modify the quantity and composition of root exudates to recruit new members and cope with the presence of pathogens (Cardoni & Mercado-Blanco, 2023), others can cause diseases.

The olive agroforestry system has a more diverse microbiome in terms of composition and functionality than monocultures. Olive tree agroforestry can attract bacteria such as *Rhizobium*, *Sphingomonas* and *Streptomyces*, which improve nitrogen fixation and phosphate solubilisation, as well as the formation of bioactive constituents for the suppression of soil pathogens (Ahmad et al., 2026).

We already know that some species of *Pseudomonas* and *Bacillus* have an antagonistic effect on the fungus that causes Verticillium wilt of olive trees (VWO), *Verticillium dahliae*, inhibiting its growth through the solubilisation of phosphate and nitrogen (Aranda et al., 2011; Fausto et al., 2018; Fernández-González et al., 2019). In particular, the presence of *P. simiae* PICF7 or *P. polymyxa* PIC73 stimulates the recruitment from the rhizosphere of bacteria and fungi with antagonistic activity towards *V. dahliae* (Cardoni et al., 2023).

Other bacterial species such as *Streptomyces* spp. promote plant development by increasing ammonia and indole-3-acetic acid, phosphate solubilisation, siderophore production and secondary metabolites (Dede et al., 2020). The classes *Thermoleophilia*, *Bacilli*, and *Chloroflexi* and the genera *Micrococcus* and *Steroidobacter* are important for the adaptation of olive trees to arid and dry conditions (Fernández-González et al., 2019; Marasco et al., 2021).

As their names suggest, the bacteria *Nitrososphaera* spp, *Nitrospira* spp. and *Nitrosovibrio tenuis* are related to the nitrification process, while *Pseudomonas corrugata* and *Denitrobacter* spp. are related to denitrification (Caliz et al., 2015; Fausto et al., 2018; Kakagianni et al., 2023; Sofo et al., 2014; Wentzien et al., 2023).

Several species of the genus *Glomus* spp. improve olive tree crop performance by enhancing mineral nutrition, defence systems and tolerance to water stress (Bizos et al., 2020). Many

arbuscular mycorrhizal fungi (AMFs) can harbour endobacteria within fungal hyphae and spores that influence host function and plant-fungus interactions. Some interactions may be beneficial to fungal and plant hosts, but some endobacteria, such as *Burkholderia rhizoxinica*, can induce the host fungus to cause disease in the plant. However, studies on the associations between bacteria, AMF and plant hosts in olive trees still need further investigation to clarify aspects related to (a)biotic stress resilience (Chialva et al., 2021).

2.2.2 The role of phyllosphere-associated microbial communities

Research on the plant microbiome has always focused on the rhizosphere compartment and on microbiota-root interactions or soil pathogen dynamics. Recently, thanks to molecular and genomic technologies, the study of microbiota has also extended to the aerial compartment, phyllosphere, carposphere and flowers (Zhu et al., 2022).

Although the rhizosphere and phyllosphere are linked by physiological processes, the different selective pressures and distinct environmental filters to which they are subjected give rise to functionally and compositionally divergent microbiomes (Crucitti et al., 2025b).

Since the phyllosphere is an open system, the assembly of its associated microbiome depends on many factors that interact in synchrony. Certainly, the composition of the phyllosphere microbiome will depend on the genotype of plant species, leaf structures (roughness), release of primary and secondary metabolites, seasonality, microbial interactions themselves or interactions with phytophagous organisms (Crucitti et al., 2025b; Zhu et al., 2022). As with the underground part, the phyllosphere also performs important functions related to plant productivity and fitness, some of which are linked to leaf longevity, seed mass, apical growth, flowering, fruit development and even the removal of contaminants (Zhu et al., 2022). Some microbial communities can induce the formation of biofilms that protect leaf surfaces from dehydration and pathogen attack, while others can even cause antimicrobial products and systemic resistance in the host (Ahmad et al., 2026). It has been shown that some bacteria that inhabit these aerial niches (such as *Pseudomonas*, *Acinetobacter*, *Micrococcus* and *Cutibacterium*) promote plant growth and inhibit leaf pathogens (Gayathry et al., 2024; Subrahmanyam et al., 2020)

For example, some olive cultivars appear to host fungal communities with a greater presence of leaf endophytes belonging to the genera *Cladosporium* or *Alternaria*, whose physiological

role varies from mutualistic to potentially pathogenic, depending on the ecological context (Crucitti et al., 2025b).

The study by Vergine et al. (2019) shows that resistance to *Xylella fastidiosa* subsp. *pauca* is closely linked to the stability of the microbial community in the leaves. The cultivar 'Leccino', known for its tolerance, is distinguished by a naturally more diverse microbiota than the 'Cellina di Nardò' cultivar, which is more susceptible and suffers from drastic dysbiosis when they are affected by *X. fastidiosa*. 'Leccino'-specific taxa include species known to exert beneficial effects on host plants, such as *Rhizobium*, *Burkholderiaceae*, *Sphingomonas* and *Enterobacter*, while *Pseudomonas* and *Sediminibacterium* were reduced in the infected 'Cellina di Nardò'. This highlights that the functional capacity of the plant microbiome goes beyond the simple aggregation of its constituents, as they engage in robust interactions to respond to external stress (Cardoni & Mercado-Blanco, 2023).

Significant correlations are also known between the presence of *Saccharomycetales* spp in the phyllosphere of olive trees and a lower susceptibility to anthracnose (Cardoni & Mercado-Blanco, 2023).

In a recent study on the diversity of endophytes isolated from leaves and twigs of Sicilian olive trees, Crucitti et al. (2025a) demonstrated that many of the bacterial isolates exhibit plant growth promoter (PGP) properties, enzymatic activity and inhibitory activity against the fungal pathogen *Neofusicoccum vitifusiformea* by the bacterium *Bacillus licheniformis*.

These studies indicate that plants respond to pathogen attack by recruiting specific beneficial soil microorganisms and modulating their composition to confer immunity against a wide range of diseases; these carefully selected microbial groups can activate plant defences and reduce plant susceptibility to pathogen attack.

2.2.3 Plant host genetics, soil, and environmental features as drivers of microbial assembly

The biodiversity of the microbiota is shaped by genotype and environmental factors. Regarding plant features, the anatomical and physiological traits of the host species, such as wood density, leaf nitrogen content, secondary metabolites, or leaf morphology (Laforest-Lapointe et al., 2016; Unterseher et al., 2016) can influence microbiota profile. The genetics of the plant determine the composition of root exudates and consequently the recruitment of the microbiome in the rhizosphere.

The microbiome of a collection of European 36 olive cultivars maintained at the World Olive Germplasm Collection (WOGC, Córdoba, Spain) was recently analyzed to identify both a core microbiome and cultivar-dependent microbial patterns including the Italian cultivars like 'Frantoio', 'Grappolo', and 'Leccino' (Fernández-González et al., 2019). The study shows that olive belowground microbial communities are mainly shaped by cultivar genotype under identical environmental conditions and that *Actinophytocola* may be key for stress adaptation. Other olive microbiome studies have focused on a restricted set of cultivars, such as 'Kalamata', 'FS17', 'Picual', 'Arbequina', 'Acebuche', 'Ocal', 'Cobrançosa', 'Verdeal Transmontana', 'Leccino', 'Cellina di Nardò', and 'Ogliarola' salentina, primarily targeting endophytic, rhizosphere, xylem, and phyllosphere communities (Crucitti et al., 2025b; Giampetruzzi et al., 2020). Some olive varieties tend to host a richer and more diverse microbiota, others have simpler communities dominated by a few taxa, and still others select more specific microbial communities, suggesting a genotype-specific effect in the recruitment and modulation of microorganisms. For example, some cultivars are enriched with bacteria of the genus *Pseudomonas* or *Bacillus* known as PGP and in resistance to biotic stresses; others may host *Cladosporium* or *Alternaria*, which, depending on the ecological context, may play a mutualistic or potentially pathogenic role (Crucitti et al., 2025b).

Although these studies, despite being based on an extensive and substantial research effort, have significantly advanced our understanding of the olive microbiome (also in response to abiotic and abiotic stress), the cultivars analyzed are not fully representative of the overall genetic diversity of olive germplasm. Moreover, they provide limited insight into site-specific conditions and into the influence that agronomic practices exert on the cultivar–microbiome relationship at the rhizospheric level.

The biodiversity of the microbiota is closely dependent on the anatomical and physiological traits of the host species, such as wood density, leaf nitrogen content, secondary metabolites, or leaf morphology (Laforest-Lapointe et al., 2016; Unterseher et al., 2016). The genetics of the plant determine the composition of root exudates and consequently the recruitment of the microbiome in the rhizosphere. Metabolomic and ionomic analyses reveal different concentrations of aspartic acid, phenylalanine, sodium and iron in xylem sap, and different levels of proline, carotenoids and pigments in fresh leaves from different olive cultivars (Anguita-Maeso et al., 2021).

The different microbial communities into the soil of olive groves in the Mediterranean depend, among other things, on the relatively higher organic matter content of the soil and on temperature regimes compared to those of olive groves in more arid regions. Wet winters and dry summers affect the stability of the microbial community: copiotrophic growth is promoted by these conditions and taxa adapted to drought become enriched during dry summers, supporting plant hydration and nutrient uptake (Ahmad et al., 2026).

Microbiomes associated with the Mediterranean features promote functional traits for nutrient mobilisation and balanced growth, while the characteristics of microbiomes in arid regions favour survival under conditions of extreme stress; these associations result from strong interaction with the environment and represent an ecological compromise induced by resource scarcity (Ahmad et al., 2026; Kumar & Droby, 2021).

Soil structure, texture, porosity, together with temperature, irradiation and precipitation, are factors that naturally influence the microbial composition of the soil.

Sandy soils, typical of arid regions, with their low organic carbon and low water retention capacity, select drought-resistant oligotrophic microbes (Melloni & Cardoso, 2023). Heat and salinity stress, common in irrigated arid areas, selects for thermotolerant and halophilic microbial groups capable of balancing their ions and osmoprotection. In contrast, moderately acidic soils enriched with organic matter, typical of the Mediterranean, support diverse communities with functional redundancy, including mycorrhizal fungi that improve phosphorus uptake and bacterial groups involved in nitrogen fixation (Ahmad et al., 2026).

2.3 Agricultural management and microbiome modulation

Research on soil-plant interactions is gaining interest with regard to the influence of cultivation practices on microbiota and soil functions as agricultural management affects the structure, stability and function of olive tree-associated microbiomes (Ahmad et al., 2026; Melloni & Cardoso, 2023).

Several studies aim to understand and apply new knowledge about the microbiome to farming practices in a sustainable way, especially when olive trees are grown in places with abiotic stresses, including high or low temperatures, high or low humidity, poor and/or degraded soils, and the presence of pests and diseases (Melloni & Cardoso, 2023).

2.3.1 Main olive grove management systems in the Mediterranean

Olive growing in the Mediterranean basin is characterised by an agricultural mosaic ranging from traditional extensive rain-fed farming to biological farming and intensive and super-intensive olive growing (conventional) (Raz et al., 2024).

In recent decades, olive growing has seen the adoption of irrigation systems, artificial fertilisers, herbicides and pesticides to meet the growing global demand for olive oil. In addition, new super-intensive olive groves have been planted, characterised by high-density plantings of dwarf varieties, the use of mechanical harvesters (Raz et al., 2024), synthetic fertilisers and agrochemicals (Verrascina & Reda, 2022); these are features that distinguish conventional agriculture.

In contrast, traditional extensive olive groves are managed with low intensity, with limited or no irrigation and low chemical inputs that do not alter biodiversity in the agroecosystem. In fact, traditional systems host a greater diversity of birds, bats and herbaceous plants than conventional management. Reduced (conservative) tillage practices, as well as no-till farming, are considered a more sustainable alternative (Fekete et al., 2023). Their advantages include lower energy costs, soil and water conservation by reducing soil disturbance and returning part of the plant residues to the soil (Khmelevtsova et al., 2022). In addition, traditional olive groves have also been used for centuries as pasture for livestock, increasing the livelihood of local communities (Fekete et al., 2023).

To meet the growing global demand for olive products, each growing region must respond by including new olive groves, changing land use, or modifying olive grove cultivation and management methods. The transition from traditional to conventional agriculture can potentially affect the exposure of trees to a range of established and emerging stresses (Cardoni & Mercado-Blanco, 2023).

The intensification of olive growing systems, although intended to increase productivity, has raised concerns about environmental degradation, especially in drought-prone areas such as the Mediterranean. Fortunately, European policies propose the development of organic olive growing, which is more environmentally friendly. This type of management is becoming increasingly popular among growers because it allows farms to compete with large production systems (Raz et al., 2024).

We can define biological farming as a system that aims to obtain high nutritional quality products while respecting the environment through the rational use of local resources and without the addition of chemicals that are harmful to the environment (Benitez et al., 2006).

The biological management of an olive grove is achieved through a series of agronomic techniques, defined by legislation Reg. (EEC) n. 2092/91, Reg. (EC) 834/07 e Reg. (EU) 848/2018), that avoids altering the environment. Some of these involve increasing and maintaining the natural fertility of the soil by adding organic materials of animal origin (cattle manure, sheep or horse manure, livestock waste, poultry manure) or vegetable origin (green manure or fertilisation) or through grassing and green manure. The latter is particularly useful in areas with slopes greater than 5% to prevent dangerous surface runoff. No recurrent and deep mechanised tillage is planned. Certified plant nursery material is used, as well as predatory insects against pests, following sampling and monitoring of the main olive tree pathogens. Pruning is the intervention which, together with other cultivation operations (fertilisation, irrigation, plant protection), helps to maintain the right balance. Olive trees need pruning both during the cultivation phase, to shape the tree and ensure correct planting, and during the production phase, including any thinning to promote a correct balance between the qualitative and quantitative requirements of production (Verrascina & Reda, 2022).

2.3.2 Effects of agricultural management on the microbial community of olive

The various cultivation methods, particularly biological farming compared to conventional farming, contribute differently to the composition of microbiomes. The application of sustainable management techniques can improve the quality of soil ecosystems and protect the presence and activity of important bacterial taxa with physiological and protective functions for plants (Giordani, 2021).

Confirming this, Sofo et al. (2019) demonstrated the positive effect that biological management has on the bacterial community and soil fertility through biochemical and metagenomic analyses. They studied the soil of Maiatica olive groves in southern Italy, characterised by a hot, dry climate, under two management processes applied for over 18 years: sustainable vs conventional. The sustainable management of these olive groves involved drip irrigation, light winter pruning (the prunings were shredded and left along the rows as mulch), ground cover with self-seeding weeds, fertigation with N-NO_3^- ; instead, conventional management was characterised by frequent tillage and included severe pruning with residues removed from the

orchard and mineral fertilisation. The organic input of plant origin increased the levels of Soil Organic Matter (SOM) and Total Organic Carbon (TOC) in the surface layers of the soil managed with sustainable practice. This input improved soil biodiversity, promoting the formation of an active litter layer, which is absent in conventional systems. Significant differences were found between the abundances of free N-fixing bacteria and symbiotic N-fixing bacteria. The former were significantly more abundant in the conventional system, as nitrogen fixation is carried out by free cyanobacteria in the soil and not by bacteria associated with root nodules (e.g. *Rhizobiales*) of cover crops such as Fabaceae, which were much more abundant in sustainable systems.

The use of cover crops is one of the most recommended agricultural practices for improving the sustainability of Mediterranean woody crops such as olive groves. There is a trend in sloping olive production systems (slope > 10%) to reduce bare soil management through the implementation of permanent or temporary cover crops (Arias-Giraldo et al., 2021). However, there is a wide range of cover crop types, and while their effects on the physical/chemical properties of the soil are well known, only recently have they also been studied in relation to the structure of microbial communities. Arias-Giraldo et al., (2021) highlighted that the composition of the microbial community in olive groves with cover crops consisting of spontaneous vegetation (CC-NAT) differed from that with cover crops consisting of a mix of plant species (CC-MIX), grasses only (CC-GRA) and bare soil (TILL) under conventional management.

They found the lowest bacterial diversity in highly disturbed soils (TILL), average diversity in cover crop treatments (CC-MIX and CC-GRA), and the highest diversity in minimally disturbed soils (CC-NAT). In the soil of olive groves, natural cover crops (CC-NAT) hosted *Bacilli* and *Clostridia*, known antagonists and biocontrol agents and the genus *Massilia*, which plays an important role in plant growth thanks to the synthesis of secondary metabolites (i.e. auxin and siderophores) and the mobilisation of elements such as phosphorus and heavy metals in phytoremediation processes. Other cover crops (CC-GRA and CC-MIX) shared *Proteobacteria*, a good indicator of high nutrient content, and *Actinobacteria*, which contribute to the carbon, nitrogen, phosphorus, potassium and other element cycles. The study revealed that the presence of cover crops (CC-MIX and CC-GRA) improved both the physicochemical properties (organic carbon and cation exchange capacity) and the biological parameters related to soil quality (e.g., soil respiration, enzymatic activity, and soil metabolic activity) compared

to tillage management (TILL), achieving values closer to those of physically undisturbed soil (CC-NAT) with a cover crop of herbaceous vegetation present spontaneously in the orchard. These are all relevant parameters relevant to supporting a highly diverse microbial community. The results showed that the use of a mixture of plant species (CC-MIX) or grasses (CC-GRA) as temporary cover in a non-organic olive grove soil induced significant changes in indicators associated with soil quality compared to tilled soil (TILL) and that properties associated with poor soil quality can be restored to a level similar to that of undisturbed soil that has maintained natural spontaneous vegetation cover for decades (CC-NAT) (Arias-Giraldo et al., 2021).

Although the ecology of epiphytic microorganisms is important from both a scientific and economic point of view, little research has been conducted on changes in the microbiota of the phyllosphere and carposphere in response to the adoption of different cultivation practices. However, Pascasio et al., (2015) studied the effect of sustainable and conventional management on bacterial endophytes in the carposphere of the 'Maiatica' cultivar in southern Italy. They demonstrated, using 16S rRNA fingerprinting by PCR-DGGE, that, phyllosphere and carposphere of sustainable agriculture hosted a greater number of bacteria (*Rahnella* spp., *Kluyvera intermedia*, *Averyella dalhousiensis*, *Pantoea* sp. and *Serratia* sp. (*Enterobacteriaceae*)) than conventional agriculture. In addition, the carposphere is also home to a community of yeasts that play specific roles in softening the fruit, activating its defences and performing other biochemical functions that lead to improved aroma and taste (Ahmad et al., 2026).

In accordance with the studies cited thus far and other related research, typical conventional farming practices tend to simplify and impoverish the soil microbiome, while typical traditional or organic farming practices promote its complexity and functionality. Deep tillage practised in conventional agriculture interferes with soil aggregates and AMF connectivity; in contrast, traditional or biological agriculture, which limits tillage, encourages the development of fungal communities. Furthermore, the addition of mineral nitrogen induces the growth of heterotrophic bacteria at the expense of diazotrophs; the use of organic amendments such as compost, manure and recycled pruning debris enhances the development of Santos et al., (2022) PGPR; mulching and plant cover on the soil lower the soil temperature and conserve moisture, creating a microclimate suitable for the survival of the microbiota even in unfavourable conditions (Ahmad et al., 2026).

2.4 Drought response mechanisms in olive

Water shortage is the stress condition that most negatively affects agricultural production, reducing the quality of agricultural products (Grillo et al., 2006).

In plants, water deficits occur when there is not enough water in the soil to replace water losses due to evapotranspiration. Other conditions that limit water absorption include saline soils, low temperatures and flooding. Under these unfavourable conditions, plants trigger physiological, biochemical and molecular responses at the cellular and organismal levels to limit the resulting damage (Brito et al., 2019). The response mechanisms depend on the duration and intensity of the stress and on the ability to induce short- and long-term cellular responses that limit damage and preserve cellular structures (response to shock conditions) and restore a new homeostatic balance (adaptive response) (Grillo et al., 2006).

2.4.1 *Physiological and biochemical responses*

The waxy leaf surface, trichome density/diameter and petal elasticity are morphological characteristics that improve drought tolerance in olive trees by reducing transpiration. Some olive varieties are predisposed to developing deep root systems to capture more water in dry soil conditions, but root growth is conditioned by the structural characteristics of the soil (Nteve et al., 2024).

Mainly, the regulation of water loss in a plant depends on leaf characteristics. For example, an increase in stomatal density is a disadvantageous trait for drought tolerance in many plant species as it leads to greater water loss through transpiration. In olive trees, however, stomatal density is negatively correlated with stomatal size, thereby reducing water consumption (Nteve et al., 2024). Some traits can be macroscopic, such as leaf size and shape, leaf margin or cuticle thickness, while others are microscopic, such as those associated with hydraulic connectivity, including xylem anatomy, aquaporins, carbon pathway metabolism and stomatal size (Coleman et al., 2023).

The establishment of a water potential gradient in the leaves and roots of olive trees through osmoregulation facilitates water absorption even when the water potential of the soil solution drops to -2.5 MPa (Karimi et al., 2018). Osmoregulation or “osmotic adjustment” refers to the ability to synthesise and accumulate osmotically active and metabolically compatible solutes, which allows the plant to lower its osmotic potential in order to absorb water from the soil even

when it is scarce (Gucci, 2006; Karimi et al., 2018). There are two types of osmotic adjustment: active, when it depends on the synthesis and accumulation of osmolytes within the cells; passive, when it depends on the loss of water from the cells, resulting in an increase in the concentration of osmolytes present (Sofo et al., 2008). This physiological process is due to the accumulation, in plant cells, of osmotically active compounds such as carbohydrates (glucose and mannitol), certain amino acids and derivatives (proline), organic acids (malic acid and citric acid) and inorganic acids, cations and anions (Karimi et al., 2018; Sofo et al., 2008). One of the osmoprotectants that accumulates under abiotic stress conditions is proline. Proline is the first metabolite produced in response to dehydrated tissues. It can act as a signal to modulate mitochondrial functions, influence cell proliferation or cell death, and trigger specific gene expression (essential for recovery from stress) (Anjum et al., 2011). It can also reduce the oxidation of lipid membranes and prevent photoinhibition. This phenomenon allows olive trees to tolerate severe soil dehydration conditions and preserve cell turgidity (Karimi et al., 2018).

In general, olive tree leaf tissue has a good capacity for rehydration even after losing 40% of its water content and up to a water potential of -8 MPa (Karimi et al., 2018). However, in conditions of severe drought, as the water content decreases, the cell wall relaxes, resulting in a loss of turgor and a reduction in water potential (Ψ). If water deficiency occurs when the leaves are developed, they will tend to abscission; if, instead, the deficit occurs at the beginning of leaf development, then leaf expansion will be inhibited and the surface area will be reduced. This results in a limitation of the synthetic activity of the canopy. When the soil is particularly dry and the evapotranspiration rate exceeds the capacity to transport water from the roots to the canopy, the water potential of the xylem decreases. Under these conditions, the plant is susceptible to cavitation, i.e. the suction of air into the transpiration stream. Embolism phenomena may occur, during which air blocks the xylem conduit, increasing hydraulic resistance (Brito et al., 2019). One method for assessing the level of plant dehydration is relative water content (RWC). Under physiological conditions, the RWC is $\geq 90\%$, but in the case of mild drought stress it is between 60 and 70%, in the case of moderate stress between 40 and 60%, and if the dry conditions persist, it will fall below 40% (Bandurska, 2022).

For tree species, the relationship between stomatal responses and leaf water status is important given their large size and long life cycle (Coleman et al., 2023). One of the fundamental mechanisms by which plants limit water loss is the regulation of stomatal conductance (g_s), which is still not fully understood. A study conducted by Hernandez-Santana et al., (2016)

found that one of the variables most strongly correlated with g_s modulation during a period of water deficit is leaf hydraulic conductance (K_{leaf}). K_{leaf} values decrease immediately at the onset of drought, while g_s decreases only after a significant decrease in K_{leaf} (Hernandez-Santana et al., 2016). Other studies have shown that, during water deficit, the decrease in g_s values in olive trees was more correlated with the loss of hydraulic function in the distal organs of the plant (i.e. roots and leaves) than with the increase in abscisic acid (ABA) levels in leaves, stems and roots (Torres-Ruiz et al., 2015). In any case, ABA induces stomatal closure both through a direct biochemical action on the guard cells of the stomata and through a decrease in leaf water permeability within the leaf vascular tissues (Pantin et al., 2013).

In addition to ABA, other phytohormones are also stimulated by drought, such as ethylene. This hormone is involved in leaf abscission, induces the remobilisation of minerals from the leaves, reduces oxidative stress damage and may inhibit plant growth (Brito et al., 2019). Furthermore, ABA and ethylene can act antagonistically: ethylene prevents the accumulation of ABA and consequently inhibits stomatal closure, whereas high concentrations of ABA slow down ethylene and allow plant growth even under conditions of water stress (Brito et al., 2019). Auxins, growth hormones, are also affected by drought. It has been shown that auxin concentration and transport are not uniform within the plant during water stress, which would allow a balance to be maintained between vegetative growth and survival (Shen et al., 2010). It should be noted that the dialogue between different plant hormones results in synergistic or antagonistic interactions that play crucial roles in the response of plants to abiotic stress (Brito et al., 2019).

Water deficiency increases reactive oxygen species (ROS) and consequently photo-oxidative damage. If the water deficit is light or moderate, the plant can increase the level of enzymatic and non-enzymatic antioxidants, but under conditions of severe water stress, the antioxidant defence system cannot balance ROS production, resulting in damage to lipids, proteins, carbohydrates, pigments and deoxyribonucleic acid (DNA), cell membrane lesions and electrolyte loss (Brito et al., 2019).

2.4.2 Molecular responses

We can understand the ability of plants to adapt to water scarcity as their ability to maintain a growth rate and, therefore, production under sub-optimal water conditions. This ability is closely associated with changes in the expression of entire sets of genes that code for proteins

involved in cell structure repair mechanisms (dehydrins, chaperones), enzymes for the synthesis of osmoregulatory molecules and for the detoxification of reactive oxygen species (ROS). Plant cells have therefore evolved signalling pathways to perceive and integrate different signals from the environment and respond by modulating the expression of gene sets (Grillo et al., 2006).

From a molecular point of view, one of the most studied gene expressions concerns the role of membrane aquaporins in hydraulic conductance. Water flow through membranes occurs not only through the lipid bilayer, but also through aquaporins (AQPs), which are major intrinsic membrane proteins (MIPs) with molecular masses of 26-30 kDa, capable of increasing the osmotic hydraulic conductivity of the membrane by 10 to 20 times. In plants, AQPs are classified into different groups based on their sequence identity or structural characteristics, some groups include: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins, and small basic intrinsic proteins (Secchi et al., 2007).

Water stress influences the expression of aquaporins: under drought conditions, increased AQP regulation enhances membrane water permeability (Yamada et al., 1997); conversely, decreased AQP gene expression tends to conserve water in the cell (Li et al., 2000; Smart et al., 2001). Secchi et al., (2007) previously characterised the expression pattern of three genes encoding olive aquaporin, demonstrating that gene expression in roots, shoots and leaves is reduced under water stress conditions and that lower gene expression in drought-affected shoots is associated with greater hydraulic resistance.

During the water stress phase, there is an increase in genes encoding LEA (Late Embryogenesis Abundant) proteins involved in the protection of cellular structures and metabolic functions (Grillo et al., 2006). The expression of these proteins, in addition to accumulating in the final stages of embryogenesis, also occurs in other plant tissues in response to water shortage, low temperatures, excess salts or exogenous ABA treatments. Given their hydrophilic nature, they intervene to prevent incorrect protein folding and prevent protein denaturation (Xiong & Zhu, 2002).

Within the LEA superfamily are DHNs, classified as “dehydration-induced proteins”. These proteins are also involved in various cellular functions, including membrane protection against lipid peroxidation, chaperone activity, ROS removal and, in addition, inducing pleiotropic effects on various stress-sensitive genes by modulating jasmonate signalling (Piccini et al., 2025).

Several findings suggest that specific members of the DHN family play a critical role in enabling olive trees to withstand drought stress. For example, in the study on Tripepi et al., (2011) on young olive leaves, two different DHNs showed increased expression under drought conditions. Chiappetta et al., (2015) also isolated and characterised a DHN gene, OesDHN, which showed upregulation at both the transcriptional and post-transcriptional levels, leading to increased accumulation of DHN proteins in leaf tissues under mild water deficit conditions. These increases were correlated with good preservation of tissue organisation, suggesting that DHNs contribute to the maintenance of both cellular and tissue integrity (Chiappetta et al., 2015).

Recently, several transcription factors have been discovered that regulate the expression of various stress-related genes. One such class of transcription factors is DREB/CBF, which binds to cis-elements that respond to drought (Agarwal et al., 2006). Studies conducted on olive leaves have confirmed that the expression level of these genes is higher in drought-tolerant cultivars (Pascali et al., 2019). The role of DREBs in drought responses has been further confirmed by the fact that increased DREB expression increases the expression of downstream target genes encoding late embryogenic abundant (LEA) proteins (Battaglia et al., 2008).

Transcription factors also include those related to myeloblastosis (MYB), a large subfamily of the MYB family (Li et al., 2021). The MYB family is one of the largest families of transcription factors in plants and has the largest number of members with the most diverse functions. Several studies have shown that MYB transcription factors are involved in drought stress responses related to ABA signalling and light signalling (Zhao et al., 2024).

2.5 Priming as adaptive strategy in *Olea europaea*

Current efforts to increase crop resistance in unfavourable environmental conditions are often aimed at improving or reducing loss of crop productivity without resorting to pesticides and fertilisers (Singh et al., 2024), which damage the already fragile agroecological system of a territory. Until now, traditional agriculture, which exploits natural genetic diversity, has been fundamental in improving crops and strengthening their resilience against pathogens (Singh et al., 2024) and abiotic factors, selecting cultivars and varieties with more competitive phenotypic traits.

Plant priming is a process whereby prior exposure to abiotic stress alters a plant's response to subsequent stress events, leading to faster and/or stronger defensive reactions that enhance stress tolerance (Ben Abdallah et al., 2017). This improved responsiveness, often referred to as stress memory, has been observed in a wide range of plant species (Li & Liu, 2016).

Priming, often referred to as stress hardening or acclimation, constitutes a pivotal physiological strategy in olive cultivation to enhance the plant's capability to withstand abiotic constraints such as prolonged drought or high salinity (Ben Abdallah et al., 2017). This acquired tolerance is mediated by complex molecular and metabolic adjustments (including the transcriptional up-regulation of defense-related genes (Skodra et al., 2023), the accumulation of compatible solutes and carbohydrates (such as fructose and galactose) for osmotic adjustment, and the reinforcement of antioxidant systems for effective Reactive Oxygen Species (ROS) which give the plant a somatic memory of the stressful event. Whether achieved through controlled water deficit, mild saline pre-treatment (e.g., moderate NaCl exposure), or the application of chemical elicitors (such as silicon) and beneficial microorganisms, priming triggers systemic signaling pathways that mitigate cellular damage, preserve photosynthetic efficiency, and limit necrosis. Consequently, the integration of priming protocols offers a sustainable agronomic approach to safeguard yield integrity and fruit quality against the increasing pressures of climate change, specifically in regions characterized by recurrent water scarcity and soil salinization.

From an agronomic perspective, priming represents a promising tool for sustainable olive production in environments increasingly affected by recurrent drought, heat waves, and soil or water salinization. The main benefits include reduced leaf damage and tip necrosis under salinity stress, improved water-use efficiency and photosynthesis during drought, and decreased yield losses with improved fruit quality under repeated stress conditions (Skodra et al., 2023).

With regard to the triggering and storage process in drought conditions, scientific research has focused on herbaceous species. There is little information available on woody species such as olive trees (Ben Abdallah et al., 2017).

However, a study on Tunisian olive cultivars showed that olive trees had the ability to undergo some form of “stress imprinting” due to previous exposure to water stress. Changes in the morpho-physiological and biochemical parameters of primate (PP) and non-primate (NPP) plants were compared during repeated phases of water stress. In particular, primed plants (PP) were prepared by prior exposure to water deficit and only subsequently exposed to a second

cycle of drought and compared with non-primed plants (NPP). Ben Abdallah et al., (2017) confirmed that primed olive trees have a better ability to retain water, a more active antioxidant system, and are able to reduce oxidative damage by safeguarding the integrity of photosystem II, resulting in higher photosynthetic rates and greater biomass accumulation compared to non-primed plants. This result suggests that olive trees are able to maintain a stress footprint after a long recovery period.

Chapter 3 OBJECTIVES

Based on the state of the art outlined in the previous chapters, it is evident that olive trees should be considered as a holobiont, whose structure and functioning are shaped by both anthropogenic factors, such as orchard management practices, and by the specific environmental features of the cultivation areas, including soil characteristics.

Plants constantly interact with microorganisms naturally present in the soil, which in turn are influenced by the management practices used. The management practices applied during cultivation directly affect the soil, its characteristics and properties (structure, texture, fertility), which in turn are reflected in the microbial communities.

Several microorganisms or microbial groups and their processes are used as indicators of soil quality due to their sensitivity. Sustainable soils management in orchards can have positive effects on the soil itself and, consequently, can promote plant productivity by increasing microbial biomass and their complex activity. Differential soil management also has positive effects on its chemical and microbiological fertility, which in turn improves the physiological status of plants, even affecting the composition of rhizospheric, endophytic and even phyllospheric microbial communities.

However, the current literature focuses individually on the microbe-genotype or microbe-environment association without taking into account the synergistic influence of these two factors. This knowledge gap stems from the fact that microbial interactions are highly dependent on the specific environmental context and, above all, on the host genotype. Consequently, results obtained in one region or with one cultivar cannot be easily transferred to others, making localised and specific studies for each given agroecosystem essential. It is rare to be able to clearly separate how much of the microbiome really depends on the genotype of the olive tree and how much is due to the type of soil, climate or agronomic practices adopted. For this reason, it is difficult to understand whether there are “universal” patterns of the soil microbiome, or whether the patterns observed are always strongly linked to the local context (site, management, climate) (Labouyrie et al., 2023).

Further research is required to better understand how agricultural practices and environmental factors shape the biodiversity of plant-associated microbial communities within a holobiont framework.

Given these premises, this research was initially based on the hypothesis that sustainable management systems in olive groves in southern Italy not only modify soil composition but also actively reshape the taxonomic and functional profiles of the rhizosphere and phyllosphere of olive trees, with potential implications for the overall health of the plant.

Although olive trees are considered well suited to Mediterranean conditions, future climate projections indicate that the region will become hotter and drier. Rising temperatures and reduced rainfall will reduce the resilience of olive groves, especially in areas that are already arid. This will result in more frequent and prolonged droughts, putting pressure on local cultivars and traditional cropping systems that have developed under different climatic conditions. This will pose new challenges for olive growers, who will need to modify their management techniques with ecologically sustainable adaptation strategies, both in the short and long term. In this context, innovative technologies such as biostimulants and the manipulation of plant-associated microbiota are emerging as alternatives to the use of chemical fertilisers and pesticides.

Recent studies (refer to chapter 2.5) on stress memory confirm the ability of primed plants to respond quickly and efficiently to recurring abiotic and biotic stress conditions. Primed plants show faster and/or stronger activation of defensive responses when subsequently challenged by biotic stress (infections, insects) or abiotic stress (such as water stress). However, few studies have investigated the effect of priming by specific microbial groups on certain tree cultivars (let alone olive trees) when subjected to drought.

Therefore, given the potential of bacteria to modulate the physiological state of plants, we hypothesised that certain groups of beneficial rhizobacteria (PGPR), inoculated into olive tree roots, could activate the plant's defensive metabolism before the stress challenge, inducing a priming state. Specifically, we hypothesised that these bacterial consortia could improve the fitness of the holobiont olive tree by “training” the plant to respond adequately to environmental changes without losing fitness and therefore productivity.

Therefore, the general objectives of this thesis are:

- a) Characterise the microbiome associated with olive trees and its responses to different agronomic practices, identifying key microbial taxa and functions essential to the holobiont.

b) Evaluate the effects of beneficial microbial consortia as sustainable adaptation strategies to improve drought tolerance in climate-sensitive olive cultivars in southern Italy.

In order to achieve these aims, the experimental activity was divided into the following specific objectives:

i) Evaluate changes in these communities based on different olive grove management techniques (organic, traditional and conventional).

ii) Identify the composition and function of microbial communities associated with olive tree roots and leaves.

iii) Investigate the adaptive responses, through the physiological, biochemical and molecular response patterns, of the three selected cultivars ('Ortice', 'Ortolana' and 'Racioppella') to recurrent drought cycles, and evaluate cultivar-specific resilience strategies.

iv) Evaluate the priming effect of a PGPR-based microbial consortium as a sustainable biotechnological strategy to mitigate water stress in different olive genotypes.

Chapter 4 RESULTS

4.1 Management affects the diversity and functions of root and leaf associated microbiomes

The results of this section correspond to the article "*Management affects the diversity and functions of root and leaf-associated microbiomes: implications for olive resilience*," published in the peer-reviewed journal *Frontiers in Plant Science*.

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Management affects the diversity and functions of root and leaf associated microbiomes: implications for olive resilience

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Abstact

This study explores the impact of organic, conventional, and traditional agricultural management on the aboveground and belowground microbiomes of *Olea europaea* L. cv. 'Ortice', a cultivar widely cultivated in southern Italy's agricultural landscape. Through metabarcoding analyses (16S rRNA and ITS), we assessed the influence of farming approaches on the microbiome traits of the olive holobiont. Our findings demonstrate that agricultural management practices significantly shape microbiome composition both aboveground and belowground. The conventional management was associated with the highest number of microbial biomarkers aboveground, mainly belonging to *Rhizobiaceae* and *Rhodocyclaceae* families. Instead, *Fusarium* (family *Nectriaceae*) was the most abundant taxon under organic treatment. Regarding root-associated microbiome, organic management supported a greater

number of microbial biomarkers, including the bacterial genera *Actinophytocola* and *Streptomyces*, both known for their roles in promoting plant health and protecting against pathogens. In traditional systems, biomarkers included taxa from the order *Burkholderiales* and the species *Nocardioides islandensis*. Functional analysis of the aboveground fungal community revealed a higher capacity for endophytic interactions in traditional management, predominantly involving known pathogenic species such as *Alternaria alternata*, *Aureobasidium* spp., and *Cladosporium* spp. Similarly, traditional management was associated with significant enrichment of phototrophic functions belowground, mainly attributed to the bacterium *Rhodopseudomonas palustris*. Conversely, the potential for endophytic interactions was significantly greater under conventional management and was primarily linked to fungi within the class *Sordariomycetes*. Management practices shape distinct microbial communities both aboveground and belowground of olive groves, potentially influencing the resilience of Mediterranean agroecosystems and underscoring the importance of sustainable strategies.

Keywords: agricultural management, olive microbiome, olive root-associated microbiome, phyllosphere, leaf associated microbiome, rhizosphere

4.1.1 Introduction

The olive tree (*Olea europaea* L.) has been a symbol of the Mediterranean for thousands of years, deeply impacting the region's economy, society, and culture, while shaping its landscapes and identity (Brunori et al., 2019; Raz et al., 2024). Moreover, olive oil production is a vital economic sector in countries such as Italy, Spain, and Greece (Martins et al., 2024). Olive trees are well adapted to conditions of the Mediterranean climate, characterized by low rainfall and rocky, nutrient-poor soils, and they have evolved to thrive in these environments (Erel et al., 2013). Nevertheless, the health and productivity of olive trees are profoundly influenced by the management practices employed in olive groves, which can have significant effects on soil quality as well as plant growth (Orlandi et al., 2020). In this context, the type of management has changed profoundly over time to meet the growing product demand and to address climate challenges and olive farming has undergone significant intensification, marked by the adoption of irrigation systems, artificial fertilizers, and pesticides (Infante-Amate et al., 2016).

Olive groves are typically managed under three main systems: organic, traditional, and conventional farming (Cárdenas et al., 2015). The organic system follows strict guidelines established by legislation for biological agriculture, which prohibit the use of synthetic

chemicals (EC, 2007). Organic farming practices focus on promoting biodiversity, enhancing soil fertility through crop rotation, composting, and natural pest control methods (the fungicides are mineral or natural as approved under EU regulation), and supporting a sustainable farming model (Mäder et al., 2002). In contrast, traditional olive farming represents a low-intervention system in which management practices are minimal and mostly limited to essential activities such as pruning and basic soil maintenance. Farmers typically avoid the use of synthetic fertilizers, or other chemical inputs, relying instead on natural ecosystem processes to sustain plant health. This approach seeks to maintain the ecological balance of the orchard, promoting long-term resilience of the trees and preserving the native microbial communities associated with both soil and plant tissues (Fekete et al., 2023). Unlike organic and traditional management, in conventional olive farming, the use of synthetic chemicals, including fertilizers, pesticides, and herbicides, to maximize yields and manage pests is adopted. In conventional management foliar spray of synthetic fungicides are often used to control olive leaf spot and olive scab in early spring to protect the new emerging leaves, providing both preventive and early curative activity. In addition, soil tillage is carried out more frequently and more deeply than in traditional and organic treatments. This approach is designed to maximize productivity, but it may have adverse effects on the soil ecosystem and plant associated microbiome (Garbeva et al., 2008). Organic farming is typically associated with a greater diversity of soil microorganisms and a higher prevalence of beneficial microbes. For example, it has been observed that this system often foster the presence of nitrogen-fixing bacteria, mycorrhizal fungi, and microorganisms involved in nutrient cycling and disease suppression (Mäder et al., 2002; Iqbal et al., 2023). Conventional farming, by contrast, with its reliance on chemical inputs and more intensive soil management practices, can lead to a shift of microbial communities, which may have negative consequences for soil fertility and plant health (Kannan et al., 2024). Traditional farming systems, which involve limited interventions (such as pruning, the soil is left untilled, with periodically cut of natural herbaceous cover) without additional chemicals or fertilizers, can strike a balance between maintaining soil quality and ensuring the sustainability of the farming system (Khmelevtsova et al., 2022). A growing body of research has highlighted the importance of soil management in determining the structure and function of microbial communities. For instance, Wentzien et al., (2023) demonstrated that different soil management practices, such as tillage and cover cropping, had significant effects on microbial diversity and functionality in olive groves. They found that organic farming systems supported a more diverse microbial community, contributing to improved soil quality and plant health.

Similarly, Montes-Borrego et al., (2013) showed that organic farming practices in olive groves promoted higher microbial diversity, especially among bacterial groups involved in nutrient cycling and disease suppression. These studies illustrate the importance of agricultural practices in shaping both microbial diversity and functionality within the agricultural ecosystem and playing a critical role in maintaining soil health and plant productivity. The olive plant hosts a complex and diverse microbiome in both its aerial and belowground compartments. In line with the broader concept of root and leaf associated microbiome, these communities, primarily bacteria and fungi, can colonize plant tissues epiphytically, on external surfaces, or endophytically, within internal tissues, forming an integrated network of plant-microbe interactions across the entire plant (Larry et al., 2016; Sinha et al., 2025; Zhu et al., 2022). Microorganisms engage in intricate interactions with each other and with their host, influencing key plant traits, including growth, nutrient uptake, and disease presence or resistance (Berendsen et al., 2012). The phyllosphere harbors a wide range of microbial taxa, including both pathogenic and beneficial microorganisms. While some members of the phyllosphere microbiota can act as pathogens and compromise plant health, others contribute positively to the host by suppressing disease. Beneficial phyllosphere microbes can limit pathogen infection through direct mechanisms, such as the secretion of antagonistic metabolites and competition for space and nutrients, or indirectly, by priming or inducing plant immune responses (Fontana et al., 2021; Morales-Cedeño et al., 2021; Binyamin et al., 2019). Similarly, root-associated microbiome can play a pivotal role in shaping plant health by modulating nutrient cycling, enhancing stress tolerance, and mediating pathogen suppression (Berendsen et al., 2012). The majority of studies on the olive tree microbiome have investigated its composition in individual plant compartments (Fernández-González et al. 2019; Malacrinò et al. 2022). While some authors have explored the connections between aboveground and belowground microbiomes (e.g., Kakagianni et al., 2023; Martins et al., 2024), more research to understand how agricultural management practices may shape such interactions is needed. Given the crucial role of the plant microbiome in determining host fitness and function, there is increasing interest in characterizing, modeling, and managing host-microbiome interactions to optimize plant performance and resilience. This has led to the emergence of microbiome-informed strategies in agriculture, where microbiome-driven cropping systems are being developed as a means to enhance crop productivity and sustainability. Such approaches hold the potential to drive a paradigm shift in plant production, contributing to a more resilient and eco-friendly agricultural future.

With this study, we aimed to add new knowledge about whether adaptation to contrasting management systems alters the microbiome community composition and functionality of above- and below-ground olive trees, focusing on the cultivar Ortime which is highly representative of a key Mediterranean region for oil production such as Southern Italy. To achieve this target, the following objectives were pursued: assessment of the impact of management (traditional, organic and conventional) in (i) shaping the belowground root-associated (including the endorhizosphere, rhizoplane, and ectorrhizosphere) microbial (bacteria and fungi) community, (ii) shaping the aboveground leaf-associated (including both the phylloplane and internal endophytic microbial (bacteria and fungi) community and (iii) altering the potential functional traits of the root and leaf associated bacterial and fungal community. We test the hypothesis that low-impacting management has a positive influence on the belowground and/or aboveground microbial communities associated with olive trees at different levels, such as diversity and functional profile.

4.1.2 Materials and methods

4.1.2.1 Sampling activity

All samples analyzed in the study were collected from 15 olive groves of *Olea europaea* L. cv Ortime in March 2024. The study area falls within the Sannio olive cultivation area (Southern Italy, Figure 3). All fields are located in areas with similar climatic conditions and soil types (clayey and marly hill soil type, Campania Region Geoportal), at 600 m above sea level. The physical-chemical features of the soil are reported in Supplementary Table S1 (Supplementary Material). Five olive groves were selected for each type of the considered agricultural management: organic, traditional, and conventional. During the sampling period, organic groves were in the final stage of winter cover-crop growth (which consisted mainly of legume-based green manure mixtures) with no recent soil disturbance. In conventional groves, late-winter tillage and weed-control operations had recently been carried out, and preventive fungicide applications targeting newly emerging leaves occurred. In addition, nitrogen-rich fertilizers in late winter/early spring were applied to stimulate vegetative growth. Traditional farming systems had no recent mechanical inputs (e.g., cutting of the natural herbaceous cover) prior to sampling. The olive groves across the three farming systems were similar in size, each covering roughly 2,000 m². The average distance between sampled groves was 590 m, and together the 15 groves spanned a total geographic area of 0.48 km². At each olive grove, roots (including the endorhizosphere, rhizoplane, and ectorrhizosphere) and leaves (including both the

phylloplane and endophytic) samples were collected in order to perform metagenomic analyses, respectively. To ensure the representativeness of the olive grove and to consider biological variability, 6 trees of the same age were identified for each grove running along the diagonal of the grove in the NE - SO direction avoiding border interferences. To collect the belowground samples, the topsoil layer (less than 5 cm) was removed, and then the main roots of each plant were tracked until young and cork-free roots were discovered at a depth of approximately 5-40 cm. Using a core drill (or soil auger), six sub-samples consisting of roots with attached soil were collected at each site. Fully expanded leaves were sampled from the same individuals, collecting from branches with similar light exposition and position in the canopy. During sampling, all equipment was sterilized (10% bleach followed by 70% Ethanol and then distilled water) to avoid cross-contamination between sample types and between fields. The six sub-samples (per type) were homogenized to obtain a composite sample for each grove.

After collecting, all samples were transported at 4 °C to the Department of Science and Technology at the University of Sannio and then stored at -80 °C until analysis.

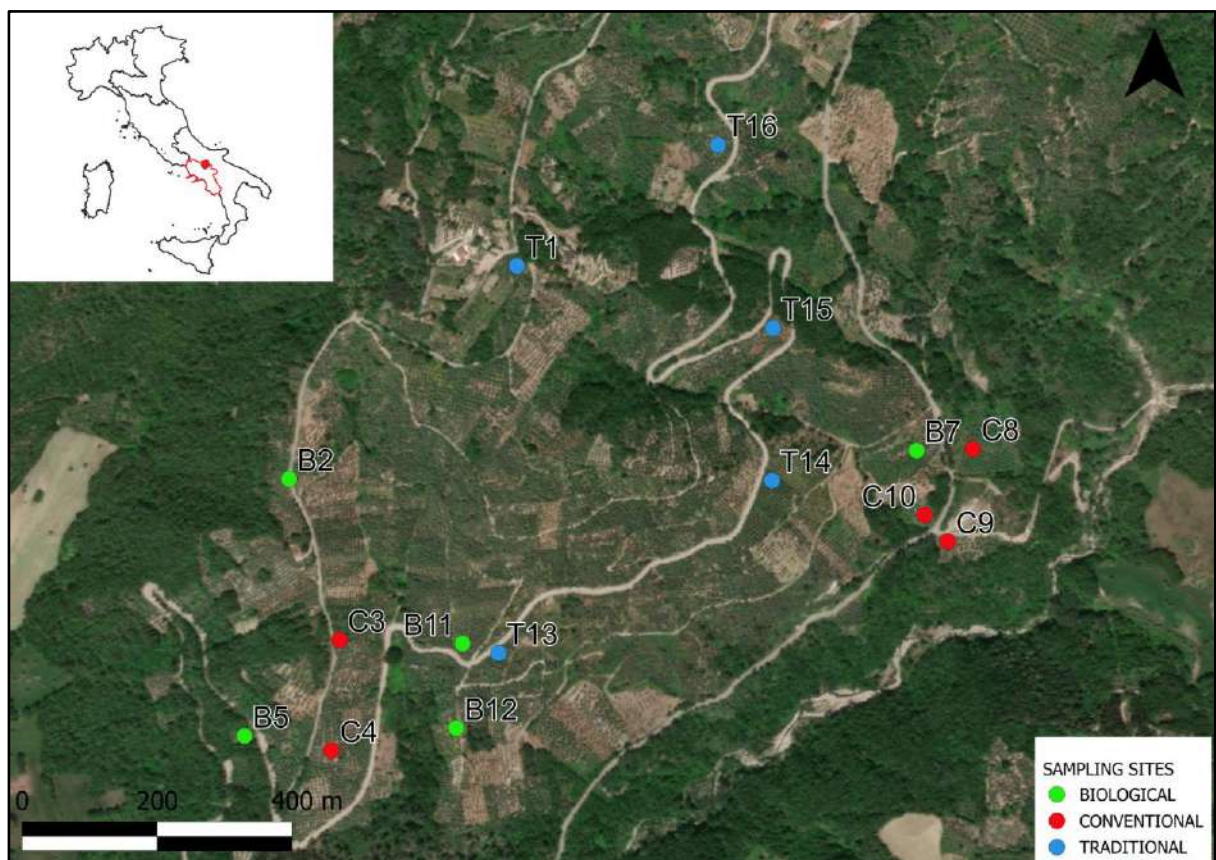


Fig.3. Study area and sampling points. O: fields with organic treatment, C: fields with conventional treatment, T: fields with traditional treatment.

4.1.2.2 DNA extraction

Microbial communities root and leaf associated were analyzed through total DNA extraction from composite plant samples. For the root-associated communities, approximately 0.2 g of roots with tightly adhering soil were collected. Root systems were gently shaken to remove loosely attached soil particles, retaining only the soil fraction in close contact with the root surface. The resulting material, consisting of root tissues and the adherent soil fraction, was homogenized under sterile conditions to ensure an even representation of microorganisms inhabiting both endorhizosphere, rhizoplane, and ectorhizosphere. This sampling strategy was designed to capture the overall microbial assemblage directly interacting with the root environment. For the leaf-associated microbiome analyses, DNA was extracted from entire leaf tissues rather than from surface washes or detached cells. This approach was adopted to include both epiphytic (surface-dwelling) and endophytic (internal) microorganisms, thereby representing the complete microbial assemblage associated with the leaf environment. Leaf tissues were processed under sterile conditions and homogenized prior to extraction to obtain a composite sample encompassing both microbial fractions.

Total genomic DNA from all samples was isolated using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-tek, Inc.) following the manufacturer's instructions. DNA concentration was quantified fluorometrically with a Qubit system (Thermo Fisher Scientific), and DNA integrity was assessed using the 4150 TapeStation system (Agilent Technologies). The purified DNA samples were subsequently used for downstream analyses of microbial community composition and predicted functional potential.

4.1.2.3 Metagenomic analysis

The bacterial community was analyzed by amplifying the 16S rRNA genes by PCR using primers for the hypervariable regions V2, V3, V4, V6-7, V8 and V9, according to the Ion 16S Metagenomics kit (Life Technologies, 5781 Van Allen Way Carlsbad, CA, USA 92008). The kit used to amplify the resulting libraries was the Ion Plus Fragment Library kit (Thermo Fisher Scientific) while with the Qubit 3.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA) using the Qubit DNA High Sensitivity Assay kit (Thermo Fisher Scientific), the libraries were quantified. The libraries were pooled with a final equimolar concentration of 40 pM and they were loaded onto chips for sequencing with the Ion Torrent[™] system, Ion S5[™], using the Ion 530[™] Chip kit. Ion GeneStudio[™] S5 was subsequently used to sequence the templates. The

Ion Reporter™ Software 5.20 platform with the Metagenomics 16S w1.1 v5.14 workflow was used to analyze the generated unaligned binary data files (Binary Alignment Map, BAM) (Ranauda et al., 2024). The Ion reporter workflow is based on both the MicroSEQ™ ID 16S Reference Library v2013.1 and Greengenes v13.5 as reference databases. Any reads shorter than 150 base pairs were excluded after primer trimming. The analysis was performed with a Minimum Alignment Coverage of 90% and a Read Abundance Filter set to 10, with a 99% identity threshold. Finally, the 16S rRNA sequence reads were assigned to operational taxonomic units (OTUs) through the closed reference method. For the analysis of the fungal community, the ITS1 and ITS2 regions were amplified using multiplex PCR. The specific primer sequences used are as follows: ITS1 Fw: GTCCCTGCCCTTTGTACACA; ITS1 Rev: TTTCGCTGCGTTCTTCATCG and ITS2 Fw: GTGAATCATCGAATCTTTGAA; ITS2 Rev: TTCCTCCGCTTATTGATATGC. The amplification reactions were carried out with the TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems™). The PCR protocol was as follows: 3 minutes at 94°C, followed by 25 cycles of 15 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 68°C, with a final extension at 72°C for 7 minutes. The generated libraries were amplified and sequenced using the same procedure as the 16S libraries. Taxonomic identification was performed using the UNITE database, applying a length filter of >10 bp. Any reads shorter than 150 base pairs were excluded after primer trimming. The analysis was conducted with a Minimum Alignment Coverage of 90% and a Read Abundance Filter set to 10, with a 99% identity threshold. The ITS sequence reads were then grouped into operational taxonomic units (OTUs) using the closed-reference method. All sequencing data have been deposited in the NCBI with accession number SUB15436629.

4.1.2.4 Data analysis

The microbial community was analyzed using R version 4.3.3 (R Development Core Team, 2024), with several key packages including *reshape2* (v.1.4.4), *ggplot2* (v.3.4.2), *plyr* (v.1.8.8) (Wickham, 2007; Wickham and Sievert, 2009, 2011), *Hmisc* (v.5.1–0) (Harrel and Dupont, 2019), and *phyloseq* (v.1.42.0) (McMurdie and Holmes, 2013). Both 16S and ITS sequences were processed into OTUs and imported into R as *phyloseq* objects for exploratory analysis and to evaluate the taxonomic composition of the samples. The *phyloseq* object includes the taxonomy table (indicating the taxonomic classification of each OTU), the OTU table (which records OTU counts per sample) and the sample data matrix (providing metadata for each sample). To homogenise the number of sample reads (read depth) and avoid bias in distance

and dissimilarity calculations (Gloor et al., 2017), we used the function `microbiome::transform` from the *microbiome* package in R with the method “compositional”; then the data was prepared following the procedure described by Zuzolo et al. (2023). To assess the dispersion of microbial communities within management systems, the *betadisper* function (*vegan* package) was used since it computes the distance of each sample to the centroid of its group, providing a measure of within-treatment variability. Statistical significance of differences in dispersion among treatments was tested using permutation tests (Permutation test for homogeneity of multivariate dispersions with 999 permutations). Mean distance-to-centroid values by treatment were extracted from the *betadisper* results to summarize the within-group variability (Maher et al., 2019). Shannon diversity index values were calculated for all microbial communities in both compartments. For each management system, mean values and standard deviations from five samples were computed and differences in alpha diversity among treatments were assessed using one-way ANOVA. To evaluate dissimilarity in microbial communities between treatments, we performed first the Permutational analysis of variance (PERMANOVA) with *adonis* function from *vegan* package and then the *post-hoc* test *pairwise.adonis* function from *pairwiseAdonis* package. The R/Bioconductor package *microbiomeMarker* (Cao et al., 2022) was employed to investigate taxa that exhibit differential abundance and to identify microbial biomarkers characterizing microbiome data of the different management type. In particular, linear discriminant analysis effect size (LEfSe) was utilized for the discovery of metagenomic biomarkers after data normalization using the total sum scaling (TSS) method. The parameters employed for the LEfSe analysis were as follows: Kruskal–Wallis test with $\alpha = 0.05$ to identify features significantly different among management systems. LDA score cutoff of ≥ 3.0 (log10 scale) to define biomarkers. Multiple testing correction: the default LEfSe workflow was used; no additional FDR adjustment was applied. Additionally, functional predictions were made using the R package *microeco* (Liu et al., 2021). The bacterial functional profile was predicted using FAPROTAX, a tool designed for the functional annotation of prokaryotic taxa. Following this, fungal OTUs were annotated with functional data by cross-referencing them with the FungalTraits database (Pölme et al., 2020). To assess differences in functional traits across various sample types, an ANOVA test ($p < 0.05$) was performed. All results were visualized using the *ggplot2* package. Both FAPROTAX (Louca et al., 2016) and FungalTraits (Pölme et al., 2020) are comprehensive databases containing taxonomic and functional descriptions derived from a selection of carefully curated publications (Wen et al., 2023).

4.1.3 Results

4.1.3.1 Sequencing results

For 16S metagenomic analysis across 15 samples (5 samples per management type) of leaf-associated microbiome, a total of 8,817,677 reads were generated, of which 7,237,936 were valid reads (ranging from a minimum of 248,470 to a maximum of 720,015 per sample). Additionally, 5,516,331 reads were successfully mapped. For the ITS metagenomic analysis, a total of 5,756,513 reads were obtained from 15 samples (ranging from 68,054 to 641,050 reads per sample), considering a blast abundance threshold >10. Regarding the sequencing data of the root-associated microbial community the 16S metagenomic analysis yielded a total of 7,030,958 reads, of which 5,882,827 were valid, across 15 samples (5 per management type), with 2,153,701 reads successfully mapped. In contrast, the ITS analysis detected a total of 7,703,087 reads distributed across the 15 samples. The sequencing depth (Supplementary Figure S1, Supplementary Material) was sufficient for both microbial communities, as all rarefaction curves approached a clear plateau, indicating adequate coverage of bacterial and fungi richness.

4.1.3.2 Composition of leaf-associated bacterial and fungal communities

From the taxonomic assignment with the NCBI database, we found that the data of the aboveground compartment overall returns 98 bacterial OTUs, divided into 6 phyla, 10 classes, 27 orders, 47 families, 67 genera and 86 species. The fungi contained 151 OTUs, divided into 3 phyla, 15 classes, 34 orders, 63 families, 84 genera and 80 species (Supplementary Table S2). In terms of relative abundance, *Pseudomonadota*, *Actinomycetota* and *Bacteroidota* are the most abundant bacterial phyla in all treatments (respectively: 0.49 ± 0.07 , 0.27 ± 0.06 , 0.22 ± 0.11 in the organic, 0.43 ± 0.04 , 0.25 ± 0.04 , 0.27 ± 0.05 in the conventional and 0.44 ± 0.11 , 0.48 ± 0.11 , 0.07 ± 0.09 in the traditional) (Supplementary Figure S3a). *Streptomyetaceae*, *Fulvivirgaceae* and *Chitinophagaceae* are the most abundant families in the organic (respectively: 0.14 ± 0.08 , 0.11 ± 0.06 , 0.07 ± 0.03) and conventional treatments (respectively: 0.15 ± 0.06 , 0.14 ± 0.02 , 0.10 ± 0.04); the traditional treatment differs with *Micrococcaceae*, *Streptomyetaceae* and *Sphingomonadaceae* being the most abundant (respectively: 0.35 ± 0.15 , 0.22 ± 0.14 , 0.21 ± 0.12) (Supplementary Figure S3b). Regarding the aboveground bacterial species, *Arthrobacter russicus*, *Sphingomonas* sp. and *Ralstonia insidiosa* are the most abundant of traditional treatments (respectively: 0.21 ± 0.15 , 0.13 ± 0.12 , 0.09 ± 0.11). Instead,

Ohtaekwangia kribbensis is the most abundant species in organic (0.11 ± 0.06) and conventional treatments (0.14 ± 0.02); the second most abundant species are *Actinacidiphila glaucinigra* for organic (0.08 ± 0.05) treatment and *Niastella yeongjuensis* for conventional treatment (0.08 ± 0.03) (Supplementary Figure S3c). *Ascomycota* mainly dominated the leaf-associated fungal community across all agricultural treatments, showing almost complete prevalence in organic (1.00 ± 0.00), conventional (0.98 ± 0.02) and traditional samples (0.98 ± 0.01). In contrast, *Basidiomycota* were present only at very low relative abundances in all treatments ($\leq 0.02 \pm 0.01$) (Supplementary Figure S4a). The *Phaeosphaeriaceae* family is the most abundant in organic (0.62 ± 0.25) and conventional (0.48 ± 0.33) treatments, while in the traditional is *Pleosporaceae* (0.59 ± 0.36), however, *Phaeosphaeriaceae* (0.34 ± 0.28) are also well represented in these samples (Supplementary Figure S4b). *Parastagonospora avenae* species is the most abundant in organic (0.63 ± 0.24) and conventional (0.46 ± 0.36) treatments, on the other hand, *Stemphylium solani* is the dominant species in traditional treatment samples (0.58 ± 0.36) (Supplementary Figure S4c). Aboveground bacterial α -diversity (Table 1) differed significantly among management systems, with the traditional system showing reduced diversity, while fungal α -diversity did not vary. The dispersion of microbial communities within treatments, evaluated using the mean distances to centroid reported in Supplementary Table S3 of the Supplementary Material, showed no significant differences within management systems. Both the centroid distances and the associated permutation tests indicated no significant variation in dispersion (betadisper, all $p > 0.05$). Instead, the pairwise PERMANOVA results (Table 2, Supplementary Figure S5) indicate significant differences of the leaf-associated bacterial community. Specifically, the traditional treatment shows significant differences compared to both the organic treatment ($p = 0.015$) and the conventional treatment ($p = 0.013$). For fungi, traditional management significantly differs from the organic treatment ($p = 0.045$), but the difference with the conventional treatment is marginal ($p = 0.093$).

Table 1 - Mean, standard deviation (sd) and p-values of one-way ANOVA of Shannon index for bacterial and fungal communities within management (Conventional, Organic, Traditional).

		Treatment	Shannon (mean±sd)
Leaf associated	Bacteria	Conventional	3.12±0.18
		Organic	3.11±0.16
		Traditional	2.01±0.59
		p value	5.33x10 ^{-0.4}
	Fungi	Conventional	1.63±0.5
		Organic	1.64±0.17
		Traditional	1.18±0.4
		p value	0.11
Root associated	Bacteria	Conventional	3.30±1.47
		Organic	4.06±1.27
		Traditional	3.59±0.98
		p value	0.64
	Fungi	Conventional	2.54±0.47
		Organic	3.43±0.08
		Traditional	3.31±0.63
		p value	0.01

Table 2 - Beta diversity results. The p value of pairwise comparisons for bacterial and fungal communities within management Conventional (C), Organic (O) and Traditional (T) for Bray-Curtis and Jaccard distances are shown.

	Leaf-associated					
	Bacteria			Fungi		
Indicators	T vs O	T vs C	O vs C	T vs O	T vs C	O vs C
Bray-Curtis	0.018	0.009	0.848	0.037	0.049	0.866
Jaccard	0.015	0.013	0.826	0.045	0.093	0.865
	Root-associated					
	Bacteria			Fungi		
Indicators	T vs O	T vs C	O vs C	T vs O	T vs C	O vs C
Bray-Curtis	0.204	0.359	0.169	0.373	0.044	0.068
Jaccard	0.169	0.302	0.167	0.383	0.045	0.043

4.1.3.3 Composition of root-associated bacterial and fungal communities

Among the 15 samples from the root-associated microbiome, we identified 785 bacterial OTUs belonging to 14 phyla, 25 classes, 62 orders, 125 families, 229 genera and 674 species. Fungal OTUs were 608 divided into 8 phyla, 33 classes, 91 orders, 184 families, 304 genera and 301 species (Supplementary Table S4).

When considering relative abundance, the root-associated bacterial community shows a clear dominance of the phyla *Pseudomonadota* and *Actinomycetota* across all treatments, with *Pseudomonadota* accounting for 0.57 ± 0.04 in the organic, 0.53 ± 0.28 in the conventional, and 0.63 ± 0.11 in the traditional treatment, and *Actinomycetota* representing 0.39 ± 0.04 , 0.23 ± 0.17 , and 0.32 ± 0.10 in the same treatments, respectively (Supplementary Figure S6a). The *Sphingomonadaceae* family is the most abundant in traditional (0.23 ± 0.09) and organic (0.21 ± 0.08) treatments, while in conventional the *Bacillaceae* family is the most abundant (0.19 ± 0.41) (Supplementary Figure S6b). Similarly, the genus *Sphingomonas* is the most abundant in all samples (respectively: 0.19 ± 0.07 in the traditional, 0.18 ± 0.09 in the organic and with 0.13 ± 0.08 treatment). In terms of species, the most abundant in traditional (0.08 ± 0.04) and organic (0.05 ± 0.02) treatments is *Enhydrobacter aerosaccus*, while *Caldibacillus thermoamylovorans* presents a relative abundance of 56% in the C8 sample of the traditional treatment. (Supplementary Figure S6c). The root-associated fungal community in all samples across the

three management practices is predominantly composed of three phyla: *Ascomycota* (with a relative abundance of: 0.65 ± 0.08 in organic treatment, 0.82 ± 0.17 in conventional and 0.75 ± 0.06 in traditional), *Basidiomycota* (with a relative abundance of: 0.23 ± 0.09 in organic treatment; 0.14 ± 0.16 in conventional and in 0.75 ± 0.15 traditional), and *Mucoromycota* (with a relative abundance of: 0.11 ± 0.02 in organic treatment; 0.04 ± 0.02 in conventional and in 0.48 ± 0.10 traditional) (Supplementary Figure S7a). The family with the highest relative abundance is *Nectriaceae* (phylum *Ascomycota*), with a relative abundance of 0.14 ± 0.09 in the traditional treatment, 0.19 ± 0.08 in the organic treatment, and 0.39 ± 0.29 in the conventional treatment (Supplementary Figure S7b). In fact, *Fusarium proliferatum* is the most abundant species in the conventionally treated fields (with 0.29 ± 0.24) (Supplementary Figure S6c). Belowground, bacterial α -diversity was similar across treatments, whereas fungal α -diversity was higher under organic management (Table 1). Both the centroid distances and the associated permutation tests indicated no significant variation in dispersion (*betadisper*, all $p > 0.05$) within treatment (Supplementary Table S3). The pairwise PERMANOVA (Table 2, Supplementary Figure S5) shows no significant differences in the composition of the root-associated bacterial community among the three treatments ($p > 0.1$). However, the fungal community in the conventional treatment differs significantly when compared to both the traditional ($p = 0.045$) and organic ($p = 0.043$) treatments.

4.1.3.4 Leaf-associated biomarkers of agricultural management types

LefSe analysis identified a total of 35 microbial biomarkers across the three management regimes (Figure 4). Among these, the earliest differentiating taxa were associated with traditional management (blue), with the class *Actinomycetes* and its parent phylum *Actinomycetota* (LDA scores > 5). In contrast, conventional management accounted for the majority of differentially abundant taxa, comprising 28 distinct biomarkers. The five most representative biomarkers under conventional management belonging to the phylum *Bacteroidota*, and included the class *Cytophagia*, order *Cytophagales*, genus *Ohtaekwangia*, species *Ohtaekwangia kribbensis*, and the family *Fulvivirgaceae*, all exhibiting LDA scores around 5. While not individually emphasized by the analysis, a considerable proportion of biomarkers associated with conventional management were taxonomically affiliated with the phylum *Pseudomonadota*, suggesting its potential relevance in shaping aboveground bacterial communities under this regime. Indeed, ranking the taxa according to their own scores, we find: the genus *Novosphingobium* and the families *Rhizobiaceae* and *Sphaerotilaceae* (LDA scores

of 4.7 and 4.6, respectively); the genus *Piscinibacter* and the species *Piscinibacter aquaticus* (LDA scores of about 4.6); the genus *Mycoplana* and the species *Mycoplana dimorpha* (LDA scores of about 4.5) and the species *Novosphingobium lentum* with an LDA of 4.5. The genus *Salinibacterium*, including the species *Salinibacterium xinjiangense*, and the genus *Yinghuangia* with the species *Yinghuangia aomiensis*, all belonging to the phylum *Actinomycetota*. Within conventional management, we also find the class *Proteobacteria*, the order *Rhodocyclales*, and the related family *Rhodocyclaceae*. Additionally, two taxa from the kingdom *Archaea*, *Candidatus Nitrososphaera* and the species *Candidatus Nitrososphaera SC11145*, with LDA scores of approximately 4.4 and 4.3, respectively, are also highlighted. The final biomarkers identified through LefSe analysis associated with conventional management include the species *Phenylobacterium immobile*, the genus *Phenylobacterium*, and the family *Caulobacteraceae*, all with LDA scores near 4. Lastly, the biomarkers shown in green in Figure 4 correspond to the third type of management: organic management. These include the genus *Methylocystis*, the family *Methylocistaceae*, as well as the species *Methylocystis heyeri* and *Rhizobium mesosinicum*, all with LDA scores around 4.3 (Figure 4).

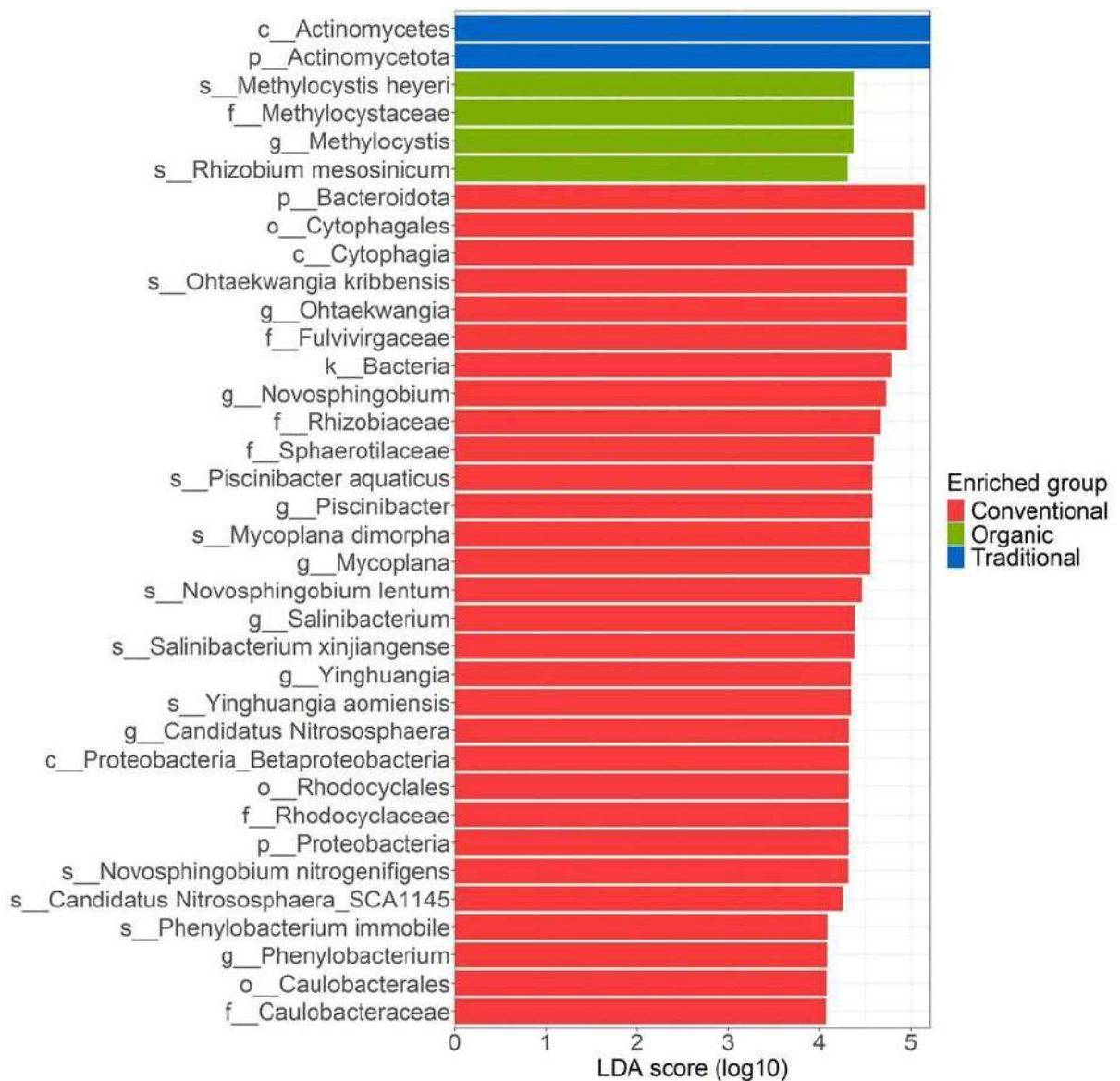


Fig. 4. LefSe analysis of the phyllosphere leaf-associated bacterial community for the three managements: conventional, traditional and organic.

Analyzing the aboveground fungal community, the LefSe analysis identified 18 differentially abundant biomarkers among the three soil treatment types (Figure 5). Regarding the fungal biomarkers of organic management, the most relevant, is the family *Nectriaceae* (*Ascomycota* phylum), with an LDA score exceeding 4.5. Within this family, the genus *Fusarium*, and the species *Fusarium proliferatum* and *Fusarium* sp. (with LDA scores slightly over 4) are identified as biomarkers. Additional biomarkers highlighted by LefSe include the class *Taphrinomycetes*, the order *Taphrinales*, the family *Taphrinaceae*, the genus *Taphrina*, and the species *Taphrina* sp., all exhibiting LDA scores just above 4. In the conventional management shown in red the most representative differentially abundant biomarkers are members of the

orders *Pleosporales* and *Dothideales*. In the order *Pleosporales*, the differentially most abundant taxa are represented by the genus *Alternaria* and the species *Alternaria alternata* with a score of around 4.5. For the order *Dothideales*, on the other hand, the family *Sacrotheciaceae*, the genus *Aureobasidium* and the species *Aureobasidium* sp. Are the representative biomarkers. Finally, regarding the traditional management (shown in blue), only two taxa resulting from the Lefse analysis belong exclusively to the phylum *Ascomycota*; the genus *Stemphylium* and the species *Stemphylium solani* with an LDA of 5 (Figure 5).

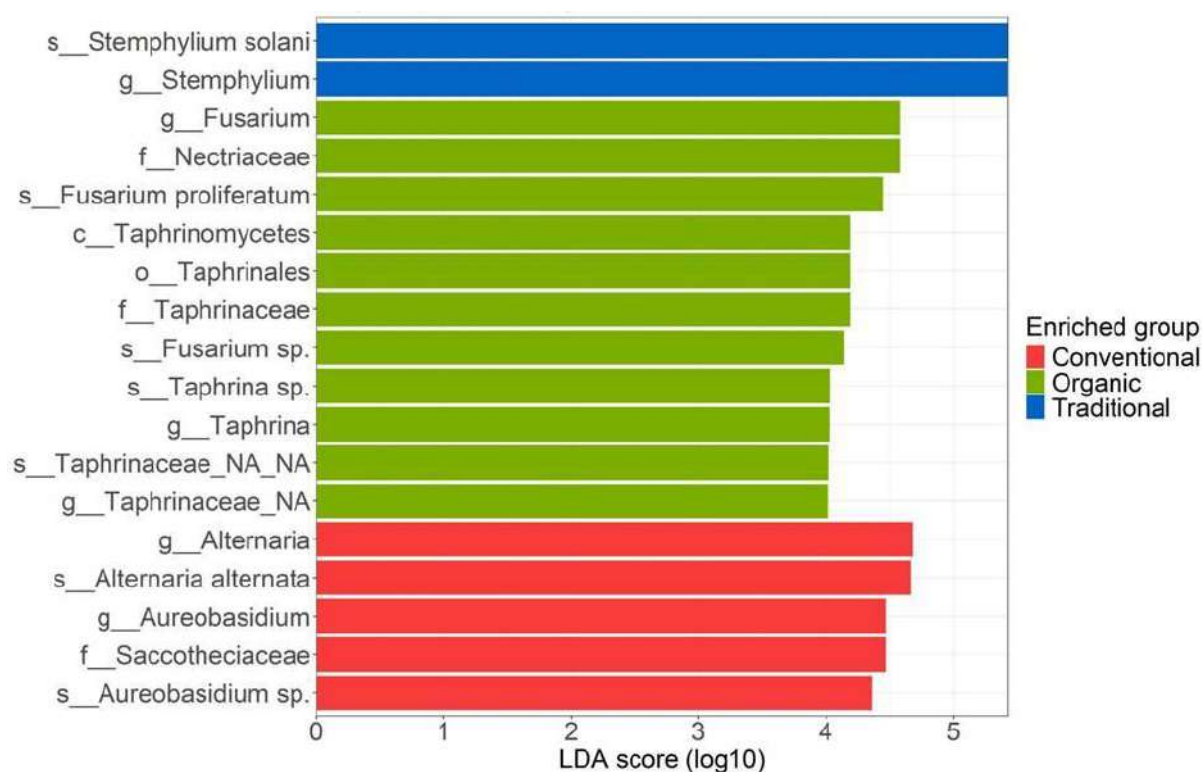


Fig. 5. LefSe analysis of the phyllosphere leaf-associated fungal community for the three managements: conventional, traditional and organic.

4.1.3.5 Root-associated biomarkers of agricultural management types

Evaluating the LefSe analysis of the root-associated bacterial community (Figure 6), the 28 biomarkers were found to belong exclusively to just two types of management: organic and traditional. Within the traditional management, the differentially abundant biomarkers are represented by the following taxa: *Nocardiodes islandensis* belonging to the phylum *Actinomycetota* with an LDA score of 4.5; the species *Rhizobacter fulvus* and an unknown family belonging to the order *Burkholderiales* with scores of about 4; and finally among the differentially abundant biomarkers in traditional management, the species *Terrimonas lutea*

(phylum *Bacteroidota*, genus *Terrimonas*) with an LDA score of more than 3.5 is evident. In the organic management 23 differentially abundant taxa were found by the LefSe analysis, all belonging to the phyla *Actinomycetota* and *Pseudomonadota*. Among the top ten biomarkers reported with the highest score we find genus *Actinophytocola* featured by the species falling within the phylum *Actinomycetota*, such as: *Actinophytocola oryzae*, *Actinophytocola xinjiangensis* and *Actinophytocola* sp. With a score of approximately 4, *Nocardioides halotolerans*, *Streptomyces* sp. And *Sporichthya jensenii* with a score higher than 3. While among the biomarkers belonging to the phylum *Pseudomonadota*, the family *Sphingosinicellaceae* with an LDA of 3.5 and the species *Hyphomicrobium* sp. And *Nitrosospora* sp. Were found to have a high score. Other biomarkers with an approximate LDA score of 3 are the species *Sporichthya* sp., *Parafrigoribacterium mesophilum*, *Actinokineospora fastidiosa*, *Paracraurococcus* and *Microbacterium favesces* (Figure 6).

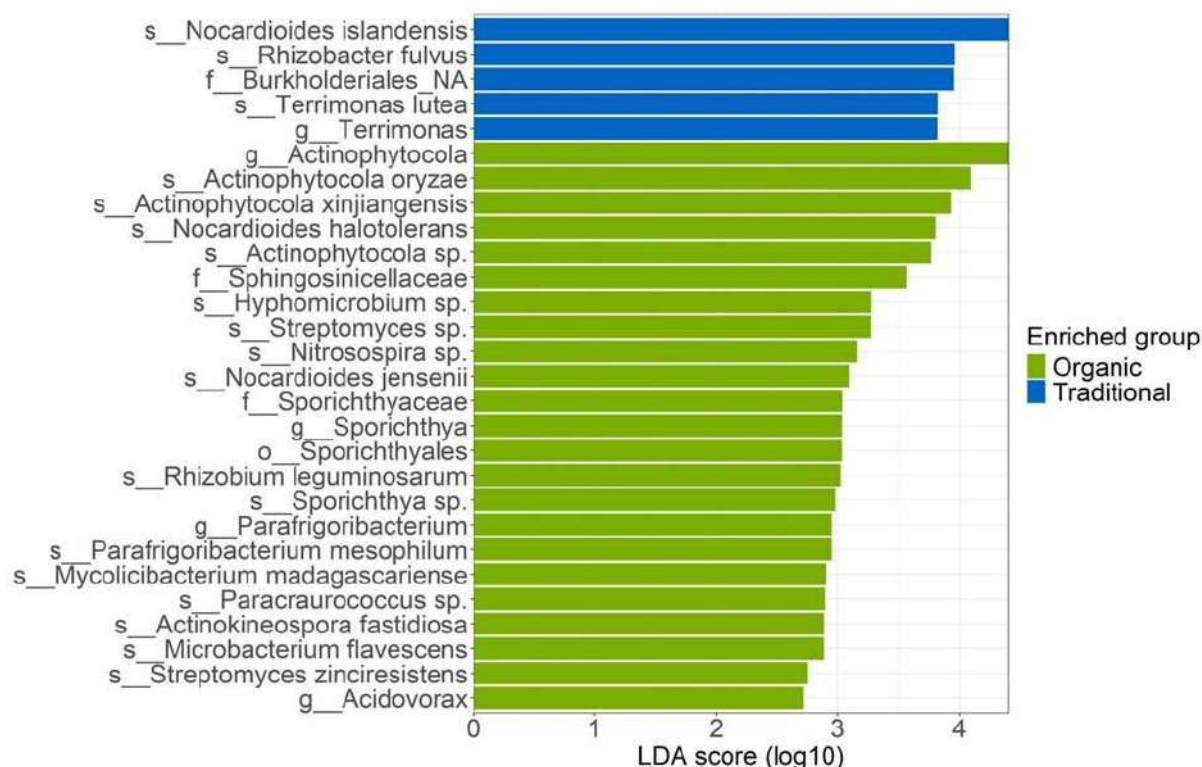


Fig. 6. LefSe analysis of the rhizosphere root-associated bacterial community for the three managements: conventional, traditional and organic.

The LefSe analysis of the root-associated fungal community (Figure 7) identified a total of 45 biomarkers across the three management practices, revealing a clear predominance of taxa from the *Ascomycota* phylum. For the traditional management, the families *Didymellaceae* and *Pyronemataceae* were most prominent, each exhibiting high LDA scores above 4. The genus

Humicola and the species *Humicola fuscoatra* showed lower LDA scores, below 4. In contrast, the order *Archaeosporales*, along with the species *Linnemannia gamsii* and *Mariannaea superimposita*, were differentially abundant, with LDA scores near 3. In conventional management, the LefSe analysis highlighted only a single biomarker: the class *Sardariomycetes* (phylum *Ascomycota*), which had a notably high LDA score greater than 5. For organic management, the majority of biomarkers also belonged to the *Ascomycota* phylum. However, the highest differentially abundant taxa were from the *Mucoromycota* phylum, with an LDA score exceeding 4.5. The next five biomarkers, which were all part of the same taxonomic lineage, exhibited LDA scores around 4.5, and included: *Mortierellamycetes* (class), *Mortierellales* (order), *Mortierellaceae* (family), *Mortierella* (genus), and *Mortierella* sp. In addition, several biomarkers had LDA scores above 3, including the families *Herpotrichiellaceae* and *Annulatascaceae*, the order *Ketothyriales*, and the genera *Exophiala*, *Oidiodendron*, *Paraphoma*, and *Piloderma*. Notably, the species *Exophiala* sp., *Oidiodendron truncatum*, and *Piloderma* sp. Were also found to be differentially abundant. Finally, the orders *Atheliales* and *Sebacinales*, both belonging to the *Basidiomycota* phylum, were also identified as differentially abundant.

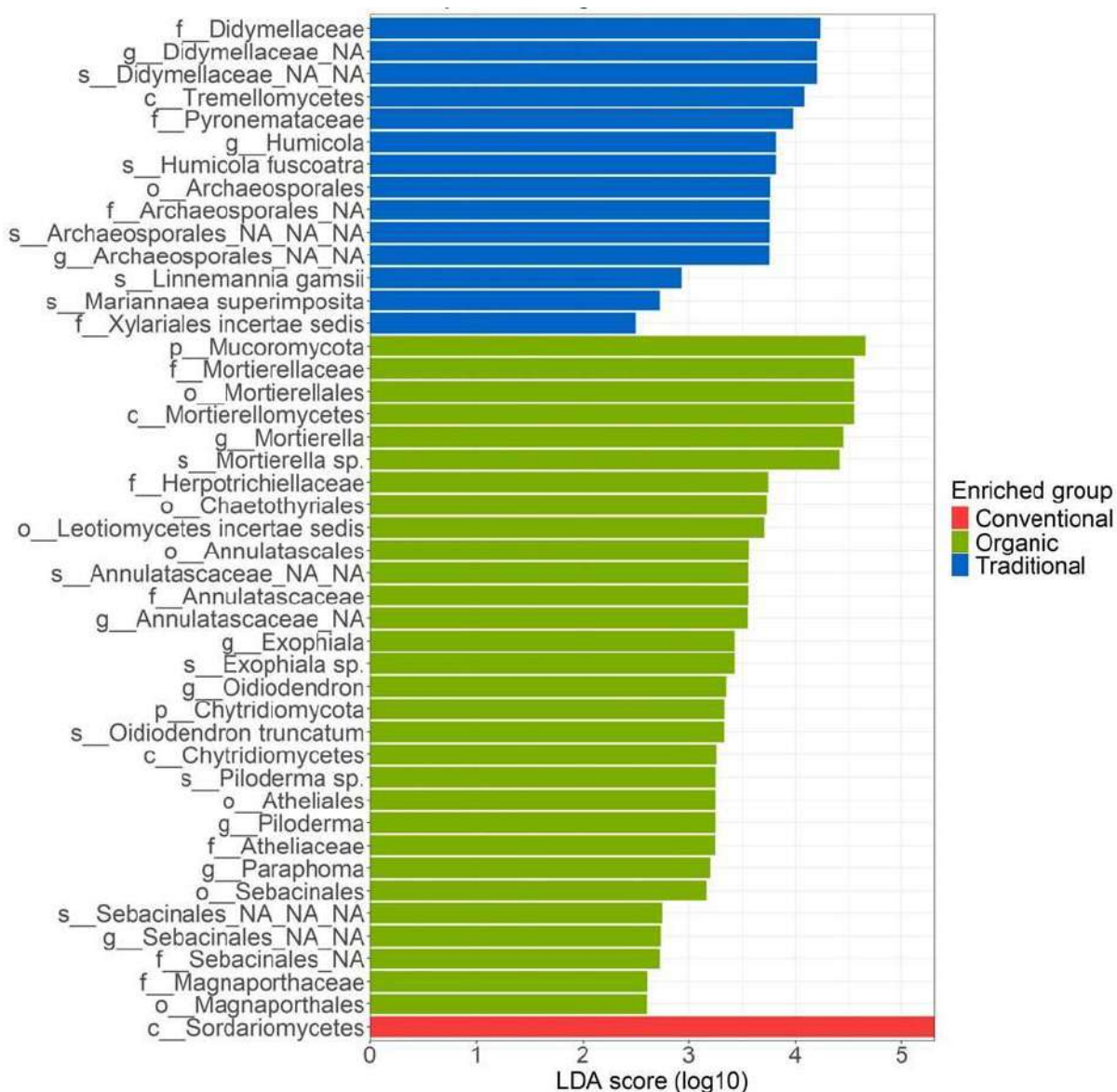


Fig. 7. LefSe analysis of the rhizosphere root-associated fungal community for the three managements: conventional, traditional and organic.

4.1.3.6 Functional classification of the leaf-associated microbiome

Concerning the aboveground bacterial community, the functional classification revealed 34 functions across 64 OTUs (Supplementary Table S5).

Figure 8 illustrates that traditional treatments show significant differences in bacterial functions, specifically anaerobic_chemoheterotrophy and nitrogen_fixation, compared to the other management types. Organic management, while only significantly different from traditional management (and not from conventional management), is most strongly associated with both of these functions. The species *Methylocystis heyeri*, identified as a biomarker for organic

management in the LefSe analysis (as previously mentioned), is responsible for carrying out both functions. On the other hand, *Rhodococcus erythropolis*, *Enterococcus cecorum* and *Erwinia oleae* are unique species for anaerobic_chemoheterotrophic function, while *Rhizobium leguminosarum* is found to be unique species for nitrogen_fixation function. These species are found to be characteristic of the organic management samples, too. Nitrate_reduction, a function not differentially expressed but relevant in terms of abundance, is realized by *Enhydrobacter aerosaccus* and *Salinibacterium xinjiangense* in both conventional and organic treatments. Functions like animal_parasite_or_symbionts, fermentation, and hydrocarbon_degradation occur more frequently for both conventional and organic methods.

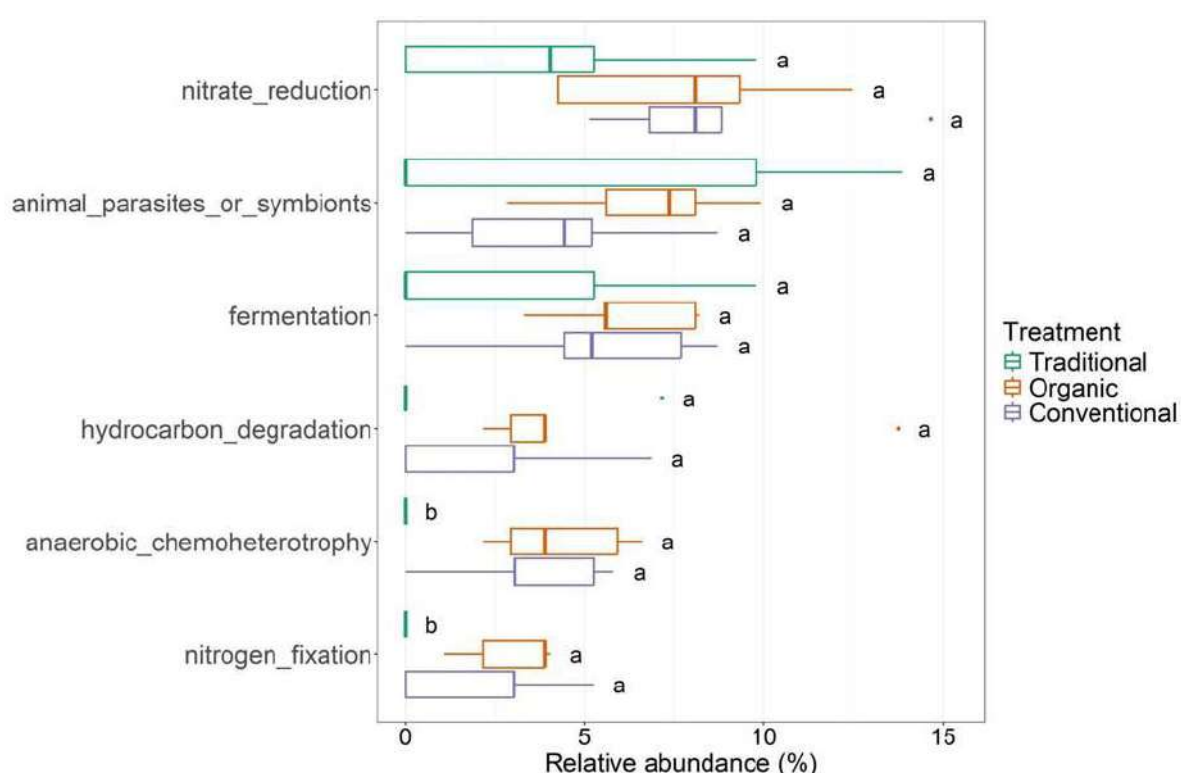


Fig. 8. Functional traits of the leaf-associated bacterial community for the three managements: traditional, conventional and organic.

The functional traits linked to the leaf-associated fungal community are 78 representative of 123 OTUs (Supplementary Table S5). The Figure 9 indicates that the foliar_endophyte function is the most abundant in the fungal community in conventional treatments and shows differences only with the communities of traditional treatments. Our data reveal that *Alternaria alternata* and *Aureobasidium* sp. Are the taxa to which the above function is associated. The same trend is visible for the growth_form|yeast function carried out mainly from *Bannozyma yamatoana* in conventional treatment, from *Malassezia* sp. In organic treatment and *Vishniacozyma*

carnescens in traditional treatment. Functional differences between traditional and organic treatments are found for the following traits: Plant_pathogenic_capacity|wood_pathogen, Secondary_lifestyle|nematophagous, Animal_biotrophic_capacity|nematophagous, Fruitbody_type|agaricoid, Hymenium_type|gills. All these functional traits, due to their unrepresentativeness in traditional treatments, show no significant differences with conventional treatments. The species belonging to the genus *Pleurotus* (*Pleurotus floridanus* and *Pleurotus ostreatus*) perform all these functions. In addition, *Biscogniauxia mediterranea*, *Dendrostoma leiphaemia*, *Ganoderma adspersum*, *Vuilleminia comedens*, which are well represented in organic treatments also perform the function of wood_pathogen. Finally, functions Decay_substrate|roots and primary_lifestyle|litter_saprotroph are well represented in terms of relative abundance in the three treatments (particularly in the conventional) although they do not show significant differences. Taxa such as *Cladosporium delicatulum*, particularly abundant in conventional treatments, performs the litter_saprotroph function.

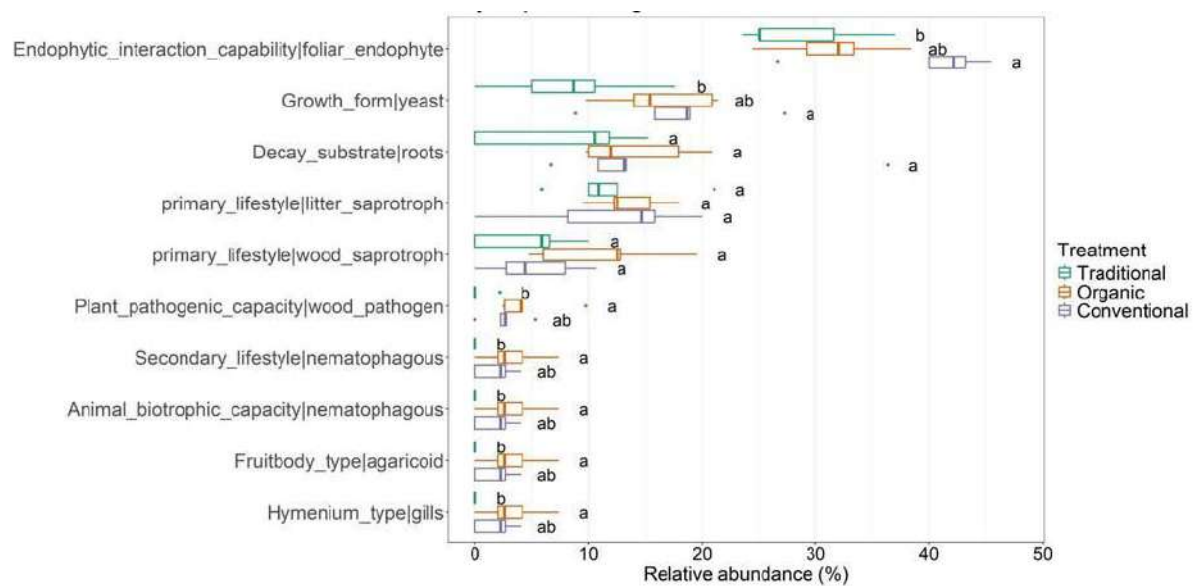


Fig. 9. Functional traits of the leaf-associated fungal community for the three managements: traditional, conventional and organic.

4.1.3.7 Functional analysis of the root-associated bacterial and fungal community

The functional analysis of the root-associated bacterial community (Figure 10) reveals 63 distinct functions across 546 OTUs (Supplementary Table S6). The first five functional traits are significantly more abundant for traditional management compared to conventional ones. The first two functions (Figure 10) are related to photoheterotrophy and phototrophy bacteria, for which *Paracraurococcus* sp. Is responsible. The next functional trait highlighted by

FAPROTAX is photoautotrophy, an activity carried out mainly by species such as *Rhodoplanes* sp., *Rhodopseudomonas palustris* and *Rubrivivax gelatinosus*. The same species, *Rhodoplanes* sp. And *Rhodopseudomonas palustris* are implicated in functional traits such as anoxygenic_photoautotrophy and anoxygenic_photoautotrophy_S_oxidising respectively. Other top abundant functions do not show significant differences among management. For instance, ureolysis (with a higher abundance in the organic treatment) is predominantly performed by *Hyphomicrobium* sp. Which is a differentially abundant species of the organic management. Furthermore, another functional trait prevailing in the organic management is nitrification for which the biomarker of biological management *Nitrosospira* sp. Is involved. The other functions highlighted by FAPROTAX are hydrocarbon_degradation, methylotrophy and methanotrophy.

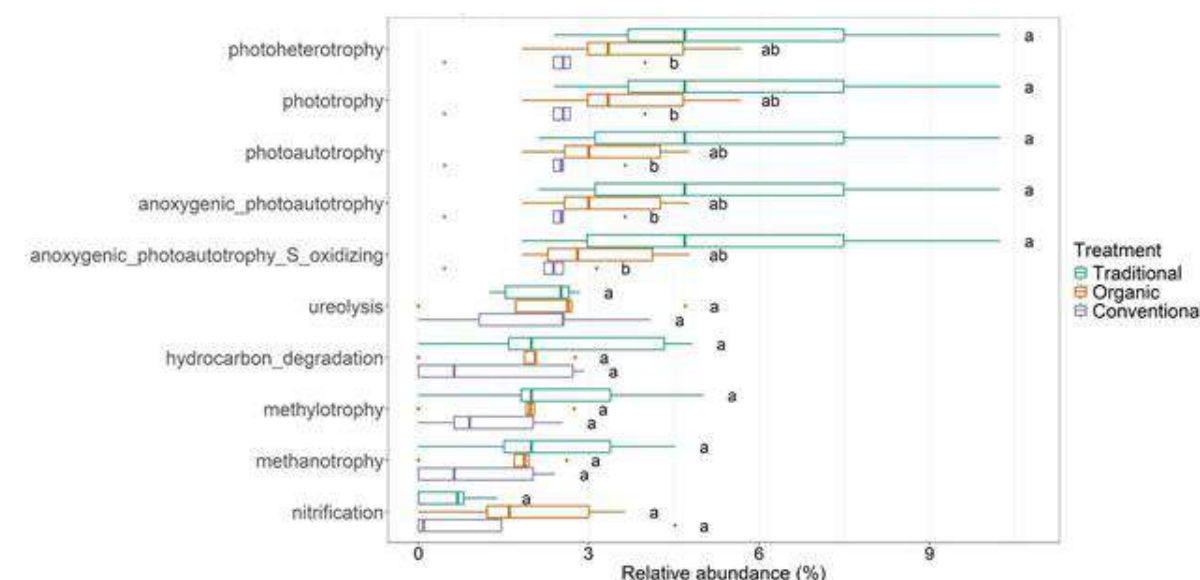


Fig. 10. Functional traits of the root-associated bacterial community for the three managements: traditional, conventional and organic; the top 10 functions are shown.

The predictive functional analysis of the root-associated fungal community revealed 191 distinct functions assigned to 473 OTUs (Supplementary Table S7). Among the most representative functions (Figure 11) we find the function foliar_endophyte in first place and is more abundantly carried out within conventional management. Moreover, it appears to be a significantly more abundant function than organic treatment, while traditional management, although well represented by this function, shows no significant differences with the other two management types. 112 taxa perform this function with 61 of these corresponding to the class *Sordariomycetes*, which is also a biomarker detected by the LefSe analysis in the conventional

treatment as shown above. Functional analysis showed that several species of the genus *Trichoderma* (belonging to the class *Sordariomycetes*) perform the foliar_endophyte function. The conventional management shows a higher abundance for the litter_saprotroph function with 140 taxa involved, mostly represented by the *Sordariomycetes* class (with genera such as *Fusarium*, *Chaetomium* and *Myrothecium*). The conventional management host also a higher abundance of nematophagous fungi, due to the presence *Pleurotus* genus. The representative fungal traits of the traditional management, are Decay_substrate|fungal_materials and Primary_lifestyle|mycoparasite. The class *Tremellomycetes*, which is differentially abundant within the traditional management, appears from the functional analysis to be a taxa involved in both of the above-mentioned functions. Specifically in the mycoparasite function, the genus *Papiliotrema* is linked to perform this function within the class *Tremellomycetes*. With regard to organic management, the function secondary_lifestyle|root_endophyte is significantly higher compared to the other types of management. The species *Oidiodendron tenuissimum* and *Oidiodendrum truncatum* are the main taxa involved in this function for organic groves.

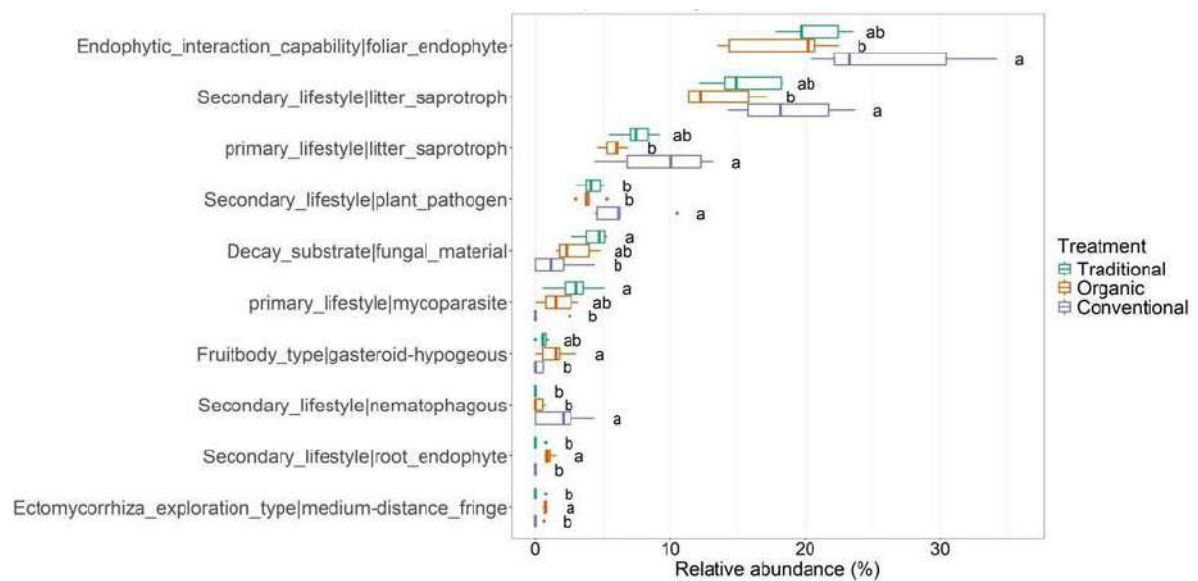


Fig. 11. Functional traits of the root-associated fungal community for the three managements: traditional, conventional and biological.

4.1.4 Discussion

In this study, we investigated to what extent different agricultural management practices could influence belowground and aboveground olive microbiome under different agricultural management. Specifically, organic, conventional and traditional management were investigated

with the hypothesis that conventional management may act as an ecological disturbance, potentially influencing both soil and plant health. While numerous studies have explored the olive microbiome (Fernández-González et al., 2019; Kacem, 2023), few of them simultaneously analyze both belowground and aboveground. In addition, we focused on Ortice cultivar (typical of southern Italy) which has not yet been investigated. The data highlighted that community composition shifted in ways that are not captured by richness alone. PERMANOVA revealed significant multivariate differences among treatments, indicating that management affected the overall structure of the communities even when alpha diversity remained largely unchanged. Biomarker analysis further highlighted taxon-specific and biologically relevant responses. Regarding the root-associated microbiome, we observed that the fungal community is significantly influenced by management practices, showing differences between conventional and organic, as well as conventional and traditional (PERMANOVA results). These results are consistent with those of Kacem (2023), who suggests that tillage, a characteristic of conventional management, exerts a greater impact on the fungal community than on the bacterial community in the rhizosphere. The management type also affects aboveground microbiome, for which both fungi and bacterial communities of traditional treatments significantly differ. Concerning root-associated bacteria, the genus *Actinophytocola* was identified as the top biomarker of organic management. This genus is particularly significant for the fitness of olive trees, contributing to drought tolerance and exhibiting antimicrobial properties that inhibit the growth of well-known human pathogens such as *B. subtilis* and *S. aureus* (Fernández-González et al., 2019). Additionally, the biomarker *Streptomyces* was also present in the organic treatment, recognized as a biocontrol agent (BCA) in various pathosystems (El-Tarabily and Sivasithamparam, 2006; Law et al., 2017). The abundance of *Streptomyces* in the organic management underscores the positive effects of these agronomic practices. In traditional treatment, biomarkers belonging to the *Burkholderiales* order (including the species *Rhizobacter fulvus*) are indicators of organic environments (Liu et al., 2022; Schmidt et al., 2019). Furthermore, another relevant biomarker detected is *Rhizobium leguminosarum*. This species belongs to the *Rhizobiaceae* family reported in literature as a root symbiont useful to the plant for nitrogen fixation activity (Harman and Uphoff, 2019). In contrast, little is yet known about *Nocardioides islandensis* and *Terrimonas* sp. role in the rhizosphere of the olive tree. Concerning the root-associated fungal taxonomy, most of the differentially abundant biomarkers are found to be related to organic management, followed by those of traditional management and finally by conventional management. The class of

Sordariomycetes, the only biomarker of conventional treatment, includes taxa that perform a wide range of functions. Some are plant pathogens that result in leaf, stem, and root diseases across a wide range of hosts, whereas others cause disease in arthropods and mammals. Many species of *Sordariomycetes* are involved in decomposition and nutrient cycling as saprophytes, in addition to several others that persist in this role (Maharachchikumbura et al., 2016). The biomarkers most abundant in traditional treatment are part of the family *Didymellaceae*. The majority of *Didymellaceae* members were plant pathogens with a wide variety of host species, primarily resulting in damage to the leaves and stems, as stated by Chen et al. (2017). The analysis of aboveground bacteria using LefSe revealed that conventional farming management shows a higher number of biomarkers compared to the other two agricultural practices. Notably, the presence of the *Rhizobiaceae* family stands out. This family includes species that form symbiotic relationships with plants at the root level, allowing them to fix nitrogen, a crucial nutrient for plant growth, while simultaneously deriving nourishment from the plant itself (Harman and Uphoff, 2019; Lindström and Mousavi, 2020). *Rhodocyclaceae* are a common family of endophytic bacteria belonging to the class *Betaproteobacteria* and are found to be dominant in leaf-associated communities. *Rhodocyclaceae* were found to be significantly enriched in the phyllosphere of plants growing in unpolluted soil, which could have an impact on the growth and survival of host plants and on bacteria-bacteria interactions (Li et al., 2020). Among biomarkers of organic management, however, we again find a species belonging to the *Rhizobiaceae* family namely *Rhizobium mesosinicum*. Therefore, it would appear that organic management too, as regarded as conventional one, promotes the growth and development of species in the family *Rhizobiaceae* known for their nitrogen fixation activity (Harman and Uphoff, 2019).

The aboveground fungal communities are characterized by distinct biomarker profiles associated with different soil management practices. Organic management promoted the enrichment of the family *Nectriaceae*, particularly *Fusarium proliferatum* and *Fusarium* sp., suggesting a potential shift toward fungi with both pathogenic and saprophytic roles. *Fusarium* spp., despite being commonly associated with olive tree dieback, can show variable pathogenicity depending on several isolates with weak or no pathogenic potential (Trabelsi et al., 2017). Mondello et al. (2019) research suggests that this fungus may suppress pathogen growth via both antibiosis and direct antagonism, as well as by priming host defense responses. These findings indicate a complex ecological role of *Fusarium*, ranging from pathogenic to potentially beneficial strains. The presence of *Taphrinomycetes*-related taxa further supports

the distinct fungal dynamics under organic inputs. In contrast, conventional management favored taxa such as *Alternaria alternata* and *Aureobasidium* sp., commonly associated with phylloplane and stress conditions (Nicoletti et al., 2020). Traditional management was characterized by just one biomarker, namely *Stemphylium solani*., which is known to cause minor foliar diseases in olives (Abdelfattah et al., 2015; Frisullo and Carlucci, 2011), indicating possible weak implications for plant health.

We observed that the conventional system shows enriched leaf-associated bacterial taxa but not root-associated taxa probably related to the above-ground management and pesticide/spray regimes. The conventional management involves the application of foliar sprays (mainly fungicides against the main pathogens of olive plants) which suppress fungal biomass, and prevents fungal biomarker taxa from increasing. When fungal competitors are reduced, new ecological space opens on the leaf surface, allowing certain bacteria to grow more easily. Many of these bacteria are tolerant or opportunistic species that take advantage of the conditions created by the fungicide. Similar enrichment of leaf-associated taxa under conventional management was observed in sugarcane (Khoiri et al., 2021). Concerning the belowground microbiome, the traditional and organic farming fosters a soil environment conducive to a thriving and diverse microbial community, leading to higher detection of both bacterial and fungal biomarkers. Conversely, conventional farming practices suppress microbial biomass and diversity through chemical and physical disturbances, resulting in lower biomarker presence. Similar observations of lower differentiation in conventional rhizosphere compared with organic systems have been reported (Hartmann et al., 2015; Zhang et al., 2025). Overall, the enriched leaf-associated taxa in the conventional system likely reflect aboveground selective pressures, whereas the conventional practices tend to homogenize and weaken microbial communities.

Concerning the functional analyses, organic farming appears to have a stronger positive effect on nutrient recycling processes (e.g., nitrogen cycling) and this result is in line with other research (Wu et al., 2024). We found that the leaf-associated microbiome under traditional management harbors a lower abundance of nitrogen-fixing bacteria compared to both organic and conventional treatments, which likely results from the reduced fertilizer inputs in traditional systems. This limitation may restrict available niches for nitrogen-fixing bacteria (Frache et al., 2009; Gattinger et al., 2012; Vitousek et al., 2002). In contrast, nitrogen fertilization, likely driven by enhanced plant growth and increased nitrogen content in the leaves due to high

nitrogen inputs (Darlison et al., 2019), may have promoted the proliferation of specific microbial groups. We also observed a significantly higher capacity for endophytic interactions within the fungal community in both organic and conventional treatments. We observed that the capacity for Endophytic_interaction_capability in the aboveground fungal community is significantly more abundant in conventional treatments. The main involved foliar endophyte taxa are *Alternaria alternata*, *Aureobasidium* sp. And *Cladosporium* sp. For which it is well known the potential pathogenic capacity. This remark involved potential pathogens in conventional treatments which may act as disturbance of natural-biological control of such pathogens. Finally, evaluating the functions in the root-associated compartment, it is interesting to note the presence of the bacterium *Rhodopseudomonas palustris* linked to a higher phototropism trait in traditional managed groves. *R. palustris* is a well-known and long-established biological control agent, and is successfully applied to suppress crop diseases and promote plant growth (Luo et al., 2019). The spontaneous occurrence of this species suggests that non-aggressive agricultural practices can support the adaptation of bacterial communities, which adjust their composition in response to abiotic stress factors. Regarding the endophytic_interaction_capability of root-associated fungi, the class *Sordariomycetes* (the most differentially abundant in the conventional treatment) is mostly involved. In agreement with Marañón-Jiménez et al., 2021, we see that saprotrophic fungi can be a major contributor to the degradation of organic substrates and the application of organic materials to land is a common practice in sustainable agriculture (Repullo et al., 2012). Using FAPROTAX (Louca et al., 2016) provided useful insights into potential functional shifts, but predictions are inherently limited by database coverage, the fraction of unassigned environmental taxa, and the variability of functional traits within taxonomic lineages. Consequently, inferred functions should be interpreted with caution rather than as confirmed activities. Even within these constraints, the analysis clearly highlights functional patterns driven by management practices. Understanding the effects of agro-technical practices on the composition and diversity of soil bacterial and fungal communities remains a complex task, particularly because field studies involve environmental variables that are difficult to fully isolate (e.g., within-orchard variability). Methodological aspects, such as the resolution of sequencing approaches and the use of predicted functional profiles, may also influence the detection and functional interpretation of some microbial taxa. Nonetheless, despite these inherent challenges, our results reveal clear patterns showing how different agricultural management practices (organic, conventional, and traditional) affect both belowground and aboveground microbial communities in the Ortime

olive cultivar. These findings provide valuable insights that can inform strategies to support more sustainable and resilient olive cultivation.

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4.2 Microbial priming and stress memory under recurrent drought

The results of this section correspond to the article "*Priming and memory in ancient olive cultivars: Enhancing drought resilience through rhizosphere microbial consortia under repeated stress cycles*," published in the peer-reviewed journal *Environmental and Experimental Botany*.

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Priming and memory in ancient olive cultivars: Enhancing drought resilience through rhizosphere microbial consortia under repeated stress cycles

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Abstract

Understanding how olive cultivars respond to recurrent drought is critical to develop climate-resilient cropping systems. This study investigated the physiological, biochemical, and molecular responses of three autochthonous cultivars ('Ortice', 'Ortolana', 'Racioppella') exposed to repeated water deficit, with and without inoculation of a PGPR consortium (*Bacillus subtilis*, *Pseudomonas fluorescens*). Under stress, 'Ortolana' maintained stable water status, 'Ortice' exhibited progressive adaptive plasticity, while 'Racioppella' showed heightened stress sensitivity with limited recovery capacity. Microbial inoculation enhanced the accumulation of osmolytes (proline, soluble sugars) and antioxidants (phenols, flavonoids), and mitigated pigment degradation. At the molecular level, drought induced strong upregulation of NCED1 and repression of PP2C, while inoculated plants displayed a tempered transcriptional response, suggesting effective priming. Upon the second stress exposure, LEA46 and DHN1 expression increased markedly, particularly in 'Ortice', indicating activation of stress memory. Expression

of OePIP2.1 aquaporin declined under drought, consistent with reduced membrane water permeability; however, cultivar-specific patterns emerged, implying differential regulatory strategies. The microbial consortium appeared to reinforce both primed and memory-based responses. These findings demonstrate that integrating tolerant genotypes with functional microbial inoculants enhances drought resilience through coordinated physiological regulation and timely modulation of stress-responsive genes, supporting sustainable olive production in water-limited environments.

Keywords: Climate change adaptation, Drought stress, Gene expression, *Olea europaea* L., Physiological traits

4.2.1 Introduction

Among the several manifestations of global climate change, the increasing frequency and severity of droughts represents one of the most pressing threats to agricultural sustainability. In the Mediterranean basin, recognized as a primary “climate change hotspot” by the Intergovernmental Panel on Climate Change (IPCC), rising temperatures and a progressive decline in annual precipitation have been accompanied by an intensification of extreme weather events, including prolonged and recurrent droughts (Lazoglou et al., 2024). These phenomena undermine the resilience of traditional cropping systems, long adapted to dryland conditions. The agricultural sector, historically based on low-input, rainfed practices, is now increasingly confronted with the challenge of maintaining productivity in the face of unpredictable water availability and deteriorating soil fertility (Brito et al., 2019).

Within this evolving context, olive (*Olea europaea* L.) cultivation, one of the most iconic and economically relevant agro-systems of the Mediterranean, is undergoing profound transformations. Traditionally established in marginal areas and managed under rainfed conditions, olive groves have long been considered resilient due to the species' intrinsic drought tolerance (Majikumna et al., 2024). However, in recent decades the expansion of high-density planting systems, combined with increasing commercial pressure, has led to the widespread introduction of irrigation regimes, even in areas historically managed without supplemental water. While such strategies may temporarily stabilize yields, they also amplify the crop's vulnerability to drought, as water availability becomes a critical limiting factor for maintaining productivity and fruit quality (Gholami and Zahedi, 2019).

Although olive trees possess well-documented adaptations to water deficit, when drought becomes prolonged and cyclic, it may severely compromise the plant's physiological integrity and its productive performance. Drought stress negatively influences both reproductive development and oil biosynthesis in olive trees, often resulting in fewer flowers, reduced fruit set, and lighter drupes, which collectively contribute to a significant decline in oil production. Concurrently, water scarcity can compromise key quality parameters of the oil, including its fatty acid composition, polyphenol content, oxidative stability, and sensory profile, features that are critical for its classification and commercial appeal as extra virgin olive oil (Trabelsi et al., 2019). These effects, particularly concerning in high-value production systems, highlight the urgent need to develop strategies that enhance the olive's drought resilience without compromising its agronomic and organoleptic excellence (Adi et al., 2025).

Recent studies have revealed that olive trees, like other perennial species, may be capable of stress memory, a phenomenon whereby exposure to a prior drought event modifies subsequent responses, often resulting in faster or stronger activation of protective mechanisms during recurring stress (Brito et al., 2019, Nteve et al., 2024). This memory is mediated by physiological adjustments, transcriptional reprogramming, and possibly epigenetic changes, and has been associated with improved resilience in certain cultivars (Nair et al., 2022, Salimonti et al., 2025). Investigating how different genotypes express this drought memory can offer critical insights into long-term adaptation strategies under climate instability (Gholami and Zahedi, 2019).

One promising approach involves the conservation and functional valorization of local genetic resources. In the Sannio area, Campania region, southern Italy, three historical cultivars, 'Racioppella', 'Ortolana', and 'Ortice', stand out for their agronomic importance and their oils exceptional quality, frequently awarded and recognized in national and international olive oil guides. 'Racioppella' is known for its balanced oil composition and moderate vegetative vigor, making it suitable for water-limited environments. 'Ortice', widely cultivated, produces oils of intense aromatic richness and high phenolic content, and is appreciated for its tolerance to variable rainfall and marginal soils. 'Ortolana', though less diffused, exhibits stable productivity under heterogeneous conditions and irregular water availability (Cirillo et al., 2023, Di Vaio et al., 2013). These cultivars offer a valuable model for exploring genotype-specific responses to water stress and identifying resilience traits that can support the development of climate-adapted agronomic strategies.

Moreover, the role of rhizosphere microorganisms, particularly plant growth-promoting rhizobacteria (PGPR), has emerged as a key factor in plant adaptation to abiotic stress. The microbial consortia that inhabit the root–soil interface actively contribute to nutrient acquisition, hormonal modulation, and activation of defense pathways (Fernández-González et al., 2019, Zarraonaindia et al., 2015). In drought scenarios, PGPR such as *Bacillus subtilis* and *Pseudomonas fluorescens* have been shown to enhance root growth, osmotic adjustment, and antioxidant capacity, thereby supporting plant function under water deficit (Hashem et al., 2019, Zia et al., 2021). PGPR can also induce a form of stress priming, whereby exposure to microbial signals conditions the plant's physiological and molecular machinery to respond more effectively to subsequent stress episodes. This phenomenon results in a faster or attenuated transcriptional activation of stress-related pathways, improved antioxidant defense, and more stable water relations during drought (Nteve et al., 2024, Yang et al., 2023). The integration of functional microbial consortia into olive cultivation may thus represent a promising tool for enhancing resilience in a sustainable and genotype-specific manner (Araújo et al., 2019, Crespo-Barreiro et al., 2025). In this context, a microbial consortium composed of *Bacillus subtilis* and *Pseudomonas fluorescens* was selected based on their complementary plant growth-promoting traits, including phytohormone modulation, ACC deaminase activity, exopolysaccharide production, and efficient rhizosphere colonization, which collectively contribute to improved water-use efficiency, osmotic adjustment, and antioxidant defense. Their combined application has therefore the potential to elicit synergistic effects on olive drought tolerance.

To address these challenges and to explore the interaction between plant genotype, environmental stress, and microbial support, this study was designed with three main aims: (i) to compare the physiological, biochemical, and molecular responses of olive plants grown under optimal irrigation with those subjected to drought stress, evaluating the potential mitigating effect of a PGPR-based microbial consortium; (ii) to assess the impact of recurrent drought cycles, simulating increasingly realistic climate conditions, and to investigate whether plants adjust their responses over time; and (iii) to determine whether the three selected landrace cultivars, 'Ortice', 'Ortolana', and 'Racioppella', exhibit differential patterns of stress adaptation and microbial responsiveness, with the goal of identifying cultivar-specific resilience strategies to support future drought-resilient olive farming systems.

4.2.2 Material and methods

4.2.2.1 Experimental design

To evaluate the effect of repeated drought stress on different cultivars of *Olea europaea* L. and to assess the potential mitigating role of a microbial consortium, a multifactorial experimental design was implemented. Three olive cultivars were selected: 'Ortice' (CR), 'Ortolana' (LR), and 'Racioppella' (RR). For each cultivar, forty certified one-year-old olive plants were used (total: 120 plants). Plants were transplanted into 2 L pots filled with a substrate consisting of 80 % field soil (collected and sieved from an olive orchard cultivated with the same cultivars in the Molinara area, Benevento, Italy) and 20 % perlite (v/v) (T0). All pots were maintained in a controlled growth chamber (© BINDER – KBWF 720) with the following environmental conditions: 16 h light / 8 h dark photoperiod, daytime temperature of 30 °C, nighttime temperature of 20 °C, and 60 % relative humidity. During an initial acclimation phase of 30 days, soil water content was adjusted to maintain the substrate at 70 % of its water holding capacity (WHC) through regular irrigation. At day 30, for the experimental conditions that required it, a microbial consortium was applied by irrigation directly to the rhizosphere of the designated pots. An additional 7-day acclimation period was allowed after inoculation, under the same growth conditions and WHC. After the acclimation period, the forty plants of each cultivar (CR, LR, RR) were randomly assigned to four experimental treatments (10 plants per group), as follows:

- CT (Control): Optimal irrigation (WHC 70 %), No Microbial consortium applied.
- S (Drought stress): Irrigation withheld until WHC reaches 30 %, No Microbial consortium applied.
- SM (Drought Stress + Microbial consortium): Irrigation withheld until WHC reaches 30 %, Microbial consortium applied.
- M (Control + Microbial consortium): Optimal irrigation (WHC 70 %), Microbial consortium applied.

All four experimental conditions were replicated for each cultivar, resulting in a total of 12 experimental groups. After the acclimatization period (T1 30+7 days), irrigation was suspended in the experimental groups as scheduled. Soil water content was monitored daily until the WHC decreased to 30 %, which was defined as the severe drought stress level (T2) for groups S and SM. At T2, plant material was collected from three randomly selected plants per

experimental group for subsequent physiological and molecular analyses. After the sampling point at T2, all plants were re-watered to restore optimal soil moisture conditions (70 % WHC) and maintained under the same environmental conditions for an additional 60 days. To simulate repeated drought stress, irrigation was then interrupted again (T3) for the S and SM groups until the WHC dropped to 30 % for a second time point (T4). At T4, samples were again collected from three randomly selected plants per group (Fig. 12). Fully expanded, physiologically active leaves from the median portion of current-year shoots were sampled between 9:00 and 10:00 a.m. to minimize diurnal variability and ensure uniform developmental and physiological status.

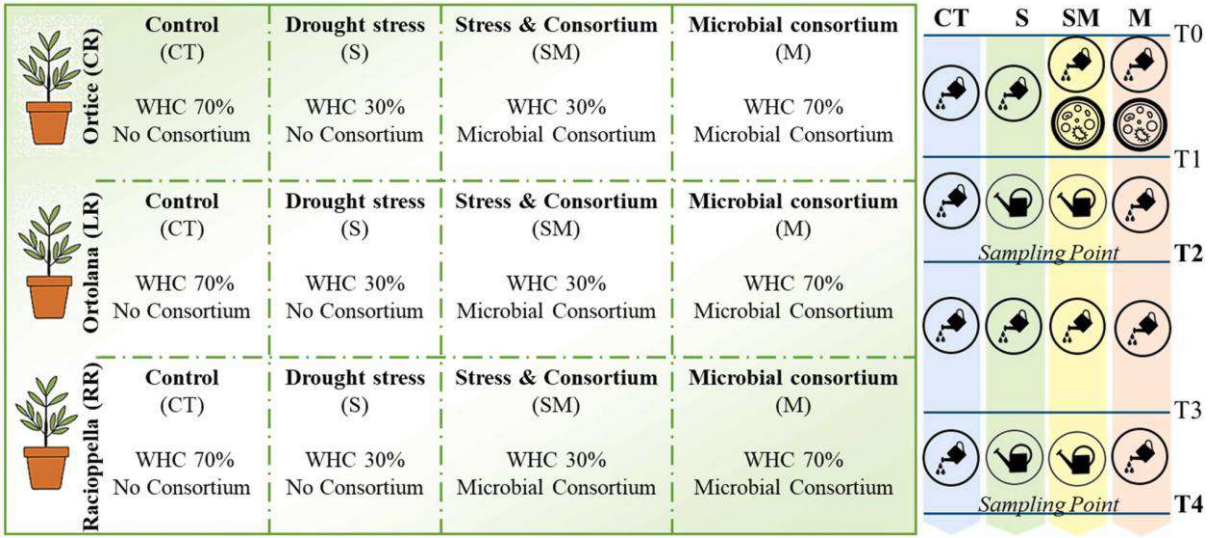


Fig. 12. Experimental set-up and workflow. Multifactorial experiment with three *Olea europaea* L. cultivars 'Ortice' (CR), 'Ortolana' (LR), and 'Racioppella' (RR), and four experimental theses: control at optimal irrigation level (CT), drought stress (S), control with microbial consortium addition (M), and stressed with microbial consortium addition (SM). Sampling points T2 and T4 correspond to the collection of plant matrices for subsequent physiological/molecular analyses.

4.2.2.2 Microbial consortium

A microbial consortium consisting of *Bacillus subtilis* (*B. subtilis*) and *Pseudomonas fluorescens* (*P. fluorescens*), both recognized for their plant growth-promoting rhizobacteria (PGPR) properties, was used to artificially contaminate the soil employed in the experimental mesocosms. The two bacterial strains were obtained from the culture collection of the University of Sannio (Benevento, Italy), where they are maintained under standard laboratory

conditions. Prior to application, each strain was individually cultured in nutrient broth at 28 °C under constant agitation (150 rpm) for 24 h to reach the exponential growth phase. The bacterial suspensions were centrifuged at $5000 \times g$ for 10 min, washed twice with sterile saline solution (0.85 % NaCl), and resuspended to a final concentration of 10^8 CFU/mL. Equal volumes of the two suspensions were mixed to prepare the microbial consortium, which was then uniformly applied to the soil substrate at a final density of 10^6 CFU/g of dry soil. The bacterial strains used to formulate the microbial consortium were obtained from the strain collection of the Department of Science and Technology, University of Sannio (Benevento, Italy), where they are maintained under controlled conditions. Both strains were previously isolated, taxonomically identified, and functionally characterized, and are preserved in glycerol stocks at -80 °C at standardized concentrations routinely verified in the departmental repository. For the present study, treatment suspensions were prepared based on the established correlation between optical density (OD_{600}) and colony-forming units (CFU/mL) previously determined for each strain, allowing accurate adjustment of inoculum concentrations. To confirm the precision of these values, serial dilutions were plated on nutrient agar (or selective media when appropriate), and CFU counts obtained after incubation were cross-checked with theoretical concentrations derived from spectrophotometric readings. This two-step calibration, combining OD-based estimation with experimental validation, ensured accuracy, reproducibility, and full reliability of bacterial concentrations across treatments and replicates.

4.2.2.3 Leaves water content and stomatal density

The relative water content (RWC) was assessed for each plant and cultivar under all experimental conditions, ensuring sampling occurred at the same phenological stage. Three fully expanded leaves were collected per sample group. After recording the fresh weight (FW), leaves were placed in vials and rehydrated in distilled water for 20 h in the dark. The following day, the turgid weight (TW) was measured, and the leaves were then oven-dried at 80 °C for 24 h to obtain the dry weight (DW). RWC was calculated using the formula: $RWC (\%) = 100 * [(FW - DW)/(TW - DW)]$.

Stomatal density was measured on three leaves per experimental group at both T2 and T4, selected at the same phenological stage. The abaxial surface of each leaf was coated with a thin layer of clear nail polish. After approximately 10 min of drying, the imprint was removed using transparent adhesive tape, which was then mounted on a glass slide. Observations were conducted under a Leica DM 2000 LED microscope at $40 \times$ magnification. For each leaf, three

micrographs ($250\ \mu\text{m} \times 250\ \mu\text{m}$) were acquired and analysed using ImageJ/Fiji software (Parri et al., 2023). Stomatal density was calculated as the number of stomata per mm^2 of leaf surface. (Xu and Zhou, 2008).

4.2.2.4 Starch, total sugars, phenolic compounds, free amino acids, flavonoids, photosynthetic pigments and proline content analysis

For metabolite extraction, three biological replicates were collected for each experimental condition at both sampling times (T2 and T4). Leaf samples were rinsed with deionized water, freeze-dried, and ground in liquid nitrogen. From each replicate, 30 mg of the resulting powdered tissue were extracted with 600 μL of cold ethanol:water solution (4:1, v/v), under stirring for 10 s. The mixture was sonicated (40 kHz, 10 min) and centrifuged (11,000 rpm, 4 min at 4 °C). The supernatant was transferred to a new tube, and the pellet was re-extracted using an additional 600 μL of the same ethanol:water solution. The combined supernatants were used for the quantification of multiple metabolite classes, according to López-Hidalgo et al. (2021) and Tartaglia et al. (2024). Hence, free amino acids were quantified spectrophotometrically at 570 nm after ninhydrin reaction; phenolic compounds were assessed at 720 nm after reaction with Folin-Ciocalteu reagent, and flavonoids were quantified at 415 nm following complexation with 10 % AlCl_3 in methanol. Photosynthetic pigments were extracted in 95 % ethanol and absorbance was recorded at 470, 649, and 664 nm. The pellets resulting from metabolites extraction were analysed for starch and total soluble sugars content. After hydrolysis with 30 % perchloric acid, their determination was performed after reaction with anthrone solution, with absorbance measured at 625 nm. For each metabolite group, appropriate standard compounds, glucose (sugars), proline (amino acids), gallic acid (phenolics), and quercetin (flavonoids), were used to generate calibration curves. Proline content was assessed separately using 30 mg of powdered tissue macerated and sonicated in 3 % (w/v) trichloroacetic acid. After centrifugation (14,000 rpm, 10 min, 4 °C) the supernatant was collected and reacted with ninhydrin reagent according to the same protocols (López-Hidalgo et al., 2021). Quantification was carried out by comparison with a calibration curve obtained from serial dilutions of proline standard, with absorbance measured at 520 nm. All spectrophotometric measurements were performed using a Thermo Scientific Evolution 201 UV-Visible spectrophotometer.

4.2.2.5 RNA extraction, cDNA synthesis, and gene expression analysis

For each experimental condition, three biological replicates were obtained by collecting plant material from three randomly selected plants per group. Total RNA was extracted from 150 mg of frozen leaf tissue using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich), following the manufacturer's instructions. RNA concentration was measured using a Qubit™ Fluorometer (Thermo Fisher Scientific), and RNA integrity was assessed with the Agilent 4150 TapeStation System (Agilent Technologies). Only samples with high RNA quality were used for downstream applications. First-strand cDNA synthesis was performed from 1 µg of total RNA using SuperScript™ II Reverse Transcriptase (Invitrogen™), according to the manufacturer's protocol. To further eliminate the possibility of genomic DNA carryover, all RNA samples were treated with DNase I (Thermo Fisher Scientific), following the manufacturer's instructions. For each biological replicate, first-strand cDNA synthesis was performed using 1 µg of total RNA and the SuperScript™ II Reverse Transcriptase kit (Thermo Fisher Scientific), which utilizes M-MLV reverse transcriptase and RNaseOUT™ recombinant ribonuclease inhibitor to safeguard RNA integrity during the reaction. Synthesis was primed with oligo(dT) primers to ensure reverse transcription of mature mRNA transcripts. All reactions were incubated at 42 °C for 50 min, followed by enzyme inactivation at 70 °C for 15 min. Quantitative real-time PCR (RT-qPCR) was carried out to analyze subsequent gene expression levels. Gene expression levels were normalized using *alpha-TUBULIN 1* (*α-TUB1*) and Glyceraldehyde-3-Phosphate Dehydrogenase 1 (*GAPDH1*) as the internal reference genes. Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method. We analyzed the expression of the following genes: *DREB1B* (Dehydration-Responsive Element-Binding protein 1B); *MYB8* (myeloblastosis viral oncogene homolog 8); *NCED1* (9-cis-epoxycarotenoid dioxygenase 1); *PP2C* (Protein Phosphatase 2 C); *PIP1-4* (Plasma membrane-intrinsic protein 1–4); *LEA6* (Late Embryogenesis Abundant protein 6); *LEA46* (Late Embryogenesis Abundant protein 46); *DHNI* (Dehydrin 1); *P5CS* (Pyrroline-5-carboxylate synthase); *SOD* (Zn-Cu) (Superoxide dismutase). The specific sequences of all primer pairs used in this study are listed in Table S1. Quantitative real-time PCR (RT-qPCR) was carried out using the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems), which allows for high-throughput, high-resolution detection of fluorescence during amplification. The reactions were performed in 96-well plates, and each gene-specific reaction was assembled using the SYBR™ Green PCR Master Mix (Applied Biosystems) in a final volume of 20 µL per well. Each reaction contained: 10 µL of SYBR Green Master Mix, 1 µL of forward primer, 1 µL of reverse primer (each at a final

concentration of 200 nM), 2 μ L of diluted cDNA template, and 6 μ L of nuclease-free water. Importantly, each biological replicate was analyzed in technical triplicate, meaning that the same cDNA sample was pipetted into three independent wells to account for any pipetting errors, well-to-well variation, or other technical inconsistencies. The mean Ct value of the three technical replicates was used as the representative Ct for that biological replicate. The thermal cycling conditions began with an initial enzyme activation and denaturation step at 95 °C for 10 min, followed by 40 amplification cycles consisting of denaturation at 95 °C for 15 s, and annealing/extension at a temperature optimized for each primer pair for 1 min. Unlike fixed-temperature protocols, this study employed a temperature gradient approach to account for variability in primer annealing temperatures across different target genes. Specifically, the QuantStudio™ 5 instrument's six-zone thermal block was utilized to apply a thermal gradient ranging from 56 °C to 62 °C across the 96-well plate, allowing simultaneous optimization of primer annealing conditions within a 10 °C range. The optimal annealing temperature for each gene-specific primer pair was pre-determined based on gradient tests to maximize amplification efficiency and specificity. After amplification, a melting curve analysis was performed from 60 °C to 95 °C with a ramp rate of 0.1 °C/s to confirm the presence of a single, specific amplicon and absence of primer-dimer formations or non-specific products. Negative controls included no-template controls (NTCs) for each primer set to monitor potential contamination, and no-RT controls to detect genomic DNA amplification. Normalization of gene expression was carried out using two stably expressed housekeeping genes: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and α -TUBULIN. These genes were selected based on prior stability assessments using geNorm and NormFinder algorithms, which confirmed their invariant expression across all cultivars and experimental conditions. The $\Delta\Delta$ Ct method ($2^{-\Delta\Delta$ Ct) was used to calculate relative expression levels, with normalization against the geometric mean of the Ct values from the two reference genes for each sample. All data were compiled and analyzed using GraphPad Prism version 8. All data were processed and analyzed as described in (Fosso et al., 2023).

4.2.2.6 Statistical analysis

Statistical analyses were conducted in R (version 4.3.3). Two-way analyses of variance (two-way ANOVA) were performed to assess the effects of treatment, time, and their interaction. When significant main or interaction effects were detected ($p < 0.05$), Tukey's post hoc test was applied to determine pairwise differences between groups. Biochemical analysis barplot were

represented using *ggplot2* package (Wilkinson, 2011), and gene expression results were represented using *pheatmap* package (Kolde, 2019).

4.2.3 Results

4.2.3.1 Leaves water content and stomatal density

RWC values confirmed the impact of drought stress across all cultivars, with marked reductions in S plants, especially in 'Ortice', which showed the lowest values at both T2 ($58.56 \pm 11.65 \%$) and T4 ($63.75 \pm 0.37 \%$). The SM moderately improved water retention under stress, particularly in 'Ortice' and 'Racioppella', where RWC remained around 70 %, compared to lower values in the corresponding S groups. Among cultivars, 'Ortice' was the most sensitive to drought, 'Ortolana' maintained relatively stable RWC regardless of treatment, and 'Racioppella' was the only cultivar showing a significant increase in RWC from T2 to T4 under stress ($p < 0.05$), suggesting a possible recovery response (Table 3). In summary, drought stress markedly reduced leaf water content in all cultivars, but microbial inoculation effectively mitigated water loss, especially in 'Ortice' and 'Racioppella', revealing cultivar-dependent modulation of drought resilience.

Table 3. Analysis of variance for RWC (%). Mean $\pm$ SD of RWC for each time point (T2 and T4) and cultivar are shown. Statistical differences identified by Tukey's post hoc test are indicated by letters while ANOVA results between time points are represented as "ns" when non-significant and "*" when $p < 0.05$.

	Treatment	T2		T4		Treatment	Cultivars	T2 vs T4
		RWC (%)		RWC (%)				
'Ortice'	CT	87.63 $\pm$ 4.61	a	a	89.92 $\pm$ 1.12	a	a	ns
	M	89.44 $\pm$ 2.49	a	a	90.79 $\pm$ 0.17	a	a	ns
	S	58.56 $\pm$ 11.65	b	a	63.75 $\pm$ 0.37	c	a	ns
	SM	70.86 $\pm$ 5.12	ab	a	69.61 $\pm$ 0.77	b	a	ns
'Ortolana'	CT	89.03 $\pm$ 1.75	a	a	88.91 $\pm$ 0.91	a	a	ns
	M	84.33 $\pm$ 11.44	a	a	92.48 $\pm$ 4.37	a	a	ns
	S	64.79 $\pm$ 0.44	b	a	65.32 $\pm$ 1.30	b	ab	ns
	SM	67.39 $\pm$ 0.60	b	a	69.84 $\pm$ 3.44	b	a	ns
'Racioppella'	CT	81.03 $\pm$ 1.05	a	b	85.64 $\pm$ 4.84	a	a	ns
	M	80.03 $\pm$ 1.06	a	a	80.34 $\pm$ 0.64	a	b	ns
	S	60.70 $\pm$ 0.89	c	a	62.71 $\pm$ 0.61	c	b	*
	SM	67.27 $\pm$ 2.47	b	a	68.66 $\pm$ 0.56	b	a	ns

When comparing stomatal density between T2 and T4 drought events, only 'Ortice' showed a significant reduction over time under both S and SM treatments, while 'Ortolana' and 'Racioppella' remained unchanged (Fig. 13 and Table S2). At T2, 'Ortolana' exhibited significantly lower stomatal density in S and M compared with the control, whereas the SM did not induce further differences. In both stress cycles, 'Ortice' and 'Racioppella' showed no significant treatment-related variations (Fig. 13). At T2, stomatal density in 'Ortice' significantly differed from 'Ortolana' under the M and S treatments, while no significant differences among cultivars were observed under SM or control conditions. At T4, no cultivar-based differences were detected across treatments. Overall, stomatal density changes were minor and cultivar-specific: 'Ortice' displayed a reduction over time under stress, while 'Ortolana' and 'Racioppella' preserved stable stomatal patterns, indicating distinct adaptive strategies in stomatal regulation.

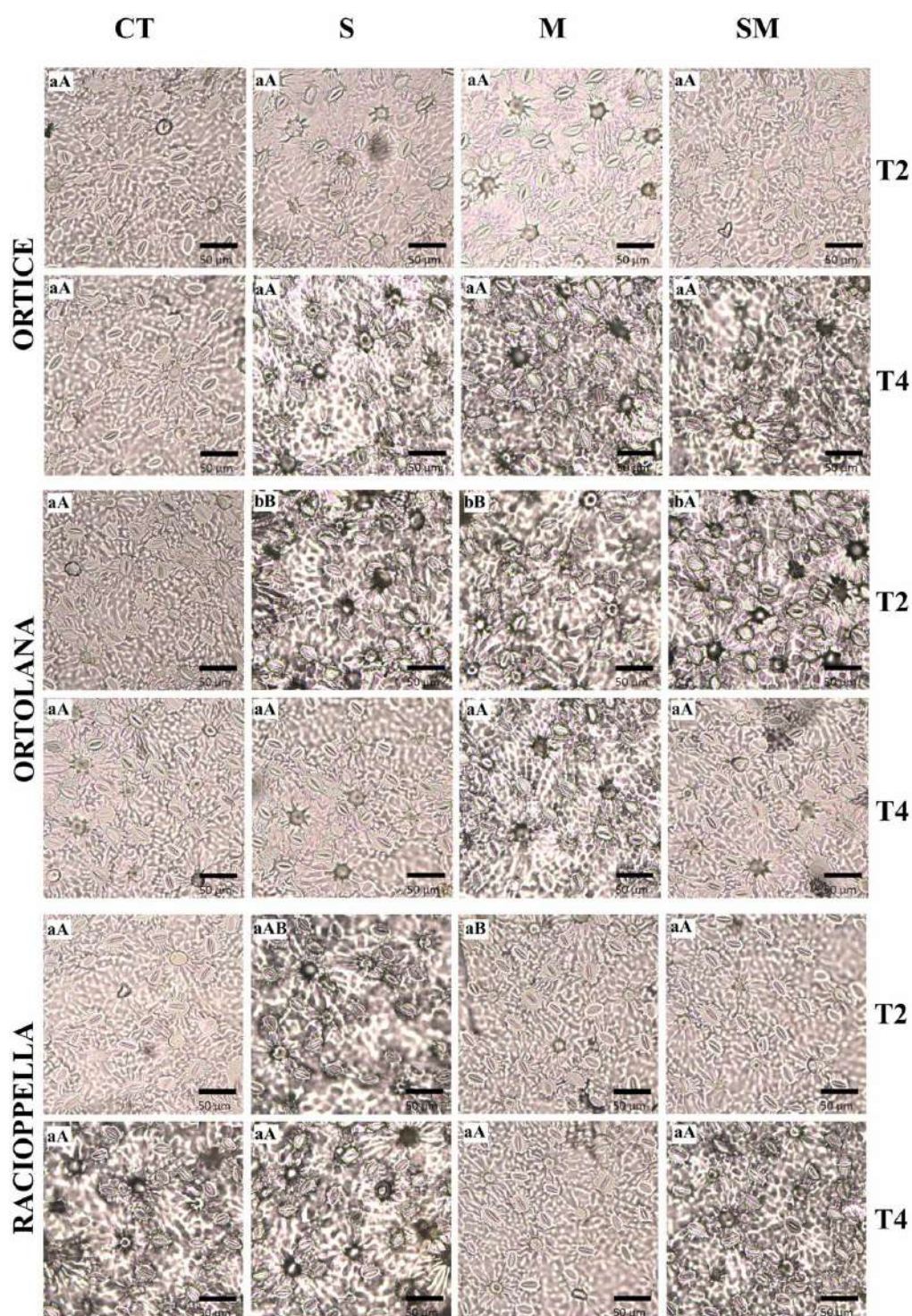


Fig. 13. Stomata of the leaves of 'Ortice', 'Ortolana' and 'Racioppella' cultivars during the two drought periods (T2 and T4), under control conditions (CT), drought stress (S), with the addition of the microbial consortium during the drought phase (SM), and during the irrigation phase (M). Image size 250 µm X 250 µm. Lowercase letters in the upper left corner of each image indicate statistically significant differences among treatments within the same cultivar, whereas uppercase letters denote differences among cultivars under the same treatment.

4.2.3.2 Starch, total sugars, phenolic compounds, free amino acids, flavonoids, photosynthetic pigments and proline content analysis

The biochemical response of three olive cultivars to water stress, in the presence or absence of the microbial consortium, was evaluated through the analysis of starch, total sugars, phenolic compounds, free amino acids, flavonoids, proline, and photosynthetic pigments, at two time points, T2 and T4. The results for 'Ortice' are shown in Fig. 14, while results for 'Ortolana' and 'Racioppella' are shown in Fig. S1 and S2 respectively. Overall, the effect of water stress was evident across all cultivars, particularly at T2, with several parameters negatively affected, while the M showed a cultivar-dependent ability to modulate the plant's metabolic response over time.

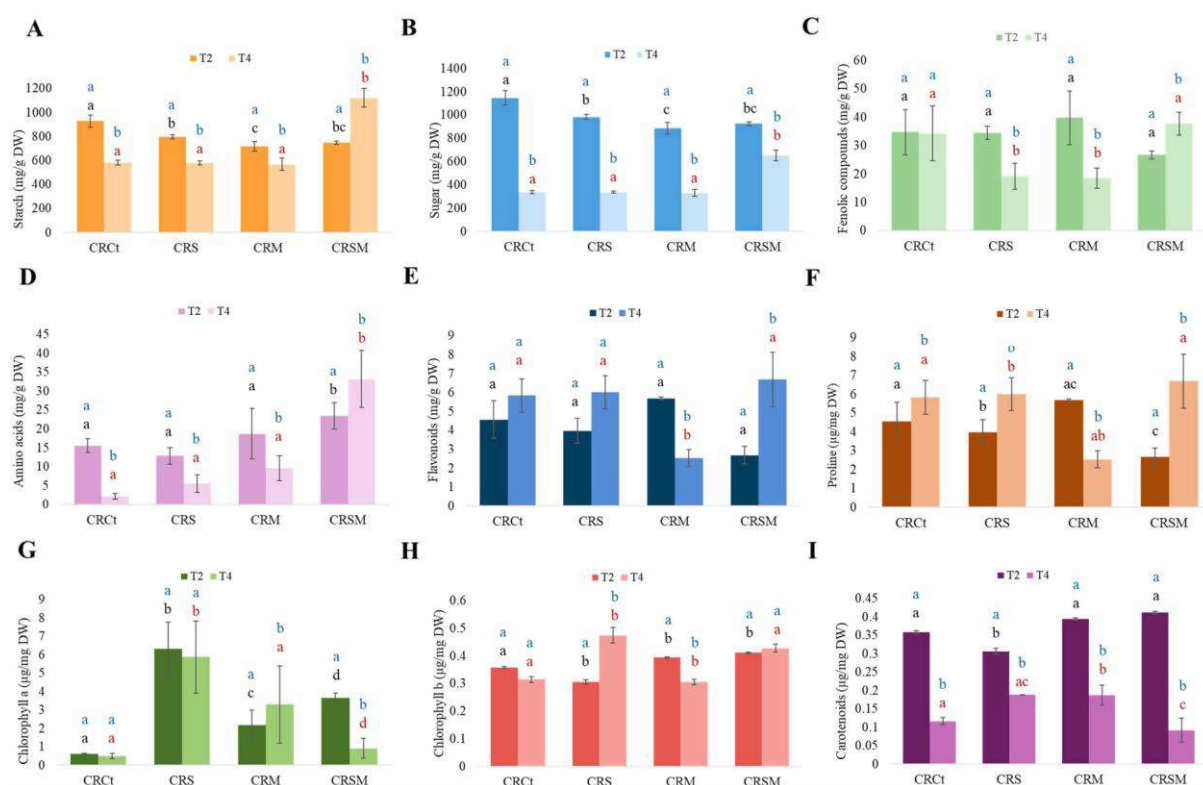


Fig. 14. Biochemical assays for 'Ortice' cultivar. A) starch; B) total sugars; C) fenolic compounds; D) amino acids; E) flavonoids; F) proline; G) chlorophyll a; H) chlorophyll b; I) carotenoids. The error was assessed as the standard deviation between the biological triplicates used for the analyses. ANOVA tests were performed between the different treatments in T2 (black), between treatments in T4 (red), and between the same treatments between T2 and T4 (blue).

In terms of starch and sugars, 'Ortice' showed a significant reduction in all treatments at T2 compared to the CT, followed by a recovery at T4, with SM displaying a marked increase. 'Ortolana', instead, showed no variation at T2 but a significant accumulation at T4 in M and SM. 'Racioppella' exhibited a slight increase in S and SM at T2, followed by a generalized increase across all treatments at T4. These results indicate a time-dependent reactivation of carbon storage mechanisms, particularly enhanced by the microbial consortium in SM treatments. These patterns indicate a time-dependent reactivation of carbon storage mechanisms, particularly enhanced by the microbial consortium under SM treatments.

Phenolic compounds followed contrasting patterns. In 'Ortice', levels remained stable at T2 and decreased in S and M at T4, while SM maintained values comparable to the control. 'Ortolana' showed no change in S across time, but a significant reduction in M and SM at T2. In 'Racioppella', phenolic compounds were drastically reduced in S and M at T2, but dramatically increased at T4, especially in SM (up to ten times higher than the control). The trend in free amino acids also varied among cultivars. In 'Ortice', a significant increase was observed only in SM at both time points. 'Ortolana' showed increased levels in S and SM at T2 and T4, though all treatments decreased from T2 to T4. In 'Racioppella', data confirmed amino acid modulation but suggested a stronger response in later stages when microbial inoculum was combined with stress. Flavonoid content increased in 'Ortolana' under S and SM at T2 but remained stable at T4. In 'Racioppella', S and M treatments caused alterations at both time points, while SM aligned with control levels, suggesting a balancing effect of the microbial treatment. In 'Ortice', flavonoids followed the general pattern of phenolic compounds. For proline, 'Ortice' showed a significant reduction in S at both time points, while M and SM maintained levels comparable to the control, with a general increase from T2 to T4. In 'Ortolana' and 'Racioppella', although specific quantitative data were not detailed, the trend suggested cultivar-specific regulation, not always directly responsive to microbial treatment. Concluding, for photosynthetic pigments, in 'Ortice' the chlorophyll *a* increased in S, while SM maintained values similar to the control, and chlorophyll *b* was reduced at T2 but recovered at T4, especially in SM; carotenoids decreased in S but remained stable in M and SM. In 'Ortolana', chlorophyll *a* levels were significantly higher in S and SM at both time points, while carotenoids dropped in all treatments at T2, stabilizing at T4 with a doubling observed in SM. 'Racioppella' showed the most striking pigment response in SM at T4, with chlorophyll *a* four times higher and carotenoids twice as high as the control, reinforcing the synergistic effect of stress and microbial inoculation on the photosynthetic apparatus.

In summary, the three cultivars displayed distinct biochemical profiles and responsiveness to water stress and microbial treatment. 'Ortice' responded with delayed but coordinated recovery of sugars, amino acids, and pigments, particularly in SM at T4, indicating a gradual stabilization supported by microbial inoculation. 'Ortolana' showed a more selective, time-dependent activation of metabolic pathways, with microbial effects becoming more pronounced in T4 phase, especially in pigment and sugar accumulation. 'Racioppella' emerged as the most responsive cultivar under combined stress and microbial inoculation, exhibiting robust T4 activation of phenolic compounds synthesis and photosynthetic pigment accumulation, particularly under SM at T4.

Collectively, biochemical data reveal that microbial inoculation supported osmotic balance, antioxidant activity, and pigment stability under recurrent drought, with 'Ortolana' and 'Racioppella' showing the strongest integrative metabolic recovery.

4.2.3.3 Gene expression analysis

4.2.3.3.1 Gene expression response to drought stress

Olive plants (*Olea europaea* L.) belonging to the cultivars 'Ortice', 'Ortolana', and 'Racioppella' were subjected to two distinct drought stress events (S), and transcriptional responses were evaluated via quantitative real-time PCR (RT-qPCR), in comparison to CT. The analysis focused on key genes associated with drought signaling, osmoprotection, and cellular defense. A first transcriptional activation was observed at time point T2, corresponding to the initial exposure to S. All three cultivars exhibited a marked induction of NCED1, a key gene in abscisic acid (ABA) biosynthesis, suggesting an early activation of the ABA-dependent signaling cascade. In contrast, expression of PP2C, a negative regulator of ABA signaling, consistently decreased under S conditions, and this trend was inversely proportional to that of NCED1. The repression of PP2C was evident in all three cultivars and at both time points, consistent with its known inhibitory role within the ABA signaling pathway. This transcriptional repression was statistically significant, ranging from approximately 30 % reduction up to about 80 % decrease in 'Racioppella' compared to CT. Concurrently, stress-responsive transcription factors DREB1B and MYB8 appeared strongly upregulated in S relative to CT, potentially indicating the initiation of broader transcriptional reprogramming. The expression of PIP1–4, an aquaporin gene critical for water transport, also increased consistently under S conditions, as did P5CS, involved in proline biosynthesis and osmotic

adjustment. Genes encoding Late Embryogenesis Abundant proteins, LEA6 and LEA46, alongside DHN (dehydrins) and the antioxidant gene SOD (Zn-Cu), showed elevated expression levels in S plants, pointing to the activation of both structural and enzymatic defense mechanisms across all cultivars. Notably, the transcriptional response appeared to intensify at time point T4, corresponding to a second drought exposure following a recovery phase. In this condition, nearly all assessed genes including NCED, DREB1B, MYB8, PIP1–4, P5CS, LEA6, LEA46, DHN, and SOD (Zn-Cu) showed further upregulation compared to T2 as can be seen in Fig. 15. Furthermore at both time points T2 and T4, plants subjected to stress exhibited a statistically significant upregulation in the transcription of all the analyzed genes. In the cultivar 'Ortice', the fold change ranged from a minimum of 1.5-fold for DREB1B to approximately 13-fold for LEA46 compared to CT plants (Fig. S3A and B). In the cultivars 'Ortolana' and 'Racioppella', the gene NCED1 showed the most pronounced increase, with transcription levels rising up to 40-fold relative to controls at T2 (Fig. S4E and Fig. S5E respectively). Following a second exposure to stress at T4, this upregulation was even more pronounced, with NCED1 expression reaching up to 60-fold higher than the control in both 'Ortolana' and 'Racioppella' (Fig. S4F and Fig. S5F respectively). These results would suggest a strong transcriptional response to repeated stress exposure. Complete quantitative data and statistical analyses for all the genes studied are presented in the supplementary materials. The observed patterns were consistent across the three cultivars, although with variable expression amplitudes, suggesting a conserved but genotype-modulated molecular response to recurring drought stress.

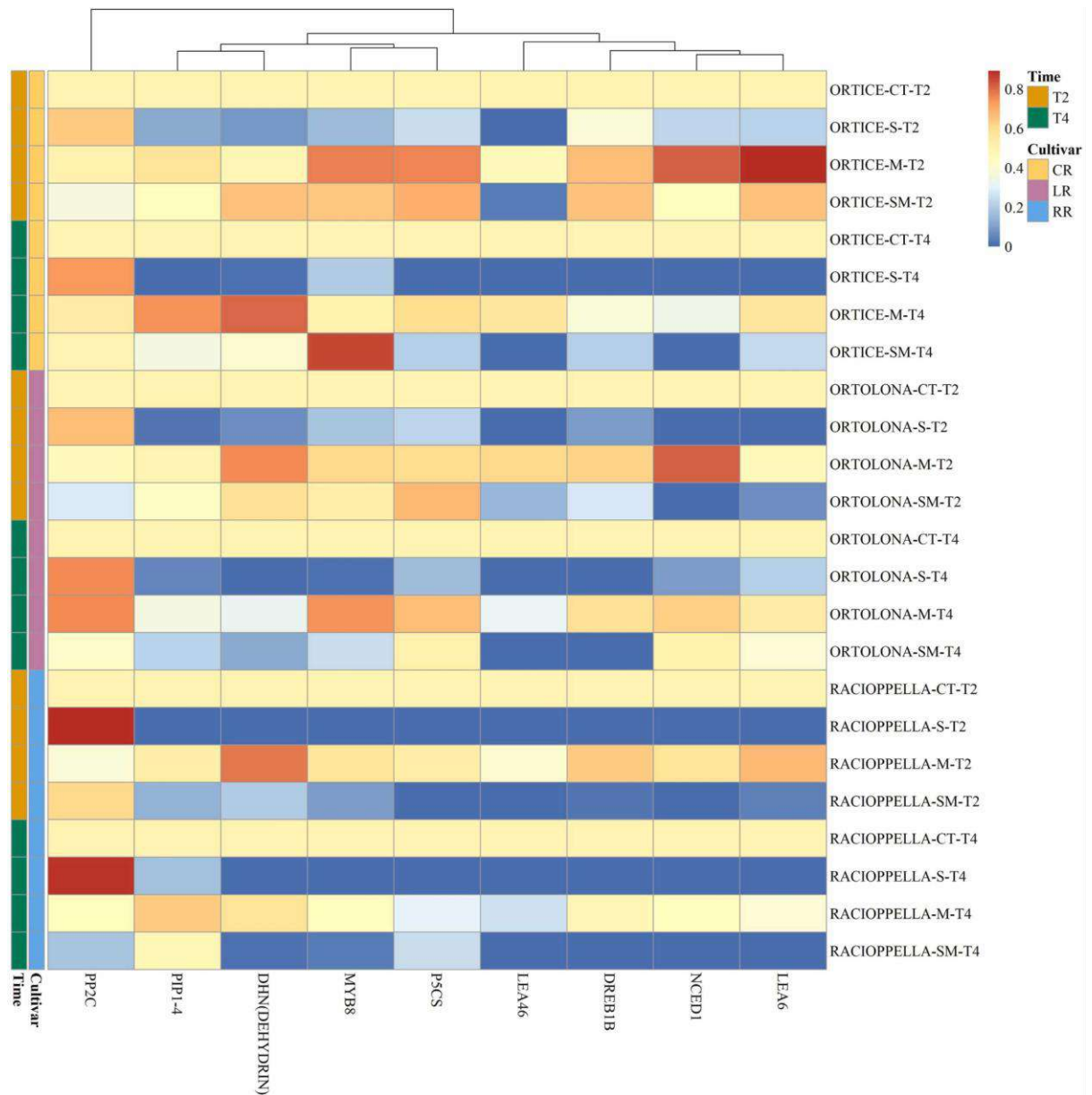


Fig. 15. Heatmap showing gene expression profiles across samples from three cultivars ('Ortice' in yellow, 'Ortolana' in pink and 'Racioppella' in blue) at time points T2 (orange) and T4 (green) under four experimental conditions: control (CT), stress (S), microbial consortium (M), and combined stress + microbial consortium (SM). Each column represents a gene, and each row corresponds to a sample, categorized by cultivar and time point. Gene expression levels are displayed using a color scale, with values normalized and centered per gene (Fold-change). The heatmap illustrates gene expression patterns modulated by stress and/or microbial consortium treatment over time and among cultivars.

Overall, drought induced a strong activation of ABA- and stress-responsive genes, which intensified after recurrent exposure (T4), indicating the establishment of stress memory.

4.2.3.3.2 Molecular analysis of drought-stress gene modulation by a microbial consortium

To evaluate the effect of the microbial consortium independently of drought stress, plants of the three *Olea europaea* cultivars 'Ortice', 'Ortolana', and 'Racioppella' were treated with M and maintained under well-watered conditions. Gene expression was quantified by RT-qPCR at both T2 and T4 time points and compared to the CT. In general, the M condition did not lead to activation of drought-responsive pathways. Expression levels of NCED1, DREB1B, MYB8, P5CS, DHN, and SOD (ZN-Cu) were either comparable to CT or showed moderate downregulation, particularly at T4 as clearly shown in Fig. 15. However, some exceptions were observed. Notably, in the cultivar 'Racioppella', a slight transcriptional increase was recorded for multiple genes, especially at T4 (Fig. S5D, F, H, L and N). This modest upregulation under M affected genes such as PIP1–4 and LEA46 also in 'Ortolana' (Fig. S4J and N). While 'Ortice' maintained expression levels largely similar to CT. The expression profile of PP2C, the negative regulator of ABA signaling, followed an opposite trend compared to NCED, as expected. While 'Ortice' and 'Ortolana' generally showed stable or slightly reduced PP2C transcript levels under M, in 'Racioppella' a slight increase was consistently observed at both T2 and T4. This cultivar-specific behavior aligns with the physiological role of PP2C in suppressing ABA-dependent stress responses under non-stress conditions. Given the minimal impact of the microbial consortium M on the transcriptional levels of the evaluated genes, no statistically significant differences were observed between M and the CT. In contrast, significant differences were detected between S and M for nearly all genes, at both time points and across all three cultivars, as shown in the supplementary materials.

Under microbial inoculation, transcriptional activation was moderate and well-regulated, suggesting priming rather than stress overreaction.

4.2.3.3.3 Gene expression response to microbial consortium under drought stress

To further investigate how the microbial consortium might impact the transcriptional levels of the genes evaluated by RT-qPCR under water stress conditions, we introduced an additional treatment combining stress and microbial inoculation (SM) for comparison. For PP2C, the situation would be different: the SM would result in a statistically significant increase in transcription compared to S, particularly at T2, with fold changes of approximately 1.5, 1.7, and 2.5 in 'Ortice', 'Ortolana', and 'Racioppella', respectively. For the other genes, when plants

of all three cultivars were inoculated with the microbial consortium and subjected to water stress, the SM would induce a clear upregulation at both time points. However, gene expression levels under SM would consistently be lower than those observed in plants exposed to S as indicated in Fig. 15. Statistically significant differences between S and SM samples would be detected for nearly all genes across all cultivars. Notably, at time point T4 in the cultivar 'Racioppella', fold changes for MYB8 and P5CS would be approximately 5–6 times lower in SM compared to S, indicating a moderated transcriptional response in the presence of the microbial consortium during stress (Fig. S5D and R). These results would suggest that while the microbial consortium stimulates gene expression under stress conditions, it may attenuate the overall magnitude of the stress-induced transcriptional activation. All quantitative data and statistical analyses mentioned are provided in the supplementary materials.

The SM combination confirmed a balanced transcriptional pattern, highlighting the consortium's role in fine-tuning stress responses.

4.2.4 Discussion

This study investigated the effects of recurrent drought stress on three autochthonous olive cultivars ('Ortice', 'Ortolana', and 'Racioppella') and evaluated the potential of microbial consortium inoculation to mitigate stress conditions. A multifactorial experimental design and an integrated approach combining physiological, biochemical, and molecular analyses were applied. The microbial consortium, composed of *Bacillus subtilis* and *Pseudomonas fluorescens*, is well known for its plant growth-promoting and stress-alleviating properties. Despite the olive tree's well-documented drought tolerance, linked to anatomical and physiological traits such as thick cuticle, water-storing parenchyma, and conservative stomatal regulation strategies (Brito et al., 2019, Majikumna et al., 2024), the two drought cycles revealed distinct cultivar-specific responses, suggesting the existence of divergent resilience mechanisms.

4.2.4.1 Relative water content (RWC) and stomatal density

The three cultivars exhibited distinct physiological responses to repeated drought stress, reflecting genotype-specific adaptation strategies. The RWC revealed vulnerability in 'Ortice', whereas 'Ortolana' maintained a more conservative and stable water status. 'Racioppella' demonstrated a modest yet significant RWC recovery between the stress cycles, suggesting functional plasticity. The SM treatment mitigated water loss and preserved higher RWC values

under drought conditions. These observations mirror those of Karimi et al., who reported severe reductions in RWC across four olive cultivars under drought and an improved moisture stability and osmolyte accumulation due to the PGPR inoculation (Karimi et al., 2018). Our results suggest that 'Ortolana' and 'Racioppella', particularly under microbial inoculation, better sustain the RWC recover, paralleling the behavior of drought-tolerant cultivars in the literature. This supports the proposed mechanism in which the physiological maintenance of leaf hydration, combined with the transcriptional priming of genes such as PIP1-4, P5CS, and LEA46, enhances olive resilience under recurrent water stress (Karimi et al., 2018, Salimonti et al., 2025).

Stomatal density showed variable responses under drought. In 'Ortolana', only slight reductions were observed under S and SM treatments, suggesting better stomatal regulation. Conversely, 'Ortice' and especially 'Racioppella' exhibited more pronounced decreases, particularly under S conditions, indicating higher susceptibility to drought-induced stomatal loss. In 'Racioppella' cultivar stomatal density decreased drastically in S (from 228.9 control in T2–176.7 stomata/mm²), and only partially in SM, indicating a strong but energetically costly stress response that fails to fully restore water regulation. This pattern highlights three distinct adaptive strategies: 'Ortice' displays gradual plasticity, 'Ortolana' exhibits a stable conservative behavior, and 'Racioppella' shows reactive but inefficient acclimation, traits commonly associated with drought-sensitive genotypes (Gholami and Zahedi, 2019).

4.2.4.2 Osmotic metabolites, pigments, and antioxidant compounds

In 'Ortice', proline levels increased under S treatments between T2 and T4, with intermediate levels in SM (S > SM > C), suggesting a primed metabolic response. Proline functions both as an osmotic stabilizer and ROS scavenger (Ashraf and Foolad, 2007). Similarly, SM treatments led to increases in soluble sugars and free amino acids (+35–50 % vs. control), supporting osmotic stability and readily available energy. These findings agree with Kitsaki and Drossopoulos, who reported significant modulation of primary metabolites, including sugars and amino acids, as key osmoprotective strategies in olive under water stress (Kitsaki and Drossopoulos, 2005). Total phenolics also increased (+40 % in SM), indicating the activation of antioxidant defenses by the PGPR consortium.

In 'Ortolana', the consortium's effect was particularly pronounced, flavonoids and total phenolics increased by up to 70 % in SM, confirming the strong activation of the antioxidant

systems. This cultivar also exhibited the lowest proline accumulation, consistent with reduced stress perception. The starch content decreased under stress in all groups, reflecting the mobilization of energy reserves, but 'Ortolana' experienced limited starch loss, suggesting more efficient resource use. Conversely, 'Racioppella' accumulated large amounts of proline under S treatments, indicative of severe stress perception, yet this was not accompanied by effective damage containment. Starch dropped sharply from T2 to T4 in both S and SM, suggesting energetic imbalance and poor recovery capacity. Interestingly, the increase in flavonoids in M groups points to high sensitivity to microbial presence even in the absence of stress. This behavior is consistent with sensitive genotypes that activate strong responses but fail to maintain physiological homeostasis under prolonged drought (Gholami and Zahedi, 2019, Razouk et al., 2022). In agreement with Abboud et al., our results suggest that phenolic accumulation under stress is not always correlated with tolerance, but rather reflects the extent of oxidative challenge (Abboud et al., 2019). Interestingly, it has been demonstrated in other studies that PGPR-induced enhancement of antioxidant compounds, particularly phenolics and flavonoids, contributes to drought tolerance by counteracting ROS-induced damage (Ahmed et al., 2009) an effect clearly observed in 'Ortolana' and 'Ortice' under SM treatments.

Photosynthetic pigment analysis further supported these patterns: under drought, 'Ortice' and 'Racioppella' exhibited the highest total pigment levels (929 and 958 mg/g, respectively), whereas 'Ortolana' showed lower values (~830 mg/g), in line with a conservative strategy that limits photosynthetic input to reduce water loss. However, the relatively high pigment retention in 'Racioppella' did not translate into greater physiological efficiency, suggesting impaired photosynthetic function. These findings are consistent with other studies. For instance, Dias et al. reported that inoculation with *Pseudomonas reactans* in olive improved photosynthesis, chlorophyll content, and water balance under drought (Dias et al., 2024). Araújo et al. emphasized the importance of flavonoid accumulation and specific peroxidase activation in antioxidant protection under field conditions (Araújo et al., 2021). El-Saadony et al. described the combined role of PGPR in drought tolerance through phytohormone regulation, systemic resistance, and ACC deaminase production (El-Saadony et al., 2024). Overall, the activation of osmoprotectants, antioxidant compounds, and modulation of photosynthetic pigments represents a competitive advantage induced by microbial inoculation, particularly in the more resilient cultivars 'Ortolana' and 'Ortice'.

4.2.4.3 Expression of key genes related to drought stress

At the molecular level, drought stress triggered a transcriptional cascade consistent with ABA-dependent signaling. The NCED1 gene, encoding a key enzyme in ABA biosynthesis, was strongly upregulated in S treatments, especially in 'Racioppella' (up to +60-fold vs. control). In parallel, PP2C, a negative regulator of the ABA pathway, was downregulated by up to 80 %, thus releasing activation of SnRK2 kinases. This expression pattern mirrors findings in tomato by Pérez-Martín et al., where organ-specific regulation of NCED1 and PP2C played a central role under drought. In SM treatments, although stress was perceived, the intensity of the transcriptional response was markedly attenuated (Perez-Martin et al., 2014). Lower induction of NCED1, P5CS, LEA46, and DHN1 genes, together with milder repression of PP2C, suggests a fine-tuned ABA response consistent with molecular priming, as described in recent studies (Yang et al., 2023).

A particularly relevant finding in this study is the cumulative effect observed during the second stress event (T4). Compared to T2, expression of stress-responsive genes (LEA46, DHN1) was significantly increased, up to two-fold in 'Ortice', indicating an adaptive memory mechanism. This phenomenon, also described by Nair et al., is associated with epigenetic and transcriptional modifications that enable faster and stronger activation of response genes during subsequent stress events (Nair et al., 2022). Recent findings confirmed sustained upregulation of ABA- and osmoprotection-related genes (e.g., LEA, HSP, aquaporins) in resilient olive cultivars subjected to repeated drought. Aquaporins, particularly OePIP2.1, play a crucial role in regulating membrane water permeability under drought (Salimonti et al., 2025). In our study, OePIP2.1 expression was downregulated under S treatments, consistent with a conservative response to limit water loss. This trend agrees with a study that reported reduced OePIP2.1 expression in adult olive trees under drought, associated with increased xylem resistance and embolism formation (Secchi et al., 2007). However, expression patterns differed among cultivars, suggesting genotype-specific regulation. 'Ortolana', which maintained better water status, may finely regulate aquaporin activity to preserve homeostasis, whereas 'Racioppella' showed stronger repression of OePIP2.1, possibly reflecting a less efficient coping strategy. These findings highlight the potential role of aquaporins in cultivar resilience and suggest that microbial priming may enhance their transcriptional regulation, contributing to improved drought adaptation. Identifying cultivar-specific resilience strategies and validating the effectiveness of functional bioinoculants provides valuable tools for sustainable and adaptive

olive cultivation. In Mediterranean regions where drought events are projected to increase in intensity and frequency (Lazoglou et al., 2024), combining varietal selection with PGPR-based microbial consortia represents a promising strategy to support climate-resilient agriculture.

Our findings highlight the importance of stimulating osmotic and antioxidant responses, priming molecular defenses, and maintaining metabolic efficiency under adverse conditions. The experimental design adopted, based on recurrent stress and multilevel analysis, reflects realistic climate scenarios and yields crucial data to guide varietal selection and development of climate-smart agronomic technologies.

4.2.5 Conclusion

Increasing drought frequency and severity in Mediterranean regions represents a critical threat to olive cultivation. In this study, the drought resilience of three traditional cultivars ('Ortice', 'Ortolana' and 'Racioppella') was assessed through a multi-level approach integrating physiological, biochemical and molecular analyses under sequential water deficit. The three genotypes exhibited distinct adaptive strategies: 'Ortolana' maintained efficient water balance with minimal stress activation, 'Ortice' showed plastic and progressive adjustments involving stress-memory mechanisms, whereas 'Racioppella' displayed strong but energetically costly reactions. Microbial inoculation with *Bacillus subtilis* and *Pseudomonas fluorescens* improved plant water status, enhanced osmolyte and antioxidant accumulation, stabilized pigments, and modulated ABA- and drought-responsive gene expression, indicating a priming effect that promoted more controlled transcriptional responses under repeated stress. Overall, the results highlight that drought resilience in olive arises from a genotype-dependent integration of stress perception, energy conservation and microbial signaling. These findings provide a solid basis for designing climate-smart cultivation strategies that combine resilient genotypes with functional PGPR consortia to enhance the sustainability of Mediterranean olive systems.

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Chapter 5 INTEGRATED DISCUSSION

In the context of global warming, the effects of climate change pose considerable challenges for maintaining the sustainability of olive cultivation and improving its productivity and environmental performance. Water deficit is one of the most complex challenges to be addressed, and it is expected that low-density olive groves, irrigated by rain, will be the most vulnerable to climate change, particularly in the driest areas such as southern Italy (Mairech et al., 2021).

In this context, understanding the olive tree as a holobiont (i.e., the functional unit composed of the plant and its associated microbial communities) becomes crucial. This perspective highlights that the tree's health, growth, nutrient uptake, and resilience to stress are not determined by its genetics alone, but are significantly influenced by the interactions with its microbial partners (especially bacteria and fungi) in the roots, leaves, and surrounding soil. However, the rapid adaptation of microbes to changing abiotic conditions could provide plants with a crucial resource, acting as an effective buffer against environmental fluctuations (Brüssow et al., 2024). In this context, understanding how the plant microbiome responds to environmental and anthropogenic drivers is essential, as such knowledge could be harnessed to manipulate microbial communities in ways that enhance the resilience of the host plant.

This PhD thesis, in line with the hypotheses outlined above, investigated how different agronomic management techniques (organic, traditional and conventional) shape the microbial communities associated with olive trees and that the olive holobiont is able to actively respond to environmental variations. Moreover, by integrating field experiments with controlled laboratory studies, the response to drought stress was found to be genotype-dependent and significantly influenced by microbial inoculation, confirming the effectiveness of microbial inoculation as a promising biotechnological strategy to enhance olive tree resilience to climate change.

5.1 The influence of management on microbiome assembly

The literature confirms that agricultural practices have a profound impact on the microbial composition of crops and that the olive tree is also affected. In the in-field investigation (Chapter 4, Section 4.1), the PhD research focused on a single olive cultivar, 'Ortice', to minimize genotype-driven variability, and was conducted at a site with uniform pedoclimatic

conditions. This design allowed for a more precise evaluation of the effects of management practices on the soil microbial community. The results showed that, in an area purely dedicated to olive growing, sustainable management (characterised by minimal tillage, the use of cover crops and organic fertilisers) promotes greater diversity and abundance of microbial taxa. This is in clear contrast to conventional management practices, which tend to simplify and deplete the microbiome, as already observed by (Arias-Giraldo et al., 2021; Sofo et al., 2014).

Metagenomic analyses (16S rRNA and ITS) conducted on 'Ortice' in olive groves in southern Italy have revealed that organic management significantly enriches the fungal community associated with roots in terms of abundance (biomarkers) compared to conventional treatments. The bacterial community associated with the rhizosphere also presented some important genera: *Actinophytocola* and *Streptomyces*, both belonging to the order *Actinomycetales*, long known for their antagonistic behavior against root pathogenic fungi (Fernández-González et al., 2021). It was interesting to observe the presence of the saprophytic pathogen *Fusarium* spp. in the bacterial community associated with the leaves of organic treatments. Although this fungus is responsible for the rot of olive trees, Mondello et al. (2019) have shown that *Fusarium proliferatum* exerts antibiosis and direct antagonism against pathogens, making it a potential BCA, while its presence in organically managed olive groves is favoured by the absence of broad-spectrum insecticides and pesticides (Lazzaro et al., 2015). Although not particularly evident from a functional point of view, organic management seemed to favour dynamics linked to nutrient recycling, particularly nitrogen with the bacterium *Methylocystis heyeri*.

The microbiome of rhizosphere compartments of olive trees managed using traditional methods hosted bacteria that are indicators of natural environments, such as *Burkholderiales* and nitrogen-fixing symbionts (*Rhizobium leguminosarum*, *Rhizobiaceae* family). However, the functional traits associated with nitrogen are not significantly reflected in the aerial parts of traditionally managed olive trees. This condition may depend on the low input of fertilisers, which on the one hand tend to stimulate colonisation in the root area with nitrogen-fixing bacteria capable of compensating for this deficiency (Li et al., 2026), and on the other hand tend to limit the niches in the aerial part for certain functional traits to the detriment of other more suitable ones (as described in the section 4.1.4).

Another positive aspect is the enrichment of the root microbiome with the presence of phototropic bacteria, such as *Rhodopseudomonas palustris*, whose various strains are used as biofertilisers (Kantachote et al., 2016). Mild pathogens such as *Stemphylium solani* are not

absent in the phyllosphere; this fungus is often found in dry fields during periods of drought or in soils poor in potassium, or where root development is limited due to hard, compacted soil (Strayer-Scherer et al., 2023).

On the other hand, significant differences emerged, showing that conventional management simplifies the fungal communities associated with roots (with only the class of *Sordariomycetes* as biomarkers) and alters those in the aerial part, due to the application of foliar fungicide sprays. The application of these products eliminates fungal taxa and encourages the development of more tolerant and opportunistic bacteria (e.g. *Rhodocyclacea*, endophytes of polluted environments (Li et al., 2020)). However, not all pathogenic fungi are eliminated by chemical treatments. In fact, the fungus *Alternaria alternata*, a filamentous, endophytic *Ascomycete*, is still abundant in the canopy of conventionally managed olive groves (DeMers, 2022). Furthermore, functional analysis reveals the presence of other endophytic fungi such as *Aureobasidium* sp. and *Cladosporium* sp. These species are known as opportunistic pathogens that affect many field crops and also fruit and vegetables during post-harvest storage (Hortová & Novotný, 2011).

These results confirm that the microbial community responds differentially to fertiliser and pesticide inputs from organic and conventional treatments (Hartman et al., 2018; Sangiorgio et al., 2022) with potential implications for the host plant health and resilience.

5.2 Genotype-specific resilience and PGPR-mediated priming mechanisms

In addition to management practices, drought acts as a major driver of microbial community structure and function. Limited water availability directly impacts soil - the primary source of fungi and bacteria associated with plants (Díaz-González et al., 2025) - potentially altering plant-microbe interactions and affecting host performance. The second part of this PhD thesis (Chapter 4, section 4.2) investigates genotype-specific variability in responses to drought stress in the 'Ortice', 'Ortolana' and 'Racioppella' cultivars and the application of a PGPR consortium (composed by *Bacillus subtilis* and *Pseudomonas fluorescens*) to support the soil microbiota in maintaining the functional processes that promote olive tree health. This becomes particularly relevant in the context of climate change, where increasingly frequent and prolonged periods of drought can significantly impact plant health and productivity.

To simulate recurring episodes of drought, a mesocosm was created in which the three cultivars were subjected to two cycles of severe drought (30% WHC). An additional condition was

provided for with the addition of the PGPR consortium and related controls. This configuration allowed us to distinguish between genotype-specific responses to drought and PGPR-mediated priming effects. The results showed different drought resilience strategies among the cultivars.

Under water stress conditions (S), 'Ortice' proved to be the most susceptible cultivar in terms of water retention (RWC), while 'Ortolana' and Raccioppella were more dynamic. The latter, with microbial inoculation (SM), supported water recovery better during stress cycles. This observation, as described in the section 4.2.4, is in line with Karimi et al., (2018), who demonstrates a decrease in RWC values in olive trees under stress and an increase in osmolytes with the addition of PGP. The role of PGPR was also significant in the 'Raccioppella' cultivar, where it significantly attenuated drought-induced stomatal loss. It is important to note that, in addition to stomatal regulation, osmoregulation is one of the most effective and spontaneous adaptation mechanisms of the olive tree. As widely documented in the literature (Dichio et al., 2003, 2006) and confirmed by the studies of Ennajeh et al., (2008), the olive tree is capable of performing a high osmotic adjustment, which generates a strong water potential gradient between the leaves and the soil.

From a biochemical point of view, the addition of PGPR (SM) improved proline values compared to the stress-only condition (S) in 'Ortice' and increased sugar and amino acid levels compared to the control. These dynamics are described by Kitsaki & Drossopoulos, (2005) as key osmoprotective strategies in olive trees when under water stress. In the 'Ortolana' cultivar, SM treatment enhanced antioxidant defences by significantly increasing phenol and flavonoid levels. In fact, plant growth-promoting bacteria (PGPR/PGPB) are able to activate the phenylpropanoid pathway through ISR (Induced Systemic Resistance), increasing the synthesis of phenolic acids and flavonoids and improving tolerance to abiotic stresses such as drought, salinity and heavy metals (Jakubowska et al., 2025). The inoculation of the microbial consortium also made it possible to limit starch losses, optimising the plant's reserves; this metabolic balance, combined with a low proline content, indicates that the cultivar perceived the stress less severely.

Under SM treatment, both 'Ortice' and 'Raccioppella' showed significantly elevated pigment levels; however, the increase was particularly marked in the latter.

However, this response did not translate into real physiological efficiency, as confirmed by the instability of the water and metabolic balance observed in the genotype. It is well known that

loss of turgor can seriously compromise plant metabolism and growth, negatively affecting photosynthetic activity (Chaves et al., 2003). For example, it has been reported that the leaves of the Chemlali cultivar maintain substantial photosynthetic activity even below the point of turgor loss (with water potential values of -6 MPa), similarly to the Coratina cultivar (Ennajeh et al., 2008). In the case of 'Racioppella', on the contrary, the high pigment content does not seem to have been sufficient to guarantee similar metabolic resilience, highlighting a genotype-specific susceptibility despite the support of the microbial consortium. In contrast, 'Ortolana' recorded the lowest pigment values, confirming a conservative strategy: by limiting photosynthetic input, the plant reduces metabolic stress and prevents further water loss, preserving overall homeostasis.

The induction of osmoprotectants, the accumulation of antioxidant metabolites and the regulation of photosynthetic pigments constitute a decisive adaptive benefit resulting from PGPR inoculation and are evident in the most resilient cultivars 'Ortolana' and 'Ortice'.

At the molecular level, and in particular in the 'Racioppella' cultivar, the dynamics of NCED1 and PP2C gene expression under water stress (S) conditions was consistent with ABA-dependent signalling (Perez-Martin et al., 2014).

Of particular note is the significant increase in the expression of stress-responsive genes (LEA46, DHN1) observed in 'Ortice' in condition S compared to the control. This increase was even more pronounced during the second stress cycle (T4), highlighting this cultivar's ability to activate an adaptive memory mechanism. This phenomenon is attributable to epigenetic and transcriptional modifications that predispose the plant to a faster and more energetic response in the event of subsequent adverse events (Nair et al., 2022).

The expression of aquaporins is globally downregulated under stress conditions (S). This phenomenon represents a conservative strategy aimed at reducing the water permeability of membranes and limiting water loss. The responses of PIP aquaporins under drought conditions depend on the isoform, tissue, species or stress level, but in general, the downregulation of these genes prevents water loss from the plant (Šurbanovski et al., 2013). In fact, the intensity of this response varies significantly among the cultivars analysed in section 4.2. Under water stress conditions, 'Ortolana' shows moderate downregulation of OePIP2.1, consistent with its good water status, while 'Racioppella' showed drastic repression of these genes, attributable to its general inability to cope effectively with the stress situation. In 'Ortice', aquaporin closure

appeared more balanced: sufficient to contain water loss, compatible with good overall physiological function, in line with its tolerant resilience strategy.

With the addition of PGPR (SM), the repression of all other genes is further reduced and expression remains closer to control. In summary, these results demonstrated that the three olive cultivars responded with different strategies to water stress conditions and that the addition of the PGPR consortium improved the fitness and resilience of the plants themselves.

5.3 Limitations and perspectives

In general, studies on the olive tree microbiome suffer from limitations related to reduced spatial and temporal scale and difficult interpretation of functional interactions within the microbial community. Furthermore, sequencing of 16S rRNA or ITS amplicon has very limited functional resolution and overlooks many metabolically active but rare taxa (Ahmad et al., 2026).

These conditions can be bypassed by extending the time series of observations (thus monitoring environmental variations), improving sequencing resolution, and enriching reference databases to cover a wider taxonomic range and the functions associated with taxa (Ferrocino et al., 2022). Furthermore, adopting a multi-omic approach (integrating metagenomics, transcriptomics, proteomics and metabolomics) may enhance the investigation of processes underlying plant-microbe interactions and microbiome-mediated stress tolerance (Ahmad et al., 2026).

Although PGPM are a promising strategy for improving plant growth and productivity under drought conditions, their agronomic application may be constrained by certain limitations. For example, microbes may not always colonise the roots of target plants or effectively express their beneficial traits in overly compromised soil conditions. Therefore, it is essential to select PGPM strains adapted to specific agroecological contexts and test whether they are able to colonize the host plant which have species-specific response (Kaya et al., 2023).

The relationships between genotype, environment and management and the contribution of rare/specialised taxa to resistance against stress factors still need to be thoroughly understood and investigated in olive trees (Ahmad et al., 2026).

There is still a lack of targeted studies on epigenetic modifications induced by PGPMs in olive trees under drought stress. Understanding DNA methylation and histone modification profiles and their role will provide important information on regulatory mechanisms. Planning long-

term studies and monitoring with dedicated test plots would enable researchers to monitor the effects of PGPMs and changes in soil health and mechanics, microbial communities, and potential ecological consequences (Kaya et al., 2023).

Exploring transgenerational effects, clarifying molecular mechanisms, and identifying synergies with other management practices are key avenues for research.

The appropriate implementation of these microbial biotechnologies has been shown to stabilise key physiological processes, including photosynthetic performance, the regulation of leaf senescence, and the mitigation of oxidative damage to cellular membranes and proteins under conditions of extreme climatic stress (Shahid et al., 2023). The biotechnological contribution of rhizospheric microbial consortia is therefore evident, particularly in enhancing plant resilience to abiotic constraints.

However, the design and application of such consortia cannot be considered universally transferable. Microbial composition must be tailored to the intrinsic traits of the target cultivars, as well as to specific soil physicochemical properties, and should ensure the capacity to establish persistent and functionally stable associations within both the rhizosphere and the endosphere (Ahmad et al., 2026; Delitte et al., 2021). Although PGPR-mediated priming represents a promising and environmentally sustainable strategy for advancing agricultural systems under increasing demographic pressure and climate change (Singh et al., 2024), substantial gaps remain in our understanding of the underlying molecular, ecological, and functional mechanisms. Both fundamental and applied research are required to elucidate context-dependent interactions and to overcome current limitations to scalability and broad-field implementation.

Chapter 6 CONCLUSION

This PhD thesis provides an integrated framework to understand how agricultural management practices, plant-associated microbiomes, and genotype-specific traits interact to shape resilience in Mediterranean olive agroecosystems. By combining field-based microbial ecology with controlled drought experiments and multi-level plant analyses, this work bridges plant associated microbiome research with plant physiological and molecular responses to environmental stress.

Despite the challenges outlined in the previous section 5.3, the results consistently demonstrate that organic, conventional, and traditional management systems distinctly structure both belowground and aboveground microbial communities in the 'Ortice' cultivar. These findings highlight the central role of farming practices in modulating microbiome composition and reinforce the importance of microbiome-informed management to enhance agroecosystem sustainability.

Concurrently, the increasing frequency and severity of drought events in Mediterranean regions demand a deeper understanding of olive adaptive strategies. Through an integrative approach encompassing physiological, biochemical, and molecular analyses, this thesis reveals clear genotype-dependent responses among three traditional cultivars ('Ortice', 'Ortolana', and 'Racioppella') exposed to sequential water deficit. 'Ortolana' exhibited a conservative and efficient water-management strategy, 'Ortice' displayed progressive and plastic adjustments supported by stress-memory mechanisms, whereas 'Racioppella' activated rapid but energetically costly defense responses. These contrasting strategies underline the importance of intraspecific variability as a key resource for climate adaptation. Importantly, the inoculation with beneficial rhizobacteria enhanced drought tolerance across genotypes by improving plant water status, stimulating osmolyte and antioxidant accumulation, stabilizing photosynthetic pigments, and fine-tuning ABA- and drought-responsive gene expression. The observed priming effect suggests that beneficial microbes contribute to a more coordinated and energy-efficient transcriptional and physiological response under recurrent stress.

Overall, this PhD demonstrates that olive resilience emerges from the dynamic integration of agricultural management, microbial community assembly, plant physiological plasticity, and genotype-specific adaptive mechanisms. By linking plant holobiont perspective with plant stress biology, this research provides a solid scientific basis for the development of climate-

smart cultivation strategies that combine agronomic practice, resilient local cultivars with functional PGPR consortia. Such an integrated approach represents a promising pathway to enhance the sustainability, stability, and long-term productivity of Mediterranean olive systems under ongoing climate change.

OTHER PhD ACTIVITIES

The research undertaken as part of the PhD programme in Science and Technologies for Environment and Health involved my participation in the project “S.Oil” funded by the Regional Rural Development Program (PSR) of Campania region (Italy)¹. The data collected and the analyses conducted provided the fundamental analytical basis for the work presented in Chapter 4 (Section 4.1) of this thesis.

During my PhD, I contributed to a research line focused on the application of environmental DNA (eDNA) to investigate how landscape simplification influences plant–arthropod interactions. This work aimed to integrate molecular tools with ecological approaches to better understand how changes in landscape structure affect biodiversity patterns and the complexity of biotic interactions. By using eDNA-based methods, we sought to assess shifts in community composition and interaction networks across gradients of habitat simplification, providing new insights into the ecological consequences of anthropogenic landscape change.

In line with the internationalisation objectives of the doctoral programme, I spent a total of six months conducting research at the Bangor Molecular Ecology and Evolution Laboratory (MEEB) at the School of Environmental & Natural Sciences, Bangor University in Wales, UK, with the Prof. Simon Creer. During this period, I was able to first explore the theoretical and computational aspects of metabarcoding analysis, to further develop my training in bioinformatics and data analyses for processing complex datasets. Subsequently, based on the results of the sequencing analyses, the research focused on the ecological and functional aspects of the arthropod communities detected in the eDNA samples.

The results of this research ultimately led to the drafting of a manuscript entitled “*Landscape complexity shapes plant–arthropod interactions in agricultural systems: insights from an eDNA approach*” by Daniela Zuzolo; Gina Gizzi; Maria Tartaglia; Maria Antonietta Ranauda; Maria Maisto; Monica Labella-Ortega; Emanuele Fosso; Amy Ellison; Simon Creer; Carmine Guarino, currently under review by the international scientific journal *Global Ecology and Conservation*.

¹ PSR REGIONE CAMPANIA 2014-2020 Misura non connesse alla superficie e/o animali - Tipologia di Intervento 16.1.2- Azione 2 “Sostegno ai Progetti Operativi di Innovazione (POI)” B59H23000020006.

Other related activities during my PhD included presenting the following posters:

1. 'eDNA to test the functionality of hedgerows in a vineyard system' at 118° Congresso della Società Botanica Italiana (IX International Plant Science Conference).
2. 'Landscape complexity influences plant-arthropod interactions in agricultural systems: an eDNA approach' at 119° Congresso della Società Botanica Italiana (X International Plant Science Conference).

APPENDIX

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Fig. 2. Distribution of the olive orchards in the Mediterranean region according to (Caudullo et al., 2017).

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Fig. 13. Stomata of the leaves of 'Ortice', 'Ortolana' and 'Racioppella' cultivars during the two drought periods (T2 and T4), under control conditions (CT), drought stress (S), with the addition of the microbial consortium during the drought phase (SM), and during the irrigation phase (M). Image size 250 μm X 250 μm . Lowercase letters in the upper left corner of each image indicate statistically significant differences among treatments within the same cultivar, whereas uppercase letters denote differences among cultivars under the same treatment.

Fig. 14. Biochemical assays for 'Ortice' cultivar. A) starch; B) total sugars; C) fenolic compounds; D) amino acids; E) flavonoids; F) proline; G) chlorophyll a; H) chlorophyll b; I) carotenoids. The error was assessed as the standard deviation between the biological triplicates used for the analyses. ANOVA tests were performed between the different treatments in T2 (black), between treatments in T4 (red), and between the same treatments between T2 and T4 (blue).

Fig. 15. Heatmap showing gene expression profiles across samples from three cultivars ('Ortice' in yellow, 'Ortolana' in pink and 'Racioppella' in blue) at time points T2 (orange) and T4 (green) under four experimental conditions: control (CT), stress (S), microbial consortium (M), and combined stress + microbial consortium (SM). Each column represents a gene, and each row corresponds to a sample, categorized by cultivar and time point. Gene expression levels are displayed using a color scale, with values normalized and centered per gene (Fold-change). The heatmap illustrates gene expression patterns modulated by stress and/or microbial consortium treatment over time and among cultivars.

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Table 3. Analysis of variance for RWC (%). Mean $\pm$ SD of RWC for each time point (T2 and T4) and cultivar are shown. Statistical differences identified by Tukey's post hoc test are indicated by letters while ANOVA results between time points are represented as "ns" when non-significant and "*" when $p < 0.05$.

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