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Effect of soil management on rhizosphere microbial structure and function in olive grove

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MAN4HEALTH

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Abstract

Microorganisms are an important component of the rhizosphere, having a direct impact on soil health and crop yield. However, the factors that drive their assembly are not fully understood. Such knowledge will offer the opportunity to manipulate these microorganisms, in such a way, to increase beneficial plant-microbe interactions. Here was evaluated the effect of different soil managements on rhizospheric microorganisms of olive tree. Accordingly, within an olive orchard were collected rhizospheric soil samples from tilled and not-tilled soils and from soils with spontaneous cover vegetation or sowing, and used to analyse both bacterial and fungal community by using a culture dependent PCR-based identification approach. Overall, the bacterial community was dominated by the phylum Proteobacteria and genus *Pseudomonas*, while fungal community was dominated by Ascomycota and genus *Penicillium*. Tillage practices exhibited contrasting effects on fungal and bacterial communities, exerting a stronger influence on fungal community. Indeed, tillage showed to reduce significantly the fungal diversity and abundance, while bacterial richness was unaffected by tillage. Still, distinct fungal and bacterial community's composition was detected among tilled vs. not tilled soils, mainly due to the exclusive presence of Mortierellaceae and *Janthinobacterium* in tilled soils, and *Pseudogymnoascus*, *Solicoccozyma* and *Rhodococcus* in not tilled soils. The cover crop had no impact on both fungal and bacterial community´s diversity and structure. Still, a number of fungal and bacterial taxa were exclusively found either on seeded or not seeded soils. The capacity of phosphate solubilization and synthesis of indole acetic acid (IAA) of the isolates obtained was evaluated *in vitro*. Out of the 64 strains tested, all produced IAA and 47 were able to solubilize phosphate. The isolates showing the highest plant-growth-promoting proprieties, are potential inoculants for olive tree soils. However, the need remains to reveal the long-term effects of tillage practices and cover crop on rhizospheric bacteria and fungi composition and functionality.

Keywords: Tillage; soil cover vegetation; bacteria; fungi; plant growth promoting microorganisms.

Resumo

Os microrganismos são um componente importante da rizosfera, tendo um impacto direto na saúde do solo e na produção das culturas. No entanto, os fatores que determinam a sua composição não se encontram esclarecidos. Este conhecimento contribuirá para a manipulação destes microrganismos, de forma a aumentar as interações benéficas planta-microrganismo. Neste estudo pretendeu-se avaliar o efeito de diferentes práticas de gestão do solo nos microrganismos rizosféricos da oliveira. Para tal, num olival foram colhidas amostras de solo rizosférico de solos mobilizados e não mobilizados e de solos com vegetação natural ou semeados, e usadas para analisar a comunidade de bactérias e fungos através de identificação molecular dos isolados obtidos em cultura. No geral, a comunidade bacteriana foi dominada pelo filo Proteobacteria e género *Pseudomonas*, enquanto a comunidade fúngica foi dominada por Ascomycota e género *Penicillium*. A mobilização do solo teve efeitos contrastantes sobre as comunidades fúngica e bacteriana, exercendo uma maior influência na comunidade fúngica. De fato, a mobilização reduziu significativamente a diversidade e abundância de fungos, enquanto que a diversidade bacteriana não foi afetada. Ainda assim, a composição da comunidade fúngica e bacteriana foi distinta entre solos mobilizados vs. não mobilizados, principalmente devido à presença exclusiva de Mortierellaceae e *Janthinobacterium* em solos mobilizados, e *Pseudogymnoascus*, *Solicoccozyma* e *Rhodococcus* em solos não mobilizados. O tipo de coberto vegetal não teve impacto na diversidade e estrutura da comunidade fúngica e bacteriana. Ainda assim, várias espécies microbianas foram detetadas exclusivamente em solos semeados ou não semeados. A capacidade de solubilização de fosfato e síntese de ácido indolacético (IAA) dos isolados obtidos foi avaliada *in vitro*. Das 68 estirpes testadas, todas produziram IAA e 47 solubilizaram fosfato. Os isolados que exibiram as maiores propriedades promotoras do crescimento, são potenciais fontes de inóculo para usar no olival. No entanto, fica ainda por esclarecer os efeitos a longo prazo da mobilização e do coberto vegetal na composição e funcionalidade de bactérias e fungos rizosféricos.

Palavras-chave: mobilização; cobertura do solo; bactéria; fungo; microrganismos promotores de crescimento

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List of abbreviations

- ANOSIM: ANalysis Of SIMilarities
- ANOVA: ANalysis Of VAriance
- BLASTn: Nucleotide Basic Local Alignment Search Tool
- BNF: Biological Nitrogen Fixation
- CFU: Colony-Forming Units
- CIMO: Centro de Investigação de Montanha (Mountain Research Centre)
- Csa type: Hot-summer Mediterranean climate
- DAPG: DiAcetylPhloroGlucinol
- DFS-Taq: DNA Free Sensitive- Thermus aquaticus
- DNA: DeoxyriboNucleic Acid
- dNTP: DeoxyNucleotide TriPhosphates
- EU: European Union.
- HCN: Hydrogen Cyanide
- IAA: Indole Acetic Acid
- ISR: Induced Systemic Resistance
- IST: Induced Systemic Tolerance
- ITS: Internal Transcribed Spacer
- LB: Luria Broth
- NCBI: National Center for Biotechnology Information
- NMDS: Non-metric MultiDimensional Scaling
- NYIOOC: New York International Olive Oil Competition
- OTU: Operational Taxonomic Unit
- PBS: Phosphate-Buffered Saline
- PCA: Plate Count Agar
- PCR: Polymerase Chain Reaction.
- PDA: Potato Dextrose Agar
- PDB: Potato Dextrose Broth
- PERMANOVA: PERmutational Multivariate ANalysis Of VAriance
- PGPR: Plant Growth-Promoting Rhizobacteria
- PSM: Phosphate Solubilizing Microorganisms
- PVK: Pikovskaya agar

- RNA: RiboNucleic Acid

- SIMPER: SIMilarity PERcentages analysis

- TAE: Tris Acetate EDTA

- UV-Vis: UltraViolet–Visible

- V: hyperVariable regions

- v/v: volume per volume

Framework and objectives

Soil health is an essential factor affecting crop's productivity. The literature shows that rhizosphere 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. Thus, engineering of the rhizosphere microorganisms could be very useful for enhancing plant's productivity and restoring soil health. However, the factors that drive microbial community assembly and the functions played by these microorganisms are not fully understood, making it very difficult to manage.

In Mediterranean region, the olive groves have been traditionally tilled in order to reduce the competition for water and nutrients (van der Knijff et al., 2000). This practice has been leading to high erosion rates, resulting in a reduction in soil fertility (Vanwalleghem et al., 2011; Gómez et al., 2021). The use of cover crops has been suggested as a viable alternative to conventional tillage, to overcome these problems (Sastre et al., 2017). The impact of these practices on physico-chemical soil properties is already documented (Nieto et al., 2012; Duran-Zuazo et al., 2013), but it remains unclear whether they may also affect the rhizosphere microbial community (Landa et al., 2014).

Given the close linkages between rhizosphere processes and plant nutrition and productivity, understanding how soil management practices impact this critical zone is of great importance to optimize rhizosphere engineering. Therefore, this work aims to identify new sustainable approaches, relying on rhizosphere engineering via soil management practices, to enhance olive crop productivity. The specific objectives are:

1. Evaluate the impact of tillage and the use of cover crops in the microbial community (fungi and bacteria) composition inhabiting the rhizospheric soil of olive trees. Cover crops are already used in olive cropping systems, but typically with exotic plant species. However, this type of soil management has some limitations in terms of reduction of plant biodiversity and related ecological functions, and increase competition with the crop for water and nutrients. Thus, in this work will be tested an innovative soil management strategies based on the use of indigenous Mediterranean crop species as cover crop mixtures in olive groves. Native plants, as opposed to exotics, may be better adapted to the local climate, requiring fewer inputs when employed as a cover crop. The results could provide useful information for the manipulation of rhizospheric microbial

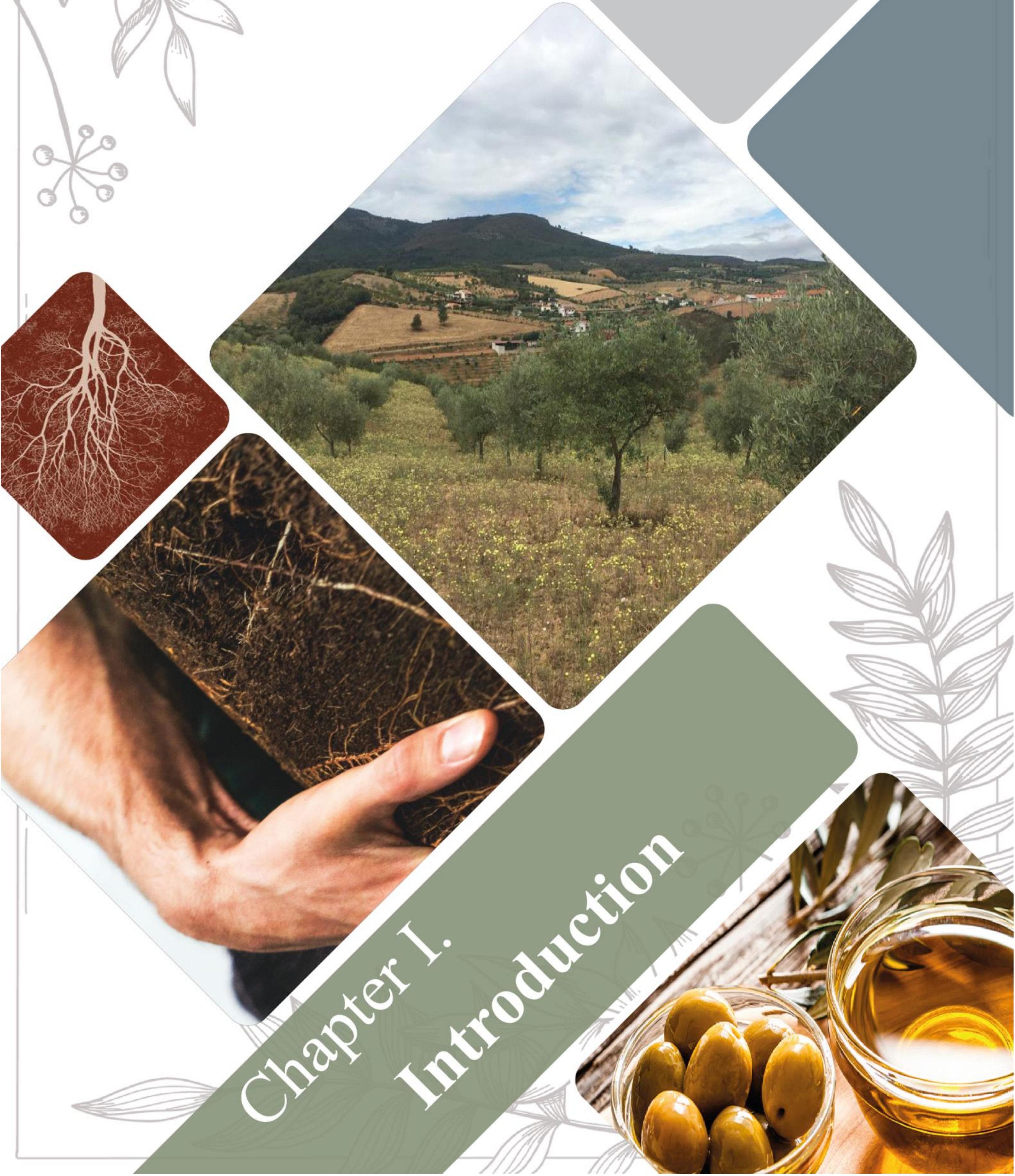
communities that are crucial to plant health and productivity, via soil management practices.

2. Identify the rhizospheric bacterial and fungal isolates with the best agronomic features that could enhance olive tree growth and performance.

This study will provide: i) new information about the best soil management practices (tillage and cover crop) to improve microbial rhizosphere-driven benefits to olive crops; and ii) a new product – microbial consortium - obtained from naturally occurring rhizospheric microorganisms, that can be used as a starting point for the development of a microbial inoculum in the future. This type of microbial consortium is cost effective and environmentally friendly for enhancing the agricultural sustainability.

Chapter I.

Introduction



1.1. Importance of olive groves in Portugal

The olive tree (*Olea europaea* L.) is one of the most significant crops in the Mediterranean basin, having high economic, social and environmental importance (Villalobos et al., 2006). The economic importance of olive tree relies mainly on their fruits, which are consumed as food either as table olives or used for oil production, a product widely appreciated in the world, especially for its health benefits (Guo et al., 2018). For generations, olive growing has been an important component of Portuguese agricultural history, culture and economy. The area under olive trees in Portugal is around 379.444 ha, being Alentejo in the South the most important producing region (56% of total area under olive trees), followed by Trás-os-Montes in the North-East (23%) and Beira in the East Centre (21%) (PORDATA, 2022). In these regions, the olive tree has been grown for many centuries under rainfed circumstances, by traditional methods, with low planting density, and thus with low production (Duarte et al., 2006). However, during the last decade, the farmers have been intensifying the production by planting and/ or converting traditional olive groves to high density olive groves, and by expanding into new crop producing areas, leading to an increase on olive oil and table olive production (Neves et al., 2013). These recent investments in olive groves are having a practical effect on the trade balance, resulting in a positive balance in the commercialization of this product. Whereas in 2017/18, national olive oil production was around 134,800 tons, making it a historic campaign, in the 2019/20 crop year, olive oil output in Portugal hit a new high record of 140,500 tons (PORDATA, 2022). With this production, Portugal currently ranks 6th in the world and 4th in the European Union(EU), just after Spain, Italy and Greece as shown in Figure 1 (IOC, 2022). Since 2014, Portugal became self-sufficient in olive oil production, producing 150 percent of its needs, making Portugal the world's fourth-largest exporter, by exporting around €705,5 millions in the 2021/22 crop year (Oil Times, 2021; INE, 2022). The high demand of Portuguese olive oil by European and world consumers is mostly due to the excellent quality of this product. Indeed, its valorisation brought the international recognition by winning international awards in several categories and important competitions, such as Mario Solinas Quality Award, Olive Japan, Olive Oil China, NYIOOC - New York Competition, and Terra Olivo Israel (Olive Oil Times, 2017).

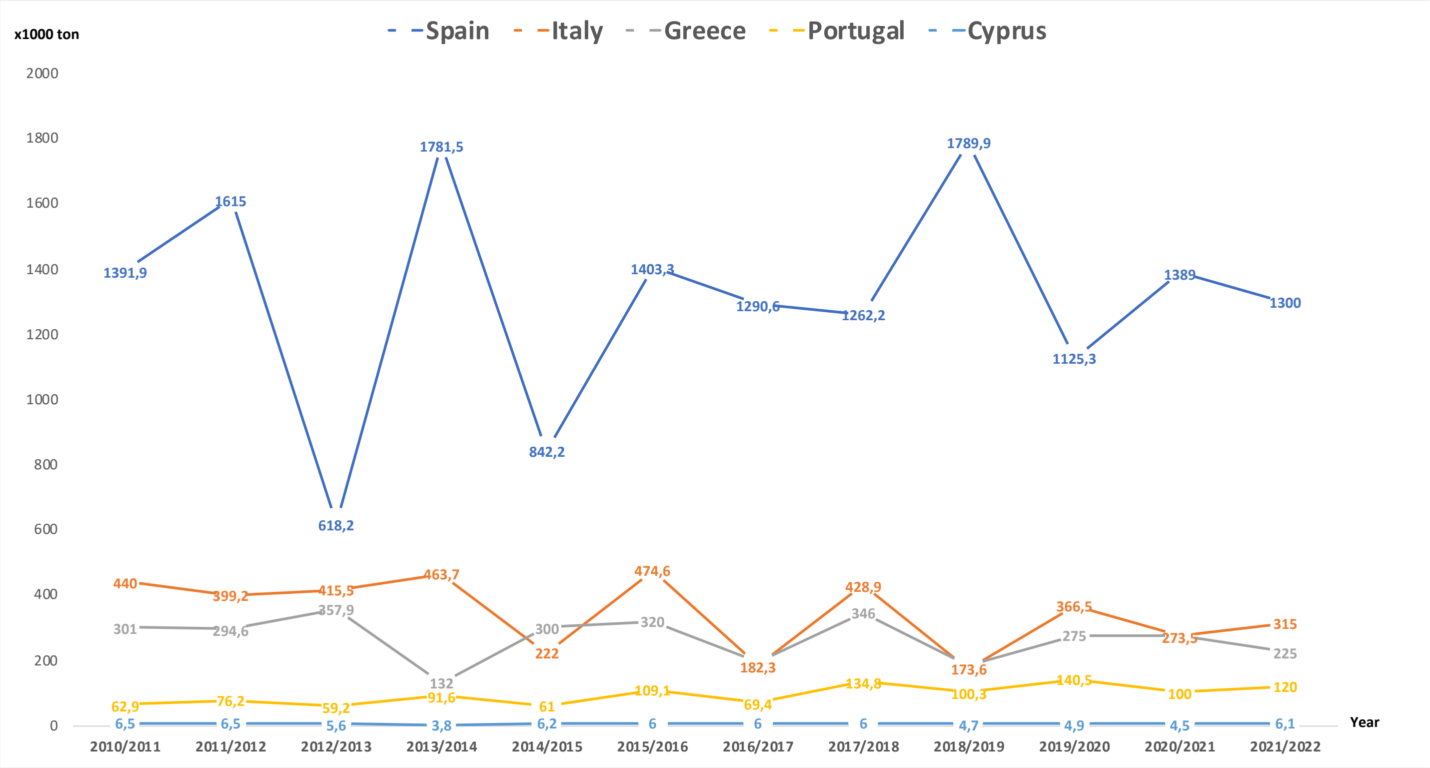


Figure 1. Olive oil production in European countries (Top 5) in 1000 tons (PORDATA, 2022).

The olive oil business also represents an important source of jobs in areas that are unappealing to other sectors of the economy due to their distance from large urban centers. More recently, it has been also valued in the tourism industry and for the dynamisation and sustainability of rural areas. Olive Oil Tourism is already recognized as a tourist product (Millán-Vazquez de la Torre et al., 2017) with a high potential to complement the activity of olive oil production and commercialization, and to create new employment opportunities (Pulido-Fernandez et al., 2019). Olive oil Tourism represents a potential for the creation and promotion of creative tourism experiences in rural regions by allowing visitors to have meaningful experiences, learn more aboutlocal skills and traditions, and engage with local people and culture while contributing to tourism's sustainable development, through gastronomic activities featuring olive oil as the main attraction (Bezerra & Correia, 2019).

Olive groves are also important to society and the environment by providing multiple ecosystem services (Bignal & McCracken, 2013) such as CO_2 sequestration, mitigation of climate change and soil erosion processes, pest control, maintenance of cultural landscape character and acting as agro-biodiversity reservoirs and thus, protecting genetic diversity. Indeed, it is thought that olive orchards host up to 25% of the species of birds, 20% of the insects, and 17% of the flora of the Iberian Peninsula (Rodriguez Sousa et al., 2021).

Despite the importance of olive farming systems as socio-ecological landscapes and sources of ecosystem services, they are threatened by a number of economic (poor farm revenue), social (rural abandonment), and environmental (soil and ecosystem deterioration) concerns that endanger their sustainability (Bidegain et al., 2020). Further environmental risks include the effects of entomological pests on agronomic productivity and the medium- to long-term effects of climate change on the distribution area of olive trees (Fraga et al., 2020). Nonetheless, soil erosion processes may be viewed as the primary hazard to olive grove agricultural sustainability (Balzan et al., 2020). In this sense, soil loss and the dragging of materials on the surface (*i.e.*, runoff) produce edaphic degradation, which hinders the economic profitability and environmental conservation of these systems (Rodriguez Sousa et al., 2021).

1.2. Soil health as key element in olive groves ecosystem

Soil health is one of the most important parameters that affect the production of resources in agricultural fields (Veerman et al., 2020). Soil health can be considered a subset of ecosystem health and is defined as soil's capacity to provide ecological functions for all forms of life (Veerman et al., 2020). Complex food webs with high microbiological and faunal variety, restricted availability of easily decomposable carbon sources, low nutrient losses, ecological resilience and resistance, and pest and disease suppression, are all characteristics of a healthy soil (van Bruggen & Semenov, 2000). Thus, soil health may be associated with biological diversity and stability (van Bruggen & Semenov, 2000) and is important for all life-sustaining processes on earth. Soil health also helps to local and global food security by laying the groundwork for the development of healthy foods (Rojas et al., 2016) (Figure 2).

'Soil health' is often used synonymously with "soil quality" (Grunwald et al, 2000), but in fact these two terms have different meanings. Indeed, soil quality concerns the capacity of a specific soil to sustain a particular use, such as biological productivity (crop production), maintain environmental quality (attenuating environmental contaminants), and promote plant and animal health (interlinking) (Laishram et al., 2012).

Global food security is a major challenge, but it should not be on the cost of depletion of nonrenewable resources such as soil. One-third of the world's soils are currently under moderate to severe degradation mainly due to soil erosion, nutrient and moisture depletion, salinity, sealing and contamination (Figure 2) (Rojas et al., 2016). These threats are related with climate change, land-use changes, and implementation of unsustainable agricultural practices (e.g., use of synthetic chemical fertilizers, soil tillage, among others) to increase crop productivity (Rojas et al., 2016).

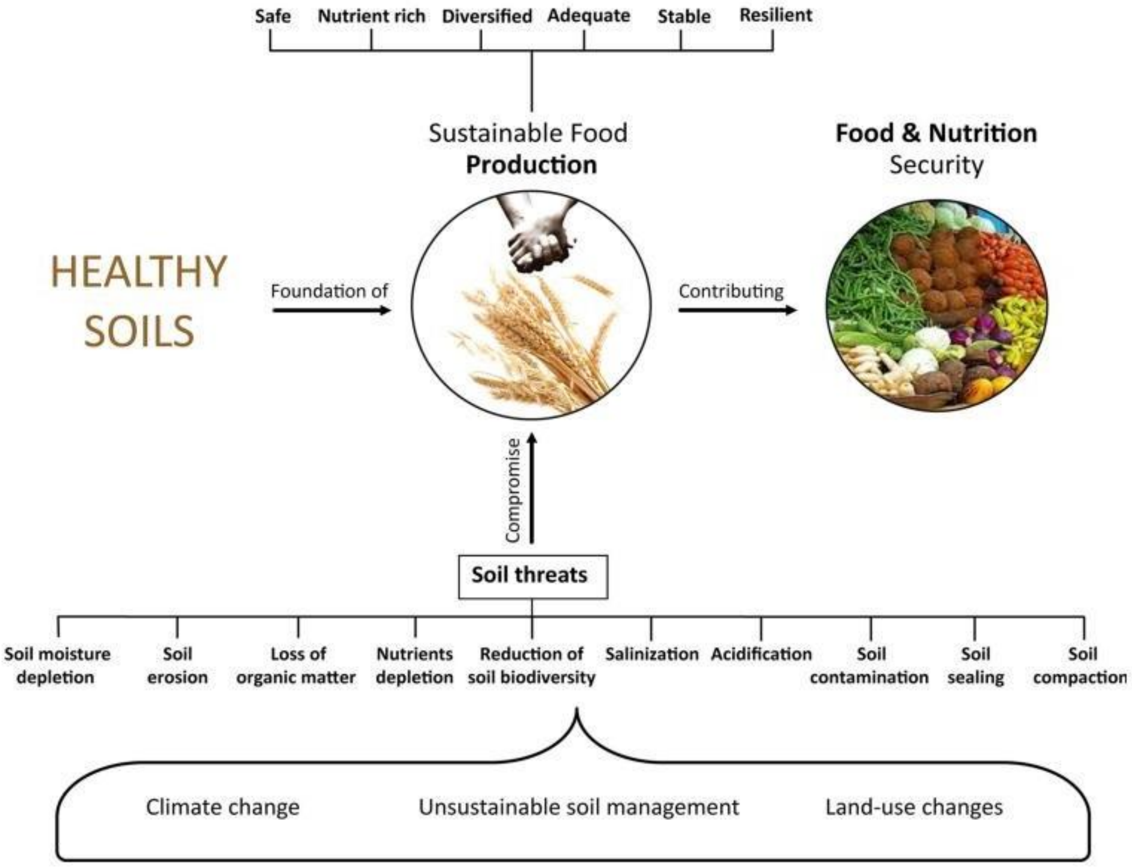


Figure 2. The importance of healthy soils in ensuring sustainable food production and food security (Rojas et al., 2016).

Soil erosion is one of the environmental key issues in Mediterranean olive groves (Gómez et al., 2021). This process of soil degradation leads to the removal of topsoil (which contains organic matter and nutrients) by water and/or wind (Rojas et al., 2016). There are several climatic and topographic factors as well as agricultural practices that make Mediterranean olive groves prone to soil erosion. These include, for instances, sloping land and sporadically intense rainfall events that are typical in Mediterranean regions, and bare soil management to ensuring olive tree survival during drought periods (Gómez et al., 2021). During the last decades, the problem of soil erosion has become worse due to olive farming intensification and intense tilling of soils (Vanwalleghem et al., 2011). Tillage has been traditionally used in rain fed olive groves aiming to reduce competition between the crop and unwanted weeds in what concerns water and nutrients (van der Knijff et al., 2000). However, this practice can result in degradation of soil structure, losing soil organic matter and accelerating soil erosion all due to the lack of ground cover. This is particularly problematic for most Mediterranean olive groves which are often established in sloping and rain-limited areas, with soils suffering from low fertility. When combined with the high temperature of the Mediterranean, tillage can also result in high losses of soil organic carbon, a major factor in soil health (Gómezet al., 2021). Yet, mechanical tillage is still the most common soil management system in many olive-growing regions (van der Knijff et al., 2000). Therefore, new soil management strategies are needed for reducing soil erosion and improving soil health in olive growing areas.

The use of cover crops has been proposed as an alternative to bare soil approaches (Gomez et al., 2008; Palese et al., 2014; Gómez et al., 2021). These cover crops, either sown or allowed to establish naturally from the soil seed bank in autumn, are mowed or grazed at some point, usually in early spring, in order to avoid competition with the crop for water and nutrients. Mulch of chopped olive-tree pruning residues is another alternative soil management measure against soil erosion (Gómez et al., 2021).

1.3. The exploitation of rhizospheric microorganisms for soil health improvement

Currently, there is an increasing interest in the exploitation of soil microorganisms, in particular fungi and bacteria, to improve soil health (Ortiz & Sansinenea, 2022). Apart from the soil formation, microbes play a pivotal role by degrading pollutants, helping in the organic matter transformation and adding more nutrients to the soil (Rebello et al., 2021). Indeed, microbes participate in various biogeochemical cycles, such as nitrogen, phosphorus, and sulphur cycles, which are essential to maintain soil fertility (Basu et al., 2021). The microorganisms inhabiting the soil are the most diverse community in the biosphere, holding at least a quarter of Earth's total biodiversity (Delago-Baquerizo et al., 2018). It has been estimated that one gram of soil may contain up to hundreds of millions of microorganisms (Roesch et al., 2007). This microbial diversity tends to be much higher in the soil immediately surrounding plant roots, an area known as the rhizosphere (Raaijmakers et al., 2009). The number of microorganisms present per gram of rhizhospheric soil is estimated to be 10^8-10^{12} microbes, which is 19-31 times higher compared to root-free soil (Ahmad et al., 2019), and it can contain more than 30 000 prokaryotic species (Mendes et al., 2011). Bacteria and, to a lesser extent, fungi, are the most abundant groups in the rhizosphere, followed by archaea, protists, nematodes, and viruses (Linget al., 2022).

The greatest richness and diversity of microorganisms in the rhizosphere is attributed mostly to the exudation of compounds by the roots (Wichern et al., 2008). These compounds serve as carbon and energy sources for the microorganisms and can also act as chemical attractants/repellents serving as communicating molecules between plants and microbes to initiate interactions (Wichern et al., 2008). Thus, their release contributes to a unique rhizosphere nutrient pool, favouring the development of selected soil microorganisms that respond to chemo taxis and thrive quickly (Hartmann et al., 2009). Root exudates compounds include primary metabolites, such as sugars, organic acids, amino acids, as well as secondary metabolites, namely fatty acids and sterols (Table 1) (Sun et al., 2021). Their composition varies, both qualitatively and quantitatively, depending on the plant species, genotype, developmental stage, and root traits, but also on the nutrient availability, management practices, and climatic conditions (Zhang et al., 2019). The growth of rhizospheric microbes on these exudates turns over all cellular functions and also releases nutrients in a form that can be used by plants (Raaijmakers et al., 2009). This rhizospheric microbiota not only helps plants in nutrient uptake, but it also helps the plant in mitigating both biotic (pests and diseases) and abiotic (drought, salinity...) (Mohanram & Kumar, 2019). The enhancement of soil fertility is mainly due to the ability of rhizosphere microorganisms to fix nitrogen, mobilize and solubilize nutrients, and decompose organic residues with the associated release of mineral nutrients (Mohanram & Kumar, 2019). These microorganisms also play a major role in stimulating plant growth, by producing phytohormones (Mohanram & Kumar, 2019). Thus, these beneficial traits can be explored to avoid further soil degradation, to restore unhealthy soils, and to improve plant growth and productivity (Thiele-Bruhn et al., 2012).

Table 1. Different compounds released by plant roots in the process of rhizodeposition (adapted from Yadav et al., 2015).

Compounds	**Exudates components**
Amino compounds	Aspargine, α-alanine, glutamine, aspartic acid, leucine /isoleucine, serine, glycine, cystine/cysteine, methionine, phenylalanine, tyrosine, threonine, lysine, proline, tryptophan, ß- alanine, arginine, homoserine, cystathionine
Fatty acids and sterols	Palmitic, stearic, oleic, linoleic, linolenic acid, campesterol, stigmasterol, sitosterol

Growth factors	Biotin, thiamin, niacin, pantothenate, choline, inositol, pyridoxine, N-methyl nicotinic acid
Nucleotides, flavonoids and enzymes	Flavanone, adenine, guanine, uridine/cytidine, phosphatase, invertase, amylase, protease, polygalacturonase
Organic acids	Tartaric, oxalic, citric, malic, propanoic, butyric, succinic, fumaric, glycolic,valeric, malonic
Sugars	Glucose, fructose, sucrose, maltose, galactose, rhamnose, ribose, xylose,arabinose, raffinose, oligosaccharide
Miscellaneous compounds	Auxins, scopoletin, fluorescent substances, hydrocyanic acid, glycosides, saponin (glucosides), organic phosphorus compounds, nematode-cyst or egg- hatching factors, nematodes attractants, fungal mycelium growth stimulants and inhibitors, zoospore attractants

1.4. Mechanisms of plant growth promotion by rhizospheric microorganisms

Among the rhizosphere microorganisms, are plant growth-promoting rhizobacteria (PGPR), but also fungi and actinobacteria that exert beneficial effects on plants through direct and indirect mechanisms. These mechanisms are variable according to the group in which the microorganisms belong: symbiotic (microorganisms that establish symbiotic association with the plant, giving rise to mycorrhizal structures or nodules), endophytic (exists inside the root of the plant), associative (exists on the root surface of the plant) and free-living (microorganisms that are present in rhizosphere and do not inhabit plant tissues) (Figure 3). The exact mechanisms by which these microorganisms promote plant growth are not fully understood. But, it is thought that they promote plant growth directly by either facilitating resource acquisition (nitrogen, phosphorus, potassium and essential nutrients) or modulating phytohormone levels, or indirectly by mitigating the deleterious effects of biotic (pathogens) and abiotic stresses (Ahmad et al.,2019).

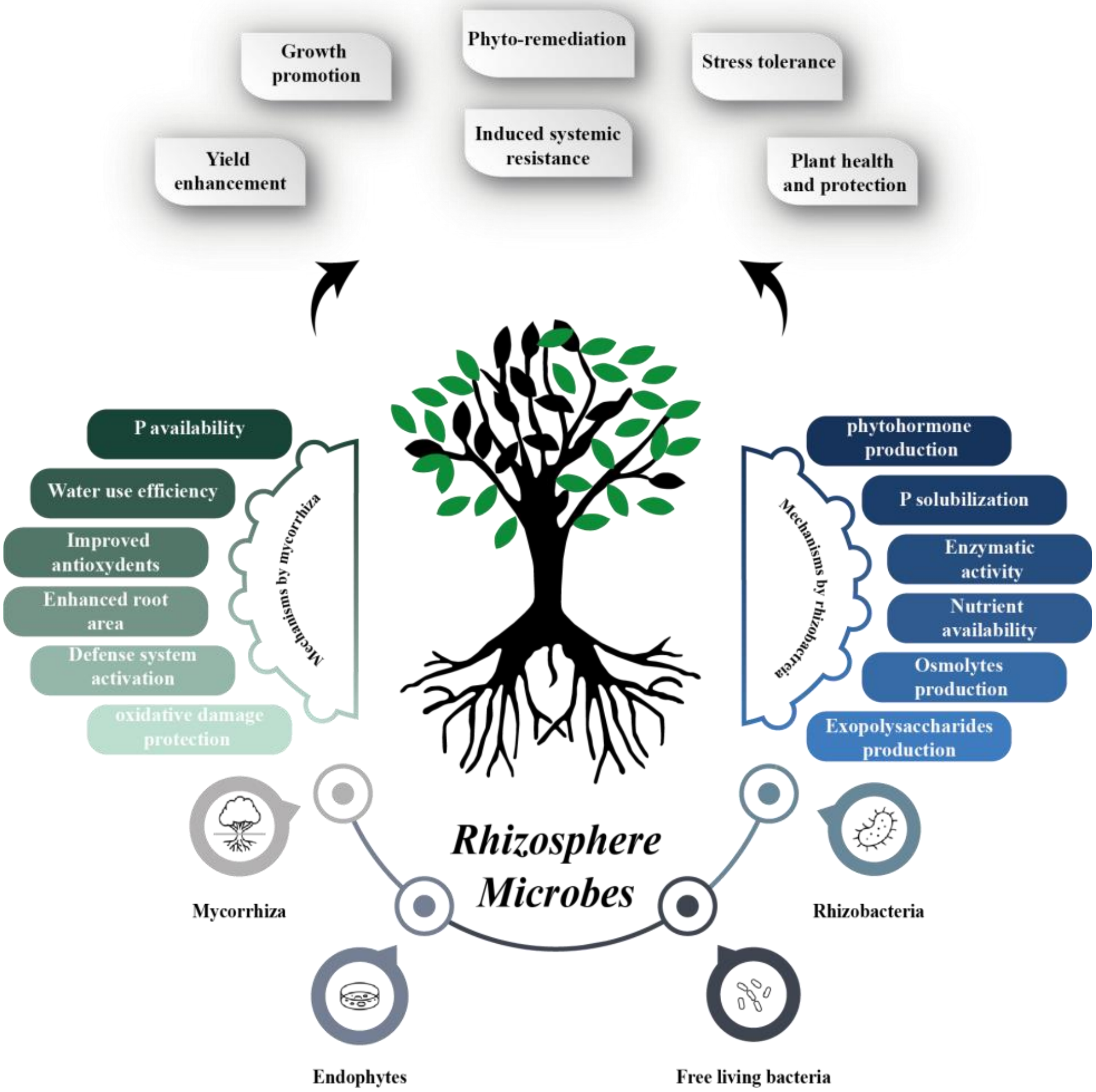


Figure 3. Common Plant-growth mechanisms displayed by rhizospheric microorganisms. Adapted from (Ahmad et al., 2019).

1.4.1. Direct mechanisms

Biological nitrogen fixation

Nitrogen is the most vital nutrient for plant growth and productivity. The majority of the nitrogen of the planet is in the atmosphere, but in a form that is unavailable to plants. It must be converted to NH_3 either artificially using the Haber-Bosch technique (Rubio & Ludden, 2008) or naturally using Biological Nitrogen Fixation (BNF). In BNF, nitrogen fixing prokaryotes convert atmospheric N_2 to NH_3 through nitrogenase activity,under a process of oxidation and reduction succession that uses a significant amount of metabolic energy (Kim & Rees, 1994). This biologically fixed N_2 accounts for approximately 60% of total fixed N_2 worldwide (Kim & Rees, 1994). Free-living,associate, and symbiotic diazotrophs are among the microorganisms implicated in BNF (Bashan & Levanony, 1990). Symbiotic nitrogen fixation has the highest efficiency of BNF, but it is limited to a few genera of the Leguminosae and nonleguminous tree and shrub families (Zahran, 2001). An array of bacteria such as *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, and *Mesorhizobium* are included in the group of symbiotic bacteria that fix nitrogen on Leguminosae family in a specialized organelle, known as the nodules (Bhat et al., 2015), while *Frankia,* an actinobacteria, establish nitrogen-fixing root nodule symbiosis on a variety of nonleguminous families (Bhat et al., 2015). Most of the crop plants are not able to form this kind of symbiotic association, but they can benefit from the nitrogen fixed by free-living and associative diazotrophs inhabiting the rhizosphere. Some examples of these diazotrophs are *Arthrobacter*, *Bacillus*, *Azospirillum*, *Azotobacter*, *Micrococcus*, *Pseudomonas*, and *Serratia* (Adesemoye & Egamberdieva, 2013).

Mobilization of nutrients

Some microorganisms inhabiting the rhizosphere have the ability to dissolve chemically fixed nutrients like phosphorus, potassium, iron, and zinc.

Phosphorus (P) is the second most important plant growth-limiting nutrient after nitrogen. Phosphorus is found in great amounts in the soil, in both organic and inorganic forms. However, only 0.1% of these P can be used by plants (Lambers & Plaxton, 2018), being necessary to convert it into monobasic and diabasic ions, which are the only two forms of P that plants can absorb (Ahemad & Kibret, 2014). Numerous microorganisms in the rhizosphere have the ability to do this conversion, and this group is referred to as Phosphate Solubilizing Microorganisms (PSM). They solubilize insoluble inorganic (mineral) phosphorus by producing organic acids that can form complexes with cations bound to phosphate, and mineralize insoluble organic phosphorus through the synthesis of different phosphatases that catalyze the hydrolysis of phosphoric esters (Dodor & Tabatabai, 2003). Examples of PSM include bacterial strains, such as *Azotobacter, Enterobacter, Erwinia, Burkholderia, Bacillus, Kushneria, Ralstonia, Rhizobium, Rhodococcus, Serratia, Bradyrhizobium, Sinomonas,* and *Thiobacillus,* and fungal strains, such as *Achrothcium, Alternaria, Arthrobotrys, Aspergillus, Cephalosporium, Cladosporium, Curvularia, Cunninghamella, Chaetomium, Fusarium, Glomus, Paecilomyces, Penicillium, Phoma, Rhizopus, Trichoderma*, and *Yarrowia* (Alori et al., 2017). Fungi are thought to be more important in the solubilization of inorganic phosphate since they normally produce and secrete more acids than bacteria (Sharma et al., 2013).

Potassium (K) is the third most essential macronutrient for plant growth. More than 90% of potassium in the soil exists in the form of insoluble rocks and silicate minerals, therefore unavailable to plants (Parmar & Sindhu, 2013). Some rhizospheric microorganisms have the ability to solubilize the insoluble K by various mechanisms. Despite the little information on these mechanisms, it is generally believed that microorganisms contribute to the release of K+ from K-bearing minerals by producing organic and inorganic acids, protons (acidolysis mechanism) as well as polysaccharides (Etesami et al., 2017). Rhizospheric microorganisms reported as K-solubilizers, include bacteria (*e.g.*, several species of the genera *Bacillus, Paenibacillus, Acidothiobacillus, Pseudomonas, Burkholderia, Enterobacter, Sphingomonas*, and *Aminobacter*), and to a lesser extent, fungi (*e.g.*, *Aspergillus, Glomus,* and *Penicillium*) and actinomycetes (*e.g.*, *Streptomyces*) (Meena et al., 2014).

Iron (Fe) is an essential nutrient for all forms of life on earth, including plants. In soil, Fe occurs in an insoluble hydroxides and oxyhydroxide form, both unavailable to plants (Liang, 2022). Certain rhizospheric microorganisms produce siderophores, a high-affinity ferric iron chelator that binds with the insoluble ferric ion (Fe^{3+}). The Fe- siderophore complex formed can be recognized and taken up by the root cells (Singh, 2020). Bacteria genera *Agrobacterium, Azospirillum, Azotobacter*, *Bacillus*, *Enterobacter, Mycobacterium, Neisseria, Pseudomonas, Rhizobium, Serratia,* and *Streptomyces*, are reported as siderophore producers (Singh, 2020).

Zinc (Zn) is a micronutrient indispensable to the plants, being involved in various physiological and biochemical functions, thus affecting plant growth, development, and yield (Kumar et al., 2019). Around 50% of the agricultural soils in the world are Zn deficient, mostly due to fixed forms of Zn compounds, making it unavailable to plants (Shaikh & Saraf, 2017). Zn is taken up by plants as the divalent Zn^{+2} cation, but in the soil Zn forms complexes with organic acids, humic substances and other types of dissolved organic carbon (Hakim et al., 2021). A range of rhizospheric microorganisms can solubilize Zn by producing organic acids (*e.g.*, acetic acid, formic acid, gluconic acid, citric acid, and oxalic acid) and chelating agents (Saravanan et al., 2011). These organic acids chelate with the cations bound to the zinc compound, enhancing zinc solubility. The majority of Zn solubilizers are bacteria from the genera *Pseudomonas, Bacillus, Enterobacter, Xanthomonas, Stenotrophomonas,* and *Acinetobacter* (Khanghahi et al., 2018).

Phytohormone production

Phytohormones are chemical messengers that, at very low concentrations, play critical roles in regulating plant growth and development, and stress tolerance to promote plant survival (Amara et al., 2015). There are several rhizospheric microorganisms with ability to produce phytohormones, including auxin, cytokinins, gibberellins, abscisic acid, and ethylene (Oleńska et al., 2020). Among all, the auxin indole acetic acid (IAA) is reported as the most abundant and secreted by rhizosphere bacteria, such as *Azospirillum*, *Pseudomonas*, *Bacillus*, *Enterobacter*, *Mycobacterium*, *Rhizobium*, *Erwinia*, *Agrobacterium*, *Bradyrhizobium* and *Klebsiella* (Oleńska et al., 2020). These auxin-producing bacteria interfere with many plant developmental processes by changing the plant auxin pool. For example, rhizobacterial IAA has been reported to be involved in root formation, growth, and yield of various crops as well as in the mitigation of deleterious effects of several abiotic (*e.g.*, salinity, drought) and biotic (pathogens) stresses (Ahemad and Kibret, 2014).

Some rhizosphere bacteria, such as *Bacillus*, *Escherichia*, *Agrobacterium*, *Methylobacterium*, *Proteus*, *Pseudomonas* and *Klebsiella*, are reported to produce cytokinins (Maheshwari et al., 2015). The release of cytokinins into the rhizosphere by these bacteria have been reported to stimulate plant growth, and to regulate the defense system of the plant against pathogens and abiotic stresses, such as drought (Hakim et al., 2021).

Gibberellins are also produced by a number of rhizosphere bacteria genera, such as *Rhizobium*, *Acetobacter*, *Azospirillum*, *Herbaspirillum*, *Bacillus*, and *Pseudomonas* (Maheshwari et al., 2015). Various studies showed that the gibberellins produced by these bacteria stimulate plant growth and yield, and mitigate abiotic stresses, such as hightemperature (Hakim et al., 2021).

1.4.2. Indirect mechanisms

Major indirect mechanisms are ascribed to biocontrol of pathogens and to the induction of plant resistance against abiotic stresses. Soil rhizosphere microorganisms show several biocontrol mechanisms against phytopathogens such as competition for nutrients and space, antagonism by production of various metabolites, and induction of systemic resistance (ISR) in plants (Lugtenberg & Kamilova, 2009). Frequently these mechanisms act in a synergistic manner to induce the plant's resistance against phytopathogens.

In the competition, the rhizosphere microorganisms prevent colonization of the host plant by pathogens by reduction the availability of nutrients around the host plant. In this regard, siderophore-producing microorganisms have been recognized as the most effective (Kim et al., 2012). These microorganisms restrict the availability of iron to pathogens by producing Fe-chelating chemicals, thereby reducing their growth (Sayyed et al., 2013). Besides nutrients, this competition between pathogens and rhizosphere microorganisms could be also for space, referred as niche exclusion (Mohanram & Kumar, 2019).

Another efficient mechanism of rhizospheric microorganisms for inhibiting pathogenic infections is through the production of antimicrobial metabolites. These include either volatile or diffusible metabolites, belonging to different groups, such as phloroglucinols, phenazines, pyoluteorin, cyclic lipopeptides, or pyrrolnitrin (Hakim et al., 2021). In particular, the hydrogen cyanide (HCN) and 2,4- diacetylphloroglucinol (DAPG), produced by *Pseudomonas*, has been associated with the biological control of various plant diseases (Meyer et al., 2016; Abd El-Rahman et al., 2019). Furthermore, rhizospheric microorganisms can fight against soil-borne phytopathogens through the production of extracellular cell wall-degrading enzymes, such as chitinases, glucanases, cellulases and proteases (Ayyadurai, Naik & Sakthivel, 2007).

Rhizosphere microorganisms, particularly *Pseudomonas* and *Bacillus*, can also interact with the plant roots inducing a systemic resistance against pathogens (Mohanram & Kumar, 2019). This phenomenon is mainly ascribed to microbial components, such as flagella, or to the release of metabolites by rhizosphere microorganisms that trigger ISR (Lugtenberg & Kamilova, 2009). Some examples of these metabolites include lipopolysaccharides, siderophores, cyclic lipopeptides, 2,4- diacetylphloroglucinol, homoserine lactones, and volatiles like, acetoin and 2,3- butanediol (Lugtenberg & Kamilova, 2009). In addition to these metabolites, jasmonate and ethylene signaling, can activate the host plant's defense systems against pathogens (Verhagen et al., 2004).

Abiotic stresses are the major constrains that are affecting both the soil quality and crop productivity (Haggag et al., 2015). Rhizospheric microorganisms can support plants to resist against these abiotic stresses, by imposing physical and chemical changes on host plants. This phenomena known as induced systemic tolerance (IST), is triggered by microorganisms by regulating the production of various metabolites such as phytohormones, antioxidants, enzymes and exopolysaccharides in host plants (Mohanram & Kumar, 2019). Several genera of *Pseudomonas, Bacillus, Achromobacter, Burkholderia, Enterobacter, Azotobacter, Methylobacterium,* and *Trichoderma* have been widely studied in plant growth promotion by mitigation of multiple kinds of abiotic stresses (Mohanram & Kumar, 2019).

1.5. Rhizosphere engineering

Due to the paramount importance of rhizospheric microorganisms for crop production and soil health, there is a growing interest in enhancing their abundance and activity to improve agricultural production making it more sustainable (Ahkami et al., 2017). In this context, rhizosphere engineering offers an exciting and powerful opportunity to reach this demand. In this process, the microorganisms are manipulated in such a way to increase beneficial plant-microbe interactions (Ahkami et al., 2017). This can be done via re-designing plant metabolism, microbial inoculation, or use of specific soil agronomical practices (Ahkami et al., 2017; Hakim et al., 2021).

Since the rhizosphere activity and functioning is shaped by the plant traits, such as root exudates, so these traits can be engineered by traditional plant breeding approaches or by gene editing techniques (Ahkami et al., 2017). These techniques can be applied to modify the expression levels of the genes controlling exudates to redesign the rhizosphere for improved features (Ahkami et al., 2017). However, the application of plant engineering to improve rhizosphere characteristics remain reduced due to the complexity of this process and to the limited knowledge about root exudates production and composition (Ahkami et al., 2017). Moreover, the use of genetically modified plants is, in general, not well accepted by the public due to concerns regarding human health and environmental sustainability.

Another alternative to engineering the rhizosphere is through exogenous application of microbial inoculants (Hakim et al., 2021). In this approach, beneficial rhizospheric microorganisms can be isolated and multiplied under laboratory conditions and used in the form of biofertilizers for the inoculation of seeds, roots or soils (Ortiz & Sansinenea, 2022). There are already some biofertilizers in the market containing one orseveral species of bacteria or fungi (Ortiz & Sansinenea, 2022). However, a number of issues have hindered their extensive and successful use. These problems are mostly associated with difficulties in the screening of rhizospheric microorganisms, which is a key step to finding and selecting an adequate and specific biofertilizer strains (Ortiz & Sansinenea, 2022). Another issue is that the efficacy of biofertilizers is dependent on a number of factors related mostly to the plant, the soil, and the products themselves. Indeed, depending on their physiological status and phenological phase of growth,plants modify the composition of the exudates released throughout the roots (Zhang et al., 2019). Root exudates contain compounds with stimulatory and inhibitory effect on rhizosphere microorganisms, and thus affecting their capacity of establishing beneficial relations with the plant (Sun et al., 2021). Soil chemical (pH, nutrient content) and physical (texture) characteristics have been found as a major driving force for structuring rhizospheric microorganisms (Deng et al., 2018), thus affecting the colonization potential and efficacy of biofertilizers strains. When applied into the soil, the biofertilizer strains must also face the competition from indigenous microorganisms and be able to survive in the local environmental conditions (*e.g.*, temperature), to be efficient and successful bioinoculants (Ortiz & Sansinenea, 2022). The production of the biofertilizer is a crucial multistep process causing great impact on its efficacy. Indeed, the formulation of an inoculant should protect the strains from the often harsh conditions during storage and transport, and enhance their establishment after introduction into soils (Malusà et al., 2016). In the formulation, one or more strains of microorganisms (inoculum) are included in a suitable carrier, with or without additives (*e.g.* sticking agents or other additives), that is used mostly to improve the shelf life of the product (Malusà et al., 2016). The technology of biofertilizers production involves sophisticated technology and qualified human resources. Therefore, to improve the efficacy of the inoculants for rhizosphere engineering, a better understanding of the interrelationships between microorganisms and plants as well as knowledge of the various challenges encountered during application of microbial inoculants is required.

Rhizosphere engineering can also be achieved through different agronomic interventions that ultimately lead to microbial community changes. Management practices such as tillage, grazing and mowing (Landa et al., 2014), as well as mineral fertilization (Llimos et al., 2021), influence the rhizosphere microorganisms of olive trees. Thus, it seems feasible to modify rhizosphere microbial population through agronomic practices. However, contradictory results on the effect of crop management practices on soil microbial communities make rhizosphere engineering very difficult to perform. For instances, in olive groves, organic amendments showed to either affect (Llimos et al., 2021) or not affect (Sosa et al., 2021), the microbial community of the soil. Therefore, an understanding of the agronomic factors that drive microbiome assembly in the rhizosphere is needed to better manage and manipulate the whole rhizosphere microbiome.

Chapter II.
Material and methods

2.1 Rhizosphere soil sampling

Sampling of the rhizospheric soil was carried out in a 10 year old rain fed olive orchard located in Suções, Mirandela, Trás-os-Montes region with the coordinates 41.488664°N, -7.248158°W (Figure 4). The site belongs in geomorphological terms to the Iberian Massif, more specifically to the Mirandela Depression, where the climate is Csa type (temperate climate with rainy winters and hot dry summers) according to the Köppen climate classification. This olive orchard comprising olive trees at spacing of 7 x 7m is managed through integrated production guidelines (Malavolta & Perdikis, 2018), and prior to the current field experiment the weeds were managed with mechanical cutting for five years. Since 2021, half of the orchard has been tilled twice a year (Spring and Autumn) using a cultivator, at a depth of approximately 10 cm from the soil surface, while the soil of the other half has been kept with natural vegetation, being, this vegetation cut, gridded and deposited on soil surface in each Spring.

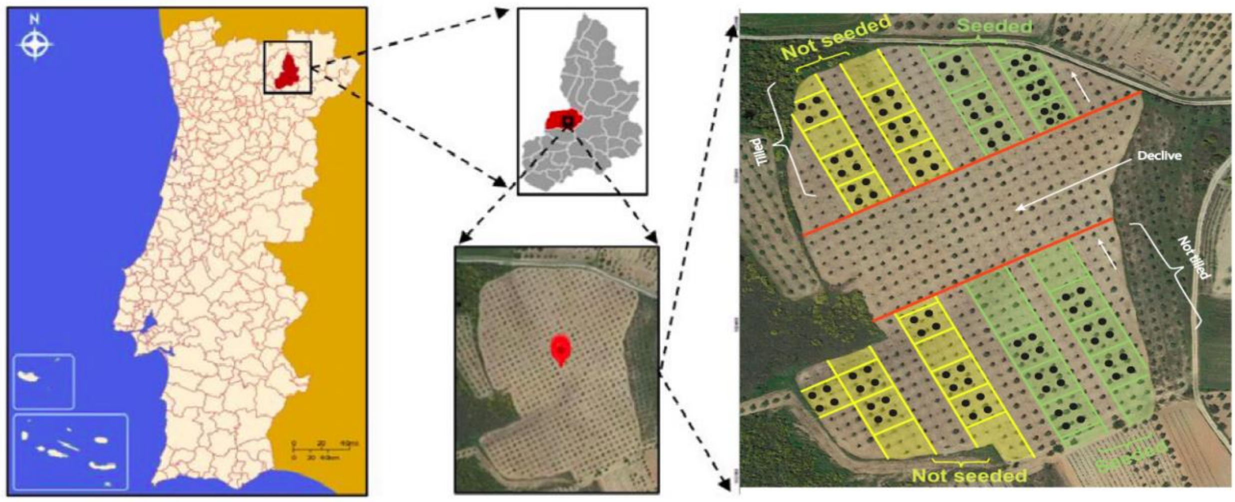


Figure 4. Location of the sampling site plots established in the olive orchard in Mirandela Trás-os-Montes region (Northeast of Portugal). The experiment comprised of two tillage systems (not tilled and tilled) and two soil cover crops (not seeded and seeded).

In each half, 12 plots, each covering an area of approximately 49 m², including four trees, were established in March 2022. From these 12 plots, six were seeded with a mixture of native plant seeds and the remaining six plots were not seeded and kept with the natural flora. Therefore, in total four treatments with six replicates each were studied (tilled and seeded; tilled and not seeded; not tilled and seeded; not tilled and not seeded) (Figure 4). The mixture of seeds sown comprised 18 plant species, ranging from *Fabaceae, Caryophyllaceae, Poaceae, Lamiaceae, Asteraceae, Primulaceae, Ranunculaceae, Papaveraceae* to *Apiaceae* (Table 2), that were selected based on their common occurrence in olive groves. The sowing was done manually using a mixture of seeds and fine sand in the ratio of 2 sand: 1 seed and the seeds were incorporated into the soil by a tractor with a superficial rotary tiller pass. The same procedure was performed in the not-seeded plots using only sand.

Table 2. Seed mixture and quantity used for cover crop sowing.

Species	**Family**	**Quantity seeds/plot (g)**
Daucus carota	Apiaceae	51.4
Centaurea cyanus	Asteraceae	59.2
Silene colorata	Caryophyllaceae	63.1
Astragalus pelecinus	Fabaceae	23.5
Medicago orbicularis	Fabaceae	2.7
Trifolium subterraneum	Fabaceae	27.6
Trifolium cherleri	Fabaceae	23.7
Trifolium lappaceum	Fabaceae	23.4
Trifolium hirtum	Fabaceae	23.4
Trifolium glomeratum	Fabaceae	23.6
Scorpiurus vermiculatus	Fabaceae	19.2
Salvia verbenaca	Lamiaceae	19.2
Papaver hybridum	Papaveraceae	27.7
Hordeum geniculatum	Poaceae	0.9
Bromus scoparius 1	Poaceae	0.8
Brachypodium distachyon	Poaceae	77.0
Anagallis arvensis	Primulaceae	16.9
Nigella damascena	Ranunculaceae	59.2

In each plot rhizospheric soil samples were collected by digging up and collecting roots from the four trees, that were further combined to form one composite sample per plot. The collection of rhizospheric soil samples was carried out twice, immediately before seed sowing (8 March 2022) and five months later in 29 July 2022. Hence, a total of 24 composed samples were obtained in each sampling time. The roots were collected to sterile bags and transported immediately to the laboratory in an icebox.

In the laboratory, the roots from each plot were manually shook to leave only the portion of the soil attached to the roots. Then, the soil adhering to these root samples was removed by stirring the roots in sterile Phosphate-Buffered Saline (PBS) solution (8 g NaCl, 0.2 g KCl, 1.44

g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.4) with 0.01% (v/v) Tween 80, in a vortex, for 5 minutes. The soil suspension was then filtered with <2.0 mm test sieve into a new sterile 50 mL tube. The rhizospheric soil was concentrated bycentrifugation at 5000 rpm for 5 minutes at room temperature. Around 0.1 g of the rhizospheric soil was dissolved in 10 mL of PBS and used for microbial isolation and assessment of diversity.

2.2 Isolation of rhizospheric soil microorganisms

For the isolation of both bacteria and fungi from the rhizospheric soil, the dilution-to-extinction approach was used, by performing ten-fold serial dilutions from 0.1 g of soil in a PBS solution. A 100 µL from the last two serial dilutions (10^{-5} and 10^{-6}) was then evenly spread over the surface of two different medias, namely Potato Dextrose Agar (PDA, HiMedia Laboratories, India) supplemented with lactic acid 1M (1mL/1L PDA), for the isolation of fungi, and Plate Count Agar (PCA, HiMedia Laboratories, India), for the isolation of bacteria. Three replicates for each dilution were prepared (in total 6 plates per culture medium). The plates were then incubated at 25ºC (for fungi) and at 30ºC (for bacteria), in the dark. Daily observations were performed in order to count and isolate the fungal/bacterial colonies with different morphology that appeared in the two medias. The size, shape, texture, color, margins of the colonies and pigmentations on both obverse and reverse of colonies were the major characteristics used to distinguish the different morphotypes. Pure cultures of each morphotype were obtained by subculture of fungal/bacterial cells on fresh medium (PDA for fungi or PCA for bacteria). The obtained morphotypes were further molecularly identified. Microbial counts were expressed as $\log_{10}$ colony-forming unit (CFU) per g of soil.

2.3 Molecular identification of fungal and bacterial isolates

The molecular identification of the different morphotypes was conducted by amplification and sequencing of the internal transcribed spacer (ITS) region and/or the protein encoding gene β-tubulin (for fungi) as well as the 16S region of the ribosomal RNA (for bacteria), according to a previously described methodology (Martins et al., 2016; Mina et al., 2020). Accordingly, DNA of both fungi and bacteria was extracted using the REDExtract-N-AmpTM Plant PCR Kit (Sigma-Aldrich, USA) according to the supplier instructions. Briefly, in a 0.5 mL microtube containing 25 µL of extraction buffer, around 0.01 to 0.02 g of microbial material from active pure colonies was added. After mixing, the samples were incubated at

95°C for 10 minutes before being diluted with 50 µL of dilution buffer. The samples were homogenized and stored at -20°C until its use for DNA amplification.

The identification of the fungi was based on the amplification of the ITS region (ITS1, 5.8S, ITS2) by using the primer pair ITS1 (forward: 5'-TCCGTAGGTGAACCTGCGG-3'), ITS4 (reverse 5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). A region of the β-tubulin gene was used together with the ITS for more reliable identification of specific fungal genera, namely *Penicillium*, by using the primer pair Bt2a (forward: 5'-GGTAACCAAATCGGTGCTGCTTTC-3'), Bt2b (reverse: 5'-ACCCTCAGTGTAGTGACCCTTGGC-3' (Glass & Donaldson, 1995). For the identification of bacteria the primer pair 27F (forward: 5'-AGAGTTTGATCCTGGCTCAG-3') (Lane et al., 1991) and 534R (reverse: 5'-ATTACCGCGGCTGCTGG-3') (Watanabe et al., 2001) were used to amplify three different hypervariable regions (V1–V3) of the bacterial 16S rRNA gene. For bacteria of the genus *Pseudomonas* we additionally amplified six extra hypervariable regions (V4–V9) of the 16S rRNA gene, for a more reliable identification. For this, two primer pairs were used, namely 341F (forward: 5'-CCTACGGGNGGCWGCAG-3') and 785R (reverse: 5'-GACTACHVGGGTATCTAATCC-3') (that amplifies V3-V4), and 515F (forward: 5'-GTGCCAGCMGCCGCGGTAA-3') and 1492R (reverse: 5'-TACGGYTACCTTGTTACGACTT-3') (that amplifies V4-V9) (Abellan-Schneyder et al., 2021). The reaction mixture for the negative (containing only sterile distilled water) and the positive (containing known DNA) samples, was performed for a final volume of 20 µL containing 0.4 µL of each primer (10 µM), 0.4 µL dNTP's (10 mM), 2 µL complete buffer (10X), 0.1 µL DFS-Taq polymerase (5 units/µL; BIORON GmbH), 14.7 µL sterile distilled water and 2 µL of DNA or sterile distilled water (negative control). Cycling conditions used for fungal identification when using ITS1 and ITS4 primers included an initial denaturation at 95°C for 5 min, 34 cycles of 95°C for 40 sec, 54°C for 40 sec and 72°C for 45 sec, followed by a final extension step at 72°C for 7 min. The PCR program, when using Bt2a and Bt2b primers, included an initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec, followed by a final extension step at 72°C for 7 min. The PCR program for bacterial samples included an initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 40 sec, annealing at 54°C for 50 sec and 72°C for 35 sec, followed by a final extension step at 72°C for 7 min.

The quality of the amplified DNA samples was determined by electrophoresis in BioRad horizontal vials using 1.25% (w/v) agarose gels in TAE buffer 1X. The molecular marker 1Kb (Promega DNA Ladder) and DNA samples were added to the gel wells, separated at 100 V, and stained for 30 minutes with GelRedTM (BIOTIUM 3X). Fluorescence under ultraviolet radiation was used to visualize DNA using a ChemiDocTMXRS transilluminator (BioRad).

The amplified products were purified and sequenced using the services of *Macrogen* Inc. (Madrid, Spain). The isolates were identified using the Nucleotide Basic Local Alignment Search Tool (BLASTn) in the NCBI database (www.ncbi.nlm.nih.gov). The DNA sequences were further examined using MEGA11.Ink software. The results were arranged in one of three categories based on query coverage of ≥ 80% and identity of ≥ 96-100% for classification up to species name: (i) species identification for sequence similarity of 100%; (ii) possibly this species (suffix cf.) for sequence similarity of 99%; and (iii) taxonomically closely related (suffix aff.) for sequence similarity of 96–98%. Classification at genus level was adopted when equal BLAST top score similarity values (ranging from 96 to 100%) were obtained for different species of the same genus or BLAST top score inferior to 96%, but with several species belonging to the same genus. Classification at higher levels was adopted when BLAST top score similarity values were < 96% for several bacteria/fungi. Each fungal and bacterial operational taxonomic units (OTUs) were classified according to Index Fungorum Database (www.indexfungorum.org) and the SILVA High Quality Ribosomal RNA Database (www.arb-silva.de), respectively. These strains were further deposited in the microbial culture collection of the Mountain Research Centre (CIMO- CC), and preserved at -80°C in 30% (v/v) glycerol.

2.4 Evaluation of biochemical features associated to plant growth promotion

Several biochemical features, in particular those associated with plant growth promotion, were evaluated. These included plant hormones synthesis, namely indole acetic acid (IAA), and availability of soil nutrients, namely phosphate solubilization. All the cultivable fungal and bacterial strains were checked for these features.

The inoculum used in these assays were obtained from fungi and bacteria cultivated in PDA and Luria Broth (LB) media (10g NaCl, 10g Tryptone and 5g yeast extract for 1L), respectively. The fungal inoculums were obtained by removing mycelia plugs (5 mm diameter) from the fungal cultures grown in PDA, at 25ºc, in the dark. Bacterial inoculums were prepared by transferring bacterial cells (2-days-old cultures grown in LBA) onto 15 mL liquid LB medium and shaken on a rotary shaker (100 rpm) for 24h at 37ºC. Bacterial cell densities were adjusted to an optical density at 600 nm (OD_{600}=0.5), corresponding to a concentration of 10^8

CFU/mL

IAA production by both fungal and bacterial strains was estimated in Potato Dextrose Broth (PDB) and LB medium, respectively, supplemented with L-tryptophan, which is the main precursor for the synthesis of IAA (Spaepen & Vanderleyden, 2011). Accordingly, in the fungal assays, 20 mL of PDB supplemented with 1g/L of L-tryptophan was inoculated with two mycelia plugs (5 mm diameter) removed from fungal cultures. In the bacterial assays, 50 µL of bacterial cell culture (OD_{600}=0.5) were transferred to 20 mL of PDB supplemented with 1g/L of L-tryptophan. After incubation at room temperature, for one week (for fungi) or 24 h (for bacteria) with shaking (100 rpm), the cultures were filtrated using a Whatman® filter paper. The production of IAA was measured by mixing 1mL of culture supernatant with 1mL of Salkowski's reagent (49mL 35% $HClO_4$, 1mL 0.5M $FeCl_3$) in 2 mL tubes. The tubes containing the mixture were incubated in the dark, at 30°C with agitation (250 rpm), for 30 min, for color development. The intensity of color was measured in a VWR®732-2535 MySPEC spectrophotometer at 530 nm using a standard curve for calibration by using authentic IAA (concentration ranging from 0 to 20 µg/mL with R^2=0.993). Three replicates per fungal/bacterial isolates were performed, being the results expressed in µg/mL.

The ability of the fungal/bacterial isolates to solubilize phosphate was assessed by using the modified Pikovskaya agar (PVK) medium containing calcium triphosphate as insoluble phosphate source (Doilom, et al., 2020). Accordingly, one mycelia plug (5 mm diameter) was removed from fungal cultures or 10 µL of bacterial inoculums (OD_{600}=0.5), was placed onto the surface of PVK medium in the center of 9 cm-Petri dishes. The plates were incubated at room temperature in the dark, and after 24h (for bacteria) or one week (for fungi), the halos that appeared around the colonies were measured with a ruler (in mm). For each microbial strain, three replicates were carried out. The results are presented as halo diameter (in mm) by subtraction colony diameter.

2.5 Data analysis

The diversity of fungal and bacterial communities in different types of soil management (tilled vs. not-tilled), planting cover crop (seeded vs. not-seeded) and soil sampling date (1st vs. 2nd soil sampling date) was estimated using abundance (log CFU/g) species richness (total number of OTUs) and by determining the Shannon-Wiener diversity index in Past4.11 software. The results are shown in box plots with the corresponding medians and quartiles. Using R version 4.1.1 (R Studio2021), one-way ANOVA was used to examine if there were significant differences in microbial diversity, abundance and richness across the three different factors (tillage, seeding and sampling time). Normality of data was checked by using Shapiro-Wilk test.

The overall microbial community of the rhizospheric soil surveyed was presented at OTU level via a Krona chart to give a general overview on the fungal and bacterial composition.

Non-metric multidimensional scaling (NMDS) was carried out to determine the similarity in fungal/bacterial community composition among types of soil management (tilled vs. not-tilled), planting cover crop (seeded vs. not-seeded) and soil sampling date (1st vs. 2nd soil sampling date). This analysis was performed by using the Bray-Curtis coefficient, that takes into account not only the presence/absence of microbial species but also their abundance (Clark, 1993). Kruskal's stress was used to estimate goodness of fit (commonly acceptable when <0.2). Significant differences on microbial community composition were tested by one-way analysis of similarity (ANOSIM) using the Bray-Curtis distance matrices, with 999 permutations. ANOSIM gives an R-value ranging from 0 (similar) to 1 (being completely dissimilar) and a p-value (significant level below to 0.05) (Clarke & Gorley, 2015). Similarity percentages (SIMPER) analysis was used to identify the OTU that contributed up to 50% to the discrimination among types of soil management, planting cover crop and soil sampling date. NMDS, ANOSIM and SIMPER analysis were performed using the Community Analysis Package v. 4.0 (Henderson & Seaby, 2019).

Finally, a permutational multivariate analysis of variance (PERMANOVA) was used to test if the type of soil management, planting cover crop and soil sampling date, play major roles in structuring rhizospheric microbial communities. This analysis was performed in R version 4.1.1 (R Studio 2021), using the *Adonis* function of the package vegan (Oksanen et al., 2018), with 999 permutations.

Chapter III. Results

3.1 Overall rhizospheric microbial community in olive orchards

Overall, the taxonomic diversity of rhizospheric fungal community of the olive orchard (40 OTUs, 24 genera, 20 families, 16 orders, 9 classes, and 3 phyla) was greater to that of bacteria (39 OTUs, 12 genera, 12 families, 8 orders, 6 classes, and 5 phyla). Most of the fungal isolates belonged to Ascomycota phylum (67% of the total of fungal isolates), and genus *Penicillium* (20%) (Figure 5A). The second most abundant phylum was Basidiomycota (22% of the total fungal isolates), being the genera *Naganishia* (5%) and *Holtermanniella* (5%) the most dominant within this phylum. The bacterial communities were predominantly composed by members of the phylum *Proteobacteria* (85% of the total bacterial isolates) and genus *Pseudomonas* (50%) (Figure 5B). Interestingly, about 3 OTUs of the bacterial isolates and 3 OTUs of the fungal isolates do not match with any reference sequence in the databases.

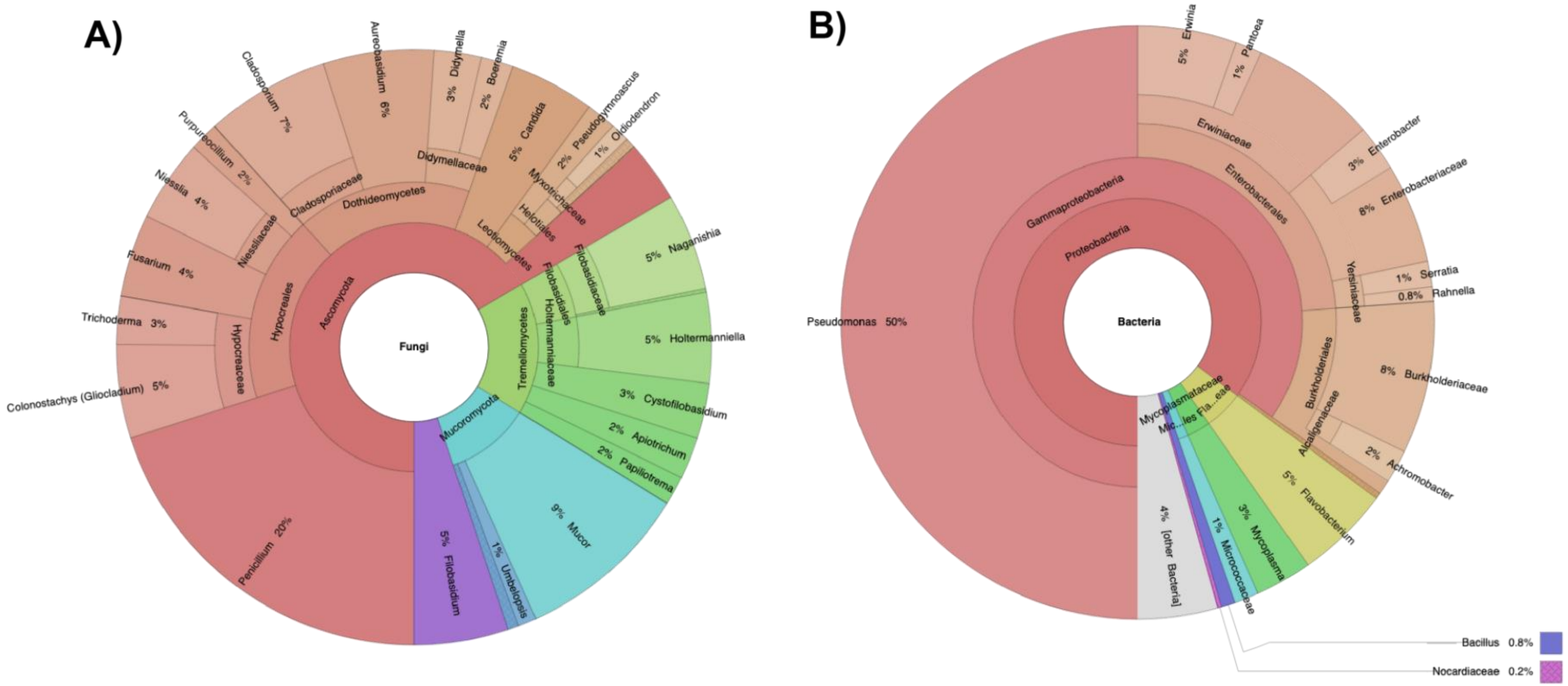


Figure 5. Krona chart showing the percentage of each operational taxonomic unit of fungal (A) and bacterial (B) of the overall rhizospheric soil communities. This chart was constructed using Krona tool (Ondov et al., 2011).

3.2 Variation in microbial diversity and composition

3.2.1 Diversity

The diversity of the rhizospheric fungal community estimated on the basis of richness, Shannon index and abundance, showed no significant differences between seeded and not seeded soils or between 1st and 2nd sampling dates (Figure 6). However, the comparison between tilled and not tilled soils showed that fungal richness, diversity and abundance were significantly higher up to 1.5-fold ($p<0.01$), 1.2-fold and 1.6-fold ($p<0.001$) in not tilled soils than in tilled soils, respectively.

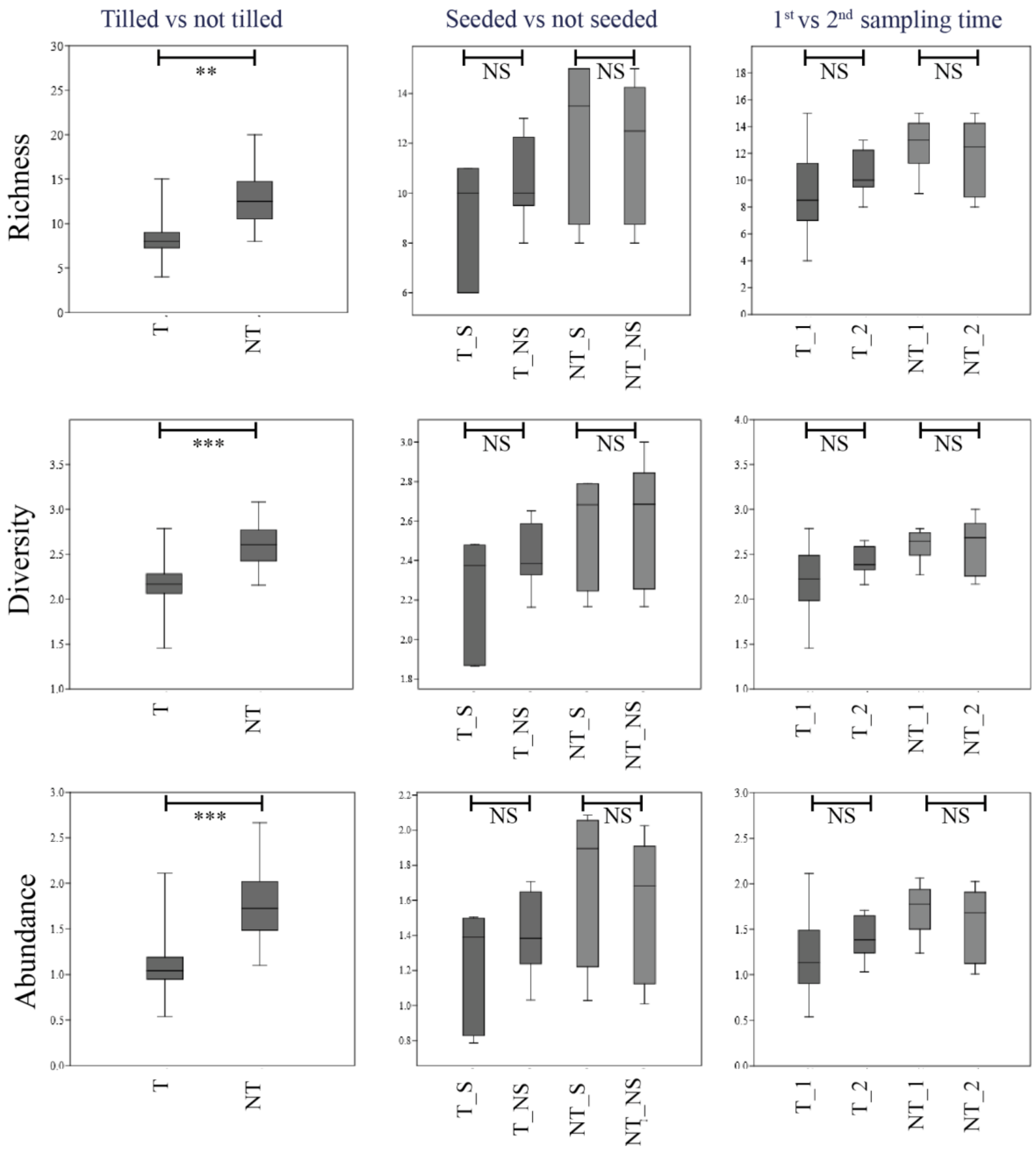


Figure 6. Comparison of fungal richness (nº of OTUs), diversity (Shannon index) and abundance ($\log_{10}$ CFU/g) between the types of soil management (tilled-T vs. not tilled-NT), planting cover crop (seeded-S vs. not seeded-NS, in each type of soil management) and soil sampling date (1st vs. 2nd soil sampling date, in each type of soil management). Box plots depict medians (central horizontal lines), the inter-quartile ranges (boxes), and 95% confidence intervals (whiskers). The letters NS denotes no significant difference among the two variables, while the **, *** denotes respectively significant differences with $p<0.01$ and $p<0.001$.

The bacterial richness, diversity (Shannon index) and abundance did not differ significantly among tilled and not tilled or seeded and not seeded soils (Figure 7). However, there was a significant difference between the two sampling dates in not tilled soils (p<0.01), being the richness, diversity and abundance higher up to 1.7-fold, 1.2-fold and 1.7-fold in the 2nd sampling date when compared to the first date, respectively.

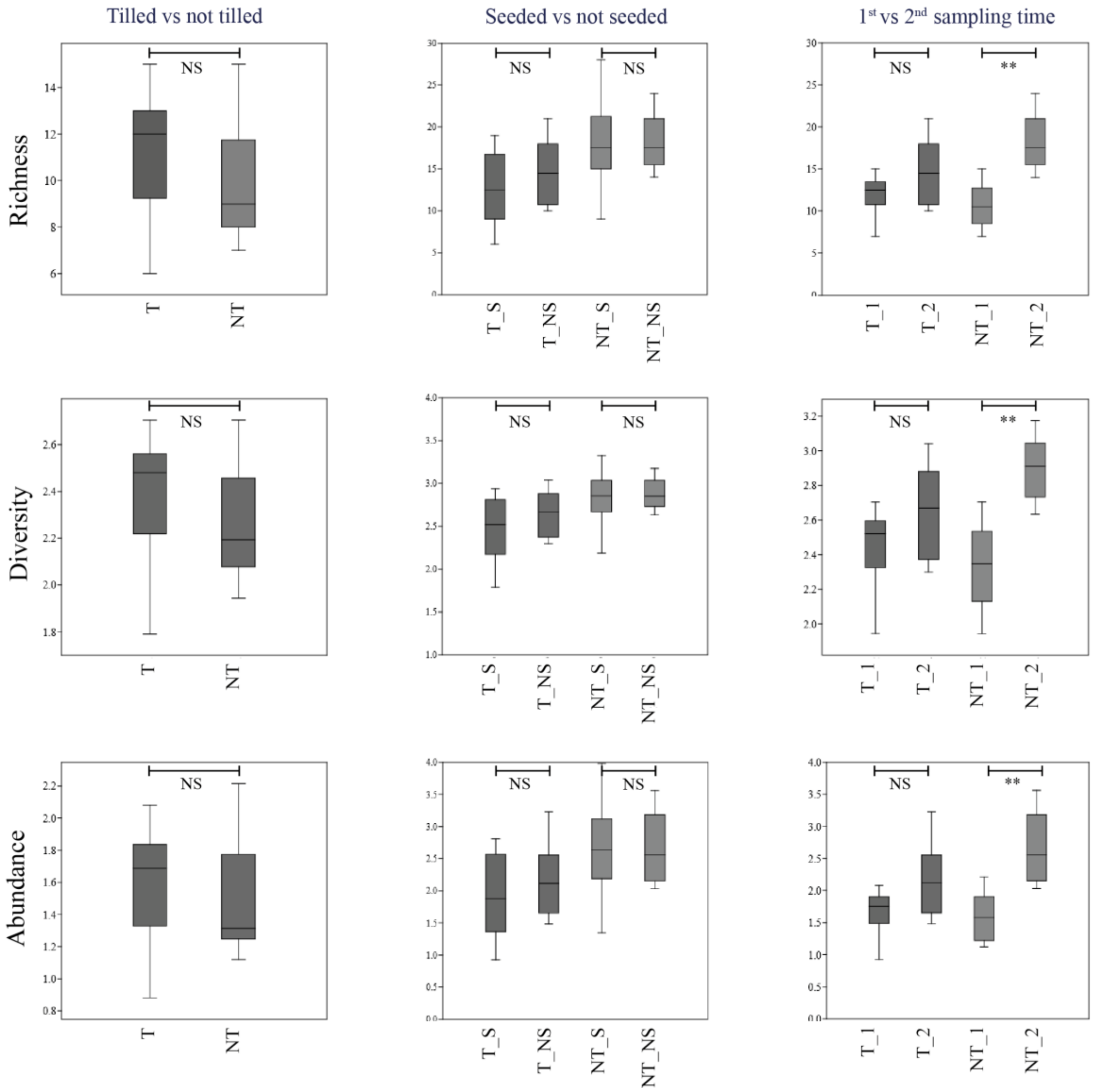


Figure 7. Comparison of bacterial richness (nº of OTUs), diversity (Shannon index) and abundance ($\log_{10}$ CFU/g) between the types of soil management (tilled-T vs. not tilled-NT), planting cover crop (seeded-S vs. not seeded-NS, in each type of soil management) and soil sampling date (1st vs. 2nd soil sampling date, in each type of soil management). Box plots depict medians (central horizontal lines), the inter-quartile ranges (boxes), and 95% confidence intervals (whiskers). The letters NS denotes no significant difference among the two variables, while the **, *** denotes respectively significant differences with $p<0.01$ and $p<0.001$.

3.2.2 Composition

The NMDS plots (Figure 8) and ANOSIM analysis (Table S1) based on Bray-Curtis similarity index, indicated that the whole fungal community composition differs significantly among tilled and not tilled soils (global R=0.361, p=0.001), and mostly between sampling dates (global R=0.591, p=0.001). Interestingly, differences between the 1st and 2nd soil sampling date were greater in not tilled soils (R=0.807, p=0.001) than in tilled soils (R=0.378, p=0.014). In contrast, the overall fungal community composition did not change significantly (global R=0.006, p=0.545) among seeded and not seeded soils. But, at the level of tilled soils (R= 0.269, p=0.021), and in a lesser extent in not tilled soils (R=0.225, p=0.04), significant differences were observed between the two types of planting cover crops. PERMANOVA analysis (Table S2) corroborates these results, by showing that the fungal communities composition was significantly affected by the type of soil management (p<0.01) and the soil sampling date (p<0.01), particularly in not tilled soils (p<0.001), explaining 11.8% and 34.5% of composition variation, respectively. The type of planting cover crop only affects significantly (p<0.01) the fungal community in tilled soils, explaining 20.6% of composition variation.

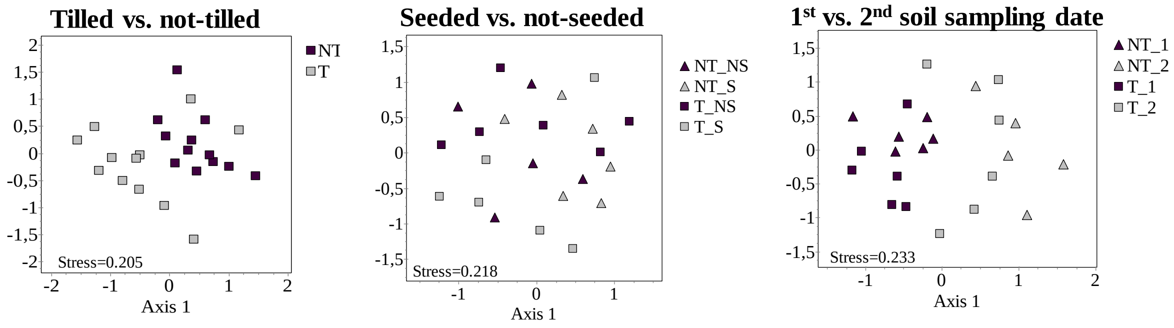


Figure 8. Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of rhizosphere fungal communities grouped by type of soil management (tilled-T vs. not-tilled-NT), planting cover crop (seeded-S vs. not-seeded-NS, in each type of soil management) and soil sampling date (1st vs. 2nd soil sampling date, in each type of soil management). Cluster analysis was performed with Bray-Curtis coefficient. Kruskal's stress values of less than 0.2 represent good ordination plots

The comparison of the fungal community composition among different types of soil management, planting cover crops or soil sampling dates, reveals differences in the abundance of specific genera/family (Figure 9; Figure S1). For example, Mortierellaceae was detected only in tilled soils, while both *Pseudogymnoascus* and *Solicoccozyma* were exclusively present in not tilled soils. Compared to not seeded soils, seeded soils showed an increased on abundance of *Botrytis* (log$_2$ fold change up to 8.6) and a decreased on *Oidiodendron* (log$_2$ fold change up to -10.1). Curiously, this result was observed in soils previously tilled, being observed an opposite trend in not tilled soils. In the 2nd sampling date, some fungal members were increased (e.g. *Botrytis* with a $\log_2$ fold change up to 8.6) or were detected for the first time (e.g., *Fusarium*, *Mortierellaceae* and *Purpureocillium*) in relation to the 1st sampling date, particularly in tilled soils. In not tilled soils, both *Solicoccozyma* and *Umbelopsis* were detected exclusively on the 2nd sampling date.

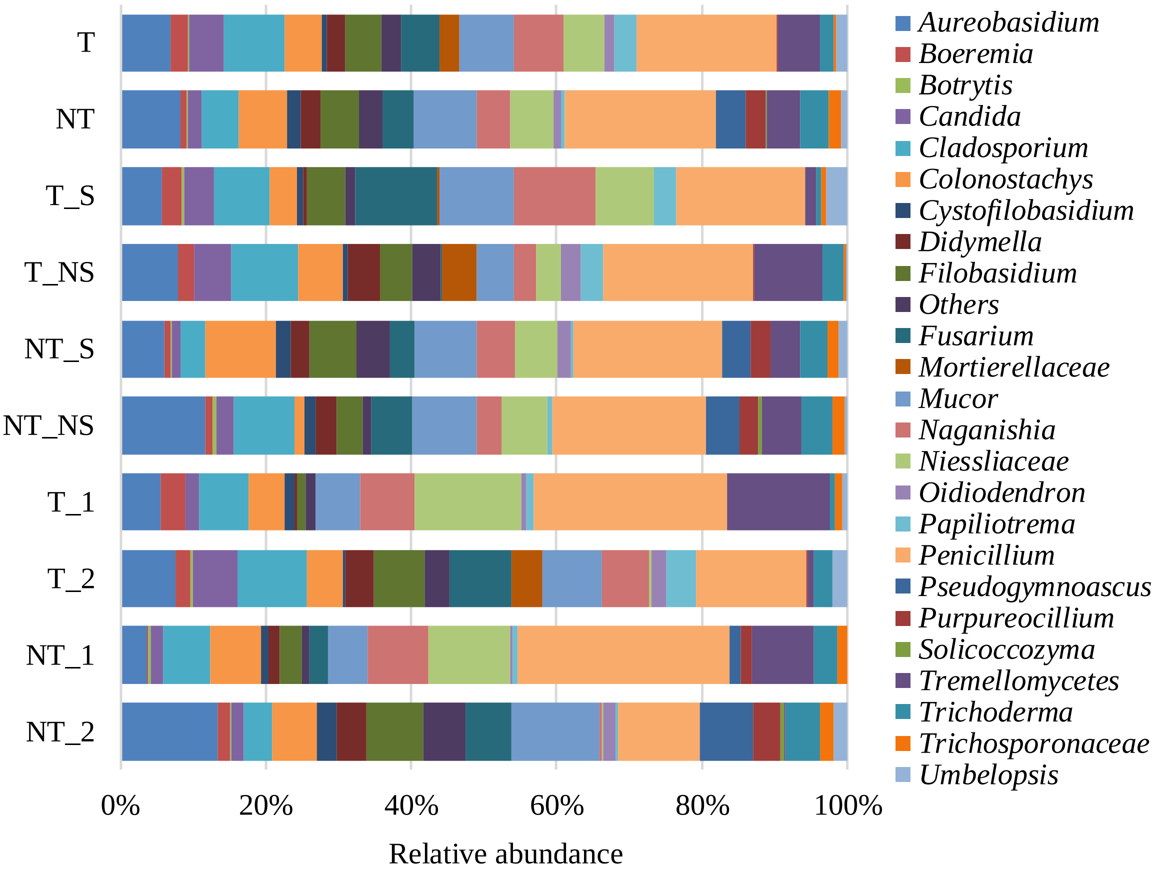


Figure 9. Relative abundance of fungi (at genus level) inhabiting rhizospheric soils in different types of soil management (tilled-T vs. not-tilled-NT), planting cover crop (seeded-S vs. not-seeded-NS, in each type of soil management) and soil sampling date (1st vs. 2nd soil sampling date, in each type of soil management).

At a lower taxonomic level, the SIMPER analysis also identified a set of fungal OTUs that explain differences among the variables studied. For example, *Penicillium* sp.1, together with *Niesslia* sp.1 and *Holtermanniella*, made the highest cumulative contribution towards differences between tilled and not tilled soils, accounting for around 30% of the observed value of dissimilarity (Table 3). These three OTUs were also the most important in distinguished fungal communities of the 1st and 2nd sampling date. *Naganishia*, *Cladosporium*, *Fusarium* *oxysporum* and *Aureobasidium pullulans* contributed the most to the differences on fungal composition between seeded and not seeded soils.

Table 3. SIMPER analysis identifying the % contribution (up to 50%) of each rhizosphere fungal OTUs to the Bray Curtis dissimilarity index between tilled and not-tilled, seeded and not-seeded, or 1st and 2nd soil sampling date.

		OTU	% Contribution
Fungi			
Tilled vs. not-tilled		*Penicillium* sp.1	11.6
		Niesslia sp.	9.51
		Holtermanniella	8.8
		Penicillium sp.4	6.8
		Naganishia	6.5
		Colonostachys rosea	5.8
		Cladosporium	5.7
Seeded vs. not-seeded	Tilled	*Naganishia*	8.6
		Cladosporium	8.2
		Fusarium oxysporum	7.0
		Colonostachys rosea	4.5
		Aureobasidium pullulans.1	4.4
		Candida membranifaciens	4.3
		Mortierella lignicola	4.2
		Filobasidium wieringae	4.1
		Penicillium raperi	4.1
		Didymella	3.9
	Not-tilled	*Aureobasidium pullulans.2*	7.1
		Colonostachys rosea	6.8
		Mucor hiemalis	6.3
		Pseudogymnoascus	6.0
		Aureobasidium pullulans.1	5.7
		Filobasidium oeirense	5.6
		Fungus sp.6	5.4
		Trichoderma	4.5
		Didymella	3.9
1st vs. 2nd soil sampling date	Tilled	*Penicillium* sp.1	7.9
		Holtermanniella	7.1
		Cladosporium	6.8
		Colonostachys rosea	6.5
		Penicillium raperi	5.2
		Didymella	5.1
		Mortierella lignicola	5.0
		Candida membranifaciens	4.5
		Penicillium sp.2	4.2
	Not-tilled	*Penicillium* sp.1	10.0
		Niesslia sp.	8.0
		Penicillium sp.4	7.7
		Aureobasidium pullulans.1	7.4
		Aureobasidium pullulans.2	6.0
		Holtermanniella	6.0
		Cladosporium	5.6

In what regards rhizospheric bacterial communities, the NMDS plots (Figure 10) and ANOSIM analysis (Table S1) based on the Bray-Curtis similarity index demonstrated that the composition of the entire bacterial population differed significantly between tilled and not tilled soils (global R=0.335, p=0.001), as well as between the 1st and the 2nd sampling times (global R=0.199, p=0.003), especially in tilled soils (R=0.667, p=0.002). In contrast, the bacterial community composition was very similar in seeded and not seeded soils, whether tilled or not (global R=0.025, p=0.666). The results of PERMANOVA (Table S2), revealed that the sampling date was the only factor that significantly influenced the bacterial composition, accounting for 21.1% and 35.2% of the community variation, in tilled and not tilled soils, respectively.

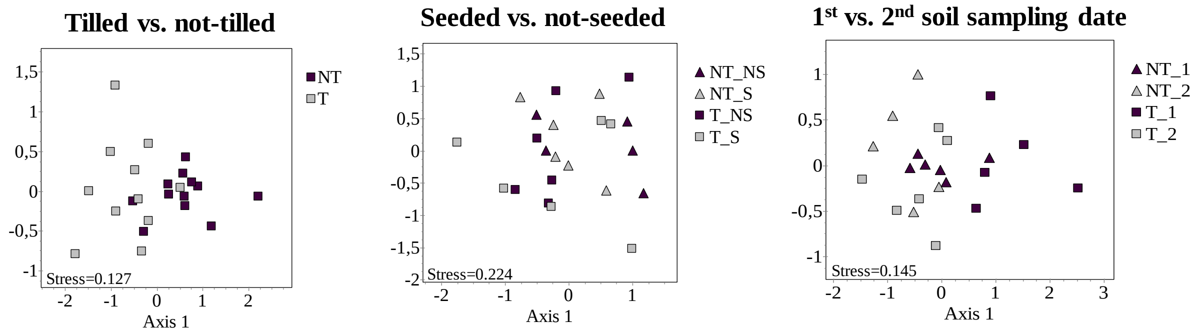


Figure 10. Non-metric multidimensional scale (NMDS) plots corresponding to the clustering of rhizosphere bacterial communities grouped by type of soil management (tilled-T vs. not-tilled-NT), planting cover crop (seeded-S vs. not-seeded-NS, in each type of soil management) and soil sampling date (1st vs. 2nd soil sampling date, in each type of soil management). Cluster analysis was performed with Bray-Curtis coefficient. Kruskal's stress values of less than 0.2 represent good ordination plots.

The comparison of the bacterial community among different types of soil management, reveals differences in the presence/absence of specific genera (Figure 11; Figure S2). For example, *Janthinobacterium* was detected only in tilled soils, while *Rhodococcus* was exclusively present in not tilled soils. Despite the lack of significant differences in ANOSIM analysis (Table S1) on bacterial composition among seeded and not seeded soils, seeded soils showed the exclusive presence of *Janthinobacterium*, *Pantoea* and *Rahnella*, in soils previously tilled, or Burkholderiales, *Rhodococcus* and *Serratia* in not tilled soils (Figure 11; Figure S2). Micrococcaceae and *Bacillus* were uniquely detected in not seeded soils, and previously tilled. Differences on bacterial composition among the two sampling dates, were mostly due to the high number of genera that occurred exclusively in the 2nd sampling date (*Achromobacter*, *Bacillus*, *Enterobacter*, Micrococcaceae, *Rahnella*, Rhodococcus and *Serratia*) (Figure 11).

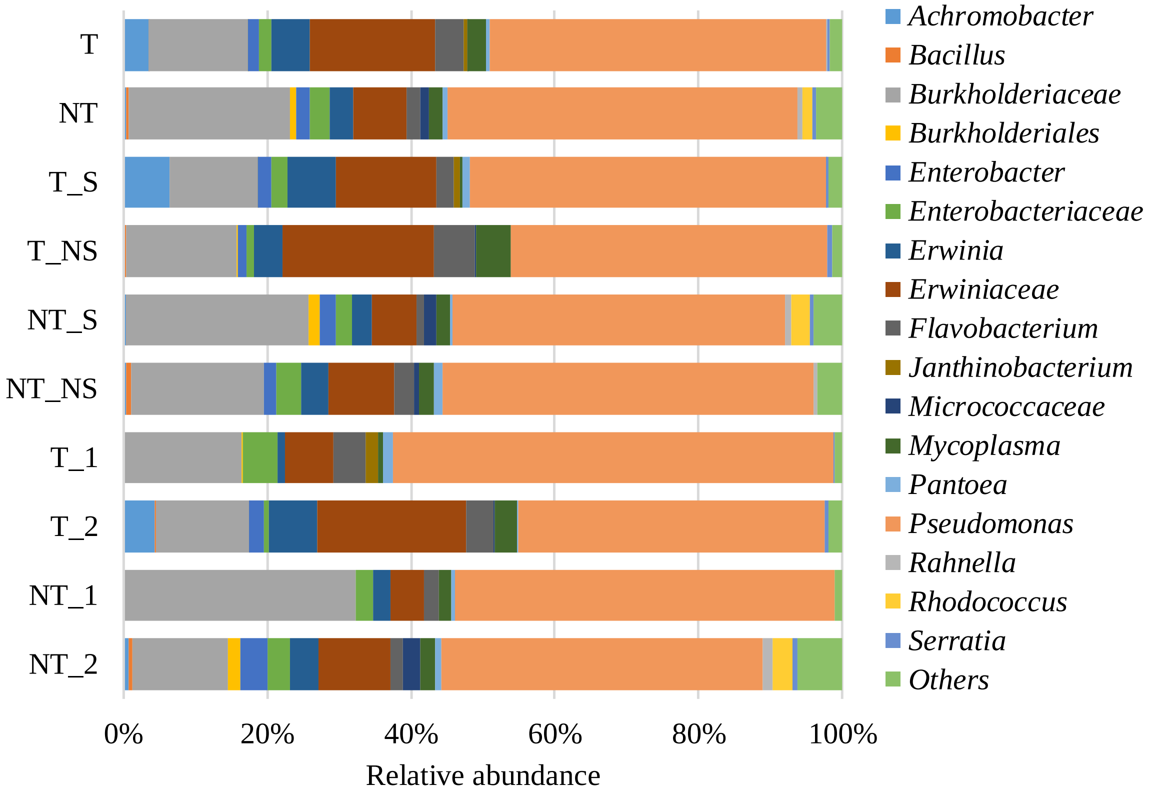


Figure 11. Relative abundance of bacteria (at genus level) inhabiting rhizospheric soils in different types of soil management (tilled-T vs. not-tilled-NT), planting cover crop (seeded-S vs. not-seeded-NS, in each type of soil management) and soil sampling date (1st vs. 2nd soil sampling date, in each type of soil management).

At lower taxonomic level, the SIMPER analysis also identified a set of bacterial OTUs that explain differences among tilled and not tilled soils (Table 4). Indeed, *Burkholderiaceae, Pseudomonas* sp.14 and *Pseudomonas* sp.10, contributed together with around 55% of the dissimilarity on bacterial composition between tilled and not tilled soils (Table 4. *Pseudomonas* sp.14, *Erwiniaceae* and *Burkholderiaceae* were identified as having the greatest effect on dissimilarity on bacterial composition (around 48% in tilled soils and 46% in not tilled soils) between the two sampling dates (Table 4).

Table 4. SIMPER analysis identifying the % contribution (up to 50%) of each rhizosphere bacterial OTUs to the Bray Curtis dissimilarity index between tilled and not-tilled, seeded and not-seeded, or 1st and 2nd soil sampling date.

		OTU	% Contribution
Bacteria			
Tilled vs. not-tilled		*Burkholderiaceae*	24.8
		Pseudomonas sp.14	20.3
		Pseudomonas sp.10	9.7
1st vs. 2nd soil sampling date	Tilled	*Pseudomonas* sp.14	18.4
		Erwiniaceae	16.1
		Burkholderiaceae	13.6
		Flavobacterium	5.5
	Not-tilled	*Pseudomonas* sp.14	25.8
		Burkholderiaceae	11.2
		Erwiniaceae	8.6
		Pseudomonas sp.10	6.4

3.3 Biochemical features analysis of isolates

In this work, a total of 30 fungal strains and 34 bacterial strains from rhizospheric soil samples were screened for their ability to produce plant hormones, namely indole acetic acid (IAA), and to solubilize phosphate.

3.3.1 Indole acetic acid production

The amount of IAA produced by fungi varied significantly ($p < 0.001$) among the 30 isolates tested ($F_{1.656} = 47.253$). All these fungal isolates showed ability to produced IAA, in amounts varying from 3.05 µg/mL (F10, *Aureobasidium pullulans*) to 17.69 µg/mL (F25, *Mucor circinelloides*) (Figure 12A). Around 40% of the tested fungal isolates (12 strains) produced more than 10 µg/mL of IAA. Likewise, significant differences ($p < 0.001$) were found on IAA production among the 34 bacterial strains tested ($F_{1.607} = 55.731$). All these bacterial isolates produced IAA, in quantities ranging from a minimum of 0.79 µg/mL (M14’, *Pseudomonas turukhanskensis*) to a maximum of 29.20 µg/mL (M14, *Pseudomonas* sp.12) (Figure 12B). The number of bacterial strains that produced more than 10 µg/mL of IAA was higher (around 56% of the total bacterial strains tested) in comparison to fungal strains. Interestingly, five bacterial strains produced more than 25 µg/mL of IAA, namely 2M13 (*Pseudomonas* sp.16), 2M8 (*Bacillus cereus*), M2 (*Enterobacteriaceae*) and M8' (*Burkholderiaceae*).

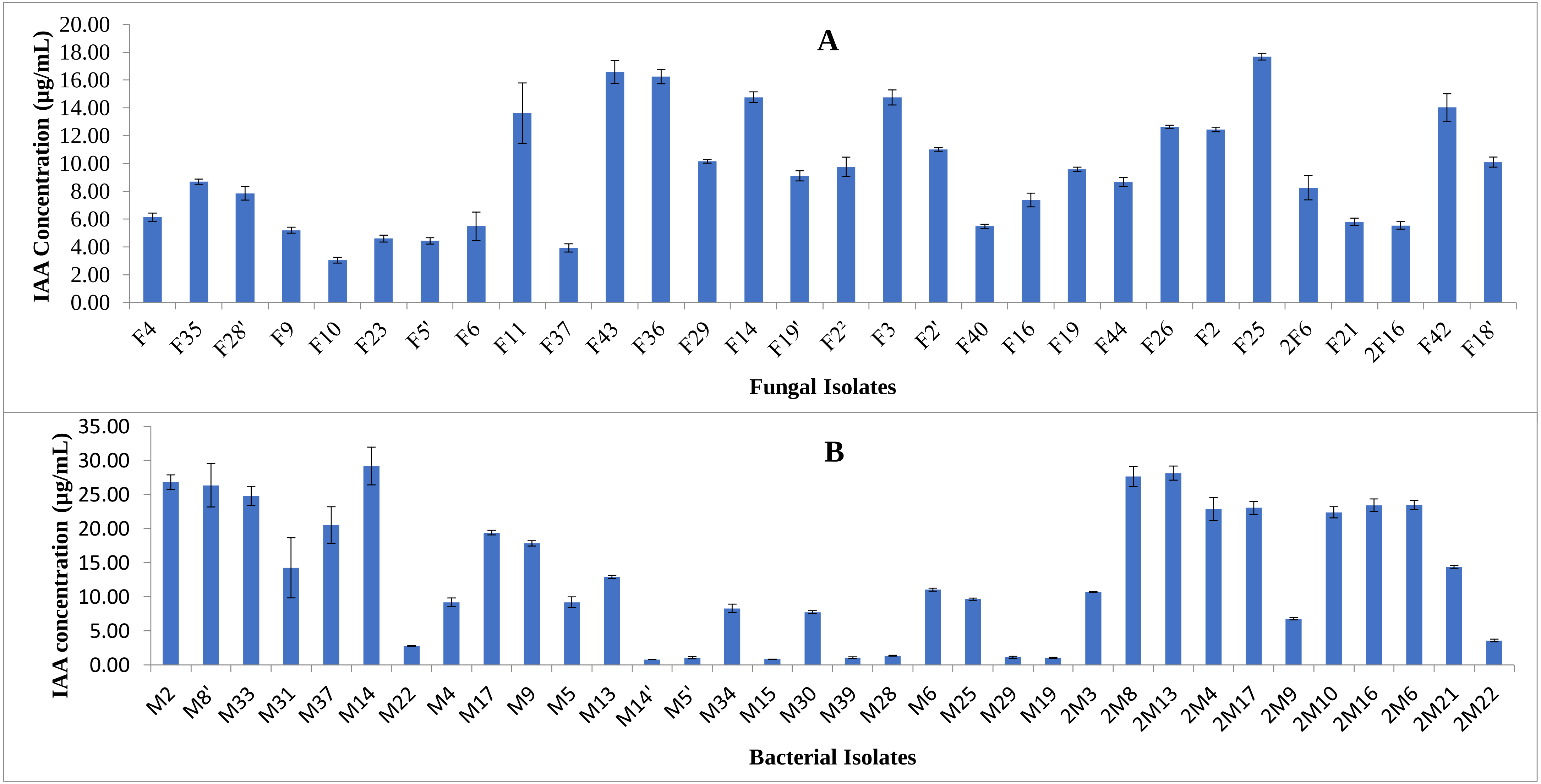


Figure 12. Quantity of indol acetic acid (IAA) in µL/mL produced by the different fungal (A) or bacterial (B) rhizospheric soil isolates. Each bar represents the mean (N = 5) ± standard error. Each fungal/bacterial code corresponds to a particular OTU, as indicated in Table S3.

3.3.2 Phosphate solubilization

Screening of phosphate solubilization ability of the 30 fungal isolates resulted in the identification of 18 isolates (Figure 13A) showing well-developed halo zones (Figure 14) after 7 days of incubation in PVK medium. The solubilization of phosphate varied significantly ($p<0.001$) among these isolates ($F_{2.232} = 31.774$), showing halo diameters ranging from 0.1 mm (F9, *Niesslia* sp.) to 7.32 mm (F5', *Solicoccozyma phenolica*) (Figure 13A). Five isolates showed halos with more than 4 mm of diameter, namely F21 (*Oidiodendron* sp.), F6 (*Naganishia* sp.), F42 (*Pseudogymnoascus* sp.) and 2F16 (*Pseudogymnoascus pannorum*), and 13 isolates produced halos with less than 4 mm of diameter.

For bacteria, around 85% of the 34 isolates tested presented phosphate solubilizing proprieties (Figure 13B; Figure 14). The diameter of the halo produced by these bacteria varied significantly among isolates ($p<0.0$; $F_{1,670} = 19.772$), ranging from 0.2 mm (M5, *Pseudomonas* sp.4) to 9.95 mm (2M3, *Rahnella* sp.). Four isolates had halo diameters higher than 4 mm, namely 2M6 (*Micrococcaceae*), M19 (*Erwinia* sp.1) and M29 (*Pseudomonas* sp.5), and 25 isolates had halo diameters of less than 4 mm.

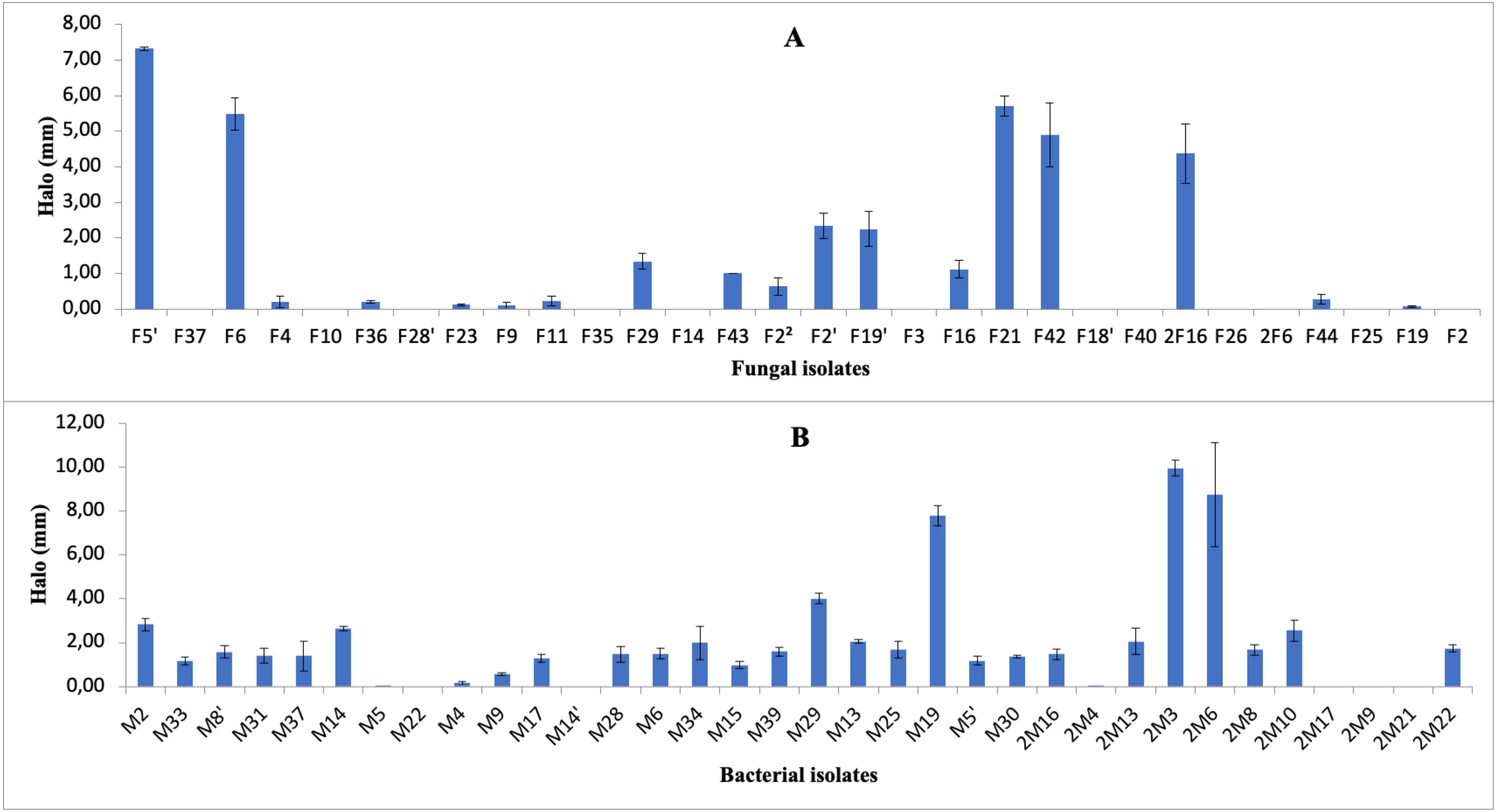


Figure 13. Phosphate solubilization halo diameter in mm produced by the different fungal (A) or bacterial (B) rhizospheric soil isolates. Each bar represents the mean (N = 3 ± standard error. Each fungal/bacterial code corresponds to a particular OTU, as indicated in Table S3.

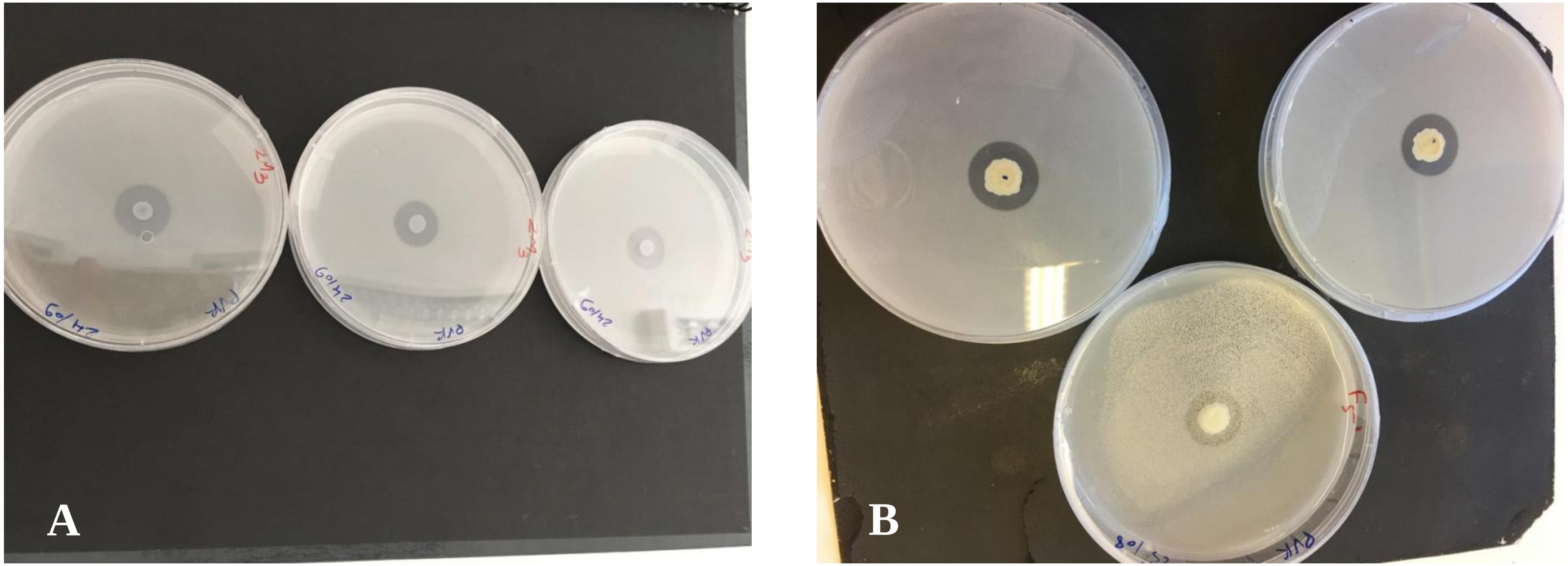


Figure 14. Representative Petri plates showing phosphate solubilization halos by the tested fungal (A) or bacterial (B) isolates in Pikovskaya agar (PVK) medium containing calcium triphosphate.

Chapter IV. Discussion

4.1 Overall microbial structure

In the recent decade, an increasing number of olive farmers have been aware of the problems related with soil erosion. Indeed, soil erosion is one of the most serious environmental problems associated with olive farming, due to the intense tilling, the use of herbicides/pesticides and the lack of vegetation cover (Dondini, 2011). It is therefore of primary importance to act in order to improve or restore soil health of olive groves. According to Prosdocimi et al. (2016), this can be achieved by a reduction in tillage and greater use of a vegetative cover, as well as an increase in grazing livestock, which can reduce the risk of soil erosion. However, little is known about the impact of these practices on the microbial communities of soils, and particularly of the rhizospheric soils. Therefore, in this work, the impact of tillage and planting cover crop on the diversity and composition of fungal and bacterial communities inhabiting the olive tree rhizospheric soils was evaluated by using a culturing approach. Overall, the results showed great diversity of both fungal (40 OTUs) and bacterial (39 OTUs) communities, which was higher than reported previously in rhizhosperic soils estimated using the culturing approach (Montes-Borregos et al.,. 2014). In our study, the microbial communities were dominated by members of the phyla Ascomycota and Proteobacteria, which is consistent with previous studies conducted on rhizospheric soils (Gonzalez et al., 2020). The presence of Proteobacteria in the soil is an indicator of high soil nutrient-content, as most inhabit nutrient-rich soils (Fierer et al., 2012; Delmont et al., 2018). *Penicillium* and *Pseudomonas* were the dominant genera found on the rhizospheric soil samples surveyed in our study, as previously reported in other studies aiming to assess microbial communities in rhizosphere soil (Zhang et al., 2014). Some members of the *Penicillium* genus have been reported to produce solubilized phosphorus, siderophores, and phytohormones, including indole acetic acid and gibberellic acid, which are important for plant growth and health (Berg et al., 2005; Elias et al., 2016; Altaf et al.,. 2018). Similarly, the *Pseudomonas* genus comprises species with a recognized ability to produce plant growth-promoting substances such as auxins, cytokinins, and enzymes, which stimulate root development, nutrient absorption, and overall plant performance (Singh et al., 2022). Additionally, some *Pseudomonas* species are known for their ability to suppress plant pathogens through the production of antimicrobial compounds and induction of systemic resistance in plants (Rifat et al., 2010). The dominance of *Penicillium* and *Pseudomonas* genera in the rhizospheric soil samples surveyed in our study aligns with their recognized role as beneficial microorganisms in promoting plant growth and health (Glick, 2012; De Faria et al., 2021).

Overall, our results showed that tillage exerted a stronger effect on the composition of the fungal community inhabiting the rhizospheric soil of olive tree than on bacterial community, which is in accordance with other studies performed in soils (Sun et al., 2018; Shanmugam et al.,. 2021). Indeed, tillage showed to significantly reduce the richness, diversity and abundance of fungi, while the diversity of bacterial community remains unchanged in comparison to not tilled soils. As tillage is known to disrupt the soil environment (De Faria et al.,. 2021), and our results suggest that bacteria seem to adapt more easily to these changes than fungi. This greater adaptation of bacteria might be due for their wider range of metabolic and nutritional strategies (Li et al., 2021) when compared to fungi. However, fungi may build more permanent mycelia networks in the absence of soil structure disruption activities (e.g., no tillage), allowing them to colonize and endure in the soil (Lombardo et al., 2019; Gonzalez, et al., 2020).

The high fungal diversity and abundance in not tilled soils could be an important benefit for host plant, as fungi are able to supply roots with mineral nutrients and phytohormones, protect host plants from root pathogens and maintain soil health (Sofo et al., 2014). In our study, both *Pseudogymnoascus* and *Solicoccozyma* were found exclusively in not tilled soils. Members of the genus *Pseudogymnoascus* are usually involved in cellulose degradation (Sigler et al., 2000), and thus they might be important in fine root turnover and nutrient cycling in olive orchards (Sarabia et al., 2018). *Solicoccozyma* members have been also reported to exhibit ability to promote plant growth (Sarabia et al., 2018) and, curiously, to be present in arid environments (Khan et al., 2020). This suggests that it may possess adaptive properties that enable it to thrive in challenging conditions, such as those often encountered in olive orchards, particularly in regions with limited water availability. Thus there is a possibility that *Solicoccozyma* members in the rhizosphere of olive trees could contribute to improved water and nutrient uptake, leading to enhanced growth, yield, and overall resilience of olive orchards. At lower taxonomic level, *Penicillium* sp.1*, Niesslia* and *Holtermanniella*, were the OTUs that most contributed for the dissimilarity between tilled and not tilled soils. These OTUs were found in highest abundance in not tilled soils and were reported to possess enzymatic activities involved in the degradation of plant cell wall components (Paul et al., 2015). They are also well known in establishing symbiotic relationships with olive trees, which could have positive effects on nutrient acquisition, disease resistance, and overall plant vigor (Chebotar et al., 2017). Although tillage was identified to have a minor effect on the structure of rhizospheric soil bacteria, some genera were detected exclusively in either not tilled (*Rhodococcus*) or tilled soils (*Janthinobacterium*). *Rhodococcus* species have been reported to possess diverse metabolic capabilities, including the ability to degrade complex organic compounds, produce plant growth-promoting substances, and enhance nutrient cycling in the soil (Liu et al., 2018). These characteristics suggest that *Rhodococcus* members in the rhizospheric soil of olive trees may contribute to improving soil fertility, nutrient availability, and overall plant health. Similarly, *Janthinobacterium* species have been associated with several beneficial traits. They have been reported to produce a wide range of antimicrobial compounds, which can contribute to plant protection against pathogens (Inoue al., 2007). Additionally, *Janthinobacterium* has shown the ability to promote plant growth and enhance the tolerance of plants to environmental stresses (Bakker et al., 2018). These properties make *Rhodococcus* and *Janthinobacterium* potential candidates for biocontrol and plant growth-promoting application in olive orchards. Their presence in the rhizospheric soil of olive trees could have positive implications for the health and productivity of olive orchards. These bacteria may contribute to nutrient cycling, disease suppression, and improved plant growth, ultimately leading to enhanced olive tree performance and yield.

In contrast to tillage, planting cover crop did not significantly affect the overall composition of both fungal and bacterial communities in the rhizosphere of olive trees. This finding aligns with several studies conducted in different agricultural systems that have also reported minimal changes in microbial community composition associated with cover crop planting. For instance, Gao et al. (2021) investigated the impact of cover crop cultivation on microbial communities in a Pepper field and they found that while cover crops influenced specific microbial taxa, the overall composition of bacterial communities remained relatively stable. Similarly, Burns et al. (2016) evaluated the effects of cover crop and tillage practices on soil microbial communities in vineyards and they observed no significant differences in bacterial community composition between cover crop and bare soil treatments. In contrast, a series of studies showed that cover cropping may increase soil microbial abundance, activity, and diversity (Kim et al., 2020; Muhammad et al., 2021). These results suggest that the effects of cover crop planting on microbial communities may vary depending on the specific context. Indeed, recent meta-analysis studies were demonstrated that the effect of cover cropping on soil microbiome is dependent on several factors, including climate, termination methods, tillage, and type of plant species used as cover crop (Kim et al., 2020; Muhammad et al., 2021). In our work, it is possible that the short-term duration of the study or other environmental factors could have limited the detectable impact of cover crop on rhizosphere microbial community composition. The establishment and impact of cover crops on microbial communities may require longer periods to be discernible, as suggested by previous studies that have shown time-dependent shifts in microbial composition with cover crop implementation (Adetunji et al., 2020). Further research with long-term assessments is needed to gain a more comprehensive understanding of the effects of cover crops on microbial communities in the rhizosphere of olive tree.

Our results showed that both fungal and bacterial of olive tree rhizosphere exhibit temporal variation, as reported previously in soil samples (Hannula et al., 2019). From the seed sowing in March (1st sampling date) to the 2nd sampling date in June (five months later), the composition of the microbial community, particularly of bacteria, change greatly. In the 2nd sampling date was detected a number of new genera (*Achromobacter*, *Bacillus, Enterobacter,* Micrococcaceae, *Rahnella*, *Rhodococcus* and *Serratia*) that were not detected in the first sampling date. These temporal changes can be attributed to several factors (Gonzalez et al., 2020). One factor influencing the rhizosphere microbial community structure is the phenological development of plants throughout the growing season. As the plant's root system expands and its patterns of rhizodeposition change, it can have a direct impact on the surrounding microbial communities (Bonkowski et al., 2021). This dynamic interaction between plants and microorganisms can lead to shifts in microbial composition over time (Hannula et al., 2019). Furthermore, variations in weather conditions during the growing season can also play a role in shaping rhizosphere microbial communities (Staley et al., 2017). Environmental perturbations like drought or heavy precipitation events can disrupt the balance between plants and soil microbiota, leading to changes in microbial composition (Padilla et al., 2019; Moravec, 2021). The close interconnection between plants and the soil microbiome suggests that any environmental perturbation affecting the plant can also impact the structure of the soil microbial community (Hartman & Tringe, 2019). In the case of our study, the shift in microbial composition between the two sampling dates may be attributed to changes in plant phenology, as well as variations in weather conditions experienced during the growing season.

4.2 Biochemical features analysis

Auxins are important chemicals produced naturally by plants and are involved in practically every aspect of plant physiology, including cell division, expansion, differentiation, and abiotic stress alleviation (Oleńska et al., 2020). Numerous studies have demonstrated the significant role of auxins, most notably indole acetic acid (IAA) in plant growth regulation

(Matsuda et al., 2017). Diverse soil microorganisms including bacteria (Park et al., 2017), filamentous fungi (Floch et al., 2003) and yeasts (El-Tarabily, 2004) are capable of producing physiologically active quantities of auxins and which have pronounced effects on plant growth and development. In our study, all the 64 strains tested (30 fungi and 34 bacteria) produced IAA. Bacteria were able to efficiently produce more IAA than fungi, similar to previously reported (Toumi et al., 1995). Both *Mucor circinelloides* and *Pseudomonas* sp.12, were the isolates that produced the greatest amount of IAA, among the fungi and bacteria tested, respectively. Studies have shown that *Mucor circinelloides* can produce IAA, although the specific mechanisms and regulatory pathways involved in its biosynthesis are not yet fully understood (Chen et al., 2021). Also, *Pseudomonas* species can produce IAA through various pathways, including tryptophan-dependent and tryptophan-independent pathways (Oberhänsli et al., 1991). The production of IAA by *Pseudomonas* species can be influenced by various factors, such as environmental conditions and the presence of specific genes involved in IAA biosynthesis (Ahmad et al., 2005). The auxin-producing bacteria and fungi found in our work have enormous potential for promoting plant growth. It would be interesting to test such effects, particularly of *Mucor circinelloides* and *Pseudomonas* sp.12, in future works.

In the natural environment numerous microorganisms in the soil and rhizosphere are effective in increasing the bioavailability of soil insoluble phosphorus for plant use (Bhattacharyya & Jha, 2012). This group of microorganisms is referred to as Phosphorus Solubilizing Microorganisms (PSM), and they solubilize insoluble inorganic (mineral) phosphorus and mineralize insoluble organic phosphorus (Sharma et al., 2011). In our study, 57% and 85% of the fungal and bacterial isolates tested were able to solubilize insoluble phosphate on agar plate assays, respectively. This result suggests that bacteria are most effective in solubilizing phosphate than fungi, which contradicts previous studies (Nahas, 1996). The mechanism of phosphate solubilization by microorganisms include the production of organic acids, which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble form (Kpomblekou & Tabatabai 1994). Fungi are known to generate and exude more acids in soils than bacteria (Alori et al., 2017), thus their capacity to solubilized phosphate is putatively higher than bacteria. In our study, the ability of the strains to solubilized phosphate was assessed in solid medium, that was reported to have some disadvantages. For example, many isolates which did not show any clear zone on agar plates solubilized insoluble inorganic phosphates in liquid medium (Louw & Webley, 1995). The direct measurement of phosphate solubilization in broth test produced more accurate results in contrast to indirect measurement of phosphate solubilization by plate assay (Nautiyal, 1999).

Thus further direct assays are needed for more reliable results. Among the isolates tested, both *Solicoccozyma phenolica* and *Rahnella* sp. showed the greatest ability to solubilize phosphate, among the total fungal and bacterial isolates tested, respectively. *Solicoccozyma phenolica* is a yeast species that has been recognized for its phosphate solubilization potential (Sarabia et al., 2018). Similarly, several strains of the bacterial genus *Rahnella* have been reported as efficient phosphate solubilizers, with ability to convert insoluble forms of phosphate into soluble forms that can be readily used by plants (Kim et al., 1997; Landa-Acuña et al., 2023).Other bacterial isolates tested in our study, were already reported in the literature for their ability to solubilize and mineralize phosphorus like *Pseudomonas* spp. (Babalola & Glick, 2012), *Bacillus* spp. (David et al., 2014; Raj et al., 2014), *Burkholderia* spp. (Istina et al., 2015), *Enterobacter*, *Serratia*, and *Erwinia* (Zaidi et al., 2009).

Chapter V. Conclusion and future perspectives

The rhizospheric soil is known to harbor a diverse microbial community capable of stimulating plant growth and development. In this work, was evaluated the effect of different soil management practices on the diversity and functionality of these microorganisms in olive groves. In particular, the rizhosphere microbial community of olive tree was compared between soils tilled and not tilled as well as well between soils with cover crop and with spontaneous vegetation. This analysis was performed immediately after sowing and five months later.

Overall, the bacterial community was dominated by the phylum Proteobacteria and genus *Pseudomonas*, while the fungal community was dominated by Ascomycota and genus *Penicillium*. The overall rhizospheric microbial communities revealed to be primarily impacted by the date of soil sampling, followed by the tillage, while the type of cover crop did not have any effect. However, fungi and bacteria communities showed to respond in different ways to these factors. Indeed, the bacterial richness and abundance was significantly enhanced from the 1st to the 2nd sampling date, while the fungal richness and abundance was unaffected. Tillage practices also exhibited contrasting effects on both microbial communities. The diversity and abundance of fungi in tilled soils were significantly reduced when compared to not tilled soils, while bacterial richness and abundance were unaffected by tillage. However, tillage showed to impacted both bacterial and fungal community composition, being some groups of microorganisms exclusively found in each type of soil management. For example, Mortierellaceae (within fungi) and *Janthinobacterium* (within bacteria) were detected only in tilled soils, while both *Pseudogymnoascus* and *Solicoccozyma* within fungal community and *Rhodococcus* within bacterial community were exclusively present in not tilled soils. Although the minor effect of the type of cover crop on the fungal and bacterial community´s structure, a number of fungal and bacterial OTUs were exclusively found in seeded (*Janthinobacterium*, *Pantoea*, *Rahnella*, Burkholderiales, *Rhodococcus* and *Serratia*) or not seeded soils (Micrococcaceae and *Bacillus*).

On the other hand, important bacterial/fungal species with physiological and beneficial functions for plants were discovered in olive tree rhizosphere. The most promising OTUs in plant growth promoting proprieties found in this study are *Bacillus cereus, Pseudomonas* sp.12 and *Pseudomonas* sp.16 in IAA production and *Erwinia*, *Micrococcaceae* and *Rahnella* in phosphate solubilization, for bacteria, and *Mucor circinelloides, Purpureocillium lilacinum* and *Umbelopsis isabellina* in IAA production and *Naganishia*, *Oidiodendron* and *Solicoccozyma phenolica* in phosphate solubilization for fungi. With immense plant growth promotion potential owing to their ability to produce auxin and solubilize phosphate studying their inoculation in olive tree or soil could provide a promising strategy to improve plant growth.

Although our results suggest that bacteria can adapt more easily to changes cause by tillage than fungi, further research is needed to fully reveal the long-term effects of tillage practices and crop cover on these two microbial communities of olive tree rhizosphere.

In future perspectives, it is essential to delve into the study of root exudates and their impact on the microbial community to gain a comprehensive understanding of the intricate interactions in the rhizosphere. Root exudates are known to play a crucial role in shaping the composition and function of microbial communities around plant roots. Investigating the specific compounds released by olive tree roots and their effects on microbial diversity and activity will provide valuable insights into how to manipulate these interactions to promote plant growth and nutrient uptake efficiently.

Furthermore, to enhance the effectiveness of cover crop studies, a thorough examination of the natural flora present in the olive orchards is essential. Comparing the microbial communities associated with the spontaneously growing vegetation to the seeded cover crops will enable researchers to identify potential beneficial microorganisms that naturally thrive in the rhizosphere. This knowledge will aid in selecting the most suitable cover crop species that can further enhance soil health, nutrient cycling, and overall ecosystem sustainability. By harnessing the power of naturally occurring microorganisms through cover crop selection, we can optimize rhizosphere engineering strategies and promote the successful implementation of sustainable practices in olive orchards.

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Supporting information

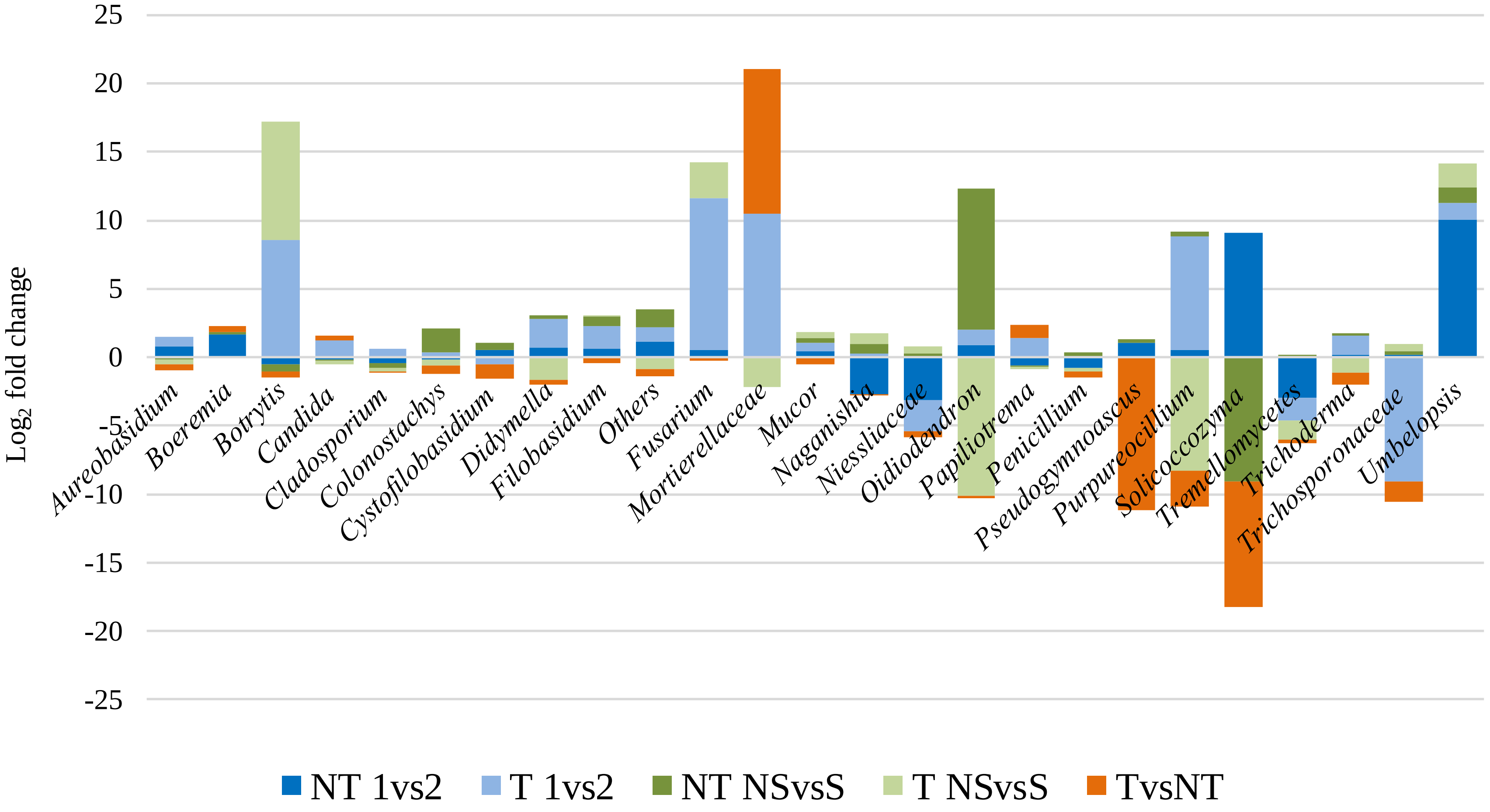

Figure S1. Log$_2$-fold changes in the abundance of rhizospheric fungal genera/family between the types of soil management (tilled-T vs. not tilled-NT), planting cover crop (seeded-S vs. not seeded-NS, in each type of soil management) and soil sampling date (1st vs. 2nd soil sampling date, in each type of soil management).

Table S1. Analysis of similarity (ANOSIM), based on Bray-Curtis distance, showing the differences in the rhizosphere fungal or bacterial community composition between tilled vs. not-tilled, seeded vs. not-seeded or 1st vs. 2nd soil sampling date.

Comparison		**Fungi**	**Bacteria**
Tilled vs. not-tilled		R=0.361 p=0.001	R=0.335 p=0.001
Seeded vs. not-seeded	Tilled	R=0.269 p=0.021	R=0.065 p=0.239
	Not-tilled	R=0.225 p=0.040	R=0.048 p=0.303
	Total	R=0.006 p=0.545	R=0.025 p=0.666
1st vs. 2nd soil sampling date	Tilled	R=0.378 p=0.014	R=0.677 p=0.002
	Not-tilled	R=0.807 p=0.001	R=0.208 p=0.041
	Total	R=0.591 p=0.001	R=0.199 p=0.003

Table S2. Relative importance of the type of soil management (tilled vs. not-tilled), planting cover crop (seeded vs. not-seeded) and soil sampling date (1st vs. 2nd soil sampling date) on the rhizospheric fungal and bacterial community composition as revealed by permutational multivariate analysis of variance (PERMANOVA).

	Variable		DF	SS	R^2	F-statistics	P value
Fungi	Tillage vs. not tillage		1	0.343	0.118	2.964	**
		Residuals	22	2.546	0.881		
		Total	23	2.889	1		
	Seeded vs not seeded:	Tilled	1	0.551	0.206	2.60	**
		Not tilled	1	0.102	0.053	0.508	n.s.
		Residuals	19	3.934	1.739		
		Total	21	4.588	2		
	1st vs 2nd Sampling:	Tilled	1	0.623	0.259	3.503	**
		Not tilled	1	0.746	0.345	4.746	***
		Residuals	19	3.194	1.394		
		Total	21	4.564	2		
Bacteria	Tillage vs. not tillage		1	0.132	0.088	2.04	n.s.
		Residual	21	1.365	0.911		
		Total	22	1.498	1		
	Seeded vs not seeded:	Tilled	1	0.105	0.095	1.058	n.s.
		Not tilled	1	0.093	0.128	1.323	n.s.
		Residuals	19	1.632	1.775		
		Total	21	1.82	2		
	1st vs 2nd Sampling:	Tilled	1	0.192	0.211	2.412	*
		Not tilled	1	0.221	0.352	4.892	**
		Residuals	18	1.125	1.435		
		Total	20	1.539	2		

Table S3. GenBank accession numbers and BLAST final match sequences of the fungal and bacterial isolates collected from the rhizospheric soil of the olive orchard.

isolate code	**Query cover (%)**	**E value**	**Identity (%)**	**Accession number**	**BAST final classification**
Fungi					
F4	100	0	100	MT573468.1	*Aureobasidium pullulans.1*
F35	100	0	100	MK267438.1	*Mucor* sp.1
F28'	100	0	99.48	MT365951.1	*Mortierella lignicola*
F9	100	0	99.34	MG826919.1	*Niesslia* sp.
F10	100	0	100	MN922124.1	*Aureobasidium pullulans.2*
F23	100	0	100	OW987795.1	*Penicillium* sp.3
F5'	100	0	100	MN299255.1	*Solicoccozyma phenolica*
F6	100	0	99.82	MK942576.1	*Naganishia* sp.
F11	100	0	100	MK186932.1	*Candida membranifaciens*
F37	100	2,00E-153	100	MT529117.1	*Penicillium* sp.1
F43	99	6,00E-123	98.46	MT530278.1	*Purpureocillium lilacinum*
F36	100	3.00E-46	100	MK883650.1	*Umbelopsis isabellina*
F29	100	0	100	OM505094.1	*Cladosporium* sp.
F14	100	0	100	MT626048.1	*Mucor hiemalis*
F19'	94	0	99.6	MF615064.1	*Mucor* sp.2
F2^2	99	0	100	MK534497.1	*Penicillium* sp.2
F3	100	0	100	MT441901.1	*Clonostachys rosea*
F2'	99	0	100	MT548672.1	*Penicillium* sp.4
F40	100	0	100	MT635312.1	*Didymella* sp.
F16	100	1.00E-145	100	MN913550.1	*Papiliotrema terrestris*
F19	100	0	100	MK451243.1	*Penicillium raperi*
F44	100	4.00E-180	100	KM023336.1	*Penicillium scabrosum*
F26	100	0	100	MT573470.1	*Botrytis cinerea*
F2	100	0	100	KU720399.1	*Penicillium* sp.1
F25	100	0	100	KY434114.1	*Mucor circinelloides*
2F6	100	8.00E-70	96.41	MT236874.1	*Fungus* sp.6
F21	100	0	99.59	EU816394.1	*Oidiodendron* sp.

2F16	100	0	100	MT573491.1	*Pseudogymnoascus pannorum*
F42	100	2.00E-107	98.67	OW986486.1	*Pseudogymnoascus* sp.
F18'	100	0	100	ON796002.1	*Fusarium oxysporum*
Bacteria					
M2	100	9.00E-177	99.43	MW089022.1	*Enterobacteriaceae*
M8'	100	0	99.01	MK373598.1	*Burkholderiaceae*
M33	100	0	99.75	NR_170539.1	*Janthinobacterium* sp.
M31	100	7.00E-127	99.23	MT631995.1	*Serratia* sp.
M37	100	0	100	MT605813.1	*Pantoea agglomerans*
M14	99	0	100	ON885267.1	*Pseudomonas* sp.12
M22	100	0	98.56	KY628892.1	*Flavobacterium* sp.
M4	100	0	100	ON885267.1	*Pseudomonas* sp.14
M17	100	3.00E-151	100	JQ977694.1	*Pseudomonas* sp.2
M9	100	0	100	MT270698.1	*Erwiniaceae*
M5	100	0	100	MT629863.1	*Pseudomonas* sp.4
M13	100	0	100	LC546431.1	*Pseudomonas baetica*
M14'	100	0	100	MT477141.1	*Pseudomonas turukhanskensis*
M5'	100	0	100	MT629866.1	*Pseudomonas* sp.6
M34	100	0	100	MT561438.1	*Pseudomonas costantinii*
M15	100	0	100	MN437598.1	*Pseudomonas* sp.9
M30	100	0	100	MT631990.1	*Pseudomonas* sp.10
M39	100	0	100	LC516494.1	*Pseudomonas* sp.7
M28	100	0	100	MT626825.1	*Pseudomonas* sp.1
M6	100	0	99.53	MK398042.1	*Pseudomonas* sp.13
M25	100	0	100	KU032865.1	*Pseudomonas rhizosphaerae*
M29	100	0	100	MT605320.1	*Pseudomonas* sp.5
M19	100	0	98.77	MT012229.1	*Erwinia* sp.1
2M3	100	0	99.18	MT094257.1	*Rahnella* sp.
2M8	100	0	100	ON795917.1	*Bacillus cereus*

2M13	100	0	100	CP048810.1	*Pseudomonas* sp.16
2M4	100	0	99.55	MH341955.1	*Enterobacter* sp.
2M17	100	6.00E-174	100	MN826595.1	*Rhodococcus corynebacterioides*
2M9	100	0	100	MN684221.1	*Achromobacter* sp.
2M10	100	0	99.44	KF785022.1	*Enterobacteriaceae*
2M16	100	0	99.77	LC041128.1	*Enterobacter cf. asburiae*
2M6	100	0	100	MN098866.1	*Micrococcaceae*
2M21	100	0	99.76	KX588589.1	*Pseudomonas* sp.17
2M22	100	0	100	MT360150.1	*Erwinia* sp.2