Università degli Studi della Tuscia di Viterbo

DIPARTIMENTO PER LA INNOVAZIONE NEI SISTEMI BIOLOGICI, AGROALIMENTARI E FORESTALI

Corso di Dottorato di Ricerca in

Scienze, Tecnologie e Biotecnologie per la sostenibilità -XXXIV ciclo

TITOLO TESI DI DOTTORATO DI RICERCA

Innovations in the mycorrhization process of *Corylus avellana* L. and *Quercus pubescens* Willd. seedlings with *Tuber melanosporum* Vittad.: development of new potting mixes and selection of MHB bacteria from host symbiome.

Settore scientifico-disciplinare

(**AGR/12 PATOLOGIA VEGETALE**)

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A.A. 2021/22

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Abstract

The black truffle (*Tuber melanosporum* Vittad.) is a highly valued non-wood forest product that has emerged as a viable agricultural alternative in rural Mediterranean regions as a result of its capacity to adapt to relatively harsh conditions and its high market value. The diminishing availability of truffles in natural habitats has encouraged truffle plant production in nursery-controlled conditions to support cultivation in plantations. The application of ectomycorrhizal (ECM) fungi to forest nursery production is regarded as part of good management practice. However, its realization is associated with the mass consumption of non-renewable natural resources and before employing large-scale inoculations in a nursery the interaction between ECM symbionts and growth substrate should be studied. The research challenge of this thesis was to meet sustainable environmental and economic issues without compromising yields of truffle inoculated seedlings. Two techniques commonly used in soil restoration: (1) soil amendments with pruning and urban waste and (2), inoculation with selected microbial strains naturally present in the *T. melanosporum* niche (i.e. *Pseuodomonas fluorescens*, *Pseudomonas jessenii*, *Priestia megaterium*, *Serratia marcescens* and *Paenibacillus polymyxa*), were evaluated for the nursery production of two of the most suitable host plants, hazel (*Corylus avellana* L.) and downy oak (*Quercus pubescens* Willd.) mycorrhized with *Tuber melanosporum* Vittad. In this context, exploiting the agroecosystem diversity of soil microbial communities appears as a promising effective approach to better understand their role in the development of the mutualistic symbiosis between *Tuber* spp. and the host trees. For this reason, a metagenomic investigation was carried out using high-throughput sequencing (HTS) to determine the diversity and richness of the bacterial and fungal community present in cultivated truffle grounds of *T. aestivum* and *T. melanosporum*. The findings suggest that on root tips colonized by *T. melanosporum* there was a significantly greater bacterial richness in terms of alfa diversity and a decreased number of fungal OTUs than *T. aestivum.* Furthermore, in the two black truffle niches, there were significant differences in terms of the abundance of bacterial and fungal species. The family *Micromonosporaceae* (Phylum *Actinobacteria*) was the most abundant bacterial class on *T. aestivum* colonized root tips, while on *T. melanosporum* mycorrhizae the *Alphaproteobacteria* class (Phylum *Proteobacteria*) was the most abundant. Regarding the fungal community, on *T. melanosporum* samples the phylum *Ascomycota* was the most abundant, while the class *Agaricomycetes* (Phylum *Basidiomycota*) predominated on root tips mycorrhized with *T. aestivum.* Significant increments of mycorrhization rate and root system development due to soil amendment were detected. Especially on *Q. pubescens* seedlings grown on a peat-based compost reached an

increase by 20% of the mycorrhization rate (60%) than control seedlings grown on forest soil (40%), and a higher number of root tips, root volume, length, and area. On *C. avellana* cuttings the mixed composted potting mix did not significantly improve the mycorrhization rate than the control substrate (40%), but significantly increased the root length and the number of root tips. The co-inoculation trial with *T. melanosporum*-associated bacteria, allowed the selection of *Pseudomonas jessenii* and *Priestia megaterium* which significantly increased the mycorrhization rate and the root growth parameters compared with seedlings that received non-bacterial inoculation treatments on both *Q. pubescens* and *C. avellana* respectively. These results stimulate the application of recycled substrates and selected microorganisms as an environmentally friendly biotechnological approach to plant management in the nursery, for promoting plant growth and ECM colonization. In this framework, the newly started project TANA exploits the results gained in this thesis to develop a new manufacturing product based on nanomaterials complexed with the inoculation of fungi of the genus *Tuber* and 'helper' organisms for the standardization and optimization of the mycorrhization process in the nursery confined environment.

1. Introduction

The term mycorrhiza is used to describe a broad range of mutualistic associations formed between plant roots and fungi (Harrison 1997). Mycorrhizal fungi are part of a diverse microbial community that inhabits the rhizosphere. The bi-directional exchange of nutrients takes place due to the fungus's intimate contact with the roots. The fungus can mobilize mineral elements from the soil such as nitrogen, phosphorus, sulfur, and zinc, which, thanks to the dense hyphal network, can be better absorbed by the plant, while the carbohydrates synthesized by the autotrophic plant are partly transferred to the heterotrophic fungus, which it thus becomes independent of the scarce and poorly distributed carbon source, allowing it to grow and reproduce. It is possible to develop a beneficial relationship for both parties. Afterward, the ectomycorrhizal symbiosis, which is considered the key element in the forest ecosystem, accounting for roughly 80% of total fungal biomass, will be fully described. In particular, the *Tuber* genus will be described with special consideration for two of the most valuable black truffle species that naturally form ectomycorrhizas, *Tuber melanosporum* Vittad. and *Tuber aestivum* Vittad. On one hand, *T. melanosporum* has a more limited distribution range with respect to *T. aestivum* but it is not so "scarce", moreover the techniques and the chances of success to cultivate it are "about" the same used for *T. aestivum*.

More recently, scientific knowledge has contributed to the taxonomy and biology of this group of Ascomycetous fungi and has contributed to the artificial cultivation of truffles in truffières (truffle plantations), supplementing natural production (Murray, 2020). The review will explore the known aspects of the ecology and physiology of fruit body production and will draw some important aspects of the current natural distribution of two of the main commercialized species of truffles related to climatic characteristics and several edaphic factors involved in their cultivation. Due to its performance in relatively harsh conditions, high market value, and dwindling output in natural areas, the cultivation of the Périgord black truffle has become an important agricultural option in rural Mediterranean regions. With the introduction of nursery procedures to promote *T. melanosporum* ectomycorrhiza formation in host seedlings, orchard cultivation began in earnest (Chevalier and Grente, 1979). In France, Italy, and Spain, productive truffle orchards are already providing rural landowners with an alternative to agricultural subsidies, promoting the regeneration of abandoned cereal fields while requiring relatively few agricultural inputs.

Despite this, criticalities persist in the mycorrhization process. The rate of root colonization by the target fungal species and possible contaminants is affected by the kind of soil used to grow inoculated plants and the quality of inoculum and growth conditions. There is evidence that various soils alter

the competitive ability of truffle species, according to Donnini et al. (2009) and Zambonelli et al. (2009). Indeed, mycorrhized plants have long been grown on substrates taken from natural soil in suitable areas. However, due to economic and environmental issues, large-scale commercial operations are not always practicable on an ongoing basis (Iotti et al., 2012). For example, nurseries very often take the soil for planting the seedlings from surrounding wooded areas. In Italy, as in the rest of the world, any movement of soil from the forests is regulated by law to safeguard the natural biodiversity. For this reason, an aspect of this thesis relates to the optimization of the mycorrhization process using alternative substrates for the growing of mycorrhized seedlings in the nursery production process. These potting mixes originate from composting sites and are based on recycled pruning plant materials as an alternative to the use of forest soils, with an economic and environmental advantage for the manufacturing company. Being truffle ectomycorrhizal (ECM) fungi growing in symbiosis with a host plant (commonly oak and hazel), an important focus of this review will be to describe the main plant species that take part in the ectomycorrhizal symbiosis regarding *Quecus pubescens* Willd. and *Corylus avellana* L., which have been chosen for the inoculation experiments of this thesis, and their physiological aspects will be highlighted above. Although the symbiosis term implies the presence of two members, the mycorrhizosphere environment is more complex and extended, in which roots and ectomycorrhizal fungi are not the only players. The soil biota is made up of thousands of different organisms. Bacteria are the most common ones, and their existence cannot be ignored. Their physicochemical interactions can improve fungal and plant growth by accessing inorganic nutrients or producing hormones and/or vitamins (Uroz et al. 2007; Deveau et al. 2010; Uroz et al. 2013). In this review, an important part will describe the recent findings on the diversity of this biotic environment characterized by the presence of truffle-associated bacteria and their involvement in the different phases of the life cycle of truffles. A large section of this thesis is reserved for the isolation and molecular characterization of the culturable microbial consortia associated with the root tips of *T. melanosporum* for their use as inoculants in the controlled mycorrhization process to improve the establishment and maintenance of a functional symbiosis. Because no commercial bacteria helper inoculants has been made, this aspect could represent an innovative biotechnological tool in forestry nursery production, particularly useful for plantation truffle production.

Furthermore, new DNA sequencing technologies allowed the study of uncultivated members, revealing the microbial diversity inhabiting the ECM of both *T. aestivum* and *T. melanosporum* orchards using the new technologies of HTS (high throughput sequencing). These new techniques

and the recent evidence will be useful in the ongoing collaboration and translational communication between scientists and truffle cultivators. Commercial truffle producers aiming to improve the sustainability and productivity of managed truffières will benefit greatly from the insights gathered in this thesis.

1.1. State of the art

1.2. Mycorrhizal symbiosis

Professor Albert Bernhard Frank, in 1885, developed hypotheses based on extensive morphological investigations of feeder rootlets from diverse plants and a wide range of ecological data. He developed a revolutionary hypothesis on tree feeding based on a symbiotic relationship between the fungus and tree roots in a single organ called "mycorrhiza". This neutral term did not imply parasitism but was simply based on the natural coexistence of dissimilar organisms, as seen in lichens. Grawitz and De Bary (1887), who contributed to the coining of this phrase, use it to describe associations in which organisms appear to help one another (Smith and Read, 2008). Mycorrhizal fungi belong to all major phyla, including *Glomeromycota*, *Ascomycota*, and *Basidiomycota* (Smith and Read, 2008). They are found in association with about 90% of eukaryotic organisms, including woody and herbaceous species, and are found in both natural and man-made habitats. They populate alpine and boreal habitats, tropical woods and grasslands, and cultivated land. They form a large network of hyphae in the soil, termed the "wood-wide web" by Helgason et al. (1998), that can connect whole plant communities by allowing for efficient horizontal nutrient transfer. In fact, both partners benefit from the relationship: mycorrhizal fungi improve the nutritional status of the host plant by transferring nutrients such as NO^{3-}, PO_4^{3-} and K^{+}, influencing water absorption, growth, and resistance to diseases while the host plant provides the fungus with sugars and the source of organic carbon necessary for its growth and disease resistance (Smith and Read, 2008). The mycorrhizal classification is mainly based on the interrelationship between plant root cells and fungal hyphae. They are classified into two common groups, which are endomycorrhizae and ectomycorrhizae (Milton et al., 2021). Moreover exist other types of mycorrhizae as orchid mycorrhizas, mycorrhizas of *Ericaceae,* and arbutoid mycorrhizas. The main differences between the two most representative groups are described.

- Ectomycorrhiza: when fungi are associated with the roots of plants only externally, they surround the young root, forming a fungal mantle (mycoclena), and penetrate between the cortical cells, forming the Hartig net.
- Endomycorrhiza: when the fungus does not form any external mantle but develops inside the cortical cells of the roots forming arbuscles and vesicles.

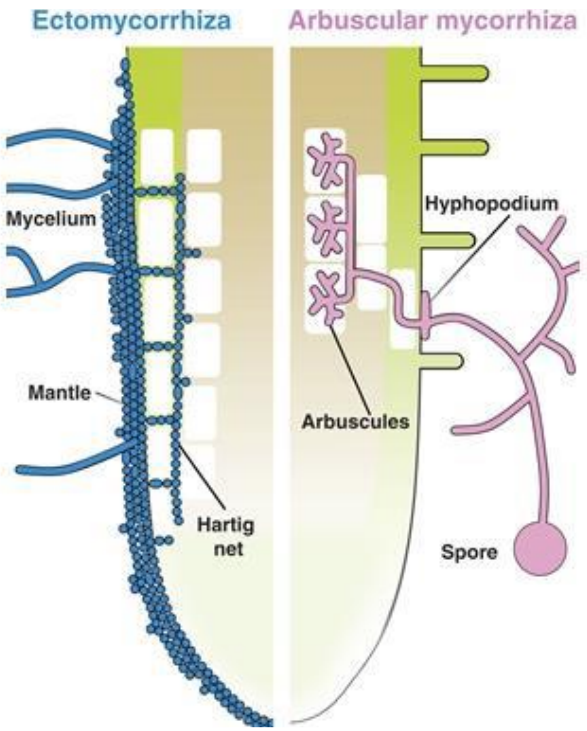


Figure 1. Illustration of root colonization structures in ectomycorrhizal interactions (in blue) and in arbuscular endomycorrhizal interactions (in pink). Source: Bonfante & Genre, 2010.

1.3. Ectomycorrhizal symbiosis

More than 20,000 species of ectomycorrhizal fungi have formed symbioses with more than 6,000 tree species, including pines, beeches, oaks, eucalypts, dipterocarps, and poplars (Pérez-Moreno et al., 2021). *Tomentella* genus is the most abundant and frequent in Basidiomycota. The other most important genera are in the *Agaricales* (*Cortinarius*, *Tricholoma*, *Inocybe*, *Amanita*, *Russula/Lactarius*), *Sebacinales* and *Boletales*. The Ascomycota belong to the genera *Tuber*, *Pachyphlodes*, and *Elaphomyces*. As a result, these symbioses have a large impact on forest ecosystems. Extensive forests in temperate, boreal, subtropical, and mountainous ecoregions of the Northern and Southern Hemispheres, for example, are made up of tree species colonized by ectomycorrhizal fungi (Smith and Read, 2008; Martin et al., 2016). Under the light microscope, in cross-section, the ectomycorrhiza is composed of an external coat of fungal hyphae present in the soil that develops around the roots (secondary rootlets), forming a felt mantle (mycoclena) whose filaments penetrate between the cortical cells of the roots, forming the Hartig net. Mycorrhized secondary roots differ in the swollen, rounded shape of the apex and the lack of root hairs. Moving to the outside in contact with the ground, the mycoclena produces simple straight (spinule) or branched hyphae or simple or branched thin-walled hyphae (peritrophic hyphae) which constitute the extra-radical mycelium, highly variable in structure (rhizomorphs, sclerotia, reproductive organs) which perform the function of root hairs in absorbing water and mineral salts to transfer them to the plant. The function of the mycoclena is to act as intermediate storage for (i) nutrients that are transported from hyphae growing in the soil destined for the Hartig net and (ii) carbohydrates that are taken from the Hartig net into the apoplast, designated for transport to the mycelium growing in the soil (Jordy et al., 1998). At the Hartig net level, the plant and the fungus are in direct contact with each other: the contact zone consists of the two juxtaposed walls (fungal hypha wall and root cell wall). Here the passage of mineral and organic compounds between the two symbionts takes place (Figure 2). A perfect mutualistic symbiosis is formed between the two parties involved in the contact. They both get nutrition from it.

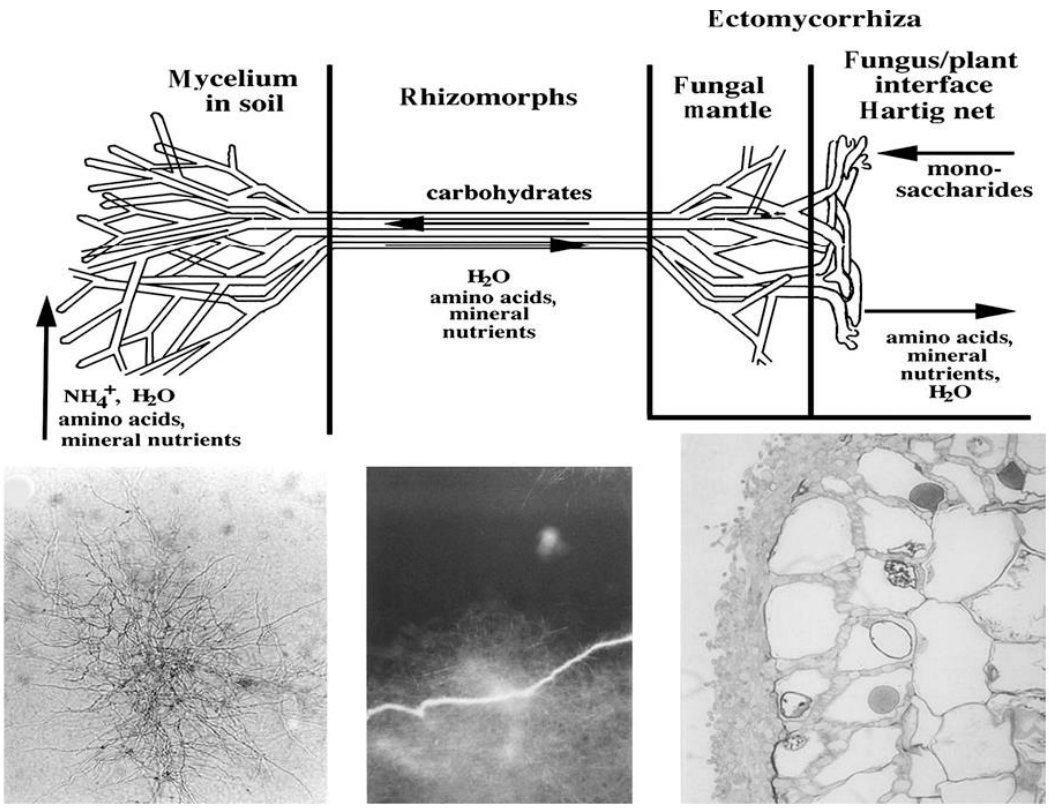


Figure 2. Scheme of an ectomycorrhizal fungal colony (without fruiting bodies). Is shown a scheme of an ectomycorrhizal fungal colony (upper part) and photographs of the respective fungal structures (lower part, from left to right: soil-growing hyphae, rhizomorph, ectomycorrhiza structure composed of mycoclena and Harting net). Source: Nehls 2008.

1.4. Sugar metabolism on ECM symbiosis

Ectomycorrhizal (ECM) fungi have a dual life cycle, living as symbionts within plant roots and as facultative saprotrophs in the soil, and many species are strictly biotrophic. This necessitates adaptations to prevent plant parasitism, such as being extremely competitive in soil nutrient exploration (Nehls et al., 2008). Major nutrients (nitrogen, phosphate) are fixed in the organic layer or held in living creatures in natural forest ecosystems, resources to which plants have only restricted access (Smith & Read 2008).

The contribution of ECM fungi to tree nutrition through mineral weathering (Landeweert et al. 2001) and/or mobilization of nutrients from organic matter (Read & Perez-Moreno 2003) is, therefore, a significant role of the ECM symbiosis, making the plant host dependent on the fungal partner. ECM fungi, on the other hand, are completely reliant on their host plant in natural conditions. Complex polysaccharides, the sole carbon source found in considerable proportions in the humus and litter layers of forest soils, are only partially used by ECM fungi (Colpaert & van Tichelen 1996; Read & Perez-Moreno 2003; Martin et al. 2008). Therefore, ectomycorrhizal symbiosis establishment is a

process dependent on the concentration of soluble sugars in the root system and on the quantity of carbohydrates released by the plant in the form of radical exudates, which constitute the stimulus necessary to attract the symbiont mycelium (Govi, 1986). The hexoses that are absorbed by the fungal hyphae will be converted into useful metabolites by increasing the glycolytic flux for ATP generation, amino acid biosynthesis, and the formation of long-term carbohydrate storage compounds (glycogen) to be used only when the short-term carbon source is not available (trehalose for *Ascomycota*, mannitol for *Basidiomycota* belonging to the order *Agaricales*) (Nehls et al., 2008). It is commonly accepted that in ECM symbiosis, sucrose is excreted by the root cells of plants in the apoplast at the plant-fungus interface and hydrolyzed by plant-derived invertase (Lewis and Harley, 1965; Salzer and Hager, 1991; Rieger et al., 1992; Schaeffer et al., 1995; Nehls, 2004). The lack of invertase in some ECM fungi (Salzer and Hager, 1991; Hatakeyama and Ohmasa, 2004; Daza et al., 2006), differentiates them from phytopathogens (Walters et al., 1996; Voegele et al., 2001) and ericoid mycorrhizal fungi (Straker et al., 1992; Hughes and Mitchell, 1996), makes them dependent on the activity of the plant partner (Smith and Read, 2008), which may be essential for the functioning of this type of symbiosis.

1.5. Importance of mycorrhizae in natural environments

It is now known that mycorrhizae play a fundamental role in the nutrient cycle in ecosystems, improving the mineral nutrition of the plant and increasing the host's ability to absorb, translocate, and accumulate these compounds. The Northern and Southern Hemisphere's temperate, boreal, subtropical, and mountainous ecoregions are mainly composed of tree species colonized by ectomycorrhizal fungi (Soudzilovskaia et al., 2015; Smith and Read 2008). The mycelium of hundreds of distinct species of ectomycorrhizal fungus colonizes and links trillions of plant rootlets in each of these forests.

1.5.1. Ecological importance of ectomycorrhizae

The ectomycorrhizal fungal mycelium represents up to 80% of the total fungal biomass and 30% of the microbial mass in forest soils (Wallander et al., 2001; Högberg and Högberg, 2002; Wallander, 2006) and is considered essential for key processes of the forest ecosystem (e.g., the nutrient cycle and the uptake of carbon sources) (Anderson and Cairney, 2007). Indeed, it is feasible to state that these fungi have shaped current forests (Smith and Read, 2008). Minerals such as phosphorus, nitrogen, sulfur, and zinc are mobilized by these fungi and transferred to plants, which then make the

absorbed carbon source accessible to the fungus for growth. Plant growth is severely limited by the scarce amount of nitrogen available in the soil. EM fungi genome analyses highlight the ability of the mycelium to import sources of organic and inorganic nitrogen, including nitrates, ammonium, and peptides, directly from the soil. A wide range of membrane proteins performs the function of transferring ammonium from the apoplast to the plant cytoplasm, including non-specific ammonium transport proteins (AMT) channels such as aquaporins (Loqué et al. 2005) and voltage-dependent cationic systems (Roberts et al., 2002). From the mycelium, the nitrogenous compounds reach the mycoclena and Hartig net to be exported to the plant; although this mechanism is poorly understood, glutamine and ammonium are the most likely molecules to cross the symbiotic interface (Martin and Nehls, 2009). This involves the assimilation of ammonium into amino acids and their translocation to the plant, or the direct discharge of ammonium ions from the hyphae of the Hartig net. A possible confirmation of the second alternative comes from the overexpression of the AMT family in the perennial species *Populus trichocarpa* (Couturier et al., 2007). Currently, recent advances in the understanding of nutrient translocation pathways in fungus-plant and fungus-soil interactions are particularly interesting, especially the priority role of carbon and nitrogen assimilation between plants and fungi (Karst et al., 2021; Bonfante and Genre, 2010). More than 95% of tree roots are coated by ectomycorrhizal fungal mantles (Taylor et al., 2000) in large boreal forests, where tree development is seriously affected by poor soil nitrogen (N) availability (Tamm, 1991). Hyphae branch out from them, efficiently scavenging the soil for nutrients like nitrogen, part of which is supplied to the host plant in exchange for carbon (C) substrates from photosynthesis (Smith & Read, 2008). The hypothesis supported by several authors is that ectomycorrhizal fungi, in part, sustain this limitation (Averill et al., 2014; Franklin et al., 2014; Gadgil and Gadgil, 1971; Näsholm et al., 2013; Smith et al., 2020). Divergent perspectives exist on explaining this natural mechanism.

As Näsholm et al. (2013) report, when the amount of N is limited, EM trees increase the allocation of C to their fungal partners to get more N, thus promoting the development of fungi. A possible explanation for symbiotic stability despite inconsistent plant benefits could be that the competition between trees for poor nitrogen sources and the competition of fungi for carbon sources provide strong incentives for symbiotic exchange at the individual level. Due to nitrogen immobilization in the biomass of mycorrhizal fungi, the symbiosis further aggravates the nitrogen limitation, ultimately reducing plant growth compared to a hypothetically non-mycorrhized forest (Franklin et al., 2014). This feedback mechanism has been proposed to maintain the strong nitrogen deficiency ubiquitous in boreal forests, which may have the ability to eliminate or even reverse the expected positive effect

of a CO_2 increase on tree growth in highly nitrogen-restricted boreal forests (Franklin et al., 2014) and could contribute to the long-term storage of soil carbon (Averill et al. 2014) because mycorrhizal fungi can contribute to nitrogen immobilization, thus limiting, rather than improving, the nitrogen uptake by the plant (Alberton et al., 2007; Corrêa et al., 2008). It is hypothesized that large carbon investments by trees in ectomycorrhizal symbiosis result in a strong limitation of ecosystem nitrogen. This nitrogen limitation is a crucial condition for coniferous trees to be competitive against tree species that are faster growing, but also more nitrogen demanding. According to this hypothesis, the vast boreal forest dominance and persistence in the environment may be due to a significant nitrogen restriction caused by trees' massive carbon investment in ectomycorrhizal symbiosis (Näsholm et al. 2013). On the contrary, according to Corrêa et al. (2012), C is not expensive for plants, and nutrient-limited plants produce excess C that is distributed belowground independent of the quantity of nutrients the host receives from its EM fungus. And finally, Prescott et al. (2020) expand on this perspective, claiming that since photosynthesis rarely restricts plant development, the objective of C allocation to EM fungi is simply to dispose of it rather than to get access to limiting nutrients. Most recently, Karst et al. (2021) conducted experiments to elucidate this mechanism, but they were unable to identify whether the putative increase in C flow from host trees to EM fungus was to dispose of extra C or to fuel EM fungi mining for a subsequent restricted resource. To understand how each partner reacts to changes in the availability of N, these points of view should be taken together. They also point out that different processes could produce the same pattern. The positive effects of ectomycorrhizal fungi on plant nutrition have traditionally been attributed to the quantitative effects of the extraradical mycelium on the uptake of dissolved nutrients from the soil solution and subsequent transport to the roots of ectomycorrhizal plants.

Ectomycorrhizal fungi might also be able to access pools of P within the mycelia of saprotrophic fungi (Lindahl et al., 1999). These types of nutrient uptake effectively represent "short cuts" to traditionally described pathways of decomposition and mineralization and highlight the important function of ectomycorrhizal fungi in forest nutrient recycling. Research by Landeweert et al. (2001) suggests that ectomycorrhizal fungi mobilize other essential plant nutrients directly from minerals through the excretion of organic acids. This enables ectomycorrhizal plants to utilize essential nutrients from insoluble mineral sources and affects nutrient cycling in forest systems. Furthermore, this could have chemical implications for measurements based on soil stock solutions. Fungi in general, and mycorrhizal fungi in particular, have often been overlooked in geochemical processes (Gadd, 2007), and their possible role in atmospheric interactions is still up in the air. On the other

hand, the ECM colonization of the root can provide protection and antagonistic action toward parasites, nematodes, and soil-borne pathogens (Duchesne et al., 1988). Indeed, most forest trees are highly dependent on their fungal partners and would be unable to survive in areas with poor or unbalanced soils (Whipps, 2004). Also, the non-nutritive benefits to plants due to changes in water relations, the level of phytohormones, the assimilation of carbon, etc. have already been verified (Smith and Read 2008). In any case, while the mechanisms regulating the bidirectional movement of nutrients within the plant-fungus symbiosis are still to be elucidated, it is believed that the presence of a common mycorrhizal network has the potential to influence the structure and functioning of the entire ecosystem through resource redistribution and thus play a critical ecological role.

1.6. General characteristics of Truffles

With their strong aromas, culinary and economic interest, high diversity, and importance to plant and animal nutrition, *Tuber* is a truffle genus that has attracted much interest (Zambonelli et al., 2016). Truffles are hypogeous fungi, belonging to the genus *Tuber* P. Micheli ex F.H. Wigg, 1780 (Bonito et al. 2013), phylum *Ascomycota*, class *Pezizomycetes*, order *Pezizales*, and family *Tuberaceae,* described for the first time in 1822 (Du Mortier, 1822). Their fruiting bodies are like tubers, more or less round in shape, but sometimes also irregular, with protuberances and cavities depending on the species, the soil in which they were formed, and the availability of water. They develop in the ground up to a depth of 40-50 cm and are covered with a rind or "peridium", which is warted or smooth, yellowish, reddish, or black.

Inside is the pulp, or more precisely, called "gleba", almost always fleshy, ranging in color from white to brown to gray up to purplish-black, sometimes pink or stained with bright red depending on the species, the degree of ripeness, and the host plant species. The gleba is composed of bundles of light mycelial filaments and darker veins that are interspersed with a sinuous course. The light veins are characterized by sterile hyphae, while the dark ones by fertile hyphae constituted by a compact mass of receptacles called "ascus" (cells in which meiospores are formed) containing one or more spores (generally two, three, five, or six) of variable shape and size depending on the species that do not actively discharge (Daniels and Trappe, 1979). The loss of direct emission of spores in fungi is related to the transition from an epigeal to a hypogeal habitus (O'Donnell, 1997; Læssøe, 2007). The epigean relatives of truffles, on the other hand, make cup-shaped ascomas (apothecia) with uniseriate elongated axes that can spread the spores into the air (Trail, 2007).

Considering this drastic morphological differentiation, it has been hypothesized that the *Tuber* genus represents an "advanced stage" in the evolutionary transition from an epigeal cup-shaped mushroom to a truffle (Singer, 1961). In this context, fungi have developed methods to attract animals by producing smells or visual attractants (Beever and Lebel 2014), coaxing them into eating, releasing, and dispersing truffle spores that are more targeted than wind or water (Frank, 2009). Many different animal species are attracted to the scents produced by truffle species (Trappe, 2009), and since truffle spores have been found in the fecal deposits of rodents, marsupials, reptiles, and gastropods, these animals are thought to be important agents of dispersal of the spores (Maser, 1978; Claridge, 2005). These last have thick walls capable of resisting and possibly benefiting from the passage through the digestive tracts of animals (Castellano, 1989), to be then left to fruit near the roots of suitable host trees with which they associate to form ectomycorrhizae starting from a primary monocaryotic mycelium (Baciarelli et al., 2006). They are completely dependent on their host and are rarely found in treeless areas. Outside these areas, they can be associated with non-tree species such as *Helianthemum* sp., *Cistus* sp., or orchids (Fontana and Giovannetti, 1982; Selosse et al., 2004). Species of the *Tuber* genus are not host-specific and form symbiotic associations with any host capable of forming ectomycorrhizae. Their strong nutritional dependence on their plant hosts has resulted in coevolution between plant and *Tuber* lineages. The *Tuber* may develop adaptive "host-generalist" or "host-specific" strategies, although most species are host generalists that may interact with a variety of plant taxa (Bonito and Smith, 2016). They show a wide geographical distribution, in the Northern Hemisphere, including the whole of Europe and Scandinavia (Gotland, an island in Sweden) (Wedén et al., 2004).

Although, as reported by the work of Bonito et al. (2013), Southern Hemisphere lineages of *Tuber* have more recently diverged than Northern Hemisphere clades, the *Tuberaceae* of Australasia have not been thoroughly studied. Some species are present in symbiotic associations with woody species in North Africa (Ceruti et al., 2003), and the *Tuber* genus is also widespread in Asia (India, China, Mongolia) and North America (Bonito et al., 2013). The distribution of species of the *Tuber* genus depends on several factors: the distribution and migration of host trees, dispersion through underground spores, dispersion through mammals, climatic conditions, and the existence of geographical barriers. Humans also contributed to moving truffle species (e.g. south hemisphere) both by truffle cultivation and the trade of forest trees. Truffles are found in temperate, Mediterranean, and continental climates. They are excluded from tropical, dry climates (annual rainfall less than 350 mm) and very cold climates (average January temperature below 10 °C), as well as areas with high

mountains, seas, and oceans that act as geographical barriers due to their dispersion (Wang et al., 2006). Despite the long history of use by humans, there are still many unanswered questions regarding the origin, biogeography, and ecology of these valuable fungi.

1.7. Phylogeny and Biogeography of the genus *Tuber*

The Index *Fungorum* recognizes 290 species, subspecies, and varieties of the *Tuber* genus. However, phylogenetic relationships, such as the classification of species within the genus *Tuber*, based on the morphological characteristics of ascomas and spores, remain controversial (Ceruti et al., 2003). The genus *Tuber*, which is among the six members of the *Tuberaceae* family (*Tuber*, *Choiromyces*, *Reddellomyces*, *Labyrinthomyces*, *Dingleya*, and Southern Hemisphere cup fungi, *Nothojafnea*) contains the most species and is estimated to include a total of at least 180 species (Bonito, 2010). In Europe, 32 species are considered valid (Ceruti et al., 2003). Unfortunately, limited geographical sampling and inconsistent phylogenetic relationships make it difficult to understand their origin, phylogeny, and diversification. To address this issue, various combinations of datasets and analysis methods were used to provide the first combined molecular phylogeny and historical biogeography of the genus *Tuber*. Phylogenetic relationships between species were recently reconstructed based on data from internal transcript spacer sequences (ITS) and various methods of phylogenetic inference derived from multiple loci (18S rRNA, 5.8S-ITS2 rRNA, and β-tubulin) (Jeandroz et al., 2008) to estimate the divergence times of the main groups of the genus *Tuber* and their biogeographical distribution (Bonito, 2013). Within the genus *Tuber*, they identified 11 main clades. According to recent estimates, there are between 180 and 220 *Tuber* species, some of which are only known through "environmental" DNA sequences extracted from rhizosphere soil. Tests related to Tajima rate (Tajima, 1993) combining the sequences of the genes 18S rRNA, 5.8S rRNA, 5.8S-ITS2 rRNA, and β-tubulin that evolved in a chronometric way to estimate divergence times, revealed that the common ancestor to all *Tuber* species (presumably ectomycorrhizal since all *Tuber* species are ectomycorrhizal) (data congruent with the results of Ducousso et al, 2004) would have developed during the Triassic or Jurassic between 271 and 140 million years ago (mA), depending on the genes used (271 mA for 5.8S, 255 mA for β-tubulin, 250 mA for 18S + 5.8S + β-tubulin, 161 mA for 18S + 5.8S, 152 mA for 18S, and 140 mA for 5.8S-ITS2). The divergence time estimates for *Tuber* clades are: *multimaculatum* (121 Mya), *aestivum* (101 Mya), *rufum* (86 Mya), *melanosporum* (79 Mya), *puberulum* (65 Mya), *japonicum* (46 Mya), *excavatum* (43 Mya), *maculatum* (67 Mya), *macrosporum* (43 Mya), *gennadii* (48 Mya), and *gibbosum* (27 Mya) (Bonito et al., 2013). From the analysis of the

phylogenetic divergence of the ITS and β-tubulin sequences calculated with the MEGA software version 2.1 (Kumar et al., 2001) using the model of the Kimura 2 (K2P) parameter with a transition-transversion ratio (Ti/Tv = 2) to return to the historical distribution of the genus *Tuber*, it is understood that the common ancestor to all the species of *Tuber* may have had two equally probable scenarios of intra and inter-continental diversification in Europe (E) or Eurasia (EA) (Jeandroz et al., 2008). Many species-specific primers designed on the ITS region are available (Parladé et al., 2016) to allow the genus *Tuber* to be identified quickly. Indeed, using these gene-specific primers, a greater number of *Tuber* species were detected by taking samples directly from the soil than those found by Murat et al. (2005), which sampled only root tips. Primers successfully cover the biodiversity of truffles by helping to understand their ecology and can be practical tools in plantation management, indicating, for example, whether a *Tuber* inoculum is naturally present.

1.8. Life cycle of truffles

The reproductive system of a fungus is the first question to be answered about its life cycle. Within the *Ascomycetes*, sexually reproducing species usually follow one of the three basic sexual productive strategies: homothallic, pseudohomothallic (or secondary homothallic), and haploid heterothallic (Leslie and Klein 1996). *Tuber* species appear to have a complex life cycle.

In the case of homotallism, the fungal cells in haploid mycelium (that possess only one copy of the genetic patrimony), are self-fertile as they can self-fertilize and thus complete the cycle of sexual reproduction without the involvement of another mycelium. On the contrary, in the case of heterotallism, sexual reproduction requires the fusion of two mycelial cells that have different but compatible genetic patrimony. Researchers have been unable to acquire direct insight into the truffle life cycle and reproductive mechanism due to the difficulty of growing and the inability of mating this fungus under controlled laboratory conditions. However, research in population genetics has been employed to address this important biological aspect. Numerous analyses carried out starting from the fruiting bodies of truffles (black truffles and white truffles) by taking the spores (gametes) and studying their genetic heritage using co-dominant markers for large-scale screening of populations of *T. melanosporum* do not highlight more than one form of DNA (or allele) for each gene studied, finding an extremely low level of polymorphism over the whole study area. All this seemd to indicate that the two mycelia at the origin of fruiting have the same genetic heritage, which is characteristic of a "homotallic" or even exclusive selfing mode of reproduction (Bertault et al., 1998).

However, population-genetics analyses coupled with methodological progress in the isolation and typing of single mycorrhizal root tips, asci, and ascospores have clarified the basic aspects of the life cycle and reproductive biology of the two most economically important truffle species worldwide, *T. magnatum* and *T. melanosporum* (Rubini et al., 2005; Paolocci et al., 2006; Riccioni et al., 2008). Following investigations using SSR (single sequence repeats) markers, several of Bertault and colleagues' results were challenged (Le Tacon et al., 2016). Murat et al. (2004) found considerable genetic differentiation within European *T. melanosporum* populations. Both Rubini et al. (2005) and Riccioni et al. (2008) found outcrossing in both *T. magnatum* and *T. melanosporum*. As Murat et al. (2013) reported, *T. melanosporum* is now considered a heterothallic species (Riccioni et al., 2008; Martin et al., 2010; Rubini et al., 2011a,b), according to evidence of recombination events and analysis of the mating-type (MAT) locus, the genomic region that drives the fungal sexual cycle (Fraser & Heitman, 2003). A single MAT locus with two alternative genes exists in all known heterothallic ascomycetes (MAT1-1-1 and MAT1-2-1) (Metzenberg & Glass, 1990; Debuchy et al., 2010). In heterothallic fungi, only haploid cells with different alleles at the mating-type locus/loci can fuse, preventing same-clone mating (Murtagh et al., 2000; Kronstad, 2007; Paoletti et al., 2007; Billiard et al., 2012).

These findings have confirmed the concept that the gleba and mycorrhizas of this species are haploid structures while proving the presence of paternal alleles among meiotic spores inside the asci (Paolocci et al., 2006; Rubini et al., 2007). Furthermore, it is assumed that the haploid mycelium that forms the ascocarps of ECMs serves as the maternal partner in the cross and that it must have come into contact with an opposing mating-type strain that serves as the paternal partner (Rubini et al., 2011b; Murat et al., 2013). The male partner is of uncertain origin: it may be a mycelium developing from germinated ascocarps or nearby ECMs (Rubini et al., 2014; Taschen et al., 2016; De la Varga et al., 2017), or free-living mycelia in the soil (De la Varga et al., 2017), or mycelia that exist as endophytes in non-host plants (Le Tacon et al., 2016). The most probable hypothesis relies on the spores as the male partner (Leonardi et al., 2020; Ori et al., 2021). As reported in the review by Qin et al. (2022), *T. melanosporum* and *T. aestivum* can endophytically colonize roots of non-ectomycorrhizal plants (Schneider-Maunoury et al., 2020; Schneider-Maunoury et al., 2018; Gryndler et al., 2014). Endophytisms in *T. melanosporum* and *T. aestivum*, on the other hand, have recently been shown to be maternal features (Schneider-Maunoury et al., 2020). Even if endophytism in the non-host plant likely makes no direct contribution to the formation of ascocarps, it can improve the production of truffles by reducing the germination and growth of weeds (Selosse 2020).

The same process is possibly involved in all species of the genus *Tuber* (Le Tacon et al., 2016). As reported by Qin et al. (2022), other species were recently shown to be heterothallic based on genome sequences (*T. aestivum*) (Murat et al., 2018), or sequences of mating type genes (*T. indicum* species complex) (Belfiori et al., 2013) and *T. borchii* (Belfiori et al., 2016). Therefore, as shown in Figure 3, the biological cycle of fungal species belonging to the genus *Tuber* begins with the release of ascus and ascospores from mature ascocarps or faeces of animals, which, remaining in the soil, undergo natural degradation by the microorganisms that are present there. The germination of the spore, regulated by the perception of chemical signals coming from the host plants, develops a haploid primary mycelium (monocaryotic, vegetative phase) capable of colonizing the roots. The mycorrhized roots (symbiotic phase) modify their morphological appearance, assuming a club-like and often branched shape, and from them develop the dense extra-radical hyphal network which explores the surrounding soil and absorbs nutrients. When the mycorrhized plant becomes productive, mycelial cords develop from the roots that can differentiate the ascomata: initially, the primordium depends on the mother plant from the nutritional point of view, it seems that at maturity, when the differentiation of sterile and fertile tissues on the inside of it is advanced, it moves towards autonomous growth, of a "saprophytic" type (Le Tacon et al., 2016). Inside the carpophore, the development of the gleba is completed following the gamia between two opposing "mating type" mycelia (fruiting phase) aimed at the differentiation of ascus and ascospores for fungal dissemination. Parguey-Leduc et al. (1984) have shown that when the ascocarp reaches the size of 1 mm in diameter and about 3 mg in weight, it already has a formed peridium and a gleba made up of fertile and sterile veins.

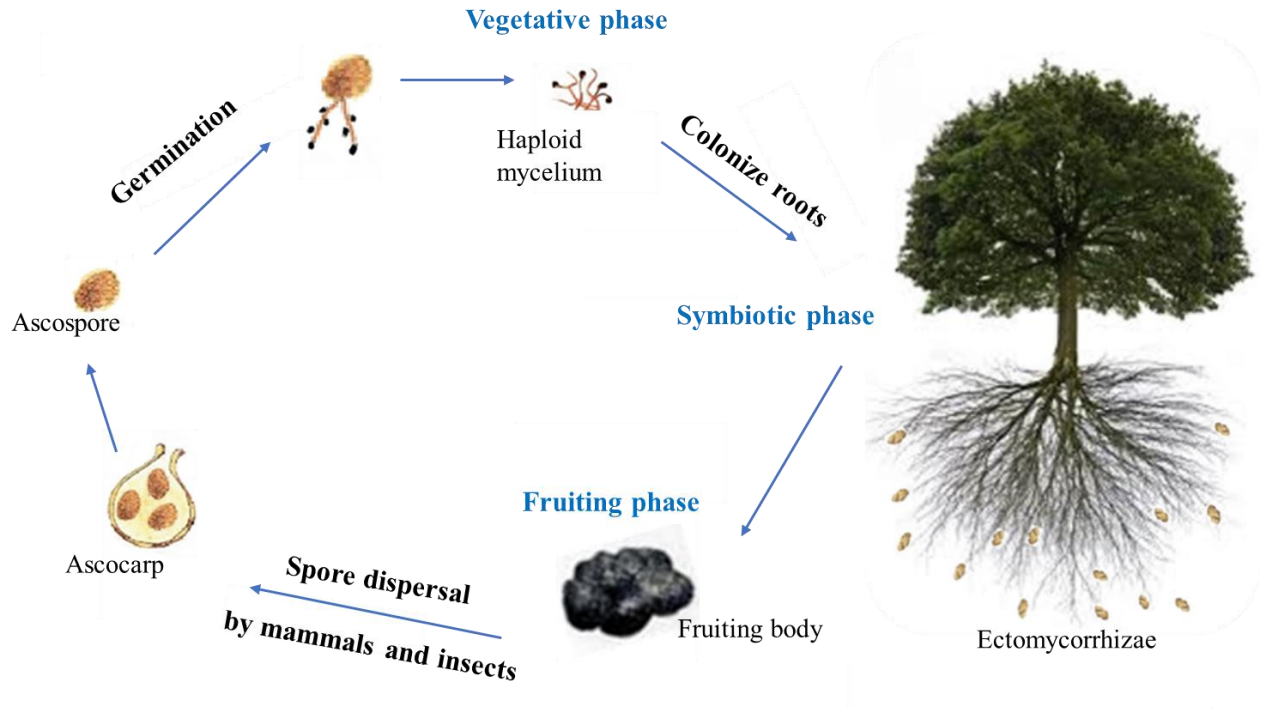


Figure 3. A scheme showing the life cycle of truffles (adapted from Daba et al., 2019).

1.9. The main edible truffle species in Europe

The edible and aromatic quality of more than seventy types of edible truffles produced across the world varies greatly. Several species with exceptional organoleptic qualities are collected for their culinary value. European species of greater economic interest based on their aromatic characteristics are (Reyna 2007): *Tuber melanosporum* Vittad., *Tuber aestivum* Vittad. (=*Tuber uncinatum* Chat.), *Tuber borchii* Vittad. (=*Tuber albidum* Pico), *Tuber brumale* Vittad., *Tuber macrosporum* Vittad., *Tuber magnatum* Pico., *Tuber mesentericum* Vittad. and *Tuber rufum* Pico.

The considerable variety of the over 60 edible species of truffles is mainly due to the presence of volatile organic compounds (VOCs) which, in their various forms, help to create the unmistakable aroma of truffles. The difference between species is largely due to the different proportions of VOCs, although some possess unique compounds. Researchers have detected and characterized about 250 fungal VOCs classifiable as a mixture of simple hydrocarbons, heterocycles, aldehydes, ketones, alcohols, and phenols, including benzene derivatives and cyclohexanes (Chiron and Michelot, 2005; Korpi et al., 2009; Ortiz-Castro et al., 2009), many of which have characteristic scents and are produced during primary and secondary metabolism.

The following sections will be dedicated to the detailed description of the climate, natural distribution, ecological requirements, and economic importance of two of the most important truffle species, *T. melanosporum* and *T. aestivum*, the main characters of the studies conducted in this thesis on the controlled and mycorrhization process and metagenomic investigations.

1.9.1. *Tuber melanosporum*: The Black Périgord Truffle

The Périgord black truffle *Tuber melanosporum* is a well-defined species reported by Carlo Vittadini, a renowned Italian mycologist, in his *Monographia Tuberacearum* (1831). It varies from the size of a walnut to that of an orange, but sometimes even larger. It has a rounded shape but can also be irregular if grown on pebbly ground. The peridium is black, sometimes with ferruginous spots, formed by regular warts up to 4 mm in diameter. The gleba is first clear, if unripe, then gray-brownish, and finally black-purplish when fully ripe, with white, fine, well-defined veins. The ascus is rounded, with 4-6 spores, but often even less, thickly aculeate, not alveolate, ovoid, elongated, opaque, yellow or brown. *Tuber melanosporum* is obtained naturally throughout Europe between 40° and 48° north latitude, particularly in France, Italy, and Spain (Ceruti et al. 2003). It can be found in a variety of climates, including warmer Mediterranean locations like southern Spain and Italy, as well as colder continental areas like Alsace (northern France). This species forms sporocarps which ripen in the late autumn and winter and are collected with the assistance of trained dogs. This species has been found in non-native woodlands in Croatia, Slovenia, Serbia, Portugal, Switzerland, Germany, Hungary, Bulgaria, Greece, and Turkey on rare occasions (Reyna 2007). Due to the high value of black truffle and the yields obtained from its cultivation, plantations are now found in all the Mediterranean-climate regions worldwide (Australia, USA, Chile, Argentina, Morocco, South Africa, New Zealand, China, and Sweden (Buntgen et al., 2017; Reyna and Garcia-Barreda, 2014**;** Wedèn et al., 2009), where they have a high economic impact (Büntgen et al., 2017; Oliach et al., 2021; Samils et al., 2008).

The main factors limiting the natural distribution of *T. melanosporum* are the summer drought and the winter frost. *Tuber* ECMs appear to be very resistant to summer drought and winter frost, while the fruiting process is very sensitive. It is the reason why *T. melanosporum* is rare or not present in Mediterranean regions where annual rainfall is less than 600 mm. The *T. melanosporum* ECMs are more resistant to frost than the ascomata and are capable of vegetative propagation. While this might allow for *T. melanosporum* to be present in the form of mycorrhizas in Northern latitudes, ascoma formation may not be possible.

Nevertheless, Zampieri et al. (2011) demonstrated that cold temperatures modify *T. melanosporum* gene expression, mainly heat shock protein-coding genes and genes involved in the cell wall and lipid metabolism. These genes could be involved in the adaptation of *T. melanosporum* to low temperatures. All life cycles, such as mycorrhizas, mycelia, and fruiting bodies of *T. melanosporum* occur within the soil environment. *Tuber melanosporum* may leave the soil only for a short time if ascomata are eaten and digested by an animal before their spores return to the soil. Soil physico-chemical characteristics are a determining factor in truffle production. Even when a plant is well-mycorrhized and the climate is favorable, fruiting cannot take place if certain soil characteristics are inappropriate (Bonito et al., 2013). *Tuber melanosporum* grows and fruits best in well-structured, porous, and aerated soils (Delmas and Poitou 1973; Poitou 1990). Soils that are good for *T. melanosporum* tend to be on the tops of hills, ridges, or slopes where free water can run off, bringing fresh air with it (Jaillard et al., 2016). This ecological requirement leaves *T. melanosporum* to grow on marginal lands occupied by shrubs and forests or abandoned agricultural areas previously planted with vineyards, olive trees, or almond trees, the least demanding crops in terms of water and fertilisers. The truffle fungus does not need rock fragments to grow, but it frequently grows on stony soils because of their location on the landscape and the induced good aeration of the soil. For this reason, many authors have associated stony soils with truffle soils because *T. melanosporum* fruits often and well in stony soils (Sourzat 1997; Oliach et al. 2005). Balanced textures, those where the soil is formed by roughly equal proportions of clay, silt, and sand (Delmas and Poitou 1973), are the most appropriate for the growth and fruiting of the black truffle. The soil structure in *T. melanosporum* truffieres is always granular. The structure of the horizons where truffles develop is characterized predominantly by fine-to-medium granular and coagulated aggregates, rather than fine subangular polyhedric aggregates. The fine earth, even in the presence of abundant skeletal material (over 80%), is never compact, but rather well organized, supple and friable. As a result, the soil has good permeability and drainage (Chevalier and Sourzat 2012). Soil physical analysis is important to clarify the functionality of the soil in terms of drainage, aeration, and biological activity, but this alone is not sufficient to assess soil for truffle production. The elements classically considered are pH (water), richness in total and active limestone, total organic matter, C/N ratio, and exchangeable micro and macronutrients (Chevalier and Sourzat 2012). Organic matter improves the soil structure significantly by increasing soil porosity, water-holding capacity, and structural stability of soil to water. Organic matter also increases exchange capacity, which, in turn, increases the soil's capacity to retain mineral elements.

The contents of organic matter in soils where the black truffle thrives range from 8 to 180 g kg-1, with a mean near 50 g kg-1 and a reported maximum value of over 370 g kg-1 (Delmas et al. 1981; Bencivenga and Granetti 1988). These are high values of organic matter content in the soil. The C/N ratio provides information on the evolution of organic matter. In France, the C/N ratio in truffières varies from 8.57 to 13.7. *Tuber melanosporum* grows and fruits best in alkaline soils (Delmas and Poitou 1973; Poitou 1990). The pH of productive truffle beds ranges from 7.0 to 8.9, with a median of 7.9 and a standard deviation of nearly 0.4 units; that means that the pH of 95% of truffle soils ranges between 7.5 and 8.3 (Bencivenga and Granetti 1988; Bencivenga et al. 1990; Raglione et al. 1992; Sáez and De Miguel 1995; Garcìa-Montero et al. 2007; Colinas et al. 2007). So, the soils favorable to *T. melanosporum* include neutral (7.0) As reported by Jaillard et al. (2014), with a high content of organic matter, the dolomitic limestone soils and the calcareous sandstone soils are among the most favorable soils for *T. melanosporum*. Only suitable soil and climate conditions in a friendly environment will produce the valuable black truffles, the "black diamond" of Mediterranean gastronomy (Jaillard et al.,2016). For centuries, this delicacy of the soil has been harvested and consumed in Europe. Following the phylloxera crisis towards the end of the nineteenth century, the annual production of *T. melanosporum* in France may have reached 1000 t (Chatin 1869). However, since the beginning of the twentieth century, studies have shown that the output of the Périgord black truffle has rapidly dropped in France (Le Tacon et al. 2014).

In recent years, annual truffle production has not exceeded 30–50 tons. Two world wars, rural desertification, changes in forest use, and agricultural modernism are among the sociological factors that have contributed to this loss (Olivier et al. 1996; Le Tacon et al. 2014). Since that time, plantations have gradually overtaken natural areas as a source of production. Black truffle plantations are being established in all Mediterranean climatic regions around the world, including Spain, France, Italy, and Australia (Reyna and Garcia-Barreda, 2014). Global production is currently at 120,000 kg per year, generating an annual turnover of around 50 million euros for truffle growers (Oliach et al., 2021). In addition to direct income, the production of black truffles has a positive impact in the collection areas, favoring the development of auxiliary economic activities such as the nursery production of mycorrhizal seedlings with *T. melanosporum*, technical consultancy, trade-in truffle products, agritourism, and research (Büntgen et al., 2017). Today, nursery production is one of the keystones of black truffle farming. Factors that influence colonization success in a nursery include the amount, quality, and treatment of inoculum as well as watering, fertilization, temperature, light levels, pH, and potting medium formulation (Hall et al., 1998). This last aspect is crucial in terms of

the company's financial outlay. One of the activities carried out in this thesis concerned the optimization of the controlled mycorrhization process in the nursery to offer a valid alternative from an economic and, above all, environmental point of view to the use of forest land as a substrate for the growth of mycorrhized seedlings. This requirement stems from two major bottlenecks in the process. The first is that soils used as potting medium are frequently taken from wooded and agricultural areas adjacent to truffle orchards. However, soil is a limited resource and rapid human population growth, coupled with its increasing consumption, is placing unprecedented pressure on soil ecosystems (Kopittke et al., 2019). Secondly, as reported by Italian law (L.R. 28 Ottobre 2002, n. 39, art. 26), in the woods it is forbidden to remove any organic material which forms the ground cover (leaves, humus, organic soil, turf); it is also forbidden to remove soil or rock, as well as modest surface levies for the realization on site or in the immediate vicinity of small works. Furthermore, a serious loss of biodiversity occurs in all transformations of land use that involve the removal of the surface horizons of the soil that are rich in organic matter (www.ispraambiente.it, suolo e territorio 2011). In addition, the success of mass production of quality mycorrhized seedlings with *T. melanosporum* has significantly increased the quantities of soil used to fill the pots, requiring large volumes of material.

Good quality soil supplies are becoming scarcer and more expensive. Several studies have been carried out to find alternative, efficient and sustainable components of growing substrates for soilless cultivation (Ferlito et al., 2020; Fascella et al., 2017). An alternative tested was substrates derived from the composting process of vegetation waste. Composting is the biological decomposition process of organic waste under aerobic conditions (Greco et al., 2021). The compost produced from biowaste can be reused as nutrient-rich fertilizers or growing substrates (Greco et al., 2020; Klock-Moore et al., 2000). Decomposition processes belong to a sustainable waste management strategy, which is almost in line with the zero-waste concept: the resource flow is circular so that the resources themselves are conserved and recovered to be reused in other processes. In this way, the company can gain an economic, social, and environmental advantage using sustainable growth substrates.

1.9.2. The Burgundy Truffle (*Tuber aestivum* Vittad. syn. u*ncinatum* Chatin)

Tuber aestivum Vittad. was first described in detail by Vittadini (1831), even though in 1729 Micheli in "*Nova Plantarum Genera*" and in 1801 Persoon in "*Synopsis Methodica Fungorum"* had already reported the existence of this specie (Micheli 1729; Persoon 1801) without adding their names, as instead did Vittadini, presenting himself as the first detailed descriptor (Zambonelli et al., 2016). The

shape of this *Tuber* species can be spherical or irregularly round, with a brown blackish peridium with warts 3–12 mm in size and a pyramidal structure. The gleba is light brown, which darkens as it matures, with numerous white veins that give the section a marbled appearance. Spherical axes with 1–4 elliptic spores, irregularly alveolate and brown. The name "*Tuber aestivum*" comes from observations on the ascomata maturation time (*aestivum* means summer in Latin). In 1887, the French botanist Adolphe Chatin described a new *Tuber* species, *T. uncinatum*, and considered this a new species because morphologically ripe fruiting bodies exhibit a darker gleba and more hooks in the spore reticulum than *T. aestivum* (*uncinatum* means hooked in Latin) (Chatin 1887). For almost two centuries, it has been debated about the fact that *T. aestivum* and *T. uncinatum* may belong to the same species.

The development of molecular tools in biology during the twentieth century has allowed botanists and scientists to approach species delimitations from a new perspective (Molinier et al., 2016). The use of restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) region could not show differentiation between *T. aestivum* and *T. uncinatum* (Henrion et al. 1994). In 2004, Paolocci and colleagues studied the ITS region, as well as sequences of the beta-tubulin and the elongation factor-alpha genes and concluded that *T. aestivum* and *T. uncinatum* fruiting bodies collected in Italy belong to a single species (Paolocci et al., 2004). In 2005, Weden and collaborators studied samples from a broader geographical range using the ITS region and concluded that the phylogenies of *T. aestivum* and *T. uncinatum* were mixed up. As Molinier et al. (2016) reported, for botanical nomenclature of Melbourne rules (Hawksworth et al., 2011), the name *T. aestivum* has priority because it was officially described by Vittadini in 1831, long before *T. uncinatum* was described (Chatin 1887). For most of the scientific community, the conspecificity of the two taxa has now been recognized, but they are still marketed as different species. Most researchers consider them to be ecological forms of the same species, having different maturation times (autumn for *T. uncinatum*, summer for *T. aestivum*) and ecological preferences (Chevalier et al. 1979, 1994; Chevalier 2010b; Gregori 1991, 2010; Mello et al. 2002). *T. aestivum* (uncinatum morphotype), also known as "Burgundy truffle" in France or "Tartufo nero di Fragno" in Italy, is the most widely distributed truffle species in Europe, being native to 26 of the 27 European countries (possibly except for Finland) and recorded also in Azerbaijan (Chevalier 2010a), Iran (Jamali et al., 2017) and China (Wang and Liu 2009), even if Zambonelli et al. (2012) reported it as a separate sister species that form a separate clade with respect to the European *T. aestivum* (*sensu stricto*). *T. aestivum* is known in Europe and North Africa from 10° West longitude (Ireland) to 37° East longitude, and from 33°

(Morocco) to 58° (Sweden) of North latitude (Chevalier 2009). The wide geographic distribution of this species is explained by different factors (Chevalier 2009). In contrast to *T. melanosporum*, *T. aestivum* develops in sedimentary terrain of very different geologic ages, from the Paleozoic Era to the Quaternary and recent alluvial. With respect to *T. melanosporum*, *T. aestivum* is less thermophilic and requires an oceanic, semi-continental, or continental climate with sufficient rain in summer and not too much low temperature in autumn. Annual precipitation totals in *T. aestivum* habitats range from 400 to 1500 mm, making rainfall alone a very variable factor (Molinier et al., 2016). This *Tuber* species can grow in soils exhibiting various physico-chemical characteristics.

Thanks to the permanent presence of limestone and the high organic matter content, the soil structure of *T. aestivum* truffle grounds in northeastern France is of good to excellent aggregation. The soil texture is extremely variable, from silty–clay to clayey–silt, and more rarely, silty, silty–sandy, or clayey. Textures are therefore generally "heavy" (up to 52.8 % clay) (Chevalier and Sourzat, 2012). *T. aestivum* appears to occur in soils with higher carbon and total nitrogen concentrations than *T. melanosporum*. The C/N ratio is generally between 8.9 and 12, indicative of an excellent capacity for mineralization of organic matter and good availability of mineral nitrogen, principally in nitric form (Chevalier and Sourzat, 2012). The soil of most truffle habitats is calcareous, with pH levels > 7 (Chevalier and Frochot 1990), although exceptional sites with a pH of 5.9 and an absence of active carbonate have been reported (Gogan Csorbaine et al. 2012). Generally, *T. aestivum* soils are less rich in limestone than those of *T. melanosporum*. The amount of total limestone does not affect the quality of truffière soils. Only its presence at the surface is important (Chevalier and Sourzat, 2012). The wide range of possible soils, climates, and host plants, together with its market value and its long harvest season, makes this species interesting for cultivation. In France, *T. aestivum* has been cultivated for exactly 30 years, and it is cultivated in various countries such as Italy, Spain, Sweden, Hungary, and Austria. Its cultivation in unfavorable soils (even very acidic ones) by liming the soil allows us to expand the possible cultivation areas of this economically important truffle. In fact, it is one of the most sought-after truffles in the world (Bonito et al. 2010), with prices ranging from 200 to 600 € per kg, making it commercially interesting for traders and consumers thanks to its cheaper prices compared to the most prized species of truffle, *T. melanosporum* (1.000 € per kg, approximately) and *T. magnatum* (4.000 € per kg, approximately).

1.10. Host plant diversity of genus *Tuber*

A mycorrhizal symbiosis is a form of coexisting between the root of the host plant and a specific fungus. As reported by Smith and Read (2008), ectomycorrhizal symbiosis requires both partners to make extensive physiological and ecological adaptations, resulting in physiological and ecological specialization in the interaction. For this reason, not all combinations of potential hosts with ECM fungus are possible in the field.

Instead, each fungus can only live in symbiosis with a limited number of host plant species (Gryndler 2016), and the knowledge of their diversity is of crucial importance. Truffles are not restricted to a single host tree species, and their natural host range is quite diverse.The host plants of the black truffles, *T. melanosporum* and *T. aestivum*, are generally *Quercus pubescens*, *Q. ilex*, *Q. cerris*, Q. *robur*, *Tilia platyphyllos*, *Tilia cordata*, *Corylus avellana*, *Ostrya carpinifolia*, *Cistus* spp., *Castanea sativa*, *Populus* spp., *Pinus* spp., *Abies alba* (Benucci et al. 2012; Hall et al. 2007; Chevalier and Frochot 1997; Riousset et al. 2001; Granetti et al. 2005). Other species, in addition to those listed above, that establish symbiosis with other species of truffles include *Salix* spp., *Fagus* spp., *Picea* spp., and *Ulmus* spp., and the formation of arbutoid mycorrhizas with *Arbutus unedo*. In a nursery, it is possible to obtain mycorrhized plants with *Tuber* belonging to almost all possible hosts (*Populus* spp., *Tilia* spp., *Ostrya carpinifolia*, *Quercus robur*, *Quercus pubescens*, *Populus* spp., *Salix* spp., and *Corylus avellana*), even if the common use in commercial truffle is attested above all for species of *Carpinus*, *Quercus* and *Corylus* (in particular, *C. avellana* and *C. corilus*). Such a wide range of hosts for many *Tuber* species represents a factor which probably enhanced the domestication of *T. melanosporum* and *T. aestivum.* For example, *C. avellana* L., with its vigorous growth and tendency to produce a well-developed root system, is considered "an excellent carrier of mycorrhizae" (Chevalier, 1998), which is behind its popularity. It also frequently produces truffles several years earlier than other hosts (Hall et al., 1994; Giovanetti et al., 1994). Hall et al. (1994) noted that *Quercus* spp. produced truffles for several decades longer than *C. avellana*, so some plantations are established at a density of ca. 250–300 trees per hectare with a mixture of *C. avellana* and *Quercus* spp. and other species like *Ostya carpinifolia* Scop. to take advantage of early harvests from the *Corylus* and longer harvests from the longer-lived species. In fact, this thesis used *C. avellana* L. and *Q. pubescens* Willd. as plant hosts for mycorrhization, with *T. melanosporum* and truffle orchards as places to collect mycorrhized root tips for metagenomics studies.

These root tips belonged to *O. capinifolia*, *Q. ilex*, *Q. pubescens*, and *C. avellana* species, and they were used in the study. In addition to the host plants of *Tuber*, in most productive truffle grounds

there is a shrub component typical of mixed forest ecosystems formed by plant species that do not form ectomycorrhiza. The most frequently detected species are *Cornus mas*, *Cornus sanguinea*, *Crataegus monogyna*, *Rosa canina*, *Rubus caesius*, and *Sambucus nigra*.

These species, known as indicator plants or "wives", live in the same environments required for the development of truffle plants and probably play a role in maintaining the soil conditions suitable for the establishment of symbiosis. In fact, HTS technology now reveals unexpected fungi in diverse environments (McLaughlin et al., 2009; Nilsson et al., 2019). Some ECM fungi also colonize the living roots of non-ECM plants as endophytes, that is, they form loose associations without symptoms or ECM morphology (endophytism, *sensu* Wilson, 1995; Rodriguez et al., 2009). As reported by Schneider-Maunoury et al. (2020), endophytism was suggested for *Tuber* species in several non-ECM families, for the Burgundy truffle (*Tuber aestivum*; Gryndler et al., 2013, 2014) and the Périgord black truffle (*T. melanosporum*; Schneider-Maunoury et al., 2018). Instead, the growth of herbaceous plants is actively controlled by *Tuber*, which protects the host plant from possible competitors for nutrients by forming the "pianello," an area around the plant that is almost free of plants.

1.10.1. The hazelnut plant, *Corylus avellana* L

The hazelnut belongs to the order of *Fagales*, *Corylaceae* family, *Corylus* genus, which includes 25 species. The most important are *C. avellana* (the common hazel), *C. maxima* (the giant hazel), *C. colurna* (the Turkish hazel) and *C. pontica*. In its natural environment, the hazelnut tree has a characteristic bushy habit due to the numerous suckers that emerge from the stump, with a very extensive bundled root system and a depth that can vary depending on the characteristics of the soil. The trunk has a smooth, thin, and compact bark; the branches are provided with numerous lenticels; and the leaves, simple and alternate, of a dark green color, are ovoid, with a serrated margin and an underside covered by down (Figure 4A) (Cristofori, 2005). On the same plant, there are both male flowers, called catkins, pollen producers, and female flowers, recognizable by the characteristic red sprout (Figure 4B). The pollination of the hazel is anemophilous, that is, it occurs thanks to the wind. The fruit, with oily edible seeds, is spheroidal, grows in groups of 3–4 units, and is characterized by a thin, but leathery, light green shell which, as it ripens, darkens until it reaches the typical brown color.

The root system extends beyond the projection of the canopy and develops in-depth, mainly in the first 50 cm, depending on the nature of the soil and the cultivation techniques used (Tombesi, 1985). Hazelnuts are widely distributed in Europe, in natural stands ranging from Scandinavia to the south

of the continent (Palmè et al., 2002). Hazel generally grows as an understorey species in mixed deciduous forests (Kull and Niinemets 1993). Due to several adaptations at the leaf level, it can grow both in the sun (in full light) and in shade conditions (Catoni et al., 2015). It grows best on fertile and nutrient-rich, slightly acidic or neutral soils, but it can also thrive on dry calcareous soils (Savill 2013). Hazelnut prefers moderate climates with high temperatures during the growing season, but it can resist cold temperatures or even frosts (Savill 2013). In Turkey, an average temperature of 13-16°C and rainfall of over 700 mm is considered to provide optimal conditions for hazelnut cultivation (Ustaoglu and Karaca 2014). The most valuable part of this species is the fruit. From ancient times, hazelnuts have been among the main food components of our ancestors (Kubiak-Martens 1999). In present times, it is cultivated for its nuts and is one of the most economically important tree nut crops worldwide (Köksal et al., 2006). The nuts are rich in protein and contain significant amounts of vitamin E, thiamine, and magnesium. The most extensive corilicolous crops are located in Turkey, Italy and Spain, where a total area of 620,255 hectares is reached, distributed among 68% in Turkey, 11% in Italy, 3% in Azerbaijan and Georgia, 2% in Spain, and the USA; these surfaces constitute over 85% of the world's hazelnut groves, and a production of 858,700 tons is covered by 68% in Turkey, 11% by Italy, 4% by the USA, 3% by Azerbaijan and Georgia, and 2% each in Spain and Iran (Ismea elaboration of 2017 Faostat data).

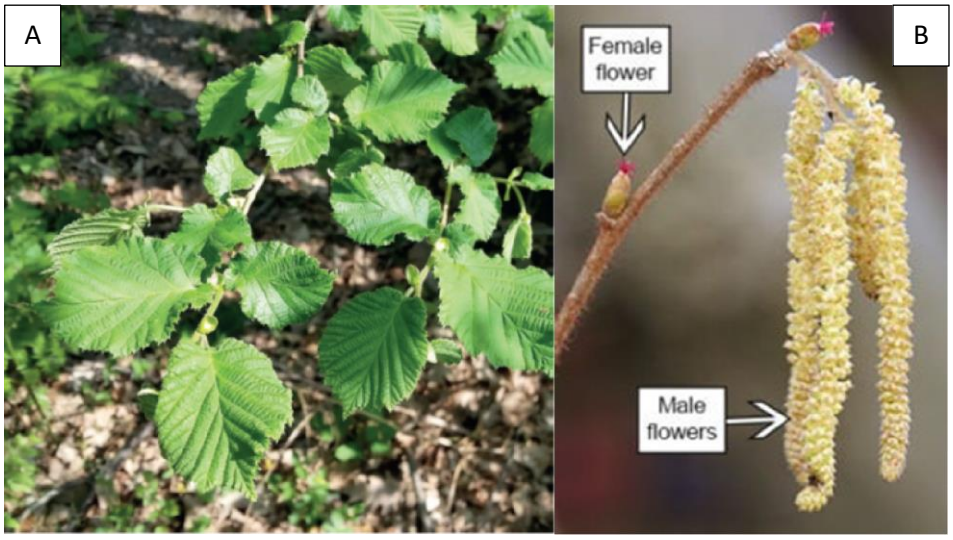


Figure 4. (A) Hairy leaves with double-serrated margins (Copyright Daniele de Rigo: CC-BY) and (B) male and female flowers (photo credit: Melissa McMasters, cc-by 2.0).

1.10.2. The downy oak, *Quercus pubescens* Willd.

The downy oak belongs to the order *Fagales*, the family of the *Fagaceae*, genus *Quercus*. It is a middle-sized (15–20m, rarely 25m tall) deciduous or semi-deciduous tree. Its alternate leaves are mostly ovate-oblong, (3) 5–10 cm long, with a short petiole (5 to 20 mm) and 5-6 deep lobes (Krüssmann 1978). Leaves are densely pubescent (the reason for their common names) and green-greyish when they start developing (Figure 5A); soon after, all the hairs on their upper side fall down and the leaves become leathery and dark green (Bussott 1998). Male flowers have 6–10 stamens and are grouped to form pubescent catkins, which grow up along with new leaves at the base of new shoots. The short-stalked female flowers have greenish stigmata and develop in the axil of the distal leaves. The fruits, often grouped in clusters of 3-4 acorns, have a short hairy petiole, are mostly elliptic (2–3.5 cm long), and are enclosed for ¼ to ½ of their total length by cupules, which are covered by imbricate triangular hairy scales (Figure 5B).

The species concept is particularly hard to apply to downy oak, primarily because of frequent hybridization with other sympatric deciduous oaks such as Pyrenean oak (*Quercus pyrenaica*) and Portuguese oak (*Quercus faginea*) (Valbuena-Carabana et al., 2005; Salvini 2009), sessile oak (*Quercus petraea*) and Hungarian oak (*Quercus frainetto*) (Fortini et al., 2015); secondly, the complex history of its climate-driven survival and migration during Pleistocene glaciations (Dumolin-Lapègue et al., 1997; Bordács et al., 2012); and finally, the fragmentation and isolation of its populations due to millennia of deforestation. In this way, *Quercus pubescens* sensu lato has a wide range, occupying almost all of central and southern Europe, from Western Spain (do Amaral Franco 1991; Rivas-Martìnez and Sáenz-Laìn 1991) to Balkan area, Black Sea, and the Caucasus. Considering its wide ecological niche, it is possible that downy oak wood covered very extensive surfaces all over Europe. Downy oaks show a very wide altitudinal range, especially in the southern countries. Although they are more common on hillsides between 200 and 800 m, they grow on coastal plains up to 1200 m. Indifferent to pH, they prefer lime-rich and well-drained soils in the northern part of their range, while they may also be common on acidic soils in the warmer countries (e.g., Sicily and Crete). It behaves as a heliophilous and thermophilous species and is perfectly adapted to stand both moderate summer drought stress and low winter temperatures, although it avoids continental locations subject to the most frequent frost and/or drought events (López-González 2001). Downy oaks are among the most frequent hosts of all the economically important truffles, such as *T. melanosporum, T. aestivum, T. macrosporum, T. magnatum* (Boutekrabt et al., 1989; Giomaro et al., 2002; Innangi et al., 2020; Chevalier and Frochot 1997; Granetti et al., 2005; Hall et al., 2007).

Considering the remarkable increase in tree ring size experienced by the downy oak in response to the augmentation of atmospheric CO_2 during the last century (Rathgeber et al., 2000), its increased use in Southern European afforestation could be a very effective tool to combat the greenhouse effect.

Figure 5. (A) Leaves are 5-10 cm long and densely pubescent when they start developing (Copyright Stefano Zerauschek, www.flickr.com: AP) and (B) acorns are covered up to half of their length by the hairy scaled cupule (Copyright Stefano Zerauschek, www. flickr.com: AP).

1.11. Truffle-Associated Bacterial Communities

Abiotic climatic controls and host requirements are vital for truffle production, but biotic environmental elements are also very important. Indeed, improved truffle cultivation needs more awareness of both environmental and soil conditions, as well as biotic elements that may encourage ECM establishment and truffle development. In this context, soilborne bacterial communities, a third partner in the symbiosis between the fungus and the plant root, appear to play a key role in the complex biological processes of exchange involving nutrients and signals from the soil hyphae, ECMs, ascomata, and stromata (Barbieri et al., 2016). In 1991, Duponnois & Garbaye first coined the term "mycorrhization helper bacteria" to refer only to bacteria that promote the establishment of the root–fungus symbiosis.

In the previous few decades, new findings have given rise to a novel interpretation of the MHB concept. As reported by Frey-Klett et al. (2007), the term "mycorrhiza" is misleading because it suggests that the bacterium helps the functioning of the already-established symbiosis. Instead, in most instances, the use of the word "mycorrhization" is preferred, which strictly relates to the process of mycorrhiza formation in the applied context of mycorrhizal inoculation (a technique commonly known as "controlled mycorrhization"). So "mycorrhization" will strictly refer to the bacteria that

help mycorrhiza formation, and "mycorrhiza" will refer to those that interact positively with the functioning of the symbiotic organ, keeping in mind that these two functional categories might be represented by different or overlapping groups of microorganisms.

Apart from this semantic clarification of the concept of MHB, these bacteria positively impact the functioning of mycorrhizal symbiosis, promoting the establishment of symbiosis by stimulating mycelial extension; increasing root–fungus contacts and colonization; facilitating truffle production (Mello et al. 2010) and the maturation process (Antony-Babu et al. 2014); and reducing the impact of adverse environmental conditions on the mycelium of the mycorrhizal fungi (Frey-Klett et al., 2007). Some MHB can also have additional beneficial impacts, such as direct stimulation of plant growth and protection against soilborne pathogens (Deveau and Labbè 2016). So, the complex truffle microbial ecosystem includes all the organisms (plants and fungi) and microorganisms living in a particular niche in which they interact in different ways to benefit each other, establishing synergistic relationships and exchanging metabolites. They act like multicellular organisms by synchronizing the behavior of all members of the group. It has been demonstrated that *Tuber* species interact with a diverse microbial population in each step of their life cycle, whether it is mycelium (vegetative phase), emanting hyphae and ECMs (symbiotic phase), or ascomata (fructification phase).

The MHB strains that have been identified to date belong to many bacterial groups and genera, such as gram-negative Proteobacteria (*Agrobacterium*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Bradyrhizobium*, *Enterobacter*, *Pseudomonas*, *Klebsiella*, and *Rhizobium*), gram-positive Firmicutes (*Bacillus*, *Brevibacillus*, and *Paenibacillus*) and gram-positive Actinomycetes (*Rhodococcus*, *Streptomyces*, and *Arthrobacter*). Gryndler and Hršelová (2012) isolated bacteria from the communities associated to *T. aestivum* ectomycorrhizae. Sbrana et al., (2002) studied the diversity of culturable bacterial populations associated to *Tuber borchii* ectomycorrhizas. Barbieri et al. (2007) investigated the occurrence and diversity of bacterial communities in *T. magnatum* Picco ascomata. The microbial community was also identified inside the ascomata of *T. borchii* (Barbieri et al., 2005) and Benucci & Bonito (2016) compared truffle microbiomes of the glebal tissue of eight truffle species including *T. oregonense*.

Similarly, to other truffle species ascocarps, the inner and outer parts of *T. melanosporum* ascocarps are deeply colonized by complex bacterial communities whose compositions change during the maturation of ascocarps (Antony-Babu et al. 2014). Different MHB, such as *Bacillu*s sp. and *Pseudomonas* sp., have been found from the early stages of mycorrhizal formation to the complete maturation of the ascocarps of *T. melanosporum* (Deveau et al., 2016; Rivera et al., 2010; Antony-

Babu 2014; Dominguez et al., 2012; Mamoun & Oliver 1992). It has also been suggested that some bacteria may play a role in the development of the characteristic truffle aroma of *T. borchii* (Barbieri et al., 2000; Splivallo et al., 2012). Although new information about the microbial diversity of *Tuber*-associated bacteria in various stages of the truffle life cycle and the surrounding environment, the role of truffle-associated bacteria is still largely unknown. Recent findings on the helper microbial community associated with truffles clearly show the benefits of using advanced metagenomics to unravel the complex network of biotic interactions in soil ecosystems (Barbieri et al. 2005, 2007; Gryndler et al. 2013; Antony-Babu et al. 2014; Mello et al. 2013). Indeed culture-independent molecular approaches, such as high-throughput technologies, allow now the analysis of the microbial community that exists in nature as a complex and interdependent multiorganism, critical for advancing our understanding of the mysterious truffle system. This new era of predictive microbial ecology will provide us with a much deeper understanding not only of the diversity of truffle-associated microbial communities, but also their function within the truffle ecosystem (Barbieri et al., 2016).

1.11. Aims and objectives of the thesis

The mutualistic symbiosis between fungi and host plants through ectomycorrhizae is an essential and long-term stage in the life cycle of truffles, which also plays an important role in the ecological environment (Mello and Balestrini, 2018). The quality of truffle-colonized seedlings, including truffle colonization level and host plant growth and physiology, is an important factor that determines the production of truffle fruiting bodies in plantations during the early symbiotic stage (Andres-Alpuente et al., 2014), which is critical for successful truffle cultivation. This thesis has the general aim of contributing to the understanding of the critical phases of the truffle supply chain, contributing to their resolution more sustainably, and investigating the biotic factors that affect *Tuber melanosporum* and *Tuber aestivum* growth and development. Nevertheless, the rhizospheric environment, which includes both the physical-chemical component of the substrate and its biotic component (soil microorganisms), can act by stimulating and helping the mycorrhization process, such as slowing it down and preventing its satisfactory outcome, which are important aspects of being taken into consideration. Indeed, there are still several aspects of the production chain that need to be optimized, including mycorrhization techniques, the development of substrates to maintain symbiosis, and the identification of microorganisms useful for improving the mycorrhization process. For these reasons, the principal objectives of this thesis were the following:

- To examine the bacterial and fungal biodiversity associated with ECMs of *Tuber aestivum* and *Tuber melanosporum* in productive plantations with a metagenomic approach (high-throughput sequencing) and identify candidate MHBs;
- To offer a valid alternative to the use of forest soil in cultivation potting mixes, especially in consideration of the interdition of the removal from forest areas of any organic material that constitutes the soil cover (leaves, humus, organic soil, turf) (art. 64, Official Journal). Commercial substrates with different organic matrices, such as green compost and peat, were then tested to evaluate their effect on *T. melanosporum* colonization and plant root system development;
- To obtain an insight on the culturable fraction of microorganisms that colonize the ectomycorrhized root tips of *T. melanopsorum* in productive plantations;
- To test in mesocosms the efficacy of candidate bacterial taxa in supporting the mycorrhization with *Tuber melanosporum*, and the development of the root system of *Corylus avellana* and *Quercus pubescens* seedlings.

2. Material and Methods

2.1. Metagenomic analysis of bacterial community associated with *Tuber aestivum* and *Tuber melanosporum* ectomycorrhized root tips

2.1.1. Root samples

In June and July 2019, ectomycorrhized root tips were sampled from 38 host plants in two different locations in Italy. Table 1 reports the geographical locality, identification number of the plantation, ectomycorrhizal symbionts, host plant species, number of sampled plants per plantation, and the age of the implants. In Figure 6 is represented the three plantations sampled in the Umbria region.

Table 1. The scheme of the sampling implants is divided into locality; the number of plantations; ectomycorrhizal symbionts; host plant species; number of sampled plant per plantation; and age of the implant.

Location	Plantation	*Tuber* spp.	Host Plant	N° of Plants	Age of the implant (yrs)
Bracciano (RM)	1	*T. aestivum*	*Q. pubescens*	4	15
Bracciano (RM)	1	*T. melanosporum*	*Q. ilex*	4	15
Scheggino (PG)	1	*T. melanosporum*	*Q. pubescens*	7	4
Scheggino (PG)	1	*T. melanosporum*	*O. carpinifolia*	2	4
Scheggino (PG)	1	*T. melanosporum*	*Q. ilex*	1	4
Scheggino (PG)	2	*T. melanosporum*	*Q. pubescens*	7	3
Scheggino (PG)	2	*T. melanosporum*	*O. carpinifolia*	2	3
Scheggino (PG)	2	*T. melanosporum*	*Q. ilex*	1	3
Scheggino (PG)	3	*T. aestivum*	*O. carpinifolia*	8	30
Scheggino (PG)	3	*T. melanosporum*	*C. avellana*	2	30

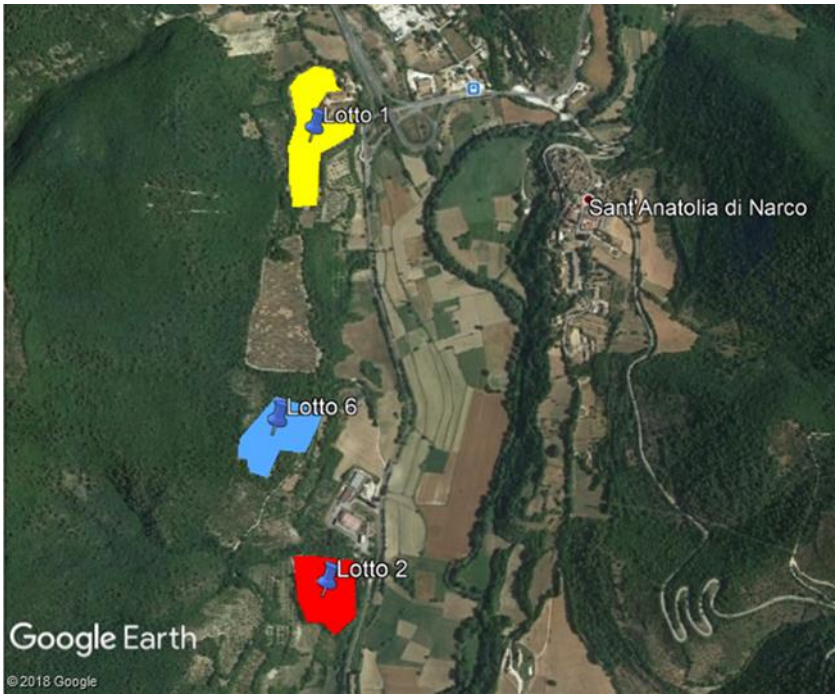


Figure 6. Map of the three sites sampled in Scheggino (PG). Site 1 is approximately 2 hectares in size and consists of 4-year-old plants (70% downy oak, 20% black hornbeam, and 10% holm oak) mycorrhized with *T. melanosporum*; Site 2 is approximately 2 hectares in size and consist of 3-years-old plants (70% downy oak, 30% black hornbeam and 10% holm oak) mycorrhized with *T. melanosporum*; Site 6 is approximately 2 hectares of 30-years-old plants most belonging to black hornbean – *T. aestivum* and a little presence of *C.avellana* – *T. melanosporum*.in a mixed orchard that produces *T. aestivum* and *T. melanosporum*.

2.2.1. Sample preparation for HTS

A total of 200 ectomycorrhized root tips for each truffle species and host plant were selected under the dissecting microscope (Sbrana et al., 2002). The roots were then surface sterilized by immersion in 30% (v/v) hydrogen peroxide for 15 s and rinsed three times in sterile distilled water (Ali and Jackson 1989). To describe the bacterial community associated with the symbiotic apparatus of the ectomycorrhiza, the root tips were homogenized in 1 mL of sterile distilled water and the homogenates were used for genomic DNA extraction using the kit "Nucleospin Soil" (Macherey-Nagel Gmbh & Co., Düren, Germany) and following the producer's instructions. For each sample, the extraction was done twice to obtain more genomic material. Every PCR mix consisted of 12,5 µl of Hot Start PCR Master Mix (2X) (Thermo Fisher Scientific, USA), 8,5 µl of distilled water, 1 µl for each primer, 1 µl of BSA (Bovine Serum Albumin), and 1 µl of template, for a total volume of 25 µl. A mock community constituted by a DNA mix of known taxa of bacteria was used as a

control to validate the HTS results. The PCR reaction was carried out using the following primers for the amplification of the 16S ribosomal RNA region: S-D-Bact-008-a-S-16 (5-xxxxAGAGTTTGATCMTGGC-3) and S-D-Bact-0343-a-A-15 (5-xxxxCTGCTGCCTYCCGTA-3) (where xxx represents the barcoding key). The internal transcribed spacer 1 (ITS1) was amplified in a multiplexing PCR using a set of barcoded primers with a dual indexing primer using the tagged primer pair ITS1F (5-xxxxCTYGGTCATTTAGAGGAAGTAA-3) and ITS2 (5-xxxxGCHRCGTTCTTCATCGDTGC-3) for fungi. For the amplification, a Multigene Gradient Thermal Cycler (Edison, NJ, USA) was used with the following thermal cycle for bacteria: denaturation cycle of 3 minutes at 95°C, denaturation of 30 seconds at 95°C, annealing at 48°C for 30 seconds with extension at 72 °C for 1 minute for 30 cycles, and a final extension at 72°C for 15 minutes. For fungi, the thermal cycle was an initial denaturation at 94°C for 10 min followed by 30 cycles of 95 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min, and a final elongation step of 72 °C for 10 min. The presence of the amplicons was determined through electrophoresis on a gel with 1% of agarose and visualized by staining with Gel Red (Biotium). Following that, the PCR products were purified according to the protocol "Agencourt Ampure XP" (Beckman Coulter, Beverly, Massachusetts, USA) and quantified via "Quant-it Assays" (Invitrogen, Eugene, Oregon, USA). After that, samples were pooled at equal concentrations for sequencing. Paired-end sequencing (2x300 bp) was done on an Illumina MiSeq V3 sequencer by Fasteris SA (Switzerland).

2.1.3. Bioinformatic analysis

To decrease the occurrence of false assignments and cross-contamination, only the reads having the combination of 5’ barcode and forward primer and the expected 3’ barcode and reverse primer were paired and considered for further analysis. The use of CLC Genomic Work bench Version 8.5.1 (QIAGEN bioinformatics, Aarhus, Denmark) allowed for quality filtration (limit = 0.05) and trimming, excluding all sequences with "N"s. Following this, the paired-end reads were merged. If some mismatches were recorded between the overlapping fragments of the forward and reverse reads, these were adjusted according to the base call with the higher sequencer-assigned quality score. After quality filtering, paired-end assembly, and demultiplexing, the sequences were processed, and similarity clustering was performed based on the UPARSE pipeline of USEARCH v8 (Edgar, 2010) using a 98 % clustering threshold. Sequences failing alignment, or identified as chimeric, were removed before downstream analysis. To identify the resulting OTUs, they were uploaded as FASTA files in the BLAST tool of the Genbank database with the following algorithm

parameters: max target sequence = 50; expect threshold = 0,05; word size = 28; max matches in a query range = 0; match/mismatch scores = 1, -2; gap cost = linear. The resulting xml file was imported together with the FASTA file into MEGAN (Huson et al., 2007) to organize and adjust the results. To support this process was employed the NCBI taxonomy. The lowest common ancestor (LCA) parameters were Min score = 150; Max expected = 0,01; Top percent = 5; Min support percent = 0,05; Min support = 1, and Percent to cover = 40. At the same time, the minimum requirements of similarity to accept the taxonomy were: Species 99 %; Genus 97 %; Family 95 %; Order 90 %; Class 85 %; and Phylum 80 %. The open-source Qiime2 (Bolyen et al. 2018) was used to perform several types of analysis for the investigation of differences within and between the samples. Alpha-diversity metrics used to calculate the diversity within the community in *Tuber* and Plant species were: OTUs richness (observed OTUs), a measure of the number of OTU found; Phylogenetic diversity (Faith's PD) that uses phylogenetic distance to calculate the diversity of a given sample; and a combination of richness and diversity (Shannon-Wiener index), that measures both the number of species and the inequality between species abundances (values start from 0 in case of a single dominant species and can exceed 1 in case of all species having equal abundance) (Iotti et al., 2010; Chiarucci et al. 2011). These indexes were analyzed using the non-parametric Kruskal-Wallis and Mann-Whitney tests. The similarity values between the communities (Beta-diversity) present in *Tuber* and Plant spp. were calculated using dissimilarity indexes not based on the phylogenetic tree (Bray-Curtis and Jaccard distance) and indexes phylogeny-based for the assessment of differences in overall bacterial community composition (Weighted and Unweighted UniFrac) (Lozupone et al. 2005; Lozupone et al. 2007). Bray–Curtis dissimilarity is based on microbial abundance between two samples (e.g., at species level); values are from 0 to 1, where 0 means both samples share the same species at exact the same abundances and 1 means both samples have completely different species abundances; Jaccard distance is based on presence or absence of species (does not include abundance information) different in microbial composition between two samples, where 0 means both samples share exactly the same species and 1 means both samples have no species in common. UniFrac is based on the fraction of branch length that is shared between two samples or unique to one or the other sample; unweighted UniFrac: purely based on sequence distances (does not include abundance information); weighted UniFrac: branch lengths are weighted by relative abundances (includes both sequence and abundance information). For all the indexes, multivariate analyses were performed using permutational analysis of variance

(PERMANOVA) with 999 permutations of residuals under a reduced model (Leonardi et al.,2013). Factors or interactions were considered statistically significant if p-value <0.05.
Significant terms were then investigated using *a posteriori* pair-wise comparisons with the PERMANOVA t statistic and 999 permutations. Venn diagrams were developed to illustrate the number of OTUs shared between the Plant and *Tuber* spp. These graphs were performed using an online application at http://bioinformatics.psb.ugent.be/webtools/Venn. A LEfSe analysis was performed to find specific taxa that could significantly affect sample structure at different levels as the basis of a strategy on the LDA threshold. (http://huttenhower.sph.harvard.edu/galaxy).

2.2. Soil-less potting mix for the production of high-quality mycorrhizal plants

2.2.1. Plant and truffle material

Acorns from *Q. pubescens* provided by the company Truffleland s.r.l. (Scheggino, Italy) were surface sterilized in a 5% sodium hypochlorite solution for 15 minutes and soaked in distilled water. Suckers from *C. avellana* var. Nocchione, provided by the nursery Vivai Michelini (Viterbo, Italy), were stripped of existing roots, brushed, and sterilized in a 5% sodium hypochlorite solution for 15 minutes and rinsed with water (Benucci et al., 2012a). After sterilization, the plant material was put in polystyrene pots containing sterile perlite and placed in a heated bed greenhouse until germination and/or rooting. A total of 180 seedlings of *Q. pubescens* and 165 saplings of *C. avellana* were transplanted in 9x9x13 cm-deep trays containing the three different sterilized potting mixes. During the winter season, fresh and mature ascomata of *T. melanosporum* were provided by Truffleland s.r.l. (https://www.truffleland.com), cleaned with a brush to remove soil, washed in water, and stored at -20 °C until use (Benucci et al., 2012b). The truffles chosen for the inoculation were examined externally and microscopically to assess their identity and the presence of mature spores. For inoculations, the truffle spore-slurry, which was prepared by blending frozen truffles with water, was dispensed to each seedling in the proportion of 1 g/plant, using a rotating dispenser that guarantees the homogeneity of the inoculum.

2.2.2. Potting mixes

The potting mixes used for this experiment were two different sterilized green compost-based substrates from i) a composting plant of the Acea group (https://www.gruppo.acea.it/) (Mix1) supplemented with perlite (30%), sand (20%), and a slow-release fertilizer called Osmocote^{TM} (2 kg/m^3), and ii) the company Mechelli (http://www.terriccimechelli.it/it/), sold under the brand Flora

Green (Mix 2) containing peat rich in organic matter, correctly fermented and supplemented with perlite (30%), sand (20%) and Osmocote^{TM} (2 kg/m^3). The potting mix used by Truffleland s.r.l. company for routine mycorrhization, containing forest soil, was used as a control thesis. All the substrates used in this assay were subjected to soil analysis at AgriBioEco Company for the following soil determinations: pH, total organic carbon (T.O.C.), organic matter, total and active limestone, granulometric analysis, total nitrogen, and trace element assessment (Table 2).

Table 2: Chemical-physical analysis of complete potting mixes: Mix 1: green compost from Acea Company; Mix 2: green composts from Mechelli Company and potting mix from Truffleland s.r.l. as control potting mix. The three substrates were used for the mycorrhization of *Q. pubescens* and *C. avellana* cuttings with *T. melanosporum*.

Physical-Chemical analysis	**Mix 1**	**Mix 2**	**CTL**
pH (unit)	7.1	7.4	7.7
Total limestone (%)	16.6	19.0	41.6
Active limestone (%)	1.7	1.2	12.1
Organic C (%)	8.03	7.18	3.43
Organic matter (%)	13.86	12.39	5.92
Sand (%)	76	76	29
Silt (%)	13	12	30
Clay (%)	11	12	41
Texture	Sandy loam	Sandy loam	Clayey
N total (%)	0.683	0.614	0.320
C/N	11.7	11.6	10.7
Calcium (Ca) (ppm)	5940	6300	5220
Magnesium (Mg) (ppm)	602	645	154
Potassium (K) (ppm)	2290	2524	355
Phosphorus (P) (ppm)	110	92	10

2.2.3. Experimental design

After mycorrhization procedures (see section 2.3.5), 20 seedlings for each substrate replicated three times (60 seedlings/thesis) were put in plastic pots. These were arranged in randomized blocks for each thesis in the Truffleland s.r.l. greenhouse, watered every day from June to August and every 2 days from September to January. The total duration of the experiment was eight months. For assessment of morphometric plant parameters and level of mycorrhization see sections 2.3.8 and 2.3.9 respectively.

2.3. Characterisation of bacteria from *Tuber melanosporum* ectomycorrhizae and their effects on root-fungus interactions and growth of *Quercus pubescens* Willd. and *Corylus avellana* L. seedlings

2.3.1. Sampling and isolation of bacteria

In July 2019, ectomycorrhizal root tips of 4-year-old *Quercus pubescens* plants (Table 1) were sampled. The root system of the plants was separated from the soil, and, under the dissecting microscope, 200 ectomycorrhizal root tips of *T. melanosporum* were selected (Sbrana et al., 2002). The roots were then surface sterilized by immersion in 30% (v/v) hydrogen peroxide for 15 s and rinsed three times in sterile distilled water (Ali and Jackson 1989). A homogenate was obtained from the roots in 1 ml of sterile physiological solution (NaCl 0.85%). Serial dilutions from 10^{-1} to 10^{-6} of the homogenates were prepared and 100 µL were plated onto three replicate plates of different culture media: Tryptic Soy Agar (TSA, Difco) for aerobic spore-forming bacteria and King's B for bacteria belonging to the genus *Pseudomonas* (Sbrana et al., 2002). The colonies grown on King's B medium showing fluorescence under UV rays and the colonies grown on TSA medium were isolated and a single colony from each plate was picked up, resuspended in 400 µL of lysis buffer PL1 (0.05 M NaOH, 0.25% SDS), heated to 95 °C for 5 min, and then total DNA was extracted using the NucleoSpin Plant II kit (Macherey-Nagel; Düren, Germany), following the manufacturer's instructions.

2.3.2. Molecular characterization of isolates

Bacterial isolates were identified using the 16S ribosomal RNA region. The PCR was performed with 1 µl of each diluted bacteria isolate in a 25 µl volume, with 1µl each of the primers 27f (5'-GAGAGTTTGACTCTGGCTCAG-3') and 1495r (5'-CTACGGCTACCTTGTTACGA-3'), 1 µl of BSA, 12,5 µl of Go Taq Hot Start (Promega; Wisconsin, United States) and distilled water. Negative controls (reaction mix with water replacing the template) were always included. The reaction mixtures were incubated in a thermocycler (C1000 Thermal Cycler) at 95 °C for 2 min and then cycled 25 times through the following temperature profile: 95 °C for 1 min, 58 °C for 45 s, and 72 °C for 30 s. Finally, the mixtures were incubated at 72 °C for 5 min (a modification of Sbrana et al., 2002). After the PCR reaction, the samples were purified using the NucleoSpin Gel and PCR Clean-up Reagent kit (Macherey-Nagel; Düren, Germany), according to the manufacturer's instructions. The purified products were loaded onto a 1.5% agarose gel.

The suitably prepared samples were sent to Eurofins genomics (Luxemburg; http://www.eurofins.com) for Sanger sequencing. The sequences obtained were analyzed using the Bioedit Sequence Alignment Editor software and were compared with those present in the NCBI (National Center for Biotechnology Information) database using the NCBI Blast function (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To confirm the identity of *Paenibacillus polymyxa*, biochemical analysis on the Phenol Red Test supplemented with glycerol as a sugar source was performed, incubating it at 35 °C for 24 hours (Schau, 1986; Von der Weid et al., 2002). Specie-specific primers were used to further analyze *Priestia megaterium* and *Serratia marcescens* strains because the amplification of the 16S ribosomal RNA region for these strains was not sufficient to achieve 100% identity. The amplification of the recA gene of *P. megaterium* (Zou et al., 2010) and the gyrB gene of *S. marcescens* (Scrascia et al., 2016), using the same protocols described above, confirmed their molecular identity.

2.3.3. Preparation of bacterial inoculum for the *in vivo* test

Bacterial strains selected (*Pseudomonas fluorescens* OL831225, *Pseudomonas jessenii* OL831226, *Paenibacillus polymyxa* OL831227, *Priestia megaterium* OL873255 and *Serratia marcescens* OL873254), were cultured in glass flasks containing 3 g L^{-1} liquid NB medium (Nutrient Broth) for 1 day at 25°C. The concentrated bacterial suspensions (more than 10^6 cells per ml) were centrifuged (2400 g, 10 min), the supernatant was eliminated, and the pellet was resuspended in 400 mL of 0.1 M $MgSO_4 \cdot 7\ H_2O$ buffer. The bacterial inoculum densities were then adjusted to $1x10^6$ cfu mL^{-1}. The concentrations were determined by measuring the absorbance (a = 600 nm) of the suspensions. Correlations between the absorbance and the living cell concentration (cfu mL^{-1}) had been established earlier. Five ml of bacterial suspension in 0.1 M $MgSO_4 \cdot 7\ H_2O$ buffer were injected into each pot with a sterile tip (Duponnois and Garbaye, 1991).

2.3.4. Plant material

Acorns from *Q. pubescens* provided by the company Truffleland s.r.l. (Scheggino, Italy) were surface sterilized in a 5% sodium hypochlorite solution for 15 minutes and soaked in distilled water. Suckers from *C. avellana* var. Nocchione provided by the nursery Vivai Michelini (Viterbo, Italy), was stripped of existing roots, brushed, and sterilized in a 5% sodium hypochlorite solution for 15 minutes, and rinsed with water (Benucci et al., 2012a). Four-month-old rooted seedlings were transplanted into 9x9x13 cm-deep trays for fungal and bacterial inoculation.

2.3.5. Fungal inoculum and inoculation

During the winter season, fresh and mature ascomata of *T. melanosporum* provided by Truffleland s.r.l. (https://www.truffleland.com), were cleaned with a brush to remove soil, washed in water, and stored at -20 °C until use (Benucci et al., 2012b). The truffles chosen for the inoculation were examined externally and microscopically to assess their identity and the presence of mature spores. For inoculations, the truffle spore-slurry, which was prepared by blending frozen truffles with water, was dispensed to each seedling in the proportion of 1 g/plant, using a rotating dispenser that guarantees the homogeneity of the inoculum.

2.3.6. Glasshouse experiment

The three components of the system (bacteria, ectomycorrhizal fungus *T. melanosporum*, *Q. pubescens* and *C. avellana* cuttings) were placed in 0.5 l pots filled with the mix used by Truffleland s.r.l. for routine mycorrhization (Table 1). Pots were inoculated following the protocol described in section 2.3.3 and 2.3.5. Control treatment consisted of the fungus and the buffer solution (0.1 M $MgSO_4 \cdot 7\ H_2O$) with no bacteria (Duponnois and Garbaye, 1991).

2.3.7. Experimental design

The experiment was designed as a randomized block factorial experiment. Each block consists of seven treatments with 20 plants per experimental unit for *Q. pubescens* s and *C. avellana*, replicated three times, for a total of 840 plants/saplings (Aspray et al., 2006). The total duration of the experiment was nine months.

2.3.8. Morphometric assessment of plants

Seedlings of experiments 2.2 and 2.3 were measured for shoot height and stem collar diameter at the moment of inoculation and at the end of the experiment to assess the extent of growth during their cultivation. After 8 months of the inoculation experiment described in section 2.2 and 9 months of the inoculation experiment described in section 2.3, the root traits of each seedling were assessed by gently washing the lateral roots to remove residual debris and severed from the shoot at the root collar.. The roots were spread into specific Plexiglas trays to minimize overlapping roots during the scanning process. A two-dimensional image of each root was collected using the scanner (Epson Perfection V700 Photo Scanner system, Epson America, Inc., Long Beach, CA, USA) to capture an image of the root sample. Scanned images were analyzed by the WinRHIZO® software

package (v. 5.0, Regent Instruments, Inc., Quebec, QC, Canada) following the program for total root length (RL, calculated using a one-pixel thinned image and multiplying the number of pixels by pixel size), average root diameter (AVD, calculated by dividing the projected area of the imaged root by the total length), number of root tips (TIP), root volume (RV, calculated using the root surface area and length), root project area (RPA) and root surfaced area (RSA, calculated by determining the root diameter and length) (Wang et al., 2009; Piñuela et al., 2020). Furthermore, after proceeding to the qualitative and quantitative evaluation of the mycorrhizae, seedling shoots and roots were oven-dried (80 °C for 2 weeks) to obtain dry weights (Rincón et al., 2005).

2.3.9. Assessment of percent of mycorrhization

To visualize ECMs, the root system was gently washed and placed in a Petri dish containing water under a stereomicroscope (Visioscope ZTL350, VWR) (Avis et al., 2003). EM colonization levels were estimated using the relative abundance valuation (RAV) following the methodology described by Bencivenga et al. (1995). To calculate the RAV, the root system was divided into two main sections: one proximal and one distal; every portion was sub-divided into 2 sections, and in each of the four sections selected, 50 root tips were counted, for a total of 200 root tips for each seedling (RAV). The abundance of ECMs and non-ectomycorrhizal root tips was calculated by dividing the number of tips counted for each group by the total number of tips (200).

2.3.10. Molecular identification of *Tuber melanosporum* ECMs

Total genomic DNA (gDNA) of 200 root tip samples of each plant was extracted using the NucleoSpin Plant II kit (Macherey-Nagel; Düren, Germany), following the manufacturer's instructions. The gDNA of the various samples was then amplified with species-specific primers, as described by Sèjalon-Delmas et al. (2000). Primers TM1b and ITS4 were used for *Tuber melanosporum* amplification. Go Taq Hot Start (Promega; Wisconsin, United States) was used for all amplifications, following the manufacturer's instructions. Both positive and negative controls were used at each stage. PCR amplifications were done according to the protocol reported by Sèjalon-Delmas et al. (2000). After the PCR reaction, the samples were purified using the NucleoSpin Gel and PCR Clean-up Reagent kit (Macherey-Nagel; Düren, Germany), according to the manufacturer's instructions. The purified products were loaded onto a 1.5% agarose gel. To further confirm their identity, the suitably prepared samples were sent to Eurofins genomics (Luxemburg; http://www.eurofins.com) for Sanger sequencing. The sequences obtained were

analyzed using the Bioedit Sequence Alignment Editor software and were compared with those present in the NCBI (National Center for Biotechnology Information) database using the NCBI Blast function (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3.11. Estimation of bacterial survival

At the end of the experiments, fifty root tips from three of each *Q. pubescens* and *C. avellana* cuttings from each treatment were pooled together for the reisolation of the bacteria inoculated. Under the flow cabinet, the ectomycorrhized apical roots were sterilized with 3% H_2O_2 and rinsed twice in sterile distilled water (Sbrana et al., 2002). The root tips were then homogenized in 1 mL of sterile distilled water and the homogenates were used for the serial dilution to plate 10^{-2}, 10^{-3}, and 10^{-4} dilutions on TSA and KB media. After one day of growth at 25 °C, the different colonies were picked up based on the morphology of the isolates used for the inoculation trail. The colonies were stained with gram staining to differentiate Gram + from Gram - strains, and the DNA of the selected colonies was extracted following the protocol described above for the molecular identification of the strains. For the amplification, were used the primers that amplified the 16S ribosomal RNA region and the specific primers for recA and gyrB genes. After the PCR reaction, the samples were purified using the NucleoSpin Gel and PCR Clean-up Reagent kit (Macherey-Nagel; Düren, Germany), according to the manufacturer's instructions. The purified products were loaded onto a 1.5% agarose gel. The suitably prepared samples were sent to Eurofins genomics (Luxemburg; http://www.eurofins.com) for Sanger sequencing. Analysis of the sequences was done with the Bioedit Sequence Alignment Editor software, and the NCBI Blast function was used to compare them to other sequences in the NCBI database (www.blast.ncbi.nlm.nih.gov).

2.3.12. Statistical Analyses

Both seedling measurements and percent of mycorrhization datasets were checked for normality distribution with the D'Agostino & Pearson test, Anderson-Darling test, and Shapiro-Wilk tests (α=0,05). In some cases, data log transformation was needed (Anscombe 1948; Freeman and Tukey, 1950). Normalized data were analyzed by one-way ANOVA using Tukey's post hoc test ($P<0.05$). All the analyses were carried out with GraphPad Prism version 8.00 (GraphPad Software, San Diego, CA, USA) (http://www.graphpad.com/).

3. Results

3.1. High throughput sequencing analyses

From the 38 samples, after filtration and trimming, 500 and 1015 OTUs were detected for fungi and bacteria, respectively. Each of them was defined at the highest taxonomic level possible: Phylum, Class, Order, Family, Genus, and Species within the ECMs of *T. aestivum* and *T. melanosporum* truffle grounds. This methodology allowed to discover that plants of *Q. pubescens*-*T. aestivum* collected on the truffle ground in Bracciano (RM) (Table 2), were mycorrhized with another species of truffle, *Tuber mesentericum* (73% of total reads), that were excluded from the analyses.

3.1.1. Alpha Diversity

Alpha-Diversity metrics were analyzed according to the category *Tuber* spp. and plant species, to evaluate the average microbial richness of every group and compare them with statistical tests.

3.1.1.1. Alpha Diversity within *Tuber* spp.

The average biodiversity richness of root samples mycorrhized with *T. aestivum* and *T. melanosporum* was performed using: Shannon diversity, Observed OTUs, Faith pd, and Pielou's evenness indices for bacterial and fungal diversity, validated with the Mann Whitney (MW) statistical test. Concerning the bacterial diversity, the value of the Faith pd index of root samples mycorrhized with *T. melanosporum* was significantly higher (MW = 9, p-value < 0,0001) than *T. aestivum* (14,86 ± 2,65 and 11 ± 0,9, respectively) while there were no significant differences with the other indexes considered (Table 3).

Table 3. Ectomycorrhizal species factor (*T. aestivum* and *T. melanosporum*) is analyzed for alpha diversity of bacterial community present in root tips. Mann-Whitney tests are used to evaluate the statistical significance of the results.

Bacteria			
Alpha diversity indexes	***T. aestivum***	***T. melanosporum***	**Significance M.W.**
Shannon diversity	4,92 ± 0,41	4,97 ±0,9	M.W.= 79 p = 0,62
Observed OTUs	130±12	133,19 ± 27,91	M.W.= 79 p = 0,61
Faith_ pd	**11 ± 0,9**	**14,86 ± 2,65**	**M.W.= 9** **p<0,0001**
Pielou's evenness	0,7 ±0,05	0,32 ± 0,18	M.W.= 81 p = 0,68

Concerning the fungal biodiversity shown in Table 4, the *T. aestivum* factor showed a higher absolute value for each of the indexes analyzed. Specifically, the number of observed OTUs of *T. aestivum* (74 ± 18) was significantly higher than of *T. melanosporum* (49,92 ± 22,95; MW= 34,5, p value < 0,01). Also, the Faith pd values of *T. aestivum* (20 ± 3,2) significantly increased with respect to *T. melanosporum* (15,18 ± 5,07; MW= 33, p value = 0,009).

Table 4. Ectomycorrhizal species factor (*T. aestivum* and *T. melanosporum*) is analyzed for alpha diversity of fungal community present in root tips. Mann-Whitney tests are used to evaluate the statistical significance of the results

Fungi			
Alpha diversity indexes	***T. aestivum***	***T. melanosporum***	**Significance M.W.**
Shannon diversity	2,4 ± 0,9	1,86 ±1,12	M.W.= 63 p= 0,23
Observed OTUs	**74 ± 18**	**49,92 ± 22,95**	**M.W.= 34,5 p=0,01**
Faith_ pd	**20 ± 3,2**	**15,18 ± 5,07**	**M.W.= 33 p=0,009**
Pielou's evenness	0,39 ± 0,14	0,32 ± 0,18	M.W.= 73 p=0,4

3.1.1.2. Alpha Diversity within plant species

Pielou's evenness and Faith-pd indexes were used to assess the bacterial and fungal alpha diversity of *C. avellana*, *Q. pubescens*, *Q. ilex*, and *O. carpinifolia* plants mycorrhized with *T. melanosporum*. The bacterial diversity composition for the Faith-pd index analyzed with the Kruskal-Wallis (KW statistical test showed that the bacterial abundance on *Q. ilex* plants was significantly higher than on *Q. pubescens* plant roots (KW H = 6.5, p value = 0.01), although for the other plants there were no statistical differences with the two indexes analyzed. Concerning the fungal biodiversity, within *C. avellana*, *Q. pubescens*, *Q. ilex*, and *O. carpinifolia* plants mycorrhized with *T. melanosporum*, the biological variety inside each category had a different magnitude. The abundance of fungal biodiversity gathered with the evenness index resulted in a significantly higher value for *Q. ilex* plants than for *O. carpinifolia* (KW H = 6.54, p-value = 0.01) and *Q. pubescens* plants (KW H = 6.96, p-value = 0.008).

3.1.2. Beta Diversity

Beta diversity analysis was carried out for the categories "Plants" mycorrhized with *T. melanosporum* (*Q. pubescens*, *Q. ilex*, *C. avellana*, *O. carpinifolia*) and inside the *Tuber* spp. (between *T. aestivum* and *T. melanosporum* orchards). Bray-Curtis, Jaccard, weighted and unweighted UniFrac similarity coefficients were used.

3.1.2.1. Bacterial Beta Diversity

Concerning the bacterial biodiversity among plants mycorrhized with *T. melanosporum*,the statistical general results are reported in Table 5.

Table 5. Two-way PERMANOVA tests: pseudo-F and p-values for bacterial biodiversity among plants mycorrhized with *T. melanosporum.*

Biodiversity indexes	**PERMANOVA significance**	
	Pseudo-F	**p-value**
Bray-Curtis	2.14	0.001
Jaccard	1.93	0.001
weighted UniFrac	1.93	0.03
unweighted UniFrac	1.92	0.002

In figure 7 is reported the PCoA of Bray-Curtis similarity coefficient, showing the differences reported in Table 5. The pair-wise results among *Q. ilex* and *Q. pubescens* (pseudo-F value = 2.66; p-value = 0.003) and between *C. avellana* and *Q. pubescens* (pseudo-F value = 2.72; p-value = 0.012) were statistically significant

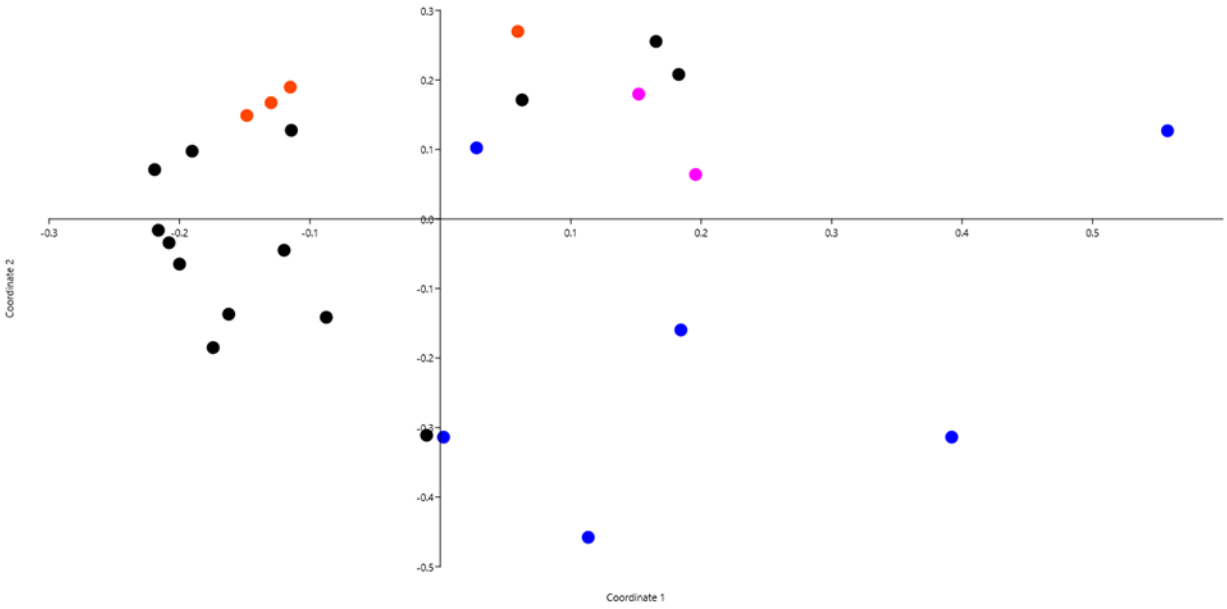


Figure 7. Principal Coordinates Analysis (PCoA) based on Bray-curtis similarity coefficient of the bacterial community of *Q. pubescens*, *Q. ilex*, *O. carpinifolia* and *C. avellana* mycorrhized with *T. melanosporum*. Black dots indicate *Q. pubescens*, blue dots *Q. ilex*, orange dots *O. carpinifolia* and pink dots *C. avellana* plants.

The results of bacterial diversity inside the category of *Tuber* spp. showed statistically significant differences between the two species for all the coefficients analyzed with the PERMANOVA statistical test except for the weighted UniFrac index (Table 6). In Figure 8 the PCoA of the unweighted UniFrac similarity coefficient shows these differences.

Table 6. Two-way PERMANOVA tests: pseudo-F and p-values for bacterial biodiversity between *T. aestivum* and *T. melanosporum.*

Biodiversity indexes	**PERMANOVA significance**	
	Pseudo-F	**p-value**
Bray-Curtis	1.82	0.01
Jaccard	2.2	0.002
weighted UniFrac	1.06	0.3
unweighted UniFrac	2.2	0.003

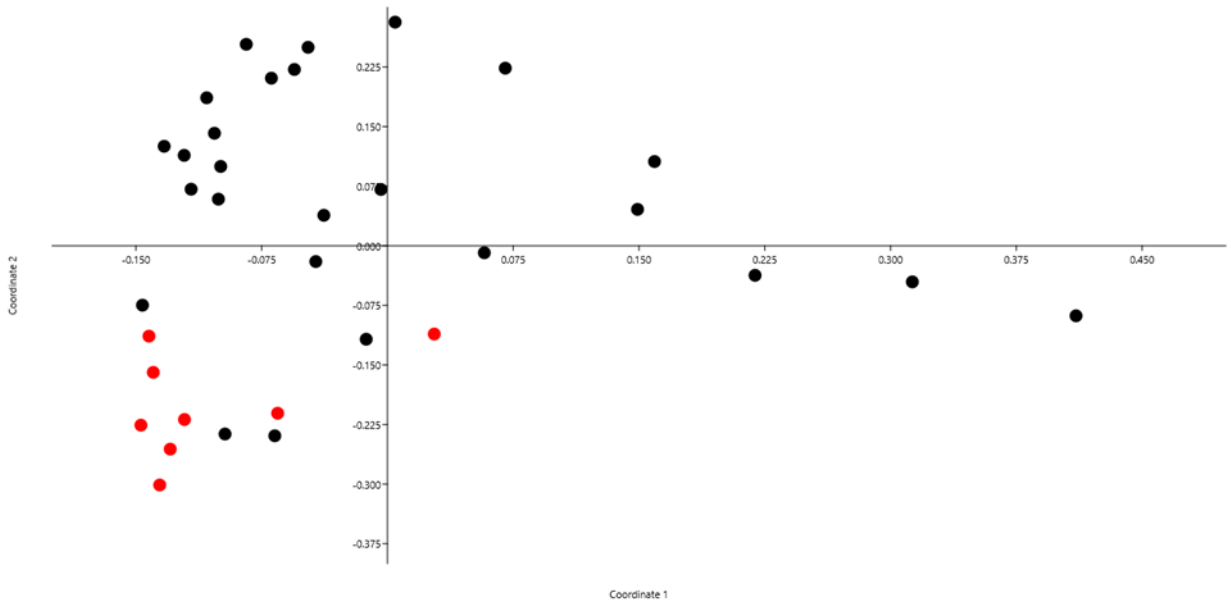


Figure 8. Principal Coordinates Analysis (PCoA) based on unweighted UniFrac similarity coefficient of the bacterial community of *T. aestivum* and *T. melanosporum*. Red dots indicate *T. aestivum*, black dots *T. melanosporum*.

3.1.2.2. Fungal Beta Diversity

For the fungal biodiversity on plants mycorrhized with *T. melanosporum*, the statistical general result using are shown in Table 7. In Figure 9, is represented the PCoA of Jaccard coefficient, where the pair-wise result showed a significant difference between *Q. ilex* and *Q. pubescens* (pseudo-F value = 2,4; p-value = 0,002).

Table 7. Two-way PERMANOVA tests: pseudo-F and p-values for fungal biodiversity among plants mycorrhized with *T. melanosporum.*

Biodiversity indexes	**PERMANOVA significance**	
	Pseudo-F	**p-value**
Bray-Curtis	3	0.001
Jaccard	1.54	0.003
weighted UniFrac	5.7	0.001
unweighted UniFrac	1.75	0.001

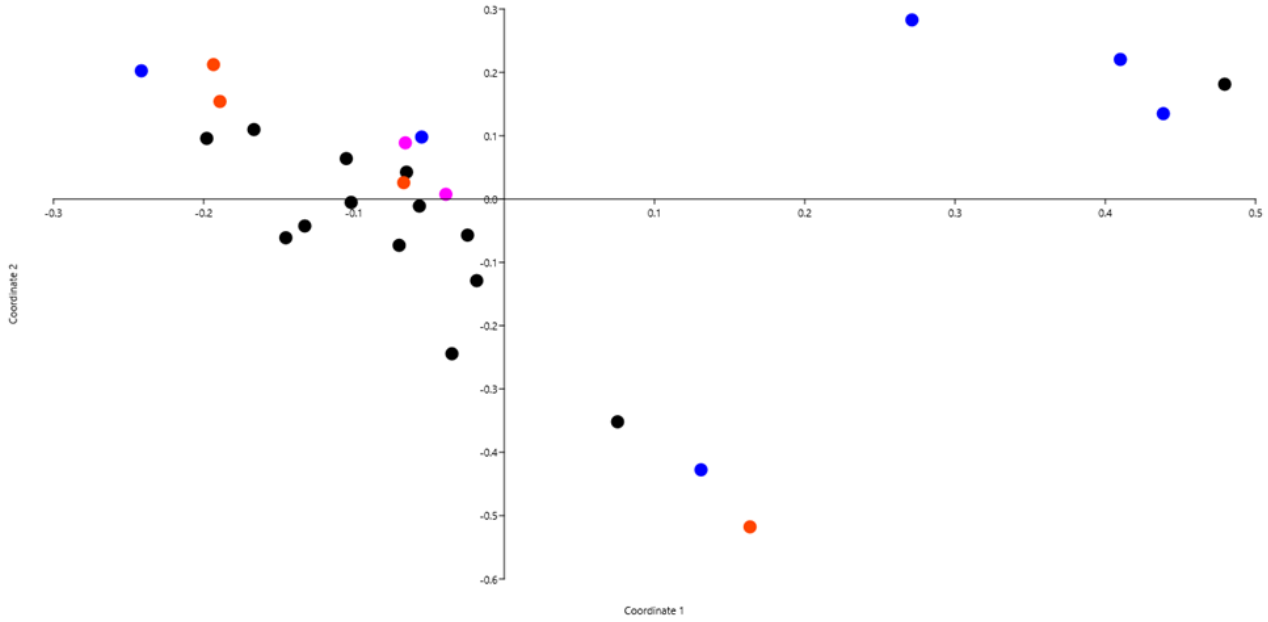


Figure 9. Principal Coordinates Analysis (PCoA) based on Jaccard similarity coefficient of the fungal community of *Q. pubescens*, *Q. ilex*, *O. carpinifolia* and *C. avellana* mycorrhized with *T. melanosporum*. Black dots indicate *Q. pubescens*, blue dots *Q. ilex*, orange dots *O. carpinifolia* and pink dots *C. avellana* plants.

Also, the fungal community varied among the two *Tuber* spp. The statistical general result are shown in Table 8. In Figure 10, the PCoA of the Bray-Curtis similarity coefficient shows these differences.

Table 8. Two-way PERMANOVA tests: pseudo-F and p-values for fungal biodiversity between the two *Tuber* spp.

Biodiversity indexes	**PERMANOVA significance**	
	Pseudo-F	**p-value**
Bray-Curtis	3.6	0.001
Jaccard	2	0.001
weighted UniFrac	7	0.001
unweighted UniFrac	2.1	0.003

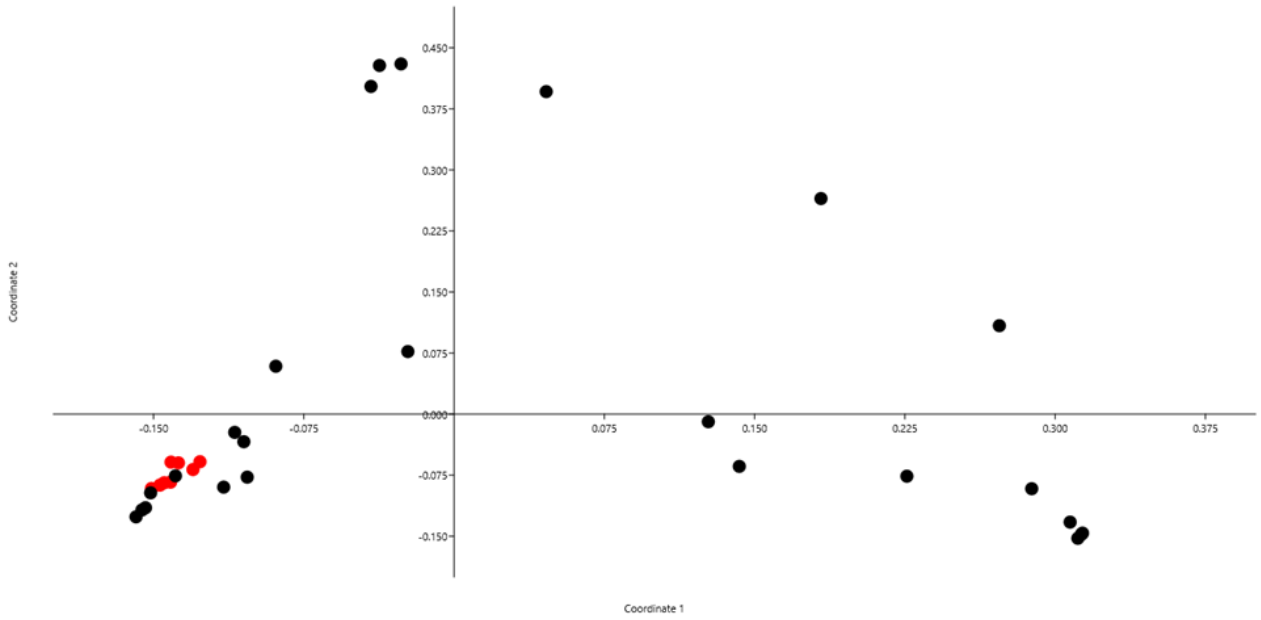
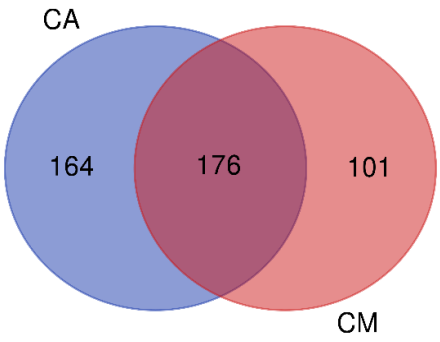
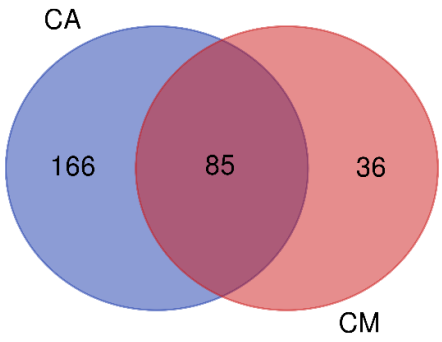


Figure 10. Principal Coordinates Analysis (PCoA) based on Bray-Curtis similarity coefficient of the fungal community of *T. aestivum* and *T. melanosporum*. Red dots indicate *T. aestivum*, black dots *T. melanosporum*.

3.1.3. OTUs distribution

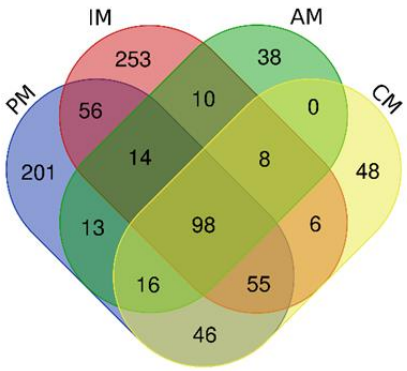
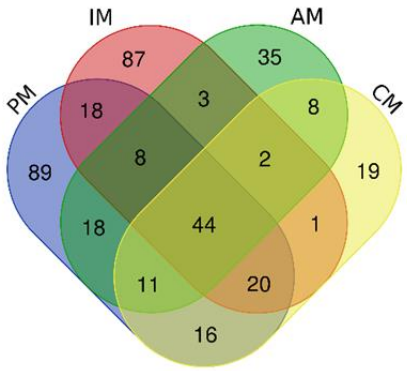
The total number of OTUs detected for each category was plotted in the Venn diagrams for both fungi and bacteria. For what concerns the plant species *O. carpinifolia* mycorrhized with *T. aestivum* (Table 9), the bacteria community possess a higher total number of OTUs (617) than fungi (372). The bacteria are the group that shares proportionally the highest number of OTUs (28%) between the two symbionts (*T. aestivum* and *T. melanosporum*) than the fungi group (22%). The root tips of *O. carpinifolia-T. aestivum* has the highest number of unique OTUs, both for bacteria and fungi.

Table 9. Venn diagram showing the number of fungal and bacterial OTUs detected in the root tips of *O. carpinifolia* mycorrhized with *T. aestivum* (CA) and *T. melanosporum* (CM).

BACTERIA	FUNGI
	

On the plants ectomycorrhized with *T. melanosporum*, the bacterial OTUs shared was 98 while the fungal ones were 44 (Table 10). For the bacterial group, *Q. ilex* plants had the highest number of unique OTUs (253) followed by *Q. pubescens* (201), *O. carpinifolia* (48) and finally *C. avellana* (38). Furthermore, *Q. ilex* and *Q. pubescens* shared the higher number of OTUs (56). For the fungal group, the two oak species (*Q. pubescens* and *Q. ilex*) had the same number of unique OTUs (89 and 87, respectively) followed by *C. avellana* (35) and *O. carpinifolia* (19). Also, for the bacteria, the two oak species shared a higher number of OTUs (18).

Table 10. The following table shows the Venn diagrams built on the distribution of each bacterial and fungal OTUs group of plant mycorrhized with *T. melanosporum*, where PM: *Q. pubescens-T. melanosporum*; IM: *Q. ilex- T. melanosporum*; AM: *C. avellana-T. melanosporum* and CM: *O. carpinifolia-T. melanosporum*.

	BACTERIA	FUNGI
Tuber melanosporum.		

3.1.3.1. Most representative and shared bacterial OTUs

The two categories of *Tuber* spp. and plants mycorrhized with *T. melanosporum* were analyzed for the most representative and shared OTUs of bacteria for each group. Concerning the bacterial communities shared by *T. aestivum* and *T. melanosporum*, the most common members belong to the genus *Cyanobacteria*; the family *Bradyrhizobiaceae*, with the genus *Bradyrhizobium*; the family *Steroidobacteraceae*; the genus *Micromonospora*.

All the plant species mycorrhized with *T. melanosporum* shared bacteria belonging to the genus *Cyanobacteria*, the family *Bradyrhizobiaceae*, with the genus *Bradyrhizobium*, and the families *Pseudonocardiaceae* and *Steroidobacteraceae*. Some host trees shared different taxa among them. For example, *Q. ilex* and *O. carpinifolia* plants shared bacteria belonging to the order *Streptomycetales*, while the order *Hyphomicrobiales* was shared more on *Q. ilex*, *Q. pubescens* and *O. carpinifolia* samples. *O. carpinifolia* and *Q. pubescens* shared the bacterial order *Rhodospirillales*. Members of the genus *Actinoplanes* were predominant in *C. avellana* and *Q. pubescens* samples. Finally, *C. avellana*, *O. carpinifolia*, and *Q. pubescens* plants had a higher representation of the family *Steroidobacteraceae*.

Also, within *Q. ilex-T. melanosporum* plants in the two different geographical sites (Bracciano and Scheggino), the most common bacterial members belong to the genus *Cyanobacteria*, the family *Bradyrhizobiaceae*, the genus *Bradyrhizobium*, the genus *Streptomyces*, and the family *Steroidobacteraceae.*

3.1.3.2. Most representative and shared fungal OTUs

The two categories of *Tuber* spp. and plants mycorrhized with *T. melanosporum* were analyzed for the most representative and shared fungal OTUs.

On *T. aestivum* and *T. melanosporum* root tips samples, the most common fungal members belong to the genus *Dactylonectria*, the order *Telephorales*, the genus *Talaromyces*, the order *Helotiales* and the related genus *Tetracladium.* However, *T. melanosporum* and the genus *Dactylonectria* were the most common fungal species in all the black truffle mycorrhized individuals. Each plant species differentially shared fungal members. For example, *Q. ilex* and *Q. pubescens* shared OTU of the *Talaromyces* and *Cyphellophora* genera; *Q. ilex*, *O. carpinifolia* and *C. avellana* shared OTU of the genus *Fusarium*; *Q. ilex*, *O. carpinifolia* and *Q. pubescens* shared OTU of the genus *Cercospora*; *O. carpinifolia* and *Q. pubescens* shared more the species *Hymenogaster olivaceus* and *Epicoleosporium ramularioides; O. carpinifolia* and *C. avellana* shared members of the order *Pezizales*.

The *Q. ilex-T. melanosporum* root tips in the two different geographical sites shared the following fungal members: the species *T. melanosporum*, the genera *Dactylonectria* and *Cercospora*, the species *Epicoleosporium ramularioides, Knufia tsunedae, Fusarium oxysporum* and the genus *Neopestalotiopsis.*

3.1.4. LEfSe analysis for bacteria

LEfSe analysis showed that bacteria from phylum to species level exhibited significant differences among different *Tuber* and tree species (LDA score > 1.0). The non-parametric factorial Kruskal-Wallis (KW) sum-rank test to detect features with significant differential abundance for bacteria among *T. aestivum* and *T. melanosporum* samples and within the host tree colonized by *T. melanosporum* was statistically significant ($p < 0,05$). The most abundant taxa that statistically differentiate the *T. aestivum* samples from those of *T. melanosporum* were the genus *Micromonospora*, the family *Steroidobacteraceae* (class *Gammaproteobacteria*), and the genus *Pseudoxanthomonas* (*Gammaproteobacteria*). In *T. melanosporum*, the significantly more abundant class was *Alfaproteobacteria*, followed by the family *Chitinophagaceae* (phylum *Bacteroidetes*), the order *Rhizobiales*, and the syn. family *Hyphomicrobiaceae* (*Alphaproteobacteria*) (Figure 11A-11B).

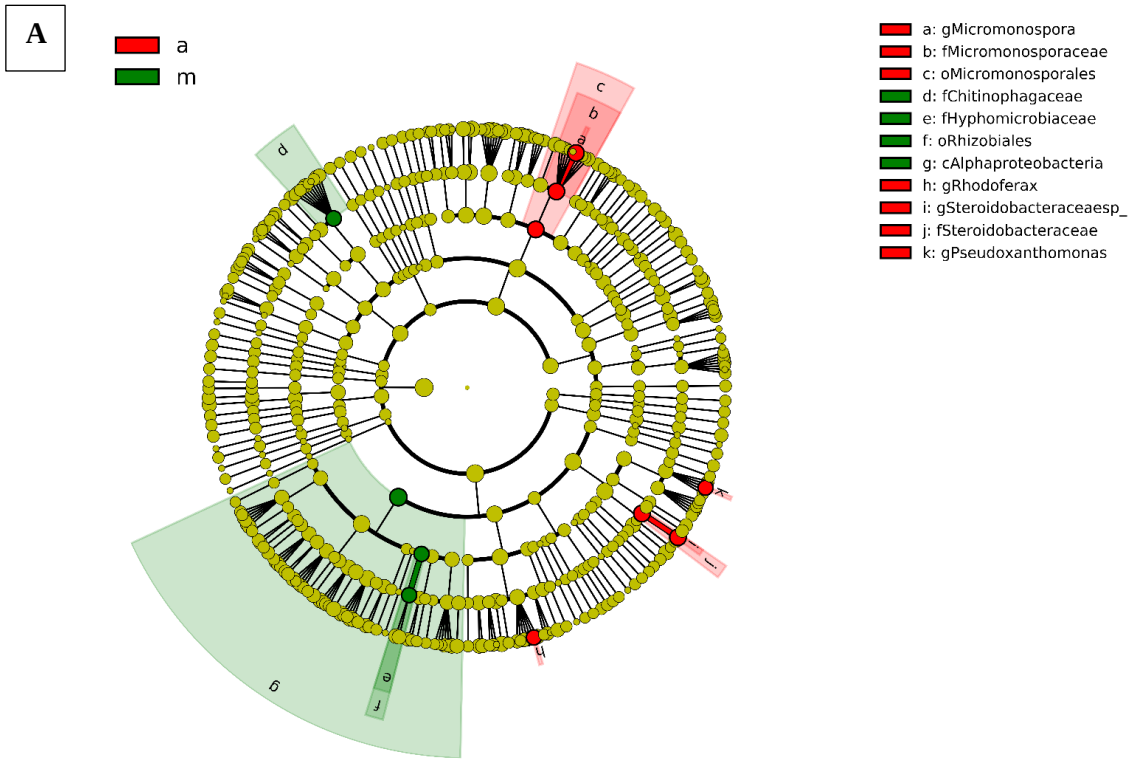


Figure 11A. Cladogram of different bacterial taxa from phylum to genus identified between two *Tuber* spp. (a: *Tuber aestivum*; b: *Tuber melanosporum*). Different color nodes represent significantly enriched and important taxa among samples, except for yellow which means no significant difference between different categories.

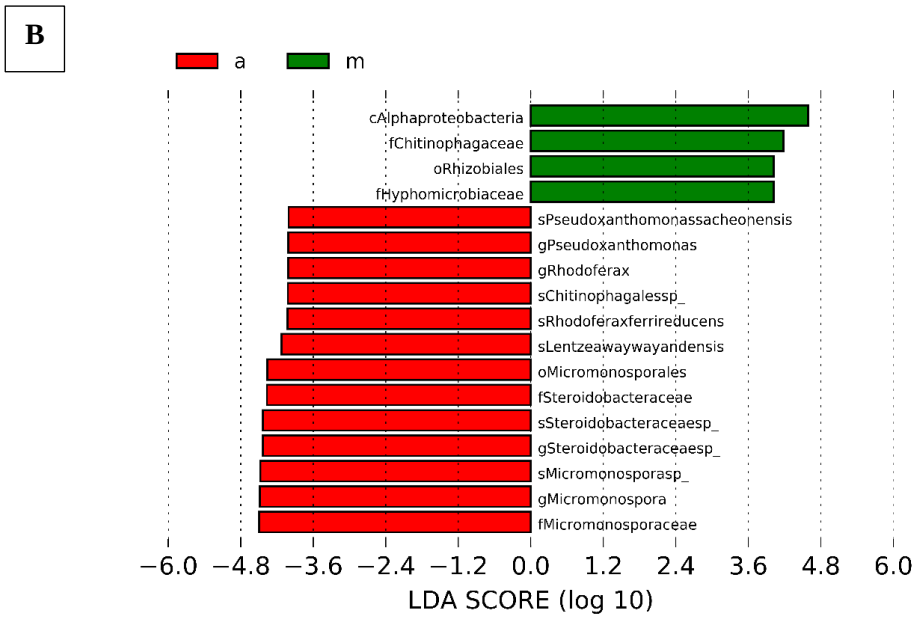


Figure 11B. LDA graph; detected taxa ranked by effect size, the higher (in case of m: *T. melanosporum*) or lower (in case of a: *T. aestivum*) LDA scores the taxa are, the more effect the taxa have. Alpha value <0.05.

Significantly more abundant taxa in different host trees were the following: on *Q. pubescens-T. melanosporum,* the family *Rhizobiaceae,* genus *Rhizobium,* family *Hyphomicrobiales* (class *Alphaproteobacteria*), and genus *Vicinamibacter* were significantly more abundant. On *Q. ilex-T. melanosporum,* the significantly more abundant taxa belong to the *Bacteroidetes* phylum, to the family *Chitinophagaceae* (phylum *Bacteroidetes*), the family Brayrhizobiaceae (*Alphaproteobacteria*) and the genus *Povalibacter* (class *GammaProteobacteria*). On *C. avellana-T. melanosporum,* the phylum *Actinobacteria*, the specie *Streptomyces canus* (*Actinobacteria*) and the order Cryptosporangiales (*Actinobacteria*) were the most abundant microorganisms ($\alpha < 0{,}05$). Finally, on *O. carpinifolia-T. melanosporum,* the species *Vicinamibacter silvestris* (phylum *Acidobacteria*) and the order *Caulobacterales* (class *Alphaproteobacteria*) were the significantly more abundant taxa (Figure 12A-12B).

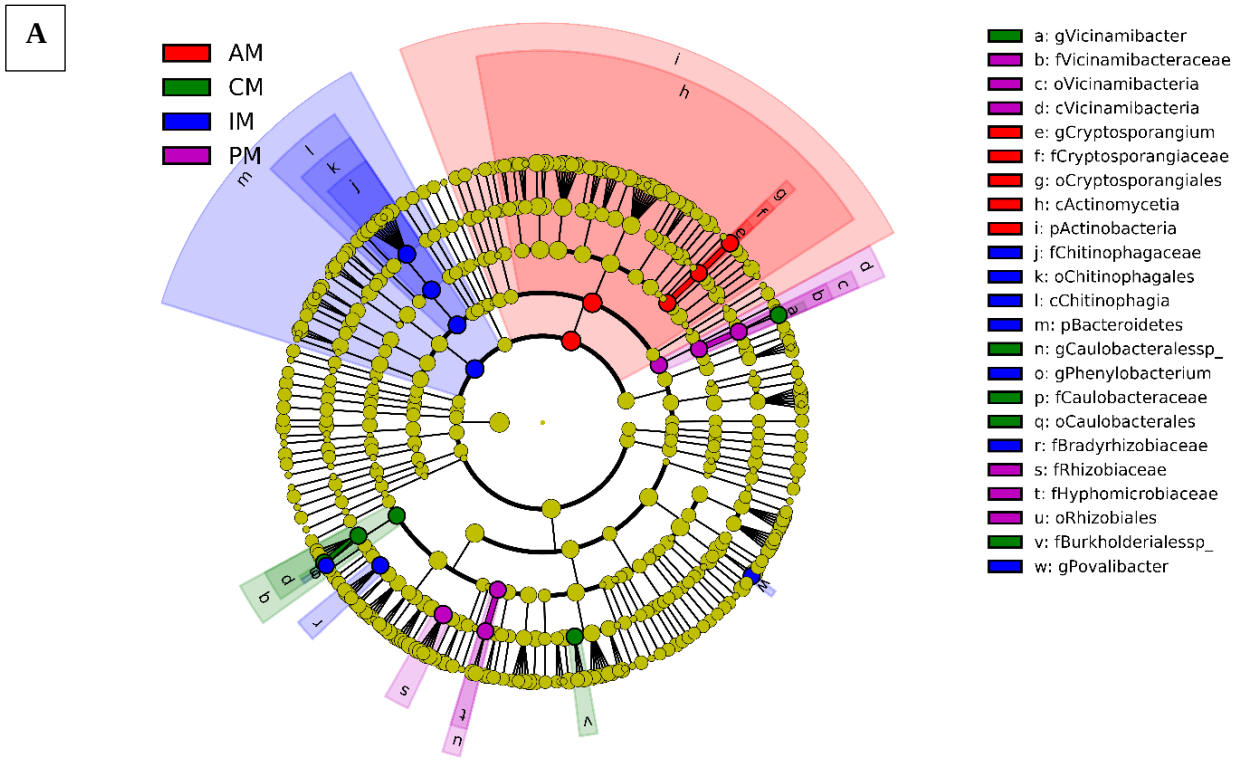


Figure 12A. Cladogram of different bacterial taxa from phylum to genus identified between different plant species. Different colour nodes represent significantly enriched and important taxa among samples, except for yellow which means no significant difference. AM= *C. avellana-T. melanosporum*; CM= *O. carpinifolia-T. melanosporum*; IM= *Q. ilex-T. melanosporum*; PM= *Q. pubescens-T. melanosporum.*

B

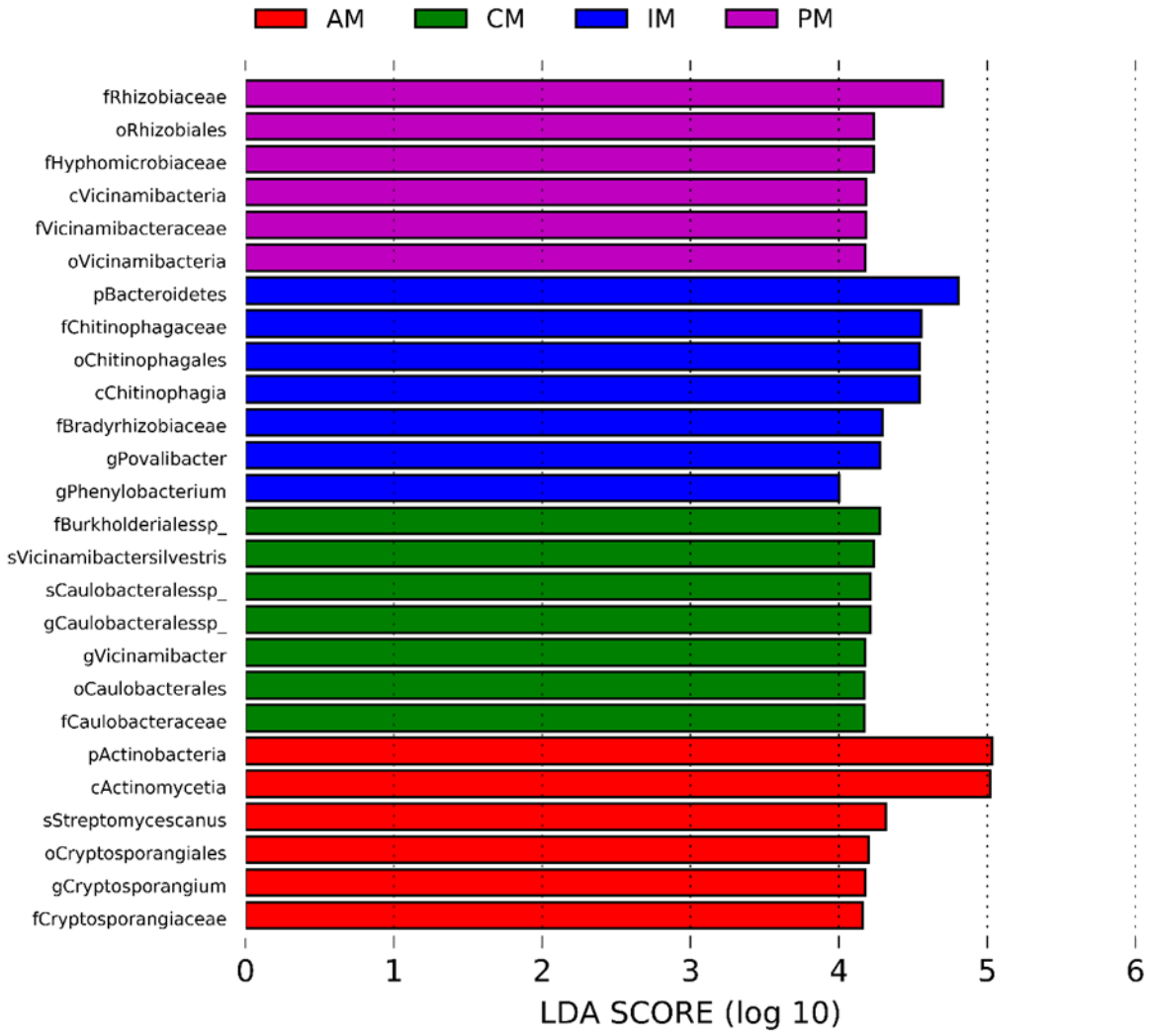


Figure 12B. LDA graph; detected taxa ranked by effect size, the higher LDA scores the taxa are, the more effect the taxa have. AM= *C. avellana-T. melanosporum*; CM= *O. carpinifolia-T. melanosporum*; IM= *Q. ilex-T. melanosporum*; PM= *Q. pubescens-T. melanosporum*. Alpha value <0.05.

On Figure 13 are plotted the bacterial taxa that stignificantly differentiated the *Q. ilex-T. melanosporum* plants collected in Lazio and Umbria regions. From the results, the bacterial taxa significantly more abundant on samples from Scheggino (PG) belong to the genus *Actinophytocola* and the species *Actinophytocola oryzae*; the genus *Agrobacterium* and the specie *Agrobacterium vitis;* the species *Niastella gongjuensis* and *Vicinamibacter silvestris*.

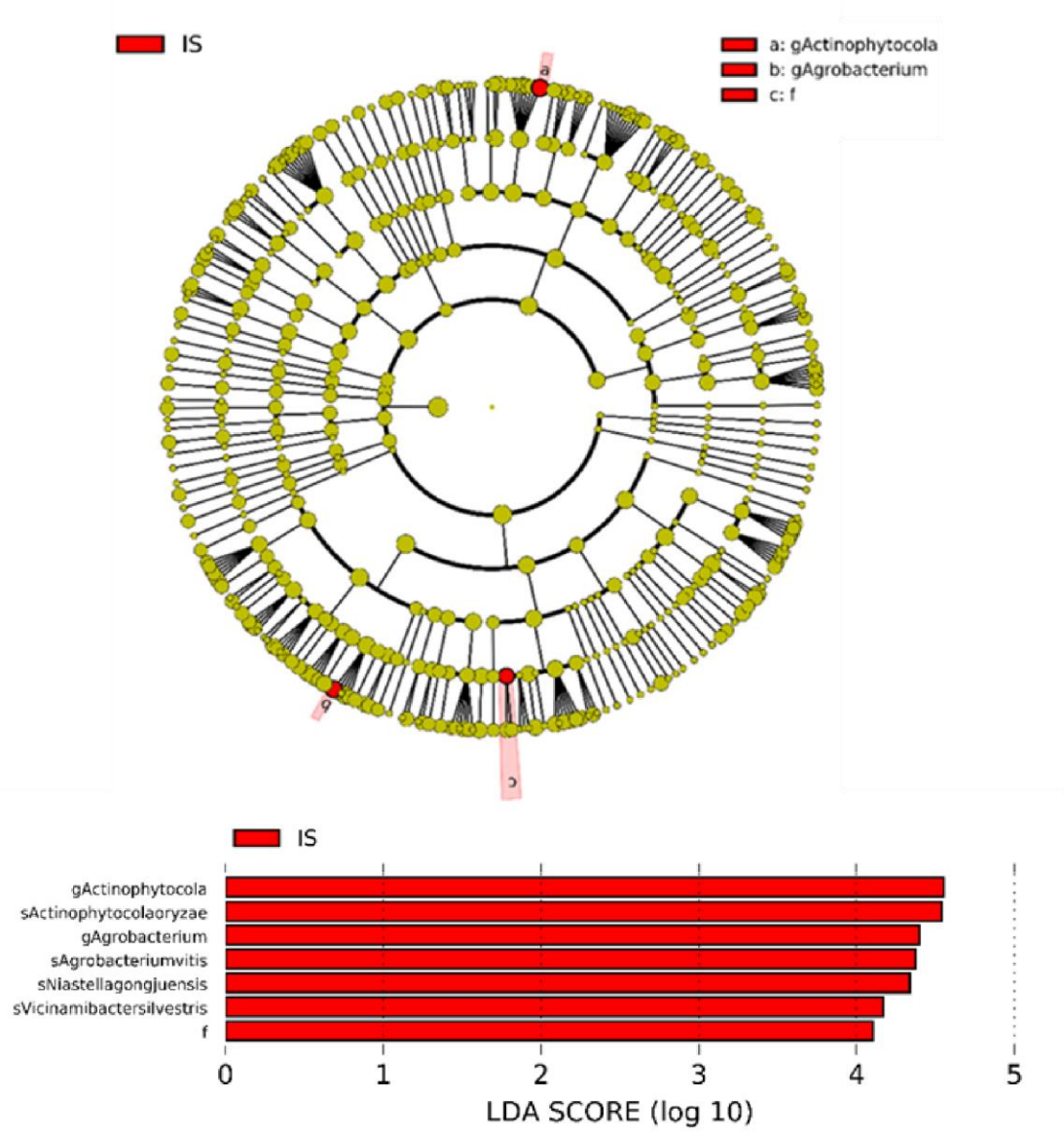


Figure 13. LDA graph; detected taxa ranked by effect size, the higher LDA scores the taxa are, the more effect the taxa have between *Q. ilex-T. melanosporum* plants collected in Bracciano (RM) and Scheggino (PG). The statistical test (alpha value <0.05) only detected more abundant bacterial taxa on plants from Scheggino (IS).

3.1.5. LEfSe analysis for fungi

LEfSe analysis showed that from phylum to species level, fungi exhibited significant differences among different *Tuber* and plant species (LDA score > 1.0). The non-parametric factorial Kruskal-Wallis (KW) sum-rank test to detect features with significant differential abundance for fungi among *T. aestivum* and *T. melanosporum* samples and plant ectomycorrhized with *T. melanosporum* was statistically significant ($p < 0,05$). On *T. aestivum* root tips the three significantly most abundant taxa were the phylum *Basidiomycota*, the class *Agaricomycetes*, and the genus *Pseudotomentella*. On *T. melanosporum* samples, the phylum *Ascomycota* and the species *T. melanosporum* were the taxa that were significantly predominant with respect to *T. aestivum* samples (Fig.14A-14B).

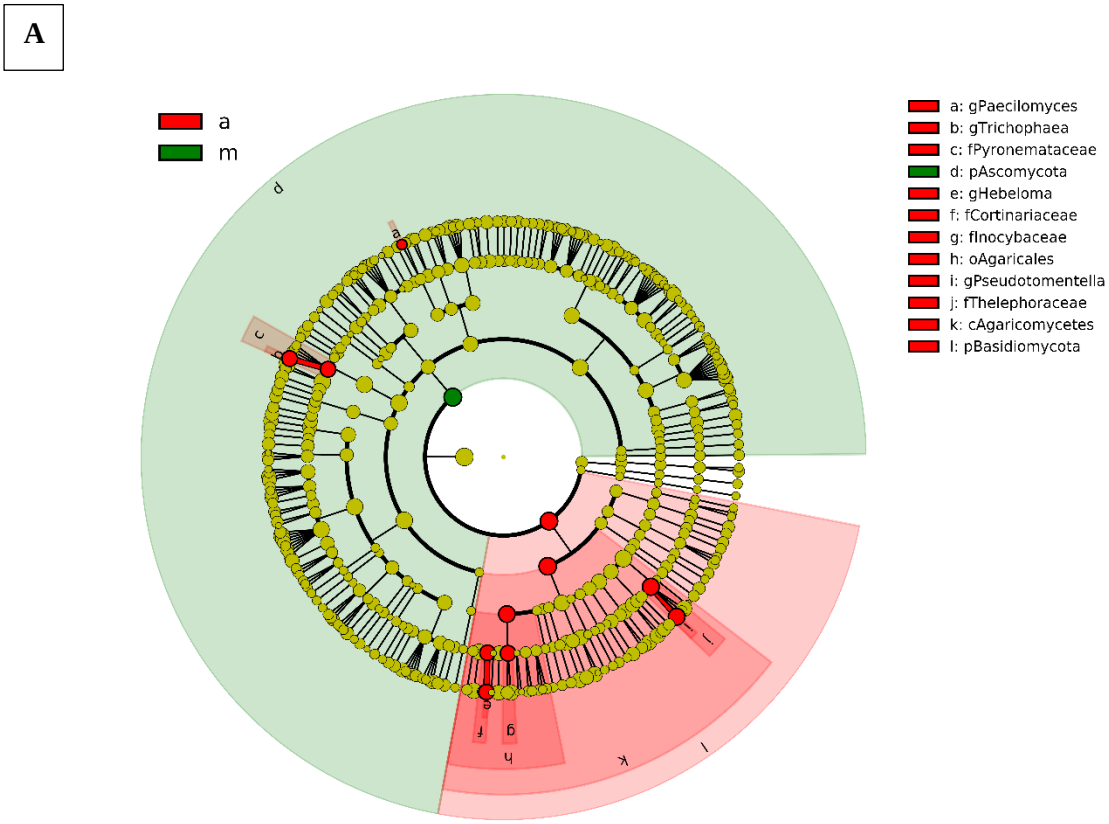


Figure 14A. Cladogram of different fungal taxa from phylum to genus identified between two *Tuber* spp. (a: *Tuber aestivum*; b: *Tuber melanosporum*). Different colour nodes represent significantly enriched and important taxa among samples, except for yellow which means no significant difference between different categories.

B

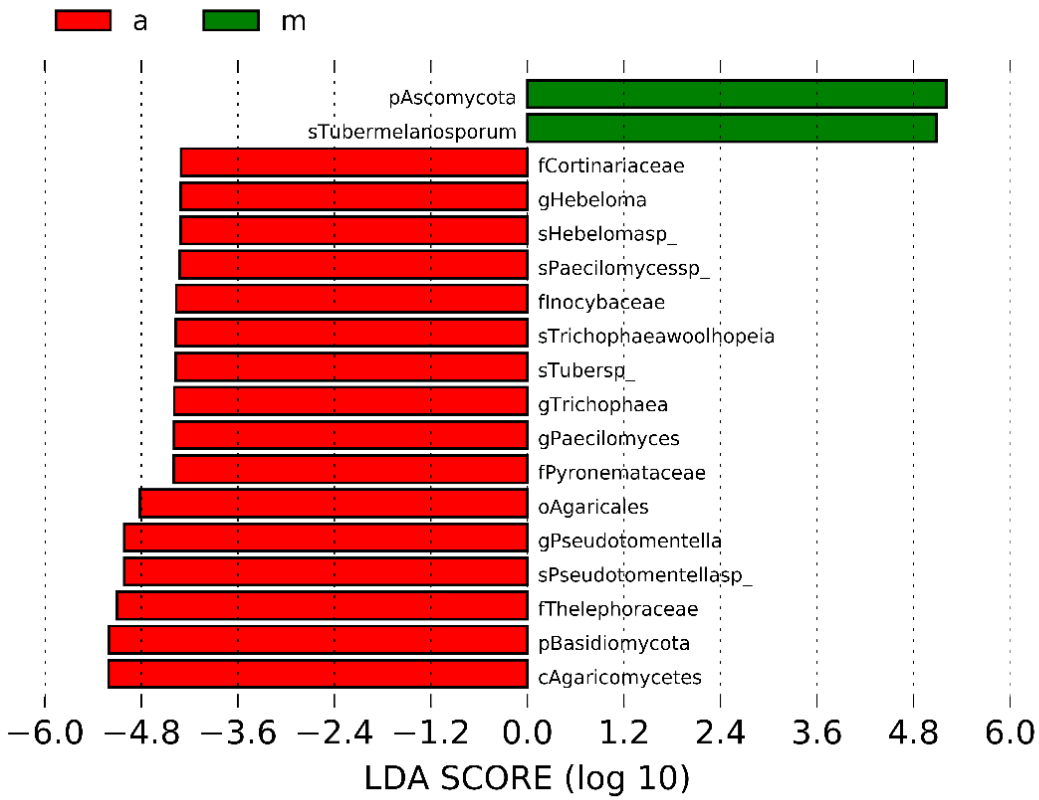


Figure 14B. LDA graph; detected taxa ranked by effect size, the higher (in case of m: *T. melanosporum*) or lower (in case of a: *T. aestivum*) LDA scores the taxa are, the more effect the taxa have. Alpha value <0.05

On different host trees, significantly more abundant taxa were the following: on *Q. pubescens-T. melanosporum*, the phylum *Ascomycota* and the genus *Exophiala* were the fungal taxa significantly more abundant ($\alpha < 0,05$). On *Q. ilex-T. melanosporum*, the class *Agaricomycetes* was the significantly most abundant, followed by members of the Boletaceae family, the specie *Xerocomellus chrysenteron* (*Agarycomicetes*). On *C. avellana-T. melanosporum*, the significantly predominant classes were *Dothidiomycetes*, *Pleosporales*, and the family *Inocybaceae*. Finally, on *O. carpinifolia-T. melanosporum*, the species *Scytalidium lignicola* (class *Leotiomycetes*) and the species *Microdochium bolleyi* (class *Sordariomycetes*) were the significantly more abundant taxa (Figure 15A-15B).

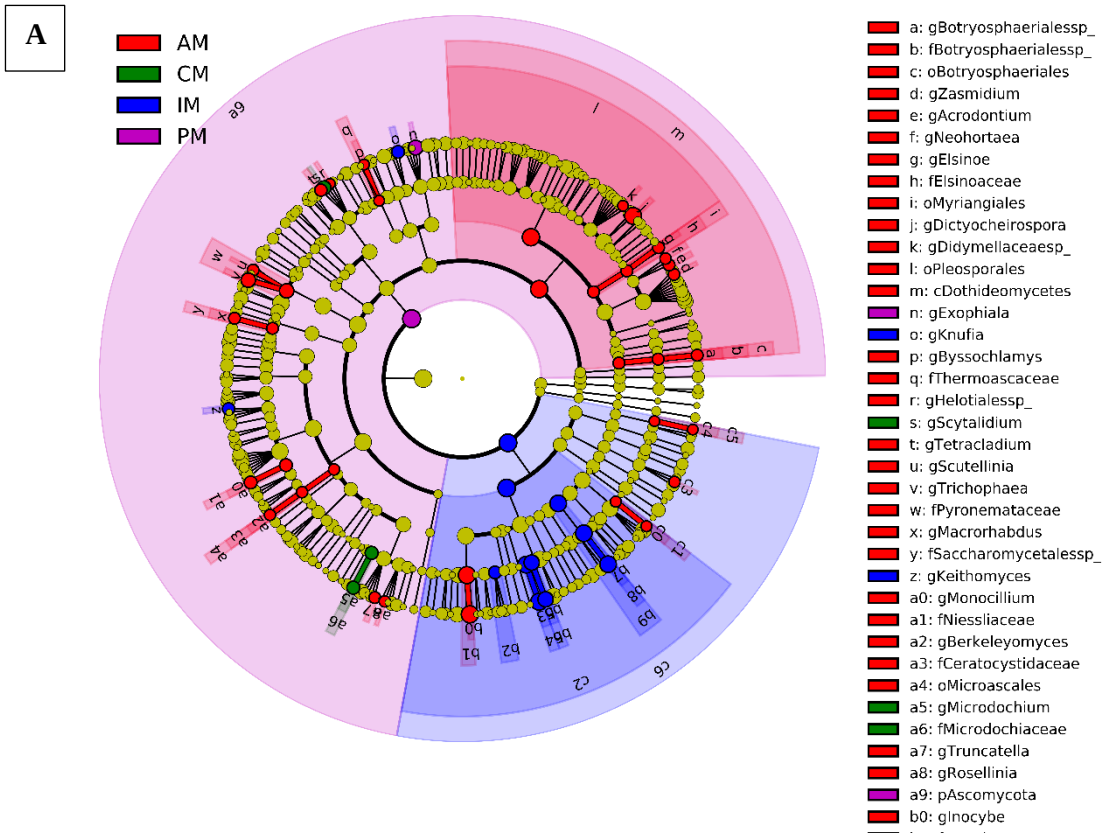


Figure 15A. Cladogram of different bacterial taxa from phylum to genus identified between different plant species. Different colour nodes represent significantly enriched and important taxa among samples, except for yellow which means no significant difference. AM= *C. avellana-T. melanosporum*; CM= *O. carpinifolia-T. melanosporum*; IM= *Q. ilex-T. melanosporum*; PM= *Q. pubescens-T. melanosporum*.

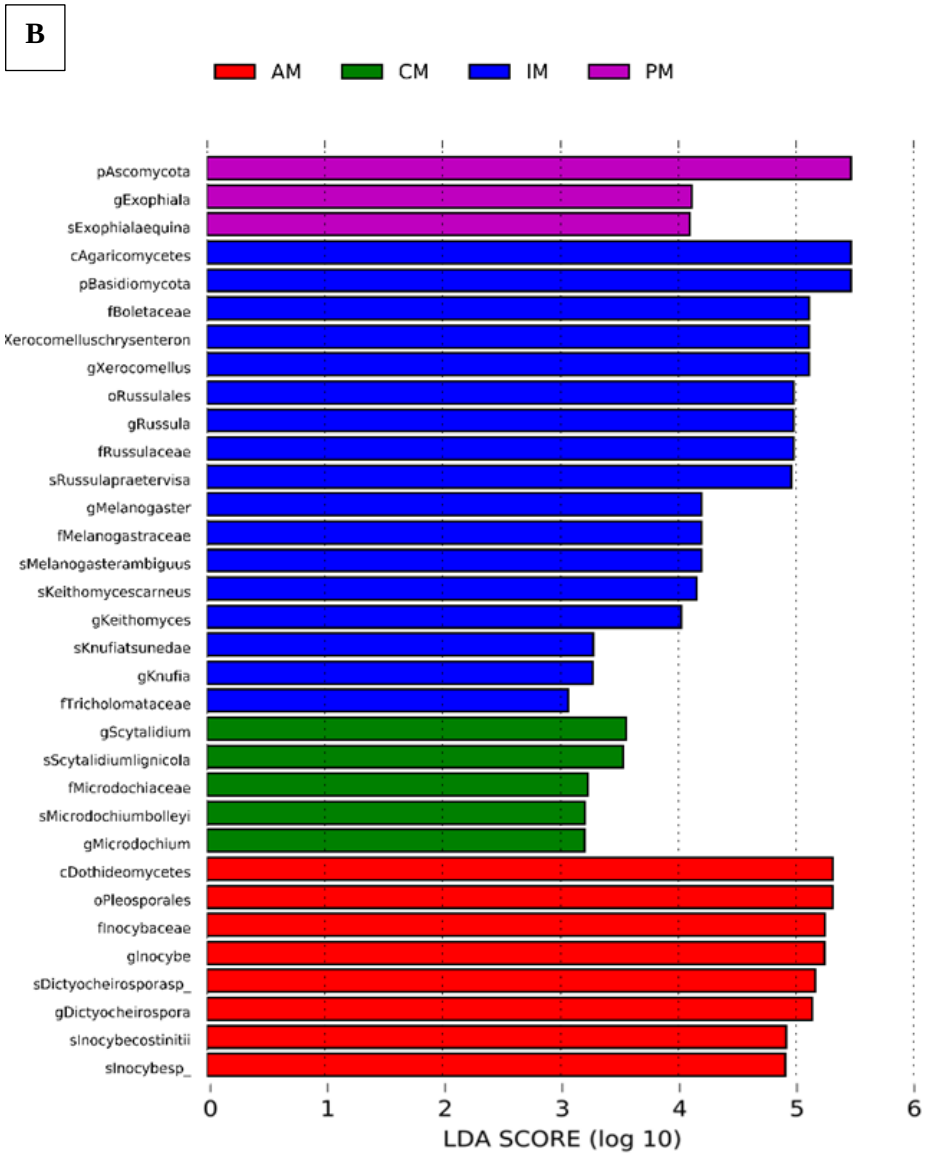


Figure 15B. LDA graph; detected taxa ranked by effect size, the higher LDA scores the taxa are, the more effect the taxa have. AM= *C. avellana-T. melanosporum*; CM= *O. carpinifolia-T. melanosporum*; IM= *Q. ilex-T. melanosporum*; PM= *Q. pubescens-T. melanosporum*. Alpha value <0.05.

On Figure 16 are showed the results of the comparision between the two *Q. ilex* host trees ectomycorrhized with *T. melanosporum* and collected in the two regions (Lazio and Umbria). As for bacteria, also for fungi, the holly oaks root tips sampled in Scheggino differed significantly from those of Bracciano for the following fungal taxa: the genus *Neopyrenochaeta* (order *Pleosporales*), the order *Sordariales*, the specie *Fusarium equiseti* (order *Hypocreales*) and the family *Chaetomiaceae* (order *Sordariales*).

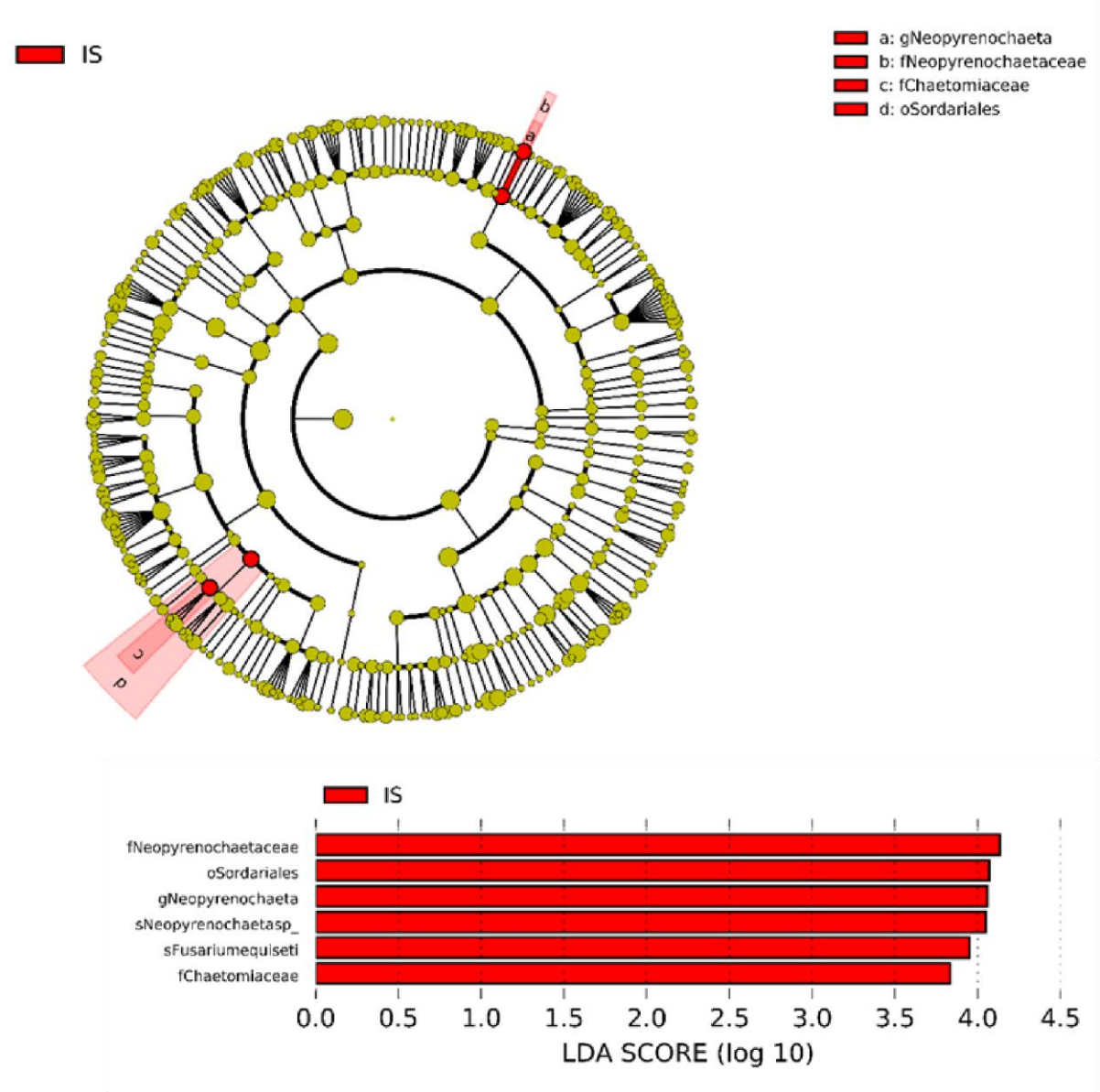


Figure 16. LDA graph; detected taxa ranked by effect size, the higher LDA scores the taxa are, the more effect the taxa have between *Q. ilex-T. melanosporum* plants collected in Bracciano (RM) and Scheggino (PG). The statistical test (alpha value <0.05) only detected more abundant fungal taxa on plants from Scheggino (IS).

3.2 Mycorrhizal colonization in recycled potting mixes

3.2.1 Seedlings survival

For the mortality assessment of the plants, the usual levels for the control potting mix used by the nursery Truffleland s.r.l. were 25% for *Q. pubescens* and 40% for *C. avellana* seedlings and these values were used as a starting point to calculate the mortality of the treatments. Of the 180 seedlings of *Q. pubescens* inoculated, the percentage of mortality of the control was 25%. As regards the treatments, the percentage of mortality in Mix 1 was 25%, while in Mix 2 was -23% compared to the control, with 56 seedlings surviving. The radication survival of *C. avellana* suckers was lower than acorns germination. The seedlings used for inoculation were 55 instead of 60 for each potting mix, and at the end of the experiment, the percentage of mortality was 43% for the control, and compared to these percentages the levels for the treatments were 4% for Mix 1 and -23% for Mix 2.

3.2.2 Percentage of truffle colonization

Data obtained with the RAV method showed that the average percentage of *Q. pubescens*-*T. melanosporum* ECMs was statistically higher on potting Mix 2 with 59.7% of infection (F value = 8,739, p = 0,0003). When growing in potting Mix 1, the percentage of ectomycorrhizal short roots was 46.4%, which was comparable with that of the control, which had a percentage of 46.7% (Figure 17A). On *C. avellana* seedlings, the level of mycorrhization obtained with the RAV approach gave similar results, and the type of substrate didn't significantly influence the mycorrhization occurrence. On potting Mix 1, the percentage of mycorrhization was 44%, on Mix 2 was 43% and on the control, it was 44% (Figure 17B).

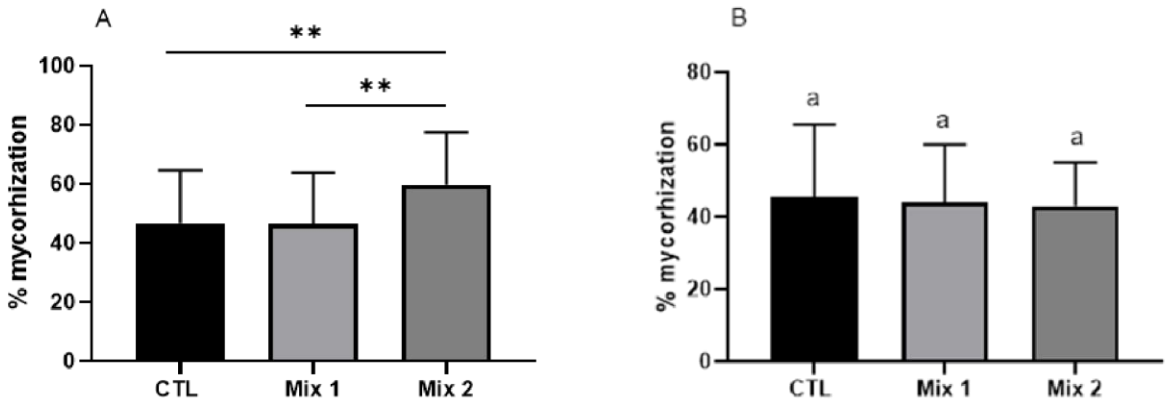


Figure 17. Percentages of ectomycorrhizal short roots obtained by inoculation of *Q. pubescens* (A) and *C. avellana* (B) with *T. melanosporum*. On *Q. pubescens* seedlings, the potting Mix 2 showed a significantly higher level of mycorrhization (60%). p-values (** $p < 0.01$) are indicated above columns to indicate specific treatments that resulted in a significantly higher percentage of mycorrhization than that of control and Mix 1. On *C. avellana* the differences are not significantly different. In each treatment, no different letters denote non-significant differences between substrates according to Tukey's test ($P<0.05$).

Genomic DNA from a pool of 10 root tips (Figure 18) of mycorrhized plants/treatment (two samples of EcMs were extracted for each potting mix) was subjected to PCR amplification using the forward primer TM1b associated with the reverse primer ITS4 (Sèjalon-Delmas et al., 2000). All of them gave an expected amplicon of ~500 bp, which perfectly matched the amplicon size obtained when DNA from *Tuber melanosporum* ascocarps was amplified with the same primers. These findings were confirmed by amplicons Sanger sequence.

Figure 18. Root tips of *Q. pubescens* mycorrhized with *T. melanosporum* under the stereomicroscope.

3.2.3 Seedlings and root system development

The growth parameters of the inoculated seedlings showed significant differences ($P < 0.05$) in the shoot height of *C. avellana* seedlings with a higher value on average height in potting Mix 2 ($F = 5.980$, $P = 0.0046$) and no statistical differences in root collar diameter. On *Q. pubescens* seedlings, there was a significantly higher value of root collar diameter in potting Mix 1 ($F = 8.487$; $P = 0.0003$) and no differences among substrates on shoot height. The type of substrate did not significantly influence the dry weight of the plants (Table 11).

Table11. Growth effect of substrate used for *T. melanosporum* inoculation of *Q. pubescens* and *C. avellana* seedlings. The signifcantly different values after the Tukey HSD post-hoc test ($P<0.05$) are annotated with a different lowercase letter.

	Quercus pubescens			***Corylus avellana***		
Growth parameter	**Mix 1**	**Mix 2**	**CTL**	**Mix 1**	**Mix 2**	**CTL**
Shoot Diameter (mm)	1.43 a	1.11 b	1.10 b	1.56 a	1.84 a	1.96 a
Shoot Height (cm)	1.38 a	1.43 a	1.30 a	1.79 b	2.08 a	1.38 b
Shoot dry weight (g)	0.72 a	0.78 a	0.78 a	2.35 a	2.49 a	2.70 a
Root dry weight (g)	1.72 a	2.16 a	1.99 a	1.57 a	1.54 a	1.53 a

Data obtained from the root parameters such as number of root tips and forks, average root diameter, root volume, surfaced area, projected area, and length of the root system performed with the WinRHIZO® software were statistically significant (ANOVA, $p<0.05$). On *Q. pubescens* seedlings, all the parameters of the root system analysed with WinRHIZO® software were significantly higher on Mix 2 than in the other treatments (number of tips: F value = 13,49, p <0,0001, Figure 19A; root length: F value = 17,84, p <0,0001, Figure 19B; number of forks: F value = 15,83, p < 0,0001, Figure 19C; root volume: F value = 19,88, p <0,0001 Figure 19D; surfaced area: F value = 21,23, p <0,0001 Figure 19E; projected area: F value = 22,03, p < 0,0001 Figure 19F), except for the average diameter of the root where Mix 1 showed the higher value (Figure 20).

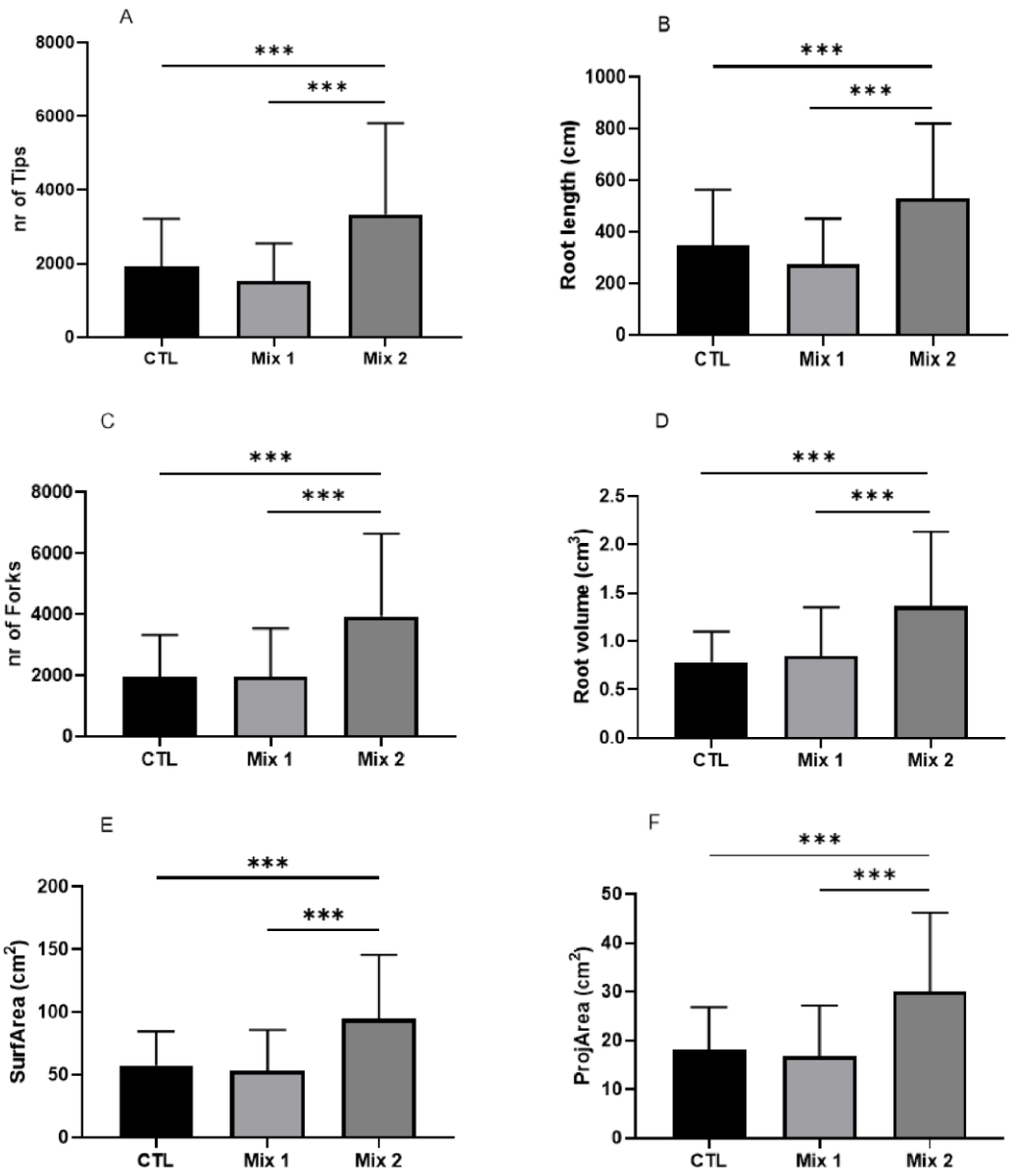


Figure 19. Root development parameters of mycorrhization of *Quercus pubescens* with *Tuber melanosporum*. (A) number of root tips; (B) root length (cm); (C) number of forks; (D) root volume (cm^3); (E) surfaced area (cm^2); (F) projected area (cm^2). p-value (*** $p < 0.001$) are indicated above columns to indicate specific treatments that resulted in significantly higher values than other treatments.

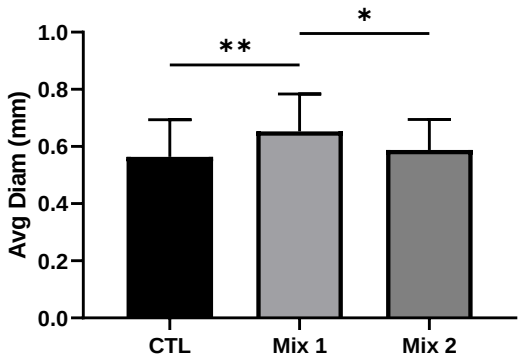
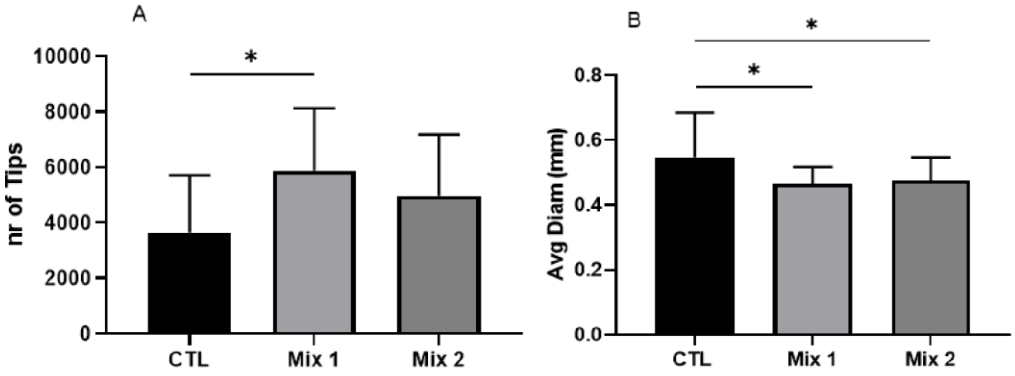


Figure 20. Root average diameter of *Q. pubescens* seedlings mycorrhized with *T. melanosporum*. p-values (* $p < 0.1$ and ** $p < 0.01$) are indicated above columns to indicate specific treatments that resulted in significantly higher values than other treatments.

On *C. avellana* seedlings, the number of root tips was higher on Mix 1 (F value = 3,652, p = 0,0319, Figure 21A). The seedlings grown on the control substrate had the highest value of the average diameter (F value = 4,010, p = 0,0232, Figure 21B), while on Mix 1, the hazelnut seedlings had a greater length of the root (F value = 3,156, p = 0,0499, Figure 21C). Regarding the number of forks, root volume surfaced, and projected area of the root system, no statistical differences were detected.



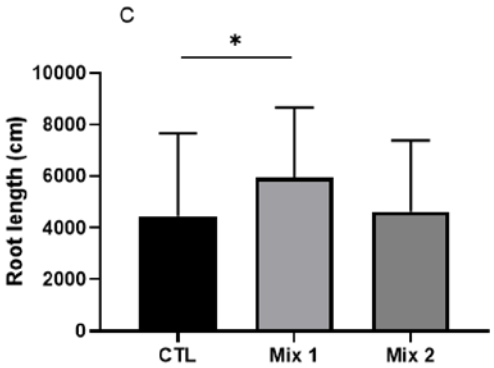


Figure 21. Root development paramenters of *C. avellana* seedlings mycorrhized with *T. melanosporum* (A) number of root tips; (B) root average diameter (mm); (C) root length (cm). p-value (* $p < 0.1$) are indicated above columns to indicate specific treatments that resulted in significantly higher values than other treatments.

3.3 Characterisation of *Tuber melanosporum*-associated bacteria and evaluation of their effect on mycorrhization of oak and hazel seedlings

3.3.1 Molecular and physiological identification of bacterial strains

The culturable fraction of bacteria associated with ectomycorrhized *Q. pubescens* root tips revealed that out of 50 isolates obtained, most of them (37%) belong to *Bacillus* (phylum *Firmicutes*) and *Psudomonas* genus (phylum *Proteobacteria*) (40%), and other genera to *Paenibacillus* (phylum *Firmicutes*), *Stenotrophomonas* (phylum *Proteobacteria*), *Serratia* (phylum *Proteobacteria*) and *Solibacillus* (phylum *Firmicutes*). The five bacteria selected for the experiment belonged to the species *Psuodomonas fluorescens, Pseudomonas jessenii*, *Priestia megaterium*, *Serratia marcescens* and *Paenibacillus polymyxa*. They were chosen for their ability to grow fast on TSA medium, for their fluorescence on King's B medium (Duponnois and Garbaye, 1991), and for their belonging to the group of Mycorrhizal Helper Bacteria (MHB, although these bacteria should rather be mentioned as Mycorrhizal Helper Bacteria) (Frey-Klett et al., 2007) and Plant Growth Promoting Rhizobacteria (PGPR).

3.3.2 Effects of bacterial inoculation on percent of mycorrhization

The analysis of the effect of individual bacteria on *T. melanosporum* root tip colonization of *C. avellana* seedlings (Figure 22A) showed that the percentage of mycorrhization ranged from a minimum of 47.9% for *P. jessenii*, to a maximum of 73% of root tips colonized by *T. melanosporum* with *P. megaterium* (F value = 4,296, p = 0,0015). On *Q. pubescens* seedlings, *P. jessenii* gave a

statistically greater percentage of mycorrhization, with 69% of colonization compared to all the other treatments (F value = 10,47, p < 0,0001) (Figure 22B).

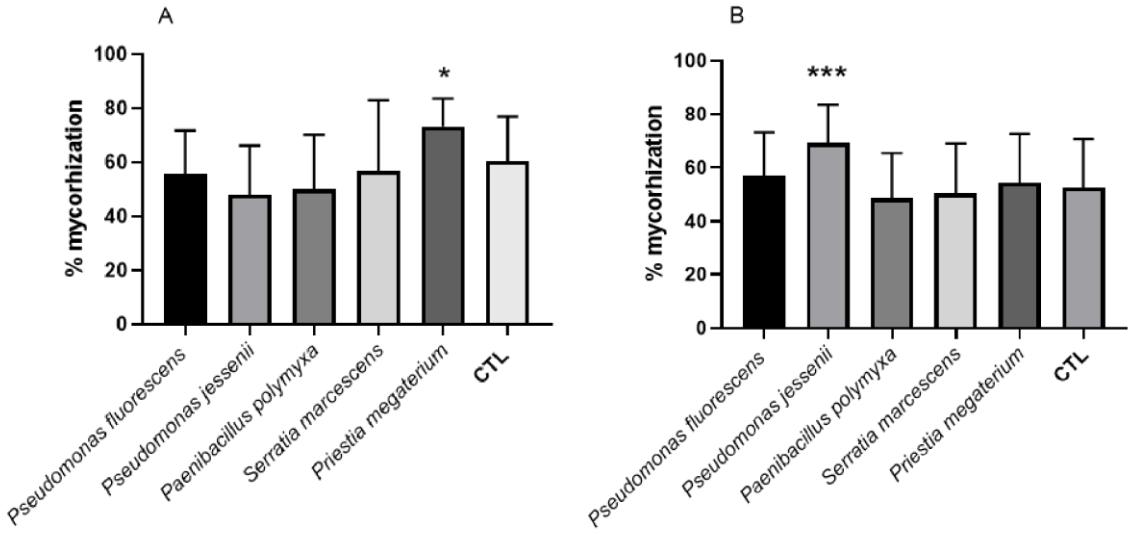


Figure 22. Mycorrhization rates of *C. avellana* (A) and *Q. pubescens* root tips by *T. melanosporum* after co-inoculation with bacterial strains and truffle inocula in a commercial nursery (Scheggino, Umbria, Italy). p-values (* p < 0.1 and *** p< 0.001) are indicated above columns to indicate specific treatments that resulted in significantly higher values than the control.

3.3.3 Effect of bacteria on seedling growth and root traits

Among the *C. avellana* seedling growth parameters measured, there was a statistically significant effect of the bacterial inoculation with *P. megaterium* strain on the shoot height of the plants (F value = 2,696, p = 0,0251) (Figure 23), while the other growth traits measured in the experiment were not affected by either the bacterial inoculation. The number of root tips, the root length, the average root system diameter, the root volume, and the surfaced and projected area of the root system analyzed with WinRHIZO® software showed that the seedlings inoculated with *P. megaterium* were not significantly different from those of seedlings that received the other treatments.

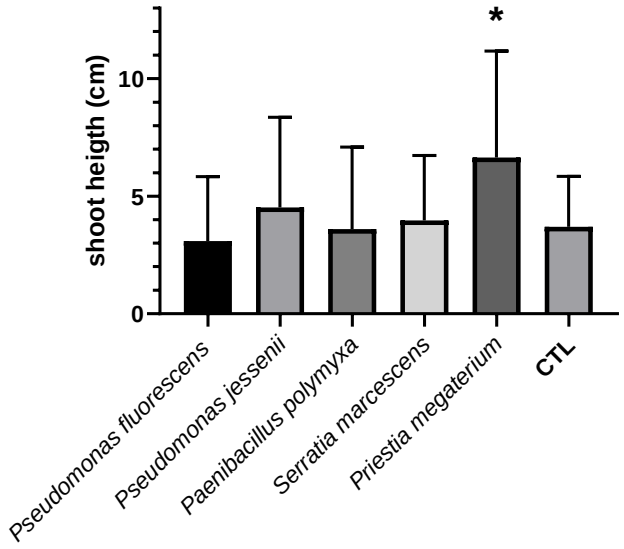


Figure 23. Shoot height of *C. avellana* seedlings in a commercial nursery (Scheggino, Umbria, Italy) co-inoculated with *T. melanosporum* and bacteria evaluated under the different treatments: *Psuodomonas fluorescens*, *Pseudomonas jessenii*, *Priestia megaterium*, *Serratia marcescens* and *Paenibacillus polymyxa*, and control seedlings (that were not inoculated with bacteria). p-value (* $p < 0.1$) is indicated above the columns to indicate treatment inoculated with *P. megaterium* that resulted in significantly higher values than the control.

On *Q. pubescens* seedlings, the root dry weight of the seedling that received the treatment with *P. jessenii* was significantly different from the control (F value = 5,812, $p < 0,0001$, Figure 24), while the other seedling growth parameters of seedlings inoculated with *P. jessenii* were not statistically different to those of seedlings that received the control treatment.

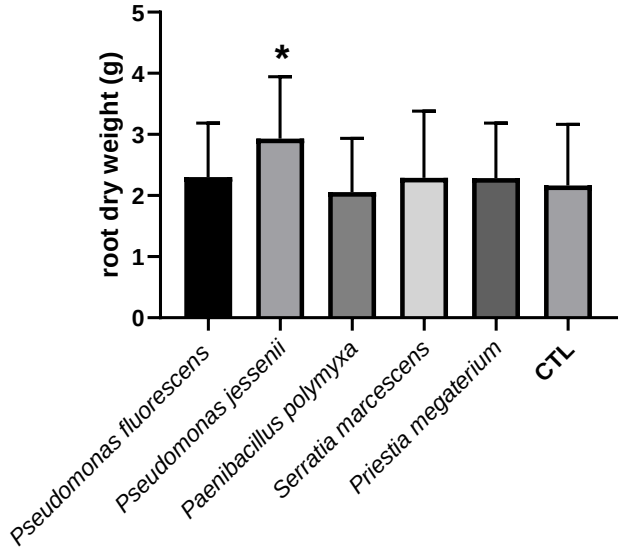


Figure 24. Root dry weight of *Q. pubescens* seedlings in a commercial nursery (Scheggino, Umbria, Italy) co-inoculated with *T. melanosporum* and bacteria evaluated under the different treatments: *Psuodomonas fluorescens, P. jessenii, Priestia megaterium, Serratia marcescens, Paenibacillus polymyxa*, and control seedlings (that were not inoculated with bacteria). p-value (* $p < 0.1$) is indicated above the columns to indicate treatment inoculated with *P. jessenii*, which resulted in significantly higher values than the control.

The root development parameters analysed with WinRHIZO® software, showed that the number of root tips, the projected and surfaced area, and the root volume were significantly greater than those of seedlings that received the control treatment (root tips: F value = 7,540, $p < 0,0001$, Figure 25A; projected area: F value = 6,312, $p < 0,0001$, Figure 25B; surfaced area: 6,312, $p < 0,0001$, Figure 25C; root volume: F value = 4,089, $p = 0,0013$, Figure 25D). Concerning the root length and average diameter, the values were not statistically different from the control. The *Q. pubescens* seedlings inoculated with the other bacterial strains, as well as in the experiment with *C. avellana* seedlings, were not statistically different from those who received the control treatment and are not reported.

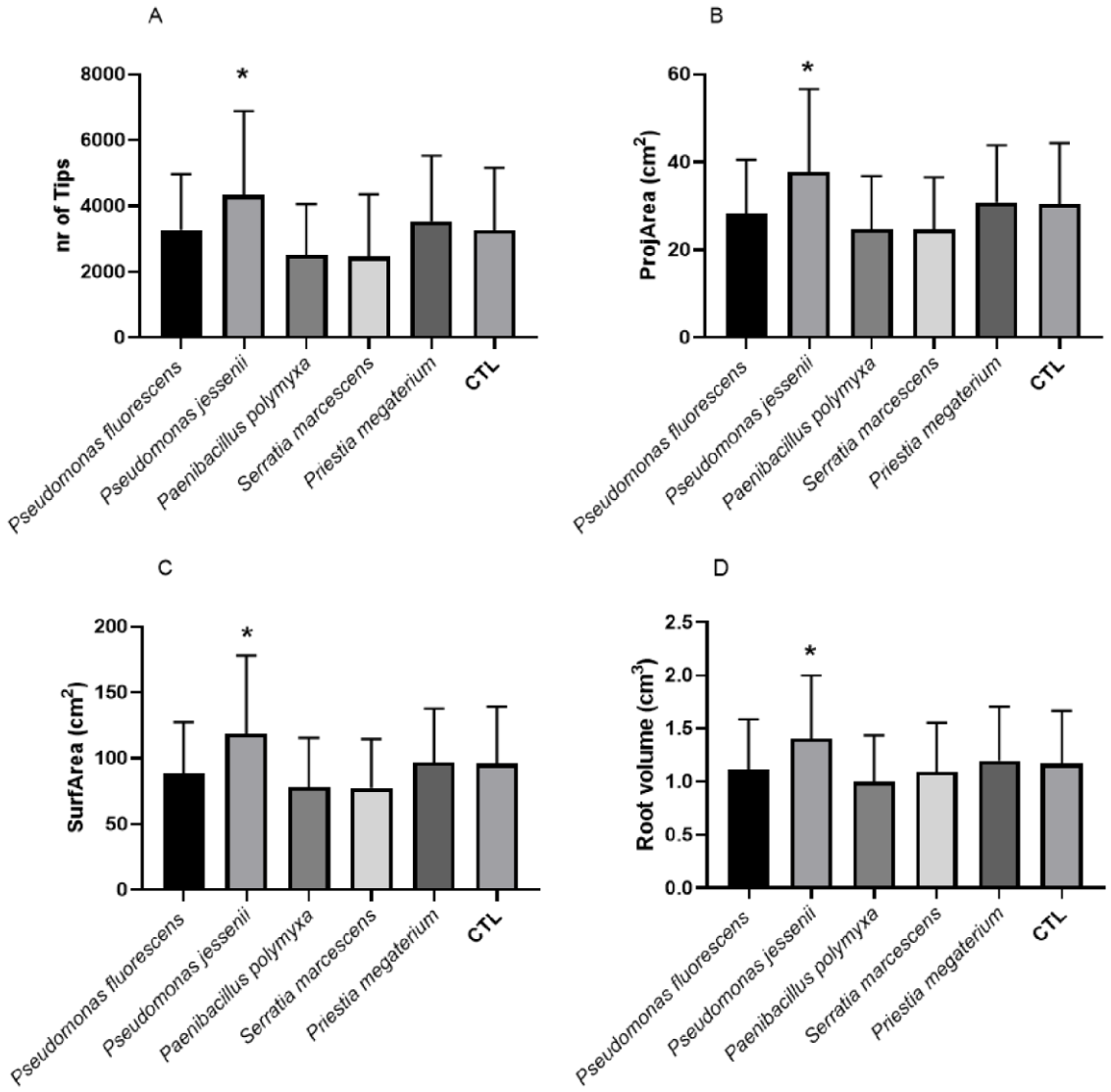


Figure 25. Root development parameters of *Q. pubescens* seedlings mycorrhized with *T. melanosporum* (A) number of root tips; (B) projected area (cm^2); (C) surfaced area (cm^2); (D) root volume (cm^3). p-values (* $p < 0.1$) are indicated above columns to indicate treatment with *P. jessenii* that resulted in significantly higher values than the control.

4. The TANA project: a great challenge for the future

To develop ad hoc biotechnology that allows the application of optimized combinations of microorganisms as effective inoculators within sustainable plant production systems, research in mycorrhizas should strive toward an improved understanding of the functional and molecular mechanisms involved in interactions in the mycorrhizosphere (Artursson et al., 2006). A polymicrobial formulation comprising a broad combination of beneficial rhizosphere microorganisms with several functions is appealing because it may exploit multiple plant growth-promoting pathways and can be used in a variety of forest systems (Avis et al., 2008; Vestberg et al., 2004). The selection and utilization of rhizosphere bacteria and fungi that are mutually compatible, have complementary functions, efficiently colonize the rhizosphere of the plant(s) of interest, and promote the growth of the plant(s) of interest in a synergistic way is a fundamental concept in designing effective polymicrobial multifunctional formulations (Azcón-Aguilar et al., 2009; Barea et al., 2005; Hata et al., 2010). As predicted, well-designed multifunctional formulations such as the one reported here would be a welcome addition to the world's rapidly developing inoculant industry. Additionally, such an inoculant is believed to be environmentally friendly and appropriate for organic farming and other integrated production systems in which synthetic fertilizer inputs are prohibited or regulated by legislation. However, developing such intricate formulas is a technological challenge. As Iotti et al. (2012) attest, entrapment of mycelial fragments in natural polysaccharide gels, such as calcium alginate, is a proven method to obtain efficient inocula for root colonization with different EM fungal species (Repáč 2011). However, this inoculant technology was unsuccessful for some EEMMs. In fact, alginate-entrapped inocula of *Lactarius deliciosus* (Parladé et al. 2004) and *Tuber* spp. (Iotti et al., 2012) completely failed in forming mycorrhizas when mixed with the plant growth substrate, although the viability of their mycelia was proven to be unaffected. Promising approaches for producing commercial mycorrhizal inocula also involve the use of various combinations of natural, semi-synthetic, and synthetic polymers (Siddiqui and Kataoka 2011), although, more studies are needed to find and test alternative polymers that do not affect the colonization ability of the ECM mycelia. The TANA (TArtuficoltura e NAnomateriali) project is part of this complex context.

Research groups from the University of Tuscia, Sapienza, in collaboration with the start-up Nanomnia, thanks to funding from LazioInnova regional funding agency for innovation of Regione Lazio, have proposed a new synergistic approach for the enhancement and optimization of truffle farming processes thanks to the combined use of nanotechnologies (Figure 26). Indeed, numerous inorganic compounds, bacteria, and viruses contribute to the complexity of the soil biota and can

stimulate the formation of mycorrhizal symbiosis and its vitality. In this context, nanomaterials can act as carriers for bioactive species and at the same time can be effective in stabilizing and protecting encapsulated molecules /microorganisms (fungi and bacteria), favoring their gradual release into the environment. The study of inorganic nanoparticles and polymer/nanoparticle composites as carriers of microorganisms in the soil has the main purpose of improving the development of the rhizosphere by providing a consistent source of fungi and bacteria capable of interacting with the root system and the microbiome of the soil. Polysaccharides are among the most stable and most efficient biomaterials to meet the needs that the encapsulation of microorganisms requires. These natural polymers are the most used because it is non-toxic to humans and the environment, inexpensive, and suitable for encapsulating living cells. It is therefore possible to prepare beads of polysaccharides with controlled diameter and, with a simple methodology, create formulations by encapsulating ectomycorrhizal fungi and MHB bacteria at the same time. Furthermore, it is possible to encapsulate inorganic silver nanoparticles (AgNPs) which at low doses show beneficial effects on the growth and development of the root system of the plants thanks to a slow controlled release.

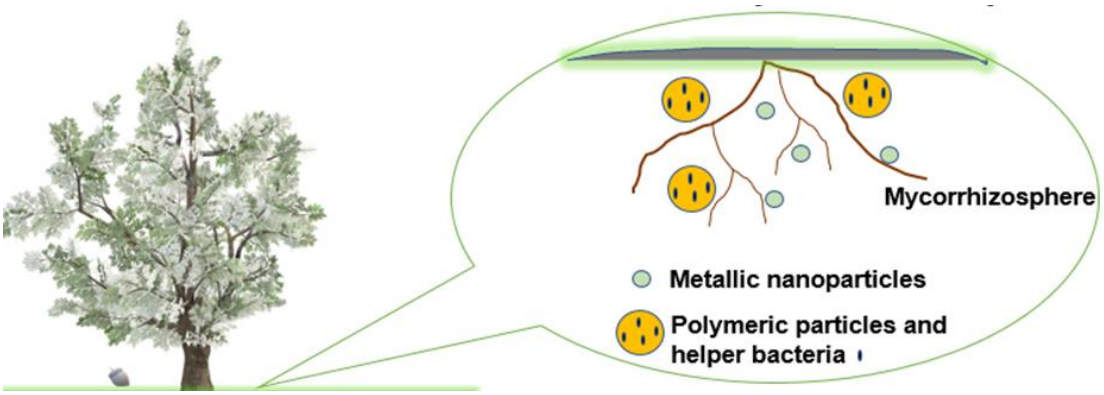


Figure 26. Representation of mycorrhizosphere environment supplemented with metallic nanoparticles and helper bacteria entrapped in polymeric particles.

4.1 Preliminary activities

4.1.1 Phytotoxicity study

The first activities carried out within the last phases of my Ph.D. project, concerned the application of silver nanoparticles (AgNPs) produced by Sapienza University (Prof. Fratoddi) to test their phytotoxicity on *Q. pubescens* Willd. plants (Di Santo et al., 2016). The seedlings were tested four different concentrations of colloidal suspensions of AgNPs (0.1, 1, 10, 50 μg/mL) that were supplemented with a ½ Hoagland nutrient solution (Smith et al., 1983) to the root system of 3-month-old seedlings in two sterile substrates: in soil and in soilless (perlite) into biodegradable plastic glasses (Figure 27). The seedlings were kept moist by covering them with another biodegradable plastic glass and watering them once a week in a controlled growth chamber (16 hours light, 8 hours dark; 25 °C / 23 °C day/night).

Figure 27. System in which *Q. pubescens* seedlings are grown after the treatment with AgNPs at different concentrations (0.1, 1, 10, 50 μg/mL) and the control without nanoparticles in soil and perlite.

During the experiment, the general status of the plants was monitored by detecting the chlorophyll and other pigment content by using a portable non-destructive device called SPAD (an acronym for "Soil Plant Analytical Development") (Dualex 4-Horta srl). After three months of growth, the plants were subjected to root analysis with the WinRHIZO® software.

From the results obtained, the chlorophyll content of the seedlings on perlite and soil (Figure 28) showed a positive effect at a concentration of 0.1 mM of AgNPs, even if it is not statistically significant compared to the control. Furthermore, there is an evident detrimental effect from the concentration of 1 mM to 50 mM of Ag NPs, and in both the substrates, at the concentration of 50 mM, the chlorophyll decreases compared to the control (0 mM).

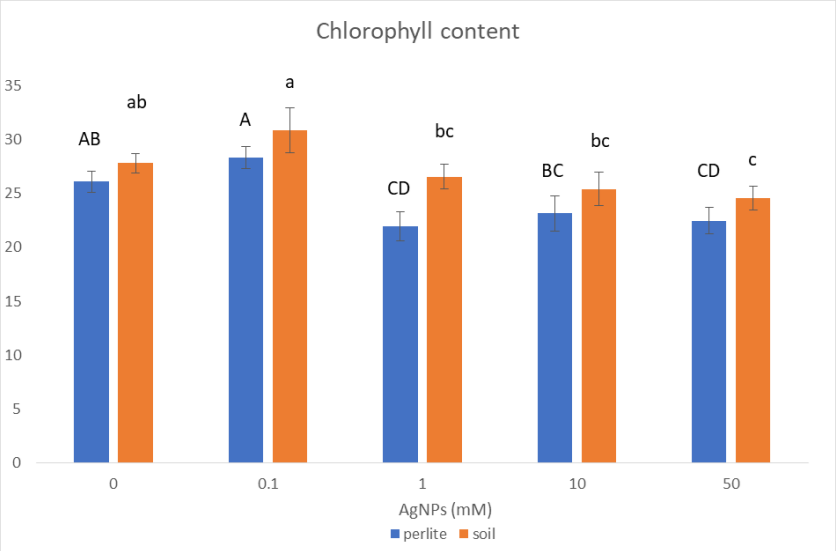


Fig.28. Chlorophyll content of *Q. pubescens* seedling after the treatment with AgNPs (0.1, 1, 10, 50 μg/mL) and the control without nanoparticles.

From the analysis of the root system with the WinRHIZO® software, there was a statistically positive effect of 0.1 mM AgNPs on the root volume and number of tips of the seedlings grown on soil (Figure 29), which increased their values significantly than the control. From these results, it is evident that AgNPs have a stimulatory effect on both seedling physiology and root development at a concentration of 0.1 mM.

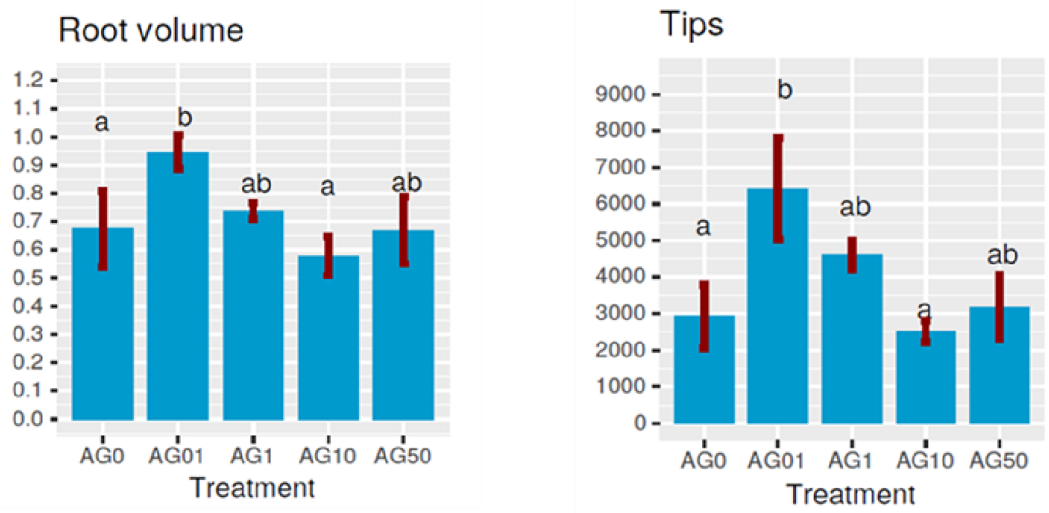


Figure 29. Root growth parameters (root volume and number of root tips) of *Q. pubescens* seedlings grown with AgNPs (0.1, 1, 10, 50 μg/mL) and the control without nanoparticles.

4.1.2 Encapsulation of MHB in alginate microparticles

The two bacteria belonging to the species *Priestia megaterium* and *Pseudomonas jessenii*, which showed a positive action on mycorrhization and the aerial and root development of the plants of *Q. pubescens* and *C. avellana* were selected to be adsorbed on different bead formulations provided by the startup Nanomina (Table 12). As described by Pereira et al. (2018), a concentration of $1x10^7$ cell/mL of the *P. megaterium* strain was prepared and absorbed into the beads. During the storage period, viable bacteria were quantified by measuring CFU/g DW of macro beads at various time intervals: at time 0, after 1 week, 2 weeks, 1 month, and two months (Perez et al., 2018).

Tab 12. Different protocols of microbeads are based on a distinct percentage of polysaccharides in which *P. megaterium* were absorbed.

N° of protocol	Potato starch	Chitosan	Alginate	Bentonite	Glycerol
30	8%	0.80%		1%	
31	8%	0.60%			
32	6%	0.80%		4%	
33	8%		0.25%	1%	
34	8%		0.25%	4%	5%
35	4%		0.25%	4%	5%

Preliminary results for *P. megaterium* showed that the protocols n° 33, 34, and 35 allowed the survival of the bacteria after 1 month quite at the same concentration as the beginning for beads n°35 and a small decrease for beads 34 (from an initial concentration of $1x10^7$ CFU/mL to $8,1x10^6$ CFU/mL after two months) and 33 (from an initial concentration of $8,1x10^6$ CFU/mL to $2,1 \times 10^6$ after two months) (Figure 30).

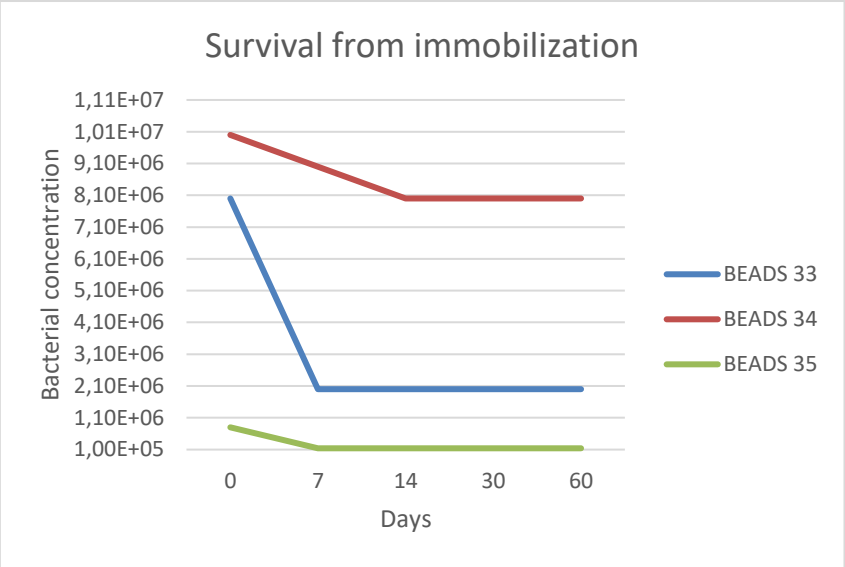


Fig.30. Survival of *P. megaterium* strain after 0, 7, 14, 30, and 60 days after the immobilization in beads

Further analysis and experiments are needed. Indeed, the activities of the project are still in progress and in the future will regard the immobilization of the other bacterial strain (*P. jessenii*) and the research of an appropriate matrix to allow the viability of the fungal inoculum (*T. melanosporum*). Finally, if all the components will be suitable and available for their application, they will be assembled to be tested on plants during the mycorrhization process in a confined environment. Analysis of mycorrhization rate, root, and shoot development of the inoculated seedlings, and their nutrient content will be carried out to assess if the system works. The final biotechnological product could be commercialized and provided to the stakeholders and used in a very easy way.

5. Discussion

5.1. High throughput sequencing analyses importance

Metagenomic technologies such as high throughput sequencing can generate huge amounts of data on the diversity of the truffle environmental community and can also be used to detect the symbiosis in implants with the *Tuber* species declared. Indeed, this methodology allowed to discover that plants of *Q. pubescens-T. aestivum* collected on a truffle ground in Bracciano (RM) were mycorrhized with another species of truffle, *Tuber mesentericum*. *T. aestivum* and *T. mesentericum* ascocarps are often mistaken for one another because of their very similar morphology and because *T. mesentericum* is a complex of species that usually lacks of the phenolic aroma (Leonardi et al. 2021) which is a unique trait of this species (Marozzi et al., 2020). Moreover, *T. aestivum* var. *uncinatum* and *T. mesentericum* can occur in the same environment and ripen at the same time of the year (Marozzi et al., 2020), so a mix of these species can be collected indistinguishably. In 2013, Bonito and colleagues (2013) showed that *T. mesentericum* and *T. aestivum* cluster into one clade, together with *Tuber panniferum* Tul. & C. Tul. and *T. magnatum* in the ITS phylogeny of the *Tuber* genus. Marozzi et al. (2020) confirmed previous findings on the presence of a "*Tuber aestivum–mesentericum* species complex" by showing that *T. mesentericum* and *T. aestivum* also have high genetic variability in the EF1α locus. The results of the HTS analysis showed unequivocal contamination with this *Tuber* specie on the root tips of *Q. pubescens* plants. This is clear evidence of how large-scale sequencing technologies are important for practical application in truffle cultivation. During this thesis work, the sample roots of *Q. pubescens* plants were not used in the tests.

5.1.1. Alpha Diversity indices

Alpha-Diversity metrics summarize the structure of an ecological community concerning its richness (number of taxonomic groups), evenness (distribution of abundances among the groups), or both (Willis, 2019). The values obtained for each sample were gathered according to their category (i.e., *Tuber* spp., Plant species) to evaluate the average microbial richness of every group and compare them with statistical tests. From the results shown in Table 3, *T. melanosporum* factor measured with the Faith pd index showed higher bacterial diversity than *T. aestivum*. This index measures the amount of the phylogenetic tree covered by the community. It is a sum of the minimum branch lengths, so a higher number means more branches and richness.

From these results, it is possible to infer that the higher bacterial phylogenetic diversity varies according to the ectomycorrhizal species and that the host *Tuber* species affects *Tuber* spp.-associated bacterial communities differently (Ye et al., 2018). Indeed, in the study of Ye et al. (2018), it was reported that bacterial compositions differed from each other, even when they were from the same *Tuber* species. Multiple biotic and abiotic factors could drive the composition of the bacterial communities colonizing the fruiting bodies of truffles. The composition of bacterial communities associated with truffles is influenced not only by the stage of the life cycle of the fungus but also by the stage of maturity of the fruiting bodies. As the biochemical composition of fruiting bodies strongly changes during the maturation of *T. melanosporum* fruiting bodies (Harki et al., 2006), the level of maturity could be an important intrinsic driver of the bacterial communities. Indeed, a correlation was noticed between the bacterial community composition and the maturity level of fruiting bodies of *T. borchii*, *T. melanosporum*, and *T. indicum* (Citterio et al., 2001; Antony-Babu et al., 2014; Ye et al., 2018). In contrast, according to Barbieri et al. (2007), the community composition of the white truffle *T. magnatum* remained stable throughout maturation. As reported in Table 4, molecular typing based on the internal transcribed spacer (ITS) region of the rDNA locus has been used to study the fungal communities in *T. melanosporum* and *Tuber aestivum* orchards (Baciarelli Falini et al., 2006; Pruett et al., 2008; Benucci et al., 2011) and in natural *Tuber magnatum* and *Tuber borchii* truffle grounds (Murat et al., 2005; Bertini et al., 2006; Iotti et al., 2010). These results are agreed with the authors that assessed the capacity of *T. melenosporum* to affect the fungal dynamics in truffle grounds. Napoli et al. (2010) assumed that *T. melanosporum*'s presence inside the brûlé (a burnt region next to truffle-colonized plants characterized by a sparse or complete absence of vegetation around a productive tree) (Fasolo-Bonfante & Fontana, 1971; Pacioni, 1991) was correlated to a reduction in the richness of ECM fungi, suggesting that its mycelium may have an inhibitory effect on ECM biodiversity. Lian et al. (2006) described a comparable situation, as they revealed that beneath the fairy rings of *Tricholoma matsutake* shiro (a solid and tight white aggregate of mycelia and mycorrhizas linked to *Tricholoma* basidiomas below the litter layer) the ECM community was dominated by this fungus. Only a few other ECM fungi with low abundance have been detected on the root tips of pine trees. However, this is not true for other *Tuber* species. As reported by Iotti et al. (2010), *T. borchii*, did not reduce the ectomycorrhizal richness, showing that there was no inhibitory effect on ectomycorrhizal biodiversity. Furthermore, according to the findings of Liu et al. (2021), the present results highlighted that the truffle-inhabiting bacterial community had more variation than those of the fungal community.

At least for the Shannon index and number of observed OTUs, there were higher absolute values of bacterial diversity in truffle-associated roots than in those of fungi. Indeed, Liu et al. (2021) in their study reported that alpha-diversity indices based on microbial richness (Chao 1, Shannon, and Simpson indices) in all the four different compartments (bulk soil, adhering soil to peridium, peridium, and gleba) of *Tuber indicum* were significantly higher for bacteria than for fungi. Instead, is interesting to note that for what concerns the richness of the microbial community (Faith pd values), the two *Tuber* spp. significantly differed from each other regarding the bacterial diversity that was higher on *T. melanosporum* and the fungal diversity that was higher on *T. aestivum*. For what concerns the different plant species mycorrhized with *T. melanosporum*, the calculations reported in section 3.1.1.2 showed that among host tree species there are different levels of similarity concerning the bacterial and fungal biodiversity. An interesting result coming from *Q. ilex* plants mycorrhized with *T. melanosporum*, showed that this plant species had the highest amount of both bacterial and fungal biodiversity compared with *Q. pubescens* and *O. carpinifolia* plants. The statistical differences between the diversity indexes that were reported in this study confirmed that the structure of the bacterial and fungal community was differentiated between the host trees. Indeed, local-specific factors such as host trees, geographical distribution, and ectomycorrhizal species may play an important role in determining the structure of the investigated truffle microbial communities, which may help to determine and maintain the balance of a unique ecosystem (Monaco et al., 2020). Fungal and bacterial taxa that were more specific to one or the other habitat helped make this ecosystem more diverse. This shows that there are complex connections between species that could help keep this ecosystem stable. As a central source of nutrients, root exudates create a niche for the growth of microorganisms (Hassan et al., 2019), thus highly contributing to shaping the soil microbiome (Wieland et al., 2001; Garbeva et al., 2004; Nunan et al., 2005). As reported by Habiyaremye et al. (2020), it can be inferred that genetically identical plants create within their root zones comparable microbial niches, resulting in a similar diversity of their root-associated microbiomes and dissimilarities between phylogenetically distant plant species. Secondly, an important explanation for the great diversity of *Q. ilex-T. melanosporum* root-associated microbiomes collected in the Lazio region rather than *O. carpinifolia* and *Q. pubescens* root tips collected in the Umbria region is the fact that the study sites did not share a similar climate with different weather variations. Temperature, the most important variable in defining the climate of a region, is one of the main factors influencing the occurrence, richness, stability, and activity of soil microorganisms (Borowik and Wyszkowska, 2016).

Temperatures in the atmosphere and the soil have been shown to affect the soil microbiome (Alkorta et al., 2017). The atmospheric temperature has a direct effect on soil temperature and indirectly affects host plant productivity as well as the availability of carbon sources for microbial growth (Anderson, 1992; Bardgett et al., 1999). Also, both directly and indirectly, soil temperature significantly shapes the conditions for the growth and development of microorganisms (Borowik and Wyszkowska, 2016). Soil temperature influences microbial metabolism, while the indirect effects are noticed via its impacts on plant productivity (Jefferies et al., 2010). Different atmospheric and soil temperatures amongst the study field sites may have also had an important contribution to the microbial diversity. These results could represent a starting point for future investigations on soil, temperature, and environmental characteristics of different habitats that are affected by fungal and bacterial biodiversity.

5.1.2. Beta Diversity indices

A Beta diversity analysis measures the similarities between samples. Overall, significant differences were discovered between different host trees and truffle groups, even though a significant heterogeneity of the ECM bacterial community was also discovered. Principal Coordinates Analysis (PCoA) based on Bray-Curtis, Jaccard, and weighted UniFrac indexes revealed that the bacterial community present in two distinctive truffle orchards, mycorrhized with *T. aestivum* and *T. melanosporum,* differed from each other creating distinctive cluster between *T. aestivum* and *T. melanosporum* (Figure 8). As shown also by the alpha-diversity analysis, in *T. melanosporum* orchards there was a greater microbial diversity (Table 3). Indeed, Monaco et al. (2020), who characterized the bacterial communities associated with *Tuber aestivum* fruiting bodies collected from truffle grounds in the Molise region (Southern Italy) by using HTS techniques, found a surprising and remarkable heterogeneity across truffle samples, in terms of microbial community composition and relative abundance of the main taxa. Furthermore, the bacterial community between plants (Figure 7) showed that host trees significantly differed from each other. Is reported in fact that the ectomycorrhizal mycelia of some fungi are sensitive to changes to host tree leaf litter composition (Ishida et al., 2007; Benucci et al., 2014). This phenomenon however is not true for all the *Tuber* spp. Liu and colleagues (2021) showed that the influence of *Pinus* and *Quercus* host tree on the bacterial community of *Tuber indicum* was not significant. These variations can be referred to the nutritional and successional status of host species that may also affect the number of microbial species associated with an individual host species.

The statistical differences detected between fungal taxa for all the diversity indices of beta diversity confirmed that the structure of the fungal community was differentiated between the different host trees and the two *Tuber* spp. (Figure 9 and 10 respectively). For what concerns the ectomycorrhizal community, Benucci et al. (2011) investigated the communities of hazels and hornbeams mycorrhized with *Tuber aestivum* and found that hornbeams showed lower levels of richness and ectomycorrhizal diversity than hazels. Indeed, another key aspect that influences the local structure of ectomycorrhizal varieties is host impacts. As reported by many authors, various fungal species can access diverse sources of nutrients in the soil with varying degrees of effectiveness in assemblages with many ectomycorrhizal species (Jonsson et al., 2001; Lilleskov et al., 2002), and plants can allocate resources selectively to "preferred and ideal" ectomycorrhizal associates (Dickie, 2007). Tedersoo et al. (2008) discovered ectomycorrhizal species that strongly preferred certain hosts in a wet sclerophyll forest in Tasmania, even though they did not exhibit a preference for a single species, and showed that these species can dominate the community underground. They found that the frequency of two-thirds of the most common ectomycorrhizal fungi was influenced by host species. Also, Ishida et al. (2007) demonstrated that the ectomycorrhizal fungus community in Japanese mixed forests is directly affected by a preference for a specific host taxon over other taxa, and this preference gets stronger with an increasing taxonomic distance of the host. Evidence of a preference for a particular host plant was also found by Iotti et al. (2010) in an area that contained natural, mixed-host *T. borchii*. The ectomycorrhizal community that was found in oak roots differed significantly from that found in pine. Morris et al. (2009) suggest that evergreen vs. deciduous habitats also influence the structure of the ectomycorrhizal community. According to this research on the ectomycorrhizal community in Californian woodlands, even congeneric oak species (*Quercus douglasii* and *Quercus wislizeni*) could produce diverse ecological niches for ectomycorrhizal fungi. In fact, in the present study, *Q. pubescens* and *Q. ilex* species developed different niches containing dissimilar fungal communities. The results of alfa- and beta-diversity indexes indicate that at least between deciduous (*Q. pubescens*) and evergreen (*Q. ilex*) oaks, there is a difference in the abundance and variety of fungal and bacterial microbiomes. Hence, some taxa are not shared, or their presence is noticeably changing between the two oaks. This variability can be explained by looking at the habitat and the geographical zones occupied by these species. Indeed, in the Mediterranean area, *Q. pubescens* co-occurs with holm oak and tends to compete for carbon assimilation with the evergreen oak specie (*Q. ilex*).

Physiological characteristics that differ between the two species, like, for example, the leaf shape and dimension, are important components of drought tolerance by *Q. pubescens* and are likely to contribute to its competitive ability with *Q. ilex* in the Mediterranean basin (Damesin et al., 1997). These ecological differences are probably affecting the diversity of the soil microbiome, favoring those organisms that are better adapted to certain conditions. Following these previous findings, our data suggest the possibility that a host-preference effect exists and is involved in the partitioning of the fungal community between *Tuber* and plant species under study.

5.1.3. Common bacterial OTUs distribution

The results revealed that exists a composition of *T. melanosporum* ectomycorrhizal bacterial communities close to that obtained in ectomycorrhizae of *T. aestivum,* in agreement with previous research (Gryndler et al. 2013). Looking at the Venn diagram displayed in Table 9 28% of bacterial OTUs are shared between samples of *O. carpinifolia* mycorrhized with *T. aestivum* and *T. melanosporum.* The overall similarity of the bacterial communities inhabiting different *Tuber* species suggests that, regardless of the species, the truffle ectomycorrhizae create a specific habitat for a core bacterial microbiome (Splivallo et al. 2015). Indeed, from the results shown in section 3.1.3.1, the most representative bacterial phylum shared between the two *Tuber* spp. belongs to the phylum *Cyanobacteria,* a group of photosynthetic, nitrogen-fixing bacteria that forms symbioses with various plants (Dixon and Kahn 2004), using sugars produced by photosynthesis to fix nitrogen. Their presence is also reported by Monaco et al (2020) on *T. aestivum* and by Herrero de Aza et al. (2022) on *T. melanosporum*. The presence of members of nitrogen-fixing bacteria also including members of the family *Bradyrhizobiaceae* and the genus *Bradyrhizobium* is frequently reported not only for *T. aestivum* and *T. melanosporum,* but also for other *Tuber* species (Le Tacon et al. 2016) as also Barbieri et al (2010) highlighted in a *T. magnatum* study. Indeed, as also Pent et al. (2017) assessed, the *Alphaproteobacteria* (mostly *Bradyrhizobiaceae*) dominated the fruitbodies of various *Tuber* species (Antony-Babu et al., 2014; Benucci and Bonito, 2016) and ECM of several fungi (Kataoka et al., 2008; Uroz et al., 2012; Deveau et al., 2016). This aspect is relevant: in fact, the ability to modify nutrient availability during their biological cycle could be of particular importance during the development of fruiting bodies which need nutrients to complete the maturation process independently of the host plant (Barbieri et al. 2010). Indeed, these bacterial genera were found to be shared also between the different plants mycorrhized with *T. melanosporum* reported in Table 10.

For example, the genus *Micromonospora* was common among the two *Tuber* spp. and has been reported to include helper bacteria in *Tuber* spp. (Gryndler et al. 2013; Benucci and Bonito 2016; Ye et al. 2018; Monaco et al., 2020). The strong selection of a specific bacterial core has been observed not only with different *Tuber* spp. but also in the host trees. Indeed, some bacterial genera like the nitrogen-fixing bacteria belonging to the genus *Cyanobacteria*, the family *Bradyrhizobiaceae*, and the genus *Bradyrhizobium*, but also bacterial families of *Pseudonocardiaceae* and *Steroidobacteraceae* were shared independently by the host trees. The members of the *Pseudonocardiaceae* (phylum *Actinobacteria*), although their direct fungal helper functions are unknown, are considered to protect the fungi from other competing fungal contaminants (Schoenian et al. 2011) due to their ability to degrade recalcitrant humic substances and lignocellulose (Stomeo et al. 2008; Porca et al. 2012). Deveau et al (2016) also reported their abundance in both *T. aestivum* and *T. melanosporum* ectomycorrhizae. Nevertheless, some bacteria were shared between specific host trees and not between all. This aspect could suggest that some host-adapted microbes may have been chosen because they provide a selective advantage for their host, and there is increasing evidence of plant recruitment of microorganisms from the ectosphere to fight pathogens (Berendsen et al., 2012), and/or to improve nutrition and growth (e.g., via mycorrhiza formation). For example, the order *Streptomycetales* shared between the host plant *Q. ilex* and *O. carpinifolia* is reported as helper bacteria fostering nutrient acquisition and enhancing the formation of mycorrhizas in filamentous fungi (Tarkka et al. 2016). The root tips of *Q. ilex*, *Q. pubescens* and *O. carpinifolia* shared the bacterial order *Hyphomicrobiales* which also includes the genus *Bradyrhizobium* retrieved at high frequency from other ectomycorrhizal fungi such as *Cenococcum geophilum* (Kataoka et al. 2008). Many of these bacteria can fix nitrogen (N), leading some to hypothesize that these bacteria contribute to the fungal nutritional requirements for N (Kataoka et al. 2008; Barbieri et al. 2010; Antony-Babu et al. 2014). The bacterial order *Rhodospirillales* was found to be shared predominantly on samples of *O. carpinifolia* and *Q. pubescens*. This order was found to be involved in the production of phenazines, which are known to inhibit bacterial and fungal growth (Mavrodi et al., 2010) thereby increasing its competitiveness and ecological fitness (Pierson et al., 2010). This could also have a positive effect as these broad-specificity antibiotics might control fast-growing bacterial and fungal pathogens. Root tips of *C. avellana* and *Q. pubescens* shared members of the genus *Actinoplanes* (phylum *Actinobacteria*) that have an endophytic lifestyle that occurs abundantly in most plants as a metal-tolerant microorganism (Govindasamy et al., 2014, Chalot et al., 2021). Indeed, the *Actinobacteria* are recognized for their ability to produce an array of antibiotics and enzymes

(Shivlata et al., 2015) that may play a role in antagonistic interactions with other soil microorganisms. Bacteria belonging to the family *Steroidobacteraceae* (order *Nevskiales*) are not reported to be truffle-associated but are known to be involved in plant growth promotion and soil nutrient cycling (Tang et al., 2022). The presence of nitrogen-fixing bacteria as the phylum *Cyanobacteria*, the family *Bradyrhizobiaceae*, the genus *Bradyrhizobium* and MHB as the genus *Streptomyces* on *Q. ilex* root tips both in Bracciano and Scheggino, indicate their important function in the mycorrhizal symbiosis. Indeed, some common trends among bacterial communities from different ectomycorrhizal fungal species and host trees also exist, suggesting a possible core bacterial community (Burke et al. 2008; Uroz et al. 2012).

5.1.4. Common fungal OTUs distribution

Ectomycorrhizal communities on root tips in natural and cultivated truffle orchards have been amply investigated (Bencivenga and Donnini, 1995; Murat et al., 2005; Baciarelli Falini et al., 2006; Pruett et al., 2008; Agueda et al., 2010; González-Armada et al., 2010; Iotti et al., 2010; Benucci et al., 2011; Garcia-Barreda and Reyna, 2012; Leonardi et al., 2013). In this study, were analyzed the unculturable microbial communities in different environments to focus on the main microbial core composition of truffle grounds. From the Venn diagram reported in Table 9, the results of the alpha diversity shown in Table 4 are confirmed. The number of fungal OTUs was drastically reduced in the root samples of *O. carpinifolia* mycorrhized with *T. melanosporum* (36) concerning samples mycorrhized with *T. aestivum* (166). This result confirms the fact that *T. melanosporum*, when present as fungal inoculum for the mycorrhization of the host tree, can break down the fungal biodiversity (Napoli et al., 2010). Also, Herrero de Aza et al. (2022) recently showed that *T. melanosporum* appeared alone in the first group without the presence of other fungal species. It might be a result of *T. melanosporum*'s ability to compete with other ectomycorrhizal fungi on the same plant. Streiblová et al. (2012) proposed that truffles may adopt an effective survival strategy by distributing their metabolites, which are thought to have allelopathic effects on herbaceous plants and microorganisms in the rhizosphere, generating a non-vegetated region surrounding the host plant known as a brûlé (Mello et al., 2013). Mello et al. (2015) investigated the composition of fungal communities in *T. melanosporum* orchards and discovered that the fungal biodiversity was lower inside the brûlé than outside the brûlée. Furthermore, Napoli et al. (2010), reported a lower diversity of fungi in the soils sampled within than outside the brûlé formed by *T. melanosporum*-colonized plants, and Belfiori et al. (2012) reported that this decrease in the richness of ECM species on the roots of plants is reflected by a parallel

decrease in soil fungi from cultivated vs. natural *T. melanosporum* truffle grounds. All these findings support the previously described results.

Despite this fact, also for fungi exist as a fraction of common species in the ectomycorrhizal environment. Looking at the Venn diagram in Table 9, between root tips of *O. carpinifolia* mycorrhized with *T. aestivum* and *T. melanosporum* a 22% of fungal OTUs were shared. Among them, the order *Thelephorales* (phylum *Basidiomycota*) was one of the most common taxa in the *T. aestivum* and *T. melanosporum* root tips samples. These findings are in accordance with results of the ectomycorrhizal community reported by other authors. The ectomycorrhizal species that were observed, which belonged among the other to the *Thelephoraceae* family are common mycobionts in various communities of ectomycorrhizal fungi in plants (Kõljalg et al., 2000; Glen et al., 2002; Selosse et al., 2002; Avis et al., 2003; Urban et al., 2003; Salerni et al., 2010) and are consistent with those reported previously for environments in which truffles are produced (Murat et al., 2005; Baciarelli Falini et al., 2006; Iotti & Zambonelli, 2006; Pruett al., 2008; Iotti et al., 2010; Leonardi et al., 2013; Salerni et al., 2014; De Miguel et al., 2015). Furthermore, the presence of both the *Tuber* spp. of the genus *Talaromyces* (phylum *Ascomycota*), fungal species known to produce metabolites with well-defined antibiotic and/or antifungal activity such as rugulosin and wortmannilactones from *Talaromyces wortmannii* (Frisvad et al. 1990; Yuesheng et al. 2006), suggests that the association with guest fungi may represent one of the strategies that *Tuber* spp. adopt to face the surrounding environment and control potential microorganisms that may antagonize their development (Pacioni et al., 2007). Indeed, most probably these fungal species can compete and protect against soil-borne pathogens like for example the genera *Dactylonectria* that was found to be widespread in the root samples. Fungi belonging to this genus are known to contain a high number of plant pathogenic species (Almario et al., 2017), albeit some authors reported that there was no evidence of pathogenicity and that can be a non-pathogenic endophyte on weeds or grapevine (Gramaje et al., 2020; Xue et al., 2019). Another common group of fungi detected in the two *Tuber* spp. was the ascomycete order *Helotiales* that includes an ecologically diverse group of plant pathogens, wood-, debris- and soil saprobes, plant endophytes, and mutualistic ericoid mycorrhizal (ERM) and ectomycorrhizal (ECM) fungi (Vrålstad et al., 2002). The genus *Tetracladium* (order *Helotiales*) was also observed as a common fungus. Members of this genus are endophytic fungi, capable of promoting plant growth (Zhong et al., 2022). Some strain (*Tetracladium setigerum*) was reported as a phosphate solubilization fungi, which has the potential to solubilize various sources of phosphates through the production of phytases and phosphatases enzymes and the organic acids (Sati et al., 2018).

Zambonelli et al. (2016) showed the capacity of the specie *Tetracladium maxilliforme* to affect the mycorrhization process and it was reported by Pacioni et al. (2007) as a truffle-inhabiting fungus. This fungal genus seems to play an important role in the formation and maturation of the *T. melanosporum* ascoma and in the production of the volatile organic compounds, which are responsible for the aroma of the truffle. Although the status of the interaction between guest fungi and their hosts may be transient, as the stability or variability of asymptomatic interaction might depend on numerous factors, all these data lead to arguing that the isolated mycelia may exert a protective rather than a saprotrophic/parasitic role in *Tuber* spp., likely controlling the bacterial growth in developing ascomata and preventing their early consumption by predators. As also reported on the Venn diagram in Table 10, the host tree mycorrhized with *T. melanosporum* differentially shared fungal taxa. The most common one in all the host trees was the species *T. melanosporum* present in two OTUs (1 and 352) for a total of 1174.230 reads (while the specie *T. aestivum* was present in three OTUs for a total of 7.715 reads). Indeed, in this case, the HTS instrument allowed to attest that this ectomycorrhizal specie was quantitively the most abundant in all the samples with respect to the other fungal species as also reported by Turco et al. (2021), where this technique associated with the qPCR assay, allowed in detecting and quantifying *Gnomoniospis castaneae* in the endophytic state, and in different host tissues. However, a preference for a certain host taxon over other taxa can affect the ectomycorrhizal fungi community directly (Ishida et al., 2007) and examples of a preference for a particular host plant have also been found. Different plant roots can attract beneficial microorganisms from surrounding soil, and those play important roles in plant performance, especially by improving plant mineral nutrition (de Dieu Habiyaremye et al., 2020). For example, Iotti et al. (2010) found that, in the case of *T. borchii*, the ECM on oak roots differed significantly from those on pine roots. Benucci et al. (2011) investigated the ectomycorrhizae of hornbeam and hazel in a *T. aestivum* orchard and found fungi belonging to the *Thelephoraceae* and *Pezizamycetes* exclusively on the roots of the hazel trees. Host effects may be important factors that determine the structure of the ectomycorrhizal community (Nantel & Neumann, 1992; Kernaghan et al., 2003), and the results shown in section 3.1.3.2 support this theory. For what concerns the plant mycorrhized with *T. melanosporum*, once again, the *Thelephorales* genus was the most common taxa on the two oak host trees that seem to share a very similar fungal community. Another fungal genus shared was the *Cyphellophora*, a yeast genus essential in ecological processes involving the mineralization of organic matter (Botha, 2011). These findings agree with the previously reported ectomycorrhizal status of oaks (Herrmann and Buscot, 2007) and the tree's ability to interact with

large microbial communities which assist in nutrient acquisition (Jumpponen and Jones, 2009; Tarkka et al., 2013).

The host trees *Q. ilex*, *O. carpinifolia* and *C. avellana* shared the presence of the genus *Fusarium* (phylum *Ascomycota*). The presence of this genus agreed with previous results reported by Herrero de Aza et al. (2022) where there was a high abundance of the genus *Fusarium* on *T. melanosporum* ECM.

Fusarium is a complex group of ubiquitous soil fungi with saprotrophic and/or pathogenic properties (Booth 1971). The *Fusarium* species are found in agricultural soils worldwide, and while some are non-pathogenic others are responsible for significant crop damage (Gordon & Martyn, 1997), and serious root rot in forest nurseries (Kim et al., 2012). It is much less frequently reported from forest soils than cultivated soils (Park, 1963; Latiffah & Azaman, 2011), and may be related to the quality of root exudates produced in the mycorrhizosphere of trees (Grayston et al., 1996). The same consideration needs to be done for the genus *Cercospora* (phylum *Ascomycota*) a phytopathogen (Toju et al., 2018) that was shared between *Q. ilex*, *O. carpinifolia* and *Q. pubescens* host trees. The hypogeous fungus *Hymenogaster olivaceus* was reported as common specie between *O. carpinifolia* and *Q. pubescens*. Indeed, this specie was observed in ectomycorrhizal association with *Tilia, Fagus, Quercus, Ostrya, Picea, Larix* (Pegler et al. 1993) and present also on root samples mycorrhized with *T. magnatum* (Leonardi et al., 2013). Another common fungal specie between *O. carpinifolia* and *Q. pubescens*, *Epicoleosporium ramularioides* (phylum *Ascomycota*), is reported as a plant pathogenic specie (Videira et al. 2016). The two *Q. ilex* host trees shared a high presence of phytopathogens fungal taxa as *Dactylonectria*, *Cercospora*, *Fusarium,* and *Neopestalotiopsis* belonging to the order *Xylariales* and generally known as pestalotioid fungi (Maharachchikumbura et al, 2014). These taxa are significant phytopathogens causing postharvest fruit rot and trunk diseases in grapevines in many countries (Arzanlou et al, 2013; Jayawardene et al, 2015). The other fungal specie *Knufia tsunedae* (phylum *Ascomycota*) was shared between the two geographical sites and is described as a soil-associated fungus (Madrid et al., 2016).

Despite these findings, the presence of these putative plant pathogens did not appear to harm the trees in the plantations, since Oliach et al. (2020) and Herrero de Aza et al. (2022) also found a greater development of the root collar in these soils with a higher abundance of *T. melanosporum* and pathogens. The tree root-associated microorganisms are well-known to serve in improving tree health and nutrition, preventing the establishment of pathogens, and adapting to specific local environmental conditions (Uroz et al., 2016; Gehring et al., 2017; Lau et al., 2017).

5.1.5. LEfSe abundance analysis for bacteria

Linear Discriminant Analysis (LDA) technique used for data classification and dimensionality reduction of HTS results, showed significantly different patterns for bacteria, in line with the results obtained by looking at alpha- and beta-diversity, and the ten most abundant OTUs for each category. The results shown in Figure 11A and 11B, confirmed earlier data for other *Tuber* species, where the phyla *Proteobacteria, Actinobacteria,* and *Bacteroidetes* formed the core components of bacterial communities (Vahdatzadeh et al., 2015; Chen et al., 2019; Wang et al., 2021). Among the two *Tuber* spp. is evident that the phylum *Proteobacteria* is the most abundant on *T. melanosporum* and the second one on *T. aestivum*. Within the two *Tuber* spp., however, this phylum differs due to the predominant presence of different classes: the *Gammaproteobacteria* in *T. aestivum* and the *Alphaproteobacteria* class in *T. melanosporum*. These results are agreed with those reported by Herrero de Aza et al. (2022), where the *T. melanosporum* plantations were enriched in the *Alphaproteobacteria* class. In *T. aestivum* root tips the family *Micromonosporaceae* (phylum *Actinobacteria*) was the most abundant.

The *Actinobacteria* phylum with genera as *Lentzea, Actinosynnema, Allokutzneria,* and the members of the suborder *Pseudonocardineae* (that resulted among the 10 most abundant fungal spp. in *T. aestivum,* Table 10) were proved to positively correlate with the presence of *T. aestivum* in ectomycorrhizae (Gryndler and Hršelová 2012). There is no information on interactions of the members of this suborder with fungi or plants available in the literature. Some members of the *Pseudonocardineae* group (including *Kibdelosporangium* and *Lentzea*) also possess "exotic" metabolic capacities such as the ability to degrade poly-Lactate (a kind of plastic), which was originally considered unique among *Actinobacteria* (Jarerat et al., 2002). Furthermore, members of the family *Micromonosporaceae* are known as plant growth-promoting rhizobacteria (Kruasuwan et al., 2016) and were obtained also by Gryndler et al. (2012) from ectomycorrhizae collected within the *T. aestivum* colony. The large numbers of *Micromonospora* populations in the roots suggest that the presence of this microorganism is not casual, and it may play an important ecological role that is not restricted to a single plant species (Carro et al. 2012). Indeed, this group of bacteria was not statistically differentiated among the host tree roots (Figures 12 A and 12 B). Another species, *Rhodoferax ferrireducens* (class *Betaproteobacteria*) was found to be highly enriched in summer truffle orchards and is of interest due to its potentially important role in carbon and metal cycling in soils and sediments and in its novel ability to convert sugars into electricity (Chaudhuri et al., 2003). It is one of the few known facultative microorganisms that can grow anaerobically by oxidizing

organic compounds to carbon dioxide with Fe (III) serving as the electron acceptor. This property, as well as its ability to grow at the low temperatures found in many subsurface environments, suggests that it could contribute to the oxidation of organic matter coupled with the reduction of Fe (III) in many soils and sediments (Risso et al., 2009). Bacteria from the family *Chitinophagaceae* (phylum *Bacteroidetes*) were found to be statistically abundant on *Tuber* spp. Members of this family have been reported to be widely distributed in diverse habitats, such as forest soil, greenhouse soil, rhizosphere soil of soybean and rice, root endophytes, vermicompost, fresh water and air samples, and associated with ectomycorrhizal fungi such as *Tuber indicum* (Urios et al., 2006; Qu et al., 2008; Lim et al., 2009; Wang et al., 2021) and possessing plant-growth-promoting traits (Madhaiyan et al., 2015). On *T. melanosporum* colonized root tips, the class of *Alphaproteobacteria* was statistically more abundant, as reported by other authors (Barbieri et al. 2007, Barbieri et al. 2010, Antony-Babu et al. 2014, Quandt et al. 2015). This class harbors a miscellaneous set of metabolisms, cellular phenotypes, and a wide range of habitats, including phototrophic genera (*Rhodobacter*) and symbionts of plants (*Rhizobium, Sinorhizobium, Mesorhizobium, and Azorhizobium*) (van Rhijn et al., 1995).

Particularly intriguing is the ability of *Alphaproteobacteria* to interact with plants, as pathogens and as nonpathogenic mutualists or commensals (symbionts or nonsymbionts) (e.g., *Rhizobium, Azospirillum*). Plant-associated bacteria *sensu lato* can be found in and around roots, in the vasculature, and on aerial tissues or in specifically developed organs (e.g., root nodules) (Danhorn et al., 2007), allowing for the categorization of strains as phyllospheric, rhizospheric, and endophytic (Pini et al., 2011). Furthermore, *Rhizobiales* (syn. *Hyphomicrobiales*, *Alphaproteobacteria*), were found to be predominantly abundant only on *Q. pubescens-T. melanosporum* root tips (Figure 12B), are well-studied associates of plants (Erlacher et al., 2015). They commonly exert beneficial functions for their hosts by providing various nutrients, phytohormones, as well as precursors for essential plant metabolites (Delmotte et al., 2009; Verginer et al., 2010). This order contains many genera of nitrogen-fixing, methanotrophic, legume-nodulating, microsymbiotic bacteria (Jourand et al., 2004; Garrity et al., 2005). Recently, nitrogen fixation was shown not to be limited to *Rhizobiales* in leguminous plants, but also to be expressed within various endophytic compartments of non-leguminous plants (Fischer et al., 2012). The family of *Bradyrhizobiaceae*, a member of the *Rhizobiales* order was significantly abundant in another *Quercus* species, *Q. ilex- T. melanosporum*, and not on *T. aestivum* ECMroot tips. Indeed, these members are reported among the bacterial genera negatively correlated with *T. aestivum* ectomycorrhizae. On the contrary, some previous studies

suggested a preferential association of *Rhizobiales* bacteria with *Tuber* spp. ascocarps besides *T. melanosporum*. For example, Barbieri and colleagues (2005) isolated bacteria closely related to *Phyllobacterium* and *Rhizobium* from *Tuber borchii* ascocarps. Cerigini and colleagues (2008) described a highly specific lectin in *T. borchii* ascocarps that recognizes surface polysaccharides of specific *Ensifer* and *Rhizobium* isolates obtained from the ascocarps. This bacterial order possesses some harmful characteristics as it did not select inside its bacterial core community.

Another genus that was statistically more abundant on downy oak and hornbeam plants in the same proportion was the *Vicinamibacter* (class *Vicinamibacteria*). This genus was reported to promote plant growth on a terrestrial well-defined model plant, *Arabidopsis thaliana* (Kielak et al., 2016; Yoneda et al., 2021) and has never been reported as *T. melanosporum*-associated bacterium. Concerning the other oak species, *Q. ilex-T. melanosporum*, their root tips were found to be more associated with taxa belonging to the phylum *Bacteroidetes*, also known as the *Cytophaga–Flexibacter–Bacteroides* group, and consists of three major groups: *Bacteroides, Flavobacteria*, and *Sphingobacteria* (Kim and Kwon 2010). Members of the *Bacteroidetes* are thought to be primarily particle-associated, and their hypothesized role is to initiate the mineralization of high-molecular-weight organic matter (Kirchman, 2002).

Within the *Bacteroidetes* phylum, the root tips of holly oak possess in abundance taxa related to the class *Chitinophagales*, which are reported as endophytic bacteria that ubiquitously inhabit most plant species and have been isolated from a variety of plants, including both monocotyledonous and dicotyledonous plants, ranging from woody tree species, such as oak and pear, to herbaceous crop plants such as sugar beet and maize (Lodewyck et al., 2002; Shi et al., 2014).

These bacteria are known to interact with plants and may exchange signaling molecules and utilize readily secreted compounds. Their proximity to the root might also be beneficial for the plant, as bacterial-reactive molecules, such as phytohormones, may act more efficiently in the vicinity of root system (Haichar et al., 2008). On *O. carpinifolia-T. melanosporum* root tips, the statistically more abundant bacterial taxon was the *Burkholderiales* order, that as abovementioned, is beneath the dominant cultivated bacteria associated with ectomycorrhizal fungi and forest soils (Bending et al., 2002, Duponnois et al., 1991, Poole et al., 2001). Furthermore, besides the specie *Vicinamibacteria silvestris*, was recorded the highest abundance of the order *Caulobacterales* that have not been detected by sequencing or cultivation methods in any *Tuber* ectomycorrhizae yet. For the first time, Rinta-Kanto et al. (2018) described the members of the prokaryotic communities colonizing common forest mushrooms and assess for the first time the presence of *Caulobacteria* in higher relative

abundances in *Cantharellus cibarius* fruiting bodies. This order may have a role in glucan degradation, as suggested by Eichorst and Kuske (2012). Bacteria belonging to these classes are adapted to low-nutrient environments, and they may have a role in supplementing the nutritional demands of the host by fixing nitrogen (Li and Castellano 1987, Barbieri et al. 2010, Sellstedt and Richau 2013), or solubilizing phosphate for the use of the fungus (Pavic et al. 2013). On *C. avellana-T. melansoporum*, among the *Actinobacteria* phylum that was found to be the most abundant taxa, the specie *Streptomyces canus* was statistically more abundant on hazel root tips than in the other plants. This species (*S. canus* BYB02) was reported as a termite-associated actinomycete that could availably produce activities against several plant pathogens thanks to antifungal compounds and could be a good source for bioactive compounds (Zhang et al., 2013). Members of the genus *Cryptosporangium* (phylum *Actinobacteria*) were also abundant on hazel root tips colonized by *T. melanosporum*. This genus is characterized by forming sporangia in substrate mycelia (Nurkanto et al., 2015). *Cryptosporangium* spp. has also been isolated from plant litter (Suriyachadkun et al., 2020) or as endophytes of plants (Himaman et al., 2017), and many of these *Actinobacteria* also including *Streptomyces,* can produce indole-3-Acetic Acid (IAA), siderophores, enzymes to solubilize tricalcium phosphate, antimicrobial compounds effective against plant pathogens, and signaling molecules for potential communication with other species (Insuk et al., 2022).

The presence of bacteria producing antimicrobial compounds on root tips of old hazelnut plants may indicate a protecting role against soil-borne pathogens which could affect the health of these plants. Besides the higher and abundant presence of the phylum *Actinobacteria* widespread in all the ECM plants, these analyses revealed the co-existence of bacterial core shared within the *Fagales* order *Quercus pubescens* and *Ostrya carpinifolia* shared the abundance of the class *Vicinamibacteria*, while other host trees revealed a distinctive bacterial community.

5.1.6. LEfSe abundance analysis for fungi

As mentioned above, it is evident that HTS reads from *T. melanosporum* samples are less abundant in terms of fungal community than *T. aestivum*. Indeed, *T. melanosporum* (Napoli et al. 2010; Belfiori et al. 2012) produces spectacular conditioning of the vegetation and microbial communities around the host plant (within the area called "brûlé" or "pianello") where its ectomycorrhizas and mycelium are prevalent. In this case study, however, when detected, ectomycorrhizae of the summer truffle always occurred alongside other ectomycorrhizal species, but its presence was not significantly higher in any samples, which were all obtained from productive trees, as reported also by Benucci et al.

(2011). The absence of *T. aestivum* ectomycorrhizae in the samples from productive host plants might reflect that the production of fruiting bodies of ectomycorrhizal fungi does not correlate with their distribution and abundance on a host root system (Horton & Bruns, 2001; Lalli et al., 2015). A situation almost like that was found with the ectomycorrhizas of *T. aestivum* in its production areas (Zambonelli et al. 2005; Benucci et al. 2011). Iotti et al. (2010) showed that *T. borchii* ectomycorrhizas accounted for 20% of the ECM fungal community, and Bonito et al. (2011) found that natural colonization of *Tuber lyonii* F.K. butters accounted for 17% of ectomycorrhizas in pecan (*Carya illinoinensis*) orchards in the USA (Bonito et al., 2013). In general, the abundance of *Ascomycota* and *Basidiomycota* phyla differed between the two *Tuber* spp. (Figure 14A, 14B). In fact, the first one occurred principally and significantly more on *T. melanosporum* orchards, while the second one was more abundant on summer truffle plantations.

Napoli et al. (2010) reported that the presence of *Basidiomycota* sequences of samples ectomycorrhized with *T. melanosporum* decreased inside the brûlé, indicating instead a higher percentage of the phylum *Ascomycota*. Even though recently, molecular methods of identification have been widely used to support classical morphological techniques in the characterization of assemblages of ectomycorrhizal species (Gardes & Bruns, 1993; Horton & Bruns, 2001), the knowledge of the diversity of truffle environment ectomycorrhizal fungi is generally poor.

Recently, Murat et al. (2005) reported that the most common ectomycorrhizal family present in natural *T. magnatum* grounds is *Thelephoraceae*, which is represented in all cases by *Tomentella*. Most recently, Benucci et al. (2011) reported that *Thelephoraceae* was the most common family with 12 species, followed by the order Pezizales. The LDA Effect Size analysis revealed that on *O. carpinifolia-T. aestivum* root tips one of the most abundant taxa belongs to the genus *Pseudotomentella* (family *Telephoraceae*), which are important contributors to the ectomycorrhizal diversity of temperate and boreal forests (Di Marino et al., 2007). Another most abundant class on *T. aestivum* samples was *Agaricomycetes* (phylum *Basidiomycota*), which is reported to be ECM fungi that together with *Tuber* (*Pezizomycetes*) species were associated with the poplar roots (Yung et al., 2021). Members of this class are a macrofungal group that has an important role in the nutrient cycling of various elements such as carbon, nitrogen, and oxygen (Miles et al., 2004). Concerning the fungal community associated predominantly with *T. melanosporum* shown in Figures 15A, 15B, on *Q. pubescens* there was a significant abundance of the *Ascomycota* phylum and the genus *Exophiala*. Members of this fungal genus are common saprobes in soil and water environments or endophytes in plant roots. Their ecological versatility could imply a capacity to produce diverse secondary

metabolites (Cheikh-Ali et al., 2015). Some root-endophytic strains of *Exophiala* have shown a capacity to confer benefits to their host plants by increasing their tolerance to abiotic stresses (Khan et al., 2011; Li et al., 2011). *Q. ilex-T. melanosporum* ectomycorrhized plants shared the significant abundance of the *Agaricomycetes* order with the horn beam plants ectomycorrhized with *T. aestivum*. Ectomycorrhizal fungi belonging to the order *Boletales* as the genus *Xerocomellus* were abundant on *Q. ilex* root tips. The specie *Xerocomellus chrysenteron* is an edible mushroom with insecticidal properties (Trigueros et al., 2003) and the presence of its genus indicate a good level of *Tuber* colonization, as also reported by De Miguel et al. (2014), where ectomycorrhizae of *T. melanosporum* were the most frequent in productive plots. In their work, *T. rufum* and members of the *Boletales* were only found in productive plots. Some authors include *Scleroderma* and *Tuber trichophea* among the species associated with good truffle sporocarp production, which have been reported to be common in truffle grounds (Pruett et al., 2008). For example, Baciarelli Falini et al. (2006) found that *T. melanosporum* ectomycorrhizae were abundant and shared the habitat with other ectomycorrhizal fungi, such as *T. borchii*, *T. aestivum*, *T. brumale*, *Scleroderma* spp., and *Cortinarius* spp.

Another genus that was found to be significantly abundant on *Q. ilex-T. melanosporum* root tips was *Russula*, which is found in the soil under hardwoods and conifers. *Russula* is an ectomycorrhizal (ECM) fungal species, and its existence relates to certain trees.

In Southeast Asia, some genera of ECM fungi such as *Russula,* together with *Scleroderma, Laccaria, Amanita, Cortinarius*, etc., are associated with the family tree of *Dipterocarpaceae* (Lancellotti et al., 2011). Moreover, other ectomycorrhizal fungi such as *Melanogaster* which was abundant in the holly oak root tips, have also been regularly detected in truffle plantations (De Miguel et al. 2014). Within the genus *Melanogaster*, ectomycorrhizal fungi associated with conifer and hardwood species (Alvarez et al. 1993), the species reported to be more abundant in this root tips sample, *M. ambiguus*, produce abundant rhizomorphs, which are considered to play an important role in water transport to host plants (Duddridge et al. 1980). The *Dothideomycets* class was significantly the most abundant taxon on the root tips of *C. avellana-T. melanosporum* and, despite not being reported to be associated with *Tuber* spp., this class is known to be very abundant endophytic fungi associated with *Holcoglossum* plants (*Orchidaceae*) (Tan et al., 2012). Another family that was significantly prevalent on hazelnut root tips was the *Inocybaceae*. If the *Basidyomicota* order decreased inside the brûlé, this taxon increased in sequences moving outside the brûlé of *T. melanosporum* (from 2% to 18%), where the most represented groups were ECM fungi belonging to the *Thelephorales* and *Agaricales* orders, as *Inocybe* (Mello et al., 2013). Furthermore, in the work of Pérez et al. (2007), *Q.*

ilex and *Q. robur* roots mycorrhized with *T. melansoporum* showed colonization of *Inocybe*. This fungal genus is widely spread also on another *Tuber* spp. as shown by Iotti et al. (2010). In their work, they reported the presence of *Thelephoraceae*, *Inocybaceae*, *Sebacinaceae*, and *Pyronemataceae* in natural *T. borchii* ground, in both fruiting and nonfruiting points, and their distribution was strongly influenced by the host plant. Members of the order *Pleosporales*, including the genus *Dictyocheirospora* were also significantly abundant on hazel root tips. This order is reported to be present both inside and outside the brûlé with the same percentage (40%) (Napoli et al., 2010). The *Dictyosporiaceae* fam. nov. was introduced to accommodate a holomorphic group of *Dothideomycetes* that are saprobes on decaying wood and plant debris in terrestrial and freshwater habitats (Ho et al. 2002, Pinnoi et al. 2006, Pinruan et al. 2007; Yang et al., 2018). Their association with *C. avellana* plants could suggest an endophytically co-existence that is beneficial for the health of this host tree without compromising the truffle production.

Finally, the analysis of the fungal taxa differentially abundant on root tips of holly oaks collected in two different regions resulted in a significant presence of fungal taxa only in the Umbria region.

First was recorded the presence of the fungal taxon belonging to the family *Neopyrenochaetaceae* (order *Pleosporales*, *Ascomycota*). Members of this family were firstly isolated from submerged decaying wood in Thailand and subsequentially in Spain (Magaña-Dueñas et al., 2021) and its ecological role is not reported yet. There was also a significant abundance of the *Ascomycota* phylum and the class of *Sordariomycetes*, which is an important class of ascomycetes that colonize diverse habitats (Hongsanan et al. 2017). It is the second-largest class of *Ascomycota* (Hyde et al. 2013, Maharachchikumbura et al. 2015, 2016). This class has a cosmopolitan distribution and can be found in almost all ecosystems (Pratibha et al. 2014, Jones et al. 2015). Some taxa of *Sordariomycetes* are economically important as biocontrol agents (Harman et al., 2004), and some species produce a wide range of important secondary metabolites (Maharachchikumbura et al., 2016; Hyde et al., 2021). Another specie significantly abundant only on the Scheggino site (Figure 16) was *Fusarium equiseti*. This specie produces a type of mycotoxin (A trichothecene) that cause serious diseases in human and animals (Marìn et al., 2015) and the genus *Fusarium* represents complex groups of ubiquitous soil fungi with saprotrophic and/or pathogenic properties (Liu et al., 2015). The role of ectomycorrhizae in protecting against common soil pathogens from the genera *Fusarium* and *Rhizoctonia* has been demonstrated in greenhouse conditions (Chakravarty & Unestam, 1987) and *in vitro* (Martın-Pinto et al., 2006). The *Fusarium* species are found in agricultural soils worldwide, and while some are non-pathogenic others are responsible for significant crop damage (Gordon & Martyn, 1997), and serious

root rots in forest nurseries (Kim et al., 2012). It is much less frequently reported from forest soils than in cultivated soils (Park, 1963; Latiffah & Azaman, 2011), and may be related to the quality of root exudates produced in the mycorrhizosphere of trees (Grayston et al., 1996). As reported by Liu et al. (2015) who explored the diversity of soil fungi found in black truffle (*Tuber melanosporum*) plantations following the introduction of the mycorrhizal-colonized host tree (*Q. ilex*), on mature truffle orchards, the genera *Fusarium* decreased as the *Q. ilex* trees matured and as *T. melanosporum* became more dominant. This phenomenon may be explained by the production of specific volatile organic compounds (VOCs) (Splivallo et al., 2011) as mechanisms for ECM fungal-mediated plant protection (Zengpu et al., 1994) by the *T. melanosporum* in the mature plantation in Bracciano, but not in the younger cultivated truffle orchard in Scheggino, where most probably the protective action towards plant pathogenic fungi it has not yet developed. The last fungal taxon recorded as significantly abundant was the family *Chaetomiaceae* (order *Sordariales*). Members of this family were also recorded on the rhizosphere of *C. avellana* mycorrhized with *T. melanosporum* (Oliferchuk et al., 2021). These fungi produce a wealthy source of enzymes involved in plant protection and with diverse biotechnological and industrial applications such as PMO (polysaccharide monooxygenase), L-methioninase, β-1,3-glucanase, laccase, dextranase, lipolytic, pectinolytic, amylolytic, chitinolytic, and proteolytic enzymes (Ibrahim et al., 2021).

This exploratory study reveals significant changes in species richness and functional groups of root bacterial and fungal communities in maturing black truffle plantations. Sanger sequencing has provided insight into the changing pattern of soil fungal diversity decline as *T. melanosporum* dominated the root tips colonization and as some taxa are differentially abundant in host trees. Finally, the more significant species found in these root tips were identified. In the future, the use of high-throughput sequencing tools will enable a more in-depth examination of the diversity and composition of root bacterial and fungal communities in truffle orchards using larger sample sizes and broader geographic distribution.

5.2 Mycorrhization in potting mix compost-based

5.2.1 Chemical-physical analyses

It is known that ectomycorrhiza occurrence is a function of the photosynthetic potential of the host and the physical, biological, and chemical characteristics of the substrate, which appear to be the main factors influencing the susceptibility of tree roots to ECM colonization (Kropp and Langlois, 1990; Marx, 1991; Johnson et al., 1997). Additionally, the soil is the medium through which host plants obtain water and nutrients essential for growth and establish the mycorrhizal relationship with fungi (Jaillard et al., 2016). As Sulzbacher et al. (2019) attested, quality mycorrhizal seedlings are not sufficient for growing truffles with the desired plant partners unless the climate, soil, and growing conditions for both symbiotic partners are met to favor their growth and fruiting. All the potting mixes were subjected to soil analysis before being used as growing substrates. Indeed, its physical, chemical and organic properties are of the utmost importance for *T. melanosporum* fruiting. *T. melanosporum* can be cultivated on a variety of substrates, ranging from the natural soil of truffle woodlands to mixtures of natural and artificial components such as soil, peat, perlite, vermiculite, sand, dolomite, and varieties of compost (Pereira et al., 2013).

In the present study, the aim was to test the substrates coming from composting processes in their natural physicochemical composition, considering the optimal values that have been reported for the cultivation of black truffles. Starting from the physical characteristics of the potting mixes (texture and structure), as reported in Table 2, the compost-based substrates used are characterized by a sandy loam texture with high permeability, while the soil used as control has a clayey texture with low permeability and high-water retention. As Chevalier and Sourzat (2012) reported, the permeability of the subsoil is essential, and the structure of the subsoil determines drainage, the speed at which a truffle grounds enter production, and the intensity of truffle production. This is because, as Callot's work (1999) has confirmed, the importance of a highly fractured substrate facilitates the development of a deep root system. Excessively sandy soils can support excellent truffle grounds if they are sufficiently rich in calcium, just as a very stony site can produce truffles. Furthermore, Chevalier and Sourzat (2012) reported that excellent drainage, because of the soil structure, permits water and gas circulation that prevents hydromorphic phenomena from developing in the upper profile of the soil. For these reasons, the physical composition of the composted potting mixes can be considered suitable for root system development and truffle fructification. Soil physical analysis is important to clarify the functionality of the soil in terms of drainage, aeration, and biological activity, but this alone is not sufficient to assess the soil for truffle production. The soil's chemical characteristics are

also fundamental, and the elements classically considered are pH (water), richness in total and active limestone, total organic matter, carbon, total nitrogen, exchangeable calcium, potassium, and magnesium, and plant-available phosphorus.

Callot (1999) reported that the natural calcareous soils of European black truffle habitats, which are frequently used pure or mixed for black truffle culture, are characterized by relatively low levels of organic matter (up to 8%). From the results obtained in this thesis, a stimulative effect of organic matter content was evident in the two compost-based amendments that contained more organic matter compared to the control soil (12,39% as organic carbon on Mix 2 and 13,86% on Mix 1, as compared to the 5,92% of the control substrate). These findings are in agreement with the results of Pereira et al. (2013) who used in their assay a substrate for the mycorrhization of *Quercus* spp. with *T. melanosporum* with a content of organic matter (31,5%) greater than the natural soils of European black truffle habitats. As Chevalier and Sourzat (2012) reported, truffles are therefore capable of fruiting in both soils with very low organic matter content as well as those with elevated levels. Indeed, organic matter improves the soil structure significantly by increasing soil porosity, water-holding capacity, and structural stability of soil in water. Organic matter also increases exchange capacity, which, in turn, increases the soil's capacity to retain mineral elements (Jaillard et al., 2016). Therefore, organic matter is the main factor in soil structuring. Although carbon levels in *T. melanosporum* truffle grounds ranged from 0.47% to 5.0% in France and from 0.55% to 3.25% in Italy (Chevalier and Sourzat, 2012), the carbon content of compost-based substrates used in this assay differed from commonly reported values (7,18% for Mix 2 and 8,03% for Mix 1 compared to 3,43% for control soil). In *T. melanosporum* truffle grounds, levels of total nitrogen vary from 0.046 % to 0.52 % (averaging 0.24%) in France, compared to 0.080 % to 0.3 % (averaging 0.15%) in Italy (Bencivenga et al. 1990, Bragato et al. 1992, 2001, Lulli et al. 1991, 1992, Lulli and Primavera 1995). The total nitrogen content of the two compost substrates is slightly higher (0.614% for Mix 2, 0.683% for Mix 1) than the control (0.320%). However, these values are in agreement with the analytical results of the soils of *T. melanosporum* burns reported by García-Montero et al. (2007), who performed a study to establish the statistical relationship between *T. melanosporum* productivity in 20 soil surface horizons and their conventional soil properties (granulometric texture, pH, calcareous fractions, organic carbon, total nitrogen, exchangeable cations). The C/N ratio provides information on the evolution of organic matter. In France, the C/N ratio in truffières varies from 8.57 to 13.7, while in Italy; C/N varies from 8.2 to 16.9 (averaging 10.8). The results obtained confirm these values, with an average value of 11 for the two compost amendments and an average of 10 for the control.

In general, soils with a C/N ratio of between 9 and 11 have an organic substance that is well humified and quantitatively stable enough over time as the release of nitrogen and its reorganization is in equilibrium. This is indicative of an excellent capacity for mineralization of organic matter and good availability of mineral nitrogen, principally in nitric form (Chevalier and Sourzat 2012). Soil pH is another important chemical parameter and is recognized as influencing soil biology (Danielson and Visser, 1989; Wallander, 2001). Both observational and manipulative studies suggest that soil pH can exert a strong influence on the growth and reproduction of ECM fungi (Dennis 1985, Petersen 1985). French soils producing *T. melanosporum* are generally alkaline, with pH ranging between 7.7 and 8.35. However, exceptions exist, with a pH of 7.0 in a truffle ground in southwestern France on sandy soil with 0% total limestone but with 0.38% exchangeable calcium (CaO), as Sourzat (2008) points out. In Italy, productive truffle grounds are also found on soils with a pH as low as 7.05, although normally the pH is 8.00. In the present study, the pH value ranged from 7.1 for Mix 1 to 7.4 for Mix 2, and the control substrate had a pH of 7.7. All of them are classified as slightly alkaline and suitable for the development of *T. melanosporum* ectomycorrhizae.

Indeed, also García-Montero et al. (2007) reported that most of the soil burns to produce *T. melanosporum* associated with *Q. ilex, Q. faginea, C. avellana* and *Tilia platyphyllos* plants host had moderately basic pH values (from 7.0 to 8.17). *T. melanosporum* productivity is also influenced by the overall action of active limestone. Active limestone is a finely divided fraction of calcareous rock measuring <50 µm, susceptible to rapid mobilization, and very chemically active. Active limestone indicates the extent and reactivity of the limestone surfaces and constitutes an important reserve of exchangeable Ca^{2+}. Continuous formation of active limestone preserves high levels of Ca^{2+} in the soil solution and maintains the exchange complex, as it counterbalances losses from leaching and other processes (Chevalier and Sourzat 2012). Although in the two substrates used in this study, the amount of active limestone is lower (1.7 for Mix 1, 1.2 for Mix 2) than the control (12.1), the formation of ectomycorrhizae does not seem to be influenced by this parameter. Both *Q. pubescens* and *C. avellana* seedlings developed good levels of mycorrhization, indicating that the quantity of active limestone present in soil alone is not a sufficient parameter to establish successful mycorrhization and truffle production. In fact, many other biotic and abiotic factors play an important role in truffle productivity in truffle grounds and natural brûlés (García-Montero et al., 2006).

As Ricard (2003) indicated, it is difficult to judge the impact of active limestone on truffle culture because several factors must be considered. Authors like Callot (1999), García-Montero et al. (2009a, b) and Demerson (2012) have even suggested that active carbonate could play a direct role in the

nutrition of mycelium or fruiting of *T. melanosporum* ascomata. However, Chevalier and Sourzat (2012) reported *T. melanosporum* growing and fruiting in neutral soils with null carbonate content, claiming that the level of limestone is highly variable, and the amount of total limestone has no effect on the quality of the truffle ground soils. Only its presence at the surface is important. Also, Jaillard et al. (2014) observed wild truffle beds on soils whose exchange complex was just close to saturation, with no carbonate in them. These observations contradict the hypothesis of a possible role played by active carbonate in the fructification of *T. melanosporum*. The data only agrees with the mean pH value, indicating that *T. melanosporum* grows and fruits in neutral and dolomitic soils as well as calcareous soils. On exchangeable calcium, the level of calcium on the two compost-based substrates was found to be high in absolute value and medium about the cation exchange capacity, while on the control potting mix, both the level and the cation exchange capacity are high. Nevertheless, the values of the potting mixes fall within the range of values reported by García-Montero et al. (2007) about the exchangeable cations values of burns soils producing *T. melanosporum* carpophores.

The exchangeable magnesium levels of mixes 1 and 2 are high both in absolute value and about the cation exchange capacity, while on the control substrate the absolute value is high but is low if related to the cation exchange capacity. As Sourzat (2008) has concluded, this parameter is not crucial, because lower or higher values of magnesium than normal levels do not seem to affect truffle ground production. The levels of exchangeable potassium are high on both the compost-based substrates, and no addition of this element is required. The phosphorus levels only in the control mix is low and an adequate supply would be necessary both to meet the nutritional needs of the seedlings and to improve soil fertility levels. These findings agree with what is reported by Nuñez et al. (2006) that in Mediterranean ecosystems, forest soils, generally limy, are usually deficient in phosphorus. Therefore, the compost substrates rich in phosphorus can cause a very important improvement in mycorrhizal settlement plantations, and, with *T. melanosporum*, which itself is very efficient at increasing phosphorus uptake. As Cameleyre and Olivier (1993) attested, probably *T. melanosporum* mycorrhizae release phosphatases and organic ions, which help move and absorb phosphorus from the soil.

5.2.2 Seedling rooting

As could be expected, germination rates of *C. avellana* seedlings were lower than those of *Q. pubescens* seedlings. Because of the different stages of development of the two plant materials used (suckers and acorns), hazelnut suckers are probably more sensitive to drought than acorns, and this is the most likely explanation for the lower germination of hazel. Furthermore, since the low survival rate at rooting of the hazel was known, the Nocchione variety was chosen, which, from previous tests, had a higher survival rate than the Tonda Gentile Romana and Giffoni varieties. As has been summarized by Vander Wall (1990), acorns and nuts are particularly vulnerable to damage by drying, intense sunlight, or low temperatures. This fits with the results of Kollman and Schill (1996), where germination rates of *Corylus* spp. were lower than those of *Quercus* spp. in all field experiments done. In their tests, germination of *Quercus* spp. was surprisingly high in all field tests, like in our experiments. Moreover, the composition of the rooting substrate for cutting propagation of broad-leaved species has been widely acknowledged (Tchoundjeu and Leakey, 2001; Tchoundjeu et al., 2002; 2004). Different substrates may have a potential effect on the root system quality (Hartmann and Kester, 1998) by way of differences in water holding capacity (Mesén et al., 1997), among other factors.

A plant suffering during rooting was then confirmed in the survival rate of treatments once transplanted into the different substrates. The mortality of the plants inoculated occurs very often in nurseries where there is an average rate of 40% for hazelnuts and 25% for downy oaks (personal communication). This phenomenon may be due to several factors. First of all, the inability of young seedlings to recover from the stress of transplanting can be the first obstacle to survival. Furthermore, other variables that take part in the increased mortality are the climate, the spatial localization of the plants inside the greenhouses, and the time of the season in which the mycorrhization is performed. The mortality of *Quercus* seedlings in all the treatments was much lower than that of *Corylus* seedlings. However, within the treatments, it is possible to highlight a clear effect of the substrate on the survival rate of the plants. In both *Quercus* and *Corylus* seedlings, the treatment containing compost from Mix 2 shows a lower mortality rate than both the control and the treatment with compost from Mix 1 as reported in section 3.2.1.

5.2.3 Truffle colonization

Inoculation using ECM symbionts with a high mycorrhizal host-symbiont specificity and efficacy has the greatest potential for enhancing the early establishment of tree seedlings at the nursery stage (Brundrett et al. 2005; Chen et al. 2006). When grown in different potting mixes, the results of the mycorrhization experiment reported in Figure 17, showed that *T. melanosporum* ECMs were formed on both *Q. pubescens* and *C. avellana* seedlings after spore slurry inoculation. The synthesis of ectomycorrhizae of the genus *Tuber* with natural hosts such as *Q. ilex* and *Q. robur* is very often reported in the literature (Taschen et al., 2020; Pérez et al., 2007) and are considered the most efficient trees in truffle culture (Reina 2000). However, other woody plant species like *Q. pubescens* and *C. avellana* are considered suitable hosts for the fructification of *T. melanosporum* because they could be easily managed in semiculture (Gryndler 2016; Zambonelli et al., 1993). Indeed, Mamoun and Oliver (1997) documented for the first time that the use of a *C. avellana* clone H219 increased receptiveness toward *T. melanosporum* and enhanced resistance to colonization by competing ECM fungi. However, Boutekrabt et al. (1990) documented that the results in the field of plants carrying truffle mycorrhizae are limited by the heterogeneity of plant material, which has originated from seedlings. These disadvantages could be reduced using selected oak clones, multiplied *in* vitro, and that of clonal "fungal" material. Despite all these factors, the results of this thesis highlighted the possibility of obtaining high-quality mycorrhized seedlings. The results of truffle colonization on *C. avellana* seedlings were in agreement with what Santelices et al. (2010) obtained. They achieved a mycorrhization level of 33% in sucker shoots of *C. avellana* infected with *T. melanosporum*. Even if their percentage of mycorrhization was one-third less than the results obtained by similar experiments realized in Chile with plants propagated from seeds (Ramírez et al., 2004), the absolute numbers of colonized root tips more than doubled those of the study by Ramírez et al. (2004).

This conforms to the present work, in which the number of root tips and the length of the root system of seedlings grown in Mix 1 were significantly higher referring to the other treatments, even if the percentage of mycorrhization is not significantly different. This aspect could be explained by the fact that the differentiation of ectomycorrhizal structures formed by *T. melanosporum* could not keep pace with the development of fine root tips. Most likely, these results are due to the presence of more and longer fine roots in the suckers at the moment of inoculation, compared to sexually propagated plants, which are usually used for mycorrhization. The *C. avellana* cuttings on the other hand, had a low survival rate and a lower number of ectomycorrhizae than seedlings of oaks. This is likely because the suckers were in a state of distress before or after being transplanted into the new substrates, which

caused the seedlings to grow a longer root system but with fewer root tips than oaks (61% and 84% ratio of tips along the entire length of the root system, respectively), as also reported Ramírez et al. (2004). Furthermore, the ectomycorrhizal percentage above 30% can be considered excellent according to Reyna (2000), and matches the quality standards of European producers.

5.2.4 Growth and development of the root system

A mutualistic symbiosis between fungi and host plants through the ectomycorrhizae is a critical and long-term stage of the life cycle of truffles, which also plays a significant ecological role (Mello and Balestrini, 2018). In the early stages of the symbiotic relationship, the quality of truffle-colonized seedlings, including the level of truffle colonization and the growth and physiology of host plants, is a critical factor determining truffle fruiting body production in plantations (Andres-Alpuente et al., 2014), which is crucial for successful truffle cultivation. The interactions between truffles and host plants were reflected in the transport of substances through the ectomycorrhizae as well as the metabolic signals produced by truffles and hosts (Daba et al., 2019). Numerous studies have demonstrated that mycorrhizal symbiosis enhances nutrient transport between host plants and mycorrhizal fungi, resulting in developmental and physiological benefits for the hosts (Xiu et al., 2019; Álvarez-Lafuente et al., 2018; Liese et al., 2017). As reported in Table 13, plants inoculated with *T. melanosporum* grown on compost-based potting mixes had greater biomass than those grown on a control mix containing forest soil under nursery conditions. *Q. pubescens* seedlings showed a higher shoot diameter on both the compost-based mixes and *C. avellana* seedlings developed a higher height if grown on compost-based potting mixes compared to the control.

The addition of organic matter in the form of composted urban refuse and vegetal substrates fermented has been widely reported to have positive effects on the proliferation and colonization capacity of mycorrhizal fungi (Lunt and Hedger, 2003). For example, Oliveira et al. (2010) performed an ectomycorrhizal inoculation of *Q. ilex* with *Paxillus involutus*, *Hebeloma mesophaeum*, and *Cenococcum geophilum* grown in three different substrates commonly used in forest nurseries: peat-based compost, forest soil, and composted pine bark. From their results, *Q. ilex* seedlings exhibited a growth response to inoculation with *H. mesophaeum* when cultivated on peat-based compost, which, if compared with forest soil and composted pine bark, was the superior growth substrate regardless of inoculation treatment (Oliveira et al., 2010). The addition of organic amendment was also reported by Ríncon et al. (2006) to improve by over 20% the height and diameter of *Pinus halepensis*, and volume and dry biomass by more than 43%, compared to plants in the non-amended soil. Similar

results have been obtained for *P. halepensis* and other tree and shrub species in field experiments where different strategies of soil fertilization with composted urban waste and organic amendments have been followed (Roldán and Albaladejo, 1994; Querejeta et al., 1998; García et al., 2000; Román et al., 2003; Caravaca et al., 2005).

On both plant species, it was evident that the two different compost-based substrates have a greater effect also on root development than the control. In *Q. pubescens* seedlings (Figure 19 and 20), almost all the root parameters analysed with the software WinRHIZO®, including apical root tips, forks, and root system length, agreed with the percentage of mycorrhization observed, indicating that the use of compost Mix 2, the greater the number of tips the plant has, the more ectomycorrhizae can form. On *C. avellana* (Figure 21) besides the assessed lower percentages of ECM colonization, significant effects on plant growth were obtained for the number of tips and the root length with the compost Mix 1, showing that a low ECM colonization is not always indicative of the inoculation's failure. The positive effect of ECM inoculation on root plant system growth, found in this study, may be due to enhanced nutrient uptake as well as induced phytohormone production in the plant and/or the production of growth regulators and hormones by the ECM fungi. All these can positively change root physiology, growth, development, and morphogenesis (Barker and Tagu 2000; Chalot et al. 2002; Luo et al. 2009). Interestingly, the two plant species reacted differently to the two substrates because the most appropriate combination of substrate for plant growth and root development of *Q. pubescens* seedlings was blond peat rich in organic matter and organic substances of vegetable origin fermented, while for *C. avellana* seedlings, the best performance was achieved with the compost substrate coming from the plant and urban wastes. Regardless, both the compost-based potting mixes could optimize the conditions for regular plant growth. These results indicate that inoculation with *T. melanosporum* spores and the use of alternative, less expensive, and recycled substrates are compatible nursery practices to produce mycorrhizal *Q. pubescens* and *C. avellana* seedlings in containers.

5.3 Characterization of Périgord black truffle associated bacteria and effect of the inoculation of helper strains on ectomycorrhizal colonization

5.3.1 Molecular characterization of bacterial strains

Soil colonization by microorganisms depends on their ability to access and metabolize the organic and inorganic nutrients present in the soil. In the rhizosphere of forest trees, ectomycorrhizal fungi,

microfungi, and bacteria physically interact to form multitrophic ectomolecular complexes that contribute to tree nutrition (Frey-Klett et al., 2015). HTS results on the analysis of the biodiversity of the bacterial community led to the selection of specific bacterial genera that are well-represented in terms of biodiversity in the mycorrhizosphere of the samples collected, which was crucial as it allowed their practical application to test their single effects on the mycorrhization. Indeed, isolating a bacterium directly from a specific compartment of the target fungus (e.g., ectomycorrhizal root tips, or sporocarps), cultivating it, and then inoculating on non-colonized seedlings with the isolated bacterium and the target fungus is essential to observe MHB effects on fungi (Tarkka et al., 2018). The culturable fraction of bacteria isolated from downy oak ectomycorrhizal root tips (belonging to the phyla *Firmicutes* and *Proteobacteria*), agreed with what was found and experimented by Bending et al. (2002). Bacteria extracted from *Pinus sylvestris-Suillus luteus* interactions and plant growth of bacteria extracted from *P. sylvestris-S. luteus* ectomycorrhizas were isolated, characterized, and investigated. They found that *Bacillus* sp. was the dominant member of the culturable bacterial community associated with ectomycorrhizas of *P. sylvestris-S. luteus*. Timonen et al. (1998) found that Gram-negative bacteria were more abundant in the mycorrhizas of *P. sylvestris-S. bovinus* and *Paxillus involutus*, although *Bacillus* spp. were the dominant Gram-positive species found. In another work by Uroz et al. (2007), 61 bacterial isolates from *Scleroderma citrinum* mycorrhizae, the mycorrhizosphere, and the adjacent bulk soil in an oak forest were characterized.

They discovered that most of them are from the genera *Burkholderia*, *Collimonas, Pseudomonas*, and *Sphingomonas*. Strains closely related to known species of the genera *Burkholderia* and *Pseudomonas* have been found previously to be beneath the dominant cultivated bacteria associated with ectomycorrhizal fungi and forest soils (Bending et al., 2002, Duponnois et al., 1991, Poole et al., 2001). As shown from the HTS data results, these results may reveal that ectomycorrhizal bacterial communities are quite diverse in their composition and are mediated by several factors: the host tree (Calvaruso et al., 2010), the fungal symbiont (Izumi and Finlay, 2011), the soil characteristics, and the composition of the soil bacterial communities (Deveau et al., 2016). However, some common trends among bacterial communities from different ectomycorrhizal fungal species and host trees also exist, suggesting a possible core bacterial community (Burke et al. 2008; Uroz et al. 2012). Indeed, the five bacterial strains selected for the inoculation experiment are commonly reported in the literature to have the effect of promoting root colonization and growth of plants (Piñuela et al., 2020; Schreiter et al., 2014; Frey-Klett et al., 2007, Marulanda et al., 2009; Dey et al., 2014; Almaghrabi et al., 2012). Some of these are bacterial strains that positively impact the functioning of mycorrhizal

symbiosis by stimulating mycelial extension, increasing root–fungus contacts and colonization; and reducing the impact of adverse environmental conditions on the mycelium of the mycorrhizal fungi (Frey-Klett et al., 2007). Some MHB can also have additional beneficial impacts, such as direct stimulation of plant growth and protection against pathogens (Deveau and Labbè 2016). Since the effect of MHB is not always limited to mycorrhiza formation and/or functioning, some MHB also behaves as Plant Growth Promoting Rhizobacteria (PGPR). Kloepper and Schroth, in the 1980s, introduced the term "PGPR" to indicate those microorganisms which, colonizing the rhizosphere, promote seed germination, accelerate growth in the early stages, induce root initiation, enhance the formation of roots and root hairs, facilitate root regeneration, and help control pathogens in some forest species (Heinonsalo et al. 2004). The MHB strains that have been identified to date belong to many bacterial groups and genera, such as gram-negative *Proteobacteria* (*Agrobacterium, Azospirillum, Azotobacter, Burkholderia, Bradyrhizobium, Enterobacter, Pseudomonas, Klebsiella* and *Rhizobium*), gram-positive *Firmicutes* (*Bacillus, Brevibacillus,* and *Paenibacillus*) and gram-positive *Actinomycetes* (*Rhodococcus, Streptomyces*, and *Arthrobacter*).

The presence of MHB has been identified in ectomycorrhizal root tips colonized by economically important truffle species. For example, Gryndler and Hršelová (2012) isolated bacteria from the communities associated with *T. aestivum* ectomycorrhizae. Sbrana et al. (2002) studied the diversity of culturable bacterial populations associated with *T. borchii* ectomycorrhizas. Barbieri et al. (2007) investigated the occurrence and diversity of bacterial communities in *T. magnatum* Pico. Bacterial strains belonging to *Bacillus* sp. and *Pseudomonas* sp. have been found at different stages of the *T. melanosporum* life cycle, e.g., from mycorrhiza formation to sporocarp development (Rivera et al., 2010; Antony-Babu et al., 2014; Deveau et al., 2016). However, the influence of specific MHB during the early stages of root tip colonization by *T. melanosporum* is still to be elucidated. For this reason, this experiment with the help of the knowledge of the genera of bacteria present on large scale deriving from HTS analysis aimed to test their effect *in vivo* on plant growth and mycorrhization occurrence. The positive effect on the mycorrhizal colonization of *P. halapensis* and *C. avellana* by the *P. fluorescens* strain was also reported by Mamoun and Oliver (1992), Labbé et al. (2014), Hrynkiewicz et al. (2009), and Domínguez-Núez et al. (2013). Based on these results, *P. fluorescens* OL831225 was selected for co-inoculation with *T. melanosporum*. The fluorescent strain *P. jessenii* OL831226 was chosen because it is reported to be an important rhizobacterium that acts as a biocontrol agent in protecting against several soil pathogens (Deora et al., 2010) and since it has been isolated from the root tips of ectomycorrhizal plants, its effect was tested. The strain *S. marcescens*

OL873254 was selected as it is a bacterial species described by Almaghrabi et al. (2012) as PGPR for its positive effect on tomato plant growth and yield. In their work, the highest number of shoot dry weights was detected in the plant treated with *S. marcescens*. Based on the results of Bidondo et al. (2016), *Bacillus megaterium* (SJ5R7) (now renamed *Priestia megaterium*) acts as an MHB, stimulating the germination of propagules and pre-symbiotic mycelial growth of arbuscular mycorrhizal (AM) fungi, both directly and by exudates. These plant-growth-promoting capacities and mycorrhizal helper effects were for the first time tested in this work on ectomycorrhizal colonization of *T. melanosporum* on *Q. pubescens* and *C. avellana* seedlings. The last strain selected for this experiment is another commonly reported to be MHB as *Paenibacillus polymyxa* (Muthukumar and Udaiyan 2010) that behaved as mycorrhizal helper bacteria because it promoted root colonization by mycorrhizal fungi, confirming previous findings involving other mycorrhizal combinations (Garbaye 1994). As a control, was decided to test the effect of seedlings inoculated only with *T. melanosporum* ascocarps without co-inoculation with bacteria.

5.3.2 Effect of bacteria on seedling growth and root system development

From the results highlighted in Figures 22 and 23, on *C. avellana* seedlings, the strain *P. megaterium* promoted both *T. melanosporum* mycorrhization and shoot and height which results in significantly higher average values compared to the control. The other parameters of the development of the seedlings and the root system were statistically not significant in absolute value, but they too were superior in the treatment with *P. megaterium*. To the best of my knowledge, these results represent the first report on the use of *P. megaterium* as co-inoculants of *C. avellana* and *Q. pubescens* mycorrhization with *T. melanosporum*. Bending et al. (2002) and Poole et al. (2001) identified strains of *Bacillus*, *Burkholderia*, and *Rhodococcus* that can help plants' growth and short root tips, which is a common feature of MHB. These strains can also help plants grow more quickly than non-inoculated seedlings. This effect could indeed stimulate ectomycorrhiza formation by increasing the number of lateral roots that can be colonized by the fungus. The results of this thesis are agreed with those of Navarro-Ródenas et al. (2016), who isolated mycorrhizal roots of *Helianthemum almeriense* colonized by the desert truffle *Terfezia claveryi*, mycorrhizosphere soil, and peridium of *T. claveryi* to evaluate their effect on mycorrhizal plant production. In these ectendomycorrhizal associations, the bacterial strains *P. megaterium* and *Pseudomonas* genus were present, which best represented the PGPR group in the greatest number of isolated strains (40.8% for *Pseudomonas* and *Bacillus* 12.2%). Vivas et al. (2003) reported that a rhizosphere bacterium isolated from an arid soil and identified as

Bacillus sp. positively influenced the development and activity of two *Glomus* spp., stimulating not only the activity of intraradical mycelium from *Glomus* sp. but also the development of extraradical mycelium from *G. intraradices*. Most recently, Marulanda et al. (2009) reported that the inoculation with *P. megaterium* increased shoot and root biomass and water content in *Trifolium* plants. Under drought conditions, these bacterial strains were compatible and effective in increasing the benefits of arbuscular mycorrhizal symbiosis on plant growth with each fungal isolate tested. For the first time, the same effect on shoot height and mycorrhiza formation was observed with the ectomycorrhizal fungus *T. melanosporum*. Results from *Q. pubescens* seedlings showed that the co-inoculation with the *P. jessenii* strain promoted both *T. melanosporum* mycorrhizal development (Figure 22), shoot, and root development the most effective when compared to the control (Figures 24 and 25). These results reported for the first time a stimulating effect on the ectomycorrhizal occurrence of this bacterial species. Numerous studies, mostly on crops, have demonstrated that strains of *Pseudomonas* sp. are beneficial for plant growth (Gamez et al., 2019, Batista et al., 2018) and other research has been conducted to study its influence on the growth of forest species (Piñuela et al., 2020; Rincón et al., 2007; Ouahmane et al. 2009). However, the results gained in this thesis showed for the first time that the combined inoculation of both *P. jessenii* OL831226 and *T. melanosporum* could have synergistic effects and positively influence the *Q. pubescens* seedling physiology, thereby improving the plant's quality at early developmental stages. And interestingly, these bacterial species that resulted to be beneficial for the mycorrhization of *T. melanosporum* are shared within all the host tree species analyzed for the HTS, suggesting the importance of the two *Bacillus* and *Pseudomonas* species as MHB, maybe on several other forest species forming mycorrhizae for black truffle.

In the same way as with *C. avellana* seedlings, was observed that the effect of *P. fluorescens* inoculation was not statistically different from the control and had a slight effect on promoting mycorrhizal development or root growth, at least with the specific strain used for inoculation in the present trial (*P. fluorescens* OL831225). These findings are in contrast with what is reported from the study of Piñuela et al. (2020), where *Quercus faginea* Lam. seedlings were co-inoculated at two different times with bacterial strains including *P. fluorescens, P. putida* and *Bacillus amyloliquefaciens* with *T. melanosporum*. Only *P. fluorescens* had a significant mycorrhizal promoter effect, increasing the truffle inoculation rates of root tips by more than 10% compared with seedlings that received non-bacterial inoculation treatments. Simultaneously, the co-inoculation of *P. fluorescens* with *T. melanosporum* improved seedling root growth parameters compared with those of seedlings that received non-bacterial inoculation treatments. Different bacterial inoculation times

did not affect the root traits analyzed or the root mycorrhization rates. Also, Dominguez et al. (2012) and Mamoun and Oliver (1992) reported positive effects of *P. fluorescens* on the mycorrhizal colonization of *Pinus halapensis* and *C. avellana*. Strain *Pseudomonas* sp. GM41 stimulated the formation of secondary roots of *Populus trichocarpa* and *P. deltoides* (Labbé et al., 2014), while strain *P. fluorescens* CECT 5281 modified the response of roots of *P. halepensis* to drought (Hrynkiewicz et al., 2009; Domínguez-Núez et al., 2013). Most likely, as reported by Frey-Klett et al. (2007), the fact that under optimal glasshouse conditions, such as well-irrigated soil or pot culture in a controlled environment, the effect on promoting plant growth and/or on the intensity of mycorrhiza formation of the presence of the MHB *P. fluorescens* in these trials was not observed, does not rule out the fact that this endosymbiotic bacteria could behave as true MHB when the environmental soil conditions are not favorable to fungal survival. The intracellular colonization of *T. melanosporum* by soil bacteria would confer on the fungal host the ability to adapt to changing environments, especially during its presymbiotic life in the soil. Also, Brulé et al. (2001) previously made the same suggestion in the case of the MHB effect of *P. fluorescens* BBc6R8 on the ectomycorrhizal Douglas-fir–*Laccaria bicolor* symbiosis. Otherwise, the mycorrhizal promoter effects of this strain may not have been seen in these experiments due to the non-survival of the bacterial inoculum during the growth phase of the seedlings.

It is widely reported that unfavorable environmental factors such as soil moisture and temperature regimes, pH and texture, as well as 0_2 and nutrient availability can be the major abiotic factors that govern the survival of newly introduced bacteria in the soil (Vandenhov et al., 1991; Kubat et al., 1980; Dupler and Baker, 1984; Davies and Whitbread, 1989). *P. fluorescens* OL831225 was the only strain that was difficult to re-isolate due to the scarce presence of fluorescent colonies coming from the survival experiment, while the other bacterial species have been successfully re-isolated. This explication matches with the findings of van Elsas et al. (1992), who reported that the *P. fluorescens* survival in non-rhizosphere and rhizosphere soil in different experiments (van Elsas et al. 1986, 1989, 1991) was often very poor. In the co-inoculation experiment with *T. melanosporum*, other strains were also included as they were isolated from the same ectomycorrhizal root tips of *Q. pubescens* and its co-occurrence in natural habitats at the *T. melanosporum*–root interface (Rivera et al., 2010, Antony-Babu et al., 2014, Deveau et al., 2014, Muthukumar et al., 2010). However, the results of this thesis showed that co-inoculating *P. polymyxa* and *S. marcescens* on both *C. avellana* and *Q. pubescens* did not influence either seedling mycorrhization rates or seedling growth and root development, at least not at this mycorrhizal establishment phase and not the specific strains that were

isolated. However, as shown in previous studies of other ECM species, such a co-existence might not indicate a functional interaction between organisms. For instance, in the case of truffles, Gryndler and Hršelovà (2012) observed that selected bacteria (among other organic and inorganic compounds tested) isolated from ECM root tips of *T. aestivum* showed no stimulation and one isolate induced the damage of hyphae on the growth of *T. aestivum* mycelium under culture conditions. Conflicting reports have been made regarding the natural colonization of the mycorhizosphere by MHB. Ectomycorrhizae from some species, such as *Pinus radiata/Rhizopogon luteolus* (Garbaye and Bowen, 1989) or *Acacia holosericea-Pisolithus* sp. (Founoune et al., 2002), seem to be preferentially colonized by MHB strains. However, most bacterial strains isolated from ectomycorrhizae between *Laccaria bicolor* and *Pseudotsuga menziensii* harmed ectomycorrhizae formation (Frey-Klett et al., 2005). Other possible explanations could be that even though *P. polymyxa* and *S. marcescens* have been isolated from the same root mycorrhized with *T. melanosporum* and are reported to possess PGPR activity and to be MHB, their presence does not seem to be a result of a fungal-bacterial interaction. Otherwise, these results may be due to an incompatibility between the plant species and the bacterial microorganism, which resulted in a neutral effect on the growth of the seedling and the mycorrhization with *T. melanosporum*, which is equal to the control treatments.

It is interesting to note that different plant species like *C. avellana* and *Q. pubescens* evolved different relationships with the bacterial strains co-inoculated during the mycorrhization process. The *Bacillus* genus was beneficial for root growth and mycorrhization formation in hazel seedlings but not influenced *Q. pubescens* seedlings that gave results like the control treatment, whereas the *P. jessenii* strain was beneficial for root growth and mycorrhization in the downy oak but not in *C. avellana* seedlings. In all cases, ectomycorrhizae present in the roots of the inoculated plants were colonized by *T. melanosporum*, and the overall effects of all the bacterial strains on both *Q. pubescens* and *C. avellana* seedlings were not negative. All the treatments allowed the establishment of the ectomycorrhizal symbiosis and the growth of the plants, even if slightly lower than that of the control treatments. Also, the mortality of the plants was not a noteworthy parameter. Is widely reported that the fungus makes a strong selection of specific bacterial communities in the ectomycorrhizosphere of many other ectomycorrhizal fungi (Mogge et al. 2000; Assigbetse et al. 2005; Frey-Klett et al. 2005; Uroz et al. 2007; Izumi et al. 2008; Calvaruso et al. 2010). They also select a specific bacterial core from among all the bacteria present in its microbiome that is potentially beneficial for a specific function during its lifecycle (Deveau et al., 2016, Tarkka et al., 2018), indicating that focusing investigations on biological interactions in the rhizosphere is more complex than it appears.

As also reported by Deveau and Labbè (2016), the most frequent niches of MHB are still undocumented. However, future perspectives on understanding the molecular mechanisms and their specificities will help us to learn more about the ecology of the MHB. Indeed, some MHB might naturally be selected in the mycorrhizosphere or the hyphosphere and coevolve with their fungal and plant partners; they would then use quite specific mechanisms of interaction. In contrast, other MHBs would most probably use fewer specific mechanisms to stimulate mycorrhiza formation, as they have not coevolved with the beneficiary of their activity.

6. Conclusions and Future Perspective

The major problems that are still detected in the truffle plant sector are related to the poor quality of the mycorrhizal nursery material currently available and the difficulty of these plants to maintain mycorrhization in the subsequent phases of planting. Other problems depend on numerous variables attributable to the methods of root inoculation in the nursery and development of the plant, in addition to the knowledge of the right agronomic planting and cultivation practices aimed at ensuring rapid and consolidated productivity. During the thesis, an innovative biotechnological application was developed in which an attempt was made to solve practically the various problems in truffle plant production (Fischer et al., 2017). Since the removal of forestland and any other organic material that constitutes the ground cover (leaves, humus, and organic soil) in the woods is prohibited, an attempt was made to evaluate the effectiveness of the use of alternative organic matrices to the use of forestland for the maintenance of mycorrhization. As described above, this is one of the critical factors in the mycorrhization process and the production of truffle plants. The use of compost-based amendments as potting mixes for the establishment and maintenance of the mycorrhizal symbiosis represents an encouraging starting point for future truffle cultivation. From the results obtained in this thesis, it is clear how the use of alternative matrices to the classic forest soil currently available on the market can bring advantages to the growth of plants and their root systems, allowing mycorrhization with *T. melanosporum* to persist even after inoculation. Furthermore, the recovery of organic waste transformed into an organic fertilizer such as green compost represents an effective system that significantly contributes to the sustainable use of agricultural and environmental resources while reducing the amount of organic waste to be disposed of in landfills or incinerated (Centemero et al. 2001). Truffle farming is synonymous with specialized plantations where the quality of the starting material, that is, the plant with the mycorrhiza, determines its productivity and economic sustainability. The mycorrhizal phase still represents a crucial point in the process, and the study of the rhizospheric microbiome in the process of mycorrhizal symbiosis through the action of helper microorganisms is of crucial importance. Soil is probably one of the most complex ecosystems in which fungus-bacteria interactions operate. There are complex communities of fungi and bacteria that surround the roots of plants and can exert deleterious, beneficial, or commensal actions.

Ectomycorrhizae interact physically and functionally with soil fungal and bacterial communities, establishing the so-called “multitrophic ectomycorrhizal complex” which affects productivity and nutrients (Frey-Klett & Garbaye, 2005). To explore the microbial community structure, an in-depth and high-quality bioinformatics study of the bacteria and fungi present in cultivated *T. melanosporum*

and *T. aestivum* plantations was performed with the adoption of high-throughput sequencing methodologies that provided insight into the truffle habitat. This knowledge is useful for future planning, management, and long-term maintenance of truffle plantations. Beginning studies of the plant-microbe system found in native environments have provided the greatest opportunity for discovering the true diversity of system functions and exploring fungal-bacterial interactions. As reviewed by Deveau and Labbè (2016), these mycorrhiza-induced interactions attract more and more interest, which has led to the development of focused research on the so-called mycorrhizosphere (Barea et al., 2013). Such interactions within the mycorrhizosphere involving MHB and PGPR can be managed as mycorrhizosphere tailoring to benefit plant growth and health, and soil quality (Barea et al., 2013). A better understanding of the effects of microbiota thriving in mycorrhizospheres is needed for agricultural purposes as much as for understanding mycorrhizal functioning (Barea et al. 2005; Artursson et al. 2006; Frey-Klett et al. 2007). For this reason, the selection of the best fungal-bacterial plant combinations obtained with the knowledge coming from the HTS analysis and the application of *Pseudomonas jessenii* and *Priestia megaterium* as co-inoculants with *T. melanosporum* ectomycorrhizal fungus on *Q. pubescens* and *C. avellana* seedlings, gave promising results for enabling the establishment of future truffle plantations. These inoculants could be engineered as biofertilizers and bio-enhancers for a sustainable environment and the nursery production of quality truffle-inoculated seedlings. In April 2021, the new project TANA based on the overall results obtained during these three years of research started. This project has the aim of solving some weaknesses that are still present in the mycorrhization process. The weakest points concern the quality and viability of the inoculum, the synchronization between inoculation and receptivity of the root system, and the excessive loss of inoculum by percolation and dispersion through the substrate (Domínguez-Núñez and Albanesi, 2019; Le Tacon et al., 1983). TANA's specific goal is to develop a new biotechnological manufacturing product that is functional to the truffle supply chain for *Tuber* spp. that involves the production of high-quality mycorrhized plants for productive plantations through the employment of nanotechnologies.

In a general conclusion, many achievements have been reached with the application of microbial biotechnology in agriculture, but many challenges, as well as opportunities, need to be explored for future sustainable agricultural developments. In part, the results that have been achieved so far and those that are still to come could help in the future implementation of truffle plant production.

References

Agueda, B., Torián, L. F., de Miguel, A. M., & Peña, F. M. (2010). Ectomycorrhizal status of a mature productive black truffle plantation. Forest Systems, 19(1), 89-97.

Alberton O., Kuyper T.W., Gorissen A. (2007) Competition for nitrogen between *Pinus sylvestris* and ectomycorrhizal fungi generates potential for negative feedback under elevated CO_2. Plant and Soil 296:159.

Ali, Nahia A., and Richard M. Jackson. "Stimulation of germination of spores of some ectomycorrhizal fungi by other micro-organisms." Mycological Research 93.2 (1989): 182-186.

Alkorta, I., Epelde, L., & Garbisu, C. (2017). Environmental parameters altered by climate change affect the activity of soil microorganisms involved in bioremediation. FEMS microbiology letters, 364(19).

Almaghrabi, Omar A., Samia I. Massoud, and Tamer S. Abdelmoneim. "Influence of inoculation with plant growth promoting rhizobacteria (PGPR) on tomato plant growth and nematode reproduction under greenhouse conditions." Saudi journal of biological sciences 20.1 (2013): 57-61.

Almario, J., Jeena, G., Wunder, J., Langen, G., Zuccaro, A., Coupland, G., & Bucher, M. (2017). Root-associated fungal microbiota of nonmycorrhizal Arabis alpina and its contribution to plant phosphorus nutrition. *Proceedings of the National Academy of Sciences*, *114*(44), E9403-E9412.

Alvarez IF, Parladé J, Trappe JM, Castellano MA (1993) Hypogeous mycorrhizal fungi of Spain. Mycotaxon 47 :201–217.

Álvarez-Lafuente, A., Benito-Matías, L. F., Peñuelas-Rubira, J. L., & Suz, L. M. (2018). Multi-cropping edible truffles and sweet chestnuts: Production of high-quality *Castanea sativa* seedlings inoculated with *Tuber aestivum*, its ecotype *T. uncinatum*, *T. brumale*, and *T. macrosporum*. Mycorrhiza, 28(1), 29-38.

Anderson I.C., Cairney JWG. (2007) Ectomycorrhizal fungi: exploring the mycelial frontier. FEMS Microbiology Reviews 31, 388-406.

Anderson, R. C., & Liberta, A. E. (1992). Influence of supplemental inorganic nutrients on growth, survivorship, and mycorrhizal relationships of *Schizachyrium scoparium* (*Poaceae*) grown in fumigated and unfumigated soil. American Journal of Botany, 79(4), 406-414.

Andrés-Alpuente, A., Sánchez, S., Martín, M., Aguirre, Á. J., & Barriuso, J. J. (2014). Comparative analysis of different methods for evaluating quality of Quercus ilex seedlings inoculated with *Tuber melanosporum*. Mycorrhiza, 24(1), 29-37.

Anscombe F. J., The transformation of Poisson, binomial and negative-binomial data, Biometrika, Volume 35, Issue 3-4, December 1948, Pages 246–254.

Antony-Babu, Sanjay, et al. "Black truffle-associated bacterial communities during the development and maturation of *Tuber melanosporum* ascocarps and putative functional roles." *Environmental Microbiology* 16.9 (2014): 2831-2847.

Artursson, V., Finlay, R. D., & Jansson, J. K. (2006). Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environmental microbiology, 8(1), 1-10.

Arzanlou, M., Bolandnazar, S., Moghbeli, E. M., & Panahandeh, J. (2013, October). Biological Control of Fusarium Wilt in Greenhouse Tomato by Mycorrhizal Fungi and Resistant Rootstock. In *II International Symposium on Organic Greenhouse Horticulture 1041* (pp. 127-132).

Aspray, Thomas J., et al. "Mycorrhization helper bacteria: a case of specificity for altering ectomycorrhiza architecture but not ectomycorrhiza formation." Mycorrhiza 16.8 (2006): 533-541.

Assigbetse K, Gueye M, Thioulouse J, Duponnois R (2005) Soil bacterial diversity responses to root colonization by an ectomycorrhizal fungus are not root-growth-dependent. Microb Ecol 50:350–359.

Averill C., Turner B.L. and Finzi A.C. (2014) Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage, Nature 505, 543–545.

Avis, P.G., McLaughlin, D.J., Dentinger, B.C. and Reich, P.B. (2003), Long-term increase in nitrogen supply alters above- and below-ground ectomycorrhizal communities and increases the dominance of *Russula* spp. in a temperate oak savanna. New Phytologist, 160: 239-253.

Avis, T. J., Gravel, V., Antoun, H., & Tweddell, R. J. (2008). Multifaceted beneficial effects of rhizosphere microorganisms on plant health and productivity. Soil biology and biochemistry, 40(7), 1733-1740.

Azcón-Aguilar C, Barea JM, Gianinazzi S, Gianinazzi-Pearson V, editors. Mycorrhizas Functional Processes and Ecological Impact. Berlin: Springer; 2009.

Baciarelli-Falini, Leonardo, et al. "Morphological and molecular analyses of ectomycorrhizal diversity in a man-made *T. melanosporum* plantation: description of novel truffle-like morphotypes." Mycorrhiza 16.7 (2006): 475-484.

Barbieri, E., Bertini, L., Rossi, I., Ceccaroli, P., Saltarelli, R., Guidi, C., & Stocchi, V. (2005). New evidence for bacterial diversity in the ascoma of the ectomycorrhizal fungus *Tuber borchii* Vittad. FEMS Microbiology Letters, 247(1), 23-35.

Barbieri, E., Ceccaroli, P., Saltarelli, R., Guidi, C., Potenza, L., Basaglia, M., ... & Stocchi, V. (2010). New evidence for nitrogen fixation within the Italian white truffle *Tuber magnatum*. Fungal biology, 114(11-12), 936-942.

Barbieri, Elena, et al. "Occurrence and diversity of bacterial communities in *Tuber magnatum* during truffle maturation." *Environmental Microbiology* 9.9 (2007): 2234-2246.

Barbieri, Elena, et al. "Phylogenetic characterization and in situ detection of a *Cytophaga-Flexibacter-Bacteroides* phylogroup bacterium in *Tuber borchii* Vittad. ectomycorrhizal mycelium." *Applied and Environmental Microbiology* 66.11 (2000): 5035-5042.

Barbieri, Elena, et al. "Truffle-associated bacteria: extrapolation from diversity to function."*True Truffle (Tuber spp.) in the World*. Springer, Cham, 2016. 301-317.

Bardgett, R. D., Mawdsley, J. L., Edwards, S., Hobbs, P. J., Rodwell, J. S., & Davies, W. J. (1999). Plant species and nitrogen effects on soil biological properties of temperate upland grasslands. Functional Ecology, 13(5), 650-660.

Barea, J. M., Pozo, M. J., Azcón, R., & Azcón-Aguilar, C. (2013). Microbial interactions in the rhizosphere. Molecular microbial ecology of the rhizosphere, 1, 29-44.

Barea, J.M., Azcón, R., Azcón-Aguilar, C. 2005. Interactions between mycorrhizal fungi and bacteria to improve plant nutrient cycling and soil structure. In: F. Buscot, A.Varma (eds). Microorganisms in Soils: Roles in Genesis and Functions. Springer-Verlag, Berlin, pp: 195-212.

Barker, S. J., & Tagu, D. (2000). The roles of auxins and cytokinins in mycorrhizal symbioses. Journal of Plant Growth Regulation, 19(2), 144-154.

Barns, S. M., Takala, S. L., & Kuske, C. R. (1999). Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. Applied and environmental microbiology, 65(4), 1731-1737.

Batista, B.D., Lacava, P.T., Ferrari, A., Sousa Teixeira-Silva, N., Bonatelli, M.L., Tsui, S., Mondin, M., Watanabe Kitajima, E., Odair Pereira, J., Azevedo, J.L., et al. Screening of tropically derived, multi-trait plant growthpromoting rhizobacteria and evaluation of corn and soybean colonization ability. Microbiol. Res. 2018, 206,33–42.

Beever, R. E., and T. Lebel. "Truffles of New Zealand: a discussion of bird dispersal characteristics of fruit bodies." Journal of the Auckland Botanical Society 69 (2014): 170-8.

Belfiori, B., Riccioni, C., Paolocci, F., & Rubini, A. (2016). Characterization of the reproductive mode and life cycle of the whitish truffle *T. borchii*. *Mycorrhiza*, *26*(6), 515-527.

Belfiori, B., Riccioni, C., Tempesta, S., Pasqualetti, M., Paolocci, F., & Rubini, A. (2012). Comparison of ectomycorrhizal communities in natural and cultivated *Tuber melanosporum* truffle grounds. FEMS microbiology ecology, 81(3), 547-561.

Belfiori, Beatrice, et al. "Mating type locus of Chinese black truffles reveals heterothallism and the presence of cryptic species within the *T. indicum* species complex." PloS one 8.12 (2013): e82353.

Bencivenga M, Calandra R, Granetti B (1990) Ricerche sui terreni e sulla flora delle tartufaie naturali di *Tuber melanosporum* Vittad. dell'Italia Centrale. In: Bencivenga M, Granetti B (eds) Proceedings of the 2nd international congress on truffle, Spoleto, 24–27 Nov 1988. Comunità Montana dei Monti Martani e del Serano, Spoleto, pp 337–374.

Bencivenga M, Donnini D, Tanfulli M, Guiducci M (1995) Tecnica dicampionamento delle radici e degli apici radicali per la valutazione delle piante micorrizate. Micol Ital 2:35–47.

Bencivenga, M., & Granetti, B. (1988). Ricerca comparativa sulle esigenze ecologiche di *Tuber magnatum* Pico e *Tuber melanosporum* Vittad. dell'Italia Centrale. Annali della Facoltà di agraria. Università degli studi di Perugia, 42, 861-872.

Bencivenga, M., and B. Granetti. "Analisi comparativa delle caratteristiche ecologiche di *Tuber magnatum* Pico e *Tuber melanosporum* Vittad. nell'Italia centrale."*Atti del II Congr Int sul tartufo. ComunitaMontana dei Monti Martani e del Serano, Spoleto* (198890): 433-434.

Bencivenga, M., R. Calandra, and B. Granetti. "Ricerche sui terreni e sulla flora delle tartufaie naturali di *Tuber melanosporum* Vittad." Dell'Italia Cebtrale. Atti "Il Congresso Internationale sul Tartufo", Spoleto. Vol. 24. No. 27. 1988.

Bendia, A. G., Callefo, F., Araújo, M. N., Sanchez, E., Teixeira, V. C., Vasconcelos, A., & Galante, D. (2021). Metagenome-assembled genomes from Monte Cristo Cave (Diamantina, Brazil) reveal prokaryotic lineages as functional models for life on Mars. Astrobiology.

Bending, G. D., E. J. Poole, J. M. Whipps, and D. J. Read. 2002. Characterisation of bacteria from *Pinus sylvestris-Suillus luteus* mycorrhizas and their effects on root-fungus interactions and plant growth. FEMS Microbiol. Ecol. 39:219–227.

Benucci, G. M. N., Raggi, L., Albertini, E., Grebenc, T., Bencivenga, M., Falcinelli, M., & Di Massimo, G. (2011). Ectomycorrhizal communities in a productive *Tuber aestivum* Vittad. orchard: composition, host influence and species replacement. FEMS microbiology ecology, 76(1), 170-184.

Benucci, G.M.N, and Gregory M. Bonito. "The truffle microbiome: species and geography effects on bacteria associated with fruiting bodies of hypogeous *Pezizales*."*Microbial ecology* 72.1 (2016): 4-8.

Benucci, G.M.N, et al. "Mycorrhization of Pecan trees (*Carya illinoinensis*) with commercial truffle species: *Tuber aestivum* Vittad. and *Tuber borchii* Vittad."*Mycorrhiza* 22.5 (2012b): 383-392.

Benucci, G.M.N., Gógán Csorbai, A., Baciarelli Falini, L. et al. Mycorrhization of *Quercus robur* L., *Quercus cerris* L. and *Corylus avellana* L. seedlings with *Tuber macrosporum* Vittad. Mycorrhiza 22, 639–646 (2012a).

Berendsen, R. L., Pieterse, C. M., & Bakker, P. A. (2012). The rhizosphere microbiome and plant health. Trends in plant science, 17(8), 478-486.

Berlanas, C., Ojeda, S., López-Manzanares, B., Andrés-Sodupe, M., Bujanda, R., del Pilar Martínez-Diz, M.,& Gramaje, D. (2020). Occurrence and diversity of black-foot disease fungi in symptomless grapevine nursery stock in Spain. Plant Disease, 104(1), 94-104.

Bertault, G., et al. "Trifling variation in truffles." Nature 394.6695 (1998): 734-734.

Bertini, L., Rossi, I., Zambonelli, A., Amicucci, A., Sacchi, A., Cecchini, M., ... & Stocchi, V. (2006). Molecular identification of *Tuber magnatum* ectomycorrhizae in the field. Microbiological Research, 161(1), 59-64.

Bidondo, L. F., Colombo, R., Bompadre, J., Benavides, M., Scorza, V., Silvani, V., ... & Godeas, A. (2016). Cultivable bacteria associated with infective propagules of arbuscular mycorrhizal fungi. Implications for mycorrhizal activity. *Applied Soil Ecology*, *105*, 86-90.

Billiard, S., et al. "Sex, outcrossing and mating types: unsolved questions in fungi and beyond." Journal of evolutionary biology 25.6 (2012): 1020-1038.

Boersma, F. G. H., Otten, R., Warmink, J. A., Nazir, R., & Van Elsas, J. D. (2010). Selection of *Variovorax paradoxus*-like bacteria in the mycosphere and the role of fungal-released compounds. Soil biology and biochemistry, 42(12), 2137-2145.

Boersma, F. H., Warmink, J. A., Andreote, F. A., & van Elsas, J. D. (2009). Selection of Sphingomonadaceae at the base of *Laccaria proxima* and *Russula exalbicans* fruiting bodies. Applied and environmental microbiology, 75(7), 1979-1989.

Bolyen, Evan, et al. QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. No. e27295v1. PeerJ Preprints, 2018.

Bonfante, P. & Genre, A. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. Nat. Commun. 1:48 doi: 10.1038/ncomms1046 (2010).

Bonito G, Smith ME, Nowak M, Healy RA, Guevara G, Cazares E, Kinoshita A, Nouhra ER, Dominguez LS, Tedersoo L, Murat C, Wang Y, Moreno BA, Pfister DH, Nara K, Zambonelli A, Trappe JM, Vilgalys R (2013) Historical biogeography and diversification of truffles in the *Tuberaceae* and their newly identified southern hemisphere sister lineage. PLoS One 8(1) e52765.

Bonito, Gregory M., and Matthew E. Smith. "General systematic position of the truffles: evolutionary theories." True truffle (*Tuber* spp.) in the world. Springer, Cham, 2016. 3-18.

Bonito, Gregory M., et al. "A global meta-analysis of *Tuber* ITS rDNA sequences: species diversity, host associations and long-distance dispersal." Molecular Ecology 19.22 (2010): 4994-5008.

Bonito, Gregory, et al. "Historical biogeography and diversification of truffles in the *Tuberaceae* and their newly identified southern hemisphere sister lineage." PloS one 8.1 (2013): e52765.

Booth, C. 1971. The Genus Fusarium. Commonwealth Mycological Institute, Kew, England

Bordács, Sándor, et al. "Chloroplast DNA variation of white oaks in northern Balkans and in the Carpathian Basin." *Forest Ecology and Management* 156.1-3 (2002): 197-209.

Borowik, A., & Wyszkowska, J. (2016). Soil moisture as a factor affecting the microbiological and biochemical activity of soil. Plant, Soil and Environment, 62(6), 250-255.

Botha, A., Bussin, M., & De Swardt, L. (2011). An employer brand predictive model for talent attraction and retention. *SA journal of human resource management*, *9*(1), 1-12.

Boutekrabt, A., Chevalier, G., Pargney, J. C., & Dexheimer, J. (1990). Mycorhization par *Tuber melanosporum* Vitt de vitroplants de *Quercus robur* L et *Quercus pubescens* Willd. Agronomie, 10(2), 127-132.

Brabcová V, Nováková M, Davidová A, Baldrian P. Dead fungal mycelium in forest soil represents a decomposition hotspot and a habitat for a specific microbial community. New Phytol. 2016; 210:1369–81.

Bragato G, Lulli L, Castrignano` A, Bencivenga M (2001) Regionalization analysis of some soil factors realted to *Tuber melanosporum* production in an experimental truffle bed. In: Actes du Ve congrés international, Science et culture de la truffe et des autres champignons hypogés comestibles, Aix-en-Provence, 4–6 Mar 1999, Fédération Francąise des Trufficulteurs, Paris, pp 5.253–5.256.

Bragato G, Lulli L, Panini T, Gardin L, Primavera F (1992) I suoli delle tartufaie naturali della zona di San Miniato (Pisa). Monti Boschi 43:17–24.

Brulé, C., Frey-Klett, P., Pierrat, J. C., Courrier, S., Gérard, F., Lemoine, M. C. & Garbaye, J. (2001). Survival in the soil of the ectomycorrhizal fungus *Laccaria bicolor* and the effects of a mycorrhiza helper *Pseudomonas fluorescens*. Soil Biology and Biochemistry, 33(12-13), 1683-1694.

Brundret, M., Bougher, N., Dell, B., Grove, T., Malajczuk, N., 1996. Working with Mycorrhizas in Forestry and Agriculture, Australian. ed. ACIAR Monograph 32, Canberra.

Bruns, T. D. (1995). Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. Plant and soil, 170(1), 63-73.

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., & Schulze-Lefert, P. (2012). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. Nature, 488(7409), 91-95.

Büntgen, U., Latorre, J., Egli, S., & Martínez-Peña, F. (2017). Socio-economic, scientific, and political benefits of mycotourism. Ecosphere, 8(7), e01870.

Büntgen, Ulf, et al. "New insights into the complex relationship between weight and maturity of Burgundy truffles (*Tuber aestivum*)." PLoS One 12.1 (2017): e0170375.

Burke DJ, Dunham SM, Kretzer AM. 2008. Molecular analysis of bacterial communities associated with the roots of Douglas fir (*Pseudotsuga menziesii*) colonized by different ECM fungi. FEMS Microbiol. Ecol. 65:299–309.

Bussotti F., Enzyklopädie der Holzgewächse: Handbuch und Atlas der Dendrologie, A. Roloff, H. Weisgerber, U. M. Lang, B. Stimm, P. Schütt, eds. (Wiley-Vch Verlag, Weinheim, 1998).

Callot, Gabriel. La truffe, la terre, la vie. Editions Quae, 1999.

Calvaruso C, TurpaultMP, Leclerc E et al (2010) Influence of forest trees on the distribution of mineral weathering-associated bacterial communities of the *Scleroderma citrinum* mycorrhizosphere. Appl Environ Microbiol 76:4780–4787.

Cameleyre, Isabelle, and Jean-Marc Olivier. "Evidence for intraspecific isozymes variations among French isolates of *Tuber melanosporum* (Vittad.)." FEMS microbiology letters 110.2 (1993): 159-162.

Caravaca, F., Alguacil, M. M., Azcón, R., Parladé, J., Torres, P., & Roldán, A. (2005). Establishment of two ectomycorrhizal shrub species in a semiarid site after in situ amendment with sugar beet, rock phosphate, and *Aspergillus niger*. Microbial Ecology, 49(1), 73-82.

Carro, L., Pukall, R., Spröer, C., Kroppenstedt, R. M., & Trujillo, M. E. (2012). *Micromonospora cremea* sp. nov. and *Micromonospora zamorensis* sp. nov., isolated from the rhizosphere of *Pisum sativum*. International journal of systematic and evolutionary microbiology, 62(Pt_12), 2971-2977.

Castellano, Michael A. "The biological component: nursery pests and mycorrhizae." The container tree nursery manual (1989): 101-167.

Catoni, R., et al. "*Corylus avellana* responsiveness to light variations: morphological, anatomical, and physiological leaf trait plasticity." *Photosynthetica* 53.1 (2015): 35-46.

Centemero, M. (2001). Il compost nei terricci per il florovivaismo. Fertilizzanti, 3, 24-29.

Cerigini, E., Palma, F., Barbieri, E., Buffalini, M., & Stocchi, V. (2008). The *Tuber borchii* fruiting body-specific protein TBF-1, a novel lectin which interacts with associated Rhizobium species. FEMS microbiology letters, 284(2), 197-203.

Ceruti, A., A. Fontana, and C. Nosenzo. "European species of the genus *Tuber*. An historical revision." European species of the genus *Tuber*. An historical revision. (2003).

Chakravarty, P., & Unestam, T. (1987). Differential influence of ectomycorrhizae on plant growth and disease resistance in Pinus sylvestris seedlings. *Journal of Phytopathology*, *120*(2), 104-120.

Chalot, M., Javelle, A., Blaudez, D., Lambilliote, R., Cooke, R., Sentenac, H. & Botton, B. (2002). An update on nutrient transport processes in ectomycorrhizas. In Diversity and Integration in Mycorrhizas (pp. 165-175). Springer, Dordrecht.

Chatin, M. Ad. "Sur Les Arbres Et Arbustes Truffiers." *Bulletin de la Société Botanique de France* 16.1 (1869): 19-26.

Chatin, M. Ad. "Une nouvelle espécé de truffe (*Tuber uncinatum*)." *Bulletin de la Société Botanique de France* 34.6 (1887): 246-248.

Chaudhuri, S. K., & Lovley, D. R. (2003). Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells. Nature biotechnology, 21(10), 1229-1232.

Cheikh-Ali, Z., Glynou, K., Ali, T., Ploch, S., Kaiser, M., Thines, M., ... & Maciá-Vicente, J. G. (2015). Diversity of exophillic acid derivatives in strains of an endophytic *Exophiala* sp. *Phytochemistry*, *118*, 83-93.

Chen, X., Zhao, Y., Zeng, C., Li, Y., Zhu, L., Wu, J., & Wei, Z. (2019). Assessment contributions of physicochemical properties and bacterial community to mitigate the bioavailability of heavy metals during composting based on structural equation models. Bioresource technology, 289, 121657.

Chen, Ying Long, Li Hua Kang, and Bernard Dell. "Inoculation of *Eucalyptus urophylla* with spores of *Scleroderma* in a nursery in south China: comparison of field soil and potting mix." Forest ecology and management 222.1-3 (2006): 439-449.

Chevalier G, Grente J (1979) Application pratique de la symbiose ectomycorhizienne: production à grande échelle de plants mycorhize´s par la truffe. Mushroom Sci 10:483–505.

Chevalier, G. "La truffe d'Europe (*Tuber aestivum*): limites geographiques, ecologie et culture."*Aust J Mycol* 19 (2010): 249-259.

Chevalier, G., & Sourzat, P. (2012). Soils and techniques for cultivating *Tuber melanosporum* and *Tuber aestivum* in Europe. In Edible ectomycorrhizal mushrooms (pp. 163-189). Springer, Berlin, Heidelberg.

Chevalier, G., and Henri Frochot. "Ecology and possibility of culture in Europe of the Burgundy truffle (*Tuber uncinatum* Chatin)." Agriculture, Ecosystems & Environment 28.1-4 (1990): 71-73.

Chevalier, G., et al. "*Tuber uncinatum* Chat. et *T. aestivum* Vittad., espèces différentes ou simples variétés de la meme espèce? "*Documents Mycologiques* 24 (1994): 17-21.

Chevalier, Gerald. "The truffle of Europe (*Tuber aestivum* Vittad.): ecology and possibility of cultivation." First Conference on the European Truffle *Tuber aestivum*/*uncinatum*. University of Vienna, Vienna, Austria. 2009.

Chevalier, Gérard, Henri Frochot, and Lucien Ballureau. *truffe de Bourgogne (Tuber uncinatum Chatin)*. Éditions Pétrarque, 1997.

Chiarucci, Alessandro, Giovanni Bacaro, and Samuel M. Scheiner. "Old and new challenges in using species diversity for assessing biodiversity." Philosophical Transactions of the Royal Society B: Biological Sciences 366.1576 (2011): 2426-2437.

Chiron, Nicolas, and Didier Michelot. "Odeurs des champignons: chimie et rôle dans les interactions biotiques-une revue." Cryptogamie-Mycologie 26.4 (2005): 299-364.

Citterio, B., Malatesta, M., Battistelli, S., Marcheggiani, F., Baffone, W., Saltarelli, R., ... & Gazzanelli, G. (2001). Possible involvement of *Pseudomonas fluorescens* and *Bacillaceae* in structural modifications of *Tuber borchii* fruit bodies. Canadian journal of microbiology, 47(3), 264-268.

Colinas, Carlos, et al. "Mapa de aptitud para el cultivo de trufa negra (*Tuber melanosporum* Vittad.) en Cataluña." (2007).

Colpaert, Jan V., and Katia K. van Tichelen. "Decomposition, nitrogen and phosphorus mineralization from beech leaf litter colonized by ectomycorrhizal or litter-decomposing basidiomycetes." New Phytologist 134.1 (1996): 123-132.

Corrêa A., Strasser R. J., Martins-Loução M. A. (2008) Response of plants to ectomycorrhizae in N-limited conditions: which factors determine its variation? Mycorrhiza, Vol 18 Issue 8, pp 413-427.

Corrêa, A., Gurevitch, J., Martins-Loução, M. A., & Cruz, C. (2012). C allocation to the fungus is not a cost to the plant in ectomycorrhizae. Oikos, 121(3), 449-463.

Couturier J., Montanini B., Martin F., Brun A., Blaudez D., Chalot M. (2007) The expanded family of ammonium transporters in the perennial poplar plant. New Phytologist 174, 137-150.

Cristofori, V. (2005). Fattori di qualità della nocciola. Tesi di dottorato in "ortoflorofrutticola" - XVIII Ciclo Settore Scientifico-Disciplinare AGR 03, 5–41.

Daba G.M., Elkhateeb W.A., Wen TC., Thomas P.W. (2019) The Continuous Story of Truffle-Plant Interaction. In: Kumar V., Prasad R., Kumar M., Choudhary D. (eds) Microbiome in Plant Health and Disease. Springer, Singapore.

Daba, A. W., Qureshi, A. S., & Nisaren, B. N. (2019). Evaluation of some Rhodes grass (*Chloris gayana*) genotypes for their salt tolerance, biomass yield and nutrient composition. Applied Sciences, 9(1), 143.

Damesin, C., Rambal, S., & Joffre, R. (1997). Between-tree variations in leaf δ13C of *Quercus pubescens* and *Quercus ilex* among Mediterranean habitats with different water availability. Oecologia, 111(1), 26-35.

Danhorn, T., & Fuqua, C. (2007). Biofilm formation by plant-associated bacteria. Annu. Rev. Microbiol., 61, 401-422.

Daniels, Barbara A., and James M. Trappe. "*Glomus epigaeus* sp. nov., a useful fungus for vesicular–arbuscular mycorrhizal research." Canadian Journal of Botany 57.5 (1979): 539-542.

Danielson, R. M., and S. Visser. "Effects of forest soil acidification on ectomycorrhizal and vesicular—arbuscular mycorrhizal development." New Phytologist 112.1 (1989): 41-47.

Davies, K.G, and R. Whitbread. "Factors affecting the colonisation of a root system by fluorescent Pseudomonads: The effects of water, temperature and soil microflora." Plant and Soil 116.2 (1989): 247-256.

Daza A., Manjón J.L., Camacho M., Romero de la Osa L., Aguilar A., Santamaría C. (2006) Effect of carbon and nitrogen sources, pH and temperature on in vitro culture of several isolates of Amanita muscaria caesarea Pers. Mycorrhiza. 16, 133-136.

De Bary, Anton. "Comparative morphology and biology of the fungi, mycetozoa and bacteria." (1887).

De la Varga, Herminia, et al. "Five years investigation of female and male genotypes in périgord black truffle (*Tuber melanosporum* Vittad.) revealed contrasted reproduction strategies." Environmental microbiology 19.7 (2017): 2604-2615.

De Miguel, A. M., Águeda, B., Sánchez, S., & Parladé, J. (2014). Ectomycorrhizal fungus diversity and community structure with natural and cultivated truffle hosts: applying lessons learned to future truffle culture. Mycorrhiza, 24(1), 5-18.

De Miguel, A. M., Román, M. D., & Etayo, M. L. (2002). Mycorrhizal fungi competing with *Tuber melanosporum* Vittad. in cultivated truffle beds in north-eastern Spain. In Edible mycorrhizal mushrooms and their cultivation. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Christchurch, New Zealand, 3-6 July, 2001 (pp. 0-5). Crop & Food Research.

De Román, M., & de Miguel, A. M. (2005). Post-fire, seasonal and annual dynamics of the ectomycorrhizal community in a *Quercus ilex* L. forest over a 3-year period. Mycorrhiza, 15(6), 471-482.

Debuchy, Robert, Véronique Berteaux-Lecellier, and Philippe Silar. "Mating systems and sexual morphogenesis in ascomycetes." Cellular and molecular biology of filamentous fungi (2010): 499-535.

Delmas, J. "[Trufficulture on the Causse de Martel. Ecological characteristics [Perigord, France; stratigraphy, geomorphology, climatology, vegetation, soils and their truffle-producing potential]]." Bulletin de la Federation Nationale des Producteurs de Truffes (France) (1981).

Delmas, J., and N. Poitou. "Contribution to the knowledge of the ecology of *Tuber melanosporum*: The Pèrigord truffle." Acad Agric Fr CR Seances (1973).

Delmotte, N., Knief, C., Chaffron, S., Innerebner, G., Roschitzki, B., Schlapbach, R.& Vorholt, J. A. (2009). Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proceedings of the National Academy of Sciences, 106(38), 16428-16433.

Demerson, J. (2012). CO2, carbonate et calcaire actif dans le sol des truffières. Le Trufficulteur, France, 81, 13-14.

Dennis J (1985) Effect of pH and temperature on *in vitro* growth of ectomycorrhizal fungi. Pacific Forestry Centre. Canadian Forestry Service Information Report BC-X.

Deora A, Hatano E, Tahara S, Hashidoko Y. Inhibitory effects of furanone metabolites of a rhizobacterium, Pseudomonas jessenii, on phytopathogenic *Aphanomyces cochlioides* and *Pythium aphanidermatum*. Plant Pathol. 2010; 59:84–99.

Deveau, A. and Labbé, J. (2016) Mycorrhiza helper bacteria, in Molecular Mycorrhizal Symbiosis (ed F. Martin), John Wiley & Sons, Inc., Hoboken, NJ, USA.

Deveau, Aurélie, et al. "Role of fungal trehalose and bacterial thiamine in the improved survival and growth of the ectomycorrhizal fungus *Laccaria bicolor* S238N and the helper bacterium *Pseudomonas fluorescens* BBc6R8." Environmental microbiology reports 2.4 (2010): 560-568.

Deveau, Aurélie, et al. "Temporal changes of bacterial communities in the *Tuber melanosporum* ectomycorrhizosphere during ascocarp development."*Mycorrhiza* 26.5 (2016): 389-399.

Dey, Rinku, Kamal Krishna Pal, and K. V. B. R. Tilak. "Plant growth promoting rhizobacteria in crop protection and challenges." Future challenges in crop protection against fungal pathogens. Springer, New York, NY, 2014. 31-58.

Di Marino, E., Kõljalg, U., & Agerer, R. (2007). The ectomycorrhizae of *Pseudotomentella humicola* on *Picea abies*. Nova Hedwigia, 429-440.

Di Santo P, Cocozza C, Tognetti R, Palumbo G, Di Iorio E, Paura B (2016). A quick screening to assess the phytoextraction potential of cadmium and copper in *Quercus pubescens* plantlets. iForest 10: 93-98.

Dickie, I. A. (2007). Host preference, niches and fungal diversity. New Phytologist, 230-233.

Dixon, R., & Kahn, D. (2004). Genetic regulation of biological nitrogen fixation. *Nature Reviews Microbiology*, *2*(8), 621-631.

do Amaral Franco J., Flora Iberica: plantas vasculares de la Peninsula Ibérica e Islas Baleares, Volume 2: Platanaceae - Plumbaginaceae (partim), S. Castroviejo, et al., eds. (Real Jardìn Botánico, CSIC, Madrid, 1991), pp. 15–36.

Dominguez, J. A., et al. "The combined effects of Pseudomonas fluorescens and *Tuber melanosporum* on the quality of *Pinus halepensis* seedlings."*Mycorrhiza* 22.6 (2012): 429-436.

Domínguez-Núez J, Muñz D, de la Cruz A and Saiz de Omeñaca J. (2013). Effects of *Pseudomonas fluorescens* on the water parameters of mycorrhizal and nonmycorrhizal seedlings of *Pinus halepensis*. Agronomy 3, 571–582.

Domínguez-Núñez, J. A. D., Serrano, J. S., Barreal, J. A. R., & de Omeñaca González, J. A. S. (2006). The influence of mycorrhization with *Tuber melanosporum* in the afforestation of a Mediterranean site with *Quercus ilex* and *Quercus faginea*. Forest ecology and management, 231(1-3), 226-233.

Domínguez-Núñez, José Alfonso, and Ada S. Albanesi. "Ectomycorrhizal fungi as biofertilizers in forestry." Biostimulants in Plant Science (2019).

Donnini D, Baciarelli Falini L, Di Massimo G, Benucci GMN, Bencivenga M (2009) Mycorrhization: *Tuber borchii* Vittad. competitivity with respect to other species of *Tuber*. Acta Bot Yunn Suppl 16:100–102

Donnini, D., Baciarelli-Falini, L., Di Massimo, G., Benucci, G. M. N., & Bencivenga, M. (2008). Competizione o sinergia di funghi ectomicorrizici in tartufaie coltivate. Micologia Italiana, 37(3), 47-51.

Du Mortier, Barthélemy-Charles. Commentationes botaniques: Observations botaniques, dédiées a la Sociéte d'Horticulture de Tournay. Ch. Casterman-Dieu, 1822.

Duchesne, Luc C., R. L. Peterson, and Brian E. Ellis. "Pine root exudate stimulates the synthesis of antifungal compounds by the ectomycorrhizal fungus *Paxillus involutus*." New Phytologist 108.4 (1988): 471-476.

Ducousso, Marc, et al. "The last common ancestor of *Sarcolaenaceae* and Asian dipterocarp trees was ectomycorrhizal before the India–Madagascar separation, about 88 million years ago." Molecular Ecology 13.1 (2004): 231-236.

Duddridge JA, Malbari A, Read DJ (1980) Structure and function of ectomycorrhizal rhizomorphs with special reference to their role in water transport. Nature 287: 834–836

Dumolin-Lapegue, S., et al. "Phylogeographic structure of white oaks throughout the European continent." *Genetics* 146.4 (1997): 1475-1487.

Dupler, Marcella, and Ralph Baker. "Survival of *Pseudomonas putida*, a biological control agent, in soil." Phytopathology 74.2 (1984): 195-200.

Duponnois, Robin, and Jean Garbaye. "Effect of dual inoculation of Douglas fir with the ectomycorrhizal fungus *Laccaria laccata* and mycorrhization helper bacteria (MHB) in two bare-root forest nurseries."*Plant and Soil* 138.2 (1991): 169-176.

Edgar, Robert C. "Search and clustering orders of magnitude faster than BLAST." Bioinformatics 26.19 (2010): 2460-2461.

Eichorst, S. A., & Kuske, C. R. (2012). Identification of cellulose-responsive bacterial and fungal communities in geographically and edaphically different soils by using stable isotope probing. Applied and environmental microbiology, 78(7), 2316-2327.

Erlacher, A., Cernava, T., Cardinale, M., Soh, J., Sensen, C. W., Grube, M., & Berg, G. (2015). Rhizobiales as functional and endosymbiontic members in the lichen symbiosis of *Lobaria pulmonaria* L. Frontiers in microbiology, 6, 53.

Fascella, Giancarlo, and Youssef Rouphael. "Influence of container volume and irrigation system on photosynthesis, water productivity and growth of potted Euphorbia× lomi."*Acta Scientiarum Polonorum Hortorum Cultus* 16 (2017): 163-171.

Fasolo-Bonfante, P., Fontana, A., & Montacchini, F. (1971). Studi sullecologia del *Tuber melanosporum*. I. Dimostrazione di un effetto fitotossico. Allionia, 17, 48-53.

Ferlito, Filippo, et al. "Scion–rootstock interactions influence the growth and behaviour of the grapevine root system in a heavy clay soil."*Australian Journal of Grape and Wine Research* 26.1 (2020): 68-78.

Finlay R. (2008) Ecological aspects of mycorrhizal symbiosis: with special emphasis on the functional diversity of interactions involving the extraradical mycelium. Journal of Experimental Botany, Vol. 59, Issue 5, pp. 1115-1126.

Fischer, C., Oliach, D., Bonet Lledos, J. A., & Colinas, C. (2017). Best practices for cultivation of truffles. Centre Tecnològic Forestal de Catalunya.

Fischer, E., & Qaim, M. (2012). Gender, agricultural commercialization, and collective action in Kenya. Food security, 4(3), 441-453.

Fortini, Paola, Piera Di Marzio, and Romeo Di Pietro. "Differentiation and hybridization of *Quercus frainetto*, *Q. petraea*, and *Q. pubescens* (*Fagaceae*): insights from macro-morphological leaf traits and molecular data."*Plant Systematics and Evolution* 301.1 (2015): 375-385.

Founoune, Hassna, et al. "Mycorrhiza helper bacteria stimulate ectomycorrhizal symbiosis of *Acacia holosericea* with *Pisolithus alba*." New Phytologist (2002): 81-89.

Frank, B. "On the nutritional dependence of certain trees on root symbiosis with belowground fungi (an English translation of AB Frank's classic paper of 1885)." Mycorrhiza 15.4 (2005): 267-275.

Frank, J. L., et al. "Rodent dispersal of fungal spores promotes seedling establishment away from mycorrhizal networks on *Quercus garryana*." Botany 87.9 (2009): 821-829.

Franklin O., Näsholm T, Högberg M.N., Högberg P. (2014) Forests trapped in nitrogen limitation – an ecological market perspective on ectomycorrhizal symbiosis, New Phtytologist Volume 203, pp. 657–666.

Fraser, James A., and Joseph Heitman. "Fungal mating-type loci." Current Biology 13.20 (2003): R792-R795.

Freeman, Murray F., and John W. Tukey. "Transformations Related to the Angular and the Square Root." The Annals of Mathematical Statistics, vol. 21, no. 4, Institute of Mathematical Statistics, 1950, pp. 607–11

Frey-Klett, P., & Garbaye, J. (2005). Mycorrhiza helper bacteria: a promising model for the genomic analysis of fungal-bacterial interactions. New Phytologist, 4-8.

Frey-Klett, P., M. Chavatte, M. L. Clausse, S. Courrier, C. Le Roux, J. Raaijmakers, M. G. Martinotti, J. C. Pierrat, and J. Garbaye. 2005. Ectomycorrhizal symbiosis affects functional diversity of rhizosphere fluorescent pseudomonads. New Phytol. 165:317–328.

Frey-Klett, Pascale, Jean Garbaye, and Mika Tarkka. "The mycorrhiza helper bacteria revisited." *New phytologist* 176.1 (2007): 22-36.

Frisvad, J. C., Filtenborg, O., Samson, R. A., & Stolk, A. C. (1990). Chemotaxonomy of the genus Talaromyces. Antonie van Leeuwenhoek, 57(3), 179-189.

Gadd G. (2007) Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycological Research, Vol. 111 Issue 1 pp. 3-49.

Gadgil, Ruth L., and P. D. Gadgil. "Mycorrhiza and litter decomposition." Nature 233.5315 (1971): 133-133.

Gamez, R., Cardinale, M., Montes, M., Ramirez, S., Schnell, S., Rodriguez, F. Screening, plant growth promotion and root colonization pattern of two rhizobacteria (*Pseudomonas fluorescens* Ps006 and *Bacillus amyloliquefaciens* Bs006) on banana cv. Williams (Musa acuminata Colla). Microbiol Res. 2019, 220, 12–20.

Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210.

Garbaye, Jean, and G. D. Bowen. "Stimulation of ectomycorrhizal infection of *Pinus radiata* by some microorganisms associated with the mantle of ectomycorrhizas." New Phytologist 112.3 (1989): 383-388.

Garbeva, P. V., Van Veen, J. A., & Van Elsas, J. D. (2004). Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annu. Rev. Phytopathol., 42, 243-270.

Garcia, C., Hernández, T., Roldan, A., Albaladejo, J., & Castillo, V. (2000). Organic amendment and mycorrhizal inoculation as a practice in afforestation of soils with *Pinus halepensis* Miller: effect on their microbial activity. Soil Biology and Biochemistry, 32(8-9), 1173-1181.

García-Barreda, S., & Reyna, S. (2012). Below-ground ectomycorrhizal community in natural *Tuber melanosporum* truffle grounds and dynamics after canopy opening. Mycorrhiza, 22(5), 361-369.

García-Montero, L. G., et al. "Soil factors that influence the fruiting of *Tuber melanosporum* (black truffle)." Soil Research 44.8 (2006): 731-738.

García-Montero, L. G., Quintana, A., Valverde-Asenjo, I., & Díaz, P. (2009a). Calcareous amendments in truffle culture: a soil nutrition hypothesis. Soil Biology and Biochemistry, 41(6), 1227-1232.

García-Montero, L. G., Valverde-Asenjo, I., Díaz, P., & Pascual, C. (2009b). Statistical patterns of carbonates and total organic carbon on soils of *Tuber rufum* and *T. melanosporum* (black truffle) brûlés. Soil Research, 47(2), 206-212.

García-Montero, Luis G., et al. "Ecological patterns of *Tuber melanosporum* and different *Quercus* Mediterranean forests: quantitative production of truffles, burn sizes and soil studies." Forest Ecology and Management 242.2-3 (2007): 288-296.

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular ecology, 2(2), 113-118.

Garrity, G. M., Brenner, D. J., Krieg, N. R., Staley, J. R., & Manual, B. S. (2005). Systematic bacteriology. The Proteobacteria, Part C: The Alpha-, Beta-, Delta-, and *Epsilonproteobacteria*, Bergey's Manual Trust, Department of Microbiology and Molecular Genetics, 2.

Gehring, C. A., Sthultz, C. M., Flores-Rentería, L., Whipple, A. V., & Whitham, T. G. (2017). Tree genetics defines fungal partner communities that may confer drought tolerance. *Proceedings of the National Academy of Sciences*, *114*(42), 11169-11174.

Giomaro, Giovanna, et al. "Comparative study and molecular characterization of ectomycorrhizas in *Tilia americana* and *Quercus pubescens* with *Tuber brumale*." *FEMS microbiology letters* 216.1 (2002): 9-14.

Giovannetti, G., and A. Fontana. "Mycorrhizal synthesis between *Cistaceae* and *Tuberaceae*." New Phytologist 92.4 (1982): 533-537.

Giovannetti, Manuela, and Vivienne Gianinazzi-Pearson. "Biodiversity in arbuscular mycorrhizal fungi." *Mycological Research* 98.7 (1994): 705-715.

Glen, M., Tommerup, I., Bougher, N., & O'Brien, P. (2002). Are *Sebacinaceae* common and widespread ectomycorrhizal associates of *Eucalyptus* species in Australian forests? Mycorrhiza, 12(5), 243-247.

Gogan, A. C., et al. "Ecological characteristic of Hungarian summer truffle (*Tuber aestivum* Vittad.) producing area." Acta Mycologica 47.2 (2012).

González-Armada, B., De Miguel, A. M., & Cavero, R. Y. (2010). Ectomycorrhizae and vascular plants growing in brulés as indicators of below and above ground microecology of black truffle production areas in Navarra (Northern Spain). Biodiversity and conservation, 19(14), 3861-3891.

Gordon, T. R., & Martyn, R. D. (1997). The evolutionary biology of *Fusarium oxysporum*. *Annual review of phytopathology*, *35*(1), 111-128.

Govi G. (1986) Introduzione alla micologia, cap. 13 Simbiosi micorrizica a cura di M.B. Branzanti pp. 231-257.

Govindasamy, V., Franco, C. M., & Gupta, V. V. (2014). Endophytic actinobacteria: diversity and ecology. In Advances in endophytic research (pp. 27-59). Springer, New Delhi.

Gramaje, D., Berlanas, C., Martínez-Diz, M., Diaz-Losada, E., Antonielli, L., Beier, S., Gorfer, M., Schmoll, M., & Compant, S. (2020). Comparative Genomic Analysis of Dactylonectria torresensis Strains from Grapevine, Soil and Weed Highlights Potential Mechanisms in Pathogenicity and Endophytic Lifestyle. Journal of fungi (Basel, Switzerland), 6(4), 255.

Granetti B, DeAngelis A, Materozzi G (2005) Umbria, terra di tartufi. Regione Umbria, Assessorato Regionale Agricoltura, Foreste, Caccia e Pesca.

Grawitz, P., & de Bary, W. (1887). Ueber die Ursachen der subcutanen Entzündung und Eiterung. Archiv für pathologische Anatomie und Physiologie und für klinische Medicin, 108(1), 67-102.

Grayston, S. J., & Campbell, C. D. (1996). Functional biodiversity of microbial communities in the rhizospheres of hybrid larch (*Larix eurolepis*) and Sitka spruce (*Picea sitchensis*). *Tree Physiology*, *16*(11-12), 1031-1038.

Greco, Carlo, et al. "Effects of vermicompost, compost and digestate as commercial alternative peat-based substrates on qualitative parameters of Salvia officinalis." *Agronomy* 11.1 (2021): 98.

Greco, Carlo, et al. "Sustainable valorisation of biowaste for soilless cultivation of Salvia officinalis in a circular bioeconomy." *Agronomy* 10.8 (2020): 1158.

Gregori, G. "L ‘espèce *Tuber uncinatum* en Italie: écologie, commercialisation et valorisation." *Österr Z. Pilzk* 19 (2010): 265-271.

Gregori, Gianluigi. *Tartufi e tartuficoltura nel Veneto*. Regione del Veneto, Giunta regionale, Assessorato agricoltura e foreste, Dipartimento per le foreste e l'economia montana, Servizio forestale, 1991.

Grimm GW, Renner SS. Harvesting Betulaceae sequences from GenBank to generate a new chronogram for the family. Bot J Linn Soc. 2013; 172:465–477.

Gryndler, M., and H. Hrselova. "Isolation of bacteria from ectomycorrhizae of *Tuber aestivum* Vittad." *Acta Mycologica* 47.2 (2012).

Gryndler, Milan, et al. "A quest for indigenous truffle helper prokaryotes." *Environmental Microbiology Reports* 5.3 (2013): 346-352.

Gryndler, Milan, et al. "*Tuber aestivum* association with non-host roots." Mycorrhiza 24.8 (2014): 603-610.

Gryndler, Milan. "True truffle host diversity." True Truffle (Tuber spp.) in the World. Springer, Cham, 2016. 267-281.

Habiyaremye, J. D. D., Goldmann, K., Reitz, T., Herrmann, S., & Buscot, F. (2020). Tree root zone microbiome: exploring the magnitude of environmental conditions and host tree impact. Frontiers in microbiology, 11, 749.

Habiyaremye, J. D. D., Goldmann, K., Reitz, T., Herrmann, S., & Buscot, F. (2020). Tree root zone microbiome: exploring the magnitude of environmental conditions and host tree impact. *Frontiers in microbiology*, *11*, 749.

Haichar, F. E. Z., Marol, C., Berge, O., Rangel-Castro, J. I., Prosser, J. I., Balesdent, J., ... & Achouak, W. (2008). Plant host habitat and root exudates shape soil bacterial community structure. The ISME journal, 2 (12), 1221-1230.

Hall, I. R., A. Zambonelli, and F. Primavera. "Ectomycorrhizal fungi with edible fruiting bodies 3. *Tuber magnatum*, *Tuberaceae*." *Economic botany* 52.2 (1998): 192-200.

Hall, I., Gordon Brown, and J. Byars. "The black truffle." *New Zealand Institute for Crop and Food Research. Christchurch, New Zealand* (1994).

Hall, Ian R., Gordon T. Brown, and Alessandra Zambonelli. "Taming the truffle." *The history lore and science of the ultimate mushroom. Timber, Portland* (2007).

Harki, E., Bouya, D., & Dargent, R. (2006). Maturation-associated alterations of the biochemical characteristics of the black truffle *Tuber melanosporum* Vittad. Food Chemistry, 99(2), 394-400.

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) *Trichoderma* species–opportunistic, avirulent plant symbionts. Nature Reviews in Microbiology 2: 43–56.

Harrison M.J. (1997) The Arbuscular Mycorrhizal Symbiosis. In: Stacey G., Keen N.T. (eds) Plant-Microbe Interactions. Springer, Boston, MA.

Hartmann, H. T., D. E. Kester, and F. T. Davies Jr. "Techniques of *in vitro* culture of micropropagation." H. Hartmann T, D Kester (eds). Prentice Hall. New Jersey, USA. pp (1998): 549-608.

Hassan, M. K., McInroy, J. A., & Kloepper, J. W. (2019). The interactions of rhizodeposits with plant growth-promoting rhizobacteria in the rhizosphere: a review. Agriculture, 9(7), 142.

Hata S, Kobae Y, Banba M. Interactions between plants and arbuscular mycorrhizal fungi. In: Kwang WJ, editor. International Review of Cell and Molecular Biology, Vol. 281. San Diego, USA: Academic Press; 2010. pp. 1-48.

Hatakeyama T., Ohmasa M. (2004) Mycelial growth of strains of the genera *Suillus* and *Boletinus* in media with a wide range of concentrations of carbon and nitrogen sources. Mycoscience 45, 169-176.

Hawksworth, D. L. (2011). A new dawn for the naming of fungi: impacts of decisions made in Melbourne in July 2011 on the future publication and regulation of fungal names. IMA fungus, 2(2), 155-162.

Heinonsalo, Jussi, et al. "Fate, tree growth effect and potential impact on soil microbial communities of mycorrhizal and bacterial inoculation in a forest plantation." Soil Biology and Biochemistry 36.2 (2004): 211-216.

Helgason, T., Daniell, T. J., Husband, R., Fitter, A. H., & Young, J. P. W. (1998). Ploughing up the wood-wide web? Nature, 394(6692), 431-431.

Henrion, Bénédicte, Gérard Chevalier, and Francis Martin. "Typing truffle species by PCR amplification of the ribosomal DNA spacers." *Mycological research* 98.1 (1994): 37-43.

Herrero de Aza, C., Armenteros, S., McDermott, J., Mauceri, S., Olaizola, J., Hernández-Rodríguez, M., & Mediavilla, O. (2022). Fungal and Bacterial Communities in *Tuber melanosporum* Plantations from Northern Spain. Forests, 13(3), 385.

Herrero de Aza, C., Armenteros, S., McDermott, J., Mauceri, S., Olaizola, J., Hernández-Rodríguez, M., & Mediavilla, O. (2022). Fungal and bacterial communities in *Tuber melanosporum* plantations from northern Spain. *Forests*, *13*(3), 385.

Herrmann, S., & Buscot, F. (2007). Cross talks at the morphogenetic, physiological and gene regulation levels between the mycobiont *Piloderma croceum* and oak microcuttings (*Quercus robur*) during formation of ectomycorrhizas. *Phytochemistry*, *68*(1), 52-67.

Hilszczańska, D., Szmidla, H., Horak, J., & Rosa-Gruszecka, A. (2016). Ectomycorrhizal communities in a *Tuber aestivum* Vittad. orchard in Poland. Open Life Sciences, 11(1), 348-357.

Himaman, W., Suksaard, P., Mingma, R., Matsumoto, A., & Duangmal, K. (2017). Cryptosporangium eucalypti sp. nov., an actinomycete isolated from Eucalyptus camaldulensis roots. *International Journal of Systematic and Evolutionary Microbiology*, *67*(8), 3077-3082.

Ho, W. H., Hyde, K. D., & Hodgkiss, I. J. (2002). Seasonality and sequential occurrence of fungi on wood submerged in Tai Po Kau Forest Stream, Hong Kong. Fungal Diversity.

Hongsanan, S., Maharachchikumbura, S. S., Hyde, K. D., Samarakoon, M. C., Jeewon, R., Zhao, Q. & Bahkali, A. H. (2017). An updated phylogeny of *Sordariomycetes* based on phylogenetic and molecular clock evidence. Fungal diversity, 84(1), 25-41.

Horton, T. R., & Bruns, T. D. (2001). The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Molecular ecology, 10(8), 1855-1871.

Hrynkiewicz K, Baum C and Leinweber P. (2009). Mycorrhizal community structure, microbial biomass P and phosphatase activities under Salix polaris as influenced by nutrient availability. European Journal of Soil Biology 45, 168–175.

Hughes E., Mitchel D.T. (1996) Properties of invertases in mycelium of *Hymenoscyphus ericae* and in endomycorrhizal association with cranberry seedlings. Mycological Research 10, 1197-1203.

Huson, Daniel H., et al. "MEGAN analysis of metagenomic data." Genome research 17.3 (2007): 377-386.

Hyde, K. D., Bao, D. F., Hongsanan, S., Chethana, K. W., Yang, J., & Suwannarach, N. (2021). Evolution of freshwater *Diaporthomycetidae* (*Sordariomycetes*) provides evidence for five new orders and six new families. Fungal Diversity, 107(1), 71-105.

Hyde, K. D., Jones, E. B., Liu, J. K., Ariyawansa, H., Boehm, E., Boonmee, S., & Zhang, M. (2013). Families of *Dothideomycetes*. Fungal diversity, 63(1), 1-313.

Högberg M.N., Högberg P. (2002) Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces, together with associated roots, half the dissolved organic carbon in a forest soil. New Phytologist 154, 791-795.

Ibrahim, S. E., et al. "QTL analysis of drought tolerance for seedling root morphological traits in an advanced backcross population of spring wheat." International Journal of Agricultural Science 2 (2012): 619-629.

Ibrahim, S.R.M., Mohamed, S.G.A., Sindi, I.A. et al. Biologically active secondary metabolites and biotechnological applications of species of the family *Chaetomiaceae* (*Sordariales*): an updated review from 2016 to 2021. Mycol Progress 20, 595–639 (2021).

Innangi, Michele, et al. "*Tuber aestivum* is associated with changes in soil chemistry and reduced biological quality in a *Quercus pubescens* stand in Northern Italy." *Pedobiologia* 80 (2020): 150648.

Insuk, C., Pongpamorn, P., Forsythe, A., Matsumoto, A., Ōmura, S., Pathom-Aree, W., ... & Xu, J. (2021). Taxonomic and Metabolite Diversities of Moss-Associated *Actinobacteria* from Thailand. *Metabolites*, *12*(1), 22.

Iotti, M., & Zambonelli, A. (2006). A quick and precise technique for identifying ectomycorrhizas by PCR. Mycological Research, 110(1), 60-65.

Iotti, M., Lancellotti, E., Hall, I., & Zambonelli, A. (2010). The ectomycorrhizal community in natural *Tuber borchii* grounds. FEMS Microbiology Ecology, 72(2), 250-260.

Iotti, M., Piattoni, F., & Zambonelli, A. (2012). Techniques for Host Plant Inoculation with Truffles and Other Edible Ectomycorrhizal Mushrooms. Edible Ectomycorrhizal Mushrooms, 145–161.

Ishida, T. A., Nara, K., & Hogetsu, T. (2007). Host effects on ectomycorrhizal fungal communities: insight from eight host species in mixed conifer–broadleaf forests. New Phytologist, 174(2), 430-440.

Izumi H, Cairney JWG, Killham K et al (2008) Bacteria associated with ectomycorrhizas of slash pine (*Pinus elliottii*) in south-eastern Queensland, Australia. FEMS Microbiol Lett 282:196–204.

Izumi H, Finlay RD (2011) Ectomycorrhizal roots select distinctive bacterial and ascomycete communities in Swedish subarctic forests. Environ Microbiol 13:819–830.

Jaillard, Benoit, et al. "Alkalinity and structure of soils determine the truffle production in the Pyrenean Regions." Forest Systems 23.2 (2014): 364-377.

Jaillard, Benoît, et al. "Soil characteristics of *Tuber melanosporum* habitat." True Truffle (Tuber spp.) in the World. Springer, Cham, 2016. 169-190.

Jamali, S. (2017). First report of identification and molecular characterization of *Tuber aestivum* in Iran. Agroforestry Systems, 91(2), 335-343.

Jakucs, E., & Erős-Honti, Z. (2008). Morphological-anatomical characterization and identification of Tomentella ectomycorrhizas. Mycorrhiza, 18(6), 277-285.

Jarerat, A., Pranamuda, H., & Tokiwa, Y. (2002). Poly (L-lactide) -degrading activity in various A*ctinomycetes*. Macromolecular Bioscience, 2(9), 420-428.

Jayawardena, R. S., Zhang, W., Liu, M., Maharachchikumbura, S. S., Zhou, Y., Huang, J., ... & Hyde, K. D. (2015). Identification and characterization of *Pestalotiopsis*-like fungi related to grapevine diseases in China. *Fungal Biology*, *119*(5), 348-361.

Jeandroz, S., Murat, C., Wang, Y., Bonfante, P., & Tacon, F. L. (2008). Molecular phylogeny and historical biogeography of the genus Tuber, the 'true truffles'. Journal of Biogeography, 35(5), 815-829.

Jefferies, R. L., Walker, N. A., Edwards, K. A., & Dainty, J. (2010). Is the decline of soil microbial biomass in late winter coupled to changes in the physical state of cold soils? Soil Biology and Biochemistry, 42(2), 129-135.

Johnson, Nancy C., Jim H. Graham, and Frank Andrew Smith. "Functioning of mycorrhizal associations along the mutualism–parasitism continuum." The New Phytologist 135.4 (1997): 575-585.

Jones, E. B., Suetrong, S., Sakayaroj, J., Bahkali, A. H., Abdel-Wahab, M. A., Boekhout, T., & Pang, K. L. (2015). Classification of marine *ascomycota*, *basidiomycota*, *blastocladiomycota* and *chytridiomycota*. Fungal Diversity, 73(1), 1-72.

Jonsson, L. M., Nilsson, M. C., Wardle, D. A., & Zackrisson, O. (2001). Context dependent effects of ectomycorrhizal species richness on tree seedling productivity. Oikos, 93(3), 353-364.

Jordy M. N., Azemar L. S., Brun A., Botton B., Pargney J. C. (1998) Cytolocalization of glycogen, starch, and other insoluble polysaccharides during ontogeny of *Paxillus involutus*/*Betula pendula* ectomycorrhizas. New Phytologist 140, 331-341.

Jourand, P., Giraud, E., Bena, G., Sy, A., Willems, A., Gillis, M., ... & de Lajudie, P. (2004). *Methylobacterium nodulans* sp. nov., for a group of aerobic, facultatively methylotrophic, legume root-nodule-forming and nitrogen-fixing bacteria. International Journal of Systematic and Evolutionary Microbiology, 54(6), 2269-2273.

Jumpponen, A., & Jones, K. L. (2009). Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate *Quercus macrocarpa* phyllosphere. *New Phytologist*, *184*(2), 438-448.

Junker, Robert R., and Dorothea Tholl. "Volatile organic compound mediated interactions at the plant-microbe interface." *Journal of chemical ecology* 39.7 (2013): 810-825.

Kanokratana, P., Uengwetwanit, T., Rattanachomsri, U., Bunterngsook, B., Nimchua, T., Tangphatsornruang, S., & Eurwilaichitr, L. (2011). Insights into the phylogeny and metabolic potential of a primary tropical peat swamp forest microbial community by metagenomic analysis. Microbial ecology, 61(3), 518-528.

Karst, J., Wasyliw, J., Birch, J. D., Franklin, J., Chang, S. X., & Erbilgin, N. (2021). Long-term nitrogen addition does not sustain host tree stem radial growth but doubles the abundance of high-biomass ectomycorrhizal fungi. Global Change Biology, 27(17), 4125-4138.

Kataoka, R., Taniguchi, T., Ooshima, H., & Futai, K. (2008). Comparison of the bacterial communities established on the mycorrhizae formed on *Pinus thunbergii* root tips by eight species of fungi. Plant and soil, 304(1), 267-275.

Kernaghan, G., Sigler, L., & Khasa, D. (2003). Mycorrhizal and root endophytic fungi of containerized *Picea glauca* seedlings assessed by rDNA sequence analysis. Microbial Ecology, 45(2), 128-136.

Khan, A. L., Hamayun, M., Ahmad, N., Waqas, M., Kang, S. M., Kim, Y. H., & Lee, I. J. (2011). *Exophiala* sp. LHL08 reprograms *Cucumis sativus* to higher growth under abiotic stresses. Physiologia Plantarum, 143(4), 329-343.

Kielak, A. M., Barreto, C. C., Kowalchuk, G. A., Van Veen, J. A., & Kuramae, E. E. (2016). The ecology of *Acidobacteria*: moving beyond genes and genomes. Frontiers in microbiology, 7, 744.

Kim, H. K., Cho, E. J., Lee, S., Lee, Y. S., & Yun, S. H. (2012). Functional analyses of individual mating-type transcripts at MAT loci in *Fusarium graminearum* and *Fusarium asiaticum*. FEMS microbiology letters, 337(2), 89-96.

Kim, S. J., & Kwon, K. K. (2010). *Bacteroidetes*. In Handbook of Hydrocarbon and Lipid Microbiology.

Kirchman, D. L. (2002). The ecology of *Cytophaga–Flavobacteria* in aquatic environments. FEMS microbiology ecology, 39(2), 91-100.

Klock-Moore, Kimberly A. "Comparison of *Salvia* growth in seaweed compost and biosolids compost." *Compost Science & Utilization* 8.1 (2000): 24-28.

Köksal, A. İlhami, et al. "Nutrient composition of hazelnut (*Corylus avellana* L.) varieties cultivated in Turkey." *Food Chemistry* 99.3 (2006): 509-515.

Kõljalg, U., Dahlberg, A., Taylor, A. F. S., Larsson, E., Hallenberg, N., Stenlid, J. & Jonsson, L. (2000). Diversity and abundance of resupinate thelephoroid fungi as ectomycorrhizal symbionts in Swedish boreal forests. Molecular ecology, 9(12), 1985-1996.

Kollmann, J. and H.-P. Schill. "Spatial patterns of dispersal, seed predation and germination during colonization of abandoned grassland by *Quercus petraea* and *Corylus avellana*." Vegetatio 125.2 (1996): 193-205.

Kopittke, Peter M., et al. "Soil and the intensification of agriculture for global food security." *Environment international* 132 (2019): 105078.

Korpi, Anne, Jill Järnberg, and Anna-Liisa Pasanen. "Microbial volatile organic compounds." Critical reviews in toxicology 39.2 (2009): 139-193.

Kramer, Rolf, and Wolf-Rainer Abraham. "Volatile sesquiterpenes from fungi: what are they good for?." *Phytochemistry Reviews* 11.1 (2012): 15-37.

Kronstad, J. W. "Self-fertility: the genetics of sex in lonely fungi." Current Biology 17.19 (2007): R843-R845.

Kropp, Bradley R., and Charles-Gilles Langlois. "Ectomycorrhizae in reforestation." Canadian Journal of forest research 20.4 (1990): 438-451.

Kruasuwan, W., & Thamchaipenet, A. (2016). Diversity of culturable plant growth-promoting bacterial endophytes associated with sugarcane roots and their effect of growth by co-inoculation of diazotrophs and actinomycetes. Journal of Plant Growth Regulation, 35(4), 1074-1087.

Krüssmann G., Handbuch der Laubgehölze, vol. 3 (Paul Parey, Berlin, 1978), second edn.

Kubát, J., B. Novák, and M. Ružička. "Modelling the growth of bacteria during glucose decomposition in soil under different temperatures." Plant and Soil 55.1 (1980): 77-84.

Kubiak-Martens, Lucyna. "The plant food component of the diet at the late Mesolithic (Ertebolle) settlement at Tybrind Vig, Denmark." *Vegetation history and archaeobotany* 8.1 (1999): 117-127.

Kull, Olevi, and Ülo Niinemets. "Variations in leaf morphometry and nitrogen concentration in *Betula pendula* Roth., *Corylus avellana* L. and *Lonicera xylosteum* L." *Tree physiology* 12.3 (1993): 311-318.

Kumar S., Tamura K., Jakobsen I. B., Nei M. (2001) MEGA2: molecular evolutionary genetics analysis software Bioinformatics, Volume 17, Issue 12, Pages 1244–1245.

Labbé, Jessy L., et al. "Newly identified helper bacteria stimulate ectomycorrhizal formation in Populus." Frontiers in Plant Science 5 (2014): 579.

Lalli, G., Leonardi, M., Oddis, M., Pacioni, G., Salerni, E., Iotti, M., & Zambonelli, A. (2015). Expanding the understanding of a forest ectomycorrhizal community by combining root tips and fruiting bodies: a case study of Tuber magnatum stands. Turkish Journal of Botany, 39(3), 527-534.

Læssøe, Thomas, and Karen Hansen. Truffle trouble: what happened to the Tuberales? Mycological research 111.9 (2007): 1075-1099.

Landeweert, R., Hoffland, E., Finlay, R. D., Kuyper, T. W., & van Breemen, N. (2001). Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. Trends in ecology & evolution, 16(5), 248-254.

Langenhoven, S. D., Halleen, F., Spies, C. F., Stempien, E., & Mostert, L. (2018). Detection and quantification of black foot and crown and root rot pathogens in grapevine nursery soils in the Western Cape of South Africa. Phytopathologia Mediterranea, 57(3), 519-537.

Latiffah, Z., & Azaman, R. S. (2011). Fusarium species isolated from forest soil samples. *Malays J Microbiol*, *7*(3), 171-174.

Lau, J. A., Lennon, J. T., & Heath, K. D. (2017). Trees harness the power of microbes to survive climate change. *Proceedings of the National Academy of Sciences*, *114*(42), 11009-11011.

Le Tacon, François, et al. "Certainties and uncertainties about the life cycle of the Périgord black truffle (*Tuber melanosporum* Vittad.)." Annals of Forest Science 73.1 (2016): 105-117.

Le Tacon, François, et al. "Climatic variations explain annual fluctuations in French Périgord black truffle wholesale markets but do not explain the decrease in black truffle production over the last 48 years." Mycorrhiza 24.1 (2014): 115-125.

Le Tacon, François, et al. "Efficiency in a forest nursery of an inoculum of an ectomycorrhizal fungus produced in a fermentor and entrapped in polymetric gels." (1983).

Leonardi, M., Iotti, M., Oddis, M., Lalli, G., Pacioni, G., Leonardi, P., & Zambonelli, A. (2013). Assessment of ectomycorrhizal fungal communities in the natural habitats of *Tuber magnatum* (*Ascomycota*, *Pezizales*). Mycorrhiza, 23(5), 349-358.

Leonardi, M., Salvi, D., Iotti, M., Rana, G. L., Paz-Conde, A., & Pacioni, G. (2021). Multilocus Phylogeography of the *Tuber mesentericum* Complex Unearths Three Highly Divergent Cryptic Species. Journal of Fungi, 7(12), 1090.

Leonardi, P., Murat, C., Puliga, F., Iotti, M., & Zambonelli, A. (2020). Ascoma genotyping and mating type analyses of mycorrhizas and soil mycelia of *Tuber borchii* in a truffle orchard established by mycelial inoculated plants. Environmental microbiology, 22(3), 964-975.

Leslie, John F., and Keith K. Klein. "Female fertility and mating type effects on effective population size and evolution in filamentous fungi." Genetics 144.2 (1996): 557-567.

Lewis D.H., Harley J.L. (1965) Carbohydrate physiology of mycorrhizal roots of beech. Identity of endogenous sugars and utilization of exogenous sugars. New Phytologist 64, 224-237.

Li, C. Y., & Castellano, M. A. (1987). *Azospirillum* isolated from within sporocarps of the mycorrhizal fungi Hebeloma crustuliniforme, *Laccaria laccata* and *Rhizopogon vinicolor*. Transactions of the British Mycological Society, 88(4), 563-565.

Lian, C., Narimatsu, M., Nara, K., & Hogetsu, T. (2006). *Tricholoma matsutake* in a natural *Pinus densiflora* forest: correspondence between above-and below-ground genets, association with multiple host trees and alteration of existing ectomycorrhizal communities. New Phytologist, 171(4), 825-836.

Liese, R., Alings, K., & Meier, I. C. (2017). Root branching is a leading root trait of the plant economics spectrum in temperate trees. Frontiers in Plant Science, 8, 315.

Lilleskov, E. A., Fahey, T. J., Horton, T. R., & Lovett, G. M. (2002). Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. Ecology, 83(1), 104-115.

Lim, J. H., Baek, S. H., & Lee, S. T. (2009). *Ferruginibacter alkalilentus* gen. nov., sp. nov. and *Ferruginibacter lapsinanis* sp. nov., novel members of the family '*Chitinophagaceae*'in the phylum *Bacteroidetes*, isolated from freshwater sediment. International journal of systematic and evolutionary microbiology, 59(10), 2394-2399.

Lindahl, Björn, et al. "Translocation of 32P between interacting mycelia of a wood-decomposing fungus and ectomycorrhizal fungi in microcosm systems." The New Phytologist 144.1 (1999): 183-193.

Liu, B., Fischer, C. R., Bonet, J. A., Castaño, C., & Colinas, C. Short communication Shifts in soil fungal communities in *Tuber melanosporum* plantations over a 20-year transition from agriculture fields to oak woodlands.

Liu, D., Chater, C. C., Yu, F., & Perez-Moreno, J. (2021). *Tuber pseudohimalayense* ascomata-compartments strongly select their associated bacterial microbiome from nearby pine forest soils independently of their maturation stage. Pedobiologia, 87, 150743.

LoBuglio, K. F., Berbee, M. L., & Taylor, J. W. (1996). Phylogenetic Origins of the Asexual Mycorrhizal Symbiont *Cenococcum geophilum* Fr. and Other Mycorrhizal Fungi among the *Ascomycetes*. Molecular Phylogenetics and Evolution, 6(2), 287-294.

Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E. R., Taghavi, S., Mezgeay, M., & der Lelie, D. V. (2002). Endophytic bacteria and their potential applications. Critical reviews in plant sciences, 21(6), 583-606.

López González, Ginés. *Árboles y arbustos de la Península Ibérica e Islas Baleares*. Ediciones Mundi-Prensa, 2001.

Loqué D., Ludewig U., Yuan L., Wirén N. von (2005) Tonoplast Intrinsic Proteins AtTIP2;1 and AtTIP2;3 Facilitate NH3 Transport into the Vacuole Plant Physiology, 137 (2) 671-680.

Loudon AH, Holland JA, Umile TP, Burzynski EA, Minbiole KPC, Harris RN. Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. Front Microbiol. 2014; 5:441.

Lozupone, Catherine A., et al. "Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities." Applied and environmental microbiology 73.5 (2007): 1576-1585.

Lozupone, Catherine, and Rob Knight. "UniFrac: a new phylogenetic method for comparing microbial communities." Applied and environmental microbiology 71.12 (2005): 8228-8235.

Lulli L, Bragato G, Panini T, Gardin L, Primavera F (1992) I suoli delle tartufaie naturali della bassa valle del Santerno (Mugello – Toscana). Ital Forest Montana 47(5):251–267.

Lulli L, Panini T, Bragato G, Gardin L, Primavera F (1991) I suoli delle tartufaie naturali delle Crete Senesi. Monti Boschi 42:31–39.

Lulli, L., & Primavera, F. (1995). I suoli idonei alla produzione di tartufi. Informatore Agrario, 51, 33-33.

Lunt, P. H., & Hedger, J. N. (2003). Effects of organic enrichment of mine spoil on growth and nutrient uptake in oak seedlings inoculated with selected ectomycorrhizal fungi. Restoration Ecology, 11(2), 125-130.

Luo, Z. B., Li, K., Jiang, X., & Polle, A. (2009). Ectomycorrhizal fungus (*Paxillus involutus*) and hydrogels affect performance of *Populus euphratica* exposed to drought stress. Annals of Forest Science, 66(1), 1.

Madhaiyan, M., Poonguzhali, S., Senthilkumar, M., Pragatheswari, D., Lee, J. S., & Lee, K. C. (2015). *Arachidicoccus rhizosphaerae* gen. nov., sp. nov., a plant-growth-promoting bacterium in the family *Chitinophagaceae* isolated from rhizosphere soil. International journal of systematic and evolutionary microbiology, 65(Pt_2), 578-586.

Madrid, H., Hernandez-Restrepo, M., Gené, J., Cano, J., Guarro, J., & Silva, V. (2016). New and interesting chaetothyrialean fungi from Spain. Mycological progress, 15(10), 1179-1201.

Magaña-Dueñas, V., Stchigel, A. M., & Cano-Lira, J. F. (2021). New *Coelomycetous* fungi from freshwater in Spain. Journal of Fungi, 7(5), 368.

Maharachchikumbura, S. S., Hyde, K. D., Jones, E. B., McKenzie, E. H., Huang, S. K., Abdel-Wahab, M. A., & Xu, J. (2015). Towards a natural classification and backbone tree for *Sordariomycetes*. Fungal diversity, 72(1), 199-301.

Maharachchikumbura, S. S., Hyde, K. D., Jones, E. B., McKenzie, E. H. C., Bhat, J. D., Dayarathne, M. C.,& Wijayawardene, N. N. (2016). Families of *Sordariomycetes*. Fungal Diversity, 79(1), 1-317.

Maharachchikumbura, S.S.N., Hyde, K.D., Groenewald, J.Z., Xu, J. & Crous, P.W. (2014) *Pestalotiopsis* revisited. *Studies in Mycology* 79: 121–186.

Mamoun, M., and J. M. Olivier. "Effect of soil *Pseudomonads* on colonization of hazel roots by the ecto-mycorrhizal species *Tuber melanosporum* and its competitors." *Plant and Soil* 139.2 (1992): 265-273.

Mamoun, M., Oliver, J. Mycorrhizal inoculation of cloned hazels by *Tuber melanosporum*: effect of soil disinfestation and co-culture with Festuca ovina. Plant and Soil 188, 221–226 (1997).

Manici, L. M., Caboni, E., Caputo, F., Frattarelli, A., & Lucioli, S. (2021). Phytotoxins from *Dactylonectria torresensis* involved in replant disease of fruit trees. Rhizosphere, 17, 100300.

Marín, P., Jurado, M., & Gonzalez-Jaen, M. T. (2015). Growth rate and TRI5 gene expression profiles of *Fusarium equiseti* strains isolated from Spanish cereals cultivated on wheat and barley media at different environmental conditions. International Journal of Food Microbiology, 195, 40-47.

Marozzi, G., Benucci, G. M. N., Suriano, E., Sitta, N., Raggi, L., Lancioni, H., ... & Donnini, D. (2020). *Tuber mesentericum* and *Tuber aestivum* truffles: new insights based on morphological and phylogenetic analyses. Diversity, 12(9), 349.

Martin F., Nehls U. (2009) Harnessing ectomycorrhizal genomics for ecological insights. Curr. Opin. Plant Biol. 12, 508-515.

Martin, F., Kohler, A., Murat, C., Veneault-Fourrey, C., & Hibbett, D. S. (2016). Unearthing the roots of ectomycorrhizal symbioses. Nature Reviews Microbiology, 14(12), 760-773.

Martin, Francis, et al. "Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis." Nature 464.7291 (2010): 1033-1038.

Martin, Francis, et al. "The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis." Nature 452.7183 (2008): 88-92.

Martín-Pinto, P., Pajares, J., & Díez, J. (2006). *In vitro* effects of four ectomycorrhizal fungi, *Boletus edulis, Rhizopogon roseolus, Laccaria laccata and Lactarius deliciosus* on *Fusarium damping* off in *Pinus nigra* seedlings. *New Forests*, *32*(3), 323-334.

Marulanda, Adriana, José-Miguel Barea, and Rosario Azcón. "Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments: mechanisms related to bacterial effectiveness." Journal of Plant Growth Regulation 28.2 (2009): 115-124.

Marx, D. H., J. L. Ruehle, and C. E. Cordell. "17 Methods for Studying Nursery and Field Response of Trees to Specific Ectomycorrhiza." Methods in microbiology. Vol. 23. Academic Press, 1991. 383-411.

Maser, Chris, James M. Trappe, and Ronald A. Nussbaum. "Fungal-small mammal interrelationships with emphasis on Oregon coniferous forests." Ecology 59.4 (1978): 799-809.

Mavrodi DV, Peever TL, Mavrodi OV, Parejko JA, Raaijmakers JM, Lemanceau P, Mazurier S, Heide L, Blankenfeldt W, Weller DM, Thomashow LS (2010) Diversity and Evolution of the Phenazine Biosynthesis Pathway Appl Environ Microbiol [Epub ahead of print]

McLaughlin, David J., et al. "The search for the fungal tree of life." *Trends in microbiology* 17.11 (2009): 488-497.

Mello, A., & Balestrini, R. (2018). Recent insights on biological and ecological aspects of ectomycorrhizal fungi and their interactions. Frontiers in Microbiology, 9, 216.

Mello, A., et al. "Bacterial and fungal communities associated with *Tuber magnatum*-productive niches." *Plant Biosystems* 144.2 (2010): 323-332.

Mello, Antonietta, et al. "Genetic variability of *Tuber uncinatum* and its relatedness to other black truffles." *Environmental Microbiology* 4.10 (2002): 584-594.

Mello, Antonietta, et al. "Truffle brûlés have an impact on the diversity of soil bacterial communities." *PLoS One* 8.4 (2013): e61945.

Mesén, F., Adrian C. Newton, and Robert RB Leakey. "Vegetative propagation of *Cordia alliodora* (Ruiz & Pavon) Oken: the effects of IBA concentration, propagation medium and cutting origin." Forest Ecology and management 92.1-3 (1997): 45-54.

Metzenberg, Robert L., and N. Louise Glass. "Mating type and mating strategies in Neurospora." Bioessays 12.2 (1990): 53-59.

Micheli, Pier Antonio. *Nova plantarum genera*. 1729.

Miles, P. G. & Chang, S. T. Mushrooms: cultivation, nutritional value, medicinal effect, and environmental impact. CRC press, New York 1–431 (2004).

Milton, M., Bisarya, D., Kumar, V., Kumar, S., & Singh, A. K. (2021). Mycorrhizae and their importance in agriculture.

Mogge B, Loferer C, Agerer R et al (2000) Bacterial community structure and colonization patterns of *Fagus sylvatica* L. ectomycorrhizospheres as determined by fluorescence in situ hybridization and confocal laser scanning microscopy. Mycorrhiza 9:271–278.

Molinier, Virginie, et al. "The Burgundy truffle (*Tuber aestivum* syn. uncinatum): A truffle species with a wide habitat range over Europe." True Truffle (*Tuber* spp.) in the World. Springer, Cham, 2016. 33-47.

Monaco, P., Toumi, M., Sferra, G., Tóth, E., Naclerio, G., & Bucci, A. (2020). The bacterial communities of *Tuber aestivum*: preliminary investigations in Molise region, Southern Italy. Annals of Microbiology, 70(1), 1-10.

Morris, M. H., Perez-Perez, M. A., Smith, M. E., & Bledsoe, C. S. (2009). Influence of host species on ectomycorrhizal communities associated with two co-occurring oaks (*Quercus* spp.) in a tropical cloud forest. FEMS microbiology ecology, 69(2), 274-287.

Müller, Anna, et al. "Volatile profiles of fungi–chemotyping of species and ecological functions." *Fungal Genetics and Biology* 54 (2013): 25-33.

Murat C., Vizzini A., Bonfante P. and Mello A. (2005) Morphological and molecular typing of the below-ground fungal community in a natural *Tuber magnatum* truffle-ground. FEMS Microbiol Lett 245: 307–313.

Murat, Claude, et al. "Fine-scale spatial genetic structure of the black truffle (*Tuber melanosporum*) investigated with neutral microsatellites and functional mating type genes." New Phytologist 199.1 (2013): 176-187.

Murat, Claude, et al. "*Pezizomycetes* genomes reveal the molecular basis of ectomycorrhizal truffle lifestyle." Nature ecology & evolution 2.12 (2018): 1956-1965.

Murat, Claude, et al. "Polymorphism at the ribosomal DNA ITS and its relation to postglacial re-colonization routes of the Périgord truffle *Tuber melanosporum*." New phytologist 164.2 (2004): 401-411.

Muriuki, R and Kimurto, P K and Towett, B K and Vadez, V and Gangarao, R (2020) Study of root traits of chickpea (*Cicer arietinum* L.) under drought stress. African Journal of Plant Science, 14 (11). pp. 420-435. ISSN 1996-0824.

Murtagh, G. J., P. S. Dyer, and P. D. Crittenden. "Sex and the single lichen." Nature 404.6778 (2000): 564-564.

Murray, P. R., Rosenthal, K. S., & Pfaller, M. A. (2020). Medical microbiology E-book. Elsevier Health Sciences.

Muthukumar, Thangavelu, and Karuthamuthu Udaiyan. "Growth response and nutrient utilization of *Casuarina equisetifolia* seedlings inoculated with bioinoculants under tropical nursery conditions." New Forests 40.1 (2010): 101-118.

Nahm, M., Tegel, W., & Gold, C. (2019). A search for naturally grown Burgundy truffles (*Tuber aestivum*) in hazelnut plantations in Germany: Results of a survey. Agriculture and Natural Resources, 53(1), 84-86.

Napoli, C., Mello, A., Borra, A., Vizzini, A., Sourzat, P., & Bonfante, P. (2010). *Tuber melanosporum*, when dominant, affects fungal dynamics in truffle grounds. New Phytologist, 237-247.

Näsholm T., Högberg P., Franklin O., Metcalfe D., Keel S.G., Campbell C., Hurry V., Linder S., M.N. Högberg (2013) Are ectomycorrhizal fungi alleviating or aggravating nitrogen limitation of tree growth in boreal forests? New Phytologist (2013) 198: 214–221.

Natel, P., & Neumann, P. (1992). Ecology of ectomycorrhizal-basidiomycete communities on a local vegetation gradient. Ecology, 73(1), 99-117.

Navarro-Ródenas A, Berná LM, Lozano-Carrillo C, Andrino A, Morte A. Beneficial native bacteria improve survival and mycorrhization of desert truffle mycorrhizal plants in nursery conditions. Mycorrhiza. 2016 Oct; 26(7):769-79.

Nehls U. (2004) Carbohydrates and nitrogen: nutrients and signals in ectomycorrhizas. In: Varma A, Abbott L, Werner D, Hampp R, eds. Plant surface microbiology. Berlin: Springer Verlag, 373-392.

Nehls U. (2008) Mastering ectomycorrhizal symbiosis: the impact of carbohydrates, Journal of Experimental Botany, Vol. 59, No. 5, pp. 1097-1108.

Nilsson, R. Henrik, et al. "Mycobiome diversity: high-throughput sequencing and identification of fungi." *Nature Reviews Microbiology* 17.2 (2019): 95-109.

Nunan, N., Daniell, T. J., Singh, B. K., Papert, A., McNicol, J. W., & Prosser, J. I. (2005). Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. Applied and environmental microbiology, 71(11), 6784-6792.

Núñez, J. A. D., Serrano, J. S., Barreal, J. A. R., & de Omeñaca González, J. A. S. (2006). The influence of mycorrhization with *Tuber melanosporum* in the afforestation of a Mediterranean site with *Quercus ilex* and *Quercus faginea*. *Forest ecology and management*, *231*(1-3), 226-233.

Nurkanto, A., Lisdiyanti, P., Hamada, M., Ratnakomala, S., Shibata, C., & Tamura, T. (2015). *Cryptosporangium cibodasense* sp. nov., isolated from leaf litter in Indonesia. International Journal of Systematic and Evolutionary Microbiology, 65(Pt_12), 4632-4637.

O'Donnell, Kerry, et al. "Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis." Mycologia 89.1 (1997): 48-65.

Okazaki, K., Tsurumaru, H., Hashimoto, M., Takahashi, H., Okubo, T., Ohwada, T., ... & Ikeda, S. (2021). Community Analysis-based Screening of Plant Growth-promoting Bacteria for Sugar Beet. Microbes and environments, 36(2), ME20137.

Oliach, D., et al. "Guia tecnica para el cultivo de trufa negra (*Tuber melanosporum* Vittad.)." Centre Tecnologic Forestal de Catalunya (2005).

Oliach, Daniel, et al. "Truffle Market Evolution: An Application of the Delphi Method." Forests 12.9 (2021): 1174.

Oliferchuk, V. P., & Fedorovych, D. V. (2021). Influence of *Tuber melanosporum* mycorrhystic mushrooms on the biodiversity rhisosphere micromycetes and growth and productivity of hazelnut. Scientific Bulletin of UNFU, 31(2), 28-34.

Oliveira, H., Rego, M. C., & Nascimento, T. (2003). Decline of young grapevines caused by fungi. In I International Symposium on Grapevine Growing, Commerce and Research 652 (pp. 295-304).

Oliveira, R. S., Franco, A. R., Vosátka, M., & Castro, P. M. (2010). Management of nursery practices for efficient ectomycorrhizal fungi application in the production of *Quercus ilex*. Symbiosis, 52(2), 125-131.

Olivier, J. M., Savignac, J. C., & Sourzat, P. (2012). Truffe et trufficulture FANLAC Editions. Périgueux, France.

Ori, F., Menotta, M., Leonardi, M., Amicucci, A., Zambonelli, A., Coves, H., ... & Iotti, M. (2021). Fungal Biology.

Ortiz-Castro, R., Contreras-Cornejo, H., Macias-Rodriguez, L., Lopez-Bucio, J. (2009) The role of microbial signals in plant growth and development. Plant Signaling Behav. 4, 701-712.

Ouahmane, Lahcen, et al. "Responses of *Pinus halepensis* growth, soil microbial catabolic functions and phosphate-solubilizing bacteria after rock phosphate amendment and ectomycorrhizal inoculation." Plant and soil 320.1 (2009): 169-179.

Pacioni, G. (1991). Effects of *Tuber* metabolites on the rhizospheric environment. Mycological research, 95(12), 1355-1358.

Palaniappan, P., Chauhan, P. S., Saravanan, V. S., Anandham, R., & Sa, T. (2010). Isolation and characterization of plant growth promoting endophytic bacterial isolates from root nodule of Lespedeza sp. Biology and fertility of soils, 46(8), 807-816.

Palmé, A. E., and G. G. Vendramin. "Chloroplast DNA variation, postglacial recolonization and hybridization in hazel, *Corylus avellana*." *Molecular ecology* 11.9 (2002): 1769-1779.

Paoletti, Mathieu, et al. "Mating type and the genetic basis of self-fertility in the model fungus *Aspergillus nidulans*." Current Biology 17.16 (2007): 1384-1389.

Paolocci F, Rubini A, Riccioni C, Arcioni S (2006) Reevaluation of the life cycle of *Tuber magnatum*. Appl Environ Microb 72:2390–2393.

Paolocci, Francesco, et al. "*Tuber aestivum* and *Tuber uncinatum*: two morphotypes or two species?. " *FEMS Microbiology Letters* 235.1 (2004): 109-115.

Parguey-Leduc, A., C. Montant, and M. Kulifaj. "Structure et évolution de l'ascocarpe du *Tuber melanosporum* Vittad.(truffe noire du Périgord)." Comptes rendus des séances de l'Académie des sciences. Série 3, Sciences de la vie 299.8 (1984): 315-320.

Parladé, J., Pera, J., & Luque, J. (2004). Evaluation of mycelial inocula of edible *Lactarius* species for the production of *Pinus pinaster* and *P. sylvestris* mycorrhizal seedlings under greenhouse conditions. Mycorrhiza, 14(3), 171-175.

Parladé, J., De la Varga, H., Pera, J. (2016). Tools to Trace Truffles in Soil. In: Zambonelli, A., Iotti, M., Murat, C. (eds) True Truffle (Tuber spp.) in the World. Soil Biology, vol 47. Springer, Cham.

Pavić, A., Stanković, S., Saljnikov, E., Krüger, D., Buscot, F., Tarkka, M., & Marjanović, Ž. (2013). *Actinobacteria* may influence white truffle (*Tuber magnatum* Pico) nutrition, ascocarp degradation and interactions with other soil fungi. fungal ecology, 6(6), 527-538.

Pavić, A., Stanković, S., Saljnikov, E., Krüger, D., Buscot, F., Tarkka, M., & Marjanović, Ž. (2013). Actinobacteria may influence white truffle (*Tuber magnatum* Pico) nutrition, ascocarp degradation and interactions with other soil fungi. fungal ecology, 6(6), 527-538.

Pegler DN, Spooner BM, Young TWK (1993) British truffles. Whitstable Litho

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., & Ley, R. E. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proceedings of the National Academy of Sciences, 110(16), 6548-6553.

Pent, M., Põldmaa, K., & Bahram, M. (2017). Bacterial communities in boreal forest mushrooms are shaped both by soil parameters and host identity. Frontiers in microbiology, 8, 836.

Pereira, G., Palfner, G., Chávez, D., Suz, L. M., Machuca, Á., & Honrubia, M. (2013). Using common mycorrhizal networks for controlled inoculation of *Quercus* spp. with *Tuber melanosporum*: the nurse plant method. Mycorrhiza, 23(5), 373-380.

Pérez, Francisco, et al. "Synthesis and establishment of *Tuber melanosporum* Vittad. ectomycorrhizae on two Nothofagus species in Chile." Mycorrhiza 17.7 (2007): 627-632.

Perez, J. J., et al. "A novel, green, low-cost chitosan-starch hydrogel as potential delivery system for plant growth-promoting bacteria." Carbohydrate polymers 202 (2018): 409.

Pérez-Moreno, J., Guerin-Laguette, A., Rinaldi, A. C., Yu, F., Verbeken, A., Hernández-Santiago, F., & Martínez-Reyes, M. (2021). Edible mycorrhizal fungi of the world: What is their role in forest sustainability, food security, biocultural conservation and climate change? Plants, People, Planet, 3(5), 471-490.

Persoon, Christian Hendrik. Synopsis methodica fungorum. Sistens enumerationem omnium huc vsque detectarum specierum, cum breuibus descriptionibus nec non synonymis et obseruationibus selectis. Auctore DCH Persoon... Pars prima [-secunda]. Cum tabulis aeneis. Apud Henricum Dieterich, 1801.

Petersen, Ronald H., and Karen W. Hughes. "Species and speciation." BioScience 49.6 (1985).

Pierson, L. S., & Pierson, E. A. (2010). Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Applied microbiology and biotechnology*, *86*(6), 1659-1670.

Pini, F., Galardini, M., Bazzicalupo, M., & Mengoni, A. (2011). Plant-bacteria association and symbiosis: are there common genomic traits in *Alphaproteobacteria*? Genes, 2(4), 1017-1032.

Pinnoi, A., Lumyong, S., Hyde, K. D., & Jones, E. G. (2006). Biodiversity of fungi on the palm *Eleiodoxa conferta* in Sirindhorn peat swamp forest, Narathiwat, Thailand. *Fungal Diversity*.

Pinruan, U., Hyde, K. D., Lumyong, S., McKenzie, E. H. C., & Jones, E. G. (2007). Occurrence of fungi on tissues of the peat swamp palm Licuala longicalycata. *Fungal Diversity*, *25*(1), 157-173.

Piñuela, Yasmine, et al. "Use of inoculator bacteria to promote *Tuber melanosporum* root colonization and growth on *Quercus faginea* saplings." Forests 11.8 (2020): 792.

Poitou, Christian. "René Baehrel: Une croissance: la Basse-Provence rurale de la fin du 16e siècle à 1789, 1988." Dix-Huitième Siècle 22.1 (1990): 494-494.

Poole, E. J., G. D. Bending, J. M. Whipps, and D. J. Read. 2001. Bacteria associated with *Pinus sylvestris-Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation in vitro. New Phytol. 151:553–766.

Porca, E., Jurado, V., Žgur-Bertok, D., Saiz-Jimenez, C., & Pašić, L. (2012). Comparative analysis of yellow microbial communities growing on the walls of geographically distinct caves indicates a common core of microorganisms involved in their formation. *FEMS Microbiology Ecology*, *81*(1), 255-266.

Pratibha, J., Prabhugaonkar, A., Hyde, K. D., & Bhat, D. J. (2014). Phylogenetic placement of Bahusandhika, *Cancellidium* and *Pseudoepicoccum* (asexual *Ascomycota*). Phytotaxa, 176(1), 68-80.

Prescott, Cindy E., et al. "Surplus carbon drives allocation and plant–soil interactions." Trends in Ecology & Evolution 35.12 (2020): 1110-1118.

Pruett, G. E. (2008). The biology and ecology of *Tuber aestivum* mycorrhizae establishment in the greenhouse and the field. University of Missouri-Columbia.

Qin, Jiao, and Bang Feng. "Life Cycle and Phylogeography of True Truffles." Genes 13.1 (2022): 145.

Qu, J. H., Yuan, H. L., Yang, J. S., Li, H. F., & Chen, N. (2009). *Lacibacter cauensis* gen. nov., sp. nov., a novel member of the phylum *Bacteroidetes* isolated from sediment of a eutrophic lake. International Journal of Systematic and Evolutionary Microbiology, 59(5), 1153-1157.

Qu, X., Wanner, L. A., & Christ, B. J. (2008). Using the TxtAB operon to quantify pathogenic Streptomyces in potato tubers and soil. Phytopathology, 98(4), 405-412.

Quandt, C. A., Kohler, A., Hesse, C. N., Sharpton, T. J., Martin, F., & Spatafora, J. W. (2015). Metagenome sequence of *Elaphomyces granulatus* from sporocarp tissue reveals *Ascomycota* ectomycorrhizal fingerprints of genome expansion and a *Proteobacteria*-rich microbiome. Environmental Microbiology, 17(8), 2952-2968.

Querejeta, J. I., Roldán, A., Albaladejo, J., & Castillo, V. (1998). The role of mycorrhizae, site preparation, and organic amendment in the afforestation of a semi-arid Mediterranean site with *Pinus halepensis*. Forest Science, 44(2), 203-211.

Raglione, M., et al. "Osservazioni sulle caratteristiche pedologiche di alcuni siti di Tartufo nero pregiato (*Tuber melanosporum*) in provincia di Rieti." Micologia e Vegetazione Mediterranea 7.1 (1992): 211-224.

Ramírez, R., S. Reyna, R. Santelices, G. Palfner, S. García, y R. Henríquez. 2004. Cultivo de trufa negra (*Tuber melanosporum* Vittad.) en Chile. Una alternativa productiva sustentable bajo un sistema de manejo agro silvícola. Segundo Congreso Forestal Chileno, Valdivia. Universidad Austral de Chile, Valdivia, Chile.

Rathgeber, Cyrille, et al. "Simulated responses of *Pinus halepensis* forest productivity to climatic change and CO_2 increase using a statistical model." *Global and Planetary Change* 26.4 (2000): 405-421.

Read, D. J., and J. Perez-Moreno. "Mycorrhizas and nutrient cycling in ecosystems–a journey towards relevance? " New phytologist 157.3 (2003): 475-492.

Reina, S. (2000). Handling and recovery of natural truffle plantations. In 1. Jornadas Internacionales sobre Truficultura en Aragon. Graus, Huesca (Espana). 21-22 Oct 1999.

Repáč, I. (2011). Ectomycorrhizal inoculum and inoculation techniques. In Diversity and biotechnology of ectomycorrhizae (pp. 43-63). Springer, Berlin, Heidelberg.

Reyna S (2007) Trufa, truficultura y selvicultura trufera. Ed. Mundi-Prensa, Madrid.

Reyna, Santiago, and Sergi Garcia-Barreda. "Black truffle cultivation: a global reality." Forest Systems 23.2 (2014): 317-328.

Ricard JM (2003) 'La truffe. Guide technique de trufficulture.' (Centre Technique Interprofessionnel des Fruits et L´egumes CTIFL: Paris).

Riccioni C, Belfiori B, Rubini A, Passeri V, Arcioni S, Paolocci F (2008) *Tuber melanosporum* outcrosses: analysis of the genetic diversity within and among its natural populations under this new scenario. New Phytol 180:466–478.

Rieger A., Guttenberg M., Hampp R. (1992) Soluble carbohydrates in mycorrhized and non-mycorrhized fine roots of spruce seedlings. Zeitschrift für Naturforschung 47, 201-204.

Rincón, A., De Felipe, M. R., & Fernández-Pascual, M. (2007). Inoculation of *Pinus halepensis* Mill. with selected ectomycorrhizal fungi improves seedling establishment 2 years after planting in a degraded gypsum soil. Mycorrhiza, 18(1), 23-32.

Rincón, Ana, Javier Parladé, and Joan Pera. "Effects of ectomycorrhizal inoculation and the type of substrate on mycorrhization, growth and nutrition of containerised *Pinus pinea* L. seedlings produced in a commercial nursery." Annals of Forest Science 62.8 (2005): 817-822.

Rinta-Kanto, J. M., Pehkonen, K., Sinkko, H., Tamminen, M. V., & Timonen, S. (2018). Archaea are prominent members of the prokaryotic communities colonizing common forest mushrooms. Canadian journal of microbiology, 64(10), 716-726.

Riousset, G., et al. "Truffles d'Europe et de Chine INRA." (2001).

Risso, C., Sun, J., Zhuang, K., Mahadevan, R., DeBoy, R., Ismail, W., ... & Methé, B. A. (2009). Genome-scale comparison and constraint-based metabolic reconstruction of the facultative anaerobic Fe (III)-reducer *Rhodoferax ferrireducens*. BMC genomics, 10(1), 1-19.

Rivas-Martínez, Salvador, and C. Sáenz Lain. "Enumeración de los *Quercus* de la Península Ibérica." *Rivasgodaya* 6 (1991): 101-110.

Rivera, C.S.; Blanco, D.; Oria, R.; Venturini, M.E. Diversity of culturable microorganisms and occurrence of *Listeria monocytogenes* and *Salmonella* spp. in *Tuber aestivum* and *Tuber melanosporum* ascocarps. Food Microbiol. 2010, 27, 286–293.

Rivera, Carmen Susana, et al. "Diversity of culturable microorganisms and occurrence of Listeria monocytogenes and Salmonella spp. in *Tuber aestivum* and *Tuber melanosporum* ascocarps." *Food microbiology* 27.2 (2010): 286-293.

Roberts D. M., Tyerman S. D. (2002) Voltage-Dependent Cation Channels Permeable to NH4 +, K+, and Ca^{2+} in the Symbiosome Membrane of the Model Legume Lotus japonicus. Plant Physiology, 128 (2) 370-378.

Rodriguez, R. J., et al. "Fungal endophytes: diversity and functional roles." *New phytologist* 182.2 (2009): 314-330.

Roldán, A., & Albaladejo, J. (1994). Effect of mycorrhizal inoculation and soil restoration on the growth of *Pinus halepensis* seedlings in a semiarid soil. Biology and Fertility of soils, 18(2), 143-149.

Román, R., Fortun, C., De Sá, M. G. L., & Almendros, G. (2003). Successful soil remediation and reforestation of a calcic regosol amended with composted urban waste. Arid Land Research and Management, 17(3), 297-311.

Rubini, Andrea, et al. "Genetic and phylogeographic structures of the symbiotic fungus *Tuber magnatum*." Applied and Environmental Microbiology 71.11 (2005): 6584-6589.

Rubini, Andrea, et al. "Impact of the competition between mating types on the cultivation of *Tuber melanosporum*: Romeo and Juliet and the matter of space and time." Mycorrhiza 24.1 (2014): 19-27.

Rubini, Andrea, et al. "Isolation and characterization of MAT genes in the symbiotic ascomycete *Tuber melanosporum*." New phytologist 189.3 (2011a): 710-722.

Rubini, Andrea, et al. "Troubles with truffles: unveiling more of their biology." The New Phytologist 174.2 (2007): 256-259.

Rubini, Andrea, et al. "*Tuber melanosporum*: mating type distribution in a natural plantation and dynamics of strains of different mating types on the roots of nursery-inoculated host plants." New Phytologist 189.3 (2011b): 723-735.

Sáez, R., and A. De Miguel. "Guía práctica de truficultura." ITG Agrícola SA y Universidad de Navarra, Pamplona, España (1995).

Sait, M., Davis, K. E., & Janssen, P. H. (2006). Effect of pH on isolation and distribution of members of subdivision 1 of the phylum *Acidobacteria* occurring in soil. Applied and environmental microbiology, 72(3), 1852-1857.

Salerni, E. (2010). Effects of thinning. Selective thinning, 67, 509p1-509p9.

Salerni, E., Iotti, M., Leonardi, P., Gardin, L., D'aguanno, M., Perini, C., ... & Zambonelli, A. (2014). Effects of soil tillage on *Tuber magnatum* development in natural truffières. Mycorrhiza, 24(1), 79-87.

Salvini, D., et al. "Natural hybridisation between *Quercus petraea* (Matt.) Liebl. and *Quercus pubescens* Willd. within an Italian stand as revealed by microsatellite fingerprinting." *Plant Biology* 11.5 (2009): 758-765.

Salzer P., Hager A. (1991) Sucrose utilization of the ectomycorrhizal fungi *Amanita muscaria* and *Hebeloma crustuliniforme* depends on the cell wall-bound invertase activity of their host *Picea abies*. Botanica Acta 104, 439-445.

Samils, Nicklas, et al. "The socioeconomic impact of truffle cultivation in rural Spain1." Economic botany 62.3 (2008): 331-340.

Santelices Moya, Rómulo & Palfner, Götz. (2010). Controlled Rhizogenesis and Mycorrhization of Hazelnut (*Corylus avellana* L.) Cuttings with Black Truffle (*Tuber melanosporum* Vittad.). Chilean journal of agricultural research.

Sati, S. C., & Pant, P. (2019). Evaluation of phosphate Solubilization by root endophytic aquatic *Hyphomycete Tetracladium setigerum*. Symbiosis, 77(2), 141-145.

Savill, Peter S. *The silviculture of trees used in British forestry*. CABI, 20139.

Sbrana, Cristiana, et al. "Diversity of culturable bacterial populations associated to *Tuber borchii* ectomycorrhizas and their activity on *T. borchii* mycelial growth." *FEMS Microbiology Letters* 211.2 (2002): 195-201.

Schaeffer C., Wallenda T., Guttenberg M., Hampp R. (1995) Acid invertase in mycorrhizal and non-mycorrhizal roots of Norway spruce (*Picea abies* Karst.) seedlings. New Phytologist 129, 417-424.

Schau, H-P. "JF MacFaddin, Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria, Volume I. XI+ 929 S., 163 Abb., 94 Tab. Baltimore, London 1985. Williams and Wilkins (1986): 240-240.

Schneider-Maunoury, Laure, et al. "Is *Tuber melanosporum* colonizing the roots of herbaceous, non-ectomycorrhizal plants? " *Fungal Ecology* 31 (2018): 59-68.

Schneider-Maunoury, Laure, et al. "Two ectomycorrhizal truffles, *Tuber melanosporum* and *T. aestivum*, endophytically colonise roots of non-ectomycorrhizal plants in natural environments." *New Phytologist* 225.6 (2020): 2542-2556.

Schoenian, I., Spiteller, M., Ghaste, M., Wirth, R., Herz, H., & Spiteller, D. (2011). Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants. *Proceedings of the National Academy of Sciences*, *108*(5), 1955-1960.

Schreiter, S., Ding, G. C., Heuer, H., Neumann, G., Sandmann, M., Grosch, R., & Smalla, K. (2014). Effect of the soil type on the microbiome in the rhizosphere of field-grown lettuce. Frontiers in microbiology, 5, 144.

Scrascia, Maria, et al. "Identification of pigmented Serratia marcescens symbiotically associated with *Rhynchophorus ferrugineus* Olivier (Coleoptera: *Curculionidae*)." MicrobiologyOpen 5.5 (2016): 883-890.

Séjalon-Delmas N., Roux C., Martins M., Kulifaj M., Bécard G., and Dargent R. Molecular Tools for the Identification of *Tuber melanosporum* in Agroindustry. Journal of Agricultural and Food Chemistry 2000 48 (6), 2608-2613.

Sellstedt, A., & Richau, K. H. (2013). Aspects of nitrogen-fixing *Actinobacteria*, in particular free-living and symbiotic Frankia. FEMS Microbiology Letters, 342(2), 179-186.

Selosse, M. A., WEIss, M., Jany, J. L., & Tillier, A. (2002). Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) LCM Rich. and neighbouring tree ectomycorrhizae. Molecular Ecology, 11(9), 1831-1844.

Selosse, M-A., et al. "Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (*Neottieae, Orchidaceae*) are associated with ectomycorrhizal septomycetes, including truffles." Microbial Ecology 47.4 (2004): 416-426.

Serraj, R., et al. "Variation in root traits of chickpea (*Cicer arietinum* L.) grown under terminal drought." Field Crops Research 88.2-3 (2004): 115-127.

Shi, Y., Yang, H., Zhang, T., Sun, J., & Lou, K. (2014). Illumina-based analysis of endophytic bacterial diversity and space-time dynamics in sugar beet on the north slope of *Tianshan mountain*. Applied microbiology and biotechnology, 98(14), 6375-6385.

Shivlata L, Satyanarayana T. Thermophilic and alkaliphilic *Actinobacteria*: biology and potential applications. Front Microbiol. 2015;6:1014

Siddiqui, Z. A., & Kataoka, R. (2011). Mycorrhizal inoculants: progress in inoculant production technology. In Microbes and microbial technology (pp. 489-506). Springer, New York, NY.

Siebyła, M., & Hilszczańska, D. (2020). Diversity of soil bacteria complexes associated with summer truffle (). Folia Forestalia Polonica, 62(2), 114-127.

Singer, Rolf. "Mushrooms and truffles." Mushrooms and Truffles. (1961).

Smith, G. S., Johnston, C. M., & Cornforth, I. S. (1983). Comparison of nutrient solutions for growth of plants in sand culture. New phytologist, 94(4), 537-548.

Smith, Joshua M., Matthew D. Whiteside, and Melanie D. Jones. "Rapid nitrogen loss from ectomycorrhizal pine germinants signaled by their fungal symbiont." Mycorrhiza 30.4 (2020): 407-417.

Smith, S.E., and D.J. Read. 2008. Mycorrhizal symbiosis. 3rd ed. 815 p. Academic Press, London, UK.

Soudzilovskaia, N. A., Douma, J. C., Akhmetzhanova, A. A., van Bodegom, P. M., Cornwell, W. K., Moens, E. J., ... & Cornelissen, J. H. (2015). Global patterns of plant root colonization intensity by mycorrhizal fungi explained by climate and soil chemistry. Global Ecology and Biogeography, 24(3), 371-382.

Sourzat P (2008) Les sols truffiers. Fédération Franc¸aise des Trufficulteurs, Paris.

Sourzat, Pierre. Guide pratique de trufficulture. Station d'expérimentations sur la truffe, 1997.

Splivallo, R., Deveau, A., Valdez, N., Kirchhoff, N., Frey-Klett, P., & Karlovsky, P. (2015). Bacteria associated with truffle-fruiting bodies contribute to truffle aroma. Environmental Microbiology, 17(8), 2647-2660.

Splivallo, R., Vahdatzadeh, M., Maciá-Vicente, J. G., Molinier, V., Peter, M., Egli, S., ... & Deveau, A. (2019). Orchard conditions and fruiting body characteristics drive the microbiome of the black truffle *Tuber aestivum*. Frontiers in microbiology, 1437.

Splivallo, R., Valdez, N., Kirchhoff, N., Ona, M. C., Schmidt, J. P., Feussner, I., et al. (2012). Intraspecific genotypic variability determines concentrations of key truffle volatiles. New Phytologist.

Stomeo, F., Portillo, M. C., Gonzalez, J. M., Laiz, L., & Sáiz-Jiménez, C. (2008). *Pseudonocardia* in white colonizations in two caves with Paleolithic paintings. International Biodeterioration & Biodegradation, 62(4), 483-486.

Straker C.J., Schnippenkoetter W.H., Lemoine M-C (1992) Analysis of acid invertase and comparision with acid phosphatase in the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf and Kernan. Mycorrhiza 2, 63-67.

Streitwolf-Engel, R., Boller, T., Wiemken, A., & Sanders, I. R. (1997). Clonal growth traits of two Prunella species are determined by co-occurring arbuscular mycorrhizal fungi from a calcareous grassland. Journal of Ecology, 181-191.

Streitwolf-Engel, R., Van der Heijden, M. G. A., Wiemken, A., & Sanders, I. R. (2001). The ecological significance of arbuscular mycorrhizal fungal effects on clonal reproduction in plants. Ecology, 82(10), 2846-2859.

Sulzbacher, Marcelo A., et al. "*Hysterangium atlanticum* sp. nov., forms ectomycorrhizae with *Coccoloba* species (*Polygonaceae*) from the Atlantic rainforest of Northeastern Brazil." Symbiosis 78.3 (2019): 275-286.

Suriyachadkun, C., Ngaemthao, W., Pujchakarn, T., & Chunhametha, S. (2020). Cryptosporangium phraense sp. nov., Isolated from Plant Litter. International Journal of Systematic and Evolutionary Microbiology, 70(2), 971-976.

Tajima, Fumio. "Simple methods for testing the molecular evolutionary clock hypothesis." Genetics 135.2 (1993): 599-607.

Tamm, Carl Olof. "Introduction: geochemical occurrence of nitrogen. Natural nitrogen cycling and anthropogenic nitrogen emissions." Nitrogen in Terrestrial Ecosystems. Springer, Berlin, Heidelberg, 1991. 1-6.

Tan, X. M., Chen, X. M., Wang, C. L., Jin, X. H., Cui, J. L., Chen, J., ... & Zhao, L. F. (2012). Isolation and identification of endophytic fungi in roots of nine *Holcoglossum plants* (*Orchidaceae*)

collected from Yunnan, Guangxi, and Hainan provinces of China. Current Microbiology, 64(2), 140-147.

Tan, Z., Liu, R., Hu, Y., & Lin, Z. (2012). Production of isoflavone genistein in transgenic IFS tobacco roots and its role in stimulating the development of arbuscular mycorrhiza. *Acta physiologiae plantarum*, *34*(5), 1863-1871.

Tang, Y., Liu, J., Bao, J., Chu, G., & Peng, F. (2022). Soil Type Influences Rhizosphere Bacterial Community Assemblies of Pecan Plantations, a Case Study of Eastern China. Forests, 13(3), 363.

Tarkka, Mika T., Barbara Drigo, and Aurelie Deveau. "Mycorrhizal microbiomes." Mycorrhiza 28.5 (2018): 403-409.

Taschen, E., Sauve, M., Vincent, B. et al. Insight into the truffle brûlé: tripartite interactions between the black truffle (*Tuber melanosporum*), holm oak (*Quercus ilex*) and arbuscular mycorrhizal plants. Plant Soil 446, 577–594 (2020).

Taschen, Elisa, et al. "How the truffle got its mate: insights from genetic structure in spontaneous and planted Mediterranean populations of *Tuber melanosporum*." Molecular Ecology 25.22 (2016): 5611-5627.

Taylor, A. F. S., F. Martin, and D. J. Read. "Fungal diversity in ectomycorrhizal communities of Norway spruce [*Picea abies* (L.) Karst.] and beech (*Fagus sylvatica* L.) along north-south transects in Europe." Carbon and nitrogen cycling in European forest ecosystems. Springer, Berlin, Heidelberg, 2000. 343-365.

Tchoundjeu, Z., and R. R. B. Leakey. "Vegetative propagation of *Lovoa trichilioides*: effects of provenance, substrate, auxins and leaf area." Journal of Tropical Forest Science (2001): 116-129.

Tchoundjeu, Z., Avana, M. L., Leakey, R. R. B., Simons, A. J., Assah, E., Duguma, B., & Bell, J. M. (2002). Vegetative propagation of *Prunus africana*: effects of rooting medium, auxin concentrations and leaf area. Agroforestry systems, 54(3), 183-192.

Tchoundjeu, Z., Mpeck, M. L. N., Asaah, E., & Amougou, A. (2004). The role of vegetative propagation in the domestication of *Pausinystalia johimbe* (K. Schum), a highly threatened medicinal species of West and Central Africa. Forest Ecology and Management, 188(1-3), 175-183.

Tedersoo, L., Jairus, T., Horton, B. M., Abarenkov, K., Suvi, T., Saar, I., & Kõljalg, U. (2008). Strong host preference of ectomycorrhizal fungi in a Tasmanian wet sclerophyll forest as revealed by DNA barcoding and taxon-specific primers. New Phytologist, 180(2), 479-490.

Timonen S, Jorgensen KS, Haahtela K, Sen R. 1998. Bacterial community structure at defined locations of *Pinus sylvestris-Suillus bovinus* and *Pinus sylvestris-Paxillus involutus* mycorrhizospheres in dry pine forest humus and nursery peat. Can. J. Microbiol. 44:499 –513.

Toju, H., Peay, K. G., Yamamichi, M., Narisawa, K., Hiruma, K., Naito, K., ... & Kiers, E. T. (2018). Core microbiomes for sustainable agroecosystems. *Nature Plants*, *4*(5), 247-257.

Tombesi A., (1985) - Il nocciolo Reda: pp.121.

Trail F. (2007) Fungal cannons: explosive spore discharge in the Ascomycota. FEMS Microbiology Letters 276: 12–18.

Trappe JM, Claridge AW (2005). "Hypogeous fungi: evolution of reproductive and dispersal strategies through interactions with animals and mycorrhizal plants." The fungal community. CRC Press, 2005. 633-644.

Trappe, James M. Diversity, ecology, and conservation of truffle fungi in forests of the Pacific Northwest. Vol. 772. US Department of Agriculture, Forest Service, Pacific Northwest Research Station, 2009.

Trigueros, V., Lougarre, A., Ali-Ahmed, D., Rahbé, Y., Guillot, J., Chavant, L., ... & Paquereau, L. (2003). *Xerocomus chrysenteron* lectin: identification of a new pesticidal protein. Biochimica et Biophysica Acta (BBA)-General Subjects, 1621(3), 292-298.

Turco, S., Bastianelli, G., Morales-Rodrìguez, C., Vannini, A., & Mazzaglia, A. (2021). Development of a TaqMan qPCR assay for the detection and quantification of Gnomoniopsis castaneae in chestnut tissues. *Forest Pathology*, *51*(4), 1-10.

Urban, A., WEI, M., & Bauer, R. (2003). Ectomycorrhizas involving sebacinoid mycobionts. Mycological Research, 107(1), 3-14.

Urios, L., Agogué, H., Lesongeur, F., Stackebrandt, E., & Lebaron, P. (2006). *Balneola vulgaris* gen. nov., sp. nov., a member of the phylum *Bacteroidetes* from the north-western Mediterranean Sea. International Journal of Systematic and Evolutionary Microbiology, 56(8), 1883-1887.

Uroz, Stéphane, et al. "Effect of the mycorrhizosphere on the genotypic and metabolic diversity of the bacterial communities involved in mineral weathering in a forest soil." Applied and environmental microbiology 73.9 (2007): 3019-3027.

Uroz, Stéphane, et al. "Functional assays and metagenomic analyses reveals differences between the microbial communities inhabiting the soil horizons of a *Norway spruce* plantation." PLoS One 8.2 (2013): e55929.

Ustaoglu, B., and M. Karaca. "The effects of climate change on spatiotemporal changes of hazelnut (*Corylus avellana*) cultivation areas in the Black Sea region, Turkey." *Applied Ecology and Environmental Research (AEER)* 12.2 (2014): 309-324.

Vahdatzadeh, M., Deveau, A., & Splivallo, R. (2015). The role of the microbiome of truffles in aroma formation: a meta-analysis approach. Applied and environmental microbiology, 81(20), 6946-6952.

Valbuena-Carabana, M., et al. "Gene flow and hybridisation in a mixed oak forest (*Quercus pyrenaica* Willd. and *Quercus petraea* (Matts.) Liebl.) in central Spain." *Heredity* 95.6 (2005): 457-465.

Vallad, G.E.; Bhat, R.G.; Koike, S.T.; Ryder, E.J.; Subbarao, K.V. Weedborne reservoirs and seed transmission of *Verticillium dahliae* in lettuce. Plant Dis. 2005, 89, 317–324.

van der Wall, S. B. 1990. Food Hoarding in Animals. University of Chicago Press, Chicago.

van Elsas, J. D., et al. "Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots." FEMS Microbiology Ecology 2.3 (1986): 151-160.

van Elsas, J. D., et al. "Survival of, and root colonization by, alginate-encapsulated *Pseudomonas fluorescens* cells following introduction into soil." Biology and fertility of soils 14.1 (1992): 14-22.

van Elsas, J.D., et al. "Survival of *Pseudomonas fluorescens* containing plasmids RP4 or pRK2501 and plasmid stability after introduction into two soils of different texture." Canadian Journal of Microbiology 35.10 (1989): 951-959.

van Rhijn, P., & Vanderleyden, J. (1995). The Rhizobium-plant symbiosis. Microbiological reviews, 59(1), 124-142.

Vandenhove, H., et al. "Survival of *Pseudomonas fluorescens* inocula of different physiological stages in soil." Soil Biology and Biochemistry 23.12 (1991): 1133-1142.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., & Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. New Phytologist, 206(4), 1196-1206.

Vander Wall, S. B. (2001). The evolutionary ecology of nut dispersal. The Botanical Review, 67(1), 74-117.

Venturella, G., Altobelli, E., Bernicchia, A., Di Piazza, S., Donnini, D., Gargano, M. L., ... & Zotti, M. (2011). Fungal biodiversity and in situ conservation in Italy. Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology, 145(4), 950-957.

Verginer, M., Leitner, E., & Berg, G. (2010). Production of volatile metabolites by grape-associated microorganisms. Journal of agricultural and food chemistry, 58(14), 8344-8350.

Vestberg, M., Kukkonen, S., Saari, K., Parikka, P., Huttunen, J., Tainio, L., ... & Gianinazzi, S. (2004). Microbial inoculation for improving the growth and health of micropropagated strawberry. Applied Soil Ecology, 27(3), 243-258.

Videira, S. I. R., Groenewald, J. Z., Braun, U., Shin, H. D., & Crous, P. W. (2016). All that glitters is not *Ramularia*. Studies in Mycology, 83, 49-163.

Vittadini, Carlo, and Giuseppe Moretti. Monographia tuberacearum. Ex Typographia Felicis Rusconi, 1831.

Vivas, Astrid, et al. "Influence of a *Bacillus* sp. on physiological activities of two arbuscular mycorrhizal fungi and on plant responses to PEG-induced drought stress." Mycorrhiza 13.5 (2003): 249-256.

Voegele R.T., Struck C., Hahn M., Mendgen K. (2001) The role of haustoria in sugar supply during infection of broad bean by the rust fungus *Uromyces fabae*. Proceedings of the National Academy of Sciences, USA 98, 8133-8138.

Von der Weid, Irene, et al. "*Paenibacillus brasilensis* sp. nov., a novel nitrogen-fixing species isolated from the maize rhizosphere in Brazil." International journal of systematic and evolutionary microbiology 52.6 (2002): 2147-2153.

Vrålstad, T., Myhre, E., & Schumacher, T. (2002). Molecular diversity and phylogenetic affinities of symbiotic root-associated ascomycetes of the *Helotiales* in burnt and metal polluted habitats. *New Phytologist*, *155*(1), 131-148.

Wagner, M. R., Lundberg, D. S., Coleman-Derr, D., Tringe, S. G., Dangl, J. L., & Mitchell-Olds, T. (2014). Natural soil microbes alter flowering phenology and the intensity of selection on flowering time in a wild *Arabidopsis* relative. Ecology letters, 17(6), 717-726.

Walker, J. F., K. MILLER JR, O. R. S. O. N., & Horton, J. L. (2005). Hyperdiversity of ectomycorrhizal fungus assemblages on oak seedlings in mixed forests in the southern Appalachian Mountains. Molecular Ecology, 14(3), 829-838.

Wallander H. (2006) External mycorrhizal mycelia-the importance of quantification in natural ecosystems. New Phytologist 171, 240-242.

Wallander H., Nilsson L. O., Hagerberg D., Baath E. (2001) Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. New Phytologist 151, 753-760.

Walters D.R., Cowley T., McPherson A., Marshall G., McRoberts N. (1996) Sugar transport in the light leaf spot pathogen *Pyrenopeziza brassicae*. FEMS Microbiology Letters 143, 285-289.

Wang Y. et al. "Phylogenetic and populational study of the *Tuber indicum* complex." Mycological research 110.9 (2006): 1034-1045.

Wang Yue-sheng, ZHOU Zhi-chun, JIN Guo-qin, HONG Gui-mu, WANG Hui. Growth of *Taxus chinensis* var .mairei for container seedlings in different media mixtures and for bare-root versus container seedlings in a young stand . Journal of Zhejiang A&F University, 2007, 24(5): 643-646.

Wang, Meng-Ben, and Qiang Zhang. "Issues in using the WinRHIZO system to determine physical characteristics of plant fine roots." Acta Ecologica Sinica 29.2 (2009): 136-138.

Wang, Shih-Min, and Ching-Chuan Liu. "Enterovirus 71: epidemiology, pathogenesis and management." Expert review of anti-infective therapy 7.6 (2009): 735-742.

Wang, Y., Peng, S., Hua, Q., Qiu, C., Wu, P., Liu, X., & Lin, X. (2021). The long-term effects of using phosphate-solubilizing bacteria and photosynthetic bacteria as biofertilizers on peanut yield and soil bacteria community. Frontiers in microbiology, 12.

Ward, N. L., Challacombe, J. F., Janssen, P. H., Henrissat, B., Coutinho, P. M., Wu, M., ... & Kuske, C. R. (2009). Three genomes from the phylum Acidobacteria provide insight into the lifestyles of these microorganisms in soils. Applied and environmental microbiology, 75(7), 2046-2056.

Warmink, J. A., Nazir, R., & Van Elsas, J. D. (2009). Universal and species-specific bacterial 'fungiphiles' in the mycospheres of different basidiomycetous fungi. Environmental Microbiology, 11(2), 300-312.

Wedén, Christina, Gérard Chevalier, and Eric Danell. "*Tuber aestivum* (syn. *T.uncinatum*) biotopes and their history on Gotland, Sweden." Mycological Research 108.3 (2004): 304-310.

Weden, Christina, Lina Pettersson, and Eric Danell. "Truffle cultivation in Sweden: Results from *Quercus robur* and *Corylus avellana* field trials on the island of Gotland." Scandinavian Journal of Forest Research 24.1 (2009): 37-53.

Westberg, J., & Jason, H. (2004). Fostering learning in small groups: A practical guide. Springer Publishing Company.

Whipps, John M. "Prospects and limitations for mycorrhizas in biocontrol of root pathogens." Canadian journal of botany 82.8 (2004): 1198-1227.

Wieland, G., Neumann, R., & Backhaus, H. (2001). Variation of microbial communities in soil, rhizosphere, and rhizoplane in response to crop species, soil type, and crop development. Applied and environmental microbiology, 67(12), 5849-5854.

Willis, A. D. (2019). Rarefaction, alpha diversity, and statistics. Frontiers in microbiology, 10, 2407.

Wilson, Dennis. "Fungal endophytes which invade insect galls: insect pathogens, benign saprophytes, or fungal inquilines?." *Oecologia* 103.2 (1995): 255-260.

Xiu, C. L., Pan, H. S., Liu, B., Luo, Z. X., Williams, L., Yang, Y. Z., & Lu, Y. H. (2019). Perception of and behavioral responses to host plant volatiles for three Adelphocoris species. Journal of Chemical Ecology, 45(9), 779-788.

Xu, J. (2014). Isolation and assessment of nitrogen-fixing and phosphate-solubilizing bacteria for use as biofertilizers (Doctoral dissertation).

Xue, L., Almario, J., Fabiańska, I., Saridis, G., & Bucher, M. (2019). Dysfunction in the arbuscular mycorrhizal symbiosis has consistent but small effects on the establishment of the fungal microbiota in Lotus japonicus. *New Phytologist*, *224*(1), 409-420.

Ye, L., Li, Q., Fu, Y., Sourzat, P., Tan, H., Zou, J., ... & Li, X. (2018). Host species effects on bacterial communities associated with the fruiting bodies of *Tuber species* from the Sichuan Province in Southwest China. Mycological Progress, 17(7), 833-840.

Yoneda, Y., Yamamoto, K., Makino, A., Tanaka, Y., Meng, X. Y., Hashimoto, J.,& Tamaki, H. (2021). Novel plant-associated acidobacteria promotes growth of common floating aquatic plants, duckweeds. Microorganisms, 9(6), 1133.

Yung, L., Bertheau, C., Tafforeau, F., Zappelini, C., Valot, B., Maillard, F., ... & Chalot, M. (2021). Partial overlap of fungal communities associated with nettle and poplar roots when co-occurring at a trace metal contaminated site. *Science of the Total Environment*, *782*, 146692.

Zambonelli A, Iotti M, Barbieri E, Amicucci A, Stocchi V, Peintner U, Hall IR (2009) The microbial communities and fruiting of edible ectomycorrhizal mushrooms. Acta Bot Yunn Suppl 16:81–85.

Zambonelli A, Iotti M., Zinoni F., Dallavalle E., Hall R. (2005). Effect of mulching on *Tuber uncinatum* ectomycorrhizas in an experimental truffière. New Zealand Journal of Crop and Horticultural Science 33: 65-73.

Zambonelli A, Salomoni S, Pisi A (1993) Caratterizzazione anatomo-morfologica delle micorrize di *Tuber* spp su *Quercus pubescens* Willd. Micol Ital 22(3):73–90.

Zambonelli, A., Iotti, M., Boutahir, S., Lancellotti, E., Perini, C., & Pacioni, G. (2012). Ectomycorrhizal fungal communities of edible ectomycorrhizal mushrooms. In Edible ectomycorrhizal mushrooms (pp. 105-124). Springer, Berlin, Heidelberg.

Zambonelli, Alessandra & Iotti, Mirco & Murat, Claude. (2016). True Truffle (*Tuber* spp.) in the World: Soil Ecology, Systematics and Biochemistry. 10.1007/978-3-319-31436-5.

Zampieri E., Mello A., Bonfante P. and Murat C. (2011) PCR primers specific for the genus *Tuber* reveal the presence of several truffle species ina truffle- ground. FEMS Microbiol Lett 297, 67–72.

ZengPu, L., JunRan, J., & Changwen, W. (1994). Antagonism between Ectomycorrhizal fungi and plant pathogens. *Mycorrhizas for Plantation Forestry in Asia. Brundett M, Dell B, Malajczuk N, and Mingqin G.(eds)*, 77-81.

Zhong, F., Fan, X., Ji, W., Hai, Z., Hu, N., Li, X., ... & Zhang, J. (2022). Soil Fungal Community Composition and Diversity of Culturable Endophytic Fungi from Plant Roots in the Reclaimed Area of the Eastern Coast of China. *Journal of Fungi*, *8*(2), 124.

Zou, Changsong, Zhifang Li, and Diqiu Yu. "*Bacillus megaterium* strain XTBG34 promotes plant growth by producing 2-pentylfuran." The Journal of Microbiology 48.4 (2010): 460-466.

ACKNOWLEDGEMENT

Il presente lavoro è il risultato di un impegno durato tre anni. Durante questo periodo ho avuto la fortuna di lavorare con professionisti che mi hanno trasmesso uno straordinario patrimonio di valori e conoscenze. A tutti loro vanno i miei ringraziamenti. Ringrazio innanzitutto il Prof. Andrea Vannini, mio Tutore di Tesi Triennale, Magistrale ed ora di Dottorato di Ricerca, che ha investito la sua conoscenza ed il suo tempo in interessanti confronti e determinanti contributi sull'intero lavoro svolto, consigliandomi e guidandomi costantemente. Ringrazio il Prof. Vannini soprattutto per avermi supervisionato durante il lavoro, offrendomi la capacità di affrontare rapidamente problematiche diverse. Ringrazio caramente la Prof.ssa Alessandra Zambonelli ed il Dott. Mirco Iotti, ricercatori che stimo moltissimo e che sono stati fonte di ispirazione fin dall'inizio della mia ricerca bibliografica, per aver revisionato la mia tesi di Dottorato ed avermi offerto preziosi consigli e suggerimenti. Desidero ringraziare la Dott.ssa Maria Pia Aleandri per la costante disponibilità e appoggio che mi ha fornito durante la prima parte del corso del Dottorato, che è stata la più faticosa fisicamente ed emotivamente. I suoi preziosi insegnamenti e consigli non solo sulle metodologie scientifiche, ma anche dal punto di vista umano hanno reso divertenti anche i momenti più duri. Il suo aiuto e supporto sono stati fondamentale nello svolgimento di questa ricerca. Vorrei anche ringraziare la mia carissima collega nonché compagna di studi dalla triennale, Giorgia Bastianelli senza il cui aiuto non sarebbe stata la stessa cosa! Il suo senso pratico e analitico, ma anche la sua ironia talvolta pungente nell'affrontare i molti giorni di organizzazione degli esperimenti e di smantellamento delle prove hanno reso quei giorni produttivi, divertenti ed indimenticabili…anche quelli che prevedevano sveglie all'alba e chilometri di strada!! La sua presenza ed il suo aiuto sono stati molto importanti. Vorrei ringraziare anche la Dott.ssa Carmen Morales-Rodríguez e i tesisti che nel corso degli anni sono diventati colleghi e amici come Merima Jasarevic, Marta Tatì, Leonardo Guidoni, Wajiid Zeeb e Dania Tabet, che mi hanno aiutato e supportato dedicandomi il loro tempo, a voi tutti un forte ringraziamento. La mia esperienza nell'Azienda cofinanziatrice del mio Dottorato, la Urbani Tartufi, è stata fondamentale e mi ha trasmesso la passione per il tartufo, pregiato prodotto della terra. Prima fra tutti Olga Urbani e i suoi collaboratori Riccardo Cesari, Pietro Manna, Marco Dominici e Giada hanno rappresentato un punto di riferimento, una guida e una grande risorsa durante tutta la mia esperienza di ricerca e sperimentazione. Infine, un ulteriore ringraziamento va alle persone che mi sono state vicine e hanno creduto in me per tutto questo tempo e che hanno fatto in modo che questa "avventura" assumesse il giusto significato. Un ringraziamento speciale a mia madre per essere stata al mio fianco sempre, insegandomi a non arrendermi mai. A mia nonna che con la

sua incrollabile forza ed ottimismo è una continua fonte di ispirazione e a mio padre, per avermi saputo comunicare la sua vicinanza emotiva ed il suo prezioso sostegno morale, senza i quali non avrei mai intrapreso questa strada e raggiunto questo traguardo. Ai miei amici Sara, Luca, Andrei, Giorgia, Silvia e Matteo un ringraziamento per avermi ascoltata e supportata moralmente nei periodi più difficili, grazie infinite. Un ringraziamento particolare va a Gianluca, il mio compagno, per essermi stato sempre vicino e per avermi dato la sua profonda fiducia e la partecipazione costante ad ogni mia difficoltà, come solo lui sa fare, e per avermi incoraggiata saggiamente in quei momenti in cui non si è certi di potercela fare. Condensare in qualche riga tutto quello che ho ricevuto da voi in questi anni non è umanamente possibile e sarebbe riduttivo ed ingiusto; perciò, mi limiterò a dire che avete continuato a crederci anche quando non ci credevo più nemmeno io e di questo non vi sarò mai abbastanza grata.

A tutti voi va il mio più profondo ringraziamento!