Universidade do Minho
Escola de Ciências

Ângela Moreira Soares

Saprotrophic fungi *Hypholoma fasciculare* effect on the fungal community associated to *Castanea sativa*

Tese de Mestrado
Mestrado em Biologia Molecular, Biotecnologia
e Bioempreendedorismo em Plantas

Trabalho efetuado sob a orientação de
Professora Doutora Maria Teresa Correia Guedes Lino Neto
Professora Doutora Paula Cristina dos Santos Baptista

Abril, 2015

DECLARAÇÃO

Nome: Ângela Moreira Soares

Endereço eletrónico: soaresm.angela@gmail.com

Telefone: +351 913 671 173

Nº Cartão Cidadão: 13954673

Título da Tese de Mestrado:

Saprotrophic fungi *Hypholoma fasciculare* effect on the fungal community associated to *Castanea sativa*

Orientadores:

Professora Doutora Maria Teresa Correia Guedes Lino Neto

Professora Doutora Paula Cristina dos Santos Baptista

Ano de Conclusão: 2015

Designação do Mestrado:

Mestrado em Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Universidade do Minho, 30 de Abril de 2015

Assinatura:_____

ACKNOWLEDGMENTS

Muitas foram as pessoas que, das mais variadas formas, contribuíram para a elaboração deste projeto, às quais quero manifestar a minha gratidão.

À Professora Teresa Lino Neto, gostaria em primeiro lugar agradecer por ter aceite ser minha orientadora. E mostrar o meu total reconhecimento pelos ensinamentos e conselhos, estímulos e empenho na minha evolução científica, assim como o rigor e organização científica transmitida. Obrigada ainda pelas suas palavras de apreço e encorajamento, e ainda pela compreensão e ajuda ao longo deste meu percurso académico.

À Professora Paula Baptista, pela disponibilidade total, por toda a sua atenção e incentivo para a minha progressão. Pelos conselhos, críticas e rigor transmitidos nesta experiência.

À Francisca por toda a sua disponibilidade, ajuda e amabilidade em todo o trabalho, bem como pelo precioso tempo dispendido comigo.

Aos amigos que nunca descuidaram, que apesar de toda a distância estiveram sempre presentes. Por toda amizade, boa disposição e disponibilidade interminável. Pela constante presença no meu quotidiano e pela força que sempre me transmitiram.

Ao Ricardo, pela sua presença. Por simplificar e reconhecer as palavras e gestos certos nas mais variadas situações, pela lição de vida, valores e energia dada sem condições em troca. O mais sincero obrigada pelo seu significado.

Ao Igor, pela força, coragem e loucura de todos os dias. Pelos valores e sonhos que tão orgulhosamente me transmitiu, desde as brincadeiras de criança até aos desafios atuais.

E em especial, à minha Mãe. Não há palavras para descrever o seu apoio incondicional e altruísmo excessivo. Agradeço pelo exemplo de força e de luta constante, perante as adversidades; e de alegria e sorrisos após cada batalha. Sem ela não teria conseguido.

Por tudo isto, o meu mais sincero obrigada!

Saprotrophic fungi *Hypholoma fasciculare* effect on the fungal community associated to *Castanea sativa*

ABSTRACT

The European chestnut (*Castanea sativa*) is a plant species with eminent importance, due to the high gastronomic value attributed to its fruit, as well as for being a high quality wood source. Associated to the chestnut orchards soils of the region of Trás-os-Montes (Bragança, Portugal) is commonly found the saprotrophic fungi, *Hypholoma fasciculare*. Some preliminary studies have suggested that the presence of this fungus in the soil can damage chestnut trees and reshape the soil microbial community, due to the strong antagonist activity that *H. fasciculare* displays against soil-borne fungi from orchard. In order to acknowledge the effect of *H. fasciculare* in soil fungal community, a metabarcoding project was performed by pyrosequencing the ITS1 barcodes of DNA obtained from different soil samples. Chestnut orchard soil was collected and used for chestnut plant growing. Two months after transplantation, plants were inoculated with *H. fasciculare*, being harvested after six-months or one-year upon inoculation. As controls, non-inoculated plants were used. In order to study the effect of the well-established fungal community on *H. fasciculare* constraints, sterile orchard soils were use in a parallel assay.

In this study, 458 OTUs (operational taxonomic units) were identified comprising 78,029 reads. The richest phylum was the Ascomycota (58.9%), followed by the Basidiomycota (38.9%). However, the Basidiomycota was the most abundant phylum (57.4%), followed by the Ascomycota (40.9%). In order to evaluate the habitat quality, alpha and beta diversity were evaluated, which allowed to determine the richness in species within each soil sample and the species turnover between conditions, respectively. Sterile soil samples were less rich and diverse than non-sterile soil samples, but non-sterile soil samples were more homogeneous among them. The variation of functional groups of the most well-represented OTUs was also analyzed, being the parasites the most rich and abundant, followed by saprotrophic and mycorrhizal functional groups. Correlations between functional groups were also computed and the most positive correlation was found between saprotrophs and parasites. At the end, a clear effect of *H. fasciculare* was not detected, although specific microbial interactions could have taken place. The use of sterile soils allowed the recognition of a buffering-like effect, in which microbial community is not so easily affected in its equilibrium, neither by the fungal inoculation nor by the chestnut growing. This effect could be of major importance from the agronomic point of view.

Key-words: chestnut orchard soils; *Castanea sativa*; *Hypholoma fasciculare*; metagenomics; ecological; diversity; fungal community

Efeito do fungo saprófita *Hypholoma fasciculare* na comunidade fúngica associada a *Castanea sativa*

RESUMO

O castanheiro europeu (*Castanea sativa*) é uma espécie com notória importância, devido ao elevado valor gastronómico atribuído ao seu fruto, bem como pela elevada qualidade da sua madeira. Associados aos soutos da região de Trás-os-Montes (Bragança, Portugal) encontra-se frequentemente o macrofungo saprófita *Hypholoma fasciculare*. Alguns estudos preliminares sugeriram que a presença deste fungo no solo poderia causar sérios danos aos castanheiros e remodelar a comunidade microbiana do solo, devido à elevada atividade antagonista que apresenta contra fungos presentes nos solos dos soutos. De modo a reconhecer o efeito de *H. fasciculare* na comunidade fúngica do solo foi efetuado um projeto de *metabarcoding*, no qual foram pirosequenciados *ITS1 barcodes* a partir de DNA obtido de diferentes amostras de solos. O solo de soutos transmontanos foi colhido e utilizado para o crescimento de castanheiro. Dois meses após a transplantação, as plantas foram inoculadas com *H. fasciculare*, sendo colhidas seis meses e um ano após inoculação. As plantas não inoculadas foram utilizadas como controlos. A fim de estudar o efeito da comunidade fúngica bem estabelecida na capacidade deletéria de *H. fasciculare*, os solos estéreis foram utilizados num ensaio paralelo.

Neste estudo, 458 *OTUs* (unidades taxonómicas operacionais) foram identificadas compreendendo 78,029 sequências identificadas. Ascomycota foi o filo mais rico (58.9%), seguido por Basidiomycota (38.9%). Contudo, o filo Basidiomycota foi o mais abundante (57.4%) seguido pelo Ascomycota (40.9%). De forma a avaliar a qualidade do habitat, diversidades alfa e beta foram consideradas, contribuindo para a determinação da riqueza de espécies em cada amostra de solo e a variação de espécies entre as condições, respetivamente. As amostras de solo estéril apresentaram ser menos ricas e diversas que as amostras de solo não estéril, além de que amostras de solo não estéril apresentaram ser mais homogéneas entre si. A variação dos grupos funcionais das espécies mais representadas foi analizada, sendo as espécies parasíticas as mais ricas e abundantes, seguida pelos saprófitas e micorrízicos. As correlações entre os grupos funcionais foram igualmente calculadas e a correlação mais positiva foi encontrada entre os saprófitas e parasitas. No geral, não foi evidenciado um claro efeito de *H. fasciculare*, sobre a restante comunidade microbiana do solo, apesar de específicas interações pussam ter ocorrido. O uso de solos estéreis permitiu o reconhecimento de um efeito semelhante ao efeito tampão,

em que a comunidade microbiana não é facilmente afetada no seu equilibrio, nem pela inoculação do fungo nem pela presença do crescimento do castanheiro. Este efeito pode ter importância de um ponto de vista agronómico.

Palavras-chave: solos de soutos; *Castanea sativa*; *Hypholoma fasciculare*; metagenómica; ecológico; diversidade; comunidade microbiana

INDEX

ABBREVIATIONS AND ACRONYMS

bp –Base pair

CCD – Charge-coupled device

DNA –Deoxyribonucleic Acid

dNTPs – Deoxynucleotide Triphosphates

DWD – Daily increase of shoot dry weight

EDTA – Ethylenediamine Tetraacetic Acid

FAO – Food and Agriculture Organization

ha – Hectare

IGS – Intergenic Spacer

ITS – Internal transcribed spacer

Gb – Giga base pair

m – Metre

M – Molar

MID – Multiplex Identifier

min – Minute

N - North

NGS – Next-Generation Sequencing

OTU - Operational taxonomic unit

PCR - Polymerase Chain Reaction

PDO - Protected Designation of Origin

PTP – Picotiter plates

rDNA - Ribosomal Deoxyribonucleic Acid

s – Second

SMS – 'Single-molecule' sequencing

SSU - Ribosomal small subunit rRNA gene

V - Volt

°C – Celsius degrees

CHAPTER I

INTRODUCTION

Chestnut tree is mainly distributed in three major areas: Europe (*Castanea sativa* Mill.), Asia (*Castanea crenata* Sieb. and Zucc. in Japan; *Castanea mollissima* Bl. in China and Korea) and North America (*Castanea dentata* Borkh) (table 1). The genus *Castanea* is represented by thirteen species that have a wide range of different dimensions. *Castanea dentata, C. henry* and *C. sativa* (fig. 1) comprise the largest individuals, while *C. pumila* (var. *ozarkiensis* and var. *pumila*), *C. mollissima* and *C. crenata* include adult plants with only 15-20 m (Gomes-Laranjo and Crespí, 2007). *Castanea sativa* Mill. is the predominant cultivated species, in Europe, but recently some hybrids from the genetic cross with other species of chestnut have been developed for increasing the resistance to ink disease and chestnut tree cancer, caused by *Phytophthora* spp. and *Cryphonectria parasitica*, respectively (Vasconcelos *et al.*, 2010). In order to have a good development and fruiting, the chestnut tree demands favorable weather conditions. The ideal conditions for its development are high altitudes (higher than 500 m) and winter low temperatures (Ribeiro *et al.*, 2007) and its dispersion is limited above the 40° N parallel (Gomes-Laranjo *et al.*, 2007).

Table 1 – Taxonomic identification of European chestnut according to Cronquist (1968).

Kingdom	Plantae
Sub-kingdom	Embryophyta (Cornophyta)
Phylum	Spermatophyta (Anthophyta)
Sub- phylum	Angiospermae (Magnoliophytina)
Class	Dicotyledonae (Magnoliopsida)
Sub-class	Hamamelidae
Order	Fagales
Family	Fagaceae
Sub-family	Castaneoideae
Genus	*Castanea*
Species	*Castanea sativa*

The consumption of chestnut fruits has increased in many European countries, mainly due to the recognition of their nutritional qualities and potential beneficial health effects, and in particular the protection that is provided by their antioxidant compounds (Borges *et al.*, 2008). In some rural regions of a few European countries, besides their agro-ecological importance,

chestnut trees also have the protection role against fire and erosion, as well as providing a supportive habitat for biodiversity and recreation (Blom *et al.*, 2009). The chestnut forests and groves have a notable diversity of wildlife, mainly due to their inaccessibility, density and nutritious nuts (Diamandis and Perlerou, 2001).

In Portugal, the main chestnut production area is located in Trás-os-Montes, although a high distribution of chestnut trees is also found in Beira Interior (Borges *et al.*, 2008). A lower density of chestnuts orchards is found in the Northeast Alentejo, North Algarve and Interior Minho (Ferreira-Cardoso and Pimentel-Pereira, 2007). Once it is a deciduous species (shedding of leaves and limited vegetative growth in winter), the hot temperatures can be a limitation for its growth and development, hence the area that fits better this feature is in the Northeast of Portugal (Gomes-Laranjo *et al.*, 2007).

The production of chestnuts represents a crucial role for the Portuguese economy and patrimony (Borges *et al.*, 2008). According to data available in Food and Agriculture Organization of the United Nations (FAO, 2012), Portugal presented the major chestnut producing area in Europe, with 34,800 ha, followed by Italy with 25,000 ha and France with 7,165 ha. Chestnut production in Portugal corresponded to 19,130 tons in 2012 and 24,739 tons in 2013 (INE, 2013). According to its importance, in Portugal, there are four different regions that produce high quality chestnut, which have been considered as Protected Designation of Origin (PDO) product: *Castanha da Terra Fria*, *Castanha da Padrela*, *Castanha dos Soutos da Lapa* and *Castanha de Marvão* (Ferreira-Cardoso and Pimentel-Pereira, 2007).

Two distinct types of chestnut tree plantation can be distinguished by their purpose: plantation for fruit production – "souto" – or plantation for wood production - "castinçal" (figure 1). In addition to their value for furniture industry, chestnut wood is also used for tannin production and source of renewable energy (Corredoira *et al.*, 2015). Besides the importance of fruit and timber there is also a notable interest in edible mushrooms, which is highly valued in international markets and comprise one of the main secondary products of chestnut forest. Due to the production of chestnuts and wood, as well as mushroom collection, the exploitation of "soutos" and "castinçais" has been increasing (Diamandis and Perlerou, 2001).

Figure 1 - European chestnut, *Castanea sativa* plantation as a "souto" (a) and as a "castinçal" (b).

1. Fungal communities associated to plants

Fungi are considered to be a very important component of an ecosystem due to the roles they play. The primary and best understood role of soil fungi is as decomposers. However, besides also playing a fundamental role in terrestrial ecosystems as plants mutualists and parasites, they have an important implication in the agriculture, pharmacology and other environmental technologies (Anderson *et al.*, 2003; Buée *et al.*, 2009; Toju *et al.*, 2012). Fungi are likewise capable of controlling soil water content and soil structure (Lim *et al.*, 2010; Orgiazzi *et al.*, 2012, 2013). Fungal symbionts are capable of developing themselves in the rhizosphere due to the presence of propagules, meaning spores, hyphae and rhizomorphs. Therefore they have the capacity to manipulate the nutrient fluxes in natural ecosystems by their extensive below-ground mycelial networks (Anderson *et al.*, 2003). Fungi also play an important role in plant development, since they have the important role of establishing the connection between plant roots and the soil, allowing them to take advantage from their nutrient and water uptake capacity (Klein and Paschke, 2004; Bonfante and Anca, 2009). Besides these advantageous associations to plants, fungi can also harm them, being the cause of severe diseases that can lead to their dead. For example, in the chestnut forest ecosystem, *Cryphonectria parasitica* (Murr.) Barr. is responsible for the chestnut blight, which has been the cause of the most *C. sativa* Mill. decay (Bissegger *et al.*, 1997). Also, *Phytophthora* spp. are considered harmful to woody plants, specifically *P. cambivora* (Petri) Buis. and *P. cinnamomi* Rands, which are the two most common oomycetes species responsible for the ink disease in European chestnut trees (Vannini and Vettraino, 2001; Vettraino *et al.*, 2005).

Ecological guilds of fungi

Fungi can be divided into several trophic levels, according to their ecological functions and specialization. The main ecological groups of soil fungi are mycorrizhal, parasites and saprotrophic.

Mycorrhizal associations

The mycorrhizal associations are typically found on most annual and perennial plants (Martin, 2007). The term mycorrhiza describes diverse root-fungus associations, in which both partners benefit (Deacon, 2006; Bonfante and Anca, 2009). Actually, mycorrhizal fungi have a strong impact on plant development, since they provide an wide hyphal network for the capture and transport of water and mineral nutrients to plant roots (Chang and Miles, 2004; Deacon, 2006). Besides improving the availability of nutrients to the plant, mycorrhizal fungi also enhance the rooting of host plants and improve the structure of the soil. In addition, they protect the plant from biotic and abiotic stresses by upgrading its resistance and tolerance to pathogens, dryness, nutrient deficiency, among others (Jeffries *et al.*, 2003; Barea *et al.*, 2005). The importance of these fungi is greatly recognized by their influence on plant biodiversity, by their assistance in control of pests and also enhancement of plant fitness in polluted environments (Schü β ler *et al.*, 2001). In return the fungi receive carbohydrates from the plant.

According to the plant and fungal partners, different types of mycorrhiza can be recognized, which can be generally divided between ectomycorrhizae and endomycorrhizae. Endomycorrhizae can be further divided into three sub-classes, arbuscular, orchidoid and ericoid mycorrhizae, but all of which have hyphae which penetrates the roots cells and establish an intracellular symbiosis (Bonfante and Anca, 2009). Arbuscular mycorrhizae are common among various plant taxa, while ericoid and orchidoid, mycorrhizae are confined to the Ericales order and family Orchidaceae, respectively. Arbuscular mycorrhizal (AM) fungi are included in the Glomerales order and Zygomycota phyla. The orchidoid mycorrhizal fungi belong to the Basidiomycete phyla are determinant in supplying inorganic and organic nutrients to their host (Smith and Read, 1997). An intrinsic characteristic of these mycorrhizal fungi is the penetration of their hyphae inside the cells of the root cortex forming elaborated tangles (Boldrini *et al.*, 2010). Ericoid mycorrhizae have great ecological importance due to its capacity as an efficient organic matter decomposer (Martin, 2007). They are distinguished by presenting an averagely dense coil of mycelium within the cells of the root cortex (Perotto *et al.*, 2002).

Following arbuscular mycorrhizae, the second most abundant mycorrhizae are ectomycorrhizae (ECM), whose fungi belong to Asco-, Basidio- and Zygomycete phylum (Horton and Bruns, 2001). Their extracellular hyphae induce changes to root morphogenesis, leading to subtle alterations in epidermal or cortical cells (Bonfante and Anca, 2009). Hyphae develop between the intercellular spaces of root cells, at the epidermis level, where they form the Hartig net (Smith and Read, 1997). There is still a third class of mycorrhizae, the ectendomycorrhizae, which presents fungal characteristics from both endo- and ectomycorrhizae, whose fungi belong to the Basidiomycete class. Ectendomycorrhizae can be sub-divided into arbutoid and monotropoid mycorrhizae, whose fungal species belong the Arbutoidaea and Monotropoidaea sub-families, respectively (Smith and Read, 1997; Yu *et al.*, 2001).

Parasitism

The relationship of parasitism is always at the expense of the host, from which the parasites obtain their nutrients for their development. However, the tolerance of plant host to the parasite is variable, some supporting a limited fungal growth, while others become diseased or even dye just by the presence of fungi (Chang and Miles, 2004; Baptista, 2007). This kind of fungi can damage some important crops and frenquently lead to food shortages (Chang and Miles, 2004).

Saprotrophism

The role of saprotrophic fungi as decomposers is very important for the soil ecosystem, since they release important nutrients for the sustained and promoted plant growth (Pereira *et al.*, 2012). The reason why these fungi are described as saprotrophic is due to the way they obtain nutrients, which is from dead or decaying organic matter (Chang and Miles, 2004). Saprotrophic are also important for improving the amount of mineral nutrients present in the soil (reviewed by Baptista, 2007). Some saprotrophic basidiomycetes are capable of forming large mycelia that can extend over tens centimeters or meters, making these organisms well adapted for terrestrial environments (Šnajdr *et al.*, 2011).

Castanea sativa establishes different types of interaction with soil fungi. The knowledge of fungal diversity associated to chestnut trees has been gained by macrofungal surveys and recent molecular approaches (Baptista *et al.*, 2015). The high abundance of *Hypholoma fasciculare* in

C. sativa forest soils and its influence on the fungal community is an important aspect to consider for the sustainability of chestnut orchards.

Hypholoma fasciculare: consequences for chestnut tree microbial community

The genus *Hypholoma* comprises around 30 species that are distributed worldwide from temperate to tropical zones. This fungus grows on decomposing wood, above living trees and mosses, or even on the soil (Cortez and Silveira, 2007).

The *Hypholoma* species that is commonly associated to *C. sativa* forest soils is *H. fasciculare* (Huds.: Fr.) P. Kumm (table 2), which is a saprothophic basidiomycete considered to be a litter decomposing (Šnajdr *et al.*, 2008, 2011) and a white-rot fungal species (Valásková *et al.*, 2009). *Hypholoma fasciculare* is a wood and litter decomposer that plays a major role in the forest ecosystem, acting on the biological control of phytopathogenic fungi (Cortez and Silveira, 2007). According to the Index Fungorum database (http://www.indexfungorum.org/) *H. fasciculare* has now a new designation, *H. acutum* (Sacc.) E. Horak. In this work, when referring to this fungus, the older classification will still be used.

Table 2 - Taxonomic identification of saprotrophic fungi *Hypholoma fasciculare* according to Kirk *et al.* (2001).

Kingdom	Fungi
Phylum	Basidiomycota
Class	Basidiomycetes
Sub-class	Agaricomycetidae
Order	Agaricales
Family	Strophariaceae
Genus	*Hypholoma*
Species	*Hypholoma fasciculare*

The behavior and reaction of other fungi towards saprotrophic basidiomycetes is not always constant. In some cases, when a saprotrophic mycelium meets another fungus, the saprotroph can either gain territory or be inhibited by the other or none of them gains ahead (Pereira *et al.*, 2013). *Hypholoma fasciculare* displays a high antagonist activity against other soil-borne fungi

(Pereira *et al.*, 2013). According to this, *H. fasciculare* has been investigated as a competitive fungus to be used to promote the reduction of timber losses related to the *Armillaria* root disease (Chapman *et al.*, 2001).

In chestnut agro-systems, saprotrophic and ectomycorrhizal fungi seem to interact with each other despite they occupy different soil horizons. Pereira *et al.* (2012) evaluated the effect of *H. fasciculare* and *Pisolithus tinctorius* (an ectomycorrhizal fungus) on the growth of *C. sativa* and reported the negative effect of *H. fasciculare* on the interaction of the ectomycorrhizal fungus and roots. This kind of suppression is expected to commit the ectomycorrhization of the chestnut trees thus constraining their development.

2. Evaluating soil fungal communities by metabarcoding analysis

Soil portrays a large reservoir of microorganisms biodiversity that are involved in key steps for the ecosystem functioning. In a single gram of soil it is likely to be present more than 10^{10} prokaryotes and about 1,000 Gb of microbial genome sequences (Orgiazzi *et al.*, 2013). The knowledge of soil fungal community is important to understand the ecological interactions among organisms that occur in that ecosystem.

The conventional study of fungal communities relies on the identification of fungal species following their isolation, and/or culture and due to their morphological and biochemical tests. This widely used approach turned out to be time consuming and lead to problems in identification. More recently, molecular identification recurring to the sequencing of ITS regions has been the most reliable method for fungal identification. However to overcome the weaknesses (and sometimes the impossibility) of cultured-based identification of organisms, novel detection methods have been developed discarding the need to perform isolation and culture steps (Brunner *et al.*, 2007).

An analysis with a genomic perspective of the complexity of environmental samples is becoming an important tool to understand the evolutionary history, as well as the functional and ecological biodiversity of microorganisms. The parallel sequencing of multiple templates, obtained from environmental DNA samples that contain sequences from innumerous species, was only possible by the emergence of next-generation sequencing (NGS) approaches (Shokralla *et al.*, 2012). Given the high demand for this technology, a remarkable development of the sequencing systems become evident, which turned out to be faster and cheaper, and most of all with a higher level of precision (Gupta, 2008).

DNA barcoding for evaluating fungal communities

DNA barcoding is an efficient taxonomic method for identification of species present in unknown samples. In this method the information contained on the nucleotide sequence of a target region (DNA barcode) can be used for the efficient, fast and accurate species identification (Toju *et al.*, 2012). This approach relies on the amplification of 500 to 800 bp sequences that allows the identification of different taxa. The amplification makes use of primers that fits for the widest taxonomic group possible relying on the concept of a "universal product code", and produces an amplicon containing a specific sequence unambiguously attributed to a particular species (Brunner *et al.*, 2007). Barcoding is a very useful approach to apply since it makes use of stable and unique sequences that are specific to one species (Schoch *et al.*, 2012).

The major difficulty of DNA barcode is the genomic region that should be used as barcode, since it is the central point for multitaxon ecological and biodiversity studies. There are standardized barcodes according to the species to be identified. For most animals, a fragment of the mitochondrial *COI* gene is used as a barcode, while for plants a fragment of the plastid gene ribulose 1,5-bisphosphate carboxylase gene (*rbcL*) together with a fragment of the maturase (*matK*) gene are used (Cristescu, 2014). For bacterial identification, the 16S rDNA is used (Shokralla *et al.*, 2012), while the standardized barcode for fungi is the nuclear internal transcribed spacer (ITS) of the rDNA (Schoch *et al.*, 2012; Cristescu, 2014).

The fungal nuclear ribosomal DNA contains the information for three rRNAs (28S, 18S and 5.8S), which are separated by the internal transcribed spacer (ITS) regions (figure 2). Whole transcription unit is repeated *in tandem* many times, being the repeats separated by intergenic spacers (IGS). Two variable non-coding regions (ITS1 and ITS2) are comprised within the rDNA repeat, specifically between the small subunit 5.8S rDNA (highly conserved) and each of the large subunit rRNA genes (Gardes and Bruns, 1993; Brunner *et al.*, 2007; Nilsson *et al.*, 2009; Toju *et al.*, 2012).

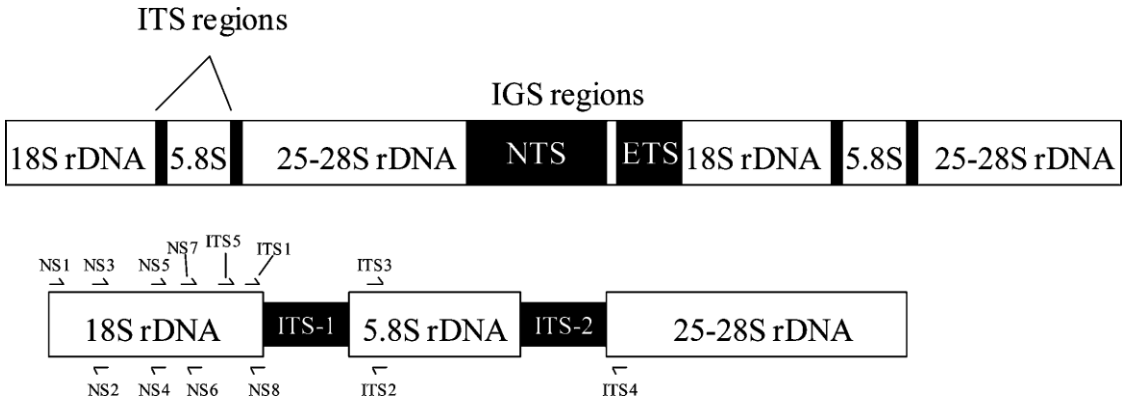


Figure 2 – Organization of the nuclear-encoded ribosomal RNA (rDNA) region including the repeating units of nuclear rDNA coding regions (Bugni and Ireland, 2004). ITS1 (ITS-1) relies between 18S and 5.8S rDNAs, while ITS2 (ITS-2) is located between 5.8S and 26S rDNA (in fungi). Universal primers used for barcoding approaches are depicted in arrows.

Since ITS regions do not contain information for proteins or RNAs, they present a great rate of evolution that turns them typically species specific. In spite of this, the 18S nuclear ribosomal small subunit rRNA gene (SSU) is usually used for phylogenetic studies in fungi. Nevertheless, ITS has been considered the most used barcoding region of fungi, in part due to the conserved regions used for primers targets. In addition, this region joins together the advantages of being a high resolution barcode at different taxa levels with the simple amplification of a multi-copy region. Indeed, a readily amplifiable product can be obtained even when studying samples with few amounts of initial DNA, like environmental samples from wood and soil, or partially degraded DNA (Nilsson *et al.*, 2008, 2009). Therefore, ITS barcodes combine the greatest resolving force for discriminating closely related fungal species with an increased PCR and sequencing efficiency through a widely range of fungi (Nilsson *et al.*, 2009; Schoch *et al.*, 2012).

For attaining ITS barcoding a set of specific primers were designed for the amplification of fungal ITS (White *et al.*, 1990). However, as many of these primers allowed the co-amplification of plant or even other eukaryotic DNAs (Anderson *et al.*, 2003), new primers (like ITSF1, based on the sequence of ITS1 primer), more specific for fungal sequences were designed by Gardes and Bruns (1993). In this way when using the pair of primers ITS1F/ITS2 the amplification become more selective towards fungi (Orgiazzi *et al.*, 2012).

The entire ITS region (≈ 650 bp) can be sequenced in a single round of Sanger sequencing, providing an extremely efficient method for the creation of DNA barcode references libraries. However, this first-generation system is only capable of sequencing individual specimens thus limiting the processing of complex environmental samples (Shokralla *et al.*, 2012). Due to the

high number of individuals present in an environmental sample, new and improved technologies for sequencing are required (Gupta, 2008; Shokralla *et al.*, 2012). This gave rise to the so-called "metabarcoding" strategy that can be described as a rapid method of biodiversity assessment that combines two technologies, DNA taxonomy and high-throughput DNA sequencing, where the purpose is to identify in just one experiment a large range of species (Hajibabaei, 2012; Taberlet *et al.*, 2012; Ji *et al.*, 2013; Ali *et al.*, 2014). This sequencing approach is capable of generating millions of sequences in just one run (Janzen *et al.*, 2005; Ji *et al.*, 2013). Therefore, next-generation sequencing (NGS) technology used for sequencing species-specific DNA barcodes is able to assist the mission of investigating the spreading of microbial communities patterns in complex environmental samples (Shokralla *et al.*, 2012; Orgiazzi *et al.*, 2013).

Next generation sequencing (NGS) in metabarcoding studies

The NGS technology extended the sequencing process across millions of reactions that occur simultaneously in a massively parallel fashion. As a result, the sequencing step is no longer limited to a single or few DNA fragments (Shokralla *et al.*, 2012). Besides the diverse chemistry and tools for base incorporation and/or detection used by the different NGS technologies, there are two key steps in common: library fragmentation/amplicon library preparation and detection of the incorporated nucleotides. Specific protocols are distinct among diverse technologies and determine the type of data that is produced in each platform.

Two types of classified NGS technologies can be distinguished: 'single-molecule' sequencing (SMS) technologies and technologies based on polymerase chain reaction (PCR) (Metzker, 2010; Shokralla *et al.*, 2012). SMS technology is an attractive approach due to its simplicity, and for being independent on a PCR amplification prior to sequencing (Pushkarev *et al.*, 2009; Shokralla *et al.*, 2012). HeliScope (Helicos BioSciences Corp., Cambridge, MA, USA) and PacBio RS SMRT system (Pacific Biosciences, Menlo Park, CA, USA) are two systems that use this technology. SMS technologies are known for being fast and cheap, producing a huge amount of data (Gupta, 2008; Harris *et al.*, 2008; Pushkarev *et al.*, 2009; Shokralla *et al.*, 2012). This technology is able of analyzing genomic information without having the need for cloning, amplification or ligation. In contrast, PCR-based NGS technology involves a PCR step that could introduce a possible bias due to amplification of DNA prior to sequencing, but also presents several advantages.

Next-Generation DNA sequencing technologies based on PCR

Nowadays four different sequencing platforms rely on technologies based on PCR: Roche 454 Genome Sequencer (Roche Diagnostics Corp., Branford, CT, USA), Applied Biosystems SOLiD™ Sequencer (Life Technologies Corp., Carlsbad, CA, USA), Illumina sequencers (Illumina Inc., San Diego, CA, USA) and Ion Personal Genome Machine (Life Technologies, South San Francisco, CA, USA) (Shokralla *et al.*, 2012). Besides generating improved sequences, these NGS sequencers are also less expensive comparatively to the conventional methods. The central point of these technologies provides a vigorous and economical workflow for better knowledge of specific genomic regions also allowing metabarcoding approaches (Harismendy *et al.*, 2009; Shokralla *et al.*, 2012; Taberlet *et al.*, 2012).

The first metabarcoding studies relied on 454 pyrosequencing. Pyrosequencing is a non-eletrophoretic method for DNA sequencing that makes use of real-time sequencing-by-synthesis technology, comprising methods dependent on DNA polymerase. This method does not need the cloning of environmental samples, therefore eliminating some of the problems that come associated with that step (Nilsson *et al.*, 2009). Sequencing based on 454 pyrosequencing is divided into three stages: sample preparation, emulsion PCR and sequencing (Carvalho and Silva, 2010; Shokralla *et al.*, 2012). When using genomic DNA, DNA is first fragmented, into 300 to 800 bp fragments, and 454 adaptors are attached to their ends. In a metabarcoding study, DNA from an environmental sample is amplified using barcode sequence primers attached to 454 adaptors. At the second stage (emulsion PCR) the fragments are hybridized to microspheres (beads), in a way that each fragment binds to just one bead. Then, fragments are submitted to amplification through emulsion PCR thermal cycling into single water:oil micro-reactors that contain the PCR mix. Here, every fragment is amplified on the surface of the corresponding bead, generating innumerous copies of the original fragment, which became fused to the surface of the bead. Finally, sequencing takes place in picotiter plates (PTP), which are designed to contain one million wells per plate, and only one DNA bead per well. Engineered beads that carry immobilized enzymes necessary for the 454 pyrosequencing process are also deposited inside each well. During pyrosequencing, every time a nucleotide is incorporated into the new chain, by DNA polymerase the release of a pyrophosphate molecule occurs. This release starts a series of enzymatic reactions that lead to the production of light by luciferase being the signal captured by the charge-coupled device (CCD) camera. The amount of light that is generated is proportional to

the number of nucleotides that were incorporated (Carvalho and Silva, 2010; Shokralla *et al.*, 2012). The emitted signals of light obtained after the introduction of each nucleotide introduction step will allow to determine the sequence produced by each bead.

3. Aims of the thesis

The main goal of this work was to study the influence of *H. fasciculare* on the microbial diversity of soils from chestnut orchards. Previous studies described high antagonist activity of *H. fasciculare* suggesting that the microbial richness and diversity will be expected to dramatically change in the presence of significant amounts of this fungal mycelium. Therefore, for determining the impact of *H. fasciculare* in this ecosystem, a metagenomic approach will be used for determining the soil fungal community by sequencing ITS1 barcodes from soil DNA samples using 454 pyrosequencing. Chestnut plants will grow on pots containing soil collected from chestnut orchards that will be then infected with *H. fasciculare*. Fungal community alterations will be followed by collecting soil samples along time (just before, six months and one year after the fungal inoculation) using a metabarcoding approach. For studying the contribution of native microbial ecosystem in *H. fasciculare* impact in plant development and microbial diversity, chestnut orchard soils will be used without or with previous sterilization.

CHAPTER II

MATERIAL AND METHODS

1. Chestnut and soil sampling

Chestnuts and soils were collected in a 100-year-old and non-tilled chestnut orchard, located in Terroso (N41° 52 W6° 50; 886 m altitude), in the Natural Park of Montesinho (74,800 ha) in Bragança (Trás-os-Montes, Northeast of Portugal). This orchard was chosen because, in contrast with others, displayed a low abundance of *H. fasciculare* carpophores between September 2002 and December 2005.

2. Experimental design

Production of *Castanea sativa* plantlets

Castanea sativa seeds were surface sterilized with sodium hypochloride (5%, v/v) during 1h and washed three times with sterile distilled water. After stratification, the germination took place in sterile moistened sand, at 5 - 10°C, for two months, following which, the radicle tips were pruned for promoting the root branching. The seedlings were then transferred into cuvettes, filled with sterile vermiculite/topsoil/sand (3:1:1, v/v/v), previously sterilized with formaldehyde at 4% (v/v). The plantlets were irrigated automatically for 30 seconds every 30 minutes and maintained under greenhouse conditions (day/night thermal regime 23°/18°±2°C, 10 h light/14 h dark photoperiod, and 70±10% relative humidity). After two months, the uniform plantlets were selected and transferred to 2 L plastic pots containing collected chestnut orchard soil, half of which were either submitted to sterilization in the autoclave (120°C for 1h) (S) or kept non-sterile (NS). Each pot was used for the growing of three chestnut plantlets.

Fungal inoculation of *Castanea sativa* plantlets

The *H. fasciculare* mycelium used for chestnut plantlets inoculation was grown in liquid-modified MMN medium, as described in Pereira (2012). The suspension cultures were kept in the dark, without agitation, at 23-25°C, for two weeks. Plant inoculation was carried out by transferring 50 mL of the *H. fasciculare* fungal suspension into a hole made at the root system level. Mock treatments were used as controls and were performed using 50 mL of sterile culture medium.

The inoculation of *H. fasciculare* into the chestnut plantlets pots (+Hf) was performed, in two different moments: two months (2M) or one year (1Y) after transplantation of plants to pots. Collection of plants and soil samples was performed six months (T6M) or one year (T1Y) after

fungal inoculation. Each treatment had three/four replicates (pots with three plants each), and for the pyrosequencing analysis some replicates of soil samples were mixed together (table 8, results and discussion). As controls, the same sampling periods were used in mock treatments. Every soil sample was thoroughly mixed and sieved using a 2 mm mesh, and stored at -80°C until DNA extraction. Although plant developmental and physiological parameters were studied in all these treatments, for metabarcoding analysis only selected treatments were used for DNA extraction (*cf.* table A 1, annex). The experimental design used in the present work are represented in figures 3 and 4, respectively.

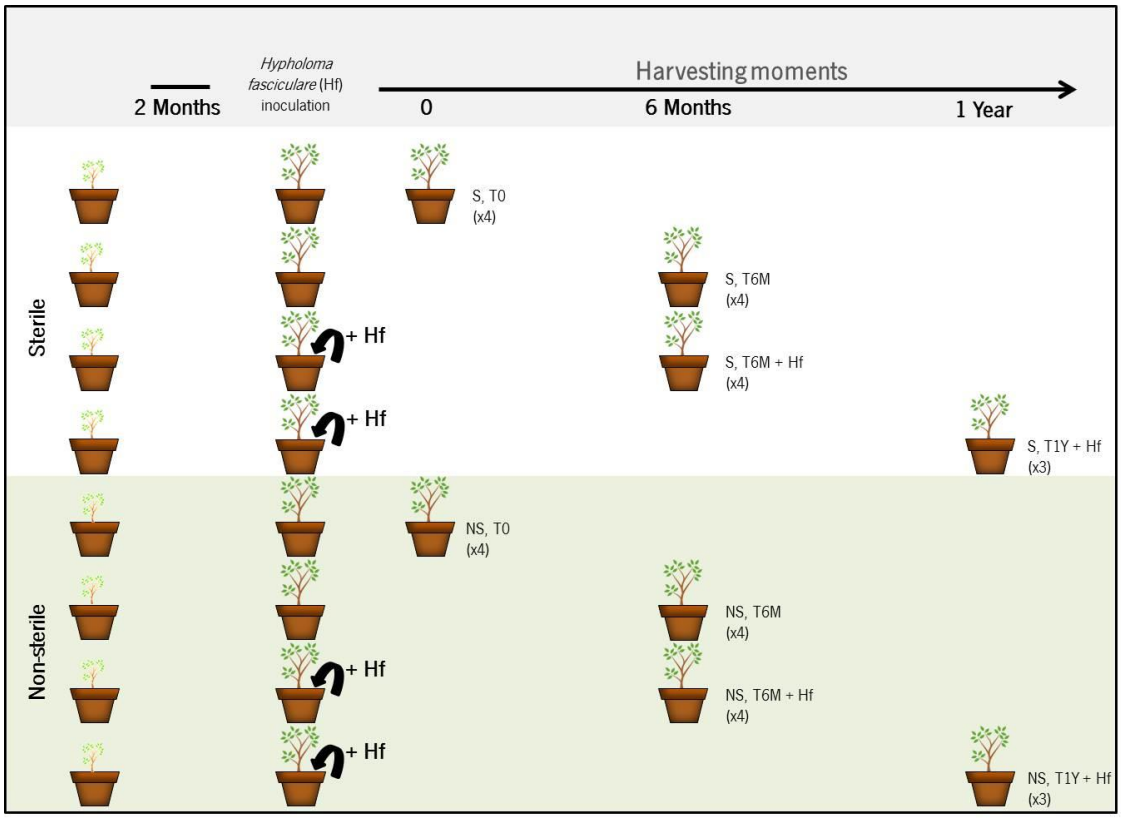


Figure 3– Experimental design used for evaluating the influence of *H. fasciculare* on the fungal community present in chestnut orchards by metabarcoding. Sterile (S) and non-sterile (NS) chestnut orchard soils were used for growing chestnut plantlets (3 plants/plot, 3-4 replicates) during two months. Inoculation of *H. fasciculare* (+Hf) was performed and soils were harvested immediately before inoculation (T0), six months (T6M) and one year (T1Y). Mock treatments were used as controls, the number of replicas used for metabarcoding are depicted in bracktes.

Figure 4 – Experimental stages. *Castanea sativa* plants after two months of growing in orchards soils that were previsously sterilized (S) or not (NS) (a) Soils preparation to perform the inoculation of *Hypholoma fasciculare* (b) and fungal inoculation (c).

3. DNA extraction

To test if the protocol of extraction worked, two simple samples were firstly tested, (S, T0 and NS, T0). DNA extraction was performed following the protocol of *Ultra Clean Soil DNA isolation kit* (MO BIO Laboratories) with few adaptations. Differents amounts of soil samples were prepared for being used for extracting DNA: 0.25 g from single soil sample or 0.125 g of different soils sample replicas to form a soil mix. A soil sample (0.25 g) was vortexed in the presence of a solution provided by the kit, containing SDS and other disruption agents to help in cell lysis. The mixture was briefly vortexed and centrifuged for 10 min. Supernatant was transferred to a new microtube and protocol proceeded as described in the instructions provided by the supplier, except the last step, where the ressuspension was performed with 100 µL of ultra pure water.

DNA samples were submitted to quantification and quality evaluation in the NanoDrop ND-1000 (NanoDrop Technologies) spectrophotometer by $A_{260\ nm}$ and $A_{280\ nm}$ readings. $A_{260\ nm}$ of 1.0 corresponds to 50 µL DNA/mL and the ratio $A_{260\ nm}/A_{280\ nm}$ was used to assess the purity of DNA samples. Dilutions were prepared from DNA stock samples, in order to get the same final concentration (10 ng/µL) of each sample for obtaining reliable results.

4. DNA amplification

DNA amplification was performed by the polymerase chain reaction (PCR), a technique which allows the production of millions of copies of a specific DNA sequence in a short time. In order to guarantee a homogenous amplification of ITS1 from environmental samples, three independent reactions of each DNA sample were performed. To perform 454 pyrosequencing it was necessary to design specific primers for the ITS regions of fungi that would be used as barcodes. The primers (FwITS1F and RvITS2) thus contained the ITS sequence primer, ITS1F (based on ITS1 sequence) and ITS2 (both depicted in figure 2), respectively, as well as adapters for the

pyrosequencing (A or B) and a four-base library "key" sequence (TCAG) (table 3). In addition, the reverse primer included multiplex identifier sequences (MID) for the identification of each soil sample (figure 5). Therefore, for every DNA sample the primer forward FwITS1F was combined with a different reverse primer, which differs according to the MID sequence that is included. For the amplification, a reaction mix to be used in the PCR reaction was prepared (table 4).

Table 3 - Set of primers used for the ITS region amplification.

Primer	Sequencing adaptor	Key	MID	Specific template
FwITS1F	A - CGTATCGCCTCCCTCGCGCCA	TCAG	-	CTTGGTCATTTAGAGGAAGTAA
RvITS2	B - CTATGCGCCTTGCCAGCCCGC	TCAG	XXXXXXXX*	GCTGCGTTCTTCATCGATGC

XXXXXXXX* - Represents the specific sequence of which sample, respectively described next: S, T0 (A) - (ACGAGTGCGT); S, T0 (B) - (ACGCTCGACA); S, T0 (C) - (AGACGCACTC); S, T0 (D) - (AGCACTGTAG); S, T6M (A) - (TGTACTACTC), S, T6M (B) - (ACGACTACAG); S, T6M (C) - (CGTAGACTAG); S, T6M (D) - (TACGAGTATG); S, T6M + Hf (A) - (TCTCTATGCG); S, T6M + Hf (B) - (TGATACGTCT); S, T6M + Hf (C) - (CATAGTAGTG); S, T6M + Hf (D) - (CGAGAGATAC); S, T1Y + Hf (A) - (ACGCGAGTAT); S, T1Y + Hf (B) - (ACTACTATGT); S, T1Y + Hf (C) - (ACTGTACAGT); NS, T0 (A) - (ATCAGACACG); NS, T0 (B) - (ATATCGCGAG); NS, T0 (C) - (CGTGTCTCTA); NS, T0 (D) - (CTCGCGTGTC); NS, T6M (A) - (TACTCTCGTC); NS, T6M (B) - (TAGAGACGAG); NS, T6M (C) - (TCGTCGCTCG); NS, T6M (D) - (ACATACGCGT); NE, T6M + Hf (A) - (ATACGACGTA); NS, T6M + Hf (B) - (TCACGTACTA); NS, T6M + Hf (C) - (CGTCTAGTAC); NS, T6M + Hf (D) - (TCTACGTAGC); NS, T1Y + Hf (A) - (AGACTATACT); NS, T1Y + Hf (B) (AGCGTCGTCT); NS, T1Y + Hf (C) - (AGTACGCTAT).

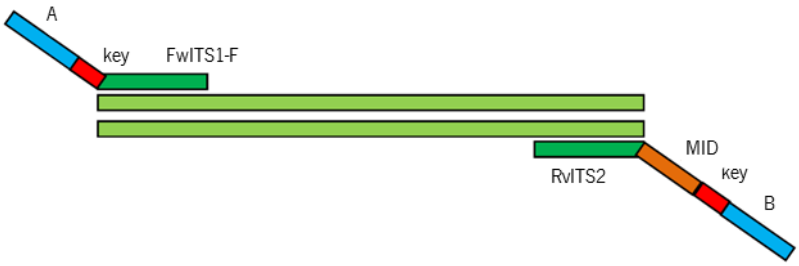


Figure 5 – Representation of the primers used for the ITS1 region amplification comprising: the adapters A and B for the pyrosequencing (blue), the four-base library "key" sequence (red), the multiplex identifier sequence (MID) (orange) and the template-specific sequence for the amplification for the ITS1 region, primer forward FwITS1-F and the reverse primer ITS2 (dark green).

Table 4 - Components used for each PCR amplification.

Components of PRC reaction	Volume (µL)
ddH_2O	22.5
PCR Buffer (10x)	10
$MgCl_2$ (25 mM)	4
dNTPs (10 mM)	1
FastStart Taq DNA Polymerase (Roche)	0.5
Primer FwITS1 (10mM)	1
Primer RvITS2 (10mM)	1
DNA (4 ng/µL)	10
Total	50

To perform the DNA amplification, a reaction mix was prepared and the amplification program presented in table 5 was used in a *MJ Mini BioRad®* thermocycler. After amplification, PCR products were kept at 4°C up to electrophoretic analysis and then stored at –20°C until 454 pyrosequencing.

Table 5 - Amplification program.

Step	Cycle number	Temperature	Time
Initial denaturation	1	94 °C	4 min
Denaturation	32	94 °C	30 s
Annealing		50 °C	45 s
Extension		72 °C	45 s
Final extension	1	72 °C	10 min

5. Agarose gel electrophoresis

Electrophoresis was performed in an agarose gel at 1.2 % (w/v) and occurred in 0.5x TAE buffer (0.01 M Tris, pH 8.0; 47.5 mM acetic acid, 25 mM EDTA), for 30 min at 100 V. For DNA visualization, 100 µL of *SYBR safe* (Invitrogen) was added to 100 mL melted agarose solution. To 10 µL of each DNA sample, 3 µL of 1x loading buffer (1.6 mM Tris-HCl, pH 7.6; 6% (v/v) glycerol; 6 mM EDTA; 0.005% (w/v) bromophenol blue) was added. A molecular marker was also loaded into the gel (Mass Ruler ™ DNA Ladder Mix, Fermentas). Observation of DNA fragments was performed on a 610 nm UV transilluminator (VWR Genosmart, VWR) coupled to an image acquisition system (VWR Genosmart, VWR).

6. High throughput sequencing - 454 pyrosequencing

Amplification products were quantified by spectrophotometry, using the Nanodrop ND-100 (NanoDrop Technologies), as previously described. DNA amounts (117 ng) from the three/four amplification replicas, obtained from each soil sample combination, were pooled together into a single microtube and stored at 4°C. All the 30 microtubes were delivered at BioCant (Cantanhede, Portugal), where the subsequent steps were performed. To guarantee that equal amounts of DNA from each sample were used in the emulsion PCR, DNA quantification by fluorescence with Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, CA, USA) was performed. The number of molecules in each and every sample was defined in accordance to the size of the predicted amplicon (250-350 bp).

Pyrosequencing occurred in a *Genome Sequencer GS FLX Titanium* (Roche-454 Life Sciences, Brandford, CT, USA), in a ¼ of a PTP sequencing plate, from adaptor B, according to the standard manufacturer's instructions at Biocant (Cantanhede, Portugal). Sequencing program was adjusted to produce an average of 7,000 reads per sample core.

7. Sequence processing and data analysis

A first step to filter the sequence data was performed at BioCant. This first quality filter aims to reduce the random sequencing errors. So, the sequences that presented less than 120 bp and displayed more than two ambiguous nucleotides (N) were discarded. The sequences in which the reverse primer was reached were also eliminated. Bases that presented low quality regions in both ends were trimmed through the use of a window of 7 bases, whose average phred score was 15. Sequences with more than 50% of low complexity regions, determined by DustMasker (Sogin *et al.*, 2006) and chimera sequences identified by UChime (Edgar *et al.*, 2011) were also discarded. Following this first treatment, the total number of quality reads was submitted to an analysis process in Metagenomics Analysis Server MG-RAST version 3.3.7.3 (Meyer *et al.*, 2008). Here, the identification cutoff parameters were: maximum e-value of $1e^{-6}$, minimum identity of 97% and 50 bp as minimum of alignment length. Operational taxonomic units (OTUs) were identified by sequence top BLAST hit compared against the SILVA LSU database.

For comparing the most well-represented OTUs between soils samples, only those OTUs that presented more than 50 reads were considered. This cutoff number was determined according to the influence of cutoff values in the resulting number of reads and identified OTUs number. The identification of trophic level of each well-represented OUT was performed by literature search, determining: which were mycorrhizal (M), parasite (P), parasite/saprotroph (PS), saprotroph (S), yeast (Y) or were unclassified (Un).

8. Diversity and statistical analysis

The species diversity is a measure of diversity of a community that takes into account the species richness and their abundance.

Species richness refers to the number of different species (in this case OTUs) present in the community, whereas species abundance refers to the number of individuals (in this case, reads) of each species. Both parameters were investigated and analyzed by *Species Diversity and*

Richness 4.1.2. (SDR) (Seaby and Henderson, 2007), *EstimateS 9.1.0.* (Colwell, 2013) and *Community Analysis Package 4.0* (CAP) (Henderson and Seaby, 2007) softwares.

Alpha diversity indices, such as Simpson diversity index (D) (in its reciprocal form), Shannon diversity index and Fisher's alpha, are usually calculated for determining the diversity of specific community taking into account both the number of species and the proportion in which each species is represented. These parameters were computed by *EstimateS 9.1.0* software (Colwell, 2013), as well as the species richness estimators (Chao1 and 1st order Jackknife). All these parameters will be shortly described:

- *Simpson's index* (Simpson, 1949) (equation 2.1) measures the probability of any two randomly chosen individuals taken from an infinitely large community belong to the same species. The value of D is indirectly proportional with the value of diversity. Therefore this index is usually expressed as 1 - D (complement) or 1/D (reciprocal). This index is more focused on the abundance of species in the sample than the species richness itself and thus retains the variance of the species abundance distribution. *Shannon-Wiener index* or *Shannon index* (H') (Shannon, 1948) (equation 2.2) is a commonly used index to characterize species diversity in a single community. This index computed the uncertainty associated with identifying species in a community. For this Shannon index assumes that all species are represented in the community and the individuals are randomly sampled from an infinitely large community (Magurran, 2004).

$$D = -\sum_{i=1}^{S} p_i^2 \qquad \text{(Equation 2.1)}$$

$$H' = -\sum_{i=1}^{S} p_i \ln p_i \qquad \text{(Equation 2.2)}$$

where:

S, number of species

p_i, proportion of the species *i* $p_i = n_i/N$

n_i, number of individuals of specie *i*

N, total number of individuals

ln *pi*, base 2 logarithm of *pi*

- *Fisher's alpha* (Fisher *et al.*, 1943) is a parametric index of diversity that accepts that species abundance follows a log series distribution and can be depermined by (equation 2.3) (Colwell, 2013).

$$S = \alpha \ln\left(1+\frac{N}{\alpha}\right) \quad \text{(Equation 2.3)}$$

where:

S, number of taxa

N, number of individuals

α – Fisher's alpha; N(1-x)/x

- *Jackknife* (1st and 2nd order) (Burnham and Overton, 1978, 1979), *Chao1* (Chao, 1984; Colwell and Coddington, 1994) and *Chao2* (Chao, 1987; Colwell and Coddington, 1994) are richness species measures that allow the estimation of the total number of species in one specific community from sample data. The 1st order Jackknife (equation 2.4) estimates the total richness using the number of species present in only one sample (Barros, 2007), whereas Chao1 (equation 2.5) uses the number of species represented by just one individual (singletons) and two individuals (doubletons) in the samples (Magurran, 2004; Barros, 2007).

$$S_J = s + Q_1\frac{n-1}{n} \quad \text{(Equation 2.4)}$$

$$S_J = s + \frac{Q_j^2}{2Q_J} \quad \text{(Equation 2.5)}$$

where:

S_J, estimated richness

s, observed richness

Q_j, number of species that occur in *j* samples

n, number of samples

The number of species/OTUs shared between soil samples was obtained by *Species Diversity and Richness 4.1.2* software (Seaby and Henderson, 2007). The coefficients of similarity (Jaccard and Sørensen indexes, as well as Bray-Curtis coefficient) between soils samples were calculated by *Community Analysis Package 4.0* software (Henderson and Seaby, 2007). For determining the variation between two sites, indexes of beta diversity were computed. These indexes measure the turnover of species between two sites, in terms of gain or loss of species, contributing for the understanding of how a community differs according to their species composition (Barros, 2007), and what manages that diversity (Condit *et al.*, 2002). Several measures appropriate for the analysis of presence and absence data were also evaluated, such

as, Whittaker ($ß_w$), Cody ($ß_c$), Routledge ($ß_r$, $ß_i$, $ß_e$), Wilson and Shmida ($ß_t$) and Harrison (1 and 2) beta diversity measures by *Species Diversity and Richness 4.1.2* software (Seaby and Henderson, 2007). All these similarity and diversity parameters will be shortly described:

- *Jaccard* (Jaccard, 1908) (equation 2.6) and Sørensen (Sørensen, 1948) (equation 2.7) indexes are similarity coefficients used for the determination of beta diversity analysis. Both indexes compare communities qualitatively (Barros, 2007).

$$ß_j = a/(a+b+c) \quad \text{(Equation 2.6)}$$

$$ß_{sor} = 2a/(2a+b+c) \quad \text{(Equation 2.7)}$$

where:

a, number of species found in both communities, A and B

b, number of species found in community B but not in A

c, number of species found in community A but not in C

- The *Bray-Curtis coefficient* (Bray and Curtis, 1957) (equation 2.8) is, also a coefficient for beta diversity, that is calculated according to the differences in abundance of each species between sites. As a consequence, the final distance will be influenced by the species with the largest differences in abundance (Kindt and Coe, 2005; Somerfield, 2008; Yoshioka, 2008).

$$S=100\frac{\sum_{i=1}^{p} 2\min(Y_{ij},Y_{ik})}{\sum_{i=1}^{p}(Y_{ij}+Y_{ik})} \quad \text{(Equation 2.8)}$$

where:

Y_{ij} and Y_{ik}, measure of species *i* in samples *j* and *k*,

Min (Y_{ij}, Y_{ik}), minimum of Y_{ij} and Y_{ik}

p, number of species

- *Whittaker* ($ß_w$) measure (Whittaker, 1960) analyzes the modification in the composition of a community, according to environmental patterns (equation 2.9). On its turn, *Cody* ($ß_c$) (1975) definies the rate of compositional turnover along a environmental gradient (equation 2.10) (Tuomisto, 2010). *Routledge* ($ß_r$) measure (equation 2.11), is quite similar to $ß_w$ measure and appears to be suitable for ecological analysis of community data, conducting to the meaning of species turnover along gradients. Besides analyzing the presence and absence data, the

Routlegde (ß) measure also appeals for the symmetry of samples sizes (equation 2.12), while Routledge ($ß_e$) refers to the community turnover (equation 2.13) (Routledge, 1977).

- *Wilson & Shmida* ($ß_i$) (Wilson and Shmida, 1984) is a measure of beta turnover, which combines the concept of species turnover with the modification due to gain and loss of species along the gradient, similar to $ß_c$ but using a standardization by average sample richness (equation 2.14).

- *Harrison 1* (Harrison, 1992) (equation 2.15) can be used in order to examinate the differentiation between sites. *Harrison 2* (Harrison, 1992) measures the value by wchich regional diversity outrun the maximum diversity attained locally (equation 2.16).

$$ß_W = \frac{S}{\acute{\alpha}} - 1 \quad \text{(Equation 2.9)}$$

$$ß_c = \frac{g(H)+l(H)}{2} \quad \text{(Equation 2.10)}$$

$$ß_r = \frac{S^2}{2r+S} \quad \text{(Equation 2.11)}$$

$$ß_i = \log(T) - \left(\frac{1}{T}\sum_i ei \log(e_i)\right) - \left(\frac{1}{T}\sum_j \alpha j \log(\alpha_j)\right) \quad \text{(Equation 2.12)}$$

$$ß_e = \exp(ß_i) - 1 \quad \text{(Equation 2.13)}$$

$$ß_c = \frac{g(H)+l(H)}{2\acute{\alpha}} \quad \text{(Equation 2.14)}$$

$$ß_1 = \left[\frac{S}{\acute{\alpha}} - 1\right]/(N-1) \quad \text{(Equation 2.15)}$$

$$ß_1 = \left[\frac{S}{\acute{\alpha}_{max}} - 1\right]/(N-1) \quad \text{(Equation 2.16)}$$

where:

S, total number of species in both samples

N, number of samples

T, total number of species

r, number of species found

g, cumulative gain in species

l, cumulative loss in species

H, range of habitat gradient

$\acute{\alpha}$, average number of species found within samples

$\acute{\alpha}_{max}$, maximum value of species richness for the two samples

α_j and e_i, total number of species found in *j* and *I*, respectively

Non-metric multidimensional scaling (NMDS) was carried out to explore the similarity of soil fungal community among treatments (sterile or non-sterile soils samples, with or without fungal inoculation and with different chestnut growing times). The NMDS plots rank fungal communities (represented by points) in ordination space in a way that the distance between two points is inversely proportional to their similarity. The correspondence of the ordination diagram to the distances is described by a stress value (Kruskal's stress), with values less than 0.2 representing good ordination plots and greater than 0.3 provides a poor representation (Clarke, 1993). NMDS was performed by using Jaccard's and Bray-Curtis similarities matrices.

A *Pearson* correlation coefficient was estimated in order to determinate the association between functional groups of fungi. This analysis was performed for both richness and abundance. Rarefaction curves were computed by *Species Diversity and Richness 4.1.2* software (Seaby and Henderson, 2007). Graphics and ANOVA statistical analysis between diversity parameters was determined by GraphPad Prism 4.0 software (San Diego, CA).

CHAPTER III

RESULTS AND DISCUSSION

Castanea sativa is a tree with major historical, economic and ecological importance in Portugal, in which high forest stands are located in the Northwest regions (Trás-os-Montes). The saprotrophic fungi *Hypholoma fasciculare* [currently designated as *H.* acutum (Sacc.) E. Horak 1971] is often found in these "soutos", where is thought to be harmful to the soil fungal community associated to *C. sativa* (Baptista, 2007). According to previous results, the plant interactions established with this alleged antagonist may also be detrimental for chestnut tree sustainability, as reported on the study of Pereira *et al.* (2012). For studying the impact of *H. fasciculare* on chestnut tree development and soil fungal community present in chestnut orchards, the abundance of *H. fasciculare* carpophores that was witnessed between September 2002 and December 2005 was determinant to select the chestnut orchards to be studied. As we pretended to inoculate the soils with this fungus, soils from the orchard that presented the lowest abundance of *H. fasciculare* carpophores (Terroso, Bragança) were collected and used all over the work.

1. Impact of *Hypholoma fasciculare* on chestnut tree development

For evaluating the plant development and physiological conditions of plants growing in the presence of *H. fasciculare* (previous work performed by Pereira *et al.* unpublished data), chestnut plantlets were subjected to *H. fasciculare* infection (+Hf). These plants were grown in sterile (S) or non-sterile (NS) chestnut orchard soils; and plant samples were taken at differents periods – immediately before (T0), six months (T6M) or one year (T1Y) after inoculation (*cf.* figure 3, material and methods). Several growth parameters, such as stem and root lengths, daily increase of shoot dry weight (DWD), and root collar diameter were determined for evaluating plant development (table 6; figure 6).

As expected, a general increase of growth parameters occurred during the period of the experiment (differences detected between T0, T6M and T1Y samples), for both inoculated and non-inoculated chestnut plants, after two months upon transplantation (2M). When considering the plants that were inoculated one year upon transplantation (1Y) this increase was not always evident (table 6). For 2M chestnut plants, such increases were always more evident after *H. fasciculare* inoculation, specifically when using sterile soils (S) rather than non-sterile soils (NS). In older inoculated soils this patterns was also evident however with inferior differences. The higher stem lengths were detected in inoculated (S, 2M, T6M), (S, 2M, T1Y) and (S, 1Y, T6M) plants, which values were significantly higher (1.5-, 1.35- and 1.42-fold, respectively) than

the corresponding (NS, 2M, T6M), (NS, 2M, T1Y) and (N, 1Y, T6M) plants. While S plants displayed evident stem lenght increases after *H. fasciculare* inoculation (up to 1.29- and 1.46-fold in relation to non-inoculated plants, for 2M and 1Y plants, respectively), NS plants were negatively affected by *H. fasciculare* (up to 0.93- and 0.77-fold decrease in relation to non-inoculated plants, for 2M and 1Y plants, respectively) (figure 6a). These results are corroborated by the analysis of the DWD, where higher increases of about 1.2-fold (not significant) and 1.16-fold (significant) were detected after *H. fasciculare* inoculation in (S, 2M, T6M + Hf) and (S, 1Y, T1Y + Hf) plants, when compared to (NS, 2M, T6M + Hf) and (NS, 1Y, T1Y + Hf) plants, respectively (figure 6b). However, for this growth parameter a positive effect of *H. fasciculare* was noticed in both S and NS plants. For this growth parameter, a major difference between younger (2M) and older (1Y) chestnuts plants was evident, which is certainly due to the plant developmental stages studied that determines a higher daily increase on the dry weight in early development (2M) than in more advanced stages (1Y). Concerning the root length, the inoculated (S, 2M, T6M) plant exhibited significant higher length (1.7-fold higher) than its correspondent non-inoculated (S, 2M, T6M) plant. On the other hand, (S, 1Y, T6M + Hf) exhibited higher length (1.28-fold higher) than the correspondent (NS, 1Y, T6M + Hf) plant. Generally, 2M inoculated plants exhibited higher growth than the corresponding non-inoculated plants. In 1Y plants the inverse pattern was observed, inoculated samples appear to be negatively affected by *H. fasciculare* since non-inoculation samples exhibited higher root length (although not significant) in NS soils (figure 6c). Regarding the root collar diameter, a gradual increase was evidenced along the different times of chestnut growing, even though the soil condition (S or NS). However this increase was significantly higher in (S, 2M, T6M + Hf) comparing to its non-inoculated correspondent (S, 2M, T6M; 2.4-fold higer), but also to its non-sterile correspondent (NS, 2M, T6M + Hf; 1.71-fold higher). For (S, 2M, T6M) plants a significant decrease was evident when comparing with the correspondent non-sterile plant (NS, 2M, T6M; 0.56-fold lower). While 1Y plants, only (NS, 1Y, T6M) exhibited significant differences in both sterile correspondent (S, 1Y, T6M; 1.44-fold higher) and inoculated correspondent (NS, 1Y, T6M + Hf; 1.64-fold higher) (figure 6d). As controls, non-infected plants were studied in the same conditions. Therefore the overall results revealed that plants growing in previously sterile soil (S), whose inoculation was perfomed in chestnut plants transplanted two months ago, presented an increased growth that was more evident in the first six months upon inoculation (S, T6M + Hf). In plants grown on non-sterile soil (NS) there was no significant differences on the growth of *C. sativa* plants in inoculated

and control plants. This suggests that soil sterilization generally improve *C. sativa* root growth in the presence of *H. fasciculare* during the first six-months after inoculation. Afterwards, this effect seems to be dissipated. As in sterile soils a lower microbial diversity was detected, more nutrients may be ultimately available for *C. sativa* growing. This effect seems to be further improved by the presence of *H. fasciculare* within the first six months after inoculation. Indeed, in non-inoculated plants no significant differences were found on plant growth between sterile and non-sterile soil, but after *H. fasciculare* inoculation a statistically difference become evident. After one year of inoculation, the supression of root growth on chestnut plantlets growing in sterile soils, could be due to the development of a new microflora in those soils that could compromise the availability of nutrients.

On the other hand, in plants whose inoculation was performed after one year of transplantation (1Y), no evident differences in growth were detected, either in previously sterile or non-sterile pots, as well as in the presence or absence of *H. fasciculare* (table 6).

The occurrence of mycorrhization in *Castanea sativa* root tips was also evaluated by direct observation of ectomycorrhized root tips, followed by barcoding with ITS sequencing. The inoculation of *H. fasciculare* reduced the number of mycorrhized chestnut root tips of plants growing in S (up to 1.1- to 1.8-fold lower) and NS (up to 1.2- to 2.5-fold lower) soils, but also the diversity of morphotypes (figure 7) (Pereira *et al.*, unpublished data), being mostly present the species *Laccaria sp.* (mainly in non-sterile soils) and *Laccaria proxima* (mainly in sterile soils). Therefore, the previous work performed by Pereira *et al.*, (unpublished) suggested that the saprotrophic *H. fasciculare* do not seem to cause harm to chestnut plants, since a reduction of growth parameters was not detected, but could be rather advantageous for chestnut plants, in particular when they are growing in soils with a poor microbial community. However, a long term effect could still be occuring, since a decrease on ectomycorrhization and ECM diversity were observed. Concerning the comparison between S and NS soils, the percentage of mycorrhized roots was generally similar or lower in the sterile soil than in non-sterile ones (up to 1.1-fold lower). This pattern was the same for inoculated and non-inoculated *C. sativa* plants, and after six-months and one year following inoculation (figure 7).

Table 6 - Growth of *Castanea sativa* plants, inoculated with *Hypholoma* fasciculare after two-months or one year following transplantation of DWD – daily increase of shoot dry weight. For each evaluated parameter, values (x±SD) followed by different letters are significantly different between treatments (sterile and non-sterile, inoculated and non-inoculated) within each harvesting date (p≤0.05) (previous work performed by Pereira *et al.*, unpublished data). Cells in grey correspond to the samples that will be further used for the metabarcoding.

	Inoculation of *Hypholoma fasciculare* two months after transplantation									
	S,T0	NS,T0	S,T6M+Hf	S,T6M	NS,T6M+Hf	NS,T6M	S,T1Y+Hf	S,T1Y	NS,T1Y+Hf	NS,T1Y
Stem lenght (cm)	14.61±1.17^{a}	13.94±0.91^{a}	21.78±1.56^{a}	20.05±1.21^{a}	14.51±0.92^{b}	17.67±1.40^{a,b}	25.86±2.24^{a}	20.03±1.58^{a,b}	19.21±1.67^{b}	20.57±1.47^{a,b}
DWD (mg/day)	0±0^{a}	0±0^{a}	2.68±0.37^{a}	1.70±0.19^{b}	2.23±0.20^{a,b}	1.86±0.24^{a,b}	2.60±0.51^{a}	2.27±0.31^{a}	2.27±0.38^{a}	1.82±0.24^{a}
Root lenght (cm)	27.10±1.95^{a}	33.07±2.88^{a}	39.30±4.32^{a}	23.07±1.50^{b}	31.8±1.65^{a,b}	31.06±2.95^{a,b}	36.85±3.56^{a}	31.6±1.47^{a}	35.54±2.72^{a}	31.49±2.25^{a}
Root collar (cm)	0.10±0.02^{a}	0.15±0.02^{a}	0.36±0.03^{a}	0.15±0.03^{c}	0.21±0.02^{b,c}	0.27±0.03^{a,b}	0.39±0.06^{a}	0.28±0.03^{a}	0.32±0.04^{a}	0.31±0.05^{a}
	Inoculation of *Hypholoma fasciculare* one year after transplantation									
	S,T0	NS,T0	S,T6M+Hf	S,T6M	NS,T6M+Hf	NS,T6M	S,T1Y+Hf	S,T1Y	NS,T1Y+Hf	NS,T1Y
Stem lenght (cm)	19.41±2.40^{a}	17.84±1.41^{a}	25.58±2.43^{a}	17.49±1.52^{b}	17.98±1.51^{b}	23.21±1.38^{a,b}	22.91±2.43^{a}	21.17±1.13^{a}	20.09±1.47^{a}	20.12±1.96^{a}
DWD (mg/day)	0.45±0.02^{a}	0.44±0.02^{a}	0.31±0.02^{a}	0.29±0.01^{a}	0.29±0.01^{a}	0.28±0.02^{a}	0.51±0.02^{a}	0.45±0.01^{a,b}	0.44±0.02^{b}	0.41±0.02^{b}
Root lenght (cm)	28.7±2.64^{a}	33.74±2.58^{a}	33.61±2.47^{a}	43.40±4.34^{a}	26.22±1.75^{b}	35.25±1.02^{a,b}	24.03±2.30^{a}	21.62±2.40^{a}	23.8±3.31^{a}	25.13±3.00^{a}
Root collar (cm)	0.18±0.03^{a}	0.18±0.04^{a}	0.44±0.03^{b}	0.41±0.03^{b}	0.36±0.04^{b}	0.59±0.04^{a}	0.46±00.4^{a}	0.37±0.05^{a}	0.45±0.03^{a}	0.41±0.04^{a}

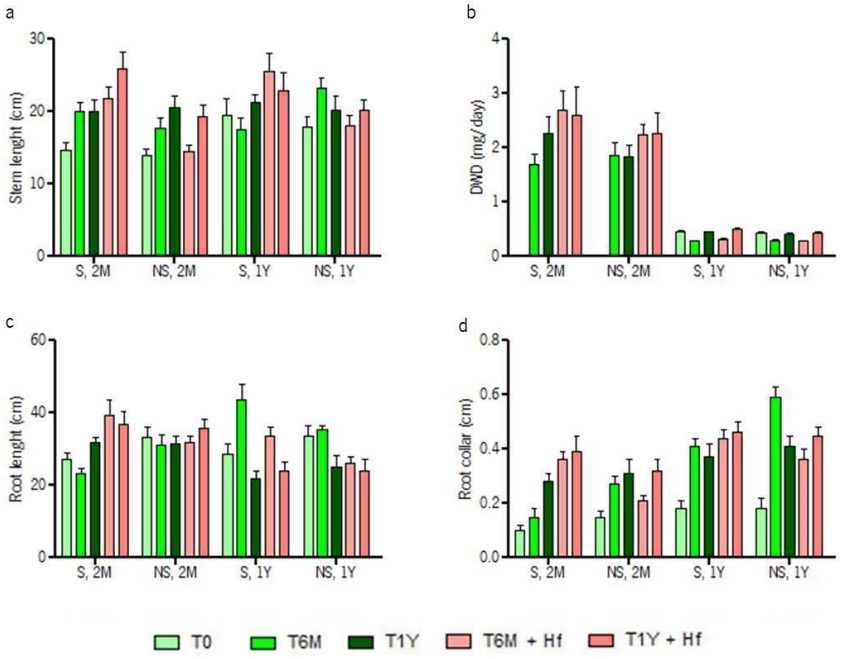


Figure 6 – Growth of *Castanea sativa* plants, inoculated two-months (2M) or one year after transplantation (1Y), in both sterile (S) and non-sterile (NS) soils samples. Sampling was performed after different periods upon inoculation - (T0 - just before inoculation, T6M – six months, T1Y – one year after inoculation). Four physiological parameters were evaluated (stem length – a, DWD - b, root length - c, and root collar diameter – d) (based on the previous work performed by Pereira *et al.*, unpublished data).

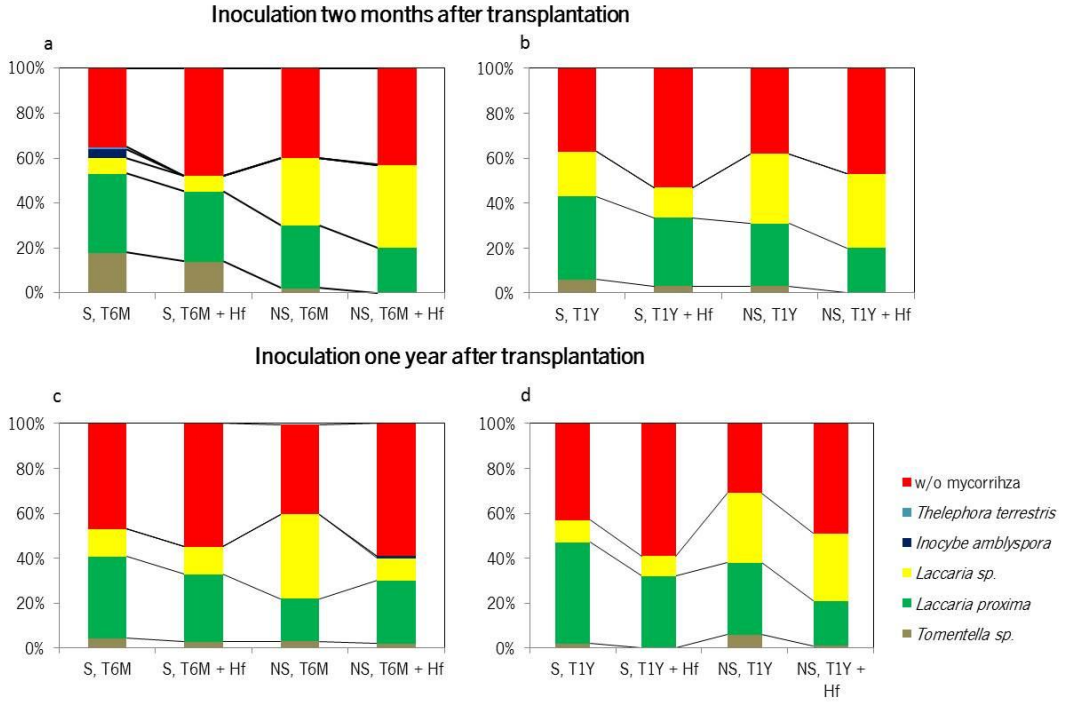


Figure 7 – Degree of *Castanea sativa* mycorrhization after six-months (6M, a and c) or one-year (1Y, b and d) of plant inoculation with *Hypholoma fasciculare* (+ Hf). Fungal inoculation occured two months (a and b) or one year after transplantation (c and d) on sterile and non-sterile (S and NS) soils (Pereira *et al.,* unpublished data).

The present work uses the same plants as used in this study, but investigates the variations on fungal community in a metagenomics perspective.

2. Analysis of soil fungal biodiversity by metabarcoding

Preparation of DNA samples for metabarcoding analysis

For determining the effect of *H. fasciculare* on fungal community the soil samples to be studied were chosen considering the results obtained for growth parameters. From the general comparison between plants whose inoculation with *H. fasciculare* was performed two months (2M) or one year after transplantation (1Y) (table 7), the older plants (one year old) were discarded for the metagenomics analysis, once no differences between growth parameters were evident. Even for those plants in which *H. fasciculare* was inoculated at two months after transplantation, no significant differences were found in plants harvested after one year, either in previously sterile or non-sterile soils, as well as with or without *H. fasciculare*. With the purpose to follow the fungal community, only (S, T1Y + Hf) and (NS, T1Y + Hf) samples were evaluated. Therefore, a total of eight different soil samples were evaluated by metabarcoding, three to four replicas of each were used, resulting in 30 different libraries that were sequenced (table A 1, annex).

In order to proceed to the 454 pyrosequencing, DNA from the 30 different libraries was extracted. The origin of each sample (mixture of soil replicas or not), as well as the obtained DNA concentration and A_{260}/A_{280} ratio, are displayed in table 7. The concentrations of DNA samples ranged between 4 – 29.7 ng/μL, A_{260}/A_{280} ratios near 2.0 revealed the good quality of samples to proceed with barcode amplification.

Table 7 – List of samples [type of soil (simple or mix), concentration of the DNA samples, and ratios estimated by NanoDrop ND-1000 (NanoDrop Technologies)] to perform DNA extraction.

Samples	Misture of soil mix	Concentration (ng/μL)	A_{260}/A_{280}
S, T0 (A)	No	4	2.88
S, T0 (B)	No	5.1	1.98
S, T0 (C)	No	11.1	1.99
S, T0 (D)	Yes	4.3	1.97

Table 7 – (Continuation)

Samples	Misture of soil mix	Concentration (ng/µL)	A_{260}/A_{280}
NS, T0 (A)	No	11.2	2.07
NS, T0 (B)	No	9.4	2.03
NS, T0 (C)	No	13.4	2.57
NS, T0 (D)	Yes	7.8	3.99
S, T6M + Hf (A)	No	18.7	1.93
S, T6M + Hf (B)	No	14.6	2.12
S, T6M + Hf (C)	No	10.7	2.36
S, T6M + Hf (D)	Yes	7.4	2.3
NS, T6M + Hf (A)	No	12.3	1.81
NS, T6M + Hf (B)	No	19.2	1.83
NS,T6M + Hf (C)	No	18.8	1.78
NS, T6M + Hf (D)	Yes	19.6	1.85
S, T6 (A)	No	17.1	2.29
S, T6 (B)	No	10.5	1.90
S, T6 (C)	No	7.8	2.50
S, T6 (D)	No	29.7	1.78
NS, T6 (A)	Yes	16	1.88
NS, T6 (B)	No	20	1.78
NS, T6 (C)	No	14.6	1.80
NS, T6 (D)	No	23.6	1.77
S, T1Y + Hf (A)	Yes	18.2	2.08
S, T1Y + Hf (B)	Yes	11.2	1.90
S, T1Y + Hf (C)	Yes	10	2.07
NS, T1Y + Hf (A)	Yes	25.6	1.74
NS, T1Y + Hf (B)	No	10	1.24
NS, T1Y + Hf (C)	No	13.7	1.76

After the extraction and quantification, the DNA stock of all samples was diluted to ensure equal concentration of sampling (10 ng/µL). The amplification of DNA samples was then performed, resulting in high quality products with the expected size (250-350 bp) (figure 8).

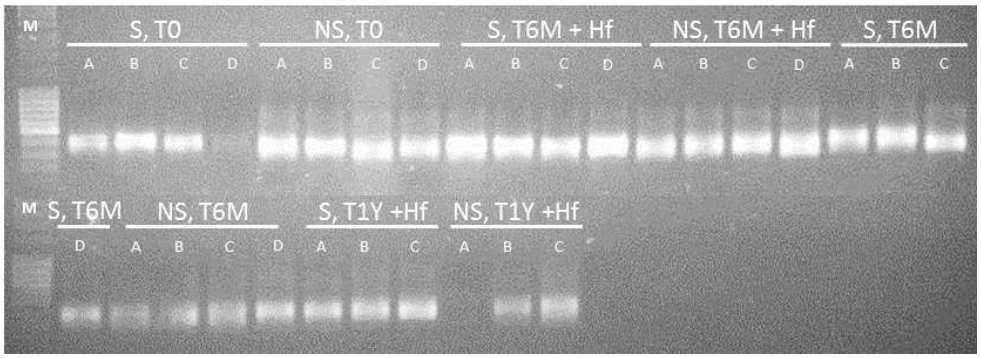


Figure 8 - Representative electrophoretic analysis (1.2% w/v agarose gel) of amplified fragments from fungal ITS1 regions. The DNA soil samples were amplified using FwITS1-F and RvITS2 as forward and reverse primers, respectively. Each DNA sample is designated according to table 8. M - Molecular marker (100 pb DNA ladder).

Reads filtering from 454 pyrosequencing data

A total of 30 soil libraries were submitted to 454 pyrosequencing and revealed a total of 176,469 ITS1 reads. After performing a first filter at BioCant that excluded sequences with less than 120 bp and with more than two ambiguous nucleotides (N), sequences containing low quality regions in both ends, sequences with more than 50% of low complexity regions and chimera sequences were discarded. At the end, 173,893 high-quality reads (98.54% of raw reads) were obtained (table 8 which is further detailed in table A 2, annex for all soil replicas). The highest number of raw reads was observed from those soils harvested six months after fungal inoculation.

In order to understand the richness of taxonomic composition and diversity of fungal community, an analysis was performed using Metagenomics Analysis Server MG-RAST version 3.3.7.3. Sequences were annotated using LSU database considering: $1e^{-6}$ as maximum e-value cutoff, 50 bp as minimum alignment length cutoff and 97% as minimum identity cutoff. As a result, 110,076 reads (63.30% of the resulting from BioCant filter) were obtained (table 8). After removing non-fungal taxa and unidentified reads, a total of 78,029 high-quality reads were obtained.

Table 8 – Number of reads obtained by 454 pyrosequencing of DNA samples taken from pot soils submitted (S) or not (NS) to sterilization and inoculated (+Hf) or not (wo/Hf) with *Hypholoma fasciculare*. Sampling was performed after different periods upon inoculation - (T0 - just before inoculation, T6M – six months, T1Y – one year after inoculation). The total raw number of reads was subjected to quality filters. BioCant filter excluded sequences less than 120 bp, containing ambiguous nucleotides (>2N) and also eliminated sequences with low quality regions in their both ends. MG-RAST filter excluded sequences that present an e-value higher than e^{-5}, an identity value higher than 97% and at least 50 bp of alignment. The excluded sequences correspond to unidentified reads. Fungal reads filter excluded the reads from non-fungal organisms and unclassified sequences. Table A 2, annex, specifies this information for each replica values.

	Sterile (S)				Non-sterile (NS)				Total
	Wo/Hf		+Hf		Wo/Hf		+Hf		
	T0 (S,T0)	T6M (S,T6M)	T6M (S,T6M+Hf)	T1Y (S,T1Y+Hf)	T0 (NS,T0)	T6M (NS,T6M)	T6M (NS,T6M+Hf)	T1Y (NS,T1Y+Hf)	
Raw reads	17,634	23,581	27,070	19,996	19,556	24,333	27,476	16,823	176,469
BioCant	17,464	23,248	26,740	19,741	19,234	23,895	26,985	16,586	173,893
MG-RAST	13,602	15,793	19,795	11,793	9,815	12,289	14,687	12,302	110,076
Fungal reads	8,101	10,559	6,919	8,668	8,684	10,427	12,886	11,785	78,029
Unidentified reads	3,862	7,455	6,945	7,948	9,419	11,006	12,289	4,284	63,817
Number of OTUs	149	206	160	145	203	227	253	198	458

Phylogenetic analysis of identified OTUs

All fungal reads were clustered into 458 OTUs, of which 270 OTUs (58.9%) belonged to Ascomycota, 178 OTUs (38.9%) to Basidiomycota and 1 (0.2%) to Zygomycota (figure 9a). The remaining 9 OTUs (2%) were unclassified, but all derived from fungi. Although Ascomycota was the richest phylum, Basidiomycota was the most abundant (figure 9b). Indeed Ascomycota OTUs (58.9%) were only represented by 40.9% of reads, while Basidiomycota OTUs (38.9%) presented 57.4% of the total number of reads. In all eight studied conditions the Ascomycota was the richest phyla, presenting always more than 57.6% of OTUs (figure 10a). Basidiomycota followed as the second most common phylum with OTUs percentages between 26.6% and 39.4%. Zygomycota was only present in one sample (NS, T0) with a very low percentage (0.5%). Unclassified OTUs were present in soil samples with 2.4% to 4% of total OTUs. When considering phyla abundance distribution among samples, four different samples presented higher abundance of Ascomycota reads [(S, T6M), (S, T6M + Hf), (S, T1Y + Hf) and (NS, T0)], whereas the remaining samples contained higher abundance of Basidiomycota reads (figure 10b).

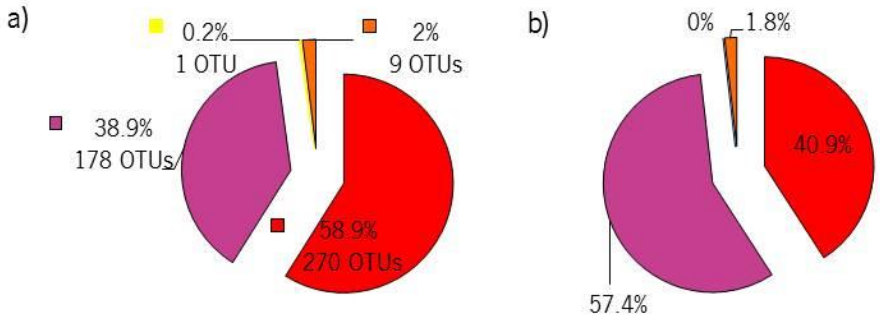


Figure 9 - Distribution of fungal phyla in all studied orchard soils, according to the obtained number of OTUs (a) and reads (b). Ascomycota (red), Basidiomycota (pink), Zygomycota (yellow) and unclassified (orange) are represented in percentage of the total of number of OTUs or reads.

The richness results were more homogeneous between the eight different soil samples than the results from abundance (figure 10). In particular, (NS, T1Y + Hf) besides presenting a similar distribution of OTUs between the most representative phyla of the other soil samples, exhibited a major difference between the number of reads (10% Ascomycota and 89.4% Basidiomycota) (figure 10b). The higer abundance of Basidiomycota phylum, rather than Ascomycota, was in agreement with other studies in chestnut orchard soils (Buée et al., 2009; Reis, 2012; Baptista et al., 2015).

To have a better view on the distribution of most rich and abundant taxa identified in all samples, a taxonomic distribution graphic representation was prepared (figure 11). As previously referred, Ascomycota and Basidiomycota were the most rich and abundant phyla. From the Ascomycota members, Sordariomycetes was the richest class (50% of the Ascomycota), exhibiting 44% abundance of Ascomycota reads. Dothiodeomycetes and Eurotiomycetes exhibited 17% and 12% of the Ascomycota OTUs, but only comprised 5% and 3% of the Ascomycota reads number, respectively. In contrast, class Leotiomycetes only present 11% of the Ascomycota OTUs, but exhibited 46% of Ascomycota reads. Concerning Basidiomycota members, the most rich and abundant class was Agaricomycetes that exhibited 74% of the Basidiomycota OTUs and 64% of the Basidiomycota reads. Tremellomycetes and Exobasidiomycetes presented 13% and 2% of Basidiomycota OTUs, respectively, and 35% and 0.04% of Basidiomycota reads, respectively.

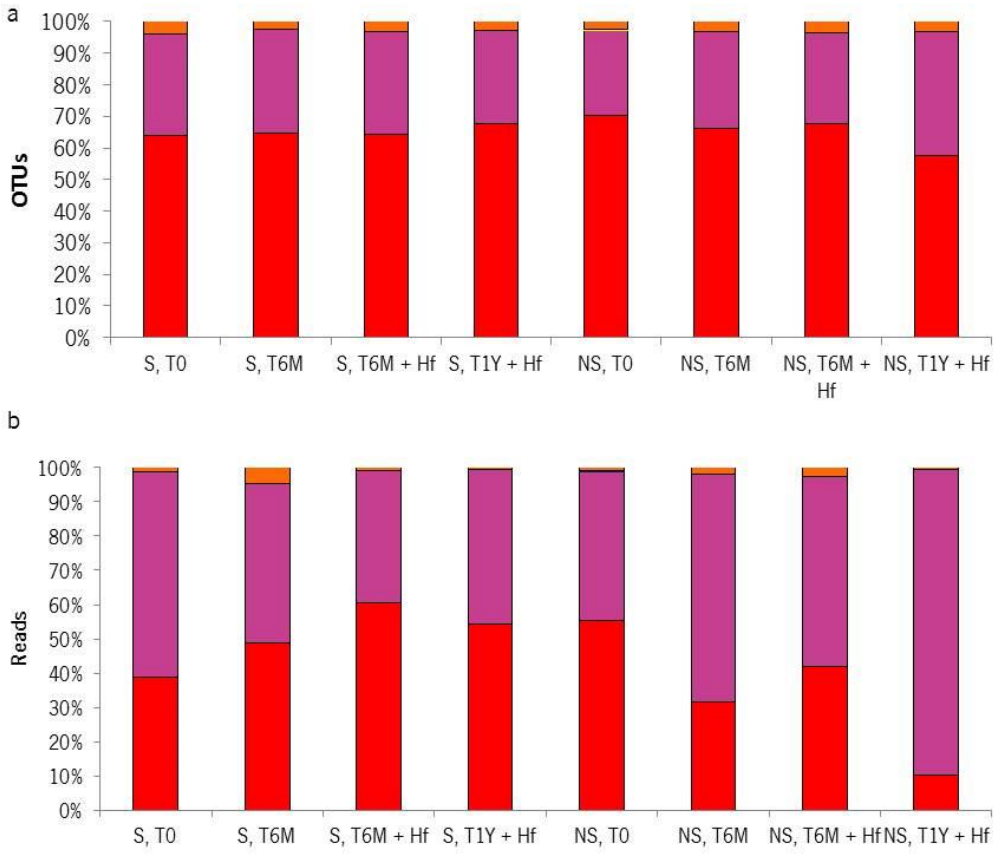


Figure 10 – Richness (number of OTUs) and abundance (number of reads) of each phylum in each soil sample. The results of Ascomycota (red), Basidiomycota (pink), Zygomycota (yellow) and unclassified (orange) phyla are represented as the percentage of number of OTUs (a) or number of reads (b) present in that sample in relation to the total. The pot soils were submitted (S) or not (NS) to sterilization and inoculated (+Hf) or not with *Hypholoma fasciculare*. Sampling was performed after different periods upon inoculation - (T0 - just before inoculation, T6M – six months, T1Y – one year after inoculation).

Trichocomaceae, Hypocreaceae and Nectriaceae were the richest Ascomycota families (8%, 7% and 7% of Ascomycota OTUs, respectively) (figure 11a), but in terms of abundance, only Nectriaceae exhibited a higher abundance (12% of Ascomycota reads), contrasting with the 3% of Ascomycota reads presented by the other families (figure 11b). Cortinariaceae is one of the richest and most abundant Basidiomycota families (15% of Basidiomycota OTUs, comprising 12% of Basidiomycota reads), followed by Tricholomataceae (8% of Basidiomycota OTUs with 7% of Basidiomycota reads). On the other hand, Tremellaceae, Ganodermataceae and Stereaceae families besides being highly abundant (15%, 13% and 10% of Basidiomycota reads, respectively) were only represented by few OTUs (1%, 2% and 2% of Basidiomycota OTUs, respectively).

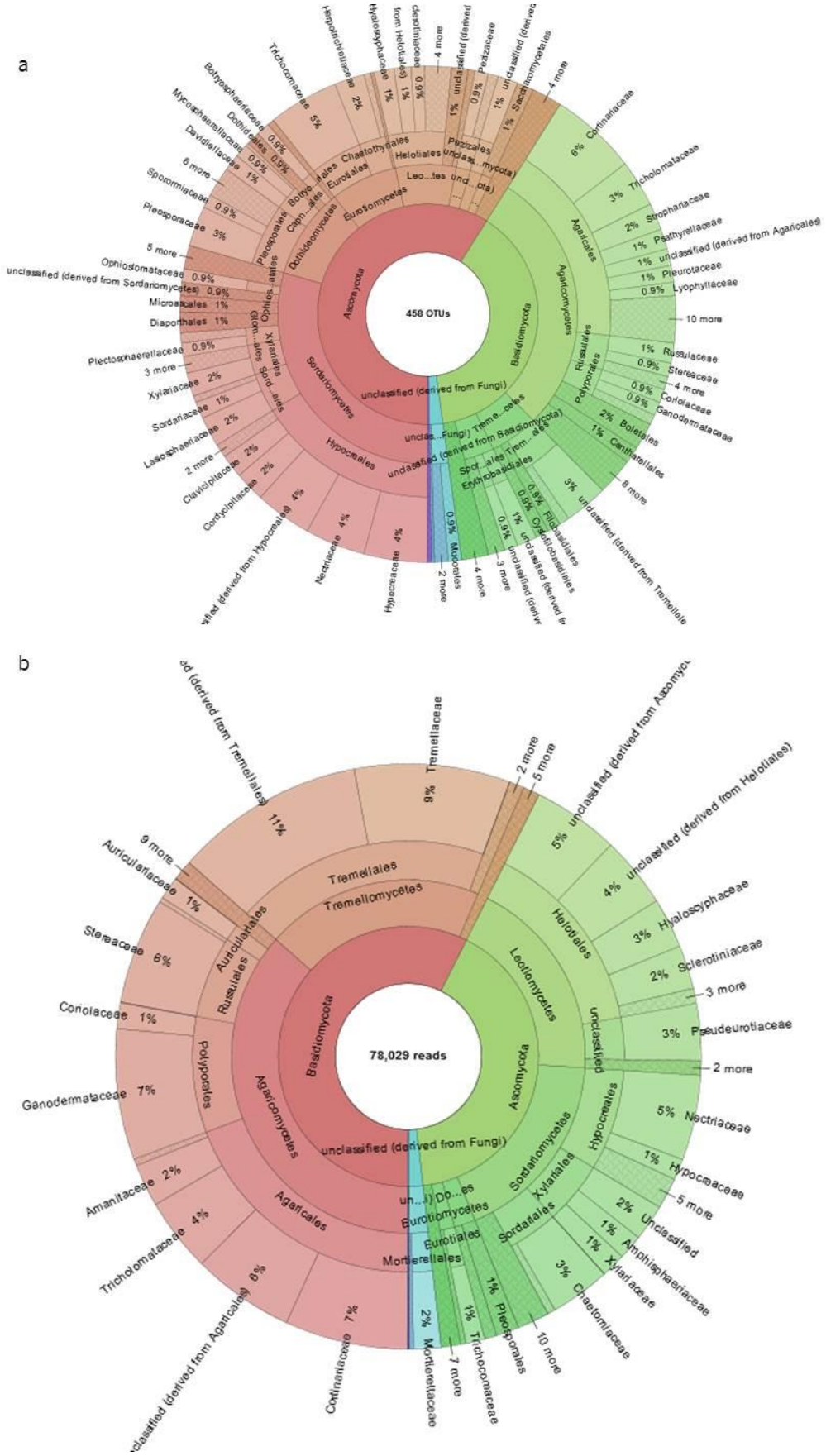


Figure 11 – Taxonomic distribution of all identified OTUs (458 OTUs) (richness - a) and all identified reads (78,029 reads (abundance - b), using krona charts with Microsoft Excel (Ondov *et al.*, 2011).

A selection of the most well-represented OTUs was performed. The influence of different read cutoff values in the number of detected reads and OTUs was determined (figure A 1, annex). The cutoff of 50 reads, in which only OTUs represented by more than 50 reads in all samples were considered, was chosen once it allows to exclude the less-represented OTUs. For this reason, some of the downstream analyses will only take into account OTUs with higher abundance. After applying the referred cutoff, only 113 of the 458 OTUs will be considered, presenting a total of 75,409 reads. The use of a cutoff value of only five reads in whole study is very common in many metabarcoding studies performed in soil communities. However, after applying such a filter, a significant number of 241 OTUs still remained, much of them were still scarcely abundant in the study (less than 0.08% of total read number). The fungi whose abundance was superior to 50 reads were identified according to their trophic group. Therefore 113 OTUs, represented by 75,409 reads were classified between mycorrhizal (M), parasite (P), parasite/saprotroph (PS), saprotroph (S), yeast (Y) and unclassified (Un) fungi (table A 3, annex).

Only nine of the 458 OTUs presented more than 2,000 reads in whole study and these nine OTUs represent most of the trophic groups. *Cryptococcus podzolicus* is the most abundant yeast (Y) with 8,049 reads, while *Tremella encephala* and *Ganoderma orbiforme* are the most abundant parasites/saprotrophs (PS) (6,704 and 5,621 reads, respectively). On the other hand *Stereum sanguinolentum* (4,305 reads) and *Pseudogymnoascus pannorum* (2,453 reads) are the saprotrophs (S) most well-represented, *Cadophora orchidicola* (3,733 reads) and *Chaetomium globosum* (2,589 reads) are the parasites (P) most present, and finally *Inocybe lacera* (3,358) and *Cortinarius lilacinovelatus* (2,287) are the most abundant mycorrhizal fungi (M). Nevertheless, there were also several OTUs scarcely represented. In the whole study, 98 OTUs (21.4%) were found as singletons, meaning that they were represented by a single read. There were also several doubletons (represented by only two single reads -50 OTUs - 10.1%). The low representativeness of some fungal taxa may be associated to the inconspicuous nature of fungi and their capacity of dispersion (Buée *et al.*, 2009).

Fungal diversity of soil samples

Biodiversity is a key concept that evaluates the habitat quality, considering the intraspecific variation (measured as genetic diversity) and also the community variation, in terms of richness, abundance and evenness of species, respectively (Unterseher *et al.*, 2011). There are three different types of diversity: alpha (α), beta (ß) and gamma (γ). Alpha diversity studies consider

the species richness within a local community (Whittaker, 1972; Whittaker *et al.*, 2001), whereas gamma diversity considers the diversity in a region, more focused on the differential overlap of species. On the other hand, beta diversity corresponds to the diversity between habitats, systems or even between environmental modifications, in which the species turnover along a complex environmental gradient is pretended to be evaluated (Whittaker, 1972; Wilson and Shmida, 1984; Barros, 2007).

To have an idea about the species diversity in the studied soil samples, several alpha diversity indexes were determined (table 9). Some of the evaluated species richness indexes (like Chao1) can predict the total number of species present in a community. Others determine the diversity within the same specific community (Simpson, Shannon and Fisher's alpha indexes). The soil libraries that exhibited the lowest values of species richness and diversity were always the libraries from (S, T0), in which chestnut harvesting was performed earlier. This was expected once the sterilization process was quite recent (two months ago), in comparison to the other soil samples, and the fungal community is still being established. However, due to the variability between libraries, when considering the diversity value obtained for the soil sample, (S, T0) not always was the less diverse sample. Instead, (S, T1Y + Hf) soil sample displayed the lowest values for the majority of diversity indexes. Indeed, this inoculated soil sample exhibited the most similar richness and diversity values to (S, T0), reflected by the fact that non-statistically difference in all studied parameters was found between both soil samples. In contrast, the richest and most diverse samples/libraries were mainly from non-sterile soil samples (NS), particularly (NS, T6M) and (NS, T6M + Hf) samples, which is probably due to the introduction of pots into a new environment, such as the greenhouse. The watering in aseptic conditions of the pots, which were disposed side-by-side to other pots containing different soil samples, as well as the probable effect of chestnut root exudates on microbial community, could have lead to an increase in fungal richness and diversity in T6M samples. However, no statistically significant differences were found between all NS samples, most probably due to the fact that a previously well-established fungal community is being studied that would be more resilient to environmental variations. The introduction of the native soil into the greenhouse environment, followed by the inoculation with *H. fasciculare*, could not have been evident enough to be supported by statistically different values.

When comparing the same soil conditions (T0, T6M, T6M + Hf and T1Y + Hf) in S and NS treatments, only statistically significant differences in diversity (D, H' and α Fisher) parameters

were found between the immediately harvested samples [(S, T0) and (NS, T0)] and those inoculated samples with six months of inoculation [(S, T6M + Hf) and (NS, T6M + Hf)]. Due to the large difference between the number of reads detected in (S, T6M + Hf, 6,919) and (NS, T6M + Hf, 12,886), only the α Fisher diversity index, which is independent of the sample size, was statistically different between both samples.

Table 9 – Species richness and diversity parameters for fungal communities from the studied pots, after surveying the fungal community by pyrosequencing methods: species richness (S), Simpson's index (D) on its inverse form, Shannon index (H'), Fisher's alpha, Chao1 and 1ª order Jacknife estimates. These parameters were determined by *EstimateS 9.1.0.* software. The highest and lowest estimates are highlighted in bold, being the highest also underlined. Statistically significant differences (at P≤0.05) were denoted by different capital letters, when analyzing sterile soil samples, or lower case letters for non-sterile soil samples. Asterisks (* and **) denote statistically significant values at P ≤0.05, and P≤0.01, respectively, when the same condition was compared between sterile and non-sterile soils. The studied soils were submitted (S) or not (NS) to sterilization and inoculated (+Hf) or not with *Hypholoma fasciculare*. Sampling was performed after different periods upon inoculation (T0 - just before inoculation, T6M – six months, T1Y – one year after inoculation).

Samples	Libraries	S	D	H'	α Fisher	Chao1	Jacknife
S, T0	A	69	4.79	2.13	13.81	115.48	69
	B	68	**1.96**	**1.5**	12.65	79.17	68
	C	108	4.60	2.42	21.92	145.04	108
	D	**34**	3.35	2.06	**9.25**	**49.12**	**34**
	Total	149^{A}	**3.58**^{A*}	**2.20**^{A**}	25.92^{A*}	216.1^{A}	149^{A}
S, T6M	A	93	**18.48**	3.41	22.71	136.12	93
	B	100	6.63	2.77	19.87	178.72	100
	C	117	14.67	3.23	23.63	156.04	117
	D	101	6.24	2.66	20.37	131.99	101
	Total	206^{A}	**20.5**^{BC}	3.55^{BC}	36.29^{A}	277.28^{A}	206^{A}
S, T6M + Hf	A	94	15.36	3.31	23.63	135.3	94
	B	88	14.08	3.09	19.74	118.98	88
	D	54	4.51	1.90	9.66	75.08	54
	C	89	11.33	3.08	21.08	131.24	89
	Total	160^{A*}	15.02^{BC}	3.23^{AC}	29.25^{A*}	221.89^{A}	160^{A*}
S, T1Y + Hf	A	106	7.84	2.90	22.28	170.97	106
	B	66	6.57	2.29	11.23	97.62	66
	C	55	4.80	2.17	10.35	74.12	55
	Total	**145**^{A}	13.13^{AC}	3.08^{AC}	**24.74**^{A}	**186**^{A}	**145**^{A}
NS, T0	A	129	12.12	3.21	29.50	164.86	129
	B	95	8.66	2.95	22.54	129.98	95
	C	134	10.09	3.09	29.57	166.99	134
	D	116	15.37	3.36	26.25	133.17	116
	Total	203^{a}	15.46^{a*}	3.42^{a**}	37.20^{a*}	264.59^{a}	203^{a}
NS, T6M	A	142	9.28	3.12	32.17	**229.32**	142
	B	151	10.54	3.22	33.33	221.48	151
	C	115	9.58	2.96	23.74	149.99	115
	D	108	7.90	2.89	25.45	160.08	108
	Total	227^{a}	12.33^{a}	3.35^{a}	40.95^{a}	**322.99**^{a}	227^{a}

Table 9 – (Continuation)

Samples	Libraries	S	D	H'	α Fisher	Chao1	Jacknife
NS, T6M + Hf	A	126	8.12	2.93	26.01	162.99	126
	B	128	16.03	**3.51**	28.96	149.56	128
	C	**169**	13.62	3.42	**35.44**	202.77	**169**
	D	143	15.21	3.38	31	190.51	143
	Total	**253**[a]*	14.32[a]	**3.56**[a]	**44.63**[a]*	310.44[a]	**253**[a]*
NS, T1Y + Hf	A	137	8.10	2.78	27.29	190.7	137
	B	114	8.84	2.86	23.95	151.04	114
	C	119	7.58	2.60	21.99	151.21	119
	Total	198[a]	11.84[a]	3.08[a]	33.81[a]	282.91[a]	198[a]

In order to compare diversities among samples, Fisher's alpha diversity index was chosen to be graphically represented (figure 12). This diversity index is very useful due to its low sensivity to sample size (Magurran, 2004). Many diversity indices are sample size dependent, which limits comparisons between sets of unequal size.

Comparing the sterile soils against the non-sterile soil samples, an evident increase of fungal diversity was detected in NS soils. Among in the different set conditions in both S and NS soil samples, a more homogenous pattern was exhibited, as also revealed by the statistical analysis that did not detect significant differences between them. The sterile soil sample (S, T0) exhibited the lowest diversity, which was an expected result since this sample had been recently subjected to sterilization. A diversity increase was then detected in (S, T6M) that displayed the highest value for sterile soil samples. As the only factor that is different in both samples is the time spent in the greenhouse and chestnut growing, these factors should have been determinant for the detected microbial community differences. *H. fasciculare* inoculation reduced the fungal diversity, as revealed by the comparison of (S, T6M) and (S,T6M + Hf), and was further reduced along time [(S, T6M + Hf) and (S, T1Y+Hf) comparison]. These results thus suggest that fungal diversity is affected by the presence of *H. fasciculare*. Nevertheless, neither of the referred variations in a α-Fisher index were supported by statistical analysis. However, the same variations were also detected for the other diversity indexes, as Simpson (D) and Shannon (H') indexes, that present significant differences, mainly in relation to (S, T0) sample (table 9).

Although less evident, due to the higher homogeneity found between samples, non-sterile soil samples also presented an evident increase of diversity from (NS, T0) and (NS, T6M). However, instead of a diversity decrease following inoculation as occurred in S soils, NS soils displayed an increase in diversity upon inoculation, as revealed by the comparison between (NS, T6M) and (NS, T6M + Hf). Only after one year of inoculation the diversity seems to be negatively

affected by *H. fasciculare.* These variations were also detected for the other diversity indexes, although they were not statistically different between them.

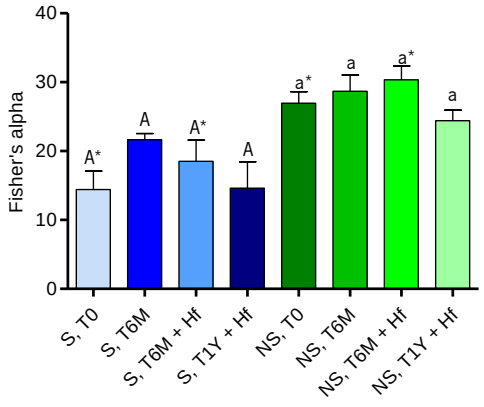


Figure 12 – Fisher's alpha diversity index for fungal communities from the studied pots, after surveying the fungal community by pyrosequencing methods. Statistically significant differences (at P≤0.05) were denoted by different capital letters, when analyzing sterile soil samples, or lower case letters for non-sterile soil samples. Asterisks (* and **) denote statistically significant values at P ≤0.05, and ≤0.01, respectively, when the same condition was compared between sterile and non-sterile soils. The studied soils were submitted (S) or not (NS) to sterilization and inoculated (+Hf) or not with *Hypholoma fasciculare*. Sampling was performed after different periods upon inoculation (T0 - just before inoculation, T6M – six months, T1Y – one year after inoculation). The different set of colors correspond to the different soil samples, as described.

In order to compare species richness between each studied soil condition, rarefaction curves were determined (figure 13). The rarefaction curves represents the species richness for a given number of individual samples (in this case, for the number of reads sequenced) with the objective of comparing different samples. As expected, all NS samples displayed a higher number of fungal species than S samples, displaying the (T6M + Hf) sample the highest value. From the rarefaction curve, the high similarity between (S, T0) and (S, T1Y + Hf) became evident, as well as their lowest richness among all other samples. All samples still presented a steeper slope at the end of their curve meaning that there is still a fraction of species diversity to discover. Therefore, the OTUs counts could increase if a more deeper sampling has been performed.

Jaccard (S_J) and Sørensen (S_S) are qualitative indexes of similarity used for beta diversity analysis that are based on the presence/absence of species. The corresponding values range between 0 - 1 (0: zero similarity; 1: maximum similarity). While Sørensen index is a statistic parameter used for the comparison of similarity between two samples, Jaccard's also compares the diversity of sample sets, besides the similarity. A similarity matrix for Jaccard/Sørensen indexes, determined between libraries of the same soil sample and between different samples,

was constructed considering all obtained OTUs and their abundance (table 10). NS soil samples always presented higher values of similary between them (ranging from 0.35 to 0.54, S_J; and from 0.51 to 0.70, S_S) than S soil samples between them (ranging from 0.18 to 0.68, S_J; and from 0.30 to 0.66, S_S). These results suggests a "buffering" effect provided by the already well-established microbial community present in NS soils. Interestingly, for NS soils the similarity between libraries of the same soil sample was not higher than between samples. In contrast, specifically (S, T6M + Hf, S_J) and (S, T6M, S_S) samples present higher similarity indexes between sample libraries than between samples. In addition, the pattern of the different NS soils samples is more homogeneous than S soil samples.

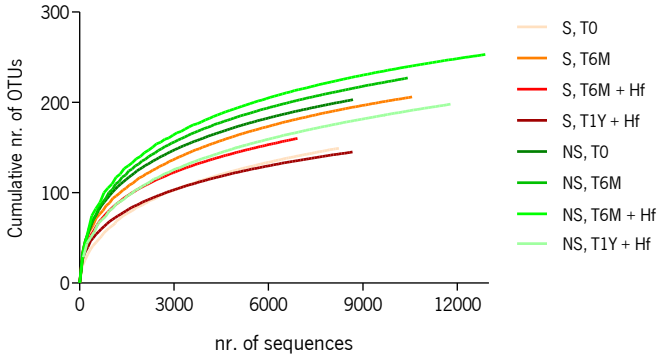


Figure 13 – Rarefaction curves for the estimated richness of microbial community of the studied soil samples. The number of identified OTUs is represented as a function of the number of sequence reads. Curves were determinated by *Species Diversity and Richness 4.1.2.* software. The studied soils were submitted (S) or not (NS) to sterilization and inoculated (+Hf) or not with *Hypholoma fasciculare*. Sampling was performed after different periods upon inoculation (T0 - just before inoculation, T6M – six months, T1Y – one year after inoculation). The different set of colors correspond to the different soil samples, as described.

For simplicity of analysis, the similarity variation of Jaccard indexes among soil samples is presented in figure 14. The same variation obtained for Sørensen index is not presented, due to their coincidence in variation to Jaccard's. Besides the previously referred higher similarity values in NS soils, a common pattern was found between S and NS samples. The similarity between (T0) and (T1Y + Hf) samples were always the lowest, meaning that they are the most heterogeneous samples, while (T6M) and (T6M + Hf) samples were always the most homogeneous samples. Although these differences were not statistically different for S soils, these comparisons in NS soils presented the statistically significant differences. These results suggest that the period of time that is spent in the greenhouse for chestnut growing has an

important impact on the microbial community. The high similarity between (T6M) samples, either inoculated or non-inoculated, could reveal the coincidence of new species establishment in the soil derived from the same environment. In agreement, the imposition of the same environment along time make the differences more notorious, as evidenced by the decrease of similarity indexes with time [(T0 *vs.* T6M + Hf) > (T0 *vs.* T1Y + Hf) or (T6M *vs.* T6M + Hf) > (T6M *vs.* T1Y + Hf)]. In addition, the sets [(T0) vs. (T6M)] and [(T0) vs. (T6M + Hf)] exhibited almost no differences between them, suggesting that *H. fasciculare* is not the most important factor for microbial community variation and appears to cause no harm on the fungal community.

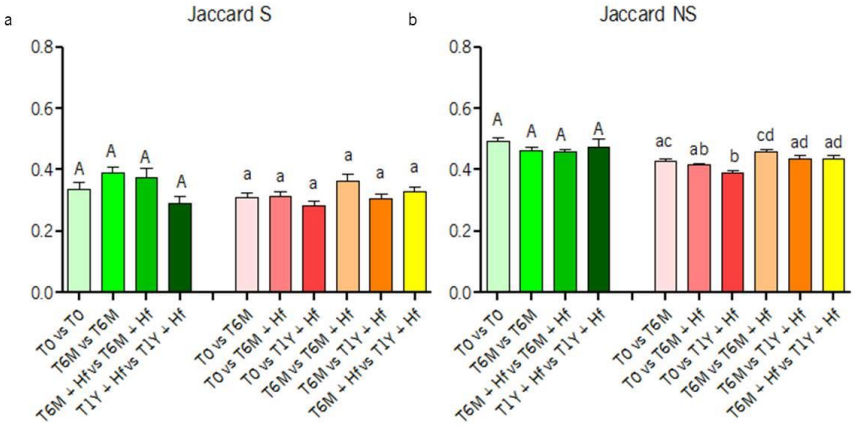


Figure 14 – Jaccard's similarity indexes established between libraries of the same soil samples and between the soil conditions studied. Soils submitted (S - a) or not (NS - b) to sterilization and inoculated (+Hf) or not with *Hypholoma fasciculare* were evaluated at different periods upon inoculation - (T0 - just before inoculation, T6M – six months, T1Y – one year after inoculation). In each analysis, different letters denote statistically significant differences (at P≤0.05) between libraries of the same soil sample (capital letters) or between different soil samples (lower case letters).

The Bray-Curtis coefficient is another ecological quantitative index that quantifies the dissimilarities between samples, being commonly used to express connections in ecology and environmental sciences. The correspondent values range between 0 and 1, where 0 indicates that both samples share exactly the same species, and 1 means a maximum of dissimilarity. OTUs within each library of the studied samples were plotted in a non-metric multidimensional scale (NMDS), according to the Jaccard index (figure 15 – b) and Bray-Curtis coefficient (figure 15 – b). Besides their different meanings, the interpretation of the libraries clusters using both measures is equivalent. The libraries from the soil samples that were not submitted to the sterilization process (NS) are all clustered, meaning that they are more similar among each other than libraries from sterile soil samples. This result is consistent with the previous suggestion that

in these soil samples the fungal community is more homogeneous and their microbial ecosystem equilibrium is not easily affected by *H. fasciculare* inoculation or chestnut growing. In contrast, different S soils samples are more dispersed among them, suggesting that sterile soils are more prone to be affected by environmental conditions, including *H. fasciculare* inoculation. The non-inoculated soil sample (S, T0) is more divergent than the others, including the other non-inoculated (S, T6M). The slight clustering of both non-inoculated (T6M) and inoculated (T6M + Hf) samples reveals that they are somewhat similar, in spite the presence of the saprothophic fungus *Hypholoma fasciculare* in the later. This result suggest that this fungus does not cause extensive alterations to the fungal community, although slight variations seem to exist. The (S, T1Y + Hf) sample is more distanced to (S, T6M) libraries, which could be due to the effect of *H. fasciculare*, since there is some closeness to (S, T6M + Hf) samples. Nevertheless, the effect of the longer period in greenhouse upon inoculation should not be neglected.

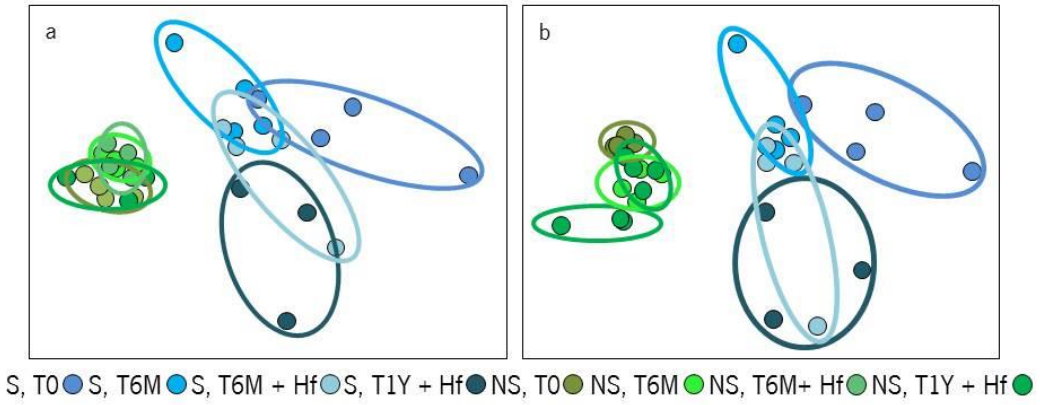


Figure 15 - Non-metric multidimensional scale (NMDS) plots corresponding to the clustering analysis among soil samples/libraries, perfomed with two different community similarity measures, namely Jaccard's index (a) and Bray-Curtis coefficient (b). Each point represents a soil library and those from the same soil sample are encircled by each different coloured ellipse. Soil samples correspond to those submitted (S) or not (NS) to sterilization, with (+Hf) or without fungal inoculation, and harvested at different periods: immediately before inoculation – T0; six months, T6M and one year after inoculation, T1Y). Both indexes were Ln(x+1) transformed data. Kruskal's stress values inferior to 0.2 represent good ordination plots and greater that 0.3 provides a poor representation. The different set of colors correspond to the different soil samples, as described.

Table 10 – Coefficients of similarity between soil samples; the Jaccard index is displayed in the lower side of the table (pink) and the Sørensen index in the upper side (blue). These qualitative indexes of beta diversity are based on the presence/absence of species, and its values range between 0 - 1 (0: zero similarity; 1: maximum similarity). A gradient pattern of colors was applied according to the similarities values (lighter color - lower similarity value; darker color - higher similarity value).

		S,T0				S,T6M				S,T6M+Hf				S,T1Y+Hf			NS,T0				NS,T6M				NS,T6M+Hf				N,T1Y+Hf		
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C
S,T0	A		0,58	0,49	0,49	0,49	0,52	0,43	0,53	0,49	0,56	0,49	0,41	0,48	0,50	0,44	0,41	0,43	0,38	0,38	0,43	0,38	0,43	0,44	0,40	0,48	0,39	0,46	0,41	0,46	0,38
	B	0,41		0,56	0,49	0,43	0,51	0,39	0,50	0,47	0,51	0,47	0,48	0,46	0,48	0,36	0,35	0,38	0,35	0,33	0,39	0,37	0,37	0,36	0,37	0,40	0,36	0,43	0,38	0,42	0,34
	C	0,32	0,39		0,39	0,53	0,57	0,48	0,60	0,55	0,55	0,61	0,42	0,54	0,45	0,36	0,47	0,46	0,41	0,44	0,50	0,44	0,48	0,43	0,48	0,43	0,48	0,47	0,44	0,45	0,39
	D	0,32	0,32	0,25		0,38	0,37	0,30	0,44	0,33	0,39	0,41	0,41	0,30	0,42	0,45	0,25	0,29	0,26	0,25	0,26	0,23	0,31	0,31	0,29	0,28	0,25	0,27	0,29	0,31	0,27
S,T6M	A	0,33	0,28	0,36	0,23		0,56	0,51	0,58	0,57	0,54	0,62	0,46	0,49	0,48	0,43	0,44	0,46	0,41	0,42	0,45	0,43	0,47	0,49	0,46	0,45	0,49	0,47	0,43	0,45	0,40
	B	0,35	0,34	0,40	0,23	0,39		0,52	0,65	0,57	0,55	0,58	0,40	0,56	0,57	0,44	0,45	0,46	0,44	0,45	0,43	0,47	0,46	0,47	0,43	0,46	0,43	0,49	0,44	0,46	0,39
	C	0,27	0,24	0,32	0,18	0,35	0,35		0,52	0,55	0,46	0,51	0,32	0,46	0,37	0,38	0,44	0,40	0,45	0,45	0,46	0,49	0,46	0,46	0,49	0,46	0,48	0,52	0,44	0,47	0,43
	D	0,36	0,33	0,43	0,29	0,41	0,48	0,35		0,65	0,56	0,66	0,46	0,50	0,51	0,40	0,48	0,51	0,45	0,49	0,48	0,52	0,52	0,49	0,48	0,50	0,52	0,52	0,50	0,55	0,45
S,T6M+Hf	A	0,33	0,31	0,38	0,20	0,40	0,40	0,38	0,48		0,55	0,51	0,31	0,55	0,53	0,42	0,48	0,50	0,46	0,49	0,50	0,51	0,52	0,50	0,47	0,50	0,49	0,51	0,52	0,54	0,42
	B	0,39	0,34	0,38	0,24	0,37	0,38	0,30	0,39	0,38		0,38	0,35	0,55	0,48	0,42	0,41	0,47	0,41	0,42	0,46	0,42	0,45	0,42	0,45	0,48	0,45	0,44	0,44	0,49	0,36
	C	0,33	0,31	0,44	0,26	0,44	0,41	0,35	0,50	0,68	0,55		0,49	0,54	0,54	0,44	0,47	0,50	0,43	0,49	0,51	0,52	0,53	0,50	0,51	0,48	0,48	0,53	0,50	0,51	0,40
	D	0,26	0,31	0,27	0,26	0,30	0,25	0,19	0,30	0,47	0,52	0,32		0,44	0,57	0,40	0,35	0,42	0,36	0,39	0,37	0,36	0,43	0,36	0,37	0,43	0,38	0,37	0,40	0,46	0,29
S,T1Y+Hf	A	0,32	0,30	0,37	0,18	0,33	0,39	0,30	0,34	0,38	0,38	0,37	0,28		0,51	0,41	0,47	0,48	0,46	0,47	0,49	0,44	0,47	0,46	0,47	0,48	0,47	0,50	0,51	0,49	0,44
	B	0,34	0,31	0,29	0,27	0,31	0,39	0,23	0,35	0,36	0,32	0,37	0,40	0,34		0,43	0,37	0,41	0,40	0,40	0,40	0,39	0,45	0,45	0,41	0,46	0,37	0,42	0,42	0,46	0,37
	C	0,28	0,22	0,22	0,29	0,28	0,28	0,24	0,25	0,26	0,27	0,29	0,25	0,26	0,27		0,40	0,40	0,33	0,40	0,37	0,35	0,38	0,37	0,39	0,38	0,33	0,38	0,39	0,40	0,37
NS,T0	A	0,26	0,21	0,31	0,14	0,28	0,29	0,28	0,31	0,32	0,26	0,31	0,21	0,31	0,23	0,25		0,63	0,64	0,68	0,59	0,59	0,55	0,59	0,60	0,57	0,58	0,56	0,56	0,56	0,56
	B	0,27	0,23	0,30	0,17	0,30	0,30	0,25	0,34	0,33	0,31	0,33	0,26	0,31	0,26	0,25	0,46		0,66	0,69	0,61	0,59	0,59	0,64	0,60	0,57	0,57	0,60	0,56	0,63	0,51
	C	0,24	0,21	0,26	0,15	0,26	0,28	0,29	0,29	0,30	0,26	0,27	0,22	0,30	0,25	0,20	0,47	0,49		0,66	0,63	0,60	0,55	0,62	0,64	0,56	0,60	0,59	0,55	0,57	0,54
	D	0,23	0,19	0,28	0,15	0,27	0,29	0,29	0,32	0,32	0,27	0,32	0,24	0,31	0,25	0,25	0,51	0,53	0,50		0,61	0,63	0,62	0,63	0,57	0,58	0,60	0,60	0,54	0,59	0,54
NS,T6M	A	0,27	0,24	0,33	0,15	0,29	0,27	0,29	0,31	0,33	0,30	0,34	0,23	0,33	0,25	0,22	0,42	0,44	0,46	0,44		0,66	0,59	0,62	0,69	0,63	0,64	0,60	0,59	0,59	0,59
	B	0,24	0,22	0,28	0,13	0,28	0,31	0,33	0,35	0,34	0,26	0,35	0,22	0,28	0,24	0,21	0,41	0,41	0,42	0,46	0,49		0,65	0,64	0,61	0,64	0,64	0,63	0,63	0,62	0,60
	C	0,28	0,23	0,32	0,18	0,31	0,30	0,30	0,35	0,35	0,29	0,36	0,27	0,31	0,29	0,23	0,38	0,42	0,38	0,45	0,42	0,49		0,63	0,61	0,65	0,65	0,66	0,66	0,67	0,59
	D	0,28	0,22	0,27	0,18	0,32	0,31	0,30	0,32	0,34	0,26	0,33	0,22	0,30	0,29	0,23	0,42	0,47	0,45	0,46	0,45	0,47	0,46		0,62	0,61	0,60	0,61	0,55	0,62	0,57
NS,T6M+Hf	A	0,25	0,23	0,31	0,17	0,30	0,28	0,32	0,32	0,31	0,29	0,34	0,22	0,30	0,25	0,24	0,43	0,43	0,47	0,40	0,53	0,44	0,43	0,45		0,59	0,60	0,64	0,59	0,59	0,56
	B	0,31	0,25	0,28	0,17	0,29	0,30	0,30	0,33	0,33	0,32	0,32	0,27	0,31	0,30	0,24	0,40	0,39	0,39	0,41	0,46	0,47	0,48	0,44	0,42		0,61	0,64	0,61	0,63	0,57
	C	0,24	0,22	0,32	0,14	0,32	0,27	0,32	0,35	0,32	0,29	0,32	0,23	0,31	0,23	0,20	0,41	0,40	0,43	0,43	0,47	0,47	0,48	0,43	0,43	0,44		0,67	0,59	0,62	0,58
	D	0,30	0,27	0,31	0,16	0,30	0,32	0,35	0,36	0,35	0,28	0,36	0,22	0,33	0,27	0,24	0,39	0,43	0,42	0,43	0,43	0,46	0,49	0,44	0,47	0,47	0,51		0,64	0,70	0,60
NS,T1Y+Hf	A	0,26	0,23	0,28	0,17	0,28	0,28	0,28	0,34	0,35	0,28	0,33	0,25	0,34	0,27	0,24	0,39	0,39	0,38	0,37	0,42	0,46	0,49	0,38	0,41	0,44	0,42	0,47		0,69	0,61
	B	0,30	0,26	0,29	0,18	0,29	0,30	0,31	0,38	0,37	0,32	0,34	0,30	0,33	0,29	0,25	0,39	0,46	0,40	0,42	0,42	0,45	0,51	0,45	0,42	0,46	0,45	0,54	0,53		0,62
	C	0,24	0,21	0,24	0,16	0,25	0,24	0,28	0,29	0,27	0,22	0,25	0,17	0,29	0,23	0,23	0,39	0,35	0,37	0,37	0,42	0,43	0,42	0,40	0,38	0,40	0,40	0,42	0,44	0,45	

For studying beta-diversity, several indexes can be computed, although these is no single coefficient appropriated for all occasions. The reason for applying or chosing any particular measure, instead of another, is still unclear. Therefore most of the studies of beta diversity use more than one measure, being the Whittaker's original measure ($ß_w$) the most commonly employed (Koleff *et al.*, 2003). In the present study more measures were also considered, such as Cody ($ß_c$), Routledge [($ß_r$), ($ß_i$), ($ß_e$)], Wilson & Shmida ($ß_t$) and Harrison 1 and 2. These measures allows the comparison between different soil samples: in sterile *vs.* non-sterile soils and inoculated *vs.* non-inoculated samples (table 11).

Table 11 - Beta diversity indexes for each studied soil sample pair. Whittaker ($ß_W$),Cody ($ß_C$), Routledge [($ß_r$), ($ß_i$), ($ß_e$)], Wilson & Shmida ($ß_t$) and Harrison 1 and 2 indexes were determined using *Species Diversity and Richness 4.1.2* software. The highest and lowest values are highlighted in bold, being the highest also underlined. Soil sample correspond to those submitted (S) or not (NS) to sterilization, with (+Hf) or without *Hypholoma fasciculare* inoculation, and harvested at different periods: immediately before inoculation, T0; six months, T6M and one year after fungal inoculation, T1Y).

	Whittaker B_w	Cody B_c	Routledge B_r	Routledge B_i	Routledge B_e	Wilson & Shmida B_t	Harrison 1	Harrison 2
S,T0 S,T6M	0.3577	63.5	0.1247	0.235	1.265	0.3577	35.77	**16.99**
S,T6M S,T6M+Hf	0.3607	66	0.1408	0.2421	1.274	0.3607	36.07	20.87
S,T6M+Hf S,T1Y+Hf	0.3836	**58.5**	0.1781	0.2647	1.303	0.3836	38.36	**31.88**
S,T0 NS,T0	**0.4943**	**87**	**0.2465**	**0.3308**	**1.392**	**0.4943**	**49.43**	29.56
NS,T0 NS,T6M	0.3535	76	0.1534	0.2435	1.276	0.3535	35.34	28.19
NS,T6M NS,T6M+Hf	**0.2583**	62	**0.0877**	**0.1776**	**1.194**	**0.2583**	**25.83**	19.37
NS,T6M+Hf NS,T1Y+Hf	0.3215	72.5	0.1128	0.2154	1.24	0.3215	32.15	17.79

As beta diversity indexes represent the variation in species composition, these results suggest that higher community variations were detected among sterile samples. Indeed, for almost all used measures (except for Cody and Harrison 2 measures), the values were mainly higher between S samples than between NS samples. However, the highest variation were

detected when comparing (S, T0) and (NS, T0) samples, whose harvesting was performed immediately before inoculation. This result suggests that even though the sterilization had occurred two months before, the effect is still noticed and a very different fungal community is present in (S, T0) sample. The samples that exhibited the lower values of beta diversity, were the NS soil samples that presented the same period in the greenhouse, (NS, T6M) and (NS, T6M + Hf). The low variation in fungal communities of these samples, suggests that the main factor that seems to contribute to microbial community variation is the period in the greenhouse environment and/or chestnut growing effect.

3. Impact of *Hypholoma fasciculare* on soil fungal community

When evaluating the richness and abundance of specific species in each soil sample, a general analysis of the distribution of the well-represented OTUs number in each soil sample indicates that previously sterile soil samples (S) are always less rich than non-sterilized (NS) ones. Besides, after 12 months of chestnut growing (T6M) an increase in OTUs richness was evident for both soils; however, after *H. fasciculare* inoculation, the observed differences were always more evident in previously sterilized soils (S) than in native soils (NS) (figure 16a). Concerning the abundance of the well-represented OTUs, while non-inoculated soil samples presented similar reads number, inoculated soils exhibited an higher difference, being non-sterile soil samples more abundant than sterile soil samples (figure 16b). When comparing the number of exclusive and shared OTUs between S and NS samples (of the well-represented OTUs), an increase on the number and abundance of shared OTUs was detected with chestnut growing (figure 17). This result could be explained by the absence of assepsia conditions in the greenhouse and by the certain contamination between pots. Specifically in NS inoculated soils the increase of exclusive OTUs number and reads appears to corroborate the possibility of contamination, and possibly suggest that along the experiment time these OTUs become more abundant. Moreover, since this increase was especially evident in inoculated samples *H. fasciculare* appears to positively effect these species.

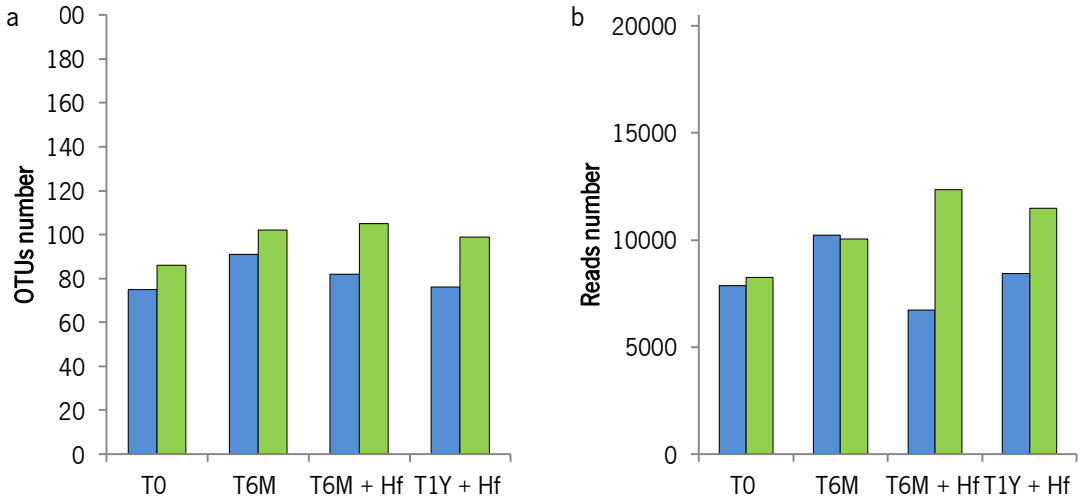


Figure 16 – Well-represented OTUs number (a) and corresponding reads number (b) detected in each soil sample, taken from plants pots containing previously sterile (S, blue) or non-sterile (NS, green) soils. Soils were inoculated (+Hf) or not with *Hypholoma fasciculare* and sampling was performed after different periods upon inoculation – (T0 – just before inoculation, T6M – six months, T1Y – one year after inoculation).

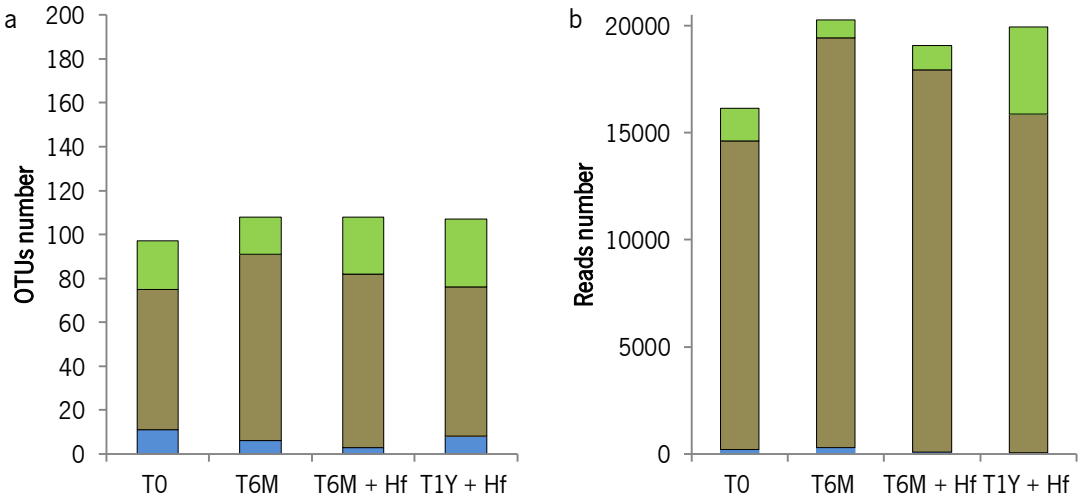


Figure 17 – Distribution of well-represented OTUs (a) and correspondent reads number (b), considering the exclusive OTUs of sterile soils samples (S – blue), non-sterile soils samples (NS – green), and common OTUs to both S and NS soil samples (brown). Different chestnut harvesting periods (T0 – just before inoculation, T6M – six months, T1Y – one year after inoculation), with (+Hf) or without *Hypholoma fasciculare* inoculation are consider.

Comparison between sterile and non-sterile soils

As the effect of *H. fasciculare* was studied in sterile and non-sterile soils, two control samples were considered: chestnut plants grown in sterile and non-sterile soils for two months, just before the fungal inoculation stage [(S, T0) and (NS, T0), respectively]. Sterile soil presented

8,101 reads distributed in 149 OTUs, while non-sterile soils exhibit 8,684 reads distributed in 203 OTUs. As expected, sterile soils were less rich than non-sterile soils, but surprisingly their abundance was comparatively equivalent (figure 18). In theory, the comparison between these samples would allow to infer about the sterilization process and a higher number of reads would be expected in non-sterile soils. However, due to the fact that all samples were sequenced at a sequence deep of 7,000 reads/replica, the number of reads does not correspond to a real comparison between samples, concerning the abundance.

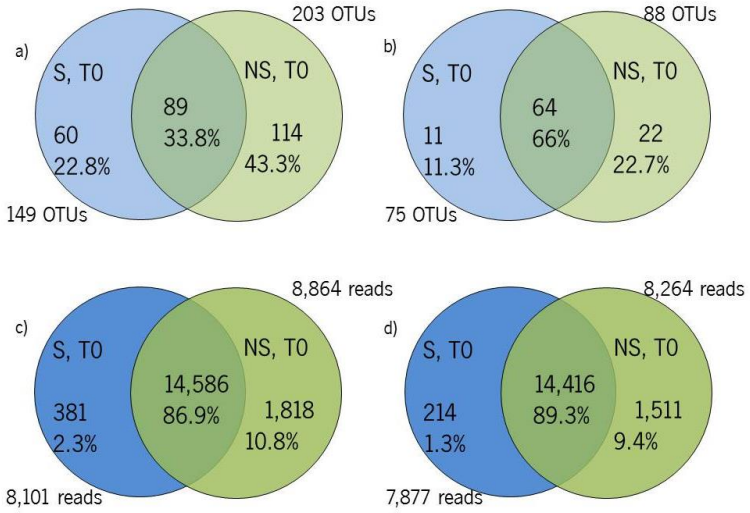


Figure 18 - Comparison of sterile (S, T0) and non-sterile (NS, T0) controls, concerning the number of OTUs (a and b), and the number of reads (c and d). Sampling was performed immediately before inoculation (T0). Analyses were performed with all obtained OTUs (a and c) and with only those that comprise more than 50 reads (b and d).

The common OTUs in both samples (89, 33.8% of total number of OTUs) gather 14,586 reads (86.9% of the reads found in both samples) (figure 18a, 18c). However, only 64 (66% of well-represented OTUs) presented more than 50 reads, with a total of 14,416 reads, corresponding to 89.3% (figure 18b, 18d). This means that there were more well-represented OTUs shared by both soils than specific to a single condition. This result is corroborated by the high percentage of shared reads, in comparison to exclusive reads from each soil condition. Of the 64 common OTUs, 31 are parasitic, 19 saprotrophics, five yeasts, four mycorrhizal, four parasitic/saprotrophic and one is an unclassified (Un) species. In both conditions, *Botryotinia fuckeliana* (P), *Chaetomium globosum* (P), *Phialocephala fortinii* (M), *Cryptococcus podzolicus* (Y), *Tremella encephala* (PS) and *Cadophora orchidicola* (P) presented high abundances, being the last three also the most represented OTUs in whole study.

Of the 60 OTUs exclusive to soil sample previously sterilized (S, T0), only 11 (11.3% of the well-represented OTUs) remained after the 50 reads cutoff (five parasitic, two mycorrhizal, two saprotrophic, two yeast) (figure 18a, 18b). From these, *Biscogniauxia nummularia* (P) was mostly present on the sterile soil with 125 reads (its abundance on the entire study was only of 167 reads). On the other hand, from the 114 OTUs restricted to non-sterile sample (NS, T0), only 22 (22.7% of well-represented OTUs) exhibited high values of abundance (more than 50 reads) in whole study, including *Pestalotiopsis besseyi* (P), *Ascocoryne cylichnium* (S), *Myrothecium roridum* (P), *Fusarium oxysporum* (P) and *Nemania serpens* (P). From these, the first two were mostly presented in (NS, T0) with 561 and 434 reads, since their abundance in the whole study was 955 and 498 reads, respectively. Parasitic species were not only the most abundant – but also the more represented (11 OTUs), followed by saprotrophic (seven OTUs), yeasts (two OTUs), one mycorrhizal OTU and one unclassified.

When comparing the fungal community of the soil sample (NS, T0) with the natural fungal population present in chestnut orchard soil, some differences became evident, in particular concerning Basidiomycota families representation. When a mushroom survey of chestnut groves was performed near the location of soil collection for this study, Baptista *et al.,* (2010) found Russulaceae, Cortinariaceae, Tricholomataceae and Boletaceae as the richest families. In a previously study performed in the same and in a near location of soil collection for this study were found the classes Agaricales, Russulales, Polyporales and Boletales as the most representative of the order Agaricomycetes (Reis, 2012). Of the most abundant class (Agaricales), there were three families most represented, Inocybaceae, Cortinariaceae and Tricholomataceae. However, in the present study Trichocomaceae, Nectriaceae, Cortinariaceae and Hypocreaceae were the richest families with 11, 10, 8 and 8 OTUs, respectively. Thus, the remained families were also found in this present study however with less abundance. Considering the genera, while *Russula*, *Inocybe* and *Cortinarius* were the more abundant in previous studies (Reis, 2012), of these, *Cortinarius* was present in the this study, being also one of the most abundant (961 reads distributed in seven OTUs). Besides this, *Tremella* (PS), *Cryptococcus* (Y), *Gibberella* (P) and *Pestalotiopsis* (P) were the more abundant OTUs (1,238, 1,102, 751 and 611 reads, respectively). The general richness and abundance of mycorrhizal species found in previous studies was not detected in this study, which could be partly explained by the location (more apart from tree trunks) and depth used for soil collection.

As expected, the results showed a reasonable difference between sterile (S, T0) and non-sterile (NS, T0) soil samples, in terms of species richness and abundance. The aim of using sterile soils in this study was to eliminate the effect of natural fungal population present in chestnut orchard soils. The effect of *H. fasciculare* on chestnut plant development and fungal community can thus be evaluated without the interference of well-established fungal ecosystem. However, when (S, T0) soil samples were collected, soils had already been used for plant growth during two-months and were no-longer deprived of fungi. Mostly probably, sterile soils became "contaminated" by greenhouse conditions, including watering water and splashing between pots, since pots were placed side-by-side in a random order. The same will most probably occur for (NS, T0) samples.

Comparison between chestnut growing times

In order to better understand fungal community progression in non-inoculated soils, a comparison between soils submitted or not to sterilization (S or NS) was performed considering the fungal community dynamics during the natural growth and development of chestnut plants. For this, S or NS soil samples were collected two months (T0) after transplantation and six months after inoculation (T6M).

In both sterile and non-sterile soil conditions, the samples whose chestnut harvesting was performed six months after *H. fasciculare* inoculation were richer (206 and 227 OTUs, in S and NS samples, respectively) and more abundant (10,559 and 10,427 reads, respectively) than samples taken two months after transplantation (149 and 203 OTUs, and 8,101 and 8,684 reads, in S and NS soils, respectively) (figure 19). Indeed, (S, T6M) presented more 2,458 reads distributed in more 57 OTUs than (S, T0). On the other hand, (NS, T6M) presented more 1,743 reads distributed in more 24 OTUs than (NS, T0).

Considering only the well-represented OTUs in whole study, 71 OTUs (74.7%) were shared between (S, T0) and (S, T6M) samples, comprising 36 parasitic, 18 saprotrophic, seven yeast, five mycorrhizal, four parasitic/saprotrophic and one unclassified species (figure 19 - i). However, from these, only 11 OTUs were represented by more than 50 reads in both samples. *Ganoderma orbiforme* (PS) was the most abundant OTU, comprising 4,091 reads in (S, T0) and 1,088 reads in (S, T6M). On the other hand, the non-sterile soils samples shared 16,663 reads (91%) distributed in 84 well-represented OTUs (80.8%) (40 parasitic, 26 saprotrophic, seven yeast, five mycorrhizal, four parasitic/saprotrophic and two unclassified species) (figure 19 – ii).

Cryptococcus podzolicus (Y) and *Tremella encephala* (P) were the most abundant OTUs in both samples, as well in whole study. Indeed, the parasitic species was the most abundant in (NS, T0) with 1,238 reads, and comprising 1,433 reads in (NS, T6M) sample. On the other hand, the yeast *C. podzolicus* presented 1,043 and 1,938 reads of abundance in the samples (NS, T0) and (NS, T6M), respectively. The abundance of many well-represented OTUs also decreased in (NS, T6M). Some OTUs were present in non-sterile control with a high abundance, but presented a low number of reads on the 12-months old soil sample, such as the saprotrophics *Lachnum virgineum* [194 reads (NS, T0) and 13 reads (NS, T6M)] and *Ascocoryne cylichnium* [434 reads (NS, T0) and seven reads (NS, T6M)] as well as the parasitic species *Pestalotiopsis besseyi* [561 and 41 reads, (NS, T0) and (NS, T6M), respectively].

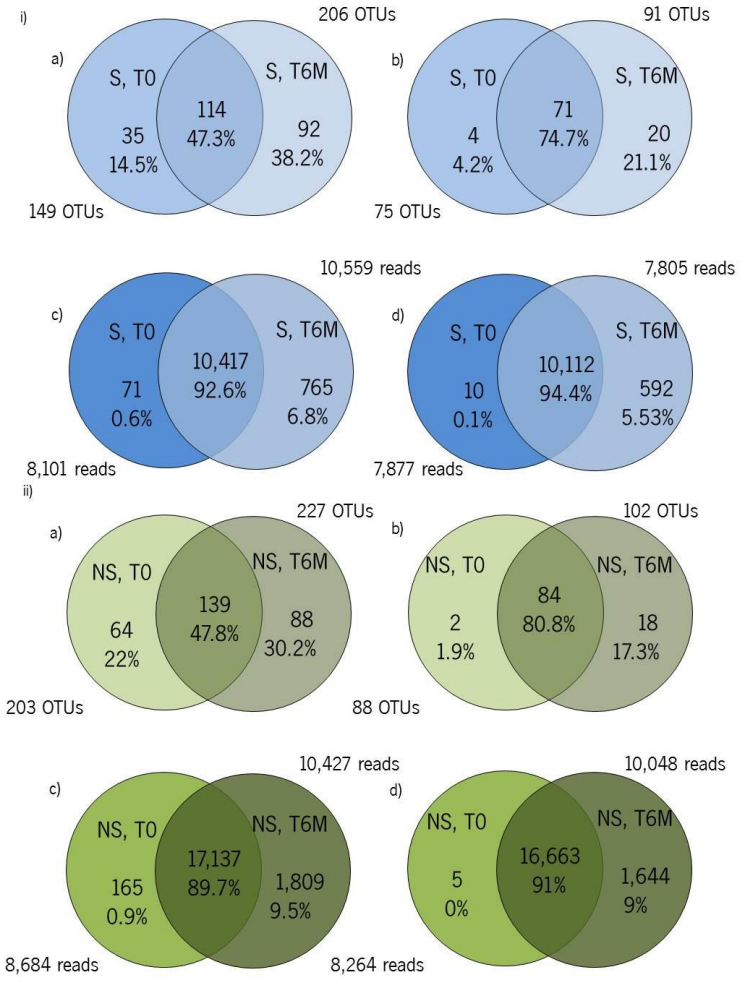


Figure 19 - Comparison between sterile (i) and non-sterile soil (ii) samples, taken from two months (T0) after transplantation or six months (T6M) after inoculation with *H. fasciculare*, concerning the number of OTUs (a and b), and the number of reads (c and d). Analyses were performed with all obtained OTUs (a and c) and with only those that comprise more than 50 reads (b and d).

The number of exclusive fungal species increased during the time of chestnut growing (figure 19). The samples collected six months after *H. fasciculare* inoculation presented 38.2% (S, T6M) or 30.2% (NS, T6M) exclusive OTUs of the total richness, in comparison to two-months of chestnut growing soils [14.5% (S, T0) and 22% (NS, T0)]. Exclusive species were also better represented by a higher number of reads after six months of fungal inoculation [6.8% (S, T6M) or 9.5% (NS, T6M) of the total number of reads] than two-months of chestnut growing soils [0.6% (S, T0) and 0.9% (NS, T0)]. These results corroborate the "contamination" effect previously suggested, since the greenhouse period will increase the number of exclusive species that in turn will be more disseminated with time.

Although (S, T0) presented 35 exclusive OTUs, only four (4.2%) were considered as well-represented in whole the study. However, in this soil sample, each OTU was only represented by one to five reads. Six months after plant inoculation (S, T6M), the number of exclusive OTUs increased (20 OTUs) corresponding to 21.1% of the total number of OTUs present in that sample. Five of these exclusive OTUs were present at a very high abundance, such as *Laccaria ohiensis* (M), whose abundance in whole study was 1,215 reads, being mostly present in the sterile sample harvested six months of fungal inoculation (1,079 reads). There were also other OTUs that, although present in both samples, were predominant after six months of plant growing inoculation, such as *Monographella cucumerina* (P) [12 and 258 reads, in (S, T0) and (S, T6M), respectively], *Mortierella hyaline* (S) (one and 376 reads, respectively) and *Leucosporidium scottii* (Y) (five and 558 reads, respectively). On the other hand, only two species significantly decreased their abundance in (S, T6M), when compared with (S, T0), namely *Biscogniauxia nummularia* (P) [125 and 23 reads, in (S, T0) and (S, T6M) respectively] and *Hypoxylon fragiforme* (S) (70 and 50 reads, respectively).

A decrease in the number of OTUs exclusive to (NS, T6M) was evident, after the 50 reads cutoff. From the 88 exclusive OTUs (30.2%), only 18 (17.3%) were well-represented in whole study, comprising ten mycorrhizae, five parasitic, two saprotroph and one yeast. *Inocybe lacera*, *Laccaria* sp. GMM1080, *Tirmania pinoyi* and *Cortinarius elegantissimus* were well-represented mycorrizhal OTUs in (NS, T6M) (937, 298, 150 and 126 reads respectively). However, *Inocybe sororia*, *Laccaria ohiensis* and *Laccaria bicolor*, also mycorrhizal OTUs, presented a low number of reads in this soil sample (12, five and three reads, respectively). From the 64 exclusive OTUs present in (NS, T0) sample, only two were well-represented [*Lecanicillium fusisporum* (P) and *Pleospora bjoerlingii* (P)], exhibiting a very low abundance (3 and 2 reads, respectively).

The general increase in the OTU number observed in soils used for growing chestnut plantlets during two months would be expected, since soil pots were joined together in the same greenhouse, and no asepsia conditions were established. Indeed, is has been recognized that the exchange of signals between plant and fungal partners begins even before any physical contact had been established (Baptista *et al.*, 2011). Plant-microbe interactions may also lead to substantial shifts in microbiome community, since plant root exudates may contribute to the enrichment of specific soil populations (Bakker *et al.*, 2014). Indeed, according to Fracchia *et al.* (2004), the exudates produced by saprotrophic fungi may influence the arbuscular mycorrhizal fungi abundance through a possible effect on the germination of AM fungal spores. Different genera of saprotrophic fungi can contribute to this influence, such as *Aspergillus*, *Penicillium* and *Trichoderma* (McAllister *et al.*, 1994, 1995; Fracchia *et al.*, 1998, 2004; García-Romera *et al.*, 1998). However, when considering the chestnut orchards soils ectomycorrhizae is more abundant. Therefore, as described in Baptista *et al.*, (2007) the root extracts had the ability to regulate ectomycorrhizal fungal growth, in early stages of *Pisolithus tinctorius* and *Castanea sativa* association. In the present work, *Trichoderma* species are the species that were well-represented in whole study, and exhibited an increase with chestnut growing. In sterile soil samples, four *Trichoderma* OTUs increased from 18 reads to 137 reads during six months of chestnut growth, while in non-sterile soil samples the five *Trichoderma* OTUs increased from 55 reads to 139 reads. In accordance with previous findings, an increase on mycorrhizal fungi was evident during plant growth, both in richness and abundance. In sterile soils, after six months of plant inoculation, mycorrhizal OTUs increased from six (108 reads) to ten OTUs (1,425 reads), while an increase of five OTUs (1,067 reads) to 15 OTUs (2,303 reads) was registered in non-sterile soils.

Comparison between *Hypholoma fasciculare* inoculation treatments

In order to study the influence of *H. fasciculare* on fungal community, soils were inoculated with the saprotrophic *H. fasciculare* after two months upon chestnut plant transplantation. Soil samples were collected six months after fungal inoculation (T6M + Hf) and compared to samples without inoculation (T6M). This procedure was performed on pots previously subjected (S) or not (NS) to sterilization (figure 20).

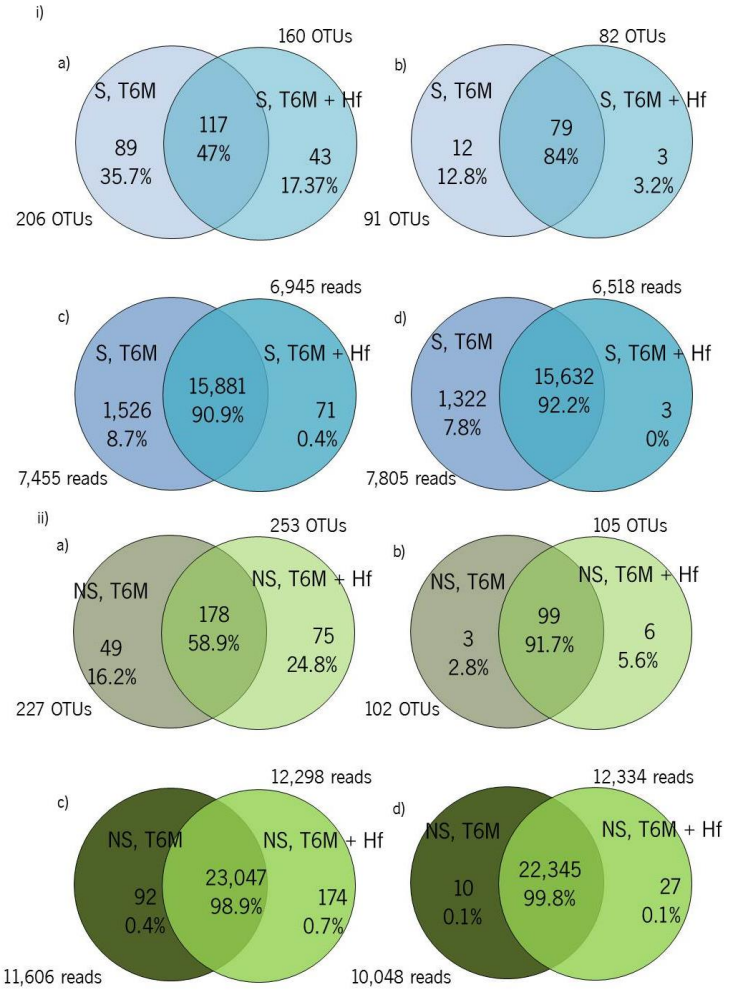


Figure 20 - Comparison between sterile (i) and non-sterile soil (ii) samples, taken from six months after inoculation (T6M + Hf) or not (T6M) with *Hypholoma fasciculare*, concerning the number of OTUs (a and b), and the number of reads (c and d). Analyses were performed with all obtained OTUs (a and c) and with only those that comprise more than 50 reads (b and d).

When previously sterile soils were used, soil samples inoculated with *H. fasciculare* were less richer (160 OTUs) than without inoculation (206 OTUs). However, an opposite trend was observed when non-sterile soils were used (253 OTUs in inoculated soils *vs*. 227 OTUs in non-inoculated soils). In both situations, a large fraction of the OTUs were shared between inoculated and non-inoculated samples. Several OTUs (117; 47% of total OTUs found in both situations) were shared between *H. fasciculare* inoculated and non-inoculated sterile soils, 79 of which were well-represented in whole study. The same pattern was detected on non-sterile soil samples, in which 178 OTUs (58.9% of total OTUs found in both situations) were maintained upon inoculation, but only 99 OTUs were well-represented in whole study. For both conditions (S and NS soils), a high abundance of common well-represented OTUs was evident (92.2% of the total number of reads in S soils; 99.8% in NS soil samples).

From the 79 well-represented OTUs in whole study that were found in both S soils (41 parasites, 20 saprotrophics, eight yeasts, five mycorrhizae, four parasitic/saprotroph and one unclassified species), only 11 OTUs presented a high read abundance (>50 reads) in both samples, 18 OTUs only presented more than 50 reads in non-inoculated soils, and just five OTUs were only well-represented in inoculated soils. All remaining OTUs (45) did not present a high abundance in reads in these soil samples. The yeast *Leucosporidium scottii* exhibited the highest difference between both samples, since it was 93-fold more abundant in the sample without fungal inoculation (S, T6M, 558 reads) than after inoculation (S, T6M + Hf, 6 reads). This trend was followed by *Leotia lubrica*, a saprotrophic species, being 61-fold more abundant in non-inoculated sample (61 reads) than inoculated one (1 reads). On the contrary, other OTUs presented higher abundance in the soil sample with *H. fasciculare* (S, T6M + Hf) than the control (S, T6M), such as, *Hebeloma mesophaeum* (M) and *Auricularia cornea* (PS) that were 145- and 51-fold, respectively, more abundante in inoculated soils [145 and 102 reads in (S, T6M + Hf)] in comparison with 1 and 2 reads in (S, T6M), respectively.

From the 99 well-represented OTUs in whole study that were found in both NS soils (43 parasites, 28 saprotrophics, 14 mycorrhizic, eight yeasts, four parasitic/saprotroph and two unclassified species), only 22 OTUs presented a high read abundance (>50 reads) in both samples, seven OTUs were only well-represented in non-inoculated soils, and 11 OTUs in inoculated soils. The remaining OTUs (59) did not present a high abundance reads in these soil samples. The mycorrhizal *Cortinarius limonius* distinguished itself by displaying the greatest difference between inoculated and non-inoculated soil samples, since it was about 12-fold less abundant in inoculated samples [107 reads in (NS, T6M + Hf) in comparison with 9 reads in (NS, T6M), respectively]. Of the 11 OTUs with higher abundance in (NS, T6M + Hf), *Auricularia cornea* (PS) was about 13-fold more abundant in comparison to non-inoculated sample [421 reads in (NS, T6M + Hf) in comparison with 32 reads in (NS, T6M), respectively].

When considering the exclusive species, an opposite trend was observed in sterile and non-sterile soils. While the sterile soil sample without fungal inoculation (S, T6M) presented a higher number of exclusive OTUs than the inoculated one, the non-sterile soil sample that presented a higher number of exclusive OTUs was the one inoculated with *H. fasciculare* (NS, T6M + Hf). An almost similar pattern was also verified when referring to the abundance. About 21-fold more reads were detected in (S, T6M, 1,526 reads) in comparison to (S, T6M + Hf, 71 reads), while a

more slight reduction in the reads number (two-fold less) was detected from (NS, T6M, 92 reads) to (NS, T6M + Hf, 174 reads).

In sterile soils without fungal inoculation (S, T6M), from the 12 exclusive OTUs that were well-represented in whole study, only three OTUs comprised more than 50 reads in that soil sample. All of which were mycorrhizal, namely: *Laccaria ohiensis* (M) (1,079 reads), *Cortinarius limonius* (Y) (98 reads) and *Paxillus involutus* (M) (51 reads). The remaining nine OTUs exhibited low abundance in the referred soil sample, but still showed high abundance in whole study, *Inocybe lacera* (M) and *Pestalotiopsis besseyi* (P). On the other hand, from the 43 OTUs exclusive to the sterile soil sample with *H. fasciculare* inoculation (S, T6M + Hf), only three OTUs (3.2%) were well-represented in the whole study, but only presented one read each in this sample, namely, *Mortierella verticillata* (S), *Neurospora africana* (S) and *Pholiota alnicola* (P). In non-sterile soil samples without fungal inoculation (NS, T6M), from the 49 exclusive OTUs, only three were well-represented in whole study, comprising in total ten reads abundance in these samples, ranging from one to six reads. On the other hand, the soils samples with fungal inoculation (NS, T6M + Hf) presented 75 OTUs exclusive, from which six were well-represented in whole study. Besides presenting low abundance in these samples (ranging between one and 12 reads), two of the six OTUs presented a high abundance in whole study, *Russula praetervisa* (M) (167 reads) and *Pleospora bjoerlingii* (P) (127 reads).

According to previous results that suggested an antagonist effect of *H. fasciculare* against other microorganisms (Pereira et al., 2012; Reis, 2012), the presence of this fungus was expected to suppress the presence of some species within the fungal community. The analysis performed on sterile soil samples corroborates this idea, since a largest number of exclusive OTUs were detected in (S, T6M), and a higher number of shared OTUs were more abundant in (S, T6M) than in (S, T6M + Hf). The results observed in sterile soils also suggested that mycorrhizal fungi could be strongly affected by the presence of *H. fasciculare*, since many were exclusively present in non-inoculated soils (*Laccaria ohiensis*, *Cortinarius limonius* and *Paxillus involutus*). Indeed, the abundance of well-represented exclusive OTUs in (S, T6M) was almost 42% from mycorrhizal fungi.

When considering native soil, which were not previously submitted to sterilization, a different scenario was detected. The number of exclusive OTUs was higher after inoculation and the number of shared OTUs were more abundant in (NS, T6M + Hf) than in (NS, T6M), thus

suggesting that *H. fasciculare* could have promoted the development of certain species. However, as the number of reads belonging to exclusive OTUs of both samples is only 0.2% of the total number of reads in both samples, *H. fasciculare* could be assumed to not display a significative effect on microbial community of native soils. The buffering effect of native soils could be due to the well-established microbial community in those soils that most probably do not present representative fluctuations along time. Indeed, when comparing the sterile soil samples against the non-sterile soil samples, major variations in the fungal community become evident, which could be explained by the initial sterilization step the equilibrium of the fungal community appears to be kept, both in richness and abundance, in contrast with sterile soils. Therefore the buffering effect detected in non-sterile soils seems to have the capacity to resist changes as detected previously (Dorioz *et al.*, 2006), in this case this effect was noticed once there was no interposing of the native established equilibrium in the orchards soils. On the contrary, sterile soils are most probably still being invaded by new microrganisms that are progressively being installed, due to the lack of assepsia of the greenhouse and cross-contamination between pots. Antagonistic interactions between fungi are most probably still occurring when *H. fasciculare* was inoculated, and could have been influenced by the presence of a new antagonist partner.

From both analyses, some species appears to be specifically affected by the presence of the saprotrophic *H. fasciculare*. The parasite/saprotroph *Auricularia cornea* appears to be positively affected by *H. fasciculare*, since it is approximately 13-fold more abundant in (NS, T6M + Hf) than in (NS, T6M), and 51-fold more abundant in (S, T6M + Hf) than in (S, T6M). In contrast, the mycorrhizal fungus, *Cortinarius limonius* is about 12-fold less abundant in non-inoculated native soils (107 reads) than in inoculated ones (9 reads) and is exclusively found in non-inoculated sterile soils (98 reads). Although the mycorrhizal fungus, *Hebeloma mesophaeum* was positively affected by *H. fasciculare* [presenting a 145-fold increase from (S, T6M + Hf, 145 reads) to (S, T6M, 1 read)], several mycorrhizal species were exclusive or more abundant in non-inoculated soils [*Laccaria ohiensis* (S, T6M, 1,079 reads; S, T6M + Hf, 0 reads) and *Paxillus involutus* (S, T6M, 51 reads; S, T6M + Hf, 0 reads)].

Evaluation of the dynamics of fungal community after inoculation

In order to further study the influence of *H. fasciculare* on fungal community, the dynamics of fungal community was evaluated in soil samples collected six months (T6M + Hf) and one year after fungal inoculation (T1Y + Hf) and compared to samples harvested immediately before

inoculation (T0). This procedure was performed on pots previously subjected or not to sterilization (S and NS) (figure 21).

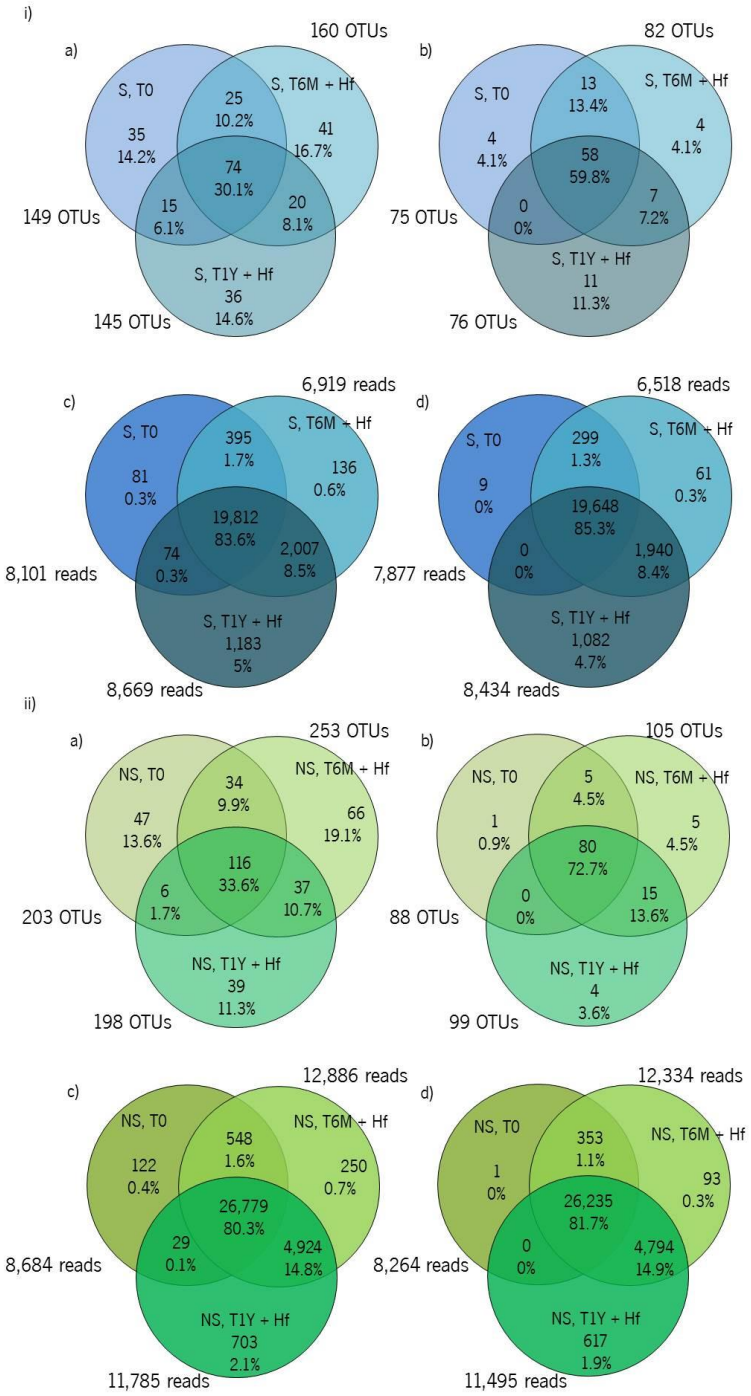


Figure 21 - Comparison between sterile (i) and non-sterile soil (ii) samples, taken after different periods upon inoculation - (T0 – just before inoculation, T6M – six months and T1Y – one year after inoculation) concerning the number of OTUs (a and b), and the number of reads (c and d). Analyses were performed with all obtained OTUs (a and c) and with only those that comprise more than 50 reads (b and d).

In both sterile and non-sterile soils a high number of OTUs was shared between all the three samples [(T0), (T6M + Hf) and (T1Y + Hf)]. In sterile samples, 74 OTUs remained following

H. fasciculare inoculation (30.1% of total number of OTUs found in all three samples), comprising 83.6% of the abundance of the total number of reads (19,812 reads) (figure 21 – i). From these 74 OTUs, 58 corresponded to well-represented OTUs (59.8% of total number of OTUs), comprising 19,648 reads (85.3%). This represents less than a half of the detected OTUs in each soil sample; although only a smaller fraction (nine OTUs) were present on the three conditions with more than 50 reads. The same pattern was detected for non-sterile soil samples (figure 21 – **ii**). From the 116 OTUs shared by all three samples, represented by 26,779 reads, only 80 (26,235 reads) were well-represented in whole study, of which only nine presented high abundance (more than 50 reads) in each sample. After removing the less-represented OTUs, in non-sterile soil samples, it was evident that the shared OTUs increased its proportion of 33.6% to 72.7%, mostly of them belonging to the trophic group of parasites (37). However, when considering the number of reads, the increase of abundance was not so evident as for richness, since only an increase of 1.4% was detected (from 80.3% to 81.7%).

When considering the common OTUs between all conditions, the soil samples harvested just before fungal inoculation (T0) and the samples harvested one year after fungal inoculation (T1Y + Hf) did not share a large percentage of OTUs (1.7%-6.1%). Only 15 OTUs (corresponding to 74 reads) were shared between both sterile samples, but none was a well-represented OTU. The same pattern was detected for non-sterile soil samples. Of the six OTUs (29 reads) shared between (NS, T0) and (NS, T1Y + Hf), none was well-represented in whole study. On the other hand, when analysing (T0) with (T6M + Hf), a higher number of well-represented OTUs and their reads was found for both sterile and non-sterile soils samples (13 OTUs and 299 reads in S soils, five OTUs and 353 reads in NS soils). Finally, between (T6M + Hf) and (T1Y + Hf) only the non-sterile soils samples presented an increase in shared well-represented OTUs (15 OTUs with 4,794 reads), in comparison with sterile soils (seven shared OTUs with 1,940 reads).

After six months of fungal inoculation (T6M + Hf), the soils samples were always richer (160 in S samples and 253 OTUs in NS samples) than six months later (145 and 198 OTUs, sterile and non-sterile soil samples, respectively). However, these samples richness values were not always accompanied by abundances values. For example, although being the richest of sterile samples (S, T6M + Hf), the same sample was also the less abundant (6,919 reads). Interestingly, although the soil sample taken one year after *H. fasciculare* inoculation, (S, T1Y + Hf), was the most abundant (8,669 reads), the correspondent sample in non-sterile soils was not the most abundant (NS, T1Y + Hf, 11,785 reads). Nevertheless, both sterile and non-sterile (T1Y

+ Hf) samples exhibited the highest number of exclusive reads one year after *H. fasciculare* inoculation (1,082 and 617 reads, respectively).

The sterile soil sample whose chestnuts were harvested immediately before the fungal inoculation (S, T0) exhibited 35 exclusive OTUs that included 81 reads. From these, only four OTUs were well-represented in whole study, but only comprised nine reads in this samples, ranging from one to five reads each. These four OTUs were identified according to their functional group: one parasitic, one mychorriza and two saprotrophs. From the exclusive OTUs present in (S, T6M + Hf) soils (41), only four remained when considering only the well-represented OTUs. From these, the saprotrophic *Ascocoryne cylichnium* in this sample (48 reads). Of the 36 OTUs that only arised on (S, T1Y + Hf), only 11 were well-represented in the whole study, four of which presented more than 50 reads in this soil sample, namely *Cortinarius limonius* (M) (804 reads), *Pestalotiopsis besseyi* (P) (78 reads), *Scytalidium cuboideum* (Un) (69 reads) and *Peziza ostracoderma* (S) (55 reads). Considering the dynamics of the fungal community in sterile soil samples, when reached one year after fungal inoculation, 13 OTUs well-represented that were previously present in S, T0 and in S, T6M + Hf no longer existed in this soil sample. Of these *Biscogniauxia nummularia* (P) singled out from the remaining 12, since was the most abundant OTU in (S, T0) sample with 125 reads. All the other OTUs besides being well-represented presented low abundance in the soil samples (S, T0) and (S, T6M + Hf).

Analysing the native soil sample harvested immediately before fungal inoculation (NS, T0), from the 47 exclusive OTUs (122 reads) only a single OTU [*Phoma cucurbitacearum* (P), represented by one read) was a well-represented OTU in whole study. From the 66 exclusive OTUs (250 reads) found in (NS, T6M + Hf) sample, only five OTUs (93 reads) were well-represented in whole study, but still presented a low abundance in the present condition (three to 42 reads). In non-sterile soil sample that were inoculated one year before (S, T1Y + Hf), from the exclusive 39 OTUs (703 reads) just five OTUs (617 reads) were well-represented in the whole study. Of these, two correspond to mycorrhizal fungi (*Cortinarius disjungendus* and *Tricholoma sejunctum,* 558 and 4 reads, respectively), one saprotrophic (*Mycetinis alliaceus*, 54 reads) and one parasitic (*Verticillium leptobactrum*, one read).

The abundance of many OTUs decreased or increased following *H. fasciculare* inoculation, but their behavior in S soils were not always the same as in NS soils. The parasitic/saprotroph *Ganoderma orbiforme* tended to disappear in the sample (S, T6M + Hf, 47 reads) when comparing with (S, T0, 4,091 reads) and (S, T1Y + Hf, 368 reads). On the other hand,

Cadophora finlandica (P) was absent in the control, but following inoculation its abundance increased (846 and 626 reads after six months and one year of inoculation, respectively). In non-sterile soil samples, OTUs were always present but with low abundance (*G. orbiforme* range from two to 12 reads and *C. finlandica* range from four to 98 reads). The mycorrhizal fungi *Amanita rubescens* presented low abundance in the S control (one read), but the number of reads in S soils increased following *H. fasciculare* inoculation (616 and 1,168 reads, after six months and one year of inoculation, respectively). In non-sterile soil samples this species also has maintained low abundance in three samples (NS, T0, 6 reads; NS, T6M + Hf, 8 reads and NS, T1Y + Hf, 6 reads). Concerning the mycorrhizal *Cortinarius lilacinovelatus*, while its abundance in sterile soils was low (ranging from one to 28 reads), in non-sterile soil samples was more abundante. This OTU presented approximately 12-fold and 14-fold more reads in (NS, T0) and (NS, T1Y + Hf), respectively, than in (NS, T6M + Hf) [NS, T0, 916 reads; NS, T1Y + Hf, 1,089 reads; NS, T6M + Hf, 77 reads]. Also, *Hebeloma mesophaeum* (M) and *Pholiota alnicola* (M) despite being present in all three NS samples, exhibited aproximmately 18- and 34-fold more abundance in (NS, T1Y + Hf, 251 and 68 reads, respectively) than on the younger chestnut sample (NS, T0, 14 and two reads, respectively), respectively. There are also several OTUs that presented a higher abundance in (T6M + Hf) soil samples. *Auricularia cornea* (PS), *Hebeloma mesophaeum* (M) and *Ilyonectria macrodidyma* (P) became predominant in (S, T6M + Hf) and then their abundance decreased in the next months [ranging between one and 15 reads in (S, T1Y + Hf) sample].

The soil conditions (NS, T6M + Hf) and (NS, T1Y + Hf) shared the highest number of OTUs and reads number. This appears to have occured due to the already referred buffering effect in NS soils, but also to the fact that these two samples were kept together in the greenhouse, subjected to the same conditions and contaminations. Analysing the dynamics of fungal community upon inoculation, the (T6M + Hf) sample is singled out by exhibiting higher or lower abundance that the remaining (T0) and (T1Y + Hf). This was particularly evident in S soils for *C. finlandica* (P) (846, 0 and 629 reads, respectively), *H. mesophaeum* (M) (145, 1 and 15 reads, respectively) and *G. orbiforme* (PS) (47, 4,091 and 368 reads, respectively).

A clear pattern of microbial community variation upon *H. fasciculare* inoculation was not evident. When evaluating the number of well-represented OTUs present in each soil condition, the arise and disappearance of new OTUs was noticed, some of which could be the result of specific fungal interactions. The presence of mycorrhizal fungi, which seemed to be enhanced by plant

development as revealed by (S, T6M) and (NS, T6M) samples, appeared to be constrained after *H. fasciculare* inoculation. Indeed, *Cortinarius limonius* was more abundant in non-inoculated soils (NS, T6M) than inoculated ones (NS, T6M + Hf) and several other mycorrhizal species were limited to non-inoculated soils, such as the mycorrhizic *Paxillus involutus*.

After 12 months of inoculation, the *H. fasciculare* promoting or inhibiting effect become weaker. For several specific OTUs, the (T1Y + Hf) sample displayed an intermediate abundance between (T0) and (T6M + Hf). This is most probably due to the establishment of a new microbial community after one year, in which *H. fasciculare* could still take part or not. Indeed, the microbial interactions could even have vanished *H. fasciculare* mycelium from the soil. In this work, the variation in abundance of specific species could be the result of antagonistic interaction displayed by co-occurring fungi. For example, the presence of *Laccaria ohiensis* could be affected by the presence of the parasite *Fusarium oxysporum* as reported by Chakravarty and Hwang (1991). In the present study, *L. ohiensis* was mostly present in the sterile soil sample (S, T6M, 1,079 reads), followed by the condition (NS, T1Y + Hf) with 120 reads. All the remaining soil conditions range between zero and nine reads of abundance of this fungus. On the other hand, *F. oxysporum* was abundant in soil conditions where this mycorrhizal fungus was absent (NS, T0, 76 reads), (NS, T6M, 59 reads), (NS, T6M + Hf, 91 reads) and almost inexistent in the presence of *L. ohiensis*. This negative effect has been described to be associated with the production of antimicrobial substances and antifungal compounds by *L. ohiensis* (Chakravarty and Hwang, 1991).

Hypholoma fasciculare effect on different fungal functional groups

The analysis of individual OTUs turns the analysis of *H. fasciculare* effect on microbial community hard and erratic, due to the high number of detected OTUs and to the low abundance that could occur in certain conditions. As ectomycorrhizal fungi, as well as parasitic fungi, correspond to functional groups with large impact in chestnut groves, an analysis of fungal functional groups was performed for depicting a more general idea of *H. fasciculare* effect.

As previously referred, the well-represented OTUs (comprising more than 50 reads in whole study) were identified according to their functional group (table 12). Parasitic species were the most rich (48 OTUs, 42.35% of total OTUs) and abundant (23,574 reads, 31.3% of total reads) functional group. Saprotrophic and mycorrhizal species followed with 32 and 18 OTUs, respectively (comprising 12,235 and 16,035 reads, respectively). Although presenting a low

richness (only four OTUs, 3.5%), parasite/saprotrophic fungi were very abundant (13,728 reads, 18.2%).

The distribution of functional fungi whithin each soil sample was quite homogeneous among the different soil samples (figure 22). However, NS soils presented a slight higher richness and abundance of mycorrhizal and saprotrophic fungi than S soils, which in turn presented a higher richness and abundance of parasites than NS soils. This incubation of sterile soils in the greenhouse environment could have led to a biased fungal community in relation to NS soils. However, due to the close proximity of all pots, it is probable that cross-contamination between samples could have led to somewhat similiarities between soil samples.

The values presented for (NS, T0) samples are quite divergent from the values obtained from previous metabarcoding study performed in chestnut orchard soils, in which a higher percentage of mycorrhizal (37%) and lower parasite (30%) and saprotrophic (20%) fungal OTUs were reported (Reis, 2012) in contrast to the 12.9%, 48.8% and 30.2% found in the present study, respectively. The same trend was also reported for abundance values, in which mycorrhizal reads attained 52% of total reads and parasitic fungal reads were only 17%, in contrast with 12.9% and 40.5% found in the present study, respectively. This divergence could be due to the site where orchard soil was collected. For metabarcoding, soil samples were collected 2 m away from the tree trunk, while for this study the soil was more distanced from the trees (>50 m).

One year after fungal inoculation with *H. fasciculare* an obvious increase on richness and abundance of mycorrhizal fungi was detected, both in S and NS soils. As previously referred, and in agreement to Fracchia *et al.*, (2004), a possible synergistic interaction between the saprotrophic *H. fasciculare* and mycorrhizal fungi may have occurred, in which the exudates produced by saprothophs may influence the development of mycorrhizal fungi (McAllister *et al.*, 1994, 1995; Fracchia *et al.*, 1998; García-Romera *et al.*, 1998). Accordingly, as evident in table 12 and figure 22, the soil conditions that have the highest number of saprotrophic OTUs also present the highest number of OTUs on the mycorrhizal trophic group.

Table 12 – Distribution of the identified OTUs number and corresponding read abundance (underlined values), according to each functional group, in each soil sample. The correspondent percentage of richness and abundance is presented in brackets. This analysis only comprises the well-represented species in the study (more than 50 reads), distributed in sterile (S) or non-sterile (NS) soils samples, with (+Hf) or without *H. fasciculare* inoculation and harvested in different times: immediately before inoculation – T0; six months T6M and one year after inoculation, T1Y.

Functional groups	Number of species (reads)								
	S,T0	S,T6M	S,T6M+Hf	S,T1Y+Hf	NS,T0	NS,T6M	NS,T6M+Hf	NS,T1Y+Hf	Total
Mycorrhizal	6 (8.0%) 108 (1.4%)	10 (11.0%) 1,425 (13.9%)	5 (6.1%) 902 (13.4%)	9 (11.8%) 3,387 (40.2%)	5 (5.8%) 1,067 (12.9%)	15 (14.7%) 2,303 (22.9%)	15 (14.3%) 798 (6.5%)	16 (16.2%) 6,047 (52.6%)	18 (15.9%) 16,037 (21.3%
Parasite	36 (48.0%) 2,407 (30.6%)	44 (48.4%) 3,652 (35.7%)	34 (51.2%) 3,856 (57.3%)	34 (44.7%) 3,430 (40.7%)	42 (48.8%) 3.346 (40.5%)	45 (44.1%) 2,227 (22.2%)	46 (43.8%) 3,668 (29.7%)	41 (41.4%) 988 (8.6%)	48 (42.5%) 23,574 (31.3%)
Parasite/ Saprotroph	4 (5.3%) 4,394 (55.8%)	4 (4.4%) 1,883 (18.4%)	4 (4.9%) 642 (9.5%)	4 (5.3%) 524 (6.2%)	4 (4.7%) 1,399 (16.9%)	4 (3.9%) 1,493 (14.9%)	4 (3.8%) 2,310 (18.7%)	4 (4.0%) 1,083 (8.6%)	4 (3.5%) 13,728 (18.2%)
Saprotroph	21 (28.0%) 842 (10.7%)	23 (25.3%) 1,964 (19.2%)	22 (26.8%) 682 (10.1%)	22 (28.9%) 904 (10.7%)	26 (30.2%) 1,315 (15.9%)	28 (27.5%) 1,869 (18.6%)	29 (27.6%) 2,884 (23.4%)	29 (29.3%) 1,775 (15.4%)	32 (28.3%) 12,235 (16.2%)
Yeast	7 (9.3%) 125 (1.6%)	8 (8.8%) 1,284 (12.6%)	8 (9.8%) 641 (9.5%)	5 (6,6%) 117 (1.4%)	7 (8,1%) 1,126 (13.6%)	8 (7.8%) 2,115 (21.0%)	9 (8.6%) 2,589 (21.0%)	7 (7.1%) 1,593 (13.9%)	9 (8.0%) 9,590 (12.7%)
Unclassified	1 (1.3%) 1 (0.0%)	2 (2.2%) 18 (0.2%)	1 (1.2%) 8 (0.1%)	2 (2.6%) 72 (0.9%)	2 (2.3%) 11 (0.1%)	2 (2.0%) 41 (0.4%)	2 (1.9%) 85 (0.7%)	2 (2.0%) 9 (0.1%)	2 (1.8%) 245 (0.3%)
Total	75 7,877	91 10,226	82 6,731	76 8,434	86 8,264	102 10,048	105 12,334	99 11,495	113 75,409

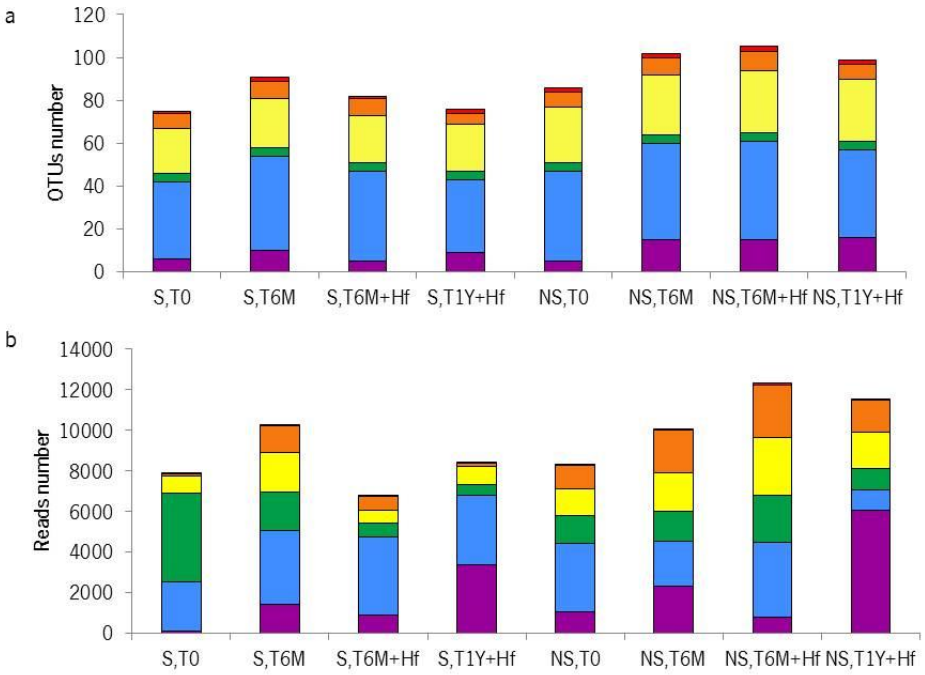


Figure 22 - Distribution of the most well-represented OTUs number (a) and reads number (b) (more than 50 reads), according to the functional group, in each soil sample. Different colors represent the different trophic groups of fungi: purple – mycorrhizal (M), blue – parasitic (P), green – parasitic/saprotrophic (PS), yellow – saprotroph (S), orange – yeast (Y) and red – unclassified (Un). Samples comprise sterile (S) or non-sterile (NS) soils samples, with (+Hf) or without *H. fasciculare* inoculation and harvested in different times: immediately before inoculation – T0; six months T6M and one year after inoculation, T1Y.

In order to detect any correlation among fungal functional groups in the different soil samples, a Pearson correlation teste was performed, considering both OTUs richness and abundance (table 13). All correlations were statistically significant eighter at $P<0.001$. Concerning the results of richness the correlation values detected were always positive, being the most positive the one determined between saprotrophs and parasites (0.750), followed by the correlation between mycorrhizal and saprotrophic fungi (0.620). These results mean that when the number of saprotrophs (or mycorrhizal fungi) increase, the number of parasites (or saprotrophs) increase. When referring to the abundance correlations a different scenario was observed. The values were mainly negative, but the correlations were not very high.

While in NS soils there was a reduction on parasites (in the number of OTUs and mainly in the number of reads) in inoculated soils, an increase of parasite OTUs and reads was detected following inoculation of S soils that attained values higher than 50% of total number of OTUs and reads in those samples.

Table 13 - Pearson correlation coefficient between fungi belonging to different functional groups identified in the studied samples. The correlation was determined between the number of well-represented OTUs (more than 50 reads) (displayed in the lower side) and the number of reads (displayed in the upper side of the table). The functional groups are labeled in the table as follows: M – mycorrhizal, P – parasite, PS – parasite/saprotrophic and S – saprotrophic fungi. Asterisks (** and ***) denote correlations that were significant at P<0.01 and P<0.001, respectively. Samples comprise sterile (S) or non-sterile (NS) soils samples, with (+Hf) or without *H. fasciculare* inoculation and harvesting in different times: immediately before inoculation – T0; six months T6M and one year after inoculation, T1Y).

	M	P	PS	S
M	1.000	-0.054**	-0.286	0.129***
P	0.556***	1.000	-0.110**	-0.122***
PS	0.069***	0.149***	1.000	-0.040***
S	0.620***	0.750***	0.191***	1.000

CHAPTER IV

CONCLUSIONS AND PERSPECTIVES

The European chestnut, *Castanea sativa* Mill., is a tree plant species that reveals a main significance at an ecological and economical level, due to the production of high quality wood and chestnuts. The present work aimed to study the impact of the saprotrophic fungi *Hypholoma fasciculare* on the soil fungal community present in *C. sativa* orchards, using a metagenomic approach. The recent methods of high-throughput sequencing have been important to allow a view of the organisms that interact in the ecological system (Bonfante and Anca, 2009).

The fungal community present is this study revealed that Ascomycota (58.9%) was the richest phylum, followed by Basidiomycota (38.9%), which in turn was the most abundant phylum (57.4%) against 40.9% Ascomycota reads. This behaviour was in conformity with other studies in chestnut orchard soils, where 55% of Basiodiomycota reads were found as evaluated by the pyrosequencing of ITS amplicons obtained from soil samples (Reis, 2012).

The dispersion capacity of fungi can lead to the low representativeness of some fungal taxa (Buée *et al.*, 2009). For example, in the whole study, 98 OTUs (21.4%) and 50 OTUs (10.1%) were found as singletons and doubletons, respectively. Thus, a selection of the most well-represented OTUs (those presenting more than 50 reads in whole study) was performed, in order to allow the exclusion of the less-represented OTUs. As a result, from the 458 OTUs (78,029 reads), only 113 (75,409 reads) were considered for evaluating the impact of *H. fasciculare* on soil fungal community.

Biodiversity is a key concept that evaluates the habitat quality. Two different types of diversity were computed: alpha diversity that considers the species richness within a local community (Whittaker, 1972; Whittaker *et al.*, 2001), and beta diversity that considers the diversity between systems or between environmental modifications, wherein the species turnover is evaluated along a complex environmental gradient (Whittaker, 1972; Wilson and Shmida, 1984; Barros, 2007). The sterile soil sample (S, T0) was the less rich and diverse , in contrast to NS soils that were the richest and most diverse samples, mainly (NS, T6M) and (NS, T6M + Hf) samples. Taking into account α and ß diversities in both types of soil (S and NS samples) a common pattern became evident: (T0) and (T1Y + Hf) samples exhibited low values of diversity (α diversity results), but were the most dissimilar among each other (ß diversity results). In contrast, both (T6M) and (T6M + Hf) samples exhibited high values of diversity (α diversity results), but were quite homogeneous (ß diversity results).

The sterile soil samples were more dissimilar among them than non-sterile samples. This result suggests that S soils are more likely to be affected by environmental conditions, namely by

H. fasciculare inoculation or during the chestnut growing than NS soils. The absence of the sterilization step in NS soils has not damaged the microbial community that was already well-established at the beginning of the assay. For this reason, NS soils seem to have a buffering-like effect, in which microbial community is not so easily affected in its equilibrium, neither by the fungal inoculation nor by the chestnut growing. Finally, S soils were less rich and abundant than NS soils.

Previous results suggested an antagonist effect of *H. fasciculare* against other microorganisms (Pereira et al., 2012; Reis, 2012). The presence of this fungus in the soil was then expectable to suppress some species within the fungal community, but an obvious pattern was not detected upon *H. fasciculare* inoculation. As the number of shared OTUs and corresponding reads increased during the incubation period, even when analyzing non-inoculated plants, *H. fasciculare* should not be entirely responsible for differences detected within fungal community. Indeed, the variations that occured could have been the result of other specific fungal interactions or could be explained by other environmental conditions applied during the study. Once in the greenhouse, the pots in which chestnut plants were growing were not kept under aseptic conditions, being watered by non-sterile water and being not protected from each other. As pot were disposed side-by-side in a randomly order, this have led to a probable cross-contamination between samples. In addition, as growing plant roots increase in contact with the soil, the chestnut root exudates could have promoted the development of previously uncommon microrganisms, increasing in this way the diversity among samples and affecting the microbial community.

At the end, *H. fasciculare* did not seem to cause considerable harm on the fungal community, but a different general trend was detected in S and NS soils. After six-months upon inoculation, sterile sample appears to be negatively affected by *H. fasciculare*, since in the absence of the fungi, (S, T6M), more exclusive species were presented and the sample was also richer and more abundant than inoculated sample (S, T6M + Hf). On the other hand, non-sterile soil samples exhibited the exactly opposite trend and seemed to be positively affected by *H. fasciculare*. Accordingly, (NS, T6M + Hf) was slight richer and more abundant than (NS, T6M) sample, even though most of the OTUs were shared between both soil samples. When evaluating the presence of well-represented OTUs in whole study, a not obvious effect was noticed. Only, some OTUs have arisen and disappeared in inoculated soil samples, which could have been the result of specific fungal interactions. For example, *Auricularia cornea* and *Hebeloma*

mesophaeum exhibited to be positively effected by the *H. fasciculare* inoculation, while *Cortinarius limonius*, *Laccaria ohiensis* and *Paxillus involutus* appear to be negatively affected by the fungus, decreasing their abundance.

All the species that were well-represented (more than 50 reads in whole study) were identified according to their functional group. Non-sterile soils were characterized for presenting higher richness and abundance of mycorrhizal and saprotrophic fungi than S soils, and S soils presented higher richness and abundance of parasites. Saprotrophs and parasites were the functional groups that were more positively correlated, in terms of richness ($r=0.750$, at $P<0.001$), but their abundance were negatively correlated ($r=0.122$, $P<0.001$). Although also presenting strong positive correlations for richness ($r=0.620$, at $P<0.001$), mycorrhizal and saprotrophic fungi were not correlated in terms of abundance. The correlation between mycorrhizal and saprotrophic species has been explained by the probable synergistic interaction that occurs involving the exudates of saprotrophs and their influence in the development of mycorrhizal fungi (McAllister *et al.*, 1994, 1995; Fracchia *et al.*, 1998; García-Romera *et al.*, 1998). In this study, *Trichoderma* species corroborate this finding by exhibiting an increase along chestnut growing, accompanied by the increase of richness and abundance of mycorrhizal species.

Altogether this study have examined the effect and influence of *H. fasciculare* on *C. sativa* orchard soils. For further studying and understanding this relation some considerations should be reformulated, in order to better understand the causes of the interactions that occurred. The conditions settled in the greenhouse should have been different, namely by considering a physical barrier between different pots and the use of sterile conditions, including during watering. Concerning the sampling, besides the physiological results that have suggested no significance differences between inoculated and non-inoculated plants, the metagenomics analysis of older plants could have result in different outcomes. The rarefaction curves also have revelead that there is still a fraction of OTUs to be uncovered in these ecosystems, which suggest a deeper sequencing in similar experiments. Finally, to take fully advantage of this study more correlations should have been perfomed, by comparing fungal diversity along the physiological development and taking into consideration the macro- and micronutrients composition of soils. Indeed, this aspect was overlooked in the present study, but has been described to contribute for the differences within the fungal community (Wardle, 2006).

The elucidation of *H. fasciculare* antagonist behaviour against the chestnut associated fungal community reveals to be of major importance, not only from the agronomic point of view, but also due to the ecological implications that it takes. The use of the recent high-throughput methodologies for detecting the microbial community of soils is an easy tool to detect constraints that could damaged community equilibrium. The inoculation with *H. fasciculare* does not seem to greatly afftect the microbial community of chestnut orchard soils, although specific microbial interactions could take place.

CHAPTER V

REFERENCES

Ali, M.A., Gyulai, G., Hidvégi, N., Kerti, B., Al Hemaid, F.M.A., Pandey, A.K., and Lee, J. (2014) The changing epitome of species identification - DNA barcoding. *Saudi J. Biol. Sci.* **21**: 204–231.

Anderson, I.C., Campbell, C.D., and Prosser, J.I. (2003) Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. *Environ. Microbiol.* **5**: 36–47.

Bakker, M.G., Schlatter, D.C., Otto-Hanson, L., and Kinkel, L.L. (2014) Diffuse symbioses: roles of plant-plant, plant-microbe and microbe-microbe interactions in structuring the soil microbiome. *Mol. Ecol.* **23**: 1571–1583.

Baptista, P. (2007) Macrofungos associados à cultura de castanheiro: aspectos da sua biodiversidade e da interacção de *Pisolithus tinctorius* e *Hypholoma fasciculare* com raízes de *Castanea sativa* Mill. *Dr. thesis* 1–267.

Baptista, P., Martins, A., Pais, M.S., Tavares, R.M., and Lino-Neto, T. (2007) Involvement of reactive oxygen species during early stages of ectomycorrhiza establishment between *Castanea sativa* and *Pisolithus tinctorius*. *Mycorrhiza* **17**: 185–193.

Baptista, P., Martins, A., Tavares, R.M., and Lino-Neto, T. (2010) Diversity and fruiting pattern of macrofungi associated with chestnut (*Castanea sativa*) in the Trás-os-Montes region (Northeast Portugal). Elsevier Ltd.

Baptista, P., Reis, F., Pereira, E., Tavares, R., Richard, F., Selosse, M., and Lino-Neto, T. (2015) Soil DNA pyrosequencing fruitbodies surveys reveal contrasting diversity for various fungal ecological guilds in chestnut orchards. *submitted*.

Baptista, P., Tavares, R.M., and Lino-Neto, T. (2011) Signaling in ectomycorrhizal symbiosis establishment. In, Rai,M. and Varma,A. (eds), *Diversity and Biotechnology of Ectomycorrhizae*. Springer-Verlag, pp. 157–175.

Barea, J.-M., Pozo, M.J., Azcón, R., and Azcón-Aguilar, C. (2005) Microbial co-operation in the rhizosphere. *J. Exp. Bot.* **56**: 1761–78.

Barros, R.S.M. (2007) Medidas de diversidade biológica. *Programa Pós-Graduação em Ecol. Apl. ao Manejo e Conserv. Recur. Nat. – PGECOL. Univ. Fed. Juiz Fora – UFJF.* 13.

Bissegger, M., Rigling, D., and Heiniger, U. (1997) Population Structure and Disease Development of *Cryphonectria parasitica* in European Chestnut Forests in the Presence of Natural Hypovirulence. *Phytopathology* **87**: 50–59.

Blom, J.M., Vannini, A., Vettraino, A.M., Hale, M.D., and Godbold, D.L. (2009) Ectomycorrhizal community structure in a healthy and a *Phytophthora*-infected chestnut (*Castanea sativa* Mill.) stand in central Italy. *Mycorrhiza* **20**: 25–38.

Boldrini, R., Santos, W., Cruz, Z., and Ramos, A. (2010) Bases da associação micorrízica orquidóide. *Nat. line* **8**: 140–145.

Bonfante, P. and Anca, I.-A. (2009) Plants, mycorrhizal fungi, and bacteria: a network of interactions. *Annu. Rev. Microbiol.* **63**: 363–383.

Borges, O., Gonçalves, B., Carvalho, J.L.S. de, Correia, P., and Silva, A.P. (2008) Nutritional quality of chestnut (*Castanea sativa* Mill.) cultivars from Portugal. *Food Chem.* **106**: 976–984.

Bray, J.R. and Curtis, J.T. (1957) An ordination of the upland forest community of southern Wisconsin. *Ecol. Monogr.* **27**: 325–349.

Brunner, K., Zeilinger S, and Mach, R.L. (2007) Molecular approaches for studying fungi in the environment. In, *The Mycota IV - Environmental and microbial relationships*. Springer, p. 350.

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R.H., Uroz, S., and Martin, F. (2009) 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytol.* **184**: 449–456.

Bugni, T.S. and Ireland, C.M. (2004) Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Nat. Prod. Rep.* **21**: 143–163.

Burnham, K.P. and Overton, W.S. (1978) Estimations of the size of a closed populations when capture probabilities vary among animals. *Biometrika* **65**: 623–633.

Burnham, K.P. and Overton, W.S. (1979) Robust estimation of population size when capture probabilities vary among animals. *Ecology* **60**: 927–936.

Carvalho, M.C.D.C.G. De and Silva, D.C.G. Da (2010) Sequenciamento de DNA de nova geração e suas aplicações na genómica de plantas. *Ciência Rural* **40**: 735–744.

Chakravarty, P. and Hwang, S.F. (1991) Effect of an ectomycorrhizal fungus, *Laccaria laccata*, on *Fusarium* damping-off in *Pinus banksiana* seedlings. **21**: 97–106.

Chang, S.-T. and Miles, P. (2004) Mushrooms Cultivation, nutritional value, medicinal effect, and environmental impact. 2nd editio.

Chao (1987) Estimating the population size for capture-recapture data with unequal catchability. *Biometrics* **43**: 783–791.

Chao, A. (1984) Nonparametric estimation of the number of classes in a population. *Scand. J. Stat.* **11**: 265–270.

Chapman, B., Emile, B., and Curran, M. (2001) Using *Hypholoma fasciculare* inoculation as a means to control Armillaria root disease on calcareous soils: trial establisment and monitoring. *Cariboo For. Reg. - Res. Sect.* 4.

Clarke, J. (1993) Non-parametric multivariate analysis of changes in community structure. *Aust J Ecol* **18**: 117–143.

Cody, M.L. (1975) Towards a theory of continental species diversities: bird distributions over Mediteranean habitat gradients. Cody,M.L. and Diamond,J.M. (eds).

Colwell, R. (2013) EstimateS 9.1.0 Statisical Estimation of Species Richness and Shared Species from samples User's Guide. 1–32.

Colwell, R.K. and Coddington, J.A. (1994) Estimating terrestrial biodiversity through extrapolation. *Philos. Trans. R. Soc. London Ser. B-Biological Sci.* **345**: 101–118.

Condit, R., Pitman, N., Leigh Jr, E.G., Chave, J., Terborgh, J., Foster, R.B., et al. (2002) Beta-diversity in tropical forest trees. *Science (80-.).* **295**: 666–669.

Corredoira, E., Valladares, S., Vieitez, A.M., and Ballester, A. (2015) Chestnut, European (*Castanea sativa*). *Methods Mol. Biol.* **1224**: 163–176.

Cortez, V.G. and Silveira, R.M.B. Da (2007) Species of *Hypholoma* (Fr.) P. Kumm. (*Strophariaceae, Agaricales*) in Rio Grande do Sul State, Brazil. *Acta Bot. Brasilica* **21**: 609–621.

Cristescu, M.E. (2014) From barcoding single individuals to metabarcoding biological communities: towards an integrative approach to the study of global biodiversity. *Trends Ecol. Evol.* 1–6.

Cronquist, A. (1968) The Evolution and Classification of Flowering Plants. *Bost. Hought. Mifflin* 396.

Deacon, J. (2006) Fungal Biology. 4th ed. Deacon,J. (ed) Blackwell Publishing Ltd.

Diamandis, S. and Perlerou, C. (2001) The mycoflora of the chestnut ecosystems in Greece. *For. Snow Landsc. Res.* **76**: 499–504.

Dorioz, J.M., Wang, D., Poulenard, J., and Trévisan, D. (2006) The effect of grass buffer strips on phosphorus dynamics - A critical review and synthesis as a basis for application in agricultural landscapes in France. *Agric. Ecosyst. Environ.* **117**: 4–21.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., and Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**: 2194–200.

FAO. (2012) Food and Agriculture Organization of the United Nations. *http://faostat.fao.org/site/* Access: 2015–01–12.

Ferreira-Cardoso, J. and Pimentel-Pereira, M. (2007) O castanheiro de fruto em Portugal. In, Gomes-Laranjo,J., Ferreira-Cardoso,J., Portela,E., and Abreu,C. (eds), *OS Castanheiros.*, p. 376.

Fisher, R.A., Corbet, A.S., and Williams, C.B. (1943) The relation between the number of species and the number of individuals in a random sample of an animal population. *J. Anim. Ecol.* **12**: 42–58.

Fracchia, S., Mujica, M.T., García-Romera, I., Garcia-Garrido, J., Martín, J., Ocampo, J., and Godeas, A. (1998) Interactions between *Glomus mosseae* and arbuscular mycorrhizal sporocarp-associated saprophytic fungi. *Plant Soil* **200**: 131–137.

Fracchia, S., Sampedro, I., Scervino, J., Garcia-Romera, I., Ocampo, J., and Godeas, A. (2004) Influence of saprobe fungi and their exudates on arbuscular mycorrhizal symbioses. *Symbiosis* 169–182.

García-Romera, I., Garcia-Garrido, J., Martin, J., Fracchia, S., Mujica, M., Godeas, A., and Ocampo, J. (1998) Interaction between saprotrophic *Fusarium* strains and arbuscular mycorrhizas of soybean plants. *Symbiosis* **24**: 235–246.

Gardes, M. and Bruns, T. (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Mol. Ecol.* **2**: 113–118.

Gomes-Laranjo, J., Coutinho, J., and Peixoto, F. (2007) Ecologia do castanheiro (*C. sativa* Mill.). In, Gomes-Laranjo,J., Ferreira-Cardoso,J., Portela,E., and Abreu,C.G. (eds), *Os castanheiros.*, p. 376.

Gomes-Laranjo, J. and Crespí, A.L. (2007) Taxonomia do castanheiro. In, Gomes-Laranjo,J., Ferreira-Cardoso,J., Portela,E., and Abreu,C. (eds), *Os castanheiros.*, p. 376.

Gupta, P.K. (2008) Single-molecule DNA sequencing technologies for future genomics research. *Trends Biotechnol.* **26**: 602–611.

Hajibabaei, M. (2012) The golden age of DNA metasystematics. *Trends Genet.* **28**: 535–7.

Harismendy, O., Ng, P.C., Strausberg, R.L., Wang, X., Stockwell, T.B., Beeson, K.Y., *et al.* (2009) Evaluation of next generation sequencing platforms for population targeted sequencing studies. *Genome Biol.* **10**: 13.

Harris, T.D., Buzby, P.R., Babcock, H., Beer, E., Bowers, J., Braslavsky, I., *et al.* (2008) Single-molecule DNA sequencing of a viral genome. *Science (80-.).* **320**: 106–109.

Harrison, S., Ross, S.J., and Lawton, J.H. (1992) Beta diversity on geographic gradient in Britain. *J. Anim. Ecol.* **61**: 151–158.

Henderson, P.A. and Seaby, R.M.H. (2007) Community Analysis Package 4.0.

Horton, T.R. and Bruns, T.D. (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Mol. Ecol.* **10**: 1855–1871.

INE (2013) Estatísticas Agrícolas 2013, Instituto Nacional de Estatística,I.P. (ed).

Jaccard, P. (1908) Nouvelles recherches sur la distribution florale. *Bull. la Société Vaudoise des Sci. Nat.* **44**: 223–270.

Janzen, D.H., Hajibabaei, M., Burns, J.M., Hallwachs, W., Remigio, E., and Hebert, P.D.N. (2005) Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **360**: 1835–1845.

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., and Barea, J. (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biol. Fertil. Soils* **37**: 1–16.

Ji, Y., Ashton, L., Pedley, S.M., Edwards, D.P., Tang, Y., Nakamura, A., *et al*. (2013) Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. *Ecol. Lett.* **16**: 1245–1257.

Kindt, R. and Coe, R. (2005) Analysis of differences in species composition. In, *Tree diversity analysis*., pp. 123–138.

Kirk, P., Cannon, J., David, J., and JA, S. (2001) Ainsworth & Bisby's Dictionary of the fungi. *Mycol. Res.* **106**: 507–511.

Klein, D.A. and Paschke, M.W. (2004) Filamentous fungi: the indeterminate lifestyle and microbial ecology. *Microb. Ecol.* **47**: 224–235.

Koleff, P., Gaston, K.J., and Lennon, J.J. (2003) Measuring beta diversity for presence –absence data. *J. Anim. Ecol.* **72**: 367–382.

Lim, Y.W., Kim, B.K., Kim, C., Jung, H.S., Kim, B.-S., Lee, J.-H., and Chun, J. (2010) Assessment of soil fungal communities using pyrosequencing. *J. Microbiol.* **48**: 284–289.

Magurran, A. (2004) Measuring biological diversity. Blackwell Science.

Martin, F. (2007) Fair Trade in the Underworld : the Ectomycorrhizal Symbiosis. *Biol. Fungal Cell, Mycota VIII* 291–308.

McAllister, C., García-Romera, I., Godeas, A., and Ocampo, J. (1994) Interactions between Trichoderma koningii, Fusarium solani and Glomus mosseae: effects on plant growth, arbuscular mycorrhizas and the saprophyte inoculants. *Soil Biol. Biochem.* **26**: 1363–1367.

McAllister, C., García-Romera, I., Martin, J., Godeas, A., and Ocampo, J. (1995) Interaction between *Aspergillus niger* van Tiegh. and *Glomus mosseae*. *New Phytol.* **129**: 309–316.

Metzker, M.L. (2010) Sequencing technologies - the next generation. *Nat. Rev. Genet.* **11**: 31–46.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E.M., Kubal, M., *et al*. (2008) The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* **9**: 386.

Nilsson, R.H., Kristiansson, E., Ryberg, M., Hallenberg, N., and Larsson, K.H. (2008) Intraspecific ITS variability in the Kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evol. Bioinforma.* **2008**: 193–201.

Nilsson, R.H., Ryberg, M., Abarenkov, K., Sjökvist, E., and Kristiansson, E. (2009) The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. *FEMS Microbiol. Lett.* **296**: 97–101.

Ondov, B.D., Bergman, N.H., and Phillippy, A.M. (2011) Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics* **12**: 385.

Orgiazzi, A., Bianciotto, V., Bonfante, P., Daghino, S., Ghignone, S., Lazzari, A., *et al*. (2013) 454 Pyrosequencing Analysis of fungal assemblages from geographically distant, disparate soils reveals spatial patterning and a core mycobiome. *Diversity* **5**: 73–98.

Orgiazzi, A., Lumini, E., Nilsson, R.H., Girlanda, M., Vizzini, A., Bonfante, P., and Bianciotto, V. (2012) Unravelling soil fungal communities from different mediterranean land-use backgrounds. *PLoS One* **7**: 1–9.

Pereira, E., Coelho, V., Tavares, R.M., Lino-Neto, T., and Baptista, P. (2012) Effect of competitive interactions between ectomycorrhizal and saprotrophic fungi on *Castanea sativa* performance. *Mycorrhiza* **22**: 41–49.

Pereira, E., Santos, A., Reis, F., Tavares, R.M., Baptista, P., Lino-Neto, T., and Almeida-Aguiar, C. (2013) A new effective assay to detect antimicrobial activity of filamentous fungi. *Microbiol. Res.* **168**: 1–5.

Perotto, S., Girlanda, M., and Martino, E. (2002) Ericoid mycorrhizal fungi: Some new perspectives on old acquaintances. *Plant Soil* **244**: 41–53.

Pushkarev, D., Neff, N.F., and Quake, S.R. (2009) Single-molecule sequencing of an individual human genome. *Nat. Biotechnol.* **27**: 847–850.

Reis, F. (2012) Influence of *Hypholoma fasciculare* in chestnut grove sustainability.

Ribeiro, B., Rangel, J., Valentão, P., Andrade, P.B., Pereira, J.A., Bölke, H., and Seabra, R.M. (2007) Organic acids in two Portuguese chestnut (*Castanea sativa* Miller) varieties. *Food Chem.* **100**: 504–508.

Routledge, R.D. (1977) On Whittaker's Components of Diversity. *Ecology* **58**: 1120–1127.

Schoch, C., Seifer, K., Huhndorf, S., Robert, V., Spouge, J., Levesque, C., *et al*. (2012) From the Cover: Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl. Acad. Sci.* **109**: 6241–6246.

Schüßer, A., Schwarzott, D., and Walker, C. (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycol. Res.* **105**: 1413–1421.

Seaby, R.M.H. and Henderson, P.A. (2007) Species Diversity and Richness IV. Pisces Conservation Ltd.

Shannon, C.E. (1948) A mathematical theory of communication. *Bell Syst. Tech. J.* **27**: 379–423 and 623–656.

Shokralla, S., Spall, J.L., Gibson, J.F., and Hajibabaei, M. (2012) Next-generation sequencing technologies for environmental DNA research. *Mol. Ecol.* **21**: 1794–1805.

Simpson, E.H. (1949) Measurement of diversity. *Nature* **163**: 688.

Smith, S. and Read, D. (1997) Mycorrhizal Symbiosis. *Acad. Press* **137**: 563–568.

Šnajdr, J., Dobiášová, P., Větrovský, T., Valášková, V., Alawi, A., Boddy, L., and Baldrian, P. (2011) Saprotrophic basidiomycete mycelia and their interspecific interactions affect the spatial distribution of extracellular enzymes in soil. *FEMS Microbiol. Ecol.* **78**: 80–90.

Šnajdr, J., Valášková, V., Merhautová, V., Cajthaml, T., and Baldrian, P. (2008) Activity and spatial distribution of lignocellulose-degrading enzymes during forest soil colonization by saprotrophic basidiomycetes. *Enzyme Microb. Technol.* **43**: 186–192.

Sogin, M.L., Morrison, H.G., Huer, J.A., Welch, D.Ma., Huse, S.M., Neal, P.R., *et al.* (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proc. Natl. Acad. Sci.* **103**: 12115–12120.

Somerfield, P.J. (2008) Identification of the Bray-Curtis similarity index: Comment on Yoshioka (2008). *Mar. Ecol. Prog. Ser.* **372**: 303–306.

Sørensen, T. (1948) A method of establishing groups of equal amplitude in plant sociology based on similaritu of species content and its application to analyses of the vegetation on Danish commons. *Videnski Selsk. Biol. Skr.* **1**: 1–34.

Taberlet, P., Coissac, E., Hajibabaei, M., and Rieseberg, L.H. (2012) Environmental DNA. *Mol. Ecol.* **21**: 1789–1793.

Toju, H., Tanabe, A.S., Yamamoto, S., and Sato, H. (2012) High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLoS One* **7**: 11.

Tuomisto, H. (2010) A diversity of beta diversities: Straightening up a concept gone awry. Part 1. Defining beta diversity as a function of alpha and gamma diversity. *Ecography (Cop.).* **33**: 2–22.

Unterseher, M., Jumpponen, A., Öpik, M., Tedersoo, L., Moora, M., Dormann, C.F., and Schnittler, M. (2011) Species abundance distributions and richness estimations in fungal metagenomics - Lessons learned from community ecology. *Mol. Ecol.* **20**: 275–285.

Valásková, V., Boer, W. de, Gunnewiek, P.J. a K., Pospísek, M., and Baldrian, P. (2009) Phylogenetic composition and properties of bacteria coexisting with the fungus *Hypholoma fasciculare* in decaying wood. *ISME J.* **3**: 1218–1221.

Vannini, A. and Vettraino, A.M. (2001) Ink disease in chestnuts: impact on the European chestnut. *For. Snow Landsc. Res.* **76**: 345–350.

Vasconcelos, M.C., Bennett, R.N., Rosa, E.S., and Ferreira-Cardoso, J. V. (2010) Composition of European chestnut (*Castanea sativa* Mill.) and association with health effects: fresh and processed products. *J. Sci. Food Agric.* **90**: 1578–1589.

Vettraino, A.M., Morel, O., Perlerou, C., Robin, C., Diamandis, S., and Vannini, A. (2005) Occurrence and distribution of *Phytophthora* species in Europea chesnut stands, and their association with Ink Disease and crown decline. *Eur. J. Plant Pathol.* **111**: 169–180.

Wardle, D.A. (2006) The influence of biotic interactions on soil biodiversity. *Ecol. Lett.* **9**: 870–886.

White, T., Bruns, T., and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In, Innis,M., Gelfand,D., Sninsky,J., and White,T. (eds), *PCR Protocols: A guide to methods and applications*. Academic Press, pp. 315–322.

Whittaker, R. (1972) Evoluation and measurement of species diversity. *Taxon* **21**: 213–251.

Whittaker, R.H. (1960) Vegetation of the Siskiyou Mountains, Oregon and California. *Ecol. Monogr.* **30**: 279–338.

Whittaker, R.J., Willis, K.J., and Field, R. (2001) Scale and species richness : towards a general , theory of species diversity hierarchical. *J. Biogeogr.* **28**: 453–470.

Wilson, M.V. and Shmida, A. (1984) Measuring beta diversity with presence-absence data. *J. Ecol.* **72**: 1055–1064.

Yoshioka, P.M. (2008) Misidentification of the Bray-Curtis similarity index. *Mar. Ecol. Prog. Ser.* **368**: 309–310.

Yu, T., Egger, K., and Peterson, L. (2001) Ectendomycorrhizal associations - characteristics and functions. *Mycorrhiza* **11**: 167–177.

CHAPTER VI

ANNEX

Table A 1 - Sample labels used to describe all work samples. The soil samples submitted to DNA extraction for metabarcoding are underlined. See text for details.

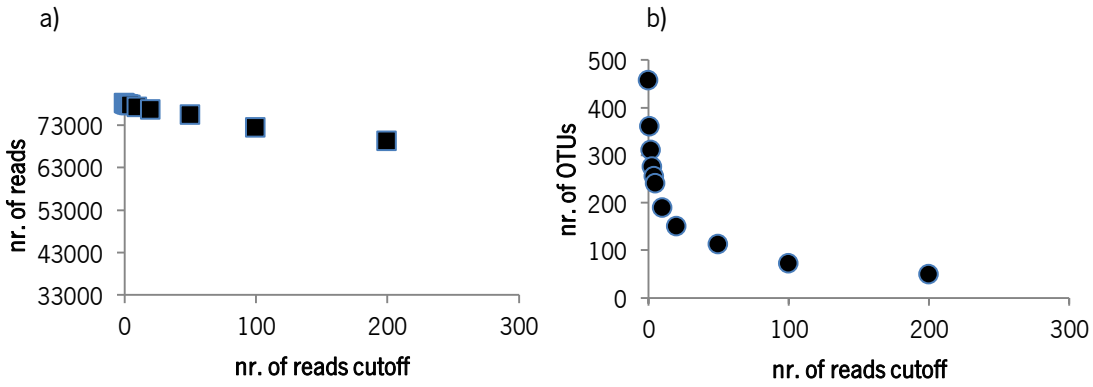
Sterile soil	Non sterile soil
S, 2M, T0 (used as S, T0)	NS, 2M, T0 (used as NS, T0)
S, 2M, T6M (used as S, T6M)	NS, 2M, T6M (used as NS, T6M)
S, 2M, T1Y	NS, 2M, T1Y
S, 2M, T6M+ Hf (used as S, T6M + Hf)	NS, 2M, T6M+ Hf (used as NS, T6M + Hf)
S, 2M, T1Y + Hf (used as S, T1Y + Hf)	NS, 2M, T1Y + Hf (used as NS, T1Y + Hf)
S, 1Y, T0	NS, 1Y, T0
S, 1Y, T6M	NS, 1Y, T6M
S, 1Y, T1Y	NS, 1Y, T1Y
S, 1Y, T6M+ Hf	NS, 1Y, T6M+ Hf
S, 1Y, T1Y + Hf	NS, 1Y, T1Y + Hf

Table A 2 - Number of reads obtained by 454 pyrosequencing of DNA samples taken from pot soils submitted to sterilization (S) or not submitted to sterilization (NS), that were inoculated with *Hypholoma fasciculare* (+Hf), or not. Sampling was performed after different periods upon inoculation, (T0 - just before inoculation, T6M – six months, T1Y – one year). The total raw number of reads was subjected to quality filters. BioCant filter excluded sequences less than 120 bp, containing ambiguous nucleotides and also eliminated sequences with low quality regions in their both ends. MG-RAST filter excluded sequences that present an e-value higher than e^{-5}, an identity value higher 97% and at least 50 bp of alignment. Fungal reads filter excluded the reads from other organisms and unclassified sequences.

Samples	Libraries	Raw reads	BioCant reads	MG-RAST reads	Fungal reads	
S, T0	A	4,109	4,079	3,529	2,026	8,101
	B	6,616	6,548	5,151	2,716	
	C	6,101	6,031	4,319	3,003	
	D	808	806	603	356	
S, T6M	A	5,772	5,715	3,886	1,340	10,559
	B	5,266	5,213	4,096	3,028	
	C	7,157	7,003	3,873	3,314	
	D	5,386	5,317	3,938	2,877	
S, T6M + Hf	A	6,896	6,822	5,304	1,239	6,919
	B	7,679	7,614	6,021	1,683	
	C	7,561	7,457	4,526	1,417	
	D	4,934	4,847	3,944	2,580	
S, T1Y + Hf	A	6,185	6,127	4,632	2,581	8,668
	B	7,222	7,106	4,752	3,998	
	C	6,589	6,508	2,409	2,089	
NS, T0	A	5,214	5,121	2,670	2,309	8,684
	B	4,452	4,387	1,699	1,504	
	C	4,997	4,917	3,023	2,718	
	D	4,893	4,809	2,423	2,153	

Table A 2 – (Continuation)

Samples	Libraries	Raw reads	BioCant reads	MG-RAST reads	Fungal reads	Samples
NS, T6M	A	6,803	6,692	2,875	2,626	10,427
	B	6,152	6,022	3,293	3,061	
	C	6,439	6,331	4,246	2,993	
	D	4,939	4,850	1,875	1,747	
NS, T6M + Hf	A	5,580	5,474	3,564	3,280	12,886
	B	5,272	5,180	2,849	2,378	
	C	8,944	8,784	4,629	4,136	
	D	7,680	7,547	3,645	3,092	
NS, T1Y + Hf	A	6,244	6,153	4,204	4,106	11,785
	B	4,368	4,321	2,928	2,772	
	C	6,211	6,112	5,170	4,907	
TOTAL		176,469	173,893	110,076		78,029



Figue A 1 - Influence of different cut off values in the number of reads (a) and OTUs (b) numbers.

Table A 3 - Identified OTUs from each chestnut soil condition. Sequences were obtained by 454 sequencing of ITS amplicons, prepared from DNA samples taken from chestnut Terroso region orchard. Sequence identification was performed with Metagenomics Analysis Server MG-RAST version 3 (http://metagenomics.anl.gov/), using default parameters (e-value under then e^{-6}, more than 97% of identity and at least 50 bp of alignment length). Besides the distribution in each soil condition, the total identified reads is also presented. Species that presented more than 50 reads were used for trophic group (TG) analysis: mycorrhizal (M), parasitic (P), saprotroph (S), parasitic/saprotroph (PS), yeast (Y) or unclassified (Un). Due to the update of the classification of species, according to Index Fungorum, it is present both designations: "actual classification"(="old classification").

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Acrostalagmus luteoalbus		0	0	0	0	0	0	11	0	11
Albonectria rigidiuscula (=*Nectria rigidiuscula*)		0	0	1	1	0	1	1	0	4
Alternaria abundans (=*Embellisia abundans*)		0	1	0	0	1	0	1	0	3
Alternaria alternariae (=*Ulocladium alternariae*)		0	1	0	0	0	0	2	0	3
Alternaria alternata	P	7	36	14	6	79	58	132	27	359
Alternaria botryospora (=*Embellisia novae-zelandiae*)		1	0	0	1	0	0	1	0	3
Alternaria leptinellae (=*Embellisia leptinellae*)		0	0	0	0	0	1	0	0	1
Alternaria sp. EAL1		0	0	1	0	0	0	0	0	1
Amanita bisporigera		0	3	0	1	0	0	0	0	4
Amanita citrina (=*Amanita virosa*)		0	0	0	0	0	0	3	0	3
Amanita rubescens	M	1	14	616	1,168	6	13	8	6	1,832
Amylostereum areolatum		1	0	0	0	1	4	3	0	9
Amylostereum chailletii	S	1	19	20	34	23	80	53	49	279
Annulohypoxylon multiforme		0	2	5	8	0	7	16	2	40
Anomoloma albolutescens		0	0	0	0	1	0	0	0	1
Antarctomyces psychrotrophicus	Un	1	10	8	3	4	35	79	8	148
Antrodia malicola (=*Trametes gibbosa*)		12	3	0	0	0	0	0	0	15
Apiospora montagnei		3	3	0	1	9	3	5	0	24
Armillaria novae-zelandiae		0	0	1	0	0	0	0	0	1

Table A 3 – (Continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Arthrinium phaeospermum		4	4	0	1	6	3	7	1	26
Arthrinium sacchari		0	0	0	0	1	0	0	0	1
Arthrobotrys musiformis		0	0	0	0	1	0	0	0	1
Aschersonia hypocreoidea		3	0	1	0	0	1	1	0	6
Aschersonia marginata		0	0	0	0	0	1	0	0	1
Ascocoryne cylichnium	S	0	3	48	0	434	7	5	1	498
Aspergillus flavus (=*Aspergillus oryzae*)		0	0	0	0	4	4	3	1	12
Aspergillus fumigatus		0	0	0	0	2	0	2	0	4
Aspergillus japonicus		0	0	0	1	0	0	0	0	1
Aspergillus terreus		1	4	4	3	6	1	4	0	23
Aspergillus versicolor		2	0	0	0	0	0	0	0	2
Aureobasidium pullulans		0	3	1	2	2	0	4	0	12
Auricularia cornea (=*Auricularia auricula-judae*)	PS	14	2	102	1	126	32	421	290	988
Ballistosporomyces xanthus (=*Sporobolomyces xanthus*)		0	0	0	0	0	1	0	0	1
Beauveria bassiana		0	2	2	1	0	3	0	1	9
Beauveria brongniartii (=*Cordyceps brongniartii*)		3	1	1	0	0	0	0	0	5
Bensingtonia subrosea		0	0	1	0	0	0	0	0	1
Bionectria ochroleuca	P	5	5	10	10	26	13	27	5	101
Biscogniauxia nummularia	P	125	23	15	0	0	1	2	1	167
Bisporella citrina	S	16	14	3	0	11	3	7	1	55
Boeremia exigua		0	6	0	0	0	0	0	0	6
Botryobasidium subcoronatum		1	1	0	0	1	1	0	0	4

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Botryosphaeria dothidea		0	0	0	0	1	0	0	0	1
Botryotinia fuckeliana	P	227	459	253	116	170	189	307	82	1,803
Botrytis fabae		1	0	0	0	0	0	0	0	1
Bovista nigrescens		0	0	0	0	2	0	3	2	7
Bulleromyces albus		0	1	0	0	0	0	0	1	2
Cadophora fastigiata		0	1	0	2	0	0	0	0	3
Cadophora finlandica	P	0	23	846	626	4	65	98	25	1,687
Cadophora malorum		0	1	1	0	0	4	9	0	15
Cadophora orchidicola (=*Leptodontidium orchidicola*)	P	227	439	831	1237	236	285	364	114	3733
Calonectria canadensis		0	0	0	0	1	0	0	0	1
Candida albicans		0	0	1	1	0	0	0	0	2
Capnodium sp. olrim506		0	0	0	0	1	6	1	1	9
Capronia pilosella		1	0	0	0	0	0	0	0	1
Capronia semi-immersa		0	0	0	0	2	0	0	0	2
Cenococcum geophilum		0	0	1	0	5	2	0	6	14
Ceratobasidium sp. AG-G		0	0	0	5	0	0	0	0	5
Ceratocystis stenoceras (=*Ophiostoma stenoceras*)		4	3	1	1	0	0	0	0	9
Ceratocystis tetropii (=*Ophiostoma tetropii*)		0	2	0	0	0	0	0	1	3
Cercospora apii		0	0	0	0	3	0	0	0	3
Cerrena unicolor		0	0	0	1	0	0	0	0	1
Chaetomium globosum	P	903	424	327	161	282	204	222	58	2,581
Chaetomium gracile		0	12	0	0	0	1	0	0	13
Cladosporium cladosporioides	S	0	66	17	45	5	11	11	20	175

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Cladosporium colocasiae		0	1	0	0	0	0	1	0	2
Cladosporium oxysporum		0	1	0	0	0	0	0	0	1
Cladosporium sphaerospermum		0	0	0	0	0	0	0	1	1
Cladosporium tenuissimum		0	3	0	1	1	3	9	0	17
Climacocystis borealis		0	0	2	0	0	1	0	0	3
Clitocybe nebularis		0	0	0	0	1	0	0	0	1
Clitocybe subditopoda		9	0	0	0	0	0	1	0	10
Clitopilus sp. VHAs07/02		0	0	0	0	0	3	2	0	5
Coccidioides posadasii		0	0	0	0	0	0	2	0	2
Colletotrichum acutatum (=*Glomerella acutata*)	P	24	4	9	9	29	39	78	11	203
Colletotrichum gloeosporioides (=*Glomerella cingulata*)	P	15	17	16	41	106	40	143	7	385
Colletotrichum musae		1	0	0	0	6	5	13	2	27
Colletotrichum trichellum		0	1	0	0	0	0	0	0	1
Colletotrichum truncatum		0	0	0	0	0	6	2	0	8
Coltricia perennis		0	0	2	0	0	0	0	0	2
Coniophora arida		0	0	0	0	0	0	0	1	1
Coprinellus micaceus		0	0	0	0	1	0	0	0	1
Coprinellus radians		0	0	0	0	0	1	0	0	1
Coprinopsis cinerea		0	0	2	0	0	0	0	0	2
Coprinopsis scobicola		0	0	0	0	1	0	0	0	1
Coprinus comatus		0	0	0	0	1	1	0	0	2
Cordyceps bassiana		0	0	0	0	0	1	1	1	3

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Cordyceps cylindrica		0	0	0	0	6	0	0	0	6
Cordyceps militaris		0	0	3	1	0	0	0	0	4
Cortinarius alboviolaceus		0	0	0	1	3	0	2	19	25
Cortinarius amoenolens (=*Cortinarius glaucopus*)		0	0	0	0	0	8	2	5	15
Cortinarius anomalus		0	0	0	0	0	0	0	2	2
Cortinarius atrovirens		0	0	0	0	0	0	0	1	1
Cortinarius claroflavus		2	1	2	1	0	0	0	0	6
Cortinarius disjungendus (=*Cortinarius brunneus*)	M	0	0	0	0	0	0	0	558	558
Cortinarius elegantissimus	M	0	0	0	0	0	126	42	187	355
Cortinarius eufulmineus		0	0	0	23	0	0	0	0	23
Cortinarius flexipes		1	0	0	12	1	1	0	0	15
Cortinarius hinnuleus		0	0	0	0	0	0	0	16	16
Cortinarius lilacinovelatus	M	1	28	3	28	916	145	77	1,089	2,287
Cortinarius limonius (=*Cortinarius callisteus*)	M	0	98	0	804	16	107	9	2	1,036
Cortinarius meinhardii (=*Cortinarius splendens*)		0	0	0	3	0	0	0	10	13
Cortinarius molochinus		0	0	0	0	0	1	0	0	1
Cortinarius odorifer		0	0	0	2	4	1	0	3	10
Cortinarius olearioides		0	1	0	0	0	0	0	0	1
Cortinarius paradoxus		1	0	0	0	0	0	0	0	1
Cortinarius praestans (=*Cortinarius infractus*)		0	0	0	0	17	3	7	7	34
Cortinarius rotundisporus		0	0	0	0	0	0	1	2	3
Cortinarius salor		0	0	0	1	0	0	1	0	2
Cortinarius semisanguineus		0	0	0	0	0	0	0	11	11

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Cortinarius traganus		0	3	3	6	4	1	2	14	33
Cortinarius variicolor		0	1	0	0	0	0	0	0	1
Corynespora cassiicola		0	0	1	0	0	0	0	2	3
Cosmospora vilior	P	1	4	1	0	0	4	42	0	52
Crepidotus autochthonus (=*Creptidotus applanatus*)		0	0	0	0	0	0	1	2	3
Cryptococcus albidus		0	1	0	0	0	0	0	0	1
Cryptococcus aureus		0	2	0	0	0	1	1	3	7
Cryptococcus laurentii	Y	0	0	0	0	19	14	35	13	81
Cryptococcus liquefaciens		0	0	0	0	0	2	3	1	6
Cryptococcus podzolicus	Y	90	615	571	78	1,043	1,938	2,227	1,487	8,049
Cryptococcus vishniacii	Y	0	2	3	0	40	105	232	60	442
Curreya pityophila	S	0	8	0	0	34	9	29	4	84
Cylindrocarpon didymum		0	3	0	0	1	0	2	0	6
Cylindrocarpon pauciseptatum		0	0	0	0	1	1	16	0	18
Cyphellophora europaea (=*Phialophora europaea*)		1	3	3	3	5	0	0	0	15
Cyphellostereum laeve		0	0	0	0	0	1	0	0	1
Cystofilobasidium capitatum		0	0	0	6	0	0	0	0	6
Dactylaria higginsii		0	0	0	0	0	0	0	1	1
Dactylellina ellipsospora		0	3	0	0	0	0	2	0	5
Dactylellina parvicolle		0	0	0	0	0	0	0	1	1
Daedalea quercina		1	0	0	0	0	0	0	0	1
Debaryomyces hansenii	Y	12	21	31	33	8	9	4	10	128
Descolea maculata		0	0	0	0	0	0	0	3	3

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Diaporthe viticola		0	0	0	9	0	0	0	2	11
Dothidea sambuci		0	0	0	0	1	1	0	1	3
Elaphocordyceps ophioglossoides	P	30	72	31	8	28	13	7	7	196
Emericellopsis minima (=*Emericellopsis microspora*)		0	0	1	2	0	3	5	1	12
Emericellopsis terricola		0	0	1	1	0	0	0	0	2
Entoloma strictius (=*Nolanea strictior*)		0	0	0	0	0	0	1	0	1
Entyloma arnoseridis		0	0	0	0	0	0	0	1	1
Entyloma calendulae		0	0	0	0	0	0	2	0	2
Epicoccum nigrum	S	3	11	5	1	21	3	11	3	58
Exidia pithya		0	0	0	0	0	1	0	0	1
Exophiala dermatitidis		1	1	0	1	0	3	0	0	6
Exophiala salmonis	P	0	10	23	2	3	16	4	2	60
Exophiala spinifera		0	0	0	6	1	10	12	5	34
Filobasidium capsuligenum	Y	6	3	8	3	6	24	31	16	97
Fonsecaea monophora		0	0	0	0	1	0	0	0	1
Fonsecaea pedrosoi	P	6	34	16	10	10	4	8	6	94
Fusarium caeruleum		1	0	0	0	0	0	1	0	2
Fusarium culmorum		0	0	1	0	0	0	0	0	1
Fusarium neocosmosporiellum (=*Neocosmospora vasinfecta*)		0	0	0	0	1	0	0	0	1
Fusarium oxysporum	P	0	4	1	1	76	59	91	28	260
Fusarium redolens		0	0	0	0	0	0	2	0	2
Fusarium sp. 2.6		0	0	0	0	1	0	0	0	1

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Fusarium sp. NRRL 22354		0	1	0	0	0	0	0	0	1
Fusarium sp. NRRL 25226		0	0	0	0	0	0	1	0	1
Fusarium sporotrichioides		0	0	0	0	0	0	0	1	1
Galerina patagonica (=*Galerina marginata*)		1	0	0	0	0	0	0	0	1
Ganoderma applanatum		1	0	0	3	0	0	0	0	4
Ganoderma fornicatum		15	1	0	2	0	0	0	0	18
Ganoderma lucidum (=*Ganoderma japonicum*)		0	0	1	0	0	0	0	0	1
Ganoderma orbiforme (=*Ganoderma lucidum*)	PS	4,091	1,088	47	368	6	2	12	7	5,621
Gautieria otthii		1	0	1	1	0	4	4	3	14
Geastrum floriforme		0	0	0	0	0	0	0	4	4
Geotrichum loubieri (=*Trichosporon loubieri*)	Y	4	64	11	0	0	0	8	0	87
Gibberella avenacea	P	5	1	5	0	98	85	190	26	410
Gibberella fujikuroi		0	0	0	0	0	0	1	0	1
Gibberella intermedia	P	4	1	3	0	653	413	577	207	1,858
Gibberella intricans (=*Fusarium equiseti*)		1	1	0	2	0	0	2	1	7
Gloeophyllum sepiarium		1	2	0	0	0	4	1	1	9
Gloeoporus taxicola		2	0	1	0	0	0	0	2	5
Gnomonia chamaemori (=*Gnomoniopsis chamaemori*)		0	0	0	0	8	3	2	1	14
Gnomoniopsis sp. CBS 121917		0	3	0	11	3	1	3	2	23
Gnomoniopsis sp. CBS 121918		0	3	0	11	3	1	3	2	23
Graphium fragrans (=*Pesotum fragrans*)		0	1	1	0	0	0	0	1	3
Grosmannia cucullata		1	0	0	1	4	1	24	5	36

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Guehomyces pullulans	Y	6	8	5	0	1	10	35	1	66
Gymnopilus penetrans	S	0	0	0	2	0	22	7	53	84
Haematonectria haematococca (=*Fusarium solani*; =*Nectria haematococca*)	P	1	3	1	0	24	5	28	7	69
Hebeloma crustuliniform (=*Hebeloma longicaudum*)		0	0	0	0	0	0	0	1	1
Hebeloma helodes	M	0	0	0	0	0	44	6	153	203
Hebeloma laterinum (=*Hebeloma sinuosum*)		0	0	0	0	0	0	0	1	1
Hebeloma leucosarx (=*Hebeloma velutipes*)		0	0	0	0	1	0	4	0	5
Hebeloma mesophaeum	M	1	1	145	15	14	135	31	251	593
Hebeloma theobrominum (=*Hebeloma truncatum*)		0	6	0	0	0	11	0	0	17
Herpotrichia pinetorum (=*Herpotrichia juniperi*)	P	1	1	0	0	48	5	6	1	62
Heterobasidion parviporum		0	0	0	0	1	0	1	0	2
Hirsutella rhossiliensis		0	2	0	0	0	0	0	0	2
Hortaea acidophila	P	1	1	3	0	30	10	32	13	90
Hortaea werneckii		0	0	0	0	0	0	1	0	1
Humicola fuscoatra		0	1	0	0	0	0	0	0	1
Hymenopellis radicata		0	0	0	0	0	1	0	0	1
Hymenoscyphus pseudoalbidus		0	0	0	0	0	8	4	0	12
Hymenoscyphus tetracladius (=*Articulospora tetracladia*)	S	6	28	27	38	56	58	125	7	345
Hypholoma acutum (=*Hypholoma fasciculares*)		0	0	6	0	3	4	18	0	31
Hypocrea lixii	S	0	1	0	0	9	20	13	16	59
Hypocrea pachybasioides		1	0	0	0	0	1	1	0	3

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Hypoxylon fragiforme	S	70	50	21	29	6	3	10	3	192
Hypsizygus ulmarius		0	1	0	0	0	0	0	0	1
Ilyonectria liriodendri		0	0	3	0	0	1	2	0	6
Ilyonectria macrodidyma	P	26	204	104	5	2	18	127	16	502
Ilyonectria radicicola	P	19	163	24	1	3	31	70	4	315
Immersiella caudata (=*Cercophora caudata*)		23	2	1	0	0	0	2	0	28
Incrucipulum ciliare (=*Lachnum ciliare*)		1	1	0	0	13	11	17	3	46
Inocybe lacera	M	0	3	0	0	0	937	407	2,011	3,358
Inocybe sindonia		3	2	0	0	0	0	0	0	5
Inocybe sororia	M	1	50	18	863	0	12	8	53	1,005
Inocybe spuria		0	0	0	0	0	0	1	0	1
Inocybe umbratica		0	0	0	0	0	8	5	25	38
Irpex lacteus		0	1	0	0	1	0	0	0	2
Isaria cateniannulata		1	0	0	0	0	1	0	0	2
Knufia chersonesos		0	0	0	0	0	1	1	0	2
Kodamaea ohmeri	P	7	16	15	34	21	42	38	35	208
Kondoa malvinella		0	5	2	0	1	0	0	0	8
Kretzschmaria deusta	P	64	353	29	17	29	18	17	10	537
Laccaria amethystina	M	1	0	0	0	0	26	7	32	66
Laccaria bicolor	M	0	0	0	20	0	3	1	1,084	1,108
Laccaria ochropurpurea		0	0	0	0	0	0	1	0	1
Laccaria ohiensis (=*Laccaria laccata*)	M	0	1,079	0	9	0	5	2	120	1,215
Laccaria sp. GMM1080	M	0	0	0	0	0	298	43	300	641

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Lachnum pulverulentum (=*Lachnellula pulverulenta*)		2	0	0	2	0	0	0	0	4
Lachnum abnorme	P	300	358	71	662	13	96	226	10	1,736
Lachnum fuscescens		0	0	0	0	8	5	2	12	27
Lachnum sp. olrim977		0	0	0	1	0	0	0	0	1
Lachnum virgineum	S	5	0	0	0	194	13	115	21	348
Lasiodiplodia pseudotheobromae		2	1	3	1	4	5	5	1	22
Lasiodiplodia theobromae		0	3	0	0	0	3	6	4	16
Lasiosphaeria ovina		0	0	0	0	0	0	1	0	1
Lasiosphaeris hispida (=*Lasiosphaeria hispida*)		2	0	1	0	0	0	0	0	3
Lecanicillium fungicola (=*Verticillium fungicola*)		0	3	2	0	0	2	0	0	7
Lecanicillium fusisporum	P	28	19	18	2	3	0	1	0	71
Lecanicillium psalliotae	P	1	13	4	0	1	25	35	12	91
Leccinum rubropunctum (=*Boletus rubropunctus*)		0	0	1	0	3	1	1	0	6
Lecythophora hoffmannii		1	2	1	3	15	6	12	3	43
Lecythophora sp. olrim22		0	0	0	1	3	2	0	0	6
Lenzites betulina		20	13	3	0	0	1	1	0	38
Leotia lubrica	S	2	61	1	1	0	0	0	0	65
Lepista nuda		0	1	0	0	0	0	0	0	1
Leptogium pseudofurfuraceum		0	0	0	0	1	0	0	0	1
Leptographium sp. HYL-2009b		2	1	0	0	0	0	0	0	3
Leucosporidiella muscorum		0	0	1	0	1	0	0	0	2
Leucosporidium scottii	Y	5	558	6	1	0	3	3	0	576
Lewia infectoria		0	0	0	0	2	0	1	0	3

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Lyophyllum sykosporum		0	2	3	0	0	0	1	0	6
Macroconia gigas (=*Cosmospora gigas*)		0	0	1	0	0	0	0	1	2
Macrophomina phaseolina		0	0	1	1	15	12	13	5	47
Marasmius oreades		0	0	0	0	0	0	0	2	2
Massarina igniaria	S	0	0	0	0	19	12	34	0	65
Megacollybia platyphylla		0	0	0	0	1	0	0	1	2
Memnoniella echinata (=*Stachybotrys echinata*)		0	1	1	0	0	0	0	0	2
Metacordyceps chlamydosporia		0	0	0	0	1	0	0	0	1
Metarhizium anisopliae	P	8	3	4	3	14	21	41	5	99
Microstroma juglandis		0	1	13	0	0	0	1	0	15
Monacrosporium drechsleri (=*Dactylellina drechsleri*)		0	1	0	0	0	0	0	0	1
Monilinia laxa	P	15	19	13	3	0	0	12	2	64
Monographella cucumerina (=*Plectosphaerella cucumerina*)	P	12	258	460	140	322	194	171	8	1,565
Mortierella alpina	S	28	86	9	12	32	59	101	11	338
Mortierella hyalina	S	1	376	13	10	26	57	93	26	602
Mortierella verticillata	S	3	0	1	12	28	67	123	6	240
Mrakia frigida		0	1	0	0	2	2	3	0	8
Mucor circinelloides		0	0	0	0	0	1	3	0	4
Mucor hiemalis		0	0	0	0	1	0	1	0	2
Mucor moelleri (=*Zygorhynchus moelleri*)		0	0	0	0	0	0	1	0	1
Muscodor albus		0	0	0	2	0	0	0	0	2
Mycena galericulata	S	0	0	0	0	0	0	1	67	68

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Mycetinis alliaceus (=*Marasmius alliaceus*)	S	0	0	0	0	0	0	0	54	54
Mycosphaerella heimii		1	1	5	0	4	0	9	4	24
Mycosphaerella punctiformis		6	0	0	0	1	0	3	0	10
Mycosphaerella tassiana (=*Davidiella tassiana*)		1	3	2	0	5	1	0	0	12
Myrothecium cinctum		2	0	0	0	0	0	0	0	2
Myrothecium roridum	P	0	0	0	6	148	5	4	0	163
Myrothecium verrucaria		0	0	0	0	3	0	0	0	3
Nemania serpens	P	0	0	0	6	58	7	29	8	108
Neofabraea alba		0	0	0	0	0	1	1	0	2
Neurospora africana	S	1	0	1	3	36	1	17	1	60
Neurospora crassa		0	0	0	0	0	0	1	0	1
Neurospora pannonica		0	1	0	0	3	0	2	0	6
Neurospora terricola		0	2	0	0	0	1	0	0	3
Oidiodendron maius		0	0	1	0	2	2	5	0	10
Olpidium brassicae	P	36	2	3	0	0	2	7	7	57
Ophiocordyceps sinensis	P	3	2	5	4	18	10	35	4	81
Ophiostoma piceae (=*Ophiostoma quercus*)		0	0	0	0	3	0	0	0	3
Paecilomyces carneus		0	0	0	0	4	0	1	0	5
Paecilomyces marquandii		0	0	0	0	2	0	0	0	2
Paecilomyces variotii	P	0	1	0	0	21	22	36	12	92
Paxillus involutus	M	0	51	0	0	0	1	0	0	52
Penicillium aculeatum		2	8	1	2	2	6	13	0	34
Penicillium brevicompactum		2	3	0	1	0	2	0	0	8

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Penicillium canescens		0	0	0	0	3	2	2	0	7
Penicillium chrysogenum		0	0	0	0	0	1	1	0	2
Penicillium citreonigrum		0	3	2	0	0	0	2	0	7
Penicillium expansum		1	0	0	0	0	1	0	0	2
Penicillium glaucoalbidum (=*Rhizoscyphus ericae*)		0	0	0	0	1	0	0	0	1
Penicillium hirsutum	S	4	5	1	2	28	28	30	25	123
Penicillium melinii		0	0	0	0	1	1	0	0	2
Penicillium restrictum		0	0	0	0	0	1	0	1	2
Peniophora cinerea		0	0	0	1	0	0	0	0	1
Pestalotiopsis besseyi	P	0	5	0	78	561	41	224	46	955
Pestalotiopsis microspora	P	0	12	9	0	43	6	16	8	94
Pestalotiopsis paeoniicola		0	0	5	6	7	0	1	3	22
Pezicula cinnamomea		0	0	1	0	3	0	6	1	11
Peziza ostracoderma	S	0	0	0	55	0	0	0	0	55
Pezoloma ericae		2	1	1	5	0	1	1	1	12
Phaeoacremonium occidentale		0	0	0	0	0	2	1	1	4
Phanerochaete sordida		0	0	0	2	0	0	0	1	3
Phialocephala dimorphospora		0	0	0	0	7	7	9	1	24
Phialocephala fortinii	M	103	83	120	450	115	301	135	33	1,340
Phialophora verrucosa		0	0	0	0	0	1	0	0	1
Phlebia radiata	S	5	49	0	0	10	7	10	1	82
Phlebopus portentosus		0	0	0	0	1	2	0	4	7
Pholiota alnicola (=*Flammula alnicola*)	P	0	0	1	0	2	29	12	68	112

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Pholiota squarrosa		0	7	0	0	0	0	0	0	7
Phoma cucurbitacearum (=Stagonosporopsis cucurbitacearum)	P	29	55	4	1	1	6	0	0	96
Phoma eupyrena		9	6	2	0	2	24	2	1	46
Phoma glomerata (=*Peyronellaea glomerata*)		0	2	0	0	2	1	4	0	9
Phoma herbarum		0	6	2	0	12	13	2	1	36
Phomopsis helianthi (=*Diaporthe helianthi*)		0	0	0	0	0	0	1	0	1
Phomopsis phaseoli (=*Diaporthe phaseolorum*)		0	0	0	0	0	0	2	0	2
Pilidiella quercicola		1	0	0	0	9	4	3	0	17
Piptoporus betulinus	P	233	223	465	101	1	15	33	3	1,074
Pleospora bjoerlingii (=*Phoma betae*)	P	2	108	8	5	2	0	2	0	127
Pleurotus australis		0	0	0	0	0	0	0	1	1
Pleurotus cystidiosus		1	1	3	0	0	0	0	0	5
Pleurotus djamor (=*Pleurotus salmoneostramineus*)		0	0	0	0	9	0	0	0	9
Pleurotus dryinus		0	0	2	0	0	1	0	0	3
Pleurotus nebrodensis		0	0	1	0	0	0	1	2	4
Pochonia bulbillosa		0	2	0	0	0	1	3	1	7
Podospora appendiculata		0	0	0	0	2	0	0	0	2
Podospora curvicolla		0	0	0	0	0	3	2	0	5
Podospora fibrinocaudata		0	0	0	0	1	0	0	0	1
Podospora myriaspora		1	33	0	0	1	0	0	0	35
Preussia minipascua		0	0	0	0	1	0	3	0	4
Psathyrella candolleana		1	0	0	0	0	0	0	0	1

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Psathyrella spadicea		0	0	1	0	0	0	0	0	1
Pseudallescheria boydii (=*Scedosporium apiospermum*)	P	6	33	22	10	19	18	58	20	186
Pseudocochliobolus verruculosus (=*Cochliobolus verruculosus*)		0	0	0	0	0	1	1	0	2
Pseudogymnoascus pannorum (=*Geomyces pannorum*)	S	637	744	398	216	19	49	353	37	2,453
Pseudogymnoascus roseus		3	3	0	0	0	4	7	1	18
Pyrenophora phaeocomes		0	0	0	0	25	8	4	4	41
Pyrenophora tritici-repentis		0	0	0	0	0	1	1	0	2
Pyronema domesticum		0	0	0	0	38	0	0	0	38
Resinicium bicolor	P	2	114	21	110	22	83	47	42	441
Rhinocladiella aquaspersa		0	0	0	0	0	2	0	4	6
Rhinocladiella atrovirens		3	0	0	0	0	1	0	0	4
Rhizopus arrhizus (=*Rhizopus oryzae*)		0	0	0	0	2	0	0	0	2
Rhodosporidium babjevae		0	3	0	0	1	0	0	0	4
Rhodosporidium toruloides		0	0	2	0	0	1	1	0	4
Rhodotorula araucariae		2	0	0	1	0	0	0	0	3
Rhodotorula calyptogenae		0	0	1	0	0	0	0	0	1
Rhodotorula laryngis		0	0	0	0	0	0	17	0	17
Rhodotorula lignophila		0	1	0	0	0	0	0	0	1
Rhodotorula minuta		0	0	0	0	0	2	0	0	2
Rhodotorula mucilaginosa	Y	2	13	6	2	9	12	14	6	64

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Rhodotorula pustula		5	1	0	0	0	0	0	0	6
Rhodotorula slooffiae		1	0	0	0	0	0	0	0	1
Rhytisma acerinum		0	0	0	0	0	0	1	0	1
Rubrinectria olivacea		0	1	0	0	0	0	0	0	1
Russula earlei		0	4	1	0	0	0	0	1	6
Russula heterophylla		0	0	0	0	0	1	0	0	1
Russula ochroleuca		1	1	12	1	0	0	0	0	15
Russula parazurea (=*Russula aeruginea*)		0	2	0	0	0	0	0	1	3
Russula praetervisa (=*Russula pectinatoides*)	M	0	0	0	0	0	0	3	164	167
Russula vesca		0	2	0	0	0	0	0	0	2
Sarocladium strictum	P	0	1	26	5	1	6	27	1	67
Schizophyllum commune		0	0	1	0	0	0	0	0	1
Schizothecium curvisporum	P	3	18	1	1	136	4	4	0	167
Schizothecium fimbriatum		0	1	0	0	2	0	0	0	3
Scleroderma bovista		0	0	0	0	1	1	3	4	9
Scleroderma citrinum		1	11	0	0	0	2	0	0	14
Sclerotinia sclerotiorum		5	1	2	2	0	0	0	0	10
Scutellinia scutellata		0	0	0	0	0	0	4	1	5
Scytalidium cuboideum	Un	0	8	0	69	7	6	6	1	97
Sebacina incrustans		3	0	9	35	0	0	3	0	50
Septobasidium sinuosum		0	0	0	0	0	0	0	2	2
Simplicillium lamellicola		0	0	2	0	0	5	2	0	9
Sordaria fimicola		6	0	2	2	4	1	12	2	29

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Spadicoides bina		0	0	0	0	0	0	1	0	1
Sporobolomyces coprosmae		0	1	0	0	0	0	0	0	1
Sporobolomyces gracilis		0	0	0	0	2	0	0	0	2
Sporobolomyces sasicola		0	0	0	0	0	0	0	1	1
Sporormia lignicola (=*Preussia lignicola*)		0	0	0	0	1	0	2	0	3
Sporormiella borealis (=*Preussia borealis*)		0	0	0	0	9	3	2	1	15
Sporothrix schenckii	S	5	66	21	10	6	10	39	2	159
Stachybotrys chartarum		0	0	0	0	0	1	0	0	1
Stereum hirsutum		0	0	0	0	0	1	1	2	4
Stereum sanguinolentum	S	4	11	9	28	213	1,181	1,534	1,325	4,305
Suillus luteus		0	0	0	0	1	0	0	0	1
Talaromyces flavus		0	2	0	2	0	0	3	0	7
Talaromyces marneffei (=*Penicillium marneffei*)		0	1	0	0	0	0	0	0	1
Talaromyces pinophilus (=*Penicillium pinophilum*)	S	12	190	27	130	50	25	45	10	489
Talaromyces trachyspermus		0	0	0	0	0	0	1	0	1
Talaromyces verruculosus (=*Penicillium verruculosum*)		0	0	0	0	3	2	1	1	7
Taphrina wiesneri		0	0	0	3	0	0	0	0	3
Tephrocybe anthracophila (=*Lyophyllum anthracophilum*)		0	0	0	0	0	0	1	0	1
Tephrocybe atrata (=*Lyophullum atratum*)		0	0	1	0	0	0	0	0	1
Terfezia boudieri		0	0	0	0	0	7	36	0	43
Terfezia claveryi		0	0	0	0	1	0	0	0	1

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Tetracladium breve		0	0	0	0	1	3	0	0	4
Tetracladium furcatum		0	1	0	0	0	0	0	0	1
Tetracladium palmatum		0	1	0	0	0	1	0	0	2
Tetracladium setigerum		0	1	0	0	0	1	0	1	3
Tetracladium sp. CCM F-10008		0	0	0	0	2	4	6	0	12
Thanatephorus cucumeris	P	0	0	0	0	1	8	5	38	52
Thelonectria lucida	PS	18	65	14	19	29	26	231	13	415
Thielaviopsis basicola	P	3	80	28	0	0	2	21	0	134
Tirmania pinoyi	M	0	0	0	0	0	150	19	0	169
Tremella encephala	PS	271	728	479	136	1,238	1,433	1,646	773	6,704
Tremellodendron schweinitzii (=*Tremellodendron pallidum*)		0	0	0	0	3	0	1	2	6
Trichoderma asperellum		1	1	0	3	0	0	0	0	5
Trichoderma aureoviride		0	0	0	0	0	4	0	1	5
Trichoderma crassum (=*Hypocrea crassa*)	S	0	0	0	3	7	20	29	12	71
Trichoderma erinaceum		0	0	0	0	0	1	1	0	2
Trichoderma gamsii	S	3	25	3	1	20	35	31	8	126
Trichoderma hamatum	S	0	95	4	262	3	2	9	4	379
Trichoderma koningii (=*Hypocrea koningii*)	S	8	5	6	0	4	8	21	3	55
Trichoderma longibrachiatum		0	1	0	0	0	0	0	0	1
Trichoderma paraviridescens (=*Hypocrea viridescens*)		0	0	0	0	0	0	1	0	1
Trichoderma parceramosum (=*Trichoderma atroviride*)		2	7	2	2	7	4	8	1	33

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Trichoderma pleuroti		0	0	0	0	0	0	1	0	1
Trichoderma pubescens		0	2	0	4	0	0	0	0	6
Trichoderma sp. DAOM 233368		0	0	0	0	0	0	1	0	1
Trichoderma sp. DAOM 237545		0	0	0	0	0	0	1	1	2
Trichoderma spirale		0	0	0	0	1	1	3	1	6
Trichoderma tomentosum		0	0	0	1	0	0	3	0	4
Trichoderma viride (=*Hypocrea rufa*)	S	7	12	5	3	21	74	18	3	143
Tricholoma equestre (=*Tricholoma flavovirens*)		5	3	6	1	0	1	1	1	18
Tricholoma saponaceum		0	3	0	0	0	0	0	0	3
Tricholoma sejunctum	M	0	18	0	30	0	0	0	4	52
Tricholoma ustale		0	0	0	0	0	1	0	0	1
Trichosporon asahii		0	0	1	0	0	0	0	0	1
Trichosporon brassicae		0	1	0	0	0	0	0	0	1
Trichosporon cutaneum		1	2	6	0	1	0	0	0	10
Trichosporon dulcitum		0	0	0	0	0	0	1	0	1
Trichosporon faecale		2	1	0	0	0	0	0	0	3
Trichosporon gracile		0	1	0	0	0	1	0	0	2
Trichosporon guehoae		3	3	1	0	0	0	0	0	7
Trichosporon moniliiforme		0	11	8	0	0	0	0	0	19
Trichosporon mucoides		5	5	0	1	0	0	0	0	11
Trichosporon sporotrichoides		0	0	0	0	0	0	0	4	4
Trichothecium roseum		0	7	9	2	0	9	0	0	27
Tuber melanosporum		1	6	0	1	1	2	2	1	14

Table A 3 – (continuation)

Species	TG	S, T0	S, T6M	S, T6M + Hf	S, T1Y + Hf	NS, T0	NS, T6M	NS, T6M + Hf	NS, T1Y + Hf	Total
Tulasnella asymmetrica		0	4	0	0	2	0	0	0	6
Ulocladium sp. CID68		0	0	1	1	5	0	1	0	8
Umbelopsis isabellina	S	21	39	42	7	0	5	10	2	126
Umbelopsis ramanniana		1	2	0	0	4	10	5	5	27
Vanderwaltozyma polyspora		0	0	0	0	0	0	1	0	1
Verticillium albo-atrum		0	0	0	0	0	2	0	0	2
Verticillium dahliae		0	3	0	0	2	0	0	0	5
Verticillium leptobactrum	P	0	13	81	1	0	3	0	1	99
Verticillium tricorpus		0	3	0	0	0	0	0	0	3
Waitea circinata	P	28	18	35	8	2	7	12	1	111
Wallemia sebi		0	1	0	0	0	0	0	0	1
Westerdykella ornata		0	0	0	0	2	0	0	0	2
Xanthoconium affine		0	0	0	0	2	0	1	1	4
Xanthophyllomyces dendrorhous		0	0	0	0	1	1	0	1	3
Xanthoria elegans		7	7	2	4	1	6	3	6	36
Xylaria cubensis		0	0	0	0	1	0	2	0	3
Xylaria curta		0	0	0	0	0	0	2	0	2