Pedro Daniel Santos de Carvalho

Studies of mycorrhizal associations in Cistaceae

from a maritime pine forest:

ecological and biotechnological approach

Dissertação apresentada à Universidade de Coimbra para a obtenção do Grau de Mestre em Biodiversidade e Biotecnologia Vegetal, especialidade em Biotecnologia, realizada sob a orientação científica da Professora Doutora Maria Teresa Gonçalves e coorientação do Professor Doutor António Manuel Santos Carriço Portugal do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Abstract

This work was developed in three chapters with three different, but complemental, work scopes: fungal growth, study of mycorrhizal associations and mycorrhizal synthesis.

In the first chapter we described the establishment of mycelial cultures of four fungi, two mycorrhizal species (*Lactarius deliciosus* and *Tricholoma portentosum)* and two saprophytic species *(Agaricus bisporus* and *Macrolepiota procera*) and their growth behaviours in five media (Potato dextrose agar (PDA), Malt extract agar number 2 (MEA), Murishage and Skoog medium (MS), Biotin-Aneurin-Folic acid medium (BAF) and Melin-Norkrans Modified medium (MNM)) at three different temperatures (4ºC, 24ºC e 30ºC). Our results showed that *L. deliciosus* grows best in MNM or MS at 24ºC and that the best media for culture maintenance at 4ºC are PDA or MNM, *T. portentosum* showed the best growth in MNM or PDA at 24ºC, for maintenance the best media are BAF or MNM.

For the saprophytes, *A. bisporus* had the best growth in PDA or BAF at 24ºC with the same media being the best for maintenance and for *M. procera* the best growth was in PDA at 30ºC and for maintenance the best media are BAF or MS.

The study of mycorrhizal associations was performed in a maritime pine forest in the Portuguese coast, the plant species studied were *Halimium halimifolium, Acacia longifolia* e *Pinus pinaster*. We found six fungal species associated with *H. halimifolium*, one associated with *A. longifolia* and six associated with *P. pinaster*, one species was found associated with all hosts and three species shared between *H. halimifolium* e *P. pinaster*.

In the last chapter, we describe a new methodology for mycorrhizal synthesis, using MS medium as substrate. The synthesis was tested between two Cistaceae, *Halimium halimifolium* and *Tuberaria lignosa*, and three fungal species of economic importance, *Lactarius deliciosus, Tricholoma equestre* and *Tricholoma portentosum*. Only the assay between *Tuberaria lignosa* and *Tricholoma equestre* didn't produce ectomycorrhizas, probably due to contamination.

Keywords: Fungal growth; Cistaceae; Common Mycorrhizal Network potential; Maritime pine forest; Mycorrhizal synthesis.

Resumo

Este trabalho foi desenvolvido em três capítulos com três diferentes, mas complementares, âmbitos de trabalho: crescimento fúngico, estudo de associações micorrízicas em campo e síntese de micorrizas.

No primeiro capítulo descrevemos o estabelecimento de culturas de micélio de quatro fungos, duas espécies micorrízicas (*Lactarius deliciosus* e *Tricholoma portentosum*) e duas espécies saprófitas (*Agaricus bisporus* e *Macrolepiota procera*), e o seu crescimento em cinco meios (Potato dextrose agar (PDA), Malt extract agar number 2 (MEA), Murishage and Skoog medium (MS), Biotin-Aneurin-Folic acid medium (BAF) e Melin-Norkrans Modified medium (MNM)) a três temperaturas diferentes (4ºC, 24ºC e 30ºC). Os resultados mostram que *L. deliciosus* cresce melhor em MNM ou MS a 24ºC e que os melhores meios para manutenção de culturas a 4ºC são PDA ou MNM, *T. portentosum* apresenta melhor crescimento em MNM ou PDA a 24ºC e para manutenção os melhores meios são BAF ou MNM.

Quanto aos saprófitas, *A. bisporus* mostra melhor crescimento em PDA ou BAF a 24ºC com os mesmos meios sendo os melhores para manutenção e para *M. procera* o melhor crescimento foi detetado em PDA a 30ºC e para manutenção os melhores meio são BAF ou MS.

O estudo de associações micorrízicas foi realizado numa floresta de pinheiro bravo na costa Portuguesa, as espécies vegetais estudadas foram *Halimium halimifolium, Acacia longifolia* e *Pinus pinaster*. Foi possível identificar seis espécies fúngicas associadas com *H. halimifolium*, uma associada a *A. longifolia* e seis associadas a *P. pinaster*, uma espécie foi encontrada em todos os hospedeiros e três espécies partilhadas entre *H. halimifolium* e *P. pinaster*.

No último capítulo, foi descrita uma nova metodologia para a síntese de micorrizas, usando o meio MS como substrato. A síntese foi testada entre duas espécies vegetais da família Cistaceae, *Halimium halimifolium* e *Tuberaria lignosa*, e três espécies fúngicas de importância económica, *Lactarius deliciosus, Tricholoma equestre* e *Tricholoma portentosum*. Apenas o ensaio de síntese entre *Tuberaria lignosa* e *Tricholoma equestre* não produziu ectomicorrízas, provavelmente devido a contaminações.

Palavras-chave: Crescimento fúngico; Cistaceae; Potencial de Rede Comum de Micorrizas; Floresta de pinheiro bravo; Síntese de micorrizas.

General Introduction

Fungal Biology and Ecology

Fungi play a cornerstone role in ecosystems. Fungi are the main decomposers and recyclers of organic matter and their symbiosis with plants allows them to relieve stress, which plays a critical role in limiting plant growth, by extending the soil area that they can reach and allowing the capture and transportation of nutrients to the plant. Indeed, the earliest known land plants had no true roots but were colonized by hyphal fungi. Another important symbiosis that fungi establish is with algae, the lichens; that are pioneer colonizers of habitats, where no other organism can grow, establishing a new favourable environment for other organisms (Deacon ,2013).

Their importance can also be seen in the species richness and in the way it alters along gradients. Fungi species richness remains unaltered even in areas where, in other groups, great diversity can be found, like in tropical rainforests, but they also maintain the same level of diversity even in environments where other groups find difficulties in establishing and have low species richness. This suggests that the role of fungi is so important in the ecosystem that they are indispensable (Morgado et al. 2015).

Fungi are a very diverse group of organisms, it's estimated that 1.5 million different species exist (Tedersoo et al., 2010). Within the fungal kingdom, there are true fungi and fungus-like organisms, that for the interest of this work will not be referred. True fungi (Mycota) are eukaryotic and typically grow as filaments (hyphae) that exhibit apical growth and branch repeatedly behind their tips forming a network called mycelium. Fungi are heterotrophs and can grow as saprophyte, parasite or symbiont. The cell wall typically contains chitin and glucans and their nuclei are typically haploid, but sometimes hyphae contain several nuclei, each of them haploid. Fungi can reproduce both by sexual and asexual means and typically produce spores. The two phyla that will be addressed in this work are the Ascomycota and the Basidiomycota, although there are three other phyla Chystridiomycota, Zygomycota and Glomeromycota, which includes arbuscular mycorrhizal fungi and their relatives (Fig. 1).
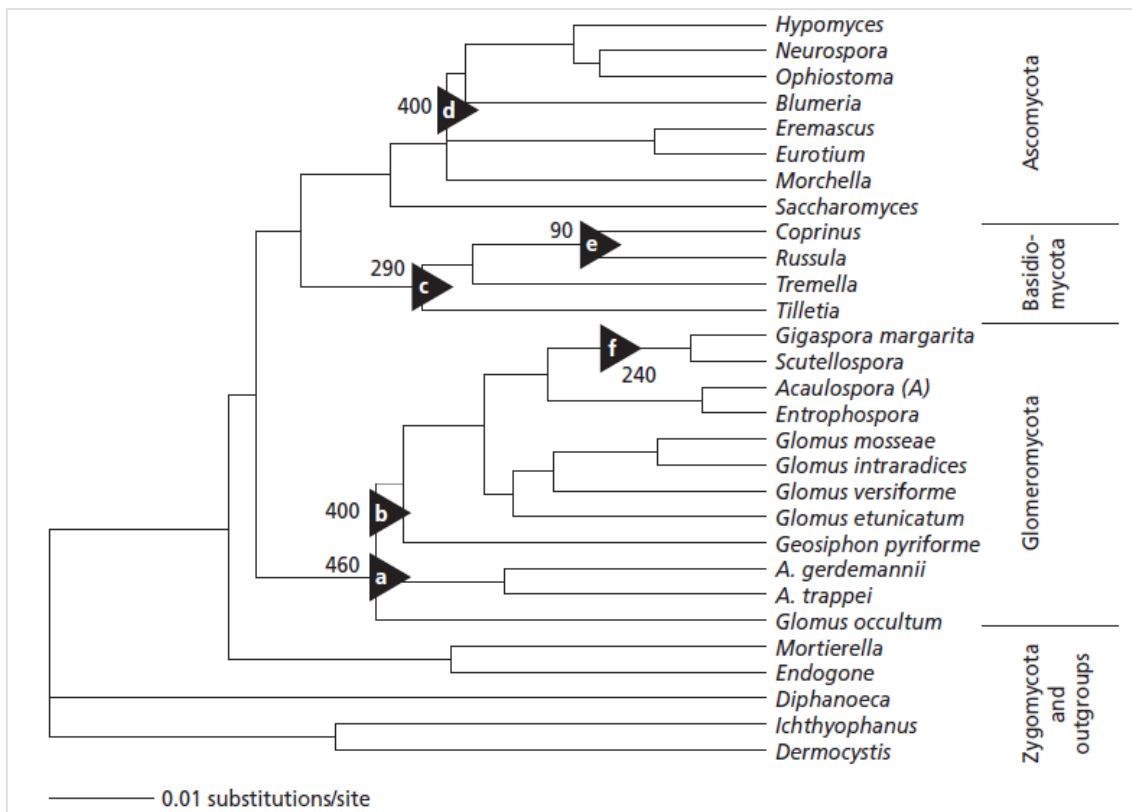
Figure 1-Phylogenetic tree based on small subunit ribosomal RNA gene sequences, here it can be seen the proximity between the fungal phyla. Triangles indicate fossil spores of important phylogenetic divergences (Deacon, 2013).

The most diverse phylum, in terms of known species, is the Ascomycota which is characterized by the ascus, a cell in which two compatible haploid nuclei of different mating types come together and fuse to form a diploid nucleus, followed by meiosis to produce haploid sexual spores, the ascospores. In more advanced species, many asci form within a fruiting body called the ascocarp. The majority of Ascomycota also produce asexual spores by mitosis, called conidiospores.

The other important phylum, for this work, is the Basidiomycota, which is characterized by the basidium, the cell that undergoes meiosis to produce the basidiospores, the sexual spores that are usually produced on short stalks called sterigmata. After the germination of the basidiospores the hypha that grows only contain one haploid nuclei, this phase is called monokaryon. After that, plasmogamy can occur if the hyphae of two monokaryons group of different mating compatibility group fuse together creating dikaryotic hyphae, that is the type found in all tissues of the mushrooms or other fruiting bodies (Fig. 2).
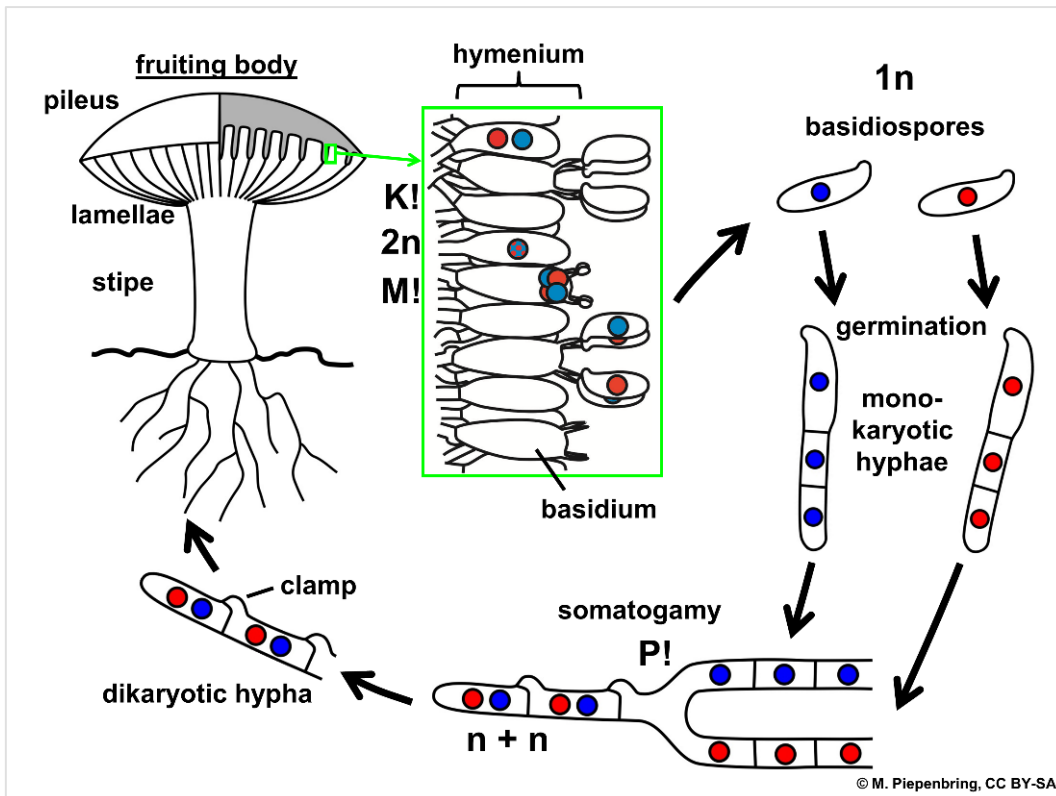
<u>Figure 2</u>-Life cycle of *Agaricus* sp. common to many mushrooms and to other species of the phylum (source:Wikipedia.com).

Most fungal species have been recognized and classified based on the morphology of their sporocarp. This has been a major obstacle to the progress of fungal ecology, being one of the major causes in the differences of advances between fungal ecology and plant or animal ecology, since fungi spend much of their life cycle as mycelia making their identification near impossible. Advances in DNA technology have enabled the identification of many fungal species. A part of the nuclear genome that has been widely used in phylogeny, identification and classification is the ribosomal DNA. This region encodes for the RNA component of the ribosomes. Unlike most genes, this particular region is arranged in repeating arrays of copies along the genome. A single copy contains the genes for the three RNA components of the ribosome, 18S, 5.8S and 28S RNA (Fig.3). The 28S and 18S have varied enough, in the course of evolution, that they can be used in works relating to phylum and domain of organisms. Between these genes, there is a region where the 5.8S gene is embedded, called the internally transcribed spacer (ITS) region. This region is removed during the processing of the mRNA. This region is less conserved than the coding regions and varies enough between species to allow the identification (Carlile et al., 2001).
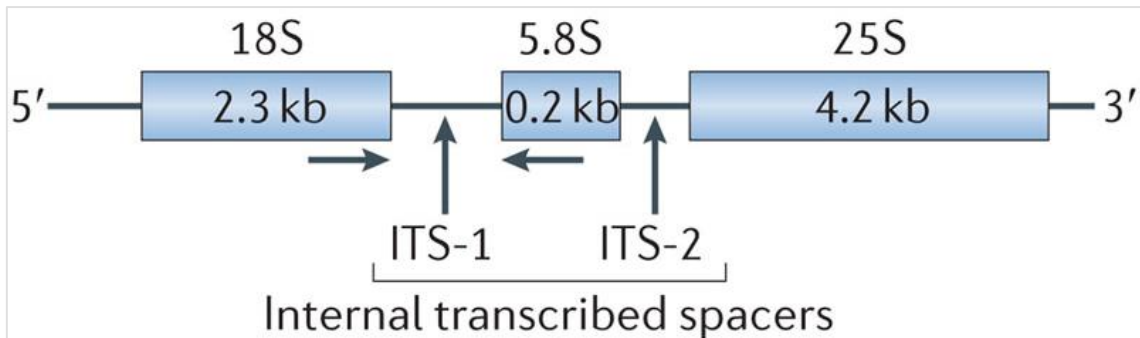
Figure 3- Diagram for a single copy of ribosomal DNA that contains the 28S, 18S and 5.8S genes for the ribosome, as well as the Internal transcribed spacer (ITS) region (Underhill and Iliev, 2014).

Spores produced by different types of fungi can undergo a process of dormancy, in which they are unable to germinate or germinate poorly. This is very important in fungal ecology because it leads to a succession of species in nature. In more detail, in the roots of mycorrhized plants there is a succession that occurs in which there are fungi called pioneers, that form mycorrhizas in young plants, and later fungi that only occurred in older plants. This is derived from the spore dormancy. Imagine a scenario where trees are planted in a new region that never had trees previously. Here there will be a larger presence of mycorrhiza with the pioneer fungi, only because the later fungi will not be able to germinate so quickly but, with the passing of time, the pioneer fungi may be replaced by the later fungi, as they will have time to germinate and grow. If we consider a case where trees are already present, the fungi that will form mycorrhiza will be the later fungi because the germination will not be a factor here. Taking this into consideration, the germination ability of the fungi will only be a factor in a natural scenario but not in an *in vitro* scenario (Deacon, 2013).

The environment in which the fungi occurs is very important, since it can influence fungal growth. These influences present themselves in the way hyphae branch, the colony growth rate, sporulation, hyphal differentiation and in other ways. Temperature, water availability, nutrient balance, concentration and spatial distribution of resources are all important environmental factors. Since fungi are, mainly, immobile, they need to produce structures that take advantage of favourable conditions, as vegetative mycelium, and others that allow them to survive when in unfavourable conditions, as spores. Changes may be triggered by concentration change in a specific element or light of a particular wavelength (Carlile et al., 2001).

A major component of fungi degradation occurs by extracellular enzyme activity. These enzymes produce soluble products that may enter the hyphae by diffusion, alongside with other soluble components. Usually, these soluble products are actively transported across the cell membrane by specific transport systems. It is characteristic of fungi that some transport systems have a high affinity for their target substrates. This allows for a rapid uptake and also facilitates scavenging activities by fungi under starvation conditions. These characteristics allow fungi to concentrate nutrients, such as sugars, amino acids and minerals, even when the external concentration is lower than in the hyphae.

The two major nutrients for fungi are carbon and nitrogen. Both of them can be obtained by a variety of different sources depending, many times, on the fungal species. As for carbon sources, fungi can use compounds such as sugars. Probably all fungi can use glucose that is transported to the cell by transmembrane carriers that are part of a constitutive transport system, but many other sugars can be used by specific species. Other compounds as polysaccharides, like starch, cellulose or even chitin, or lipids, hydrocarbons and C_1 compounds, like methanol or fuel. Amino acids and proteins are usually considered mainly as nitrogen sources, but they can also be used solely as a carbon source by some fungi. In terms of nitrogen sources, most fungi can assimilate nitrogen as nitrate or ammonia, commonly found in soils. Most fungi can also use amino acids that are transported to the cell by active transport by a range of permeases. Polypeptides and proteins are also a possible source of nitrogen.

Fungi have two main nutritional behaviours, being saprophytic or symbiotic. Saprophytes (*sapros*=death, *trophy*= feeding) are organisms that feed on dead organic matter. Fungi can produce a large array of enzymes to degrade several polymers. This group of fungi is the main responsible for recycling the components of dead plants. The most important polymer that they can degrade is cellulose, that is the main component of plant cell walls making it one of the most abundant polymers in the world. Fungi are important because they replenish the levels of carbon dioxide in the atmosphere, since they degrade plant components where carbon dioxide is stored during photosynthesis and plant growth. Not only they replenish carbon dioxide levels, but they're also important for recycling other elements like nitrogen, phosphorous and potassium. Different growing conditions for each fungi species to thrive leads to the formation of complex fungal community, in terms of enzymes present and species richness.

The symbiotic fungi live in a close contact relationship with other organisms, usually plants. The relationship may be parasitic, where only one species benefits, or mutualistic, where both intervenient benefit. Although some parasitic fungi may be addressed during this work, since many of them inhabit plant roots, the relationship is not the aim of the work. The focus of the present work is a specific mutualistic association, mycorrhizal symbiosis.

Mycorrhizal symbiosis

Mycorrhiza, from the Greek *myko*s= fungus and *rhizon*= root, refers to a symbiotic association between plant and fungi, not only with the roots of the plant but other structures of plants, as the underground organs of the gametophytes of many bryophytes and pteridophytes. The fungi improve plant mineral nutrition, allowing for a greater absorption area than what the roots alone could provide. The first description and definition of mycorrhiza and ectomycorrhiza were made by A. B. Frank in 1885 and 1887 (Frank, 2005).

Mycorrhizas can be classified on the basis of their fungal associates and the following types are recognized: Arbuscular mycorrhizas (AM), Arbutoid, Monotropoid,

Ericoid, Orchid, Ectomycorrhiza and Ectendomycorrhiza. Only Ectomycorrhiza (ECM) will be addressed in this thesis.

In ECM the fungus forms a mantle, a structure that encloses the rootlet. From the mantle hyphae or rhizomorphs radiate outwards to the substrate, and inwards between the cells of the root to form the Hartig net, a complex intercellular system that appears as a network of hyphae. There is no intracellular penetration in ECM in contrast with the ectendomycorrhizas where the hyphae penetrate the cell (Fig.4).
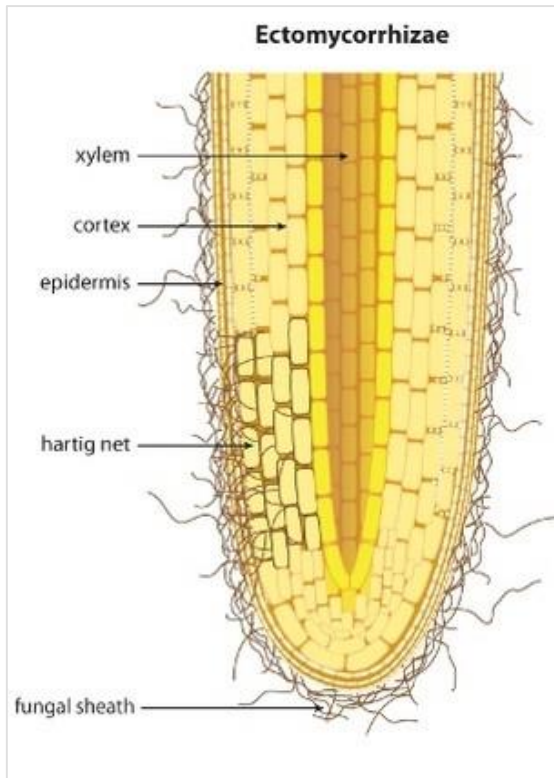
Figure 4-Diagram of ECM root where the mantle (fungal sheath) and the Hartig can be seen (adapted from: quasargroupconsulting.com).

There has been evidence of 249 fungal genera that form ECM, some of which were included based on phylogenetic data or potential of forming ECM, based on their habitat. But only 162 have been proven to form ECM associations (Tedersoo et al., 2010). It has not been possible to pinpoint the origin of ECM association and data suggest that it has occurred from several different lineages. It is estimated that about 83% of terrestrial plant species have mycorrhizal associations to maximize the nutrient uptake. From that percentage about 6000 plant species form ECM associations with around 20000-25000 fungal species (Tedersoo et al., 2010).

In this work the ECM fungi that will be used are *Lactarius deliciosus* (L.) Gray, a very esteemed edible mushroom, usually found associated with *Pinus pinaster*, easily distinguished by the orange cap and the latex it exudes of carrot colour that changes to greenish with time (Fig. 5), *Tricholoma portentosum* (Fr.) Quél., edible mushroom found in associations with conifers, grey cap with white gills and stem (Fig.5), and *Tricholoma equestre* (L.) Gillet, yellow mushroom commonly found in coniferous woods (Fig. 5),

whose edibility has been sometimes contested due to some reports of toxicity (ex: Chodorowski et al., 2001). Nonetheless, it keeps being much appreciated and widely eaten in Portugal, with no legal restriction to consumption.

<u>Figure 5</u>- ECM mushrooms that will be used in this work, from left to right, *Lactarius deliciosus, Tricholoma portentosum* and *Tricholoma equestre* (source:wikipedia.com)

Cistaceae Family

The Cistaceae family, commonly known as rockrose family, is described as shrubs, subshrubs or herbs, flowers solitary or in cymose inflorescences, petals yellow, white or reddish (maroon, pink, purplish, orange), mostly perennial but some annual (Arrington and Kubitzki, 2003).

The family comprises 8 genera, *Cistus, Crocanthemum, Fumana, Halimium, Helianthemum, Hudsonia, Lechea* and *Tuberaria*, with only three being restricted to the American continent, *Crocanthemum*, *Hudsonia* and *Lechea*. The two genera used in this work were *Halimium* (9-14 species) and *Tuberaria* (10 species), and the species used *Halimium halimifolium* and *Tuberaria lignosa*.

Tuberaria lignosa (Sweet) Samp. is a perennial herb, often woody towards the base, it reaches a height of 57 cm and branches freely, leaves are simple, 3–10 cm long and 0.9–3.4 cm wide. The inflorescence is lax, with each flower 2–3 cm in diameter (Castroviejo, 1986-2006) (Fig.6). Its distribution ranges throughout the Mediterranean basin, north Africa and the Canary Islands (Fig.7) and in Portugal it can be found in the centre-north and coastal regions (Fig. 7).

Halimium halimifolium(L.) Willk is a shrub or subshrub that can reach a height of 2 m highly branched, branches covered by stellated or peltated hairs, leafs with short petiole 1.5-6 mm, blades elliptic to oblong-elliptic with 4-48x4-18 mm, numerous flowers in terminal inflorescences, paniculated, relaxed or tense. 5 sepals, 2 external and 3 internal, petals 8-16 mm yellow pristine or rust at the base (Castroviejo, 1986-2006) (Fig. 8), occurring on sandy substrates of the South and West of the Iberian Peninsula, Italy, Greece and north of Morocco (Fig. 9). In Portugal is distributed along the coast (Fig. 9).

Figure 6-*Tuberaria lignosa* morphological characteristics, A- Flower detail immaculate yellow, B- overall aspect of the non-flowering plant, C- Flowering button, D- Leaf detail (source (A,D): flora-on.pt).

Figure 7- Distribution of *Tuberaria lignosa* in continental Portugal, left image (Carapeto et al., 2016), and worldwide, right image.

Figure 8-*Halimium halimifolium* morphological characteristics, A-Overall aspect of the shrub, B-Flower detail immaculate yellow, C- Leaf detail and D-Maturation stages of the fertilized flowering button, from the right to the left.
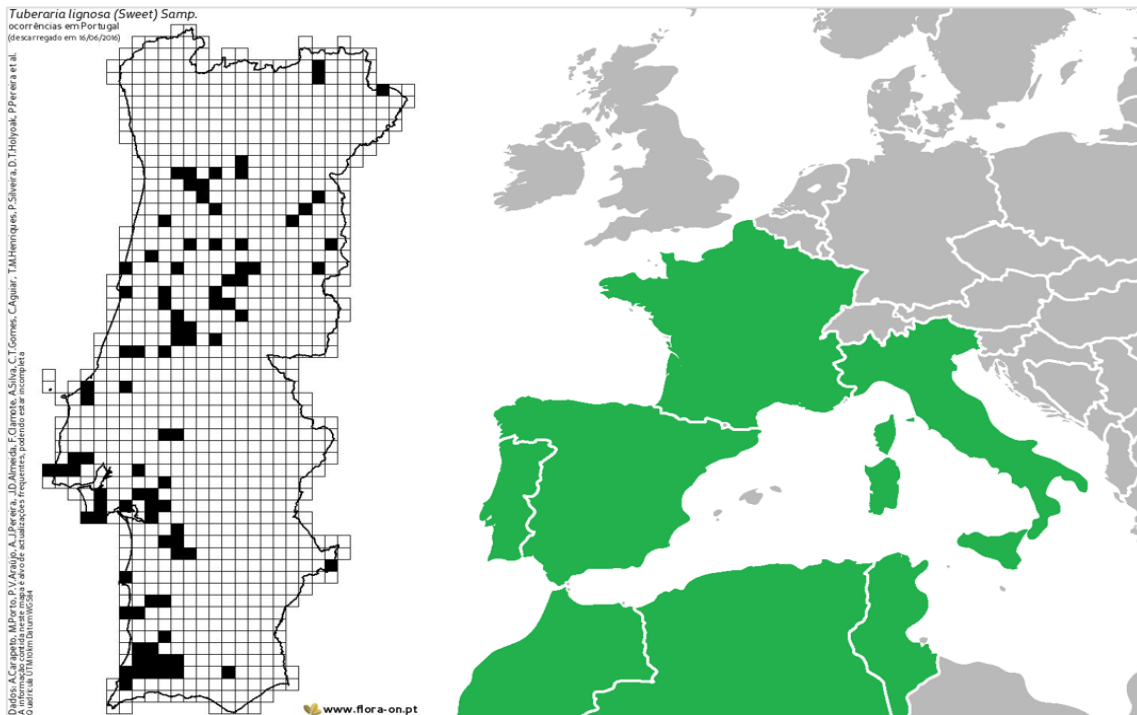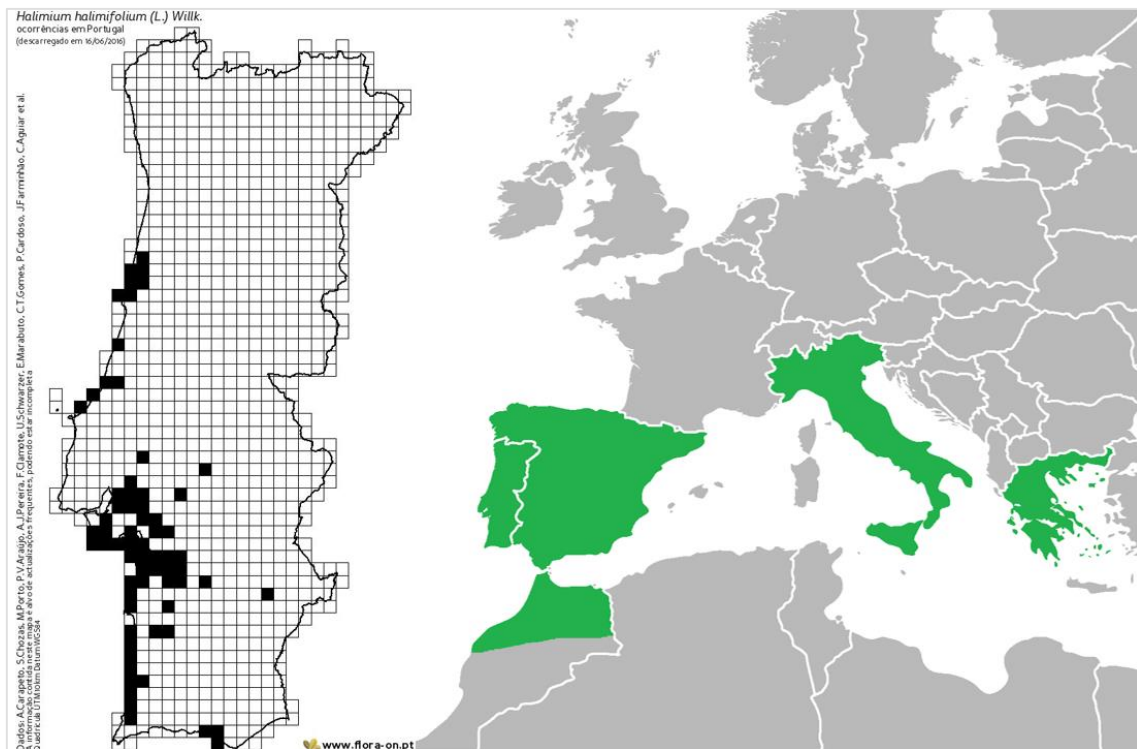

Figure 9-Distribution of *Halimium halimifolium* in continental Portugal, left image (Carapeto et al., 2016), and Worldwide, right image.

Research aim and Thesis layout

The general aim of the present work is to identify the ECM associations established in natural conditions with shrubs of the Cistaceae and to evaluate potential associations that they can form with economically important edible fungi.

This thesis is organized in three chapters, the first and the third chapter present the biotechnological approach of the thesis and the second the biodiversity perspective.

The first and second chapter provide important information and the results work as a prove of concept for the last chapter since, in the first chapter, we tested the potential of Murishage and Skoog medium to sustain fungal growth, this medium was later used as substrate for ECM synthesis, in the last chapter. In the second chapter, we investigated the ECM symbionts of *Halimium halimifolium* in natural conditions and evaluated the potential symbionts shared with *Pinus pinaster*. This information is deemed valuable for the biotechnological potential of the associations tested in the last chapter, because it proves that there can be a sharing of the symbionts tested in field conditions.

The first chapter aims to optimize the growth conditions of four fungal species, two ECM fungi and two saprophytic, and to compare the growth of the ECM fungi with the saprophytes in commercially available and commonly used mediums.

The second chapter aims to unveil some of the associations present in maritime pine forests of the Portuguese coast, specifically the associations with *Halimium halimifolium, Pinus pinaster* and *Acacia longifolia*, in order to find possible common mycorrhizal network between the two native species and to see if the invasive species has some part in this network.

The third chapter aims to test the biotechnological potential of *Tuberaria lignosa* and *Halimium halimifolium* to establish associations with three important ECM fungi, *Lactarius deliciosus*, *Tricholoma portentosum* and *Tricholoma equestre*. Afterwards, mycorrhizal plants could be used for commercially producing fruit bodies and possibly for the potential restoration of ecosystems that have been degraded.

Chapter 1

Effect of temperature and growth medium in mycelial growth of edible mushroom species (saprophytic and mycorrhizal).

Introduction

Source of food, income or even in traditional medicine and folk culture, from the shamans in ancient American cultures to the Viking berserkers, since the Palaeolithic times mushrooms have influenced cultures in many ways.

The harvest of mushrooms in the wild was, for many years, the only way to access this natural resource, but it all changed when the ability to control their growth was achieved. First reports date to around the year 1000-1100 A.D. in China with the production of *Lentinula edodes*, or has it is commonly known the Shitake mushroom. Since these early days, many other saprophytic fungi have been commercially produced around the world, the most well-known are the white bottom mushroom, *Agaricus bisporus*, and the oyster mushroom, *Pleurotus oestreatus* (Johnson, 1996).

Fungi can use a great variety of nutrient source with different complexity levels. Most fungi can be maintained in simple medium with mineral salts and glucose (see Deacon's (2013) minimal requirement medium). Fungi depend on small soluble molecules that diffuse through the wall and enter the cell by specific transport proteins. More complex molecules have to be breakdown by secreted enzymes that will depend on specific fungal species. As nitrogen source, all fungi can use amino acids. In many cases, they only required one basic amino acid since they have the capability of transforming them into all the others by transamination. Some fungi can use other sources of nitrogen as ammonia or nitrate. Phosphorous is often poorly available in natural environment but fungi can cleave phosphorous from organic sources allowing the uptake of phosphorous (Smith and Read, 2010).

Ectomycorrhizal (ECM) species get most of their carbon from sucrose given by the plant host (Nehls, 2008). It is then cleaved by plant-derived invertase into fructose and glucose that can then be used by the fungi (Nehls and Hampp, 2000) but this is not the only way fungi can obtain carbon. They also have the ability to decarboxylase amino acids and subsequently, use them as carbon and nitrogen sources (Taylor et al, 2004). In terms of nitrogen sources, studies have shown that for the ECM fungi the preferred source is ammonium-nitrogen (Finlay et al., 1992; Sarjala, 1999; Rangel-Castro et al., 2002; Hatakeyama and Ohmasa, 2004). Nevertheless, other studies showed that the majority of ECM Basidiomycetes can't use nitrate-nitrogen sources (Nygren et al., 2008; Plett and Martin, 2011).

The ECM fungi used in this study were *Lactarius deliciosus* (L.) Gray, whose nitrogen and carbon preferences have already been studied by many authors, and *Tricholoma portentosum* (Fr.) Quél., for which, to the extent of our knowledge, nothing is known about its nitrogen and carbon preferences and optimum medium and temperature for growth.

The saprophytic species used were *Agaricus bisporus* (J. E. Lange) Imbach, a cultivated species whose nitrogen and carbon preferences are well known, and *Macrolepiota procera* (Scoop.) Singer, to which to the extent of our knowledge, there are few reports on the nitrogen and carbon source preferences (Shim et al., 2005) or optimum temperature for growth.

In this study the media used were Potato Dextrose Agar (PDA), Malt Extract Agar number 2 (MEA), Melin-Norkrans Modified Medium (MNM), Biotin-Aneurin-Folic Acid medium (BAF) and Murishage-Skoog medium (MS). The first four are commonly used mediums in fungal cultures of both mycorrhizal and saprophytic fungi, but MS medium, has seldom used in the culture of mycorrhizal fungi (Sanmee et al., 2010). However, it is a commonly used for *in vitro* plant micropropagation and tissue culture.

This study aims to determine the optimal growth conditions, medium and temperature, for each fungal species and aims to find common factors in less favourable media and in more favourable ones to try and determine the factor responsible for these differences (ex. Carbon source or nitrogen source).

Another objective of this work is to determine the growth behaviour of this fungus on MS, since this is the first report for their growth on this medium. The possibility that fungi may grow on this medium opens a window, for the ECM fungi tested at least, for its usage in mycorrhization assays allowing for an easier aseptic approach and possible large-scale production of mycorrhizal plants in nursery condition.

The use of saprophytes and mycorrhizal fungi allows for a comparison between both nutritional behaviours in their growth patterns in each medium and temperature.

Materials and Methods

Fungal Isolates

Sporocarps of the two mycorrhizal fungi species were collected in a *Pinus pinaster* forest. *Lactarius deliciosus* sporocarps were collected in coastal forests and *Tricholoma portentosum* were collected in mountain forests. Sporocarps from the saprobic species *Macrolepiota procera* were collected in grassland and *Agaricus bisporus* were commercially obtained. The collection site and date of collection are registered in Table 2. The morphological identification was obtained using identification guide (Phillips, 2013).

The outside of the sporocarps were sterilized using 3% calcium hypochlorite. The sporocarps were sectioned longitudinally, small fragments were collected and placed under growth condition in PDA and sequentially cultured until a pure culture was obtained (Fig.10) (Molina and Palmer, 1982). The initial culture had to be daily observed to prevent contaminations from other organisms present inside or outside the sporocarps.

The cultures were subcultured monthly after the pure cultures were obtained. The morphological identification was confirmed by molecular methods described next.
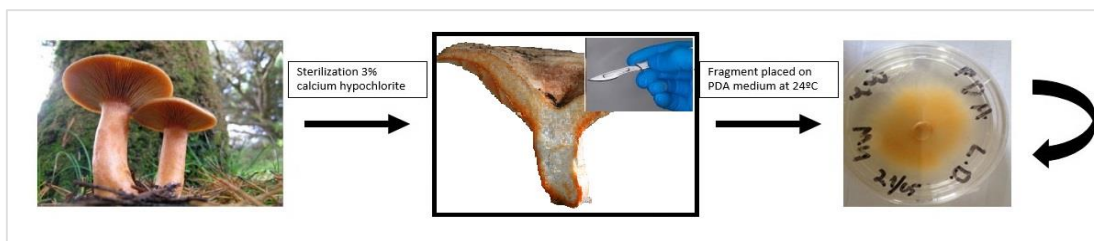
Figure 10-Steps of the methodology for mycelium isolation. Sporocarps were collected in the wild, superficially sterilized in laboratory conditions, then sectioned and small pieces of tissue placed agar growth medium.

Molecular identification

DNA extraction from pure mycelium culture was performed using REDExtract-N-Amp Plant PCR kit (Sygma-Aldrich©) using the following protocol, approximately 5 mm^2 of pure culture was placed in an Eppendorf with 10 µl of the Extract solution. We used the following temperature cycle of 65ºC for 10 minutes, 95ºC for 13 min and 90ºC for 10 minutes. After the cycle, 10 µl of the Dilution solution was added to the Eppendorf.

For the DNA amplification, NZYTaq 2x Green Master Mix (Nzytech©) kit was used. The primers used were the ITS1F and ITS4 primer region to amplify the internal spacer ribosomal DNA region (ITS) region. PCR design was initial denaturation at 94ºC for 3 min, 33 cycles of 94ºC for 45 s of denaturation, 54ºC for 45s for primer annealing and 72ºC for 45s for elongation, followed by 72ºC for 10 min for further elongation. The samples were loaded on a 2% TBE agarose gel to see the efficacy of the amplification. Samples were sent to Stabvida© for sequencing and the obtained sequences were analysed and edited using Geneious© software. Basic Local Alignment Search Tool (BLAST) was performed with the sequences available in GeneBank using sequences with >97% similarity for species identification.

Culture conditions for mycelia growth and data analyses

For this experiment, as previously stated, five media were used PDA (VWR Chemicals©), Malt extract number 2 (VWR Chemicals©), MS (Duchefa-Biochemie©), BAF and MNM (Table 1). The agar concentration used for all media was 15 g/l. The differences in composition are illustrated in Fig. 11. The composition of PDA and MEA are not included because of their complex nature, derived from their production from potato and malt extracts, respectively.

Three temperatures were tested 4ºC (±3ºC), 24ºC (±3ºC) and 30ºC (±3ºC) for each medium with five replicate each. The growth experiment had a duration of 36 days with measurements made every 3 days by delimiting the edges of the cultures using a

permanent marker. Then, at the end of the 36 days or at the time the colony has completely covered the area of the Petri dish, photographs were taken with a standardized square with 5 cm^2 and processed using Photoshop© software to obtain the colony area on the given day in pixels. The conversion to cm^2 was performed using the formula bellow, where the growth area is represented by GA, the measurement in pixel by PG and the measurement in pixels of the standardized square by SP.

$$GA = \frac{PG \times 5}{SP}$$

Growth rates (GR) were determined by calculation of growth differences in consecutive measures and dividing them by the days passed between them.

$$GR = \frac{GA2\text{-}GA1}{3}$$

Statistical analyses were performed using STATISTICA© version 7 software package (StatSoft, Inc., 2007), the data was submitted to Bartlett's test for homogeneity and normality of data. After the comprobation of normality and homogeneity the statistical analyses performed were one-way ANOVA, to compare, for each fungus, the growth between the media. These analyses were performed for each of the three temperatures. For 24ºC, a second one-way ANOVA was performed to compare within each medium the differences between the fungi tested. A two-way ANOVA was also performed to test the effect of temperature and medium on the growth differences and to see if there was an interaction between the two factors. Significant differences were accepted for a value of p≤0,05. For all the tests performed the differences were identified with Tukey´s test.

Table 1- Composition of each medium, MR- Minimum requirements as described in Deacon (2013). Composition differences are show with * for unique compounds in each medium compared with others, compounds that are present in all compared are marked with [+] (Fig.11).

Medium	Composition
PDA	Dextrose(20g/l), Potato Starch(4g/l)
MEA	Dextrin(2,75g/l), Peptone(0,78g/l), Glycerol(2,35g/l), Maltose(12,75g/l)
MS	NH_4NO_3*(1,65g/l), $CaCL_2$[+](0,33g/l), KH_2PO_4[+](0,17g/l), KNO_3*(1,9g/l) $MgSO_4$[+](0,18g/l), $CoCl_2·6H_2O$*(0,025mg/l), $CuSO4·5H_2O$(0,025mg/l), FeNaEDTA*(36,7mg/l), H_3BO_4*(6,2mg/l), Kl(0,83mg/l), $MnSO_4·H_2O$(16,9mg/l), $Na_2MoO_4·2H_2O$*(0,25mg/l), $ZnSO_4·7H_2O$(0,25mg/l), Glycine*(2mg/l), myo-Inositol(0,1g/l), Nicotinic acid*(0,5mg/l), Pyridoxine HCl*(0,5mg/l), Thiamin HCl(1mg/l)
MNM	Glucose (5g/l), $(NH_4)_2HPO_4$*(0,25g/l), $CaCl_2$[+](0,05g/l), KH_2PO_4[+](0,5g/l), $MgSO_4·7H_2O$[+](0,31g/l), NaCl*(25mg/l), $FeCl_3$(12mg/l), Yeast extract(1g/l), Malt extract* (2g/l)
BAF	Glucose (30g/l), Peptone*(2g/l), $CaCl_2·2H_2O$[+](0,1g/l), KH_2PO_4[+](0,5g/l), $MgSO_4·7H_2O$[+](0,5g/l), $FeCl_3·6H_2O$(10mg/l), $MnSO_4$(5mg/l), $ZnSO_4·7H_2O$(1mg/l), myo-Inositol(0,05mg/l), Folic acid*(0,1mg/l), Thiamin HCl(0.05mg/l), Biotin*(1µg/l), Yeast extract(0,2g/l)
MR	Glucose|Sucrose* (20g/l),$NaNO_3$*|NH_4NH_3|other Nitrogen sources(2g/l), $CaCl_2$[+](0,5g/l), KH_2PO_4[+](1g/l), $MgSO_4$[+](0,5g/l), $CuSO_4$(5-10mg/l), $FeSO_4$*(5-10mg/l), KCl*(0,5g/l), $ZnSO_4$(5-10mg/l)
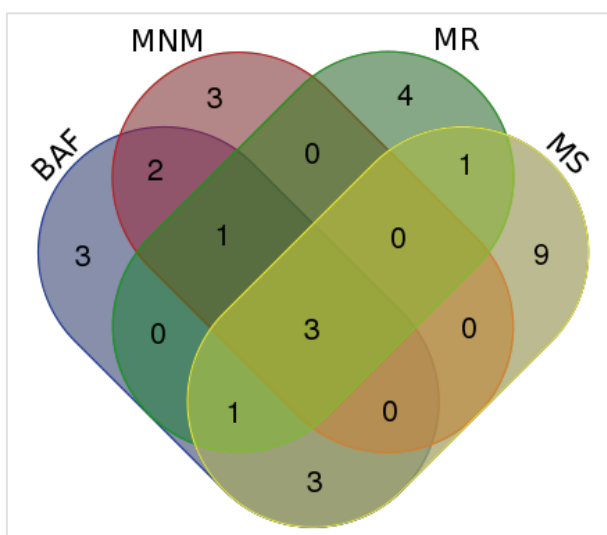
Figure 11- Comparison of BAF, MNM and MS medium with the Minimal Requirements (MR) defined in Deacon (2013). It allows the visualization of the unique compounds of each medium. Information on differences and common compounds can be found in Table 1. The numbers show the intersection of compounds to each comparison.

Results

Fungal culture

The molecular identification, collection site and GenBank reference are shown in Table 2. All sequences will be submitted to GenBank.

All morphological identifications were confirmed by the molecular analyses.

Table 2- Origin of the sporocarps collected in centre Portugal with the sequence reference in the GenBank database used for the identification confirmation. Field collection of sporocarps was performed in October 2014.

Fungal Species	Collection site	Identity (%)	GenBank reference
Lactarius deliciosus	Cantanhede, Coimbra	99	KJ769672.1
Tricholoma portentosum	Paul, Guarda	99	EU186273.1
Agaricus bisporus	Obtained commercially	99	HM561977.1
Macrolepiota procera	Paul de Arzila, Coimbra	100	JQ683121.1

Effect of culture medium and temperature in growth

The average growth rate for each medium at each temperature are shown in Fig. 12, the one-way ANOVA results are also shown in the same figure for both analyses performed. The two-way ANOVA performed showed significant differences between media and temperatures for all fungi as well as the interaction between the two factors. For *M. procera* the only media that showed no significant differences were MEA and MS but for the temperatures all showed significant differences between them. In the case of *A. bisporus,* there were no significant differences between BAF, MS and PDA and between MEA and MNM as well as no significant differences between the temperatures of 24ºC and 30ºC. For the mycorrhizal fungi, this analysis showed that for *L. deliciosus* there are no significant differences between MS and MNM but there are significant differences between all the temperatures. For *T. portentosum* there were no significant differences between PDA and MNM and no significant differences between 4ºC and 30ºC.

The first one-way ANOVA performed was to determine significant differences in the growth rates in the different mediums within each temperature allowing the determination of the best and worst medium for growth in each temperature for each fungus. For the temperature of 4ºC, the results show that *M. procera* grew better in MS but showed the lowest growth in MEA and MNM. *A. bisporus* has the best growth rate in BAF, PDA and MS but showed no growth in MNM and MEA. For *L. deliciosus* the best media were PDA, MNM and MS and no growth was obtained in BAF and MEA. For *T. portentosum* the best growth rates were achieved in BAF and MNM but no growth was obtained in MEA.

At 30ºC *T. portentosum* showed no growth in all the media, *M. procera* had the highest growth rate in BAF and the lowest in MNM. *A. bisporus* showed no growth in MEA but showed the best growth rate in BAF and PDA. For *L. deliciosus* the best growth rates were registered in PDA, MEA, MNM and MS but no growth was detected in BAF.

For the temperature of 24ºC *M. procera* showed the best growth rate in BAF and PDA and the worst in MNM and MS. *A. bisporus* showed the best rate in BAF and PDA but it had no growth in MEA. *L. deliciosus* showed the highest rate in MNM and MS and the lowest in BAF. For *T. portentosum* the best results were in PDA and MNM but no growth was obtained in MEA.

The overall results showed that 24ºC was, for the majority of the species, the best growing temperature so to determine the significant differences in growth rate of the different species within the mediums a second one-way ANOVA was performed. The results can also be seen in Fig. 13. The analyses showed that for BAF, PDA and MEA the best-growing species was *M. procera* but in MNM it was *L. deliciosus* that achieved the highest growth rate. In the other side of the spectrum in MEA both *A. bisporus* and *T. portentosum* showed no growth and both species were also the lowest growing ones in PDA. In BAF, the worst were *L. deliciosus* and *T. portentosum* and for MNM the worst species were *M. procera* and *A. bisporus*. In MS the only species to show a significantly lower rate was *T. portentosum*.

Culture morphology

The morphology of the fungal colonies grown at 24 ºC was observed after 36 days of growth is described in Table 3. Descriptions follow the methodology described by Hutchison (1991). Photographs of the cultures at 24ºC in PDA can be seen in Fig. 12 for comparison. It can be seen that some media lead to differences in morphology within the same species as well as different mycelia density.

Table 3- Morphological characteristics of the mycelia of each fungus on every medium at 24ºC.

Species	Medium	Mycelia texture	Mycelia colour	Border	Border colour	Reverse colour	Aerial growth	Medium coloration	Mycelia density
MP	BAF	Diffuse	White	Diffuse	White	White	No	No	Low
MP	PDA	Cottony	White	Clear	White	Brown	No	Yes	High
MP	MEA	Cottony	Pinkish	Clear	White	Beige	No	No	High
MP	MNM	Smooth	Beige	Clear	White	Beige	No	No	High
MP	MS	Diffuse	White	Diffuse	White	White	No	No	Low
AB	BAF	Diffuse	White	Diffuse	White	White	No	No	Low
AB	PDA	Smooth	White	Clear	White	Yellowish	No	No	High
AB	MEA	----------	----------	---------	-------------	-------------	---------	-------------	----------
AB	MNM	Smooth	White	Clear	White	Yellowish	No	No	Low
AB	MS	Diffuse	White	Diffuse	Transparent	Transparent	No	No	Low
LD	BAF	Diffuse	White	Diffuse	Transparent	Transparent	No	No	High
LD	PDA	Woolly	Orange	Clear	White	Orange	Yes	No	High
LD	MEA	Woolly	Beige	Clear	Yellowish	Beige	Yes	No	High
LD	MNM	Woolly	Orange	Clear	White	Beige	Yes	No	High
LD	MS	Diffuse	White	Diffuse	Transparent	Transparent	No	No	Low
TP	BAF	Cottony	White	Diffuse	Transparent	White	No	No	High
TP	PDA	Cottony	White	Diffuse	Transparent	Yellowish	No	No	High
TP	MEA	----------	----------	---------	-------------	-------------	---------	-------------	---------
TP	MNM	Cottony	White	Diffuse	Transparent	Yellowish	No	No	High
TP	MS	Diffuse	White	Diffuse	Transparent	Transparent	No	No	Low
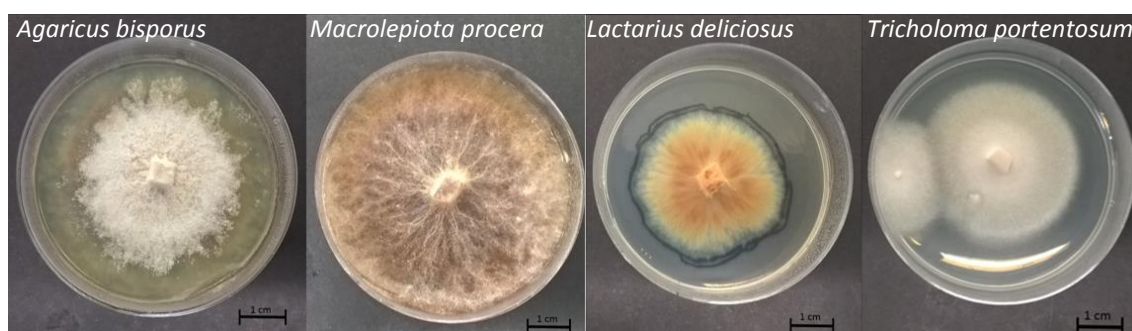
Figure 12- Culture morphology resulting from the culture in PDA at 24ºC. The description of the morphology in Table 3.
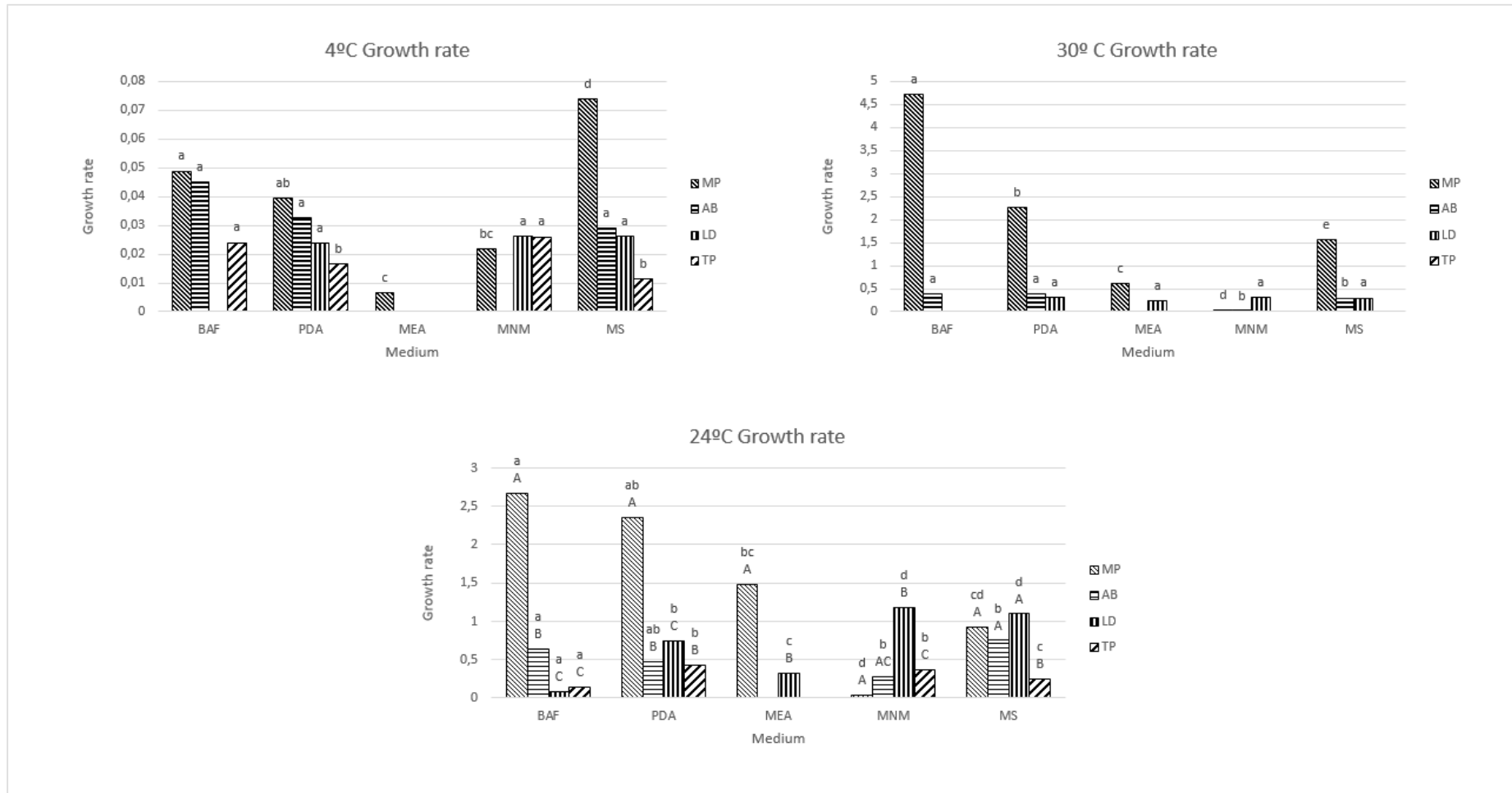
Figure 13-Medium growth rates (cm² per day) for each fungus in each medium. Uppercase letters in 24ºC graphic represent the ANOVA results for comparison of fungal species within media, lowercase letters represent ANOVA results for comparison of each medium within each fungal species in each temperature tested (p>0.05).

Discussion

Effect of culture medium and temperature in growth

The differences in the culture media can be observed in Fig. 11 and Table 2. The nitrogen sources and carbon sources differ between the media; PDA and MEA are more complex media since they are based on extracts from plants making it a more diverse and complex media than the others, making it hard to pinpoint the carbon and nitrogen sources. BAF has peptone as the main nitrogen source in opposition to MNM and MS where ammonium plays that role, MS also has KNO_3 as a possible source. As for carbon sources, BAF and MNM have glucose in their composition as the carbon source. This implies that the growth differences between BAF and MNM come from the different nitrogen source. Although there are other differences between the media they reside on differences in micronutrients and vitamins, since the data about differences at this level are scarce the discussion will only focus on differences that have been thoroughly studied before.

The two-way ANOVA results showed the overall best mediums and temperatures alone for the growth of each species. For *M. procera* the best medium was BAF, which suggests that this species prefers peptone as a nitrogen source, and the best temperature was 30ºC. For *A. bisporus* the best media were BAF, MS and PDA and the best temperatures were 24ºC and 30ºC. These results can suggest that the saprophytic studied species prefer peptone as nitrogen source and glucose as the carbon source since those are present in BAF, that lead to the best growth in both fungi, and they both presented the best growth at the temperature of 30ºC as it had already been reported (Furlan et al., 1997; Shim et al.,2005). For the ECM fungi, the media that showed the best growth for *L. deliciosus* were MNM and MS and for *T. portentosum* the media were MNM and PDA. MNM is present in both results showing a trend for the use of ammonium as a nitrogen source for this species, as it had previously been reported (Abuzinadah and Read, 1986; Finlay and Read, 1986; Finlay et al., 1992; Daza et al., 2006; Rangel-Castro et al.,2002; Itoo and Reshi, 2014), and that an increase in the carbon source presence doesn't correlate to an increase in growth since, in both cases, the media with the higher concentration didn't show increased growth rates. MS has a different source of carbon since it doesn't contain any of the common compounds used. Only vitamins possess carbon in their chemical structure making it reasonable to assume that fungi can use them as a carbon source, although there is the possibility that some carbon can also be obtained from the agar. The results show that MS can sustain the growth of all fungi broadening the range of its application, *in vitro* mycorrhization, for example. This is the first report, to the extent of our knowledge, for the growth of these species in MS, since the only report found was for *Phlebopus portentosus* (Sanmee et al., 2010).

Our results allowed for the determination of the best medium and temperature combination for large mycelial production and the best medium for culture maintenance at low temperatures. For *M. procera* the best combination seems to be BAF at 30ºC, which is in accordance with reports of other authors (Shim et al., 2005). For culture maintenance, BAF seems to be the best, although MS has a similar growth rate, however, possessing a

lower mycelia density that can jeopardise the culture replication. For *A. bisporus* the best combinations were PDA or BAF at 24ºC, these results are in line with other authors report (Wood, 1976; Rainey 1989) they're contrary, in some extent, with others (Baars et al., 1994; Furlan et al., 1997). For maintenance, the results showed the same media behaving the best, but in both situations, PDA should be preferred as it shows the largest mycelia density. For the ECM fungi, in the case of *L. deliciosus* the best combinations were MNM or MS at 24ºC. This result is in line with some works (Melin and Norkrans, 1948; Lundberg, 1970; Barros et al., 2006; Akata et al., 2012), with some showing that the best growth is achieved using a mix of amino acids as a nitrogen source. Some results with species of the same genus showed different results (Kibar and Perkensen, 2011). For maintenance, the best mediums were PDA or MNM. For *T. portentosum* the results show that the combinations of MNM or PDA at 24ºC are the best, this media differ with results obtained for other species of the genus (Kibar and Perkensen, 2011) but are in line with other works in different species in temperature and nitrogen source used (Sánchez et al, 2001; Kim et al., 2010); BAF and MNM showed the best results at 4ºC being the best ones for cultures maintenance.

In general, it can be seen that mycorrhizal fungi have better growth at 24ºC with 30ºC acting as a limiting factor for them, in accordance with results from other authors (Sánchez et al., 2001; Daza et al., 2006; Kibar and Perkensen, 2011), but for the saprophytic fungi this temperature increases their growth rate comparing with 24ºC, with the exception of *A. bisporus*. For maintenance, it seems that the media that have a broader range of usage are PDA and BAF but, as previously stated, PDA leads to a higher density of the mycelia possibly making it easier for culture replication and for the passage to a higher temperature for experimentation.

Culture morphology

Every species showed, in at least one media, differences in mycelium texture, border, border colour or reverse colour. Differences in media compositions not only change the growth rate of fungi, they also alter their morphological characteristics in culture. *M. procera* was the only species that changed the medium coloration, this was the case in PDA. *L. deliciosus* in BAF showed a particular alteration, presenting aerial growth. The composition of MS medium leads to a general modification of growth behaviour, in every fungus independently from their nutrition characteristics, all fungi showed low density of mycelium, which relates to low dry weight. Both saprobic species show a reduction in mycelium density in BAF as well.

Chapter 2

Evaluating mycorrhizal species diversity in Cistaceae shrubs, maritime pine and invasive *Acacia* in a coastal maritime pine forest in Portugal.

Introduction

Plants can obtain many benefits from a large number of fungal partners when forming ECM associations. This is possible because the majority of the ECM forming species aren't host-specific allowing them to colonize multiple hosts at the same time, from canopy trees to shrubs and even herbaceous plants (Bruns et al., 2002; Kennedy et al., 2003; Dickie et al., 2004; Ishida et al., 2007; Buscardo et al., 2012).

In nature, plants are usually colonized by more than one fungus and one fungus can have more than one hosts. This characteristic of the mycorrhizal associations leads to the formation of mycelial links that originate what is called a common mycorrhizal network (CMN), but CMN formation doesn't rely only on the same individual linking several plants. These associations can also be formed when two separated hyphae fuse creating a new connection between plants, previously not linked (Selosse et al., 2006). The identification of these structures depends on the identification of the same fungal genet, individual organism defined as a genotype, in two different hosts. Although the best way to prove the presence of a CMN would be in field observation of connection hyphae, this is a very rare event and only a few reports have published similar observation (Fitter et al., 2001; Simard et al., 2004).

Many methodologies have been used to try to characterize the diversity and the structure of ECM communities. The most commonly used are based on the survey of fruiting bodies but several ECM species, like many Ascomycota, lack or produce small or cryptic fruiting bodies that won't be accounted for in such surveys (Gardes and Bruns, 1996) leading to an inaccurate assessment of the community and its structure. Since the fruiting bodies surveys aren't the best option for this kind of characterization, the interest shifted from above-ground to below-ground surveys. This kind of surveys can be performed in two approaches: morphology or molecular analysis, or using both as is the case of our study. Most works use a combination of both, but with different goals in each methodology. Some studies use the morphological analysis to organize the structures present in the root in groups, morphotypes, and then use molecular techniques to achieve the identification of those morphotypes (ex: Horton and Bruns, 2001); others use the morphological analysis to achieve the identification of the structures and the molecular analysis serves as a confirmation of those identifications (ex: Hagerman et al., 1999). Although both methods being valid, some studies find a lack of correlation between both

methodologies (Nylund et al., 1995; Karen et al., 1997; Jonsson et al.,1999, 1999; Erland et al., 1999; Mah et al., 2001); nevertheless, this isn't always verified with some studies showing similar results for both methodologies (Sakakibara et al., 2002).

Most works on molecular ecology of ECM fungi have used the ITS region, in fungi the size of ITS can range from 600 to 900 bp. The amplification is normally performed using the universal primers ITS1 and ITS4 (White et al., 1990; Gardes et al., 1991) or fungal specific ones, ITS1F and ITS4 or ITS1F and ITS4b (Gardes and Bruns, 1993). Although it has been observed that ITS1 and ITS4 don't amplify the ITS region of the Pinaceae very well, they're still considered as universal and were first design for plant sequences, leaving the possibility of co-amplification of both host and fungi ITS regions (Horton and Bruns, 2001). This kind of analysis show some advantages, as they can accurately identify the fungi species and can also be used to analyse inter- and intra-specific genetic variability, but it also shows some challenges, as DNA extraction and amplification quality vary between species influencing the results.

Morphotyping has been used for many years as an attempt to distinguish different fungi (Zak, 1973; Haug and Oberwinkler, 1987). This type of identification relies on several characters being precisely detected and identified on both low and high magnification on a stereo microscope. Since the 1980s many works have been done to try and collect the characters identified in many studies in order to create an identification guide to facilitate such studies, resulting in the creation of several guides (Agerer et al., 1987-1988; Ingleby et al., 1990; Goodman et al., 1998). This methodology allows the examination of several roots in a relatively short amount of time, this is an advantage since the heterogeneity in the distribution of roots in the soil requires a lot of samples in order to grasp all the community that may be present (Brundrett and Abbott, 1994; Stendell et al., 1999; Birbartondo et al., 2000; Horton and Bruns, 2001), but it also presents some disadvantage, as it relies on the ability and experience of the user to achieve a precise identification. It also requires a confirmation of the identification and this can only be achieved by two methods: tracing the mycelium from the structure to a fruiting body (Agerer et al., 1987-1988) or by molecular techniques.

ECM lifestyle isn't of monophyletic origin; studies have shown that the traits have independently derived many times making the ECM fungi a polyphyletic group of organisms (Gargas et al., 1995; Hibbett et al., 1997, 2000; Bruns et al., 1998). This group contains species that span all of the phyla of true fungi (Zygomycota, Ascomycota and Basidiomycota) (Horton and Bruns, 2001).

In this work the plant species studied are: one early-successional species, *Halimium halimifolium*, a late-successional species, *Pinus pinaster*, and one invasive species, *Acacia longifolia*. Taudiere et al. (2015), in a study on the mycorrhizal associations shared by several Mediterranean species, including *Halimium halimifolium* and *Pinus pinaster*, showed that the expected number of associations shared by early-successional and late-successional species is higher than what is actually observed in the field, with the late-successional species having a lot more associations than the early-successional. From this study, we can expect a similar trend in our results with *P. pinaster* and *H. halimifolium* showing different associations and also a small number of shared associations as well.

Pinus pinaster Ait. has a great importance in forestry worldwide, and in Portugal in particular, being one of the most important species (Campelo et al., 2015). It's known to be an undermining, hardy species that grows in several habitats (Pera and Alvarez, 1995). *P. pinaster* is an obligate mutualist with ECM fungi and the absence of this type of associations leads to the inhibition of its normal growth (Read, 1998; Smith and Read, 2010). Although its widespread use in forestry, their known mycorrhizal associations come mostly from reports of fruit body collection in maritime pine forests. The work of Nieto and Carbone (2009) was the first to combine morphotyping, fruit body collection and molecular analysis to evaluate such associations; they showed that only 38% of what was surveyed by fruit body collection was present in the molecular analysis. Furthermore, they also present a review of the literature showing a total 204 species reported to be associate with *Pinus pinaster*. Other sources of possible associations are *in vitro* studies that test the compatibility between plant and fungal species. For *P. pinaster* a total of 100 works have been accounted for (Chaudhary et al., 2016).

Halimium halimifolium (L.) Wilk is a Mediterranean Cistaceae shrub that occurs in sandy soil, becoming the dominant species in sand ridges were the water table depth ranges from 2 to 4 meters (Zunzunegui et al., 2002). As for its mycorrhizal status, it's considered to be, like the rest of the Cistaceae, able to form ECM and AM associations. The first report of mycorrhizal associations being with AM fungi (de Vega et al., 2010). More recently, a total of twelve ECM partners have been linked to this species on the island of Corsica (Taudiere et al., 2015) with the majority of the findings being based on fruiting bodies surveys. It is also reported, on the same study, four mycorrhizal partners that were common between *P. pinaster* and *H. halimifolium*.

Acacia longifolia (Andrews) Wild is a native species from the southwest Australia but has been introduced in many places around the world. In Portugal, this species is one of the most prolific invasive species (Marchante et al., 2003). The *Acacia* genus are known to form AM and ECM associations, with AM being predominantly found (Reddell and Warren, 1986; Rodríguez-Echeverría et al., 2009) but, to the extent of our knowledge, there's a gap in the identification of ECM associations with this species.

This work aims to characterize part of the ECM community of a maritime pine (*Pinus pinaster*) forest in the coast of Portugal by investigating the associations with *Halimium halimifolium* and *Acacia longifolia*, one of the dominant shrubs in the area and one of the main invasive species, and of course of *Pinus pinaster*. The methodology we used combines morphological (morphotyping) and molecular analysis in an attempt to identify the fungal symbionts.

From the diversity found this study tries to re-create part of the network present in the area by using this three host and determine what mutualists they share between them.

Materials and Methods

Study Site and Root sampling

The samples were collected in an even-aged managed forest of *Pinus pinaster* within the nationally defined Perímetro Florestal das Dunas de Cantanhede. The area has a typically Mediterranean climate with oceanic influence with a marked summer drought, the soil is acidic with a sandy texture and low water-holding capacity (Campelo et al., 2015). The study area was defined as a quadrate of 10x10m having the lower left point coordinates of 40.35834º N 8.81903º W (Fig. 14). Within the area, the vegetal coverage was composed mainly of *Acacia longifolia*, *Pinus pinaster*, *Cistus psilosepalus*, *Cistus salviifolius* and *Halimium halimifolium*.

The samples were collected in November 2015; Roots of three individuals of each species were sampled in two directions, and sampling was performed following one of the main roots sampling the finer roots around them at two distances, near the plant and a bit distant from the plant. The samples were placed in plastic bags, in order to maintain humidity and avoid desiccation of the roots, and then tagged with the species name and individual number. The position of the individuals within the quadrate is approximated in Fig. 14.
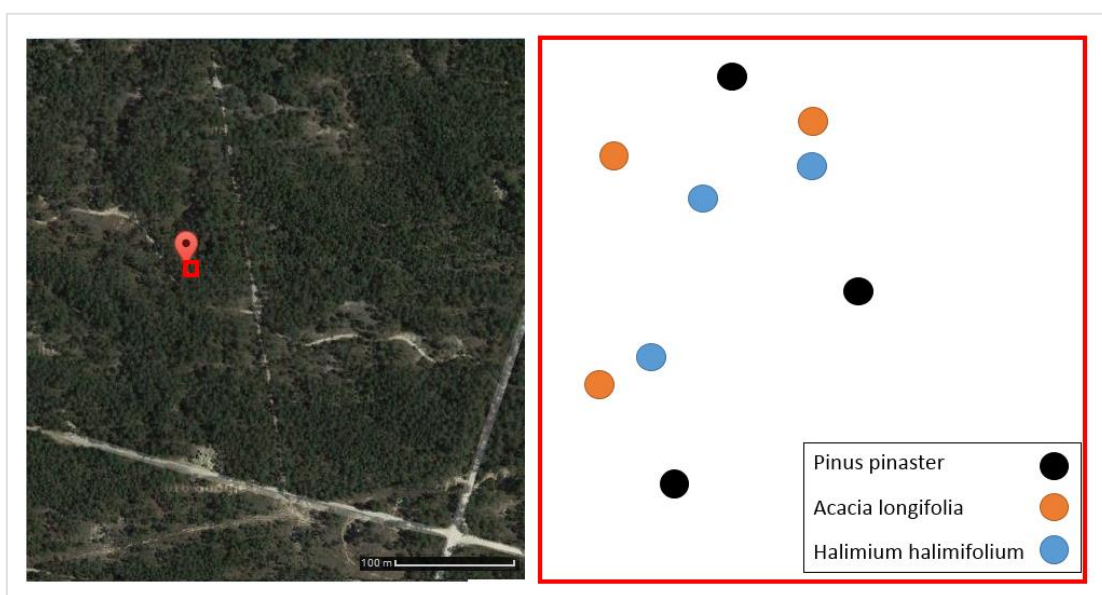
Figure 14- Study site represented on the left by a red square, the point presented has coordinates 40.35834ºN 8.81903 W. The diagram on the right indicates the position of the three individuals of each species within the quadrate.

ECM morphotyping

The sampled roots were weighed for each individual and then processed under a stereoscopic microscope for the presence of mycorrhized roots that were sorted into morphotypes based on their colour, size, texture, emanating hyphae and rhizomorphs, and distinct branching morphology (Agerer, 1997). Morphotypes were then isolated and

grouped by similarity among them (Fig. 15); after all the roots have been processed, DNA was extracted as described afterwards. Samples of each morphotypes group were kept for reference whenever more than one ECM tip was present. The reference materials were kept at -4ºC in 1 ml of Cetyltrimethylammonium bromide (CTAB) solution.

Figure 15-Steps of the methodology used for root sampling. The plant sampled were randomly selected within the quadrant and two samples were collected in two directions, one close to the plant another a bit further from it. In the laboratory, roots were examined for morphotype occurrence and ECM morphotypes were sorted.

Molecular identification

The procedure used was the same as described in Chapter 1 for DNA extraction and amplification.

For the identification of the fungal partners, the sequences obtained were used to perform BLAST analyses on GenBank; sequences with identification above 97% were considered for species identification.

Results

ECM morphotyping

A primary morphological analysis allowed for a separation of 77 morphotypes for *H. halimifolium* that were then narrowed to 73 in which DNA extraction was performed, 24 morphotypes were isolated for *A. longifolia* with only 22 having followed to DNA extraction and 38 morphotypes were isolated for *P. pinaster*, with a total of 37 being used for DNA extraction. The changes from the first identified to the ones that were selected for DNA extraction was based on the comparison of the three samples, which were individually processed, for similar morphotypes.

Pictures of the morphotypes for which the identification by molecular methods was possible are presented in Supplemental Material 1, pictures were obtained using Leica© EZ4 HD stereo microscope and Leica LAS EZ software©.

Molecular identification

The results of the identifications are shown in Table 4 along with the reference sequence, query and identification percentage. For *H. halimifolium* a total of seventeen sequences were obtained with enough quality for identification, for *A. longifolia* only three sequences were obtained and for *P. pinaster* a total of eleven sequences were

obtained and identified (with more awaiting sequencing). The sequences obtained from all the plant species were identified using the previously defined BLAST.

To determine common fungi within the hosts Google Fusion Table© software was used in order to create the network shown in Fig. 16. From the analysis, it can be seen that *H. halimifolium* and *P. pinaster* share a total of three fungal species and only one is shared between the three tested hosts.

Table 4-Identification results for every sequence obtained, the morphotypes names are composed of the first letter from the host they were collected in and a number related to the order they appeared during the process of the samples. The morphotype identification number is used to identify the pictures present on Supplemental material 1.

Morphotype	Species identification	Query (%)	Identification (%)	Reference sequence
HM1	*Russula sp.*	95	91	KF359616.1
HM2	Uncultured ECM (Pezizomycotina)	99	99	EU232106.1
HM3	*Sebacina vermifera*	99	98	JQ711843.1
HM4	*Tylospora* sp.	99	89	KF007260.1
HM5	Uncultured ECM (Pezizomycotina)	99	99	EU232106.1
HM6	Uncultured ECM (Pezizomycotina)	99	99	EU232106.1
HM7	*Humicola* sp.	85	100	DQ069025
HM8	Uncultured ECM (Helotiales)	99	85	FN565262.1
HM9	Uncultured ECM (Pezizomycotina)	91	89	KT334755.1
HM10	*Tomentella* sp.	91	96	JQ393136.1
HM11	Uncultured ECM (Pezizomycotina)	99	99	EU232106.1
HM12	*Tylospora* sp.	100	94	FN565228.1
HM13	*Tylospora* sp.	75	98	AM901986
HM14	*Mortierella* sp.	98	99	FJ553782.1
HM15	Uncultured ECM (Pezizomycotina)	99	99	EU232106.1
HM16	*Trichoderma hamatum*	59	99	KF856960.1
HM17	*Tylospora* sp.	99	89	FN565229.1
PM1	*Rhizopogon roseolus*	98	98	KF990475.1
PM2	*Russula sp.*	95	96	KT933999.1
PM3	*Russula sp*	98	93	KF359616.1
PM4	*Russula sardonia*	97	98	KT933999.1
PM5	*Oidiodendron sp.*	99	93	KJ921607.1
PM6	*Oidiodendron maius*	97	98	KF359579.1
PM7	*Rhizopogon sp.*	89	86	KF007248.1

PM8	Uncultured ECM (Pezizomycotina)	100	96	EU232106.1
PM9	*Odiodendron maius*	98	97	KF359579.1
PM10	*Archaeorhizomycetes borealis*	98	99	NR_126144.2
PM11	Uncultured ECM (Helotiales)	99	98	FN565262.1
AM1	Uncultured ECM (Pezizomycotina)	99	91	EU232106.1
AM2	Uncultured ECM (Pezizomycotina)	100	99	EU232106.1
AM3	Uncultured ECM (Pezizomycotina)	99	99	EU232106.1
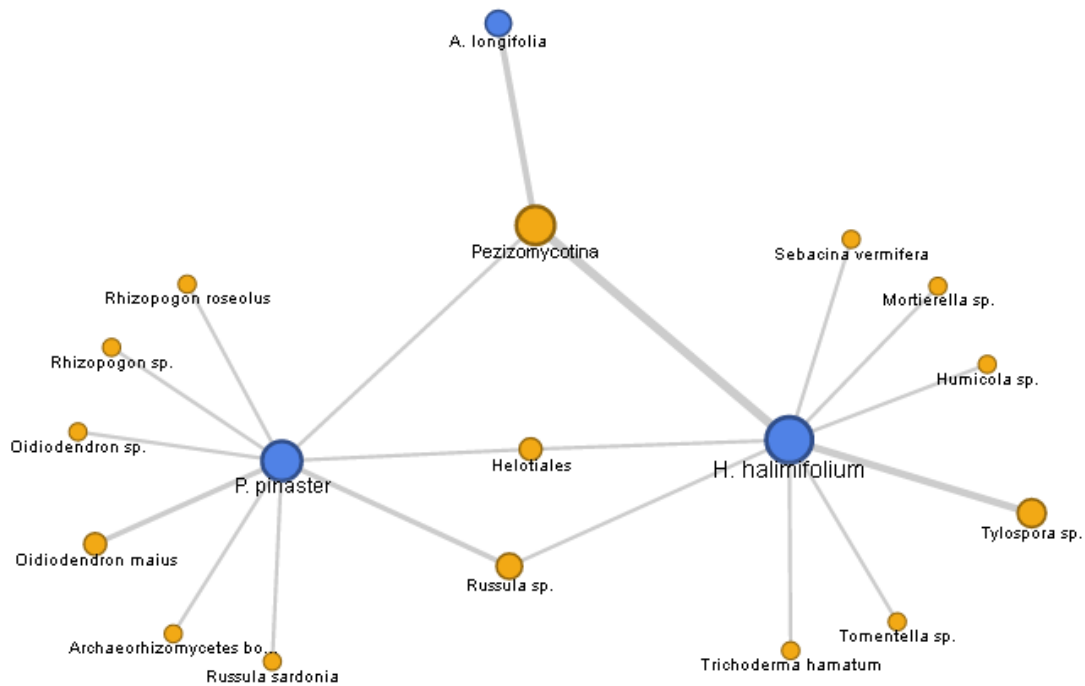
Figure 16 -Network created with Google Fusion Tables from Table 1 data. The host plants are in blue and the fungi species in yellow, the area of the circle and the connections are proportional to the number of morphotypes identified in each. Genera abbreviators are H. -*Halimium*, P. - *Pinus* and A. - *Acacia*.

Discussion

Difficulties in DNA extraction and amplification of several morphotypes were responsible for the differences between the number of morphotypes that were isolated by morphological characteristics and the number of sequences obtained.

Some species identified from the molecular data are known to be parasites of plant roots, non-ECM symbionts or saprophytic fungi, these species are *Odiodendron maius, Oidiodendron sp., Trichoderma hamatum* and the *Humicola* and *Mortierella* genera. This leads to a total of six different species identified related to *Halimium halimifolium*, six different species related to *Pinus pinaster* and one species related to *Acacia longifolia.*

Our results show a smaller diversity in *Halimium halimifolium* than a previous work by Taudiere et al. (2015) but interestingly the species found in our work don't correlate with the ones described in their work. The same occurs with *Pinus pinaster* when compared with other works (Taudiere et al., 2015; Nieto and Carbone, 2009). Our study shows a smaller diversity of symbionts, an expected result due to the small number of sampled plants (due to time limitation). As for *Acacia longifolia,* to the extent of our knowledge, our study is one of the first reports on ECM associations for this species combining both molecular data and morphology.

Although from our results only three species seem to be shared between *H. halimifolium* and *P. pinaster* most of the reference sequences used for identification were obtained in studies of *P. pinaster* related diversity, showing that, theoretically, more symbionts are shared between those hosts, which is of big importance since they are commonly found in the same habitat, and *H. halimifolium,* being an early-colonizer, may act as a "fertility islands" that can contribute for the colonization of new, or altered, habitats by *P. pinaster*.

Our results also show another identification of *Archaorhizomycetes borealis* associated with *Pinus pinaster* but on a coastal forest, which increases the number of habitats that this recently described species has been found. In this study another report of its presence on ECM-tips with the morphology shown in SM1 (Menkis et al., 2014).

For a deeper understanding of the network present in this habitat, new studies should be performed using different molecular techniques, like next-generation sequencing for example, and better extraction methods that can lead to a better quality DNA samples and more efficiency of the extraction. These kind of works are important because they allow for the connection between morphological analysis and the molecular identification of morphotypes, allowing for a widespread use of the morphological identification.

Further studies should be conducted in order to identify some species detected in several molecular studies. These species are yet to be isolated and described, leading to several fungi left unidentified, as occurs in our work with uncultured Pezizomycotina and Helotiales fungus found associated with our hosts and are yet to be properly identified and described.

Chapter 3

Ectomycorrhizal synthesis of *Halimium halimifolium* and *Tuberaria lignosa* with *Lactarius deliciosus*, *Tricholoma equestre* and *Tricholoma portentosum*.

Introduction

Ectomycorrhizal (ECM) fungi bring several advantages to plants including increased root area for absorption (Bowen ,1973, 1974; Harley and Smith, 1983), enhanced uptake of nutrients (Harley and Smith, 1983), resistance to pathogens (Marx, 1969) and drought (Duddridge et al., 1980; Boyd et al., 1986; Meyer, 1987). ECM fungi can also increase growth and nutrient content of plants (Jones et al., 1991). First works on the effect of mycorrhizae in plant growth were conducted by Frank (1894) with *Pinus* seedlings showing an increase in growth of the plants (Smith and Read, 2010).

These characteristics of the association make it very interesting for micropropagation of plants and forestry since, not only the growth of the plant is improved, most of the valuable fruiting bodies are of ECM forming fungi allowing for an extra income in forestry (Savoie and Largeteau, 2011).

The formation of ECM in the wild occurs in two ways; If the lateral root originates from a colonized root, the inoculum responsible for its colonization will come either from the Hartig net present (Robertson, 1954; Wilcox, 1968, 1968) or from the inner mantle of the subtending root. But if the lateral root originates in a non-colonized root, the colonization may occur as described before or by propagules in the soil from different fungi. These events are responsible for the differences in the space-time colonization of the roots allowing for the high diversity found in many species (Smith and Read, 2010).

The ECM association, although generally having low specificity, can sometimes be constrained by incompatibilities between the partners. Mechanisms behind the phenomena are yet to be revealed but some works have hypothesised that such events can be mediated by compounds released from the roots (Horan and Chilvers, 1990).

When the roots come in proximity with fungal hyphae the root hairs proliferate behind the apice of growing uncolonized roots to increase the area where such hyphae may contact. After contact, the hyphae may show morphological changes, as increased branching or fusion of hyphal tips. Inside the root, the hyphae of the inner mantle start to penetrate between the cells of the root cap, immediately behind the apex, and after that start to penetrate between the epidermal cells to form the Hartig net. This timeline can

differ from species to species and the Hartig net may be formed after the mantle, as described, or before.

The first report of mycorrhizal synthesis comes from Melin (1922) with the association of *Larix* with *Suillus grevillei*. The methodology used was Erlenmeyer flasks filled with sand, as the substrate for plant and fungi, with cotton used to close the flask and maintain the aseptic conditions within it. Mycorrhizae was established after several months. The methodology was very rudimental and difficult to water and to maintain many replicates. A variation of this methodology was presented by Hacskaylo (1953) where sand was replaced with Terra-lite, an expanded mica, leading to an improvement in water retaining capacity, eliminating the difficulties of watering the system. In 1965 the work of Marx and Zak improved the methodology even further with the replacement of the substrate with peat moss. This allowed the study of acidophilic species since Terra-lite has a high buffering capacity not allowing the pH to drift far from 7.

Even with all these modifications the method had some big disadvantages. In greenhouse conditions, the inside of the flasks would heat up quickly requiring the use of cooling baths; the use of cotton as a sealant limited the availability of CO_2 after a few hours of photosynthesis, limiting the plant growth in control conditions; the addition of glucose was necessary to allow the growth of the fungal mycelia in order for them to reach the roots. Moreover, the usage of glucose by the fungi increases the CO_2 levels inside the flask, apart from creating even more artificial conditions, and in this way lead to the possibility that the differences found between control and mycorrhized plants had their origin in these differences on CO_2 levels, instead of the association itself (Fortin et al., 1983). This was surpassed with the work of Trappe (1962) by using food jars and placing the plants outside the jars. In this way, the roots were kept in aseptic conditions while the plants weren't influenced by the fungal metabolism or the lack of CO_2 caused by the container. But still, the equipment needed were bulky and prevented large-scale experimenting. Moreover, the substrate made it difficult to observe the ECM formation.

In 1968 Pachlewska used test tubes with water agar medium supplemented with thiamine. This method showed some success for a large number of fungi with *Pinus sylvestris* (Pachlewska, 1968; Pachlewski and Patchlewska, 1974). This was later improved by Mason (1980) with the addition of minerals to the medium, allowing this method to be used in developmental or physiological studies. Another variation of this methodology was used by Molina (1979) with the replacement of the medium with a mix of peat and vermiculite with the supplementation of MNM medium liquid solution.

Several other methods were developed afterwards for special structural or physiological studies (Sohn, 1981; Biggs and Alexander, 1981; Nylund, 1981). Another method developed was the root-hypocotyl method, allowing for a small use of space and the observation of ECM formation. This method consisted of using agar-filled glass shell vials containing sucrose, thiamine and auxin, in order to replace the aerial part of the plant, but still allowing the root growth over a Petri dish with peat moss, it was used on *Pinus* and lead to the formation of typical ECM structures (Fortin, 1966).

In 1982 Piché and Fortin used growth pouches, commonly used to study nitrogen-fixing root symbiosis, and applied it to mycorrhizal synthesis. The method uses flat plastic polyester pouches containing a tin paper pad and nutrient solution, with the observation

of ECM formation after 3-10 days of inoculation (Fortin et al.,1983). Later, Wong and Fortin (1989) described the use of Petri dishes filled with sugar-free agar medium. Two sheets of nylon membrane sandwiched the roots and were overlaid by filter paper to keep moist. The method was also used replacing the agar medium with peat and vermiculite mix (ex: Sarjala and Taulavuori, 2004).

Halimium halimifolium has already been described in the previous chapter, and will be used again in this one, we tested the potential for *in vitro* mycorrhization with Portuguese native edible ECM fungi. Few works have used *H. halimifolium* on mycorrhizal synthesis. In fact, to the extent of our knowledge, this is the first work with ECM fungi and this species. The works already published were made with AM fungi (Camprubi et al., 2011). Although few reports on ECM associations with this particular species (Taudiere et al., 2015), some works have used other species of the genera, especially works on the association with the *Terfezia* (desert truffles) (Morte et al., 2000, 2008). With other Cistaceae, some works have been made with several edible ECM fungi (ex: Giovannetti and Fontana, 1982; Torres et al., 1995; Águeda et al., 2008) with promising results.

Tuberaria lignosa (Sweet) Samp., a perennial Cistaceae species native to the Mediterranean region, and also very common in the maritime pine forests mentioned in the previous chapter. For this species, to the extent of our knowledge, nothing has been reported about mycorrhizal synthesis with any type of association. Some field reports have associated this species with the genus *Terfezia* (Kovács et al., 2011) hinting the possibility that this species can form ECM associations. The relevance of *T. lignosa* has increased in recent years because it has shown properties that can be interesting for biomedical research. Some works have shown that it has anti-viral properties (Bedoya et al., 2010) and antioxidant activity (Pinela et al., 2012).

The plants we use are hard seed species where the dormancy of the seed has to be surpassed. Dormancy relies on a temporary suspension of visible growth of any plant structure containing a meristem (Kelly et al., 1999; Peña et al., 1987). Dormancy of the seed can be coat-imposed or embryo-imposed, or by a combination of both. In Cistaceae, it is common to find the first type of dormancy (Peña et al., 1987; Thanos et al., 1992). This type of dormancy is either due to impermeability of the coat, mechanical prevention of radicle extension, to the seed coat sending inhibitory factors or by preventing those from leaving the embryo. In order to prove that this dormancy is present the removal of the seed coat should be able to trigger germination, if not the dormancy may be embryo-imposed or a combination of both.

The present work aims to test the compatibility of these two Cistaceae species with three different edible ECM fungi. The fungal species used are *Lactarius deliciosus*, *Tricholoma portentosum* (both had already been discussed in this work on their growth behaviour) and *Tricholoma equestre*, a very sought after fungi in Portugal where it's known as "Míscaro". Of the fungi used in this study, the best known in the mycorrhization synthesis capacity is *Lactarius deliciosus*, being one of the few ECM fungi for which methods have been developed that lead to its fructification. Some works have shown the formation of fruiting bodies on artificially inoculated trees (Poitou et al., 1984; Guerin-Laguette et al., 2000, 2014; Yun and Hall, 2004).

Another objective of this work is to test a new methodology for the synthesis of mycorrhizae using MS medium, that has been shown in Chapter 1 to be able to maintain fungal growth, in different containers, to try and optimize the methodology, as it will be further explained. It also aimed to accurate methods for seed sterilization and scarification for this species, possibly showing what kind of dormancy is present in *T. lignosa* and confirming the finding for *H. halimifolium* where it was shown that it has coat-imposed dormancy linked to structures in the exotesta that contain lipid droplets that present germination.

Materials and Methods

Seed sampling

The seeds of *Tuberaria lignosa* and *Halimium halimifolium* were collected in Coimbra district in Portugal from healthy looking populations. Two areas were used to collect the seeds: a *Pinus pinaster* production forest with open spaces where *Tuberaria lignosa* can be found, and a recently burned area, where *Halimium halimifolium* is one of the dominant species along with other Cistaceae, where the last wildfire occurred three years before, being previously a production pine forest. The map of the collection sites can be seen in Fig.17. Seeds of *Tuberaria lignosa* and *Halimium halimifolium* were collected in July and August, respectively, of 2015.

Figure 17- Areas of seed collection. In red is highlighted the area where *Tuberaria lignosa* seeds were collected and in blue the area where *Halimium halimifolium* ones were collected. In the blue area, a fire had recently occurred allowing for the dominance of *Halimium halimifolium* and others Cistaceae species.

Sterilization and Scarification treatments

To determine the best treatment to achieve the least percentage of contamination of the seeds the following treatments were tested, immersion in ethanol 96% for 10 min with agitation (E), immersion in sodium hypochlorite 10% for 10 min with agitation (SH), immersion in boiling water for 10 min (W), immersion in boiling water for 10 min followed by immersion in sodium hypochlorite for 10 min (W+SH), immersion in ethanol 96% for 10 min followed by immersion in sodium hypochlorite for 10 min (E+SH), a combination of the three treatments (E+SH+W) and the control were seeds were immersed in distilled water.

Seeds were placed in PDA medium for contamination detection in growth chambers at 30ºC for 5 days. For each treatment and for each plant species within treatment 10 seeds were used.

In order to determine the best treatment applied to seed germination, three treatments were applied to *T. lignosa* and two to *H. halimifolium*. The treatments were dry heat at 140ºC for 15 min (DH) (this was only applied to *T. lignosa*) and abrasion between two layers of sandpaper, one with greater grit (HA) other with lower grit (SA). All these treatments aimed to remove or diminish the outer layer of the seed. The control seed didn't have any scarification applied, all treatments were sterilized based on the results of the sterilization treatments.

The experimental design consisted of 30 seeds per treatment for *T. lignosa* and 10 seeds for *H. halimifolium*, that were placed for germinating in Petri dishes with MS medium and incubated in a growth chamber at 25º C with 12h/12h light/dark period.

Germination was considered when there was visible radicle protrusion.

Fungi isolation

The protocol was the same as described in Chapters 1.

The isolation and obtainment of pure culture were tested for various species collected around the Coimbra district. A total of 14 species were tested and pure cultures were obtained for six of those species (Supplemental material 2). From those, three species were tested for mycorrhizal synthesis.

Mycorrhizal synthesis

In order to achieve mycorrhization using agarised medium, the first step was to choose a container that allowed the plant and fungus to interact with one another. For doing so, we tested two containers: flasks and Petri dishes. The first methodology is a flask filled with 4 to 5 cm with agarised MS medium wherein one side the medium was partially removed in order to create a space where the fungal inoculum could be placed. The fungal inoculum consisted of a cube of pure mycelium culture. After the placement of the inoculum, a circle of tin foil, previously sterilized, was placed on the medium surface to prevent light from contacting with the fungal mycelium that would develop

after. In order to place the plants, small holes were made. Two plants were placed in each flask (Fig.18 image A).

The second method consisted of Petri dishes half-filled with MS medium where plants were placed 5 to 7 days before the fungal inoculation in order to allow the acclimation of the plants. Otherwise, the fungus blocks the plant growth and leads to the death of the plant. The fungal inoculum was the same, three cubes of pure culture were placed over the MS medium. These Petri dishes were then placed on areas of the plant growth chamber where the light intensity is low in order to minimize the light that reached the mycelium, and the area used for MS medium was involved in tin foil for further prevention (Fig.18 image B).
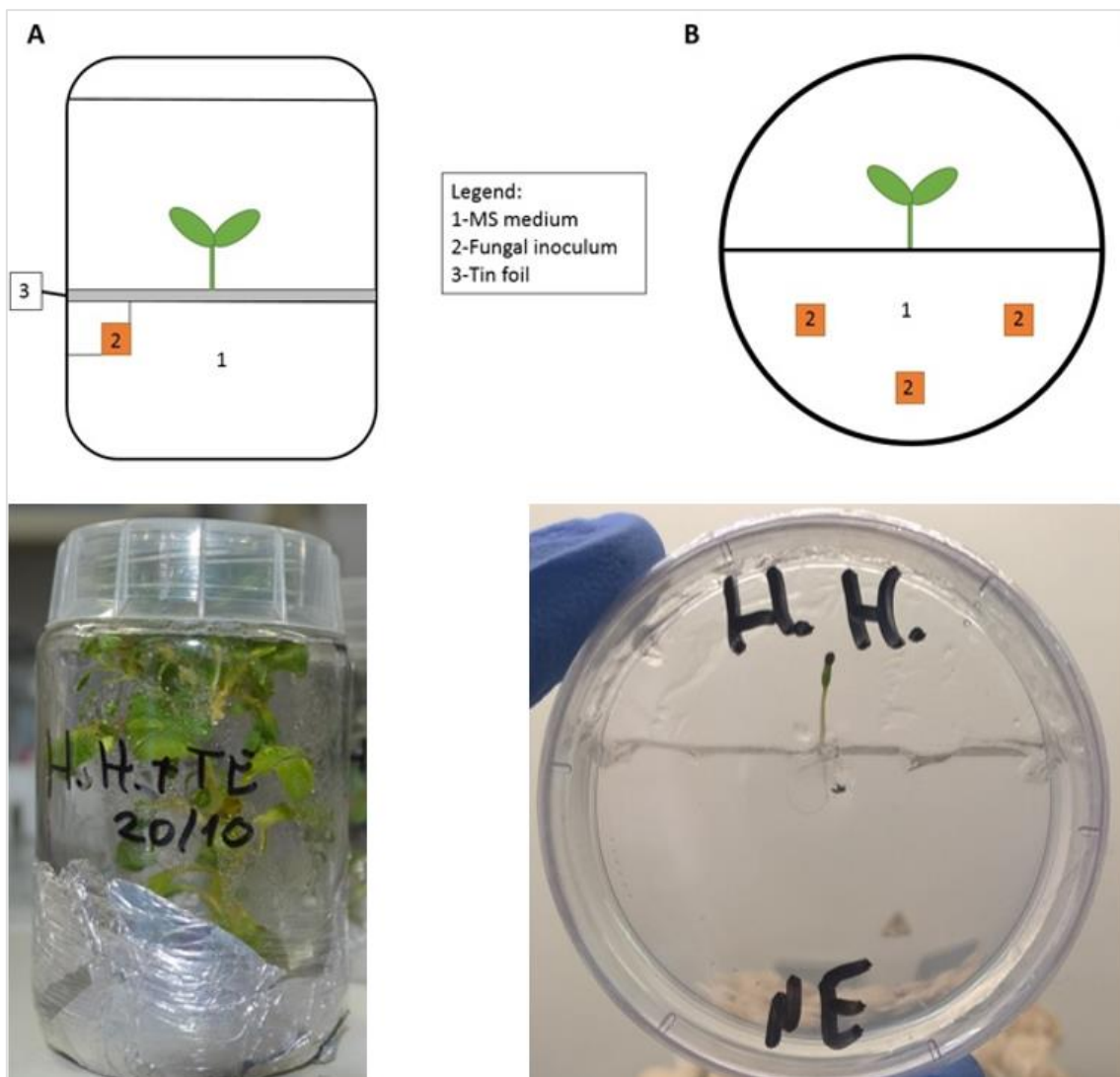

Figure 18-Methods used for mycorrhization assays; on the left the flask method where it can be seen the tin foil over the MS medium as well as the portion removed for the inoculum; on the right the Petri dish method where half the dish was kept clear to allow the growth of the plant.

Mycorrhizae synthesis confirmation

In order to confirm the success of the synthesis technique the morphotypes were assessed using Leica© EZ4 stereo microscope, and photographs were acquired with the Leica LAS EZ software© or with Nikon© D3200 when magnification wasn't required (Figure 21-22).

After sorting the morphotypes, a clearing protocol was followed to confirm the formation of the different structures needed to define the associations as ECM.

Clearing protocol begins with an acidification of the roots using 2.5% solution of KOH heated to around 90ºC for 30 minutes; samples were rinsed in distilled water and then the acidification was performed using 1% HCl for 10 minutes (Koske et al., 1989). Roots were then stained using trypan blue (results not shown).

Results

Sterilization and Scarification treatments

Percentage of contaminated seeds was accounted for the determination of sterilization treatments efficiency and the results can be seen in Fig. 19, for *H. halimifolium* the only treatment that didn't prevent contamination was the sodium hypochlorite alone, with all others having no contamination. For *T. lignosa* the best treatments were sodium hypochlorite plus water treatment and ethanol plus sodium hypochlorite plus water treatment with no contamination detected.

Germination percentage obtained with each treatment is shown in Fig. 20. The results show that the best treatment for seed germination of *H. halimifolium* is the hard abrasion since it was the only one that triggered germination, and for *T. lignosa* both methods of abrasion had similar favourable results. For both species, it is shown that they need some sort of scarification in order to achieve germination.
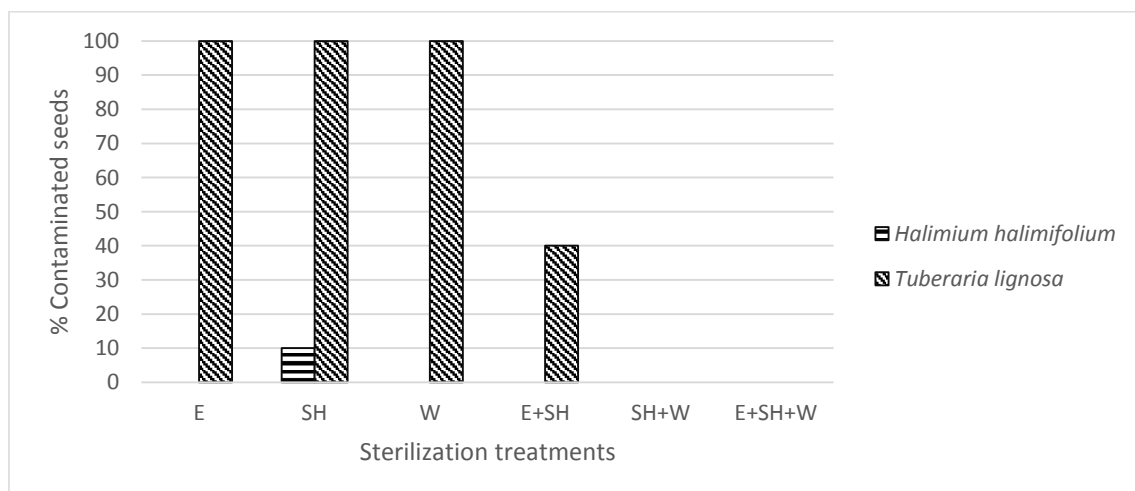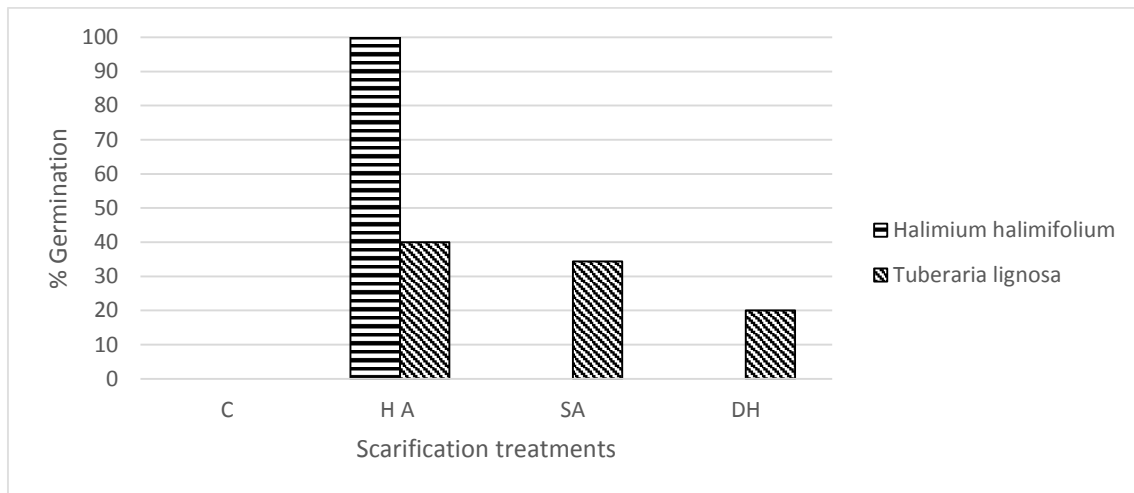
Figure 19- Efficiency of the sterilization treatments as the percentage of seed that were contaminated during the experiment. The treatments were E-Ethanol, SH-Sodium hypochlorite, W-Water at 100ºC and the combinations showed.

Figure 20- Results from the scarification treatments. The efficiency of the treatment is shown as the percentage of seeds that germinated. The treatments were C- Control, HA- Heavy abrasion, SA- Soft abrasion, DH- Dry heat.

Mycorrhizae synthesis

The only method that enabled the synthesis was the flask method. It sustained both plant growth and the fungus growth allowing for the association to occur. The Petri dish design didn't sustain plant growth long enough for the association to occur and in the case of *T. lignosa* the plant perished quickly, because of their rosette habit that only in the flasks were sustained.

The results of the synthesis are shown in Figure 21-22. For *T. lignosa* the synthesis was achieved with *Lactarius deliciosus* and *Tricholoma portentosum*. For *Tricholoma equestre* the experiment had to be restarted since the experiments were contaminated. Because of this, the results aren't yet available. For *H. halimifolium,* the synthesis was possible with all the fungi and results are shown in Figure 22. Only for *Tricholoma equestre* the ECM nature of the associations is yet to be confirmed by clearing methods but for all the others it has been (results not shown).

Figure 21- Photographs of the morphotypes obtained for the mycorrhization synthesis of *Tuberaria lignosa* with two of the species tested, A,B- *Lactarius deliciosus*; C,D- *Tricholoma portentosum*. The black arrows show the location of the primordia morphotypes. Photographs were taken with roots in the flasks, which compromises the photograph quality, with the exception of the first were the plant had become senescent and the morphotypes were extracted (Zoom B,D-2.5 and A-3.5).
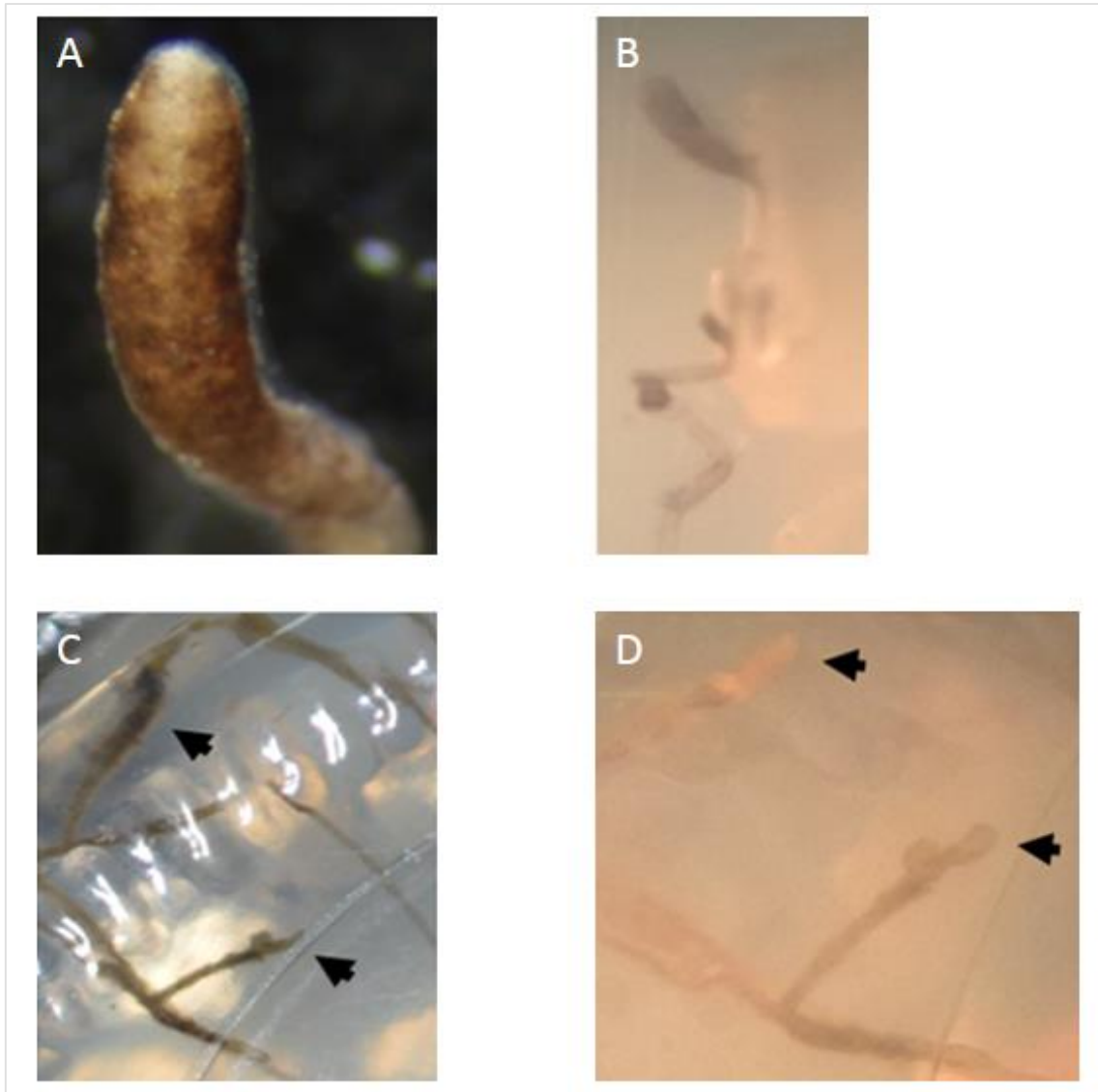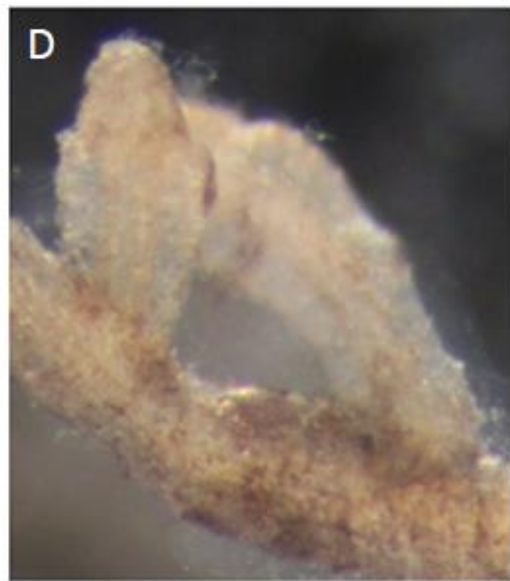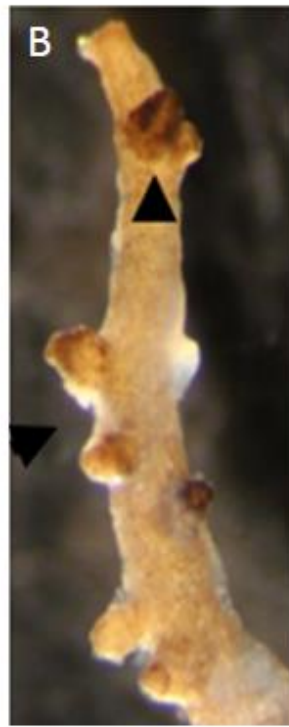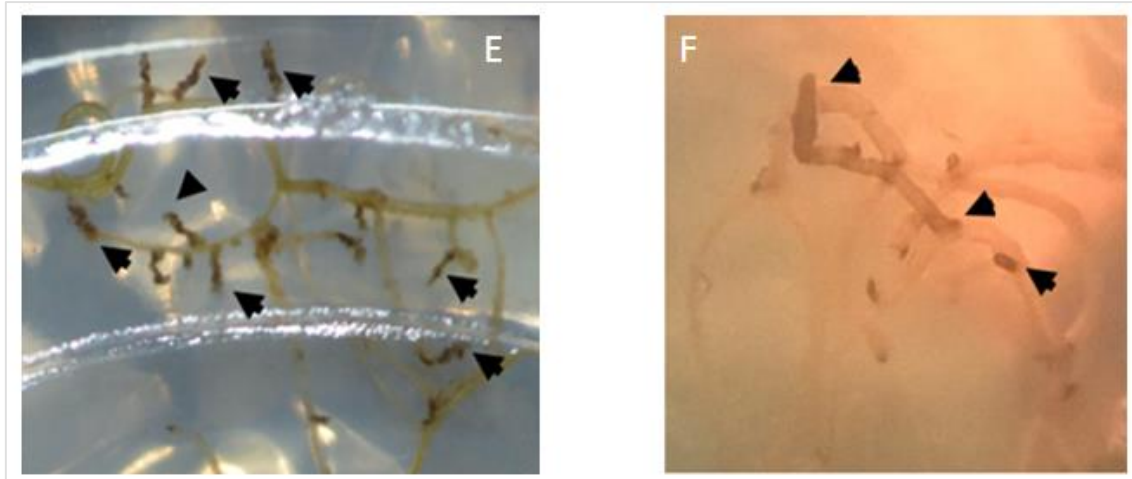
Figure 22- Photographs of the morphotypes obtained for the mycorrhization synthesis of *Halimium halimifolium* with all of the species tested. A,B- *Lactarius deliciosus*; C,D- *Tricholoma portentosum*; E,F- *Tricholoma equestre*. The black arrows show the location of the primordia morphotypes, Photographs were taken with roots in the flasks, which compromises the photograph quality, with the exception of A-D where the morphotypes were extracted from senescent plants (Zoom A-D- 3.5 and F-2.5).

Discussion

The results of this work allowed for an optimization of seed sterilization and seed germination. Our results show that, as it is typical of the Cistaceae (Thanos et al., 1992), both species require some kind of scarification in order to allow germination.

In *Halimium halimifolium*, results confirm that what has been reported for this species occurs in our seed lot as well. Once more, mechanical scarification with hard abrasion shows the best results. Thanos et al., 1992; Peña et al., 1987 and Peña et al., 1987 showed that the dormancy of the seed of this species is coat-imposed rather than embryo-imposed and our results confirm such findings. Soft abrasion not showing any germination goes in line with the finding that the lipid droplets present in the exotesta need to be removed.

For *Tuberaria lignosa* the mechanical scarification also showed the best results, with no differences between hard and soft abrasion. The pre-heat also showed increased germination, which was expected since this species is commonly found after forest fires (Ferrandis et al., 1999). This result is in line with others studies published for this species (Thanos et al., 1992) and also for other species of the genera (Gonçalves et al., 2009).

The sterilization treatments show that there are differences between the species in terms of contamination rates. In *H. halimifolium,* the seeds seemed to be less prone to contamination with only one treatment having contamination. For *T. lignosa* the results are the opposite, with only two treatments showing no contamination. As a note, treatments with sodium hypochlorite should be performed for shorter periods of time because after some time in sodium hypochlorite the seeds become bleached and inviable.

The mycorrhization synthesis was only possible using the flask methodology, mainly because in the Petri dish the plant didn't grow and became senescent after a short period of time. This is due to the habit of the plants with *T. lignosa* having a rosette habit (Castroviejo, 1986-2006) that isn't compatible with small containers, moreover, we also observe that in plants that were kept on test tubes the same senescence and lack of growth occurred. For *H. halimifolium* its shrubby habit (Castroviejo, 1986-2006) was also incompatible with the Petri dishes, but in test tubes, the plants grew well but not reaching the dimension achieved in the flasks. These results are contrary to the ones published in other works that used a modified version of MS medium, but used Petri dish and test tubes as containers with good results, although the tests were performed with *Castanea sativa* and not with these species (Martins, 2008). Our results are important because this usage of unmodified MS for mycorrhizal synthesis is, to the extent of our knowledge, the first report, since the MS medium is designed for plant maintenance (Murishage and Skoog, 1962). It's a great candidate for large-scale experiment, or even production, of mycorrhizal synthesis since it's easily obtained and we have proved in this work that it can sustain plant and fungal growth, and it's a favourable substrate for the association between them.

H. halimifolium was able to establish associations with all the fungi. These results are the first for the ECM synthesis with this plant species and are also the first report of its association with the *Lactarius* and *Tricholoma* genera (Taudiere et al., 2015.

For *T. lignosa* the associations were formed with *Lactarius deliciosus* and *Tricholoma portentosum*. The association with *Tricholoma equestre* is still to be confirmed since the experimental units were lost due to contamination and the experiments had to be restarted and the results are still not available. For *T. lignosa,* this is the first report of its association with Basidiomycetes as it only had been reported associations with the *Terfezia* genera. It is also the first work with mycorrhizal synthesis using this plant. This is very important since, as most Cistaceae, it shares its habitat with many economically important trees and it's a pioneer species, making for its possible use as "fertility islands" after forest fires, allowing for the mycelia maintenance after disturbance of this economical important fungal species.

As a note, the morphotypes found in our work are relatively small when compared with other works (ex: Águeda et al., 2008). This may be a characteristic of the associations formed by this species *in vitro* or a characteristic derived from the use of unmodified MS as substrate. More tests have to be performed in order to assure the real causes of this phenomena.

Final Remarks

In Chapter 1 our work represents the first report for the growth of the tested fungi in MS medium; our work allowed for an optimization of the fungal growth, using commercially available media, and for the determination of the best media, among the ones tested, to maintain culture in cold temperatures for longer periods of time.

The work performed in Chapter 2 is the first below-ground survey of mycorrhizal associations with *Halimium halimifolium* and one of the first reports of ECM association with *Acacia longifolia*. We also unveil novel players in the CMN between *Halimium halimifolium* and *Pinus pinaster* with the finding of four different species shared in our study site and the possibility of more being shared, since many of the sequences used for the identification of the symbionts of *Halimium halimifolium* were from data collected in *Pinus pinaster*.

Chapter 3 is a culmination of the work developed in the later chapters since in it we used fungi isolated in Chapter 1 for the possibility of establishing mycorrhizas with *Halimium halimifolium* and *Tuberaria lignosa*. In this Chapter we described a methodology for mycorrhizal synthesis using MS medium, that we showed in Chapter 1 to be able to sustain fungal growth, as a substrate for the synthesis, and flasks as containers with the addition of a tin foil to allow fungal growth in the dark, since it's known that light of specific wavelengths can modify fungal growth. This study offers several potential uses for this Cistaceae shrubs in reforestation programs or even for commercial production of mushrooms. In Chapter 2, we showed that *Halimium halimifolium* can share symbionts with an economically important tree, proving that it can be used to enrich forestry explorations with the sharing of symbionts.

As future perspectives, this work could be improved in several points. In Chapter 2 we found that a better methodology for the extraction and amplification of DNA could improve the diversity found. In Chapter 3, we believe that tests on different substrates should be performed to compare the size of the morphotypes achieved and the ability to establish the same associations.

Moreover, further works should be done to test the sustainability of the associations achieved. For example, greenhouse tests to see if the plant achieves better acclimatization to this conditions and if the associations prevail in non-sterile environments. After these tests, it would be interesting to see the behaviour of the plants in field conditions, not only to prove that these associations can withstand the field conditions, but also to see if they can be used as vectors for fungal introduction in plantations or even if they can produce mushrooms by themselves without the need of a tree.

Supplemental Materials

Supplemental materials 1

HM1- *Russula* sp.

HM2- Uncultured ECM (Pezizomycotina)

HM3- *Sebacina vermifera*

HM4- *Tylospora* sp.

HM8- Uncultured ECM (Helotiales)

HM9-Uncultured ECM (Pezizomycotina)

HM10- *Tomentella* sp.

HM12- *Tylospora* sp.

HM13- *Tylospora* sp.

HM17- *Tylospora* sp.

Figure 23- Morphotypes collected in roots of *Halimium halimifolium*, HM-morphotype number in Table 4 of Chapter 2.

AM1-Uncultured ECM (Pezizomycotina)
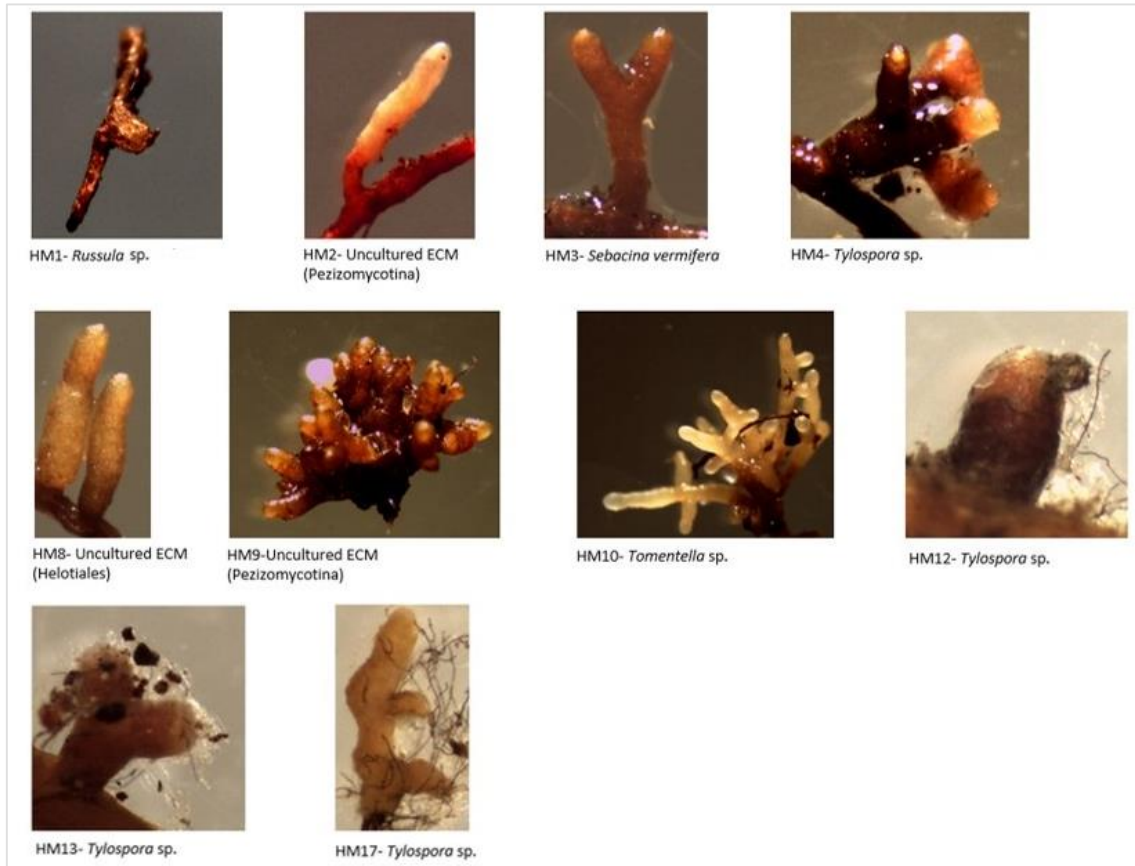
Figure 24- Morphotype collected in roots of *Acacia longifolia*, AM-morphotype number in Table 4 of Chapter 2.
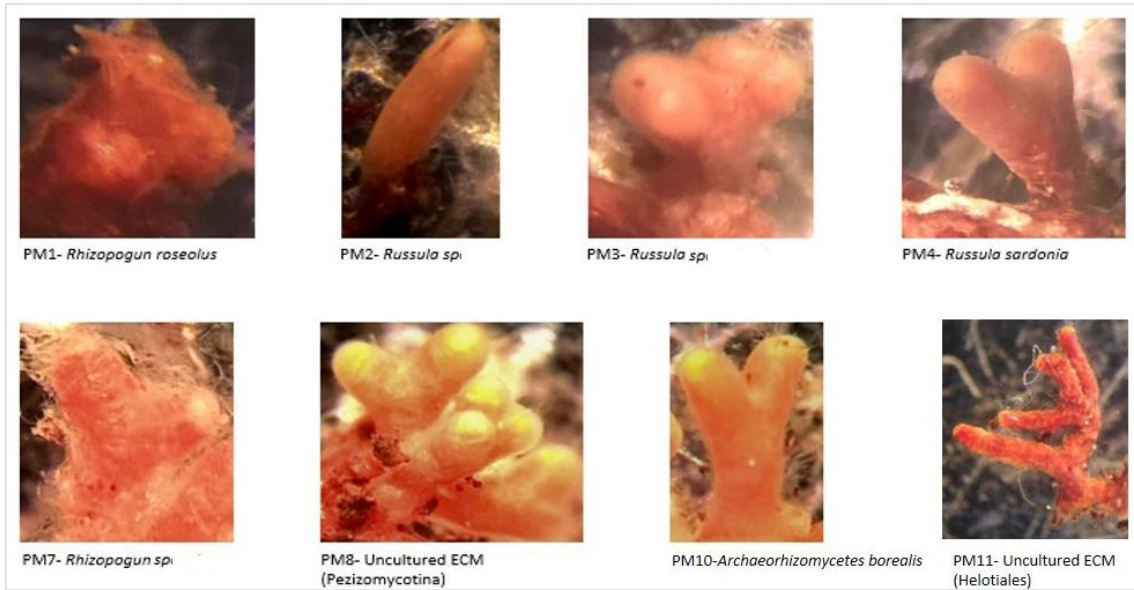
Figure 25- Morphotypes collected in roots of *Pinus pinaster*, PM-morphotype number in Table 4 of Chapter 2.

Supplemental materials 2

Table 5 -Sporocarp collected and success of its isolation. Collection site with the description in the Materials and Methods (P.A- Paul de Arzila, C.S. - Colina dos Sobreiros, O.F. - Olhos de Fervença, P.G.-Paul Guarda).

Species	Collection site	Successful isolation
Agaricus bisporus	------------------	Yes
Amanita rubescens	P.A.	Yes
Boletus aereus	C.S.	No
Boletus edulis	C.S.	No
Boletus fragrans	P.A.	Yes
Boletus reticulatus	C.S.	No
Cantharellus cibarius	C.S.	No
Cantharellus lutescens	C.S.	No
Hydnum repandum	C.S.	No
Lactarius deliciosus	O.F.	Yes
Lactarius sanguiflus	P.A.	Yes
Macrolepiota procera	P.A.	Yes
Pleurotus oestreadus	------------------	Yes
Russula cyanoxantha	C.S.	Yes
Tricholoma equestre	O.F.	Yes
Tricholoma portentosum	P.G.	Yes
Xerocomus badius	C.S.	No

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