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Ectomycorrhizal associations of edible fungi and *Cistus* spp.: from field studies to *in vitro* synthesis

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Tese submetida à Universidade de Coimbra para obtenção do grau de Mestre em Biodiversidade e Biotecnologia Vegetal (especialidade em Biotecnologia), supervisionada pelo Professor Doutor António Manuel Santos Carriço Portugal e pela Professora Doutora Maria Teresa Silva Gonçalves Serra e Silva do Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Summary

Edible mushrooms are consumed globally whether as a delicacy or due to their medicinal and nutritional proprieties. As a seasonal resource, wild edible mushrooms are not available naturally during all year, existing, therefore, a need on their cultivation. Some ectomycorrhizal (ECM) fungi produce the most sought and valuable sporocarps worldwide. Unlike saprotrophic species, a profitable cultivation of edible mycorrhizal mushrooms is very difficult due to several issues, being its association with fine roots of plants, dependency of plant partner's biomass and growth rates some of them. Several species of the Mediterranean genus *Cistus* are common shrubs in the understory of *Pinus pinaster* dominated forests in Portugal and are ectomycorrhizal species. Besides their ability of establishing ectomycorrhizas, *Cistus* are also shrubs with relative high growth rates, capable of reach their highest biomass in less time than trees, being suitable candidates for cultivation of ectomycorrhizal mushrooms.

One of the main objectives, of the present work, is the production of edible ECM mushrooms, thus were performed assays with mycelial cultures of ECM species as well as synthesis of ectomycorrhizas.

Fungal mycelial cultures were newly isolated from sporocarps of edible ectomycorrhizal species - *Boletus fragans* and *Tricholoma equestre*- and compared with the saprobic species *Pleurotus ostreatus* and *Agaricus bisporus*. The mycelial cultures were grown in several culture media - Potato Dextrose agar (PDA), Malt extract agar (MEA), Biotin-Aneurin-Folic acid (BAF), Merlin-Norkrans modified medium (MNM) and Murashige & Skoog medium (MS) - and different temperatures, 4ºC/24ºC/30ºC. Growth responses of ECM and saprotrophs fungi were compared. The experiment allowed also the establishment of optimal culture conditions for mycelial growth of each species.

Seedlings of three Portuguese native species - *Cistus psilosepalus*, *Cistus salviifolius* and *Cistus ladanifer* - were used as plant partners to axenically induce the establishment of mycorrhizas with the edible ECM fungal species *Boletus fragans*, *Lactarius deliciosus*, *Tricholoma equestre* and *Tricholoma portentosum*. Different methods for mycorrhizal synthesis were tested, including containers with different shapes and several substrates and, to our knowledge, a new method of ectomycorrhizal synthesis, adapted to these shrubs growth habit, was developed.

In order to get some insights about the ECM fungal partners of *Cistus* plants in natural conditions, a preliminary sampling was performed in a maritime pine forest. Ectomycorrhizal root tips from *Cistus psilosepalus*, *Cistus salviifolius* and *Pinus pinaster* were collected in

a natural Portuguese pinewood to identify natural fungal symbionts associated with these plants and sharing of fungal partners between *P. pinaster* and *Cistus* spp. ECM root tips were sorted into morphological groups, photographed and symbiotic fungi were molecularly identified by amplification and sequencing of fungal ITS region (Internal Transcribed Spacer).

ECM fungi showed having in general lower growth rates than saprobic fungi, as expected due to their life styles. The optimal temperature for almost all species was 24°C being subsequently the best temperature when producing spawn for cultivation of saprobic fungi and fungal inoculum for mycorrhizal synthesis. The only exception was *P. ostreatus* that grew better at 30°C. The culture medium PDA demonstrated to be the most suitable medium for axenic growth of all fungi, being a nutritional comprehensive medium fulfilling the requirements of fungi of different lifestyles. It was also proved the growth of almost all fungi in MS medium, mainly used to grow plant tissues.

Ectomycorrhizas were formed, using the novel technique, in almost all mycorrhizal fungus-plant combinations. The novel technique showed in many different ways to be very useful in testing partners compatibility as well as in monitoring mycorrhizal development. Moreover, results suggest a clear plasticity of these shrubs concerning their fungal partners diversity.

Several species were found associated naturally with *Cistus* spp. as well as sharing of fungal symbionts between *Cistus salviifolius* and *P. pinaster*. Moreover, were also found species that produce edible mushrooms associated with *Cistus* sp., showing these shrubs' potential to produce edible sporocarps.

Results are discussed in relation to optimize methods for culture and synthesis of ectomycorrhizas, to understand the future application of these relationships in using mycorrhized shrubs with edible fungi as inoculum source to naturally guide pinewoods towards cultivation of a specific edible ectomycorrhizal mushroom as well as in using *Cistus* shrubs to produce edible mushrooms.

Keywords: Ectomycorrhizas - Edible wild mushrooms - Cistaceae - Pure culture synthesis - Cultivation - Mycelial growth - Cultures optimization - ITS - Fungal diversity - ECM networks.

Sumário

Os cogumelos comestíveis são consumidos por todo o mundo ora como uma iguaria, ora devido às suas propriedades nutricionais e medicinais. Alguns dos mais apreciados e valorizados cogumelos silvestres comestíveis são os carpóforos de fungos ectomicorrízicos (ECM) pelo que o interesse económico no seu cultivo enorme. No entanto, o cultivo economicamente rentável de cogumelos micorrízicos comestíveis é muito difícil, contrariamente às espécies de fungos saprófitas, devido, nomeadamente à necessidade de associação com raízes de plantas e à dependência das taxas de crescimento e biomassa do seu simbionte vegetal.

Um dos objectivos deste trabalho é a produção de cogumelos comestíveis de espécies ECM, pelo que se realizaram ensaios com culturas de micélio das espécies fúngicas e de síntese de ectomicorrizas.

Foram isoladas culturas de micélio a partir de carpóforos das espécies ectomicorrízicas comestíveis - *Boletus fragans* e *Tricholoma equestre* - e, para comparação, das espécies saprófitas *Pleurotus ostreatus* e *Agaricus bisporus*. As culturas de micélio foram colocadas a crescer em diferentes meios de cultura - Potato Dextrose agar (PDA), Malt extract agar (MEA), Biotin-Aneurin-Folic acid (BAF), Merlin-Norkrans modified (MNM) e Murashige & Skoog (MS) - e em diferentes temperaturas, 4ºC/24ºC/30ºC. As respostas de crescimento dos fungos ECM e dos saprófitas foram comparadas. Foi também possível determinar as condições óptimas de cultura para o crescimento de micélio para cada espécie testada.

As espécies vegetais utilizadas como simbionte dos fungos ectomicorrízicos pertencem ao género mediterrânico *Cistus* (famlia Cistaceae). É conhecido serem espécies ectomicorrízicas e são comuns no sub-bosque dos pinhais dominados por pinheiro-bravo (*Pinus pinaster*) em Portugal. Para além da capacidade de estabelecerem ectomicorrizas, os *Cistus* são arbustos e como tal, capazes de atingir a sua biomassa máxima em menos tempo que as árvores, sendo, por isso, candidatos adequados para o cultivo de cogumelos ectomicorrízicos.

Para a síntese de ectomicorrizas foram usadas plântulas de três espécies espontâneas de Portugal - *Cistus psilosepalus*, *Cistus salviifolius* e *Cistus ladanifer* - e as de fungos ectomicorrízicos comestíveis *Boletus fragans*, *Lactarius deliciosus*, *Tricholoma equestre* e *Tricholoma portentosum*. Foram testados diferentes métodos para a síntese de micorrizas incluindo recipientes com diferentes formas e diferentes substratos e foi desenvolvido um novo método, tanto quanto sabemos, para a síntese de ectomicorrizas adaptado ao hábito de crescimento destes arbustos.

Para conhecer a comunidade de fungos ectomicorrízicos simbiontes de *Cistus* em condições

naturais, foi feita uma amostragem preliminar num pinhal de pinheiro-bravo. Foram colhidas raízes de *Cistus psilosepalus*, *Cistus salviifolius* e de *Pinus pinaster* num pinhal da zona costeira. A inclusão de *P. pinaster* neste estudo visou identificar os simbiontes fúngicos naturais associados, simultaneamente, com *Cistus* sp. e *P. pinaster*. No laboratório as pontas ectomicorrízicas foram separadas em grupos morfológicos e fotografadas. Os simbiontes fúngicos foram identificados molecularmente atravéz da amplificação e sequenciação da região ITS (internal transcribed spacer).

Os fungos ECM mostraram ter, em média, taxas de crescimento mais baixas comparativamente aos saprófitas, como seria de esperar devido aos seus modos de nutrição. A temperatura óptima para quase todas as espécies fúngicas foi de 24°C, sendo, desta forma, a temperatura mais adequada para a produção de inóculo para a síntese de micorrizas, assim como de "semente" para o cultivo de fungos saprófitas. A única excepção foi o *P. ostreatus* que cresceu melhor a 30°C. O meio de cultura PDA demonstrou ser o mais adequado para o crescimento axénico de todos os fungos, sendo, portanto, um meio nutricionalmente abrangente que preenche os requisitos de grupos de fungos com modos de nutrição diferentes. Foi também comprovado o crescimento de quase todos os fungos testados no meio MS - desenvolvido e usado maioritariamente para o crescimento de tecidos vegetais.

Registou-se a formação de ectomicorrizas, em quase todas as combinações fungo-planta, com o uso da nova técnica. A nova técnica mostrou ser útil, de diferentes formas, tanto na monitorização do desenvolvimento de micorrizas, como para testar a compatibilidade entre parceiros. Para além disso, os resultados obtidos sugerem uma evidente plasticidade das espécies de *Cistus* no que respeita à diversidade de parceiros fúngicos.

Relativamente à comunidade de simbiontes fúngicos ECM foi possível identificar um elevado número de espécies encontradas naturalmente associadas com *Cistus* spp., apesar da reduzida amostragem realizada. Foi também encontrada partilha de espécies fúngicas entre *Cistus salviifolius* e *P. pinaster*. A identificação (até ao momento) de uma espécie produtora de cogumelos comestíveis naturalmente associada com *Cistus* sp. evidencia o potencial destes arbustos para a produção de carpóforos comestíveis.

Os resultados são discutidos no sentido da optimização dos métodos para a cultura e síntese de ectomicorrizas e também com vista à aplicação futura destas relações no uso dos

arbustos micorrizados como fonte de inóculo para guiar, de forma natural, pinhais no sentido do cultivo de um determinado cogumelo ectomicorrízico, assim como usar os *Cistus* para produzir cogumelos comestíveis.

Palavras chave: Ectomicorrizas - Cogumelos silvestres comestíveis - Cistaceae - Síntese em cultura pura - Cultivo - Crescimento de micélio - Optimização de culturas - ITS - Diversidade de fungos - Redes de ECM.

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Chapter 1

General Introduction

True fungi, from Kingdom Fungi, are heterotrophic achlorophyllous eukaryotic organisms, which can be filamentous, exhibiting apical growth at the edge of the filaments called hyphae, or single celled, like yeasts, reproducing by budding. Fungi also exhibit cell wall, similar to other Eukaryotic organisms, like plants, but their composition primarily of chitin and glucans differs from plants, which have cell walls rich in cellulose (Deacon 2013, Itoo & Reshi 2013, 2014). Filamentous fungi can reproduce both asexually and sexually, being sexual spores of some macrofungi from Ascomycota and Basidiomycota phyla produced in fleshy sporocarps called mushrooms (Deacon 2013). Mushrooms can be hipogeous or epigeous depending if they occur below or above ground, respectively. Regarding their nutrition or lifestyle, macrofungi can be saprobic, pathogenic or mycorrhizal (Deacon 2013, Itoo & Reshi 2013) with a central role in ecosystems as nutrient recycling through decomposition of organic matter, enhancing plant health and fitness and many other positive features (Smith & Read 2008, Savoie & Largeteau 2011, Deacon 2013, Itoo & Reshi 2013, 2014). Mycorrhizal fungi establish mycorrhizal association between hyphae and plant roots. Moreover, ectomycorrhizal fungi are the ones (whithin mycorrhizal fungi) which are known to be mushrooms-producers (Tedersoo et al. 2010). Ectomycorrhizas are morphologic and anatomically distinct from the other types of mycorrhizas (endomycorrhizas *sensu lato*) and creates typical morphological modifications in root tips, which differ according with plant and fungal taxa, that can be used to sort different fungal species (Smith & Read 2008).

Edible mushrooms are very appreciated around the world and known for their gastro-

nomic, nutritional and medicinal proprieties (Wasser 2011, Wang et al. 2014, Valverde et al. 2015). Their demand has increased over time and subsequently their economic value and the interest on their cultivation (Yamanaka 1997). Mushroom cultivation has been achieved over time and worldwide with widespread edible saprobic species, such as *Agaricus* sp. and *Pleurotus* sp. (Yamanaka 1997, De Groot et al. 1998, Straatsma et al. 2000, Baysal et al. 2007, Alananbeh et al. 2014) and medicinal species as *Ganoderma lucidum* (Valverde et al. 2015).

Some species of ectomycorrhizal fungi, *Tuber melanosporum*, *Boletus edulis* and *Lactarius deliciosus*, produce very valuable (relatively) and popular sporocarps, with several studies and attempts of ectomycorrhizal sporocarps' production (Guerin-Laguette et al. 2000, Yun & Hall 2004, Águeda et al. 2008, Morte et al. 2008). One of the main problems when attempting to have a profitable cultivation of ectomycorrhizal sporocarps, besides the need of association with a plant partner, is the long period of time that takes the plant to grow enough in order to sustain fungal fruitification (Ortega-Martínez et al. 2011).This issue is evident when using trees as *Pinus* sp. and *Quercus* sp. with very low growth rates. Shrubs with relatively high growth rates and capable of establishing ectomycorrhizal association, could be the solution to this barrier, being Cistaceae a promising family that fulfils all these requirements and has been used in several experiments of cultivation and synthesis of mycorrhizas (Giovannetti & Fontana 1982, Wenkart et al. 2001, Bustan et al. 2006, Zaretsky et al. 2006, Águeda et al. 2008, Morte et al. 2008, Zitouni-Haouar et al. 2014).

Cistaceae family comprises 200 species with 8 different genus (Muñoz-Garmendía & Navarro 1993, Águeda et al. 2006, Alonso Ponce et al. 2011), being *Cistus* one of the 5 existing genus in Portugal (http://www.flora-on.pt). *Cistus* are pyrophytic, evergreen and woody shrubs represented by 20 species (Correia 2002, Comandini et al. 2006), 9 of them Portuguese native species (http://www.flora-on.pt). These ramified short-medium sized shrubs are typical members of Mediterranean maquis and garrigue, commonly called rockroses due to the morphological similarity with wild roses (Correia 2002).

Cistus are early colonizers after a disturbance, able to resist drought and to colonize degraded areas (Correia 2002). Moreover, these shrubs establish ectomycorrhizal association with approximately 230 different fungal species (Águeda et al. 2006, Comandini et al. 2006),

some of which produce highly-priced edible sporocarps, as *Tuber melanosporum* (Giovannetti & Fontana 1982, Wenkart et al. 2001, Bustan et al. 2006).

Shrubs from *Cistus* genus are common in the understory of maritime pine, *Pinus pinaster* Ait., dominated forests of Portugal and it is known that they share ectomycorrhizal fungal species with pines (Buscardo et al. 2012), functioning ecologically as a fungal natural inoculum stock (Torres et al. 1995, Águeda et al. 2006, Alonso Ponce et al. 2011). This ability of sharing fungal partners between these plants could be applied to naturally guide pinewoods towards cultivation of a specific edible ectomycorrhizal mushroom.

Besides the mentioned features, *Cistus* shrubs reach the highest biomass between 2-4 years old (Patón et al. 1998, Oñate & Munné-Bosch 2008), what means having higher growth rates. It is then expected these shrubs to have potential of sustaining ectomycorrhizal fungal fruitifications in less time than trees, being promising plants to prospectively produce edible ectomycorrhizal mushrooms.

1.1 Research objectives

The general objective of the present thesis is to evaluate the biotechnological potential of *Cistus* shrubs for producing edible ectomycorrhizal sporocarps and as inoculum source or vector in forests.

The specific aims of this study approached thereafter in each chapter are:

1) To study and compare culture conditions and requirements of edible ectomycorrhizal species with edible saprobic species in mycelial cultures as well as compare fungal morphological characterization in different culture conditions.

2) Axenically synthetize ectomycorrhizas in vitro with edible ectomycorrhizal fungal species and three Portuguese-native *Cistus* species with a technique adapted to these shrubs' requirements.

3) To record the morphological appearance of *Cistus* spp. and *Pinus pinaster* ectomycorrhizas and to identify the fungal partners using molecular tools.

1.2 Thesis content

This thesis is structured in five chapters.

In Chapter 1, are presented a general introduction, research objectives and thesis content, which contextualize the present work.

In the Chapter 2, the growth rates of two edible ectomycorrhizal fungi, in five different culture media and three different temperatures, were determined and compared with two edible saprobic species. The morphological appearance and features of mycelial cultures of each fungus were also determined for each medium.

The Chapter 3 describes the attempts to establish ectomycorrhizas between three species of *Cistus* and four edible ectomycorrhizal fungal species. Seed sterilization protocol was optimized as well as culture conditions, substrate and containers, developing a technique adapted to these shrubs' requirements. The compatibility between partners were assessed as well as the potential of each combination for future production of edible mushrooms.

In Chapter 4, a diversity survey of ectomycorrhizas of *Cistus* spp. and *Pinus pinaster* was performed by sorting ectomycorrhizal root tips within morphological groups and further molecular identification to study the sharing network of fungal partners between the studied plants. The ability of *Cistus* to establish associations with edible ectomycorrhizal mushrooms was briefly assessed as well as the ability to share those species with maritime pine in attempt of understanding the biotechnological potential of *Cistus* to be used in pinewoods as inoculum source and as edible sporocarp producer. The morphological appearance of each fungal ectomycorrhizal root tip formed in all assessed plants was also recorded.

In the Chapter 5 are presented the final conclusions of the present work.

Chapter 2

Growth rates of edible ectomycorrhizal and saprobic fungi depending on culture medium and temperature

2.1 Introduction

Mushrooms, fleshy fungi fruiting bodies, are a priceless resource granted by Nature (Alananbeh et al. 2014). Considered as a delicacy and health food, thanks to their nutritional and medical proprieties, edible mushrooms have been consumed globally since earliest history (Valverde et al. 2015). Among the many pharmacological characteristics with medical and therapeutical applications of macrofungi's primary and secondary metabolites some of them have been designated as anticancer, antiviral, antidiabetic, anticholesterolemic and some of them inhibit cancer development and proliferation (Chang et al. 2007, Yu et al. 2009, Guillamón et al. 2010, Ferreira et al. 2010, Wang et al. 2014, Alves et al. 2012, Wong et al. 2012, Finimundy et al. 2013, Carneiro et al. 2013, Valverde et al. 2015). Regarding nutritional value, mushrooms are rich in proteins, vitamins, fibres, minerals and low fat levels (Mattila et al. 2001, Barros et al. 2007, Valverde et al. 2015). Due to all this nutritional and medical

features and to their particular taste, edible mushrooms are an important food supply to many cultures globally (Wasser 2011, Wang et al. 2014). Thus, there is an increasing interest in the production of edible fungi sporocarps (Yamanaka 1997), which are naturally seasonal and unavailable on markets all year round. A few saprobic species are successfully and easily cultivated all around the World. *Pleurotus ostreatus*, for example, is cultivated using inoculated trunks (Yamanaka 1997) and organic waste like straw or banana leaves (Alananbeh et al. 2014) while wheat straw, straw-bedded horse manure, chicken manure and gypsum (Straatsma et al. 2000, Baysal et al. 2007) covered with a casing layer (De Groot et al. 1998) are very used substrates for *Agaricus bisporus* cultivation. However, the cultivation of edible ectomycorrhizal (ECM) species is far more challenging because sporocarps production depends on the stablishment of mycorrhizal association between fungus and fine plant roots (Itoo & Reshi 2014), with few successful cases reported in several species as *Lactarius deliciosus* (Guerin-Laguette et al. 2000) and *Tuber* spp. (Morte et al. 2008, Yun & Hall 2004). The difficulties on cultivation of ectomycorrhizal mushrooms are related with several factors that affect the formation of sporocarps, as the organism's genetics and environmental basis (Allen 1991, Alonso Ponce et al. 2011). This means more specifically that the fungus-plant interaction relies on the compatibility between species, the plant physiological condition, environmental requirements (van der Heijden & Sanders 2003, Alonso Ponce et al. 2011) and other biotic factors as "mycorrhization helper bacteria" (Rigamonte et al. 2010) and competition with other fungi (Savoie & Largeteau 2011). Nevertheless, abiotic elements like light, temperature, humidity and nutrient availability are also fundamental for sporocarp formation (Murat et al. 2008, Alonso Ponce et al. 2011).

Ectomycorrhizal and saprobic fungi play major roles in the ecosystems. Some saprobic fungi degrade complex polymers that compose dead organic matter like lignin and cellulose, due to the production of a wide range of extracellular enzymes (Deacon 2013). This ability is essential in recycling major nutrients (Savoie & Largeteau 2011) and in bioremediation of ecosystems (Harms et al. 2011). On the other hand, ECM fungi are important in the maintenance of the health and fitness of shrubs and trees, assisting them in the uptake of water, micronutrients and nutrients like phosphorus with low mobility in soil (Itoo & Reshi 2014, Smith & Read 2008). Moreover, ECM fungi are fundamental in increasing

and maintaining soil biodiversity and ecosystem functioning (Smith & Read 2008, Murrieta-Hernández et al. 2014), particularly in temperate and coniferous forests (Murrieta-Hernández et al. 2014); protection against pathogenic organisms and environmental stress (Smith & Read 2008, Kemppainen & Pardo 2010, Itoo & Reshi 2013, 2014). They are important in rehabilitation, regeneration (Dulmer et al. 2014) and revegetation of degraded areas (Burri et al. 2013, King & Hobbs 2006, Byers et al. 2006) as well as in protecting soil from erosion (Burri et al. 2013).

Temperature is one of the most important environmental factors for all type of organisms affecting growth and activity (Pietikäinen et al. 2005, Brock 2012). Temperature influences the metabolism of all forms of life and consequently their behaviour. In the case of fungal mycelial growth in pure culture, temperature affects the growth rates and has to be optimized for each specie that have specific requirements (Sánchez et al. 2001).

Working with mycelial cultures of fungi requires knowing about fungal nutrition. Deacon (2013) defined the Minimum Nutrient Requirements (MNR) for fungal growth as the minimal nutrient concentration and composition for the majority of fungal species. Exceptions are fungi needing to be supplemented with vitamins, with habitat-specific requirements and which cannot use nitrate or ammonium, needing to have amino acids as nitrogen (N) source, as many Basidiomycota. Nitrogen as well as carbon sources greatly influence fungal growth and establishing in controlled conditions, especially the most demanding organisms like ECM fungi (Lilleskov et al. 2002, Itoo & Reshi 2014). The utilization of carbohydrates are directly dependent on the presence and amount of nitrogen sources (Eaton & Ayres 2002, Itoo & Reshi 2014), making therefore essential taking into consideration the amount and the source of each of these nutrients when working with saprobic and ECM macrofungi.

Many ECM fungi are not able to metabolize disaccharides, such as sucrose which is produced by plants, because of the lack of an Invertase, a plant enzyme that hydrolyse sucrose into the monosaccharides fructose and glucose (Daza et al. 2006). So, the addition of monosaccharide like glucose to culture medium is fundamental for optimal fungal growth (Hatakeyama & Ohmasa 2004, Itoo & Reshi 2014).

Concerning the nitrogen source for ECM fungal growth, ammonium has been frequently reported as a readily usable inorganic N source and more suitable than organic nitrogen

sources as amino acids (Rangel-Castro et al. 2002, Sangtiean Schmidt, S. 2002, Itoo & Reshi 2014).

There are several culture media for growth of organisms, as fungi and plants, in pure cultures. The most common culture media for ECM and saprobic fungi are Potato Dextrose Agar (PDA), modified Melin-Norkrans (MMN) (Marx 1969), biotin-aneurine-folic acid (BAF) (Moser 1960) and Malt Extract Agar (MEA) reported in many studies (Barros et al. 2006, Flores et al. 2008, Díaz, Flores & Honrubia 2009, Murrieta-Hernández et al. 2014), being PDA and MEA the most nutritive and frequent media for fungal growth (Biswas et al. 2011, Deacon 2013). Murashige and Skoog medium (MS) (Murashige & Skoog 1962) is designed and very used for plant or plant cells cultivation. However, it as been used for fungal growth in only a few studies, wether modified (with addition of a carbon source) (Maia & Yano-Melo 2001, Idnurm 2010), or not (Nasim et al. 2001, Sanmee et al. 2010). Growing ECM fungi in MS medium allow to assess this medium potential as an interesting and economic method for mycorrhizal synthesis. All these culture media have different nutrient composition, as different nitrogen and carbon sources, capable of very different fungal responses.

Pleurotus ostreatus Jacq. and *Agaricus bisporus* J. E. Lange are edible saprobic species widely known for their gastronomic value, being the two most cultivated species of mushrooms in the world (Kües & Liu 2000, Hoa et al. 2015). Moreover, both species have interesting medicinal and nutritional features (Jagadish et al. 2009, Hoa et al. 2015). *Pleurotus ostreatus* sporocarps naturally occur at spring on dead trees (Lee 1993, Hoa et al. 2015). It is a white root fungus (WRF) able of degrading several organic wastes and xenobiotics, being promising species in bioremediation of ecosystems (Harms et al. 2011, Alananbeh et al. 2014). The other saprobic species, *Agaricus bisporus*, naturally occurs in grasslands around Europe and North America (Jagadish et al. 2009). It is an accumulator of some toxic heavy metals, which could lead to serious health issues when consuming sporocarps from contaminated areas (Soeroes et al. 2005).

Tricholoma equestre (L.) P. Kumm. is an edible ECM fungi, common in pine forests and known to be associated with *Pinus* spp. and with shrubs as *Cistus* spp. (Martín-Pinto et al. 2006) in coastal sandy soils. Because to its gastronomic importance, this species is very important culturally and economically in many countries as Portugal (de Román & Boa

2004, Garcia et al. 2006). However some reports of toxicity raised some controversy about the edibility of this sporocarp (Bedry et al. 2001), being prohibited its commercialization in several countries as Italy, France and Spain (Lechner & Albertó 2008).

B. fragrans Vittad. is an ECM edible fungi with great nutritional value (Barros et al. 2011). It is also a thermophilous species commonly associated with *Quercus* spp. and *Castanea* spp. trees (Rodríguez & de Ana Magán 1993, Ainsworth et al. 2013) distributed around Europe and North America. In spite of being listed in the British Fungi's Red Data List, actually considered "Endangered" (Ainsworth et al. 2013), its occurrence has been reported recently in "Serra de Sicó", Portugal (Pereira 2015).

Studying edible mushrooms species in pure culture like *Tricholoma equestre*, *Boletus fragrans*, *Agaricus bisporus* and *Pleurotus ostreatus* with great economic and gastronomic interest is a necessary step to determine their nutritional and environmental requirements, as medium composition, pH and temperature for each species (Sánchez & Honrubia 2000, Vazques-Garcíia E, Santiago-Martíinez G 2002, Pereira C et al. 2007). Understanding the behaviour of these organisms in environmental and nutritional controlled conditions is fundamental for inoculum production (Sánchez & Honrubia 2000, Murrieta-Hernández et al. 2014), that is the most important step for spawn production for saprobic fungi cultivation (Hoa et al. 2015) and for inoculum production for *in vitro* and *ex vitro* mycorrhization with ECM fungi (Sánchez & Honrubia 2000, Vazques-Garcíia E, Santiago-Martíinez G 2002, Pereira C et al. 2007, Díaz, Flores & Honrubia 2009, Murrieta-Hernández et al. 2014). Determine culture requirements of few studied species like the ECM fungi *Tricholoma equestre* and *Boletus fragrans* is also interesting to increase the knowledge about these species and to compare it and to overlap already existing data to well-known and studied species like saprobic *Agaricus bisporus* and *Pleurotus ostreatus*. So far, to our knowledge, this is the first optimization study of pure culture conditions for *T. equestre* and *B. fragrans*.

2.2 Objectives

The aim of the present work was to determine and compare the growth rates of diverse edible ectomycorrhizal and saprobic fungi on five culture media and on three different tem-

Table 2.1: Details about Macromycetes species used in this study as taxonomic information, habitat, origin and collection date.

Species	*Tricholoma equestre*	*Boletus fragans*	*Agaricus bisporus*	*Pleurotus ostreatus*
Origin	Cantanhede, Coimbra, Portugal	Ameal, Coimbra, Portugal	Acquired Commercially	Acquired Commercially
Coordinates	40°21'01.9"N 8°41'39.7"W	40°11'08.5"N 8°32'45.3"W	-	-
Habitat	*P. pinaster* /*Cistus* spp./*H. halimifolium*	*Q. faginea* /*Q. robur* /*C. psilosepalus*	-	-
Collection Date	out/14	nov/14	set/14	set/14
Collection code	TE.04.10/14.CANTNH	BF.03.11/14.AML	AB.01.9/14.COI	PO.02.9/14.COI

peratures, in order to understand the characteristics and the nutritional and environmental requirements of each species.

2.3 Materials and methods

2.3.1 Fungal species

In the present study we used sporocarps to establish mycelial cultures of four edible fungi species, ECM and saprobic. The sporocarps of *Boletus fragrans* (Fig.2.1A) and *Tricholoma equestre* (Fig. 2.1B) (ECM species) were collected under living *Quercus robur*, *Quercus faginea* and *Pinus pinaster* with *Halimium* spp. and *Cistus* spp. understory in different forests of Coimbra District (Table 2.1, Fig. 2.2). The remaining two species, the saprobic *Agaricus bisporus* (Fig. 2.1C) and *Pleurotus ostreatus* (Fig. 2.1D) were isolated from sporocarps acquired commercially. The primary identification of sporocarps was based on macro morphology, using mushrooms field guides (Moreno & Manjón 2010).

2.3.2 Isolation and Molecular Identification of pure Cultures

Sporocarps were superficially sterilized with a 3% calcium hypochlorite solution and dissected in aseptic conditions. Small tissue fragments were removed from the inner part of the stem and the cap and placed in Petri dishes with PDA medium (Difco^{TM}) with pH 5.8-6.3 in a growth chamber at 23-25°C in the dark (n=8). All mycelial cultures were subcultured every three months and periodically checked for contaminations. After the estab-

Figure 2.1: Sporocarps from *Boletus fragrans* (A), *Tricholoma equestre* (http://www.mykoweb.com) (B), *Agaricus bisporus* (http://www.mykoweb.com) (C) and *Pleurotus ostreatus* (http://www.mykoweb.com) (D).

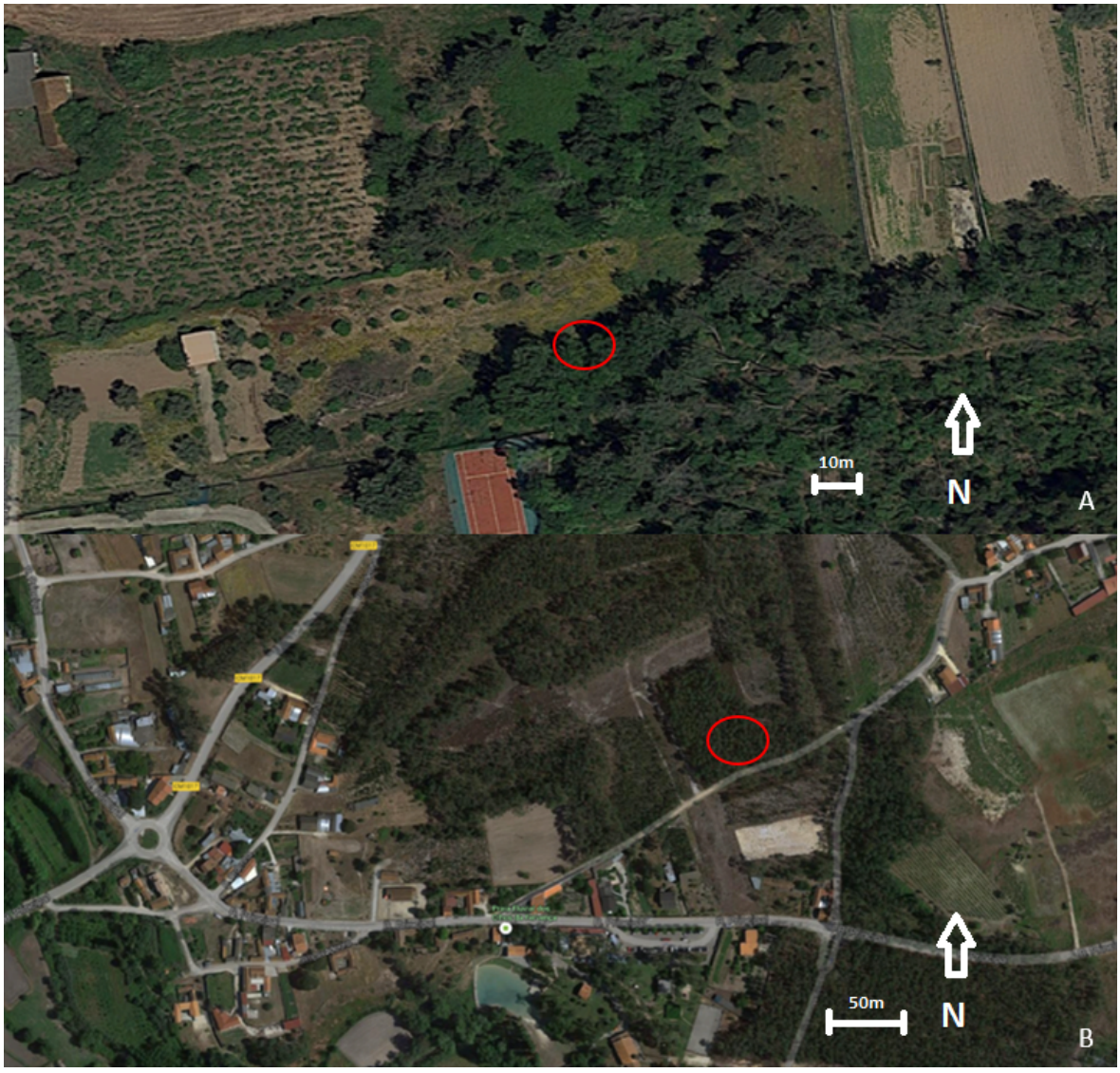

Figure 2.2: Collection site (red circle) of *Boletus fragrans* (A) and *Tricholoma equestre* (B) sporocarps (https://www.google.pt/maps).

lishment of pure cultures (1 month), species were identified at molecular level by sequencing ITS region (rDNA). The DNA was extracted with REDExtract-N-Amp^{TM} (SIGMA-ALDRICH®Company), using 1 mm^2 of a pure mycelium culture in 10μL of Extract solution and submitting it to 94^{o}C for 10 min, 60^{o}C for 13 min and 10^{o}C for 15 min. After that, an equal volume of Dilution solution was added. PCR was performed using ITS1-F/ITS4 primers pair and REDExtract-N-Amp PCR ReadyMix^{TM} (SIGMA-ALDRICH®Company) with the following cycling parameters 1 step of 94^{o}C for 5 min, 33 cycles of 94^{o}C for 45s, 56^{o}C for 35s and 72^{o}C for 35s, and 1 step of 72^{o}C for 10 min. The amplification was validated with an Electrophoresis in a 2% agaroses gel. Sequences were acquired by the modified Sanger method performed by STAB VIDA®, Portugal, and edited using Geneious®software (Biomatters). A Basic Local Alignment Search Tool (BLAST) was performed in National Center for Biotechnology Information (NCBI) database to confirm species taxonomic identification.

2.3.3 Effect of temperature and culture medium on growth rates

In this experiment five culture media and three different temperatures (4^{o}C ±1, 24^{o}C ±1 and 30^{o}C ±1) were tested for each fungal species (total 15 treatments with 5 replicates each). The culture media used were BAF (Moser 1960), MNM (Marx 1969), PDA (Difco^{TM}), MEA (Difco^{TM}) and MS (Murashige & Skoog 1962). The composition of the different culture medium as well as the Minimum Nutrient Requirements (MNR) (Deacon 2013) are described at Table 2.2. The pH was always adjusted to 5,8 with addition of KOH or HCl. For each treatment, squares with 0.25cm^2 were transferred from pure cultures (in PDA) and placed in the centre of 6 cm Petri dishes. Colonies' growth was measured every 3 days, during 36 days, by delimiting the mycelium's area with a permanent marker at the bottom of the Petri dish. The area at each 3 days was calculated with the aid of Adobe Photoshop CS5 program, Adobe Systems, Inc.®. Growth rates (cm/day) were determined for each 3 days by the following formula: $GR = \frac{FinalGrowth - InitialGrowth}{3}$. The final growth rate for each replicate was determined by the average of the growth rates every 3 days. Several morphological features as "Mycelium texture", "Mycelium colour", "Border", "Border colour", "Reverse colour", "Aerial growth", "Medium coloration", "Fruiting structures" and "Density" were used to

describe mycelial cultures, according with Barros et al. (2006).

2.3.4 Statistical analysis

All data were submitted to a Bartlett's test, in order to evaluate the Homogeneity of Variance, as to a normality test. As normality was proved, a two-way analysis of variance (ANOVA) was performed, for each fungal species, to test if the Temperature and Culture medium had effect on fungal growth rates. An Analysis of Variance (ANOVA) was also performed to test the significant differences between all culture media at the same temperature for each fungal species. The different fungi responses were compared considering the growth rates at the optimal temperature for each fungal specie. One additional analysis was performed at 24°C to test the differences between different species on the same culture medium. Significant differences ($P \leq 0.05$) between treatments were subsequently identified with a Tukey test. All statistic tests were performed with Statistica version 7 software package StatSoft Inc®.

2.4 Results

2.4.1 Species Identification

Molecular analysis of fungal mycelium confirmed the morphological identification of the wild species and the labels of the saprobic ones. After the determination of sequence similarities with those deposited in Genbank database, only sequences with high similarities (above 97%) were considered as being the same taxon. The GenBank accession numbers with which our sequences showed maximum identity as well as the query coverage and similarity values are reported in Table 2.3.

2.4.2 Effect of the Temperature on fungal growth rates

Two way ANOVA demonstrated that Temperature, culture medium and the interaction between both had significant effect ($P \leq 0.05$) on fungal growth rates of all species. Fungal growth rates in different culture medium at 4°C, 24°C and 30°C are represented in Figures 2.1, 2.2 and 2.3, respectively. Temperature had a very clear effect in fungal growth rates. At

Table 2.2: Composition of the different culture media and fungi Minimum Nutrient Requirements (MNM).

	MMR	BAF	MEA	MNM	MS	PDA
C-source	Glucose/Sacarose(20 g/L)	Glucose(30 g/L)	Dextrin(2.75 g/L)[2]	Glucose(5 g/L)		Dextrose(20g/L)[1]
N-source	NaNO3/NH4NO3/equivalent nitrogen source (2g/L)	Peptone(2 g/L)	Peptone(0.78 g/L)[2]	(NH4)2HPO4 (0.25g/L)	NH4NO3 (1.65 g/L)	
Nutrients	CaCl2 (0.5g/L)	CaCl2.2H2O (0.1g/L)		CaCl2 (0.05g/L)	CaCl2 (0.332 g/L)	
	KH2PO4 (1g/L)	KH2PO4 (0.5g/L)		KH2PO4 (0.5g/L)	KH2PO4 (0.17 g/L)	
					KNO3 (1.9 g/L)	
	MgSO4 (0.5g/L)	MgSO4.7H2O (0.5 g/L)		MgSO4.7H2O (0.31g/L)	MgSO4 (0.18054g/L)	
					CoCl2.6H2O (0.025mg/L)	
	CuSO4 (0.005–0.01g/L)				CuSO4.5H2O (0.025mg/L)	
				NaCl (25mg/L)		
	FeSO4 (0.005–0.01g/L)	FeCl3.6H2O (10mg/L)		FeCl3 (12mg/L)	FeNaEDTA (36.7mg/L)	
					H3BO3 (6.20mg/L)	
	KCl (0.5g/L)				KI (0.83mg/L)	
		MnSO4 (5mg/L)			MnSO4.H2O (16.9mg/L)	
					Na2MoO4.2H2O (0.25mg/L)	
	ZnSO4 (0.005–0.01g/L)	ZnSO4.7H2O (1mg/L)			ZnSO4.7H2O (8.6mg/L)	
Vitamins			Glycerol (2.35g/L)		Glycine(2 mg/L)	
		myo-Inositol (0.05mg/L)			myo-Inositol (0.1g/L)	
		Folic acid (0.1mg/L)			Nicotinic acid (0.5mg/L)	
					Pyridoxine HCl (0.5mg/L)	
		Thiamin HCl (0.05mg/L)			Thiamine HCl (1mg/L)	
		Biotin (1 µg/L)				
Extracts		Yeast extract (0.2g/L)		Yeast extract (1g/L)		
			technical maltose (12.75g/L)	malt extract (2g/L)		Potato Starch (4g/L)
	Agar 15g/L	Agar 15g/L		Agar 15g/L	Agar 15g/L	Agar 15g/L

Table 2.3: Details about Macromycetes species used in this study as taxonomic information, habitat, origin and collection date.

Species name	NCBI accession no. (similar sequences)	Query coverage (%)	Max identity (%)
Tricholoma equestre	EU186310.1	95%	99%
Boletus fragans	JF907800.1	91%	100%
Agaricus bisporus	HM561977.1	97%	100%
Pleurotus ostreatus	KT968340.1	100%	99%

4°C, all fungi had the lowest growth rates. At 4°C, the growth rate of both ECM fungi in all media was not measurable, although small hyphal growth was registered in some treatments of all species demonstrating some fungal activity. At 30°C, in all culture medium, none of ECM grew, considering, this way, this temperature as inhibitory for ECM fungi. Both saprobic fungi grew at 30°C, but with different behaviours. *Pleurotus ostreatus* had its maximal growth rate at this temperature, while *Agaricus bisporus* had a lower growth rate than in 24°C. The temperature of 24°C induced the highest growth rates for all fungi, with the exception of *Pleurotus ostreatus*, being therefore the most suitable temperature for the growth of *Tricholoma equestre*, *Boletus fragrans* and *Agaricus bisporus*.

2.4.3 Effect of Culture medium on fungal growth rates

Fungal responses were compared considering the growth rates at the optimal temperature for each fungal species. Culture medium had a significant ($P \leq 0.05$) and very distinct effect on fungal growth rates. PDA, followed by BAF medium allowed the highest growth rates for *Pleurotus ostreatus*. For *Agaricus bisporus*, the highest growth rates were obtained in MS, followed by BAF and PDA media. *Tricholoma equestre* was the most demanding fungi, growing only in PDA and in BAF media, although the best growth rate was obtained in the former medium. The highest growth rates of *Boletus fragrans* were obtained in MNM and PDA media.

The culture media in which fungi did not grow were MEA medium for *Agaricus bisporus* and MEA, MNM and MS for *Tricholoma equestre*. However, successful growth of *T. equestre* in MS medium was achieved in a previous experience (Fig. 2.6).

The lowest growth rates were obtained in MNM and MS media for *Pleurotus ostreatus*;

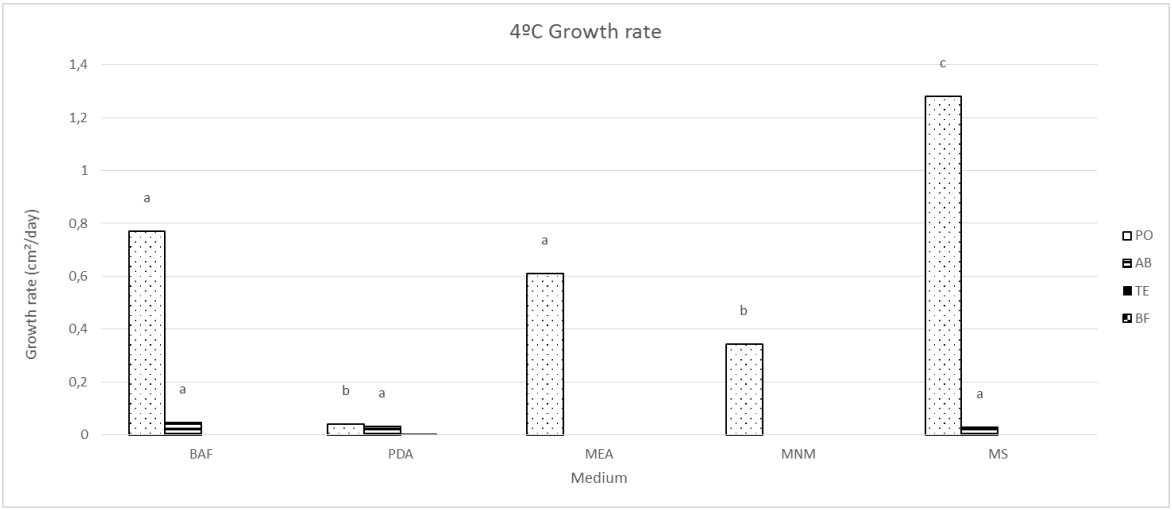

Figure 2.3: Growth rates of *Pleurotus ostreatus* (PO), *Agaricus bisporus* (AB), *Tricholoma equestre* (TE) and *Boletus fragrans* (BF) in five different culture medium at a temperature of 4ºC. Different lower case letters above the bars mean that exist significant differences among the treatments ($P < 0.05$) for the same species.

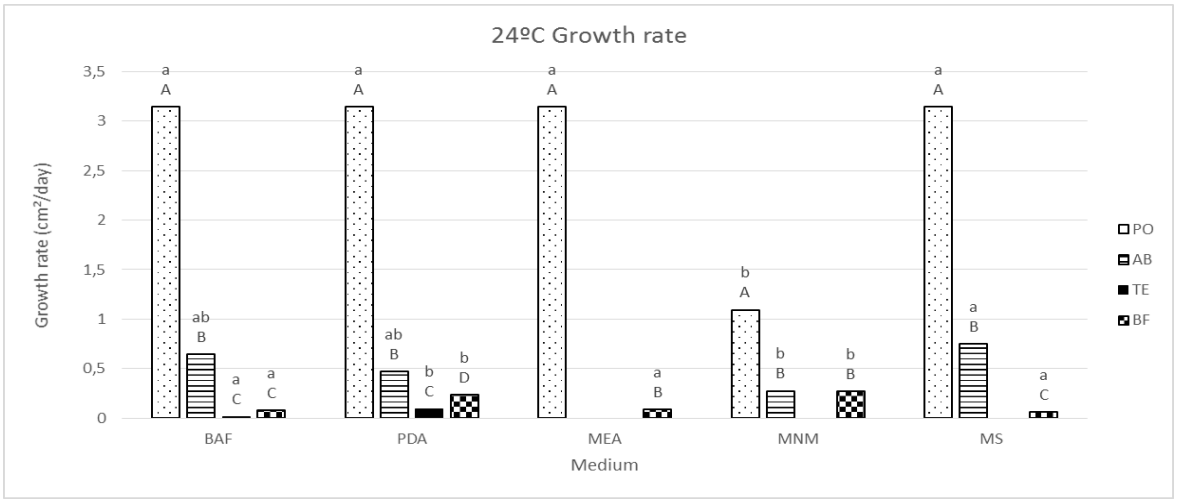

Figure 2.4: Growth rates of *Pleurotus ostreatus* (PO), *Agaricus bisporus* (AB), *Tricholoma equestre* (TE) and *Boletus fragrans* (BF) in five different culture medium at a temperature of 24ºC. Different lower case letters above the bars mean that exist significant differences among the treatments ($P < 0.05$) for the same species. Different capital letters above the bars mean that exist significant differences among the treatments different species ($P < 0.05$) for the same treatment.

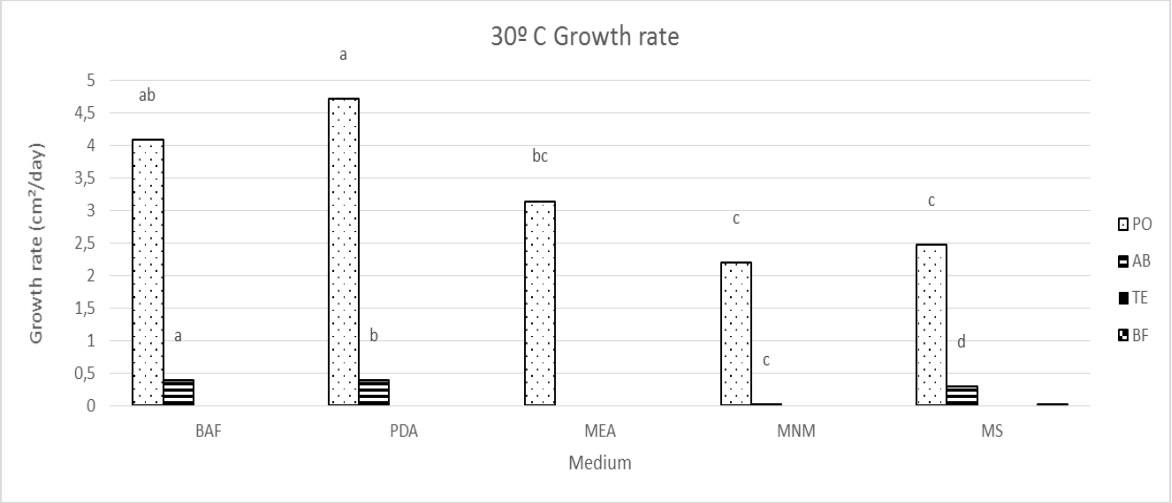

Figure 2.5: Growth rates of *Pleurotus ostreatus* (PO), *Agaricus bisporus* (AB), *Tricholoma equestre* (TE) and *Boletus fragrans* (BF) in five different culture medium at a temperature of 30°C. Different lower case letters above the bars mean that exist significant differences among the treatments ($P < 0.05$) for the same species.

MNM medium, for *Agaricus bisporus*; BAF medium for *Tricholoma equestre*; and BAF, MEA and MS media with no significant differences ($P > 0.05$) among them, for *Boletus fragrans*.

2.4.4 Effect of Culture medium on fungal Morphological features

Culture medium had an evident effect on all fungi's morphological features (Table 2.4). Aerial growth and Fruiting structures were two features that could be only observed in *Pleurotus ostreatus* (Fig. 2.7D). *Boletus fragrans* was the only fungi which caused Medium coloration (Fig. 2.7B) and in which medium coloration was different between media. Culture medium influenced also: Mycelium texture, in all fungi; Border, in all fungi except *Tricholoma equestre*; Border colour in *Agaricus bisporus* and *Boletus fragrans*; and Reverse colour in all fungi except *Pleurotus ostreatus*. All fungi had a low density mycelium in BAF and MS media. A low density mycelium was also observed for *Agaricus bisporus* growth in MNM medium. More details can be consulted in Table 2.4. Species morphology of the four species in PDA medium can be observed in Figure 2.7.

Figure 2.6: Growth of *Tricholoma equestre* in Murashige and Skoog (MS) medium.

Table 2.4: Morphological features of *Tricholoma equestre* (TE), *Boletus fragrans* (BF), *Agaricus bisporus* (AB) and *Pleurotus ostreatus* (PO) in BAF, PDA, MEA, MNM and MS media.

Specie	Medium	Mycelium texture	Mycelium colour	Border	Border colour	Reverse colour	Aerial growth	Medium coloration	Fruiting structures	Density
	BAF	smooth	white	difuse	transparent	white	no	no	no	Low
	PDA	crustaceous	white	difuse	transparent	yellowish	no	no	no	High
TE	MEA									
	MNM									
	MS									
	BAF	Difuse	white	Clear	white	yellowish	no	no	no	Low
	PDA	cottony	White	Clear	white	Brown	no	yes	no	High
BF	MEA	cottony	Cooper	clear	cooper	brown	no	yes	no	High
	MNM	cottony	white	Clear	white	brown	no	yes	no	High
	MS	Difuse	white	difuse	transparent	transparent	no	no	no	Low
	BAF	Difuse	white	difuse	white	white	no	no	no	Low
	PDA	smooth	white	clear	white	yellowish	no	no	no	High
AB	MEA									
	MNM	smooth	white	clear	white	yellowish	no	no	no	Low
	MS	Difuse	white	difuse	transparent	transparent	no	no	no	Low
	BAF	smooth	white	difuse	white	white	yes	no	no	Low
	PDA	cottony	white	clear	white	white	yes	no	yes	High
PO	MEA	cottony	white	clear	white	white	yes	no	no	High
	MNM	cottony	white	clear	white	white	yes	no	yes	High
	MS	Difuse	white	difuse	white	white	no	no	no	Low

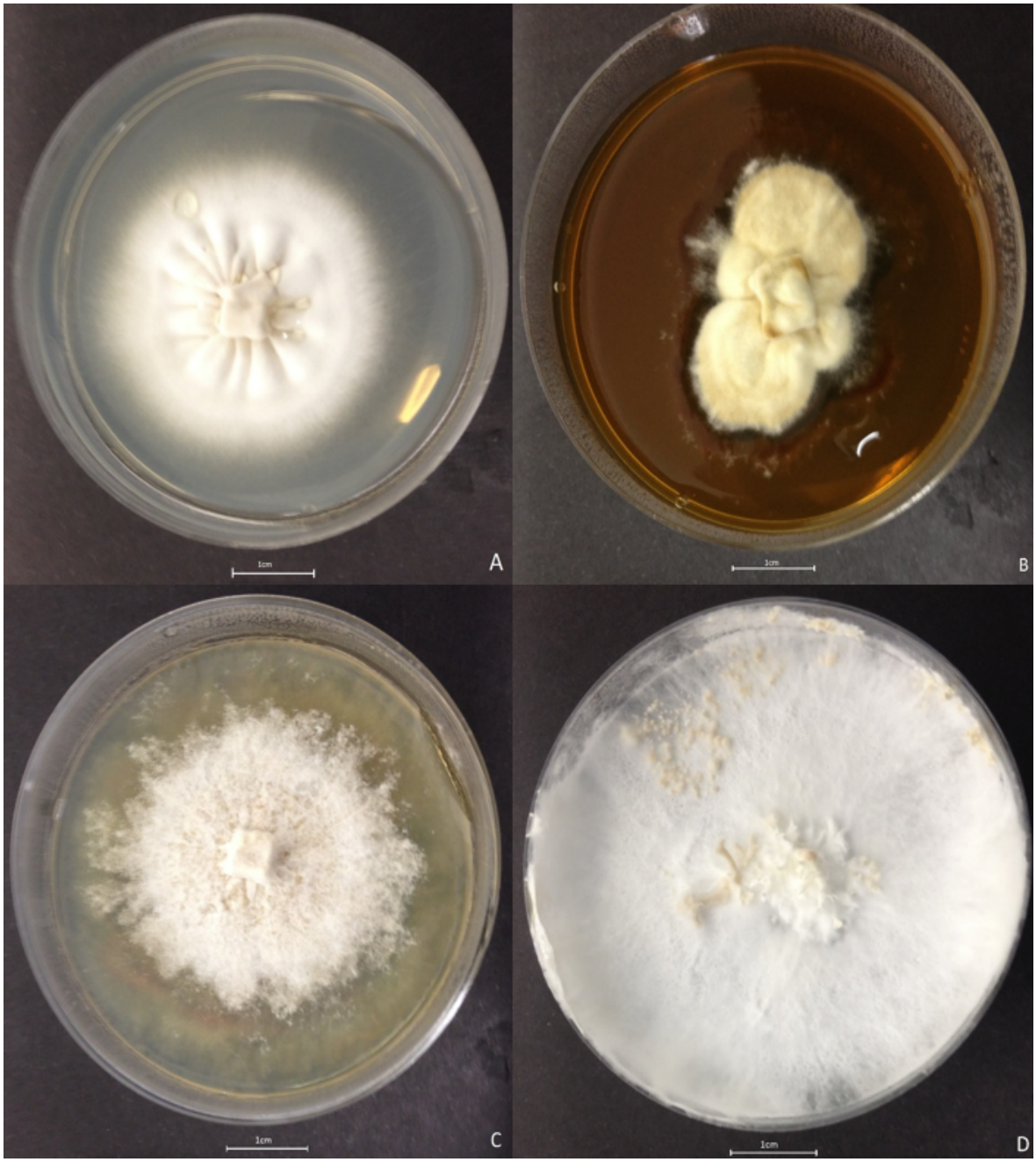

Figure 2.7: Morphology of *Tricholoma equestre* (A), *Boletus fragrans* (B), *Agaricus bisporus* (C) and *Pleurotus ostreatus* (D) in PDA medium.

2.5 Discussion

Fungal growth rates as well as fungal morphology depend on growth conditions as temperature, on culture medium and species features (Furlan et al. 1997, Sánchez et al. 2001, Barros et al. 2006, Lin & Yang 2006, Flores et al. 2008, Díaz, Flores & Honrubia 2009, Itoo & Reshi 2014).

Regarding temperature, *Pleurotus ostreatus* showed a higher growth rate at 30°C, coinciding with the findings of Furlan et al. (1997) and Hoa et al. (2015). However, *Agaricus bisporus*, showed the highest growth rate at 24°C, in accordance with the results reported by several authors (Treschow 1944, Ian M. Fraser 1958, Arrold & Blake 1966, Dijkstra et al. 1972). Nevertheless, Furlan et al. (1997), contrarily to our results, reported 30°C as the best temperature for the mycelial growth of *A. bisporus* congeneric species, *Agaricus bitorquis*. Furthermore, the best temperature for mycelial growth of the ECM species - *Tricholoma equestre* and *Boletus fragrans* - was 24°C, while 30°C inhibited their growth (Sánchez et al. 2001). Similar results have been described for *Tricholoma* spp. (Ogawa 1978, Xue et al. 2007, Lazarević et al. 2016), and for *Boletus* spp. (Coleman et al. 1989, Sánchez & Honrubia 2000, Ohta & Fujiwara 2003, Díaz, Flores & Honrubia 2009, Reczyński et al. 2013). However, 30°C has been reported as the optimal temperature for mycelial growth of a Boletales fungi, *Phlebopus portentosus* (Sanmee et al. 2010, Kumla et al. 2011). Our findings are also in accordance with Ohta (1994), who reported similar conclusions regarding the optimal temperatures for other ECM species. Low temperature (4°C) did not inhibit the mycelial growth of all fungi, delaying therefore fungal growth and metabolism, being a useful temperature for long time maintenance of fungal cultures, as described by Díaz, Flores & Honrubia (2009).

The fungi that inhabit in soil (soil-fungus) - *A. bisporus*, *T. equestre* and *B. fragrans* - had lower optimal temperatures that *P. ostreatus*, which is a wood inhabiting fungi (wood-fungus), due probably to soil-fungus being naturally exposed to more extreme conditions than wood-fungus. It is known also that soil inhabiting fungi decreased their activity and growth at temperatures above 30°C due to their ecology and ability to survive and grow in low temperatures of winter and spring (Pietikäinen et al. 2005), therefore supporting our results.

The different culture media induced different mycelium densities. This feature has to be taken into account when analyzing the effect of the culture media in the optimal fungal growth. The high density mycelium was considered as the optimal one, being representative of high fungi's biomass (Itoo & Reshi 2014).

Regarding culture media, our results showed that *Pleurotus ostreatus* had higher growth rates in PDA, followed by BAF while *Agaricus bisporus* in MS, BAF and PDA. PDA is a generalist medium composed by several organic sources of carbon, nitrogen and other compounds unlike MS and BAF media. These results could suggest peptone to be the most suitable nitrogen source for *P. ostreatus* and ammonium nitrate and peptone for *A. bisporus*. That conclusion would be in accordance with the work of Ib & Chauhan (2015), who reported higher growth rates of a White Root Fungus similar with *P. ostreatus* in a medium with peptone. However, the mycelium density was low in BAF and very low in MS medium, suggesting that fungi were not in optimal conditions. Low density mycelium in MS medium obtained in the present work could be explained by the lack of a carbon source forcing, probably, the fungus to degrade other compounds as myo-Inositol, which is molecular and structurally similar to glucose. This explanation is sustained by the work of Kumla et al. (2011) that obtained high density mycelium in modified MS medium supplemented with glucose. Another explanation for this evidence of fungal stress is possibly the fast pH decrease, typical of fungi's utilization of ammonium nitrate as nitrogen source in pure culture (Morton & Macmillan 1954, Deacon 2013). In BAF medium, an explanation to this fungal behaviour is more difficult to obtain, but could be explained by the presence of several vitamins, which are absent in fungi's MNR proposed by Deacon (2013).

Considering that MEA is a nutritive and comprehensive culture medium with peptone in its composition, like BAF, the low and null growth rates of *P. ostreatus* and *A. bisporus*, respectively, in MEA medium comparing with the growth of the same species in BAF medium, was another incongruence that is not easy to explain in the present work. Thus, the most suitable medium for the saprobic fungi *Pleurotus ostreatus* and *Agaricus bisporus* was PDA, a result similar to those of Furlan et al. (1997), Hoa et al. (2015). However, other culture media, as MEA and MS, were reported as the optimal media for growth of *P. ostreatus* and *A. bisporus* by other authors (Rainey 1989, Carrera et al. 2001, Nasim et al. 2001, Arora

2004), in contrary to our findings.

The ECM species *T. equestre* and *B. fragrans* both had their highest growth rates in PDA, and also in MNM for *B. fragrans*, with no significant difference between the two culture media. The mycelium densities obtained were always high, contrarily once more, to BAF and MS media. Similar results were obtained using other ECM species by Barros et al. (2006), Xue et al. (2007), Díaz, Flores & Honrubia (2009), Sanmee et al. (2010), Murrieta-Hernández et al. (2014). Nevertheless, the results of Barros et al. (2006), Murrieta-Hernández et al. (2014), are not completely in accordance to ours, reporting differences in ectomycorrhizal fungal growth between PDA and MNM media. Differences within fungal growth in MNM, PDA and also in MS are reported in the work of Sanmee et al. (2010). The present results are also in contradiction with the ones obtained by Kibar & Peksen (2011), Kumla et al. (2011), reporting other media to be more suitable for mycelial growth of other ECM species.

The ideal concentration of carbon source (glucose) for the congeneric species of *T. equestre*, *T. aurantium*, was stated as 20 g/l by Itoo & Reshi (2014). That finding could be extrapolated to ours obtained with *T. equestre* that have shown also the optimal growth in PDA, with a dextrose concentration of 20 g/l, and a low growth in BAF with a glucose concentration of 30 g/l.

Our results for *B. fragrans* could suggest that the preferential nitrogen source was ammonium, in accordance with Itoo & Reshi (2014), and could suggest also that the low amount of carbon source in MNM medium (5g/l) had no influence in the optimal growth, probably due to the low saprophytic condition that reflect a low expression of extracellular enzymes responsible for the degradation of organic litter in comparison to saprobic fungi (Read & Perez-Moreno 2003, Shah et al. 2015). The low expression of these enzymes can also explain the lower growth rates of the ECM fungi in pure culture, comparing with the saprobic fungi, results also reported by Barros et al. (2006).

In spite of drawn conclusions in the present work about ECM and saprobic fungi nutrition, these are no more than patterns observed, which can be confirmed or denied with further studies in which only nitrogen and carbon sources should vary separately in a standard medium, as in the study performed by Itoo & Reshi (2014).

The morphological features of fungal mycelium were influenced according with the culture

medium. The same conclusion was obtained by Barros et al. (2006), Flores et al. (2008), Díaz, Flores & Honrubia (2009) in their works.

Chapter 3

Mycorrhizal Synthesis of Edible ECM fungi in *Cistus* spp.

3.1 Introduction

The term"Mycorrhiza", a Greek word meaning"fungus root", has been defined for the first time in 1885 by Frank (Frank 2005) as a symbiotic association between plant roots and a fungus (Smith & Read 2008, Itoo & Reshi 2013). Symbiotic association can be mutualistic or non-mutualistic (Brundrett 2004). The most overarching definition of symbiosis is "a living together relationship between two or more organisms" (Lewis 1985, Brundrett 2004, Smith & Read 2008), while the wide definition of mutualism is a relationship in which the two or more individuals involved have mutual benefits (Lewis 1985, Brundrett 2004). As not all mycorrhizal associations are mutualistic, such as the association with mycoheterothrofic plants, the term "symbiosis" was adopted to define mycorrhizal association (Brundrett 2004, Smith & Read 2008). However, the widespread concept of mycorrhizal association is a beneficial relationship in which the fungi receive organic carbon and protection from the plant in exchange of helping with water and nutrient uptake to the plant, such as phosphorus (P) which has low mobility in the soil, and other nutrients like nitrogen (N), potassium (K) and several micronutrients (Smith & Read 2008, Itoo & Reshi 2013, 2014). Several mycorrhizal classifications have been proposed since the first discovery of mycorrhizal association until

nowadays. The initial classifications were mostly based in the relative position of the fungus on the root (Peyronel et al. 1969, Brundrett 2004), grouping very distinct mycorrhizal morphologies and functions as well as partners of disparate taxa (Brundrett 2004, Smith & Read 2008). There are seven categories of mycorrhizas acknowledged nowadays based on partners taxonomy and morphological features, which are arbuscular mycorrhiza, ectomycorrhiza, ericoid mycorrhiza, orchid mycorrhiza, ectendomycorrhiza, arbutoid mycorrhiza and monotropoid mycorrhiza (Finlay 2008, Smith & Read 2008). Nevertheless, some authors claimed that ectendomycorrhiza, arbutoid and monotropoid to be ectomycorrhizal subtypes due to the presence of some anatomic structures just varying only in slightly differences (Brundrett 2004, Tedersoo et al. 2010).

Ectomycorrhizal category is distinguished from the others owing to several anatomic features as: an external hyphal mantle (or sheath) that surrounds the root; an intercellular hyphal growth between epidermal and cortical root cells, nominated as Hartig net; and a septate and extraradical mycelium organized or not into tissues called rhizomorphs and mantle emanating hyphae, respectively. Functionally, the Hartig net is the fungi-plant interface, allowing trades between the two partners; extraradical mycelium is a structure that enable interaction and exchanges between the fungi and the soil; and the mantle is mainly responsible for nutrient storage and plays a key role on the nutrient exchange between the partners (Smith & Read 2008).

The definition of "ectomycorrhiza" is included in the worldwide concept of "mycorrhiza" in which the symbiosis is beneficial for both partners in natural conditions (Egger & Hibbett 2004, Tedersoo et al. 2010). On this relationship, the plant is provided with several macro and micro nutrients dissolved in the soil solution and bounded to organic matter in early stages of decomposition as well as it is assisted on the mitigation of environmental stress due to heavy toxic metals, soil acidification, drought, pathogens and chemicals or herbivory. On the other hand, the fungi is supplied with organic carbon and with habitat, vital for its survival (Smith & Read 2008, Tedersoo et al. 2010, Itoo & Reshi 2013, 2014).

Most of the fungi that form ectomycorrhizal associations are known to have the ability to reproduce sexually with macroscopic sporocarps, commonly called "mushrooms", contrarily to the fungi that form arbutoid and ericoid mycorrhizas (Tedersoo et al. 2010). Some species

of these ectomycorrhizal macromycetes, which belong mainly to Basidiomycota and also Ascomycota, are widely known for their edibility and great gastronomic and economic value. For example, the Ascomycota species *Tuber* spp. (de Román & Boa 2004, Morte et al. 2008, Thomas 2014) and *Trefezia* spp. (de Román & Boa 2004, Morte et al. 2008); and the Basidiomycota *Boletus* spp. (de Román & Boa 2004, Sitta & Floriani 2008), *Lactarius deliciosus*, *Lactarius sanguifluus* (Guerin-Laguette et al. 2000, de Román & Boa 2004), *Tricholoma* spp. (de Román & Boa 2004).

The Portuguese native ectomycorrhizal edible species *Tricholoma equestre* (L.) P. Kumm., *Tricholoma portentosum* (Fr.) Quél., *Lactarius deliciosus* (L.) Gray and *Boletus fragrans* Vittad. are considered as a delicacy and greatly used in local gastronomy of many cultures. The species *T. equestre* and *T. portentosum* have high nutritional value with several beneficial proprieties (Ferreira et al. 2007, Kalač 2009) and are highly popular species in many countries, mainly in Portugal and Spain (de Román & Boa 2004, Garcia et al. 2006). Were also used for studies of synthesis and characterization of mycorrhizas (Yamada et al. 2001) and *T. portentosum* was also used in studies of mycorrhization and sporocarp production (Yamada et al. 2007). *Lactarius deliciosus* is an appraised and nutritive species (Kalač 2009) appreciated all around the world, particularly in Spain (de Román & Boa 2006), and commonly used in several works of *in vitro* and *ex vitro* mycorrhization (Parladé et al. 2004, Díaz, Carrillo & Honrubia 2009) and cultivation of sporocarps (Poitou et al. 1989, Guerin-Laguette et al. 2000, Yun & Hall 2004). *B. fragrans* is a little studied edible species, known for its nutritional value (Barros et al. 2011), being only described its conditions for sporocarp production in artificial inoculated *Castanea* sp. (Rodríguez & de Ana Magán 1993).

These fungi are reported as mycorrhizal fungi associated with several plants species, not being associated specifically to one plant partner, denominated as "Broad host range" fungi (Molina et al. 1992), due to the fact that can form mycorrhizas with several taxa (Comandini et al. 2006).

Thus, due to growing importance and demand of these edible and seasonal sporocarps, the study of this mycorrhizal category is required in an attempt to understand and replicate the mechanisms that lead to the fruitification of these fungi's with great value in order to establish protocols of cultivation.

Even though the mechanisms that lead to sporocarp fruitification are not yet very clear (Murat et al. 2008, Ortega-Martínez et al. 2011), in order to cultivate ectomycorrhizal fungi's fruit bodies it is necessary to establish the association between the two partners and afterwards trigger the mechanisms that lead to fruitification (Ortega-Martínez et al. 2011). These steps are dependent on the organisms genetics (Allen 1991, Alonso Ponce et al. 2011), which determine the compatibility between the partners as well as the mechanisms and environmental requirements that trigger the formation of sporocarps (van der Heijden & Sanders 2003, Alonso Ponce et al. 2011); on the environmental background (Allen 1991, Alonso Ponce et al. 2011), as plant and fungi's physiological condition, plant age and size that influence the amount of carbon supplied to the fungi and also on the nutritional state of the mycelium (Ortega-Martínez et al. 2011). Depend also on biotic elements, as competition with other fungi (Savoie & Largeteau 2011), mycorrhization helper bacteria (Rigamonte et al. 2010) that aid on the establishment of the association; and on abiotic factors such as light, temperature, humidity and nutrient availability (Murat et al. 2008, Alonso Ponce et al. 2011).

As referred, ectomycorrhizal synthesis is the first and fundamental step to achieve the production of sporocarps and consists in exposing plant roots to their ectomycorrhizal fungal partner. Many studies have been developed since the description of the first technique of pure culture synthesis by Melin (1921) cit. (Molina 1979), until nowadays (Águeda et al. 2008, Zitouni-Haouar et al. 2014). There are a wide range of techniques of ectomycorrhizal synthesis as axenic, non-axenic and hydroponic systems (Peterson & Chakravarty 1991), being the axenic techniques of synthesis the most common and suitable for establishing ectomycorrhizas with a specific species of fungal partner. Several axenic techniques with different substrates, as vermiculite, peat and perlite, have been used in many works (Peterson & Chakravarty 1991, Águeda et al. 2008, Chávez M et al. 2009), being uncommon to use nutritive agar media as substrate for *in vitro* mycorrhizal synthesis, which have been only used, to our knowledge, in the "Petri Plate Method" (Peterson & Chakravarty 1991) and in "Test Tube method" (Peterson & Chakravarty 1991, Zaretsky et al. 2006). Murashige and Skoog (MS) (Murashige & Skoog 1962) medium is an agar culture medium developed and widely used for plant micropropagation and plant cell cultivation, used in many works of fungal growth in pure culture (Maia & Yano-Melo 2001, Nasim et al. 2001, Idnurm 2010,

Sanmee et al. 2010) and, after some modifications as carbon addition, in very few projects of ectomycorrhizal synthesis (Martins 2008). However, non-modified MS medium was never used to mycorrhizal synthesis, to our knowledge.

After mycorrhizal establishment, the fungus and the plant need to grow and to anchor their relationship before the fungus being able to fruit, which depends mainly on plant growth that can be a deterrent factor to a timely, realistic and profitable production of sporocarps.

Other difficulty, besides plant growth rates, is that some fungi only fructify significantly associated with old plant partners, as *Boletus edulis*, and others only with portentous plant partners with high biomass, as *Lactarius deliciosus*, for example (Ortega-Martínez et al. 2011). This probably happens due to the fact that some species of fungi are representative of late stage successions and that only plants with maximum size and biomass have enough photosynthetic rates that sustains fungal carbon requirements, respectively (Ortega-Martínez et al. 2011). Most ECM fungi are preferentially associated with trees, like *Quercus* spp. and *Pinus* spp., take several years to have enough age and size in order to be able to sustain a significant production of ectomycorrhizal mushrooms (Ortega-Martínez et al. 2011). Thus, using plants with relatively higher growth rates, which can reach the highest biomass in less time than trees, and that are able to form ectomycorrhizas, is the ideal solution to solve this issue, being shrubs from *Cistus* genus a promising candidates, which do not live usually more than 15 years and generally reach the highest biomass between 2-4 years-old (Patón et al. 1998, Oñate & Munné-Bosch 2008). Cistaceae family comprises 8 different genera and almost 200 species (Muñoz-Garmendía & Navarro 1993, Águeda et al. 2006) all capable of establishing ectomycorrhizas (Brundrett 2002, Águeda et al. 2006, Smith & Read 2008). The genera *Cistus*, *Halimium* and *Helianthemum* are the most widespread from Cistaceae family and known to be commonly associated with some ectomycorrhizal edible fungi (Águeda et al. 2006, Comandini et al. 2006, Zaretsky et al. 2006, Oria De Rueda et al. 2008, Jamali & Banihashemi 2012, Zitouni-Haouar et al. 2014). Furthermore, some works of synthesis and cultivation of ECM edible mushrooms using these genera as symbiotic partner are also known (Giovannetti & Fontana 1982, Wenkart et al. 2001, Bustan et al. 2006, Zaretsky et al. 2006, Águeda et al. 2008, Morte et al. 2008, Zitouni-Haouar et al. 2014).

Cistus is a Mediterranean native genus (Papaefthimiou et al. 2014) that harbours about

20 species (Comandini et al. 2006), being 12 of them represented in Iberian Peninsula (Águeda et al. 2006). All these species are pyrophytic, inhabit semi-arid habitats and consequently developed the ability to resist drought (Águeda et al. 2006, Comandini et al. 2006). *Cistus* are also pioneer species capable of colonize after a disturbance in degraded areas, and they function as "fertility islands" of nutrients and fungal diversity in disturbed habitats (Torres et al. 1995, Díez 1998, Simões et al. 2001, Águeda et al. 2006).

Several works about the diversity of ectomycorrhizal fungi associated with *Cistus* spp. are known, reporting sporocarps presence of several species of edible fungi near these shrubs (Comandini et al. 2006) as well as the natural association and production of edible sporocarps of species like *Boletus edulis* in *Cistus* scrublands (Águeda et al. 2006, Oria De Rueda et al. 2008). Furthermore, until nowadays have been done several successful experiences of synthesis of ectomycorrhizas with many edible ectomycorrhizal species as *B. edulis* (Águeda et al. 2008), *Laccaria laccata* (Torres et al. 1995), *Tuber melanosporum* (Giovannetti & Fontana 1982, Wenkart et al. 2001, Bustan et al. 2006) and *Terfezia* spp. (Zaretsky et al. 2006, Zitouni-Haouar et al. 2014).

The common Portuguese native species *C. psilosepalus* Sweet (Fig. 3.1), *C. salviifolius* L. (Fig. 3.2) and *C. ladanifer* L. (Fig. 3.3) are distributed almost all over the country (Figs. 3.1–3.3) in many different types of habitats as woods, pinewoods, cork-oak forests and many others (http://www.flora-on.pt), coexisting in the same habitat that many ECM fungi (Comandini et al. 2006), including the edible fungal species previously referred.

3.2 Objectives

The aim of this work is to establish novel ectomycorrhizal associations between four edible ectomycorrhizal fungi - *T. equestre*, *T. portentosum*, *B. fragrans* and *L. deliciosus* - and three shrub species from genus *Cistus* - *C. psilosepalus*, *C. salviifolius* and *C. ladanifer* -, to determine partner's compatibility, to evaluate the biotechnological potential of these novel combinations to future production of edible sporocarps and to optimize the conditions and the techniques of ectomycorrhizal synthesis aiming to develop an ectomycorrhizal synthesis technique adapted to the habits of these shrubs.

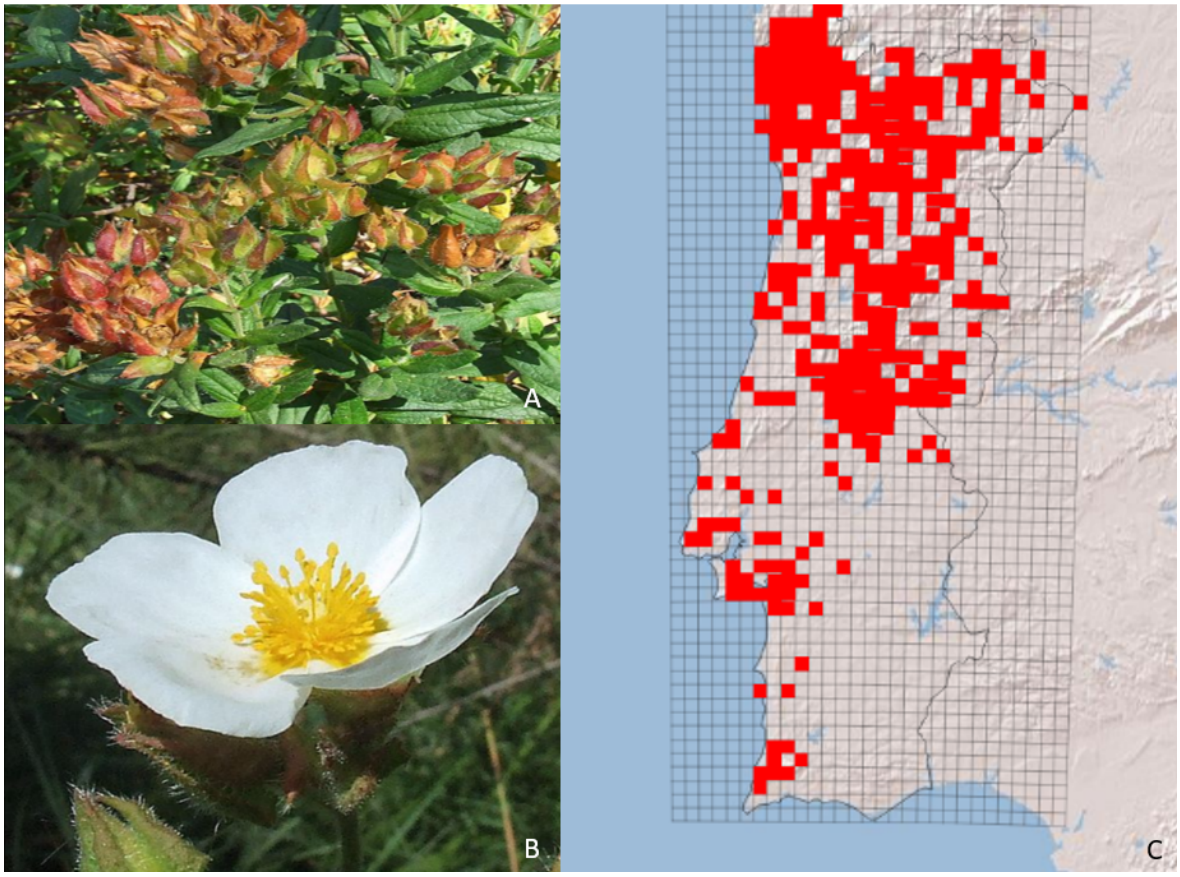

Figure 3.1: *C. psilosepalus* shoot system (A), flower (B) and distribution (C) (http://www.flora-on.pt).

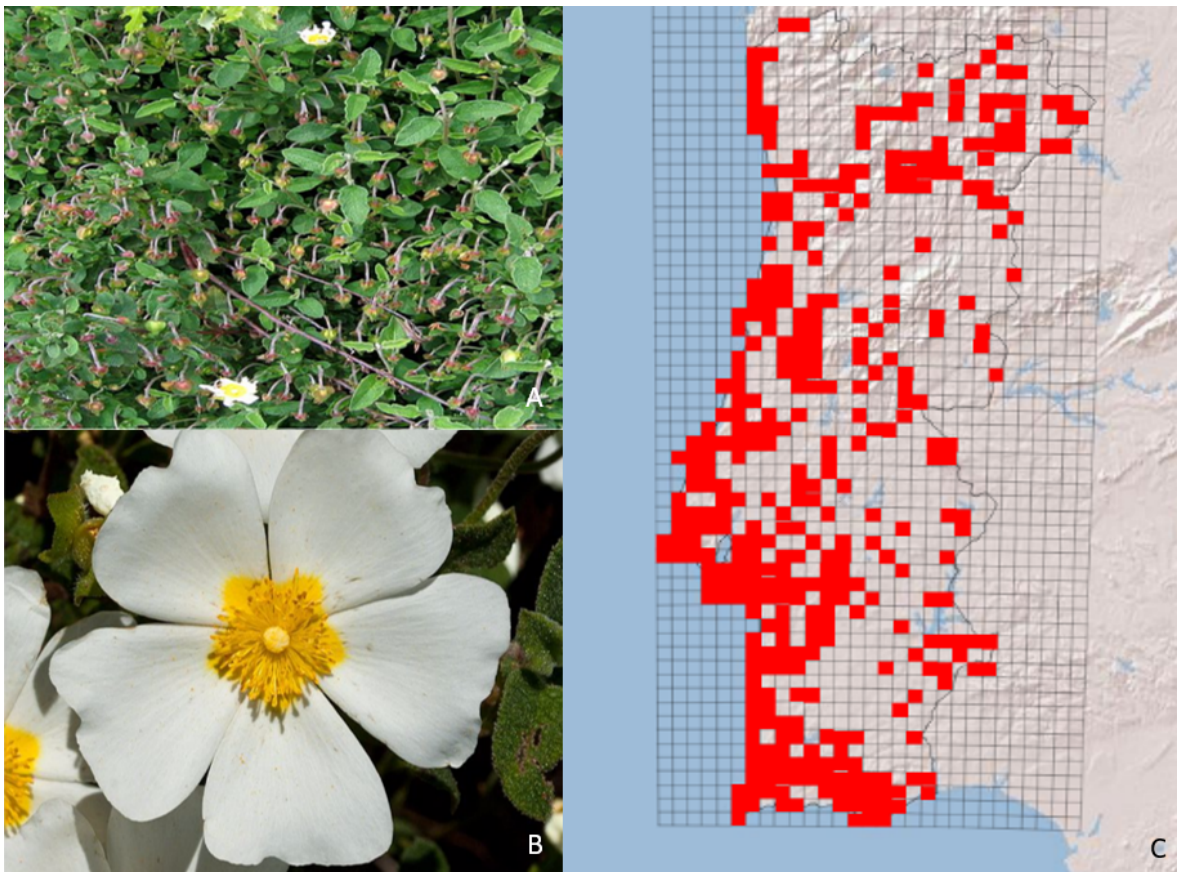

Figure 3.2: *C. salviifolius* shoot system (A), flower (B) and distribution (C) (http://www.flora-on.pt).

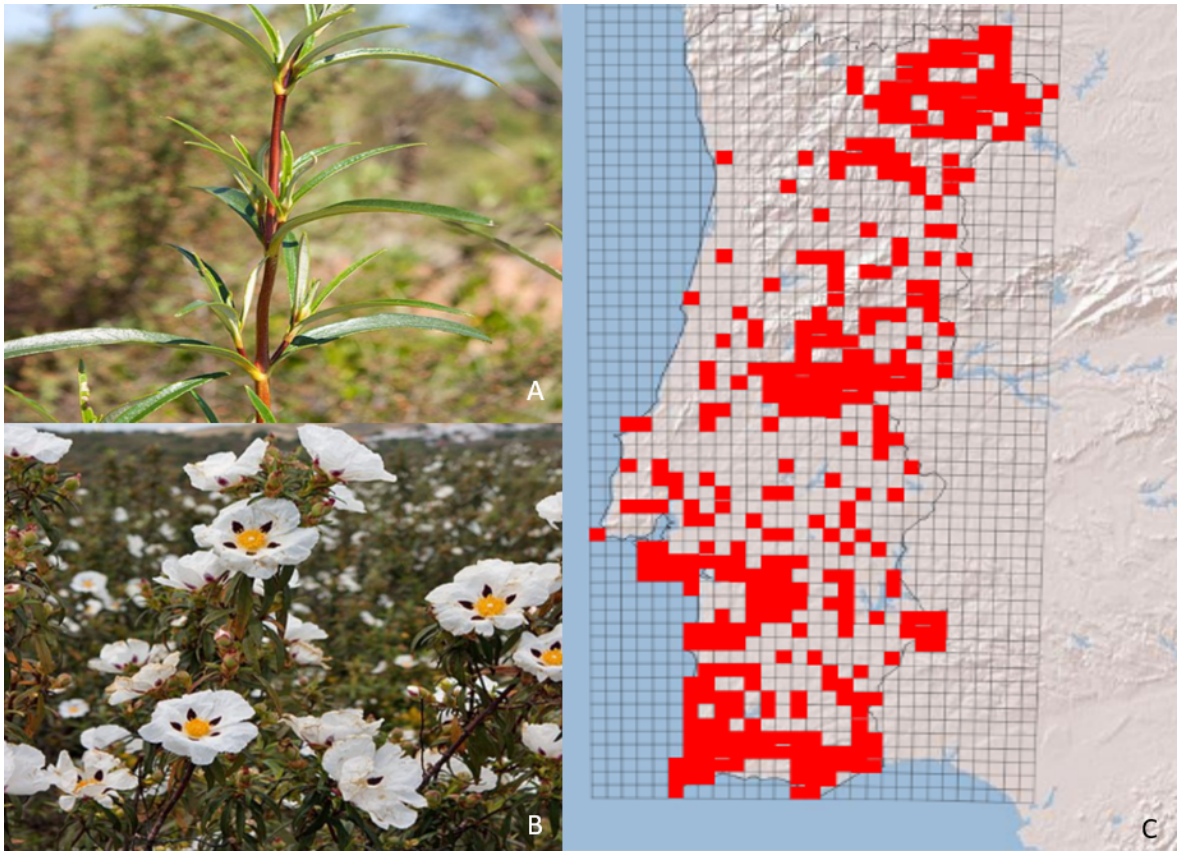

Figure 3.3: *C. ladanifer* shoot system (A), flower (B) and distribution (C) (http://www.flora-on.pt).

Table 3.1: Details about Macromycetes species used in this study as taxonomic information, habitat, origin and collection date.

Species	*Tricholoma equestre*	*Boletus fragans*	*Lactarius deliciosus*	*Tricholoma portentosum*
Origin	Cantanhede, Coimbra, Portugal	Ameal, Coimbra, Portugal	Cantanhede, Coimbra, Portugal	Acquired Commercially
Coordinates	40°21'01.9"N 8°41'39.7"W	40°11'08.5"N 8°32'45.3"W	40°21'01.9"N 8°41'39.7"W	40°10'48.9"N 7°39'40.1"W
Habitat	*P. pinaster*/*Cistus* spp./*H. halimifolium*	*Q. faginea*/*Q. robur*/*C. psilosepalus*	*P. pinaster*/*Cistus* spp./*H. halimifolium*	*P. pinaster*/*Cistus* spp.
Collection Date	out/14	nov/14	out/14	out/14
Collection code	TE.04.10/14.CANTNH	BF.06.11/14.AML	LD.03.10/14.CANTNH	TP.05.10/14.PL

3.3 Materials and methods

3.3.1 Fungal material

In the present study, sporocarps of four species of ectomycorrhizal (ECM) fungi - *Tricholoma equestre*, *Tricholoma portentosum*, *Lactarius deliciosus* and *Boletus fragrans* - were collected, isolated and molecularly identified according to the methodology already described in Chapter 2. All species were collected under living *Quercus* spp. and *Pinus pinaster* woods with *Halimium* spp., *Cistus salviifolius* and *Cistus psilosepalus* understory in different sites (Table 3.1, Fig. 3.4). The inoculum used in the synthesis of ectomycorrhizas was obtained in PDA (Potato Dextrose Agar) (Difco^{TM}) medium and in the liquid BAF (Biotin-aneurin-folic Acid) medium (Moser 1960), adjusted to pH 5.8-6.3. All cultures were maintained in dark culture chambers at 24ºC ±1. The collection sites and dates of each species can be consulted in Table 3.1. The NCBI accession numbers of our species sequence and those with which our sequences showed maximum identity as well as the similarity values are reported in Table 3.2.

3.3.2 Plant material

Seeds from *Cistus ladanifer*, *Cistus salviifolius* and *Cistus psilosepalus* were collected in areas with high diversity of ECM fungi, in different locations, near *Pinus* spp., *Quercus* spp. and *Halimium* spp. (Table 3.3, Fig. 3.5. Seed sterilization and germination protocol was developed by exposing seeds to several treatments. Seeds were selected under a stere-

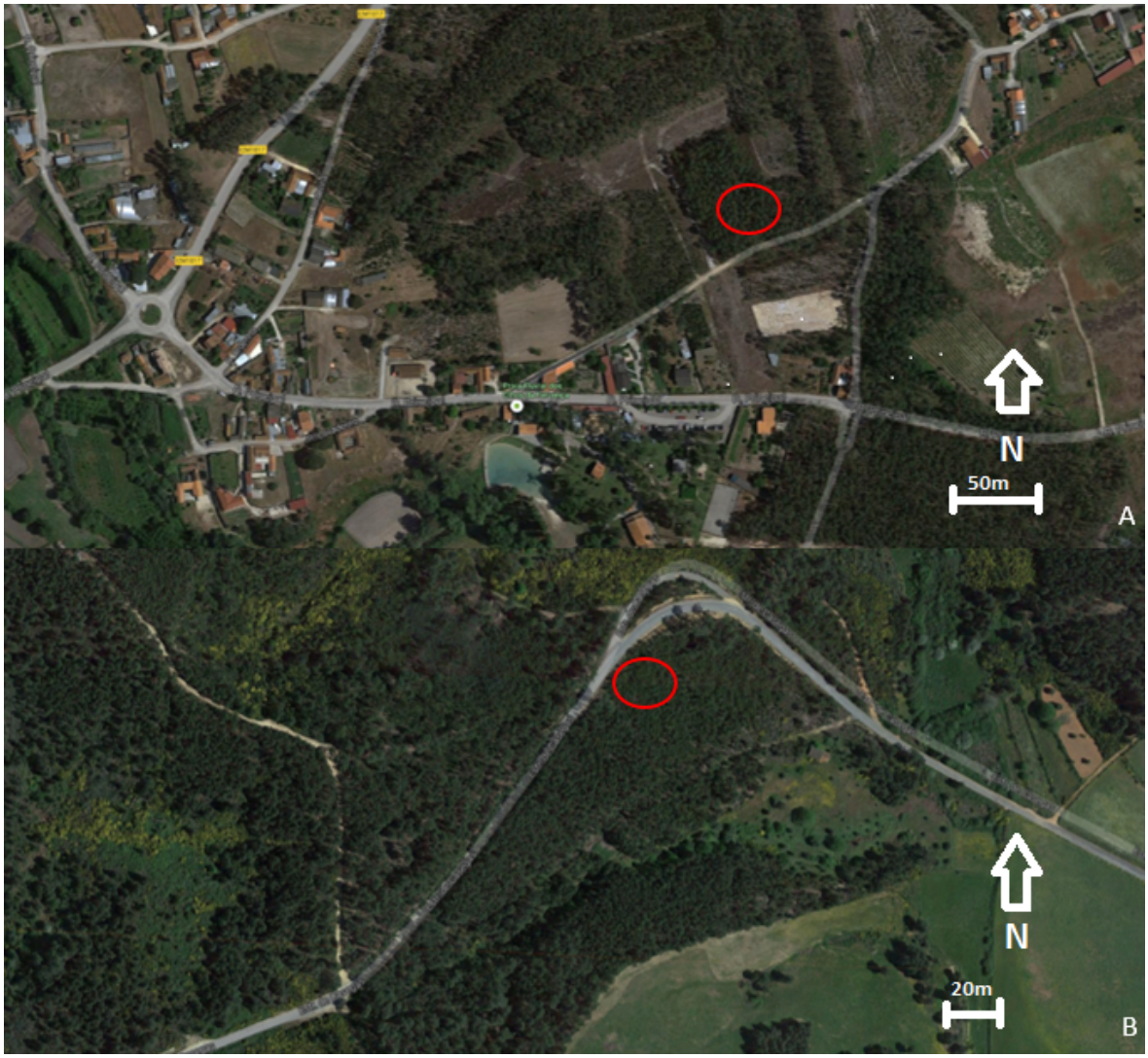

Figure 3.4: Collection site (red circle) of *Lactarius deliciosus* (A) and *Tricholoma portentosum* (B) sporocarps (https://www.google.pt/maps). (The location of the *T. equestre* and *B. fragrans* are in Fig. 2.2)

Table 3.2: Nacional Center for Biotechnology Information (NCBI) accession number of the four species sequences as well as similar sequences and the respective percentage of maximum identity.

Species	NCBI accession no. (similar sequences)	Max identity (%)
Tricholoma equestre	EU186310.1	99%
Boletus fragans	JF907800.1	100%
Lactarius deliciosus	KJ769672.1	99%
Tricholoma portentosum	EU186273.1	99%

omicroscope (only hard and viable seeds were chosen to perform the assays). Twenty seeds per treatment, for each species, were placed in 1ml Microcentrifuge tubes upon a laboratory shaker and exposed to following treatments: Distilled water at 100°C; Alcohol (96%); Sodium Hypochlorite (3%); Distilled water at 100°C + Alcohol (96%) (In this order); Distilled water at 100°C + Sodium Hypochlorite (3%) (In this order); Distilled water at 100°C + Alcohol (96%) + Sodium Hypochlorite (3%) (In this order); Alcohol (96%) + Sodium Hypochlorite (3%) (In this order). Each step within the treatments had a duration of 10 minutes. Afterwards, seeds were placed in Petri dishes with MS agar medium (Murashige & Skoog 1962). The contaminated seeds were recorder every three days during one week and discarded, while the total number of germinations was recorded every five days over a month. A seed was considered germinated when the emergence of the radicle occurred (Thanos & Rundel 1995, Cochrane et al. 1999, Pérez-Fernández & Rodríguez-Echeverría 2003). The contamination and germination percentages were determined for each species, the percentages were overlapped and, afterwards, the treatment used in seedlings obtainment for ectomycorrhizal synthesis was chosen based on combination of the lowest percentage of contamination with the highest percentage of germination. Only seedlings with developed cotyledons and radicle were used for establishment of ectomycorrhizal association.

3.3.3 Optimization of plant growth conditions

Several substrates and containers were tested in order to adjust the best methods to the growth habit of these shrubs. Seedlings (n=5) were placed in several types of sterilized sub-

Figure 3.5: Collection site (red circle) of seeds of *C. ladanifer* (A), *C. psilosepalus* (B) and *C. salviifolius* (https://www.google.pt/maps).

Table 3.3: Taxonomic information, origin, habitat and collection date of *Cistus* seeds used in this study.

Species	*Cistus ladanifer*	*Cistus psilosepalus*	*Cistus salviifolius*
Origin	Lousã, Coimbra, Portugal	Arzila, Coimbra, Portugal	Cantanhede, Coimbra, Portugal
Coordinates	40°05'24.6"N 8°13'37.9"W	40°11'08.5"N 8°32'45.3"W	40°21'01.9"N 8°41'39.7"W
Habitat	*P. pinaster*/*Cistus* spp.	*Q. faginea*/*Q. robur*/*C. psilosepalus*	*P. pinaster*/*Cistus* spp./*H. halimifolium*
Collection Date	sep/13	oct/14	oct/14

strates and in three types of containers. The tested substrates were mixtures of Vermiculite (crude) + Peat and Perlite + Peat, both with the following concentrations: 1:2, 1:1 and 2:1. MS agar (8g/l) medium (Murashige & Skoog 1962) was also used as substrate. Moreover, it was also added to the substrates with Vermiculite + Peat and Perlite + Peat about half of substrate's volume of BAF (Moser 1960) liquid medium modified reducing glucose to 15g/L. The containers tested were flasks ($\emptyset 5.5cm$), test tubes ($\emptyset 2cm$) and Petri dishes ($\emptyset 9cm$) for all substrates. Plant survival was qualitatively assessed every week, over a month. Afterwards, the best treatment was identified and modified on the basis of plant and fungal requirements, describing a novel method to synthetize ectomycorrhizas in these shrubs.

3.3.4 Ectomycorrhizal synthesis

Axenic ectomycorrhizal synthesis was performed in flasks using MS medium as substrate. Patches of fungal inoculum previously grew in PDA medium were placed on a ditch opened at the mycorrhization substrate. A sterilized aluminium disk was placed upon the culture medium, to protect roots and fungi against photo-oxidation (Martins 2008), and hollows were made for each seedling. Three plantlets were placed per flask at the inoculation time. An aluminium cover was also placed on the outside of the flask, coating fungi and roots. As control method, ectomycorrhizal synthesis in pure culture was also performed using the most Perlite + Peat (1:1) as substrate with addition of half of substrate's volume of BAF (Moser 1960) liquid medium modified reducing glucose to 15g/L and inoculated with 5ml of pure cultured fungal mycelium previously grew in liquid BAF medium (Moser 1960). All flask

were aseptically closed and placed in growth chambers at 20°C ±1 for 5-6 months under fluorescent lights (150μmol s-1 m-2 [400-700 nm], 16 h/day).

3.3.5 Mycorrhizas assessment

Plant roots were regularly checked for ECM establishment (macroscopic observation). When ECM establishment was detected (minimum 2 months), the ectomycorrhizal root tips were removed and examined under a stereomicroscope. Ectomycorrhizal roots were also photographed after and before their removal from MS medium (Fig. 3.8–3.15). Mycorrhizal root tips were fixed with an aqueous solution of lacto-glycerine (1:1:1) and further dehydrated and infiltrated with liquid paraffin sequentially in several steps: Ethanol 70% - 120min; Ethanol 70% - 120min; Ethanol 90% - 90min; Ethanol 90% - 90min; Ethanol 95% - 90min; Ethanol 100% - 90min; Ethanol 100% - 90min; Bio clear 90min; Bio clear 90min; Bio clear 90min; Paraffin (60°C) 120min; Paraffin (60°C) 120min (Rossi et al. 2006). Samples were included in paraffin blocks and sectioned with a microtome (5-7 mm thick) and stained with Toluidine blue (0.1%) (Ingleby et al. 1990), for subsequent microscopic observation of the mantle and cortical Hartig net typical from *Cistus* sp. ectomycorrhizas (Results not shown).

3.4 Results

3.4.1 Effect of several treatments on seed sterilization and germination

The different sterilization treatments had very diverse effects on seed's contamination and germination percentages of *Cistus ladanifer*, *Cistus salviifolius* and *Cistus psilosepalus*.

For *C. ladanifer*, almost all the sterilization treatments were efficient (0% contamination), with the only exception of "Alcohol (96%)" (5% of contaminated seeds). However, the maximal percentage of germinated seeds was 25% in "Distilled water at 100°C" and in "Alcohol (96%)" treatments (Table 3.4).

Seed contamination percentages for *C. psilosepalus* were lower (0%) in almost all treatments, with the most notorious exception of "Alcohol (96%)" and in "Hypochlorite (5%)" with 100% and 60% of contaminated seeds, respectively. Seed germination rates were higher at "Distilled water at 100°C" with 95% of germinations and lower (0%) in "Distilled water

at 100ºC + Hypochlorite (3%)", "Alcohol (96%) + Hypochlorite (3%)" and "Distilled water at 100ºC + Alcohol (96%) + Hypochlorite (3%)" (Table 3.5).

Seeds sterilization treatments in *C. salviifolius* was only truly efficient, with 0% of contaminations, in "Distilled water at 100ºC", "Distilled water at100ºC + Alcohol (96%)" and "Distilled water at 100ºC + Hypochlorite (3%)" treatments, and they are very inefficient in "Alcohol (96%)" with 45% of contaminations. By the other way, germination rates were the highest in "Distilled water at 100ºC" with 80% of germinations and the lowest in "Alcohol (96%)" and "Hypochlorite (3%)" with 0% of germinations (Table 3.6).

3.4.2 Effect of substrates and containers in plant behaviour for mycorrhizal synthesis purposes

Substrates had a clear effect on the behaviour of the three *Cistus* species. All shrubs died in Vermiculite + Peat substrate in all tested proportions (1:2/1:1/2:1) and in all containers, after one week, approximately. Containers had a more clear effect in the other substrates. Petri plates had the worst results, not sustaining them more than one week in Perlite + Peat in all proportions (1:2/1:1/2:1) and up to two months in MS medium. Plants behaved equally in all proportions (1:2/1:1/2:1) of Perlite + Peat and in MS medium in test tubes, not surviving more than two months. Flasks were the best containers of all, in which plants survived more than two months in MS, Perlite + Peat (1:1) and Perlite + Peat (2:1) as substrate. However, in flasks with Perlite + Peat (1:2) these shrubs survived only up to two months (Table 3.7).

Flasks using MS medium as substrate suffered some modifications, as can be observed in Figure 3.6, and were subsequently used for synthesis of ectomycorrhizas. Perlite + Peat (1:1), using flasks as containers, was chosen as a balanced substrate and used as control method for synthesis of ectomycorrhizas, as can be observed in Figure 3.7.

3.4.3 Establishment of Ectomycorrhizas

Using the proposed new methodology, ectomycorrhizas were obtained almost all plant-fungus combinations, with exception of *T. equestre* + *C. psilosepalus*, *T. equestre* + *C. salviifolius*, *T. portentosum* + *C. salviifolius* and *B. fragrans* + *C. ladanifer*, in which the

Table 3.4: Number and percentages of germination and contamination in *Cistus ladanifer* seeds exposed to several treatments.

Treatments	Contamination	Contamination %	Germination	Germination %
100ºC Distilled water	0	0%	5	25%
Alcohol	1	5%	5	25%
Hypochlorite	0	0%	2	10%
100ºC Distilled water + Alcohol	0	0%	2	10%
100ºC Distilled water + Hypochlorite	0	0%	4	20%
Alcohol + Hypochlorite	0	0%	3	15%
100ºC Distilled water + Alcohol + Hypochlorite	0	0%	3	15%

Table 3.5: Number and percentages of germination and contamination in *Cistus psilosepalus* seeds exposed to several treatments.

Treatments	Contamination	Contamination %	Germination	Germination %
100ºC Distilled water	0	0%	19	95%
Alcohol	20	100%	1	5%
Hypochlorite	12	60%	1	5%
100ºC Distilled water + Alcohol	0	0%	2	10%
100ºC Distilled water + Hypochlorite	0	0%	0	0%
Alcohol + Hypochlorite	2	10%	0	0%
100ºC Distilled water + Alcohol + Hypochlorite	0	0%	0	0%

Table 3.6: Number and percentages of germination and contamination in *Cistus salviifolius* seeds exposed to several treatments.

Treatments	Contamination	Contamination %	Germination	Germination %
100ºC Distilled water	0	0%	16	80%
Alcohol	9	45%	0	0%
Hypochlorite	2	10%	0	0%
100ºC Distilled water + Alcohol	0	0%	1	5%
100ºC Distilled water + Hypochlorite	0	0%	1	5%
Alcohol + Hypochlorite	2	10%	1	5%
100ºC Distilled water + Alcohol + Hypochlorite	1	5%	2	10%

Table 3.7: *Cistus* average response in different substrates and containers. "-" Plants died after one week ; "±" Plants survived up to two months; "+" Plants survived after more than two months.

Substrates / Conteiners	Vermiculite+Peat (1:2)	Vermiculite+Peat (1:1)	Vermiculite+Peat (2:1)	Perlite+Peat (1:2)	Perlite+Peat (1:1)	Perlite+Peat (2:1)	MS
Flasks	-	-	-	±	+	+	+
Test tubes	-	-	-	±	±	±	±
Petri plates	-	-	-	-	-	-	±

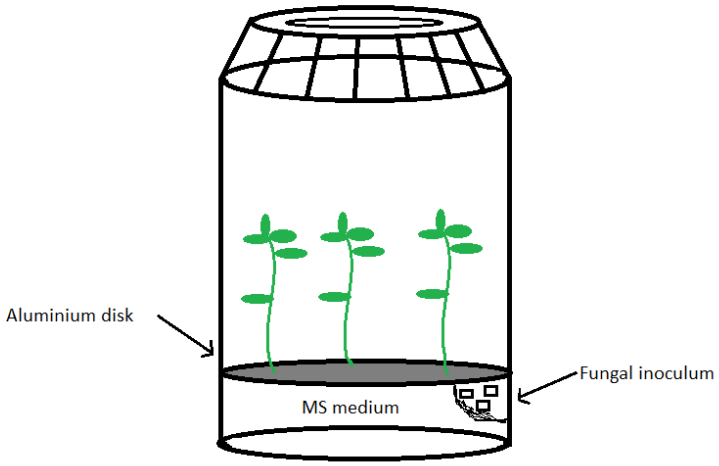

Figure 3.6: Representation of a proposed novel method adapted for the synthesis of mycorrhizas in *Cistus* spp.

association was not yet established, as can be observed at Table 3.8. External morphology of ectomycorrhizal tips of each plant-fungi combination can be observed in Figures 3.8–3.15. Because mycorrhizas were very young and in early stages of development, it was very difficult to describe their morphological features. It was not yet obtained either the ectomycorrhizas growing in Perlite + vermiculite, in which the association is not mature. Experiments are still ongoing in order to verify the compatibility the rest of plant-fungus combinations which were not yet obtained probably due to several factors, being the compatibility between partners probably one of them. Assays with Perlite + Peat as mycorrhization substrate are still ongoing.

3.5 Discussion

Overlapping the lowest contamination with the highest germination percentages, the best seeds' treatment for all the three species of *Cistus* was "Distilled water at 100°C". Alcohol (96%) was the most inefficient sterilizing agent with the highest contamination rates. In addition, alcohol is reported as being very phytotoxic (Kern et al. 2009), which was not verified

Figure 3.7: Control method based on flasks with Perlite + Peat (1:1) as substrate for synthesis of mycorrhizas in *Cistus* spp.

Figure 3.8: Ectomycorrhizal root tips of *Lactarius deliciosus* in *Cistus psilosepalus* synthetized *in vitro* using the proposed method, observed through MS culture medium (A, C) and under a stereomicroscope (B).

Figure 3.9: Ectomycorrhizal root tips of *Boletus fragrans* in *Cistus psilosepalus* synthetized *in vitro* using the proposed method, observed through MS culture medium (B) and under a stereomicroscope (A, C).

Figure 3.10: Ectomycorrhizal root tips of *Tricholoma portentosum* in *Cistus psilosepalus* synthetized *in vitro* using the proposed method (C) observed through MS culture medium (A,B).

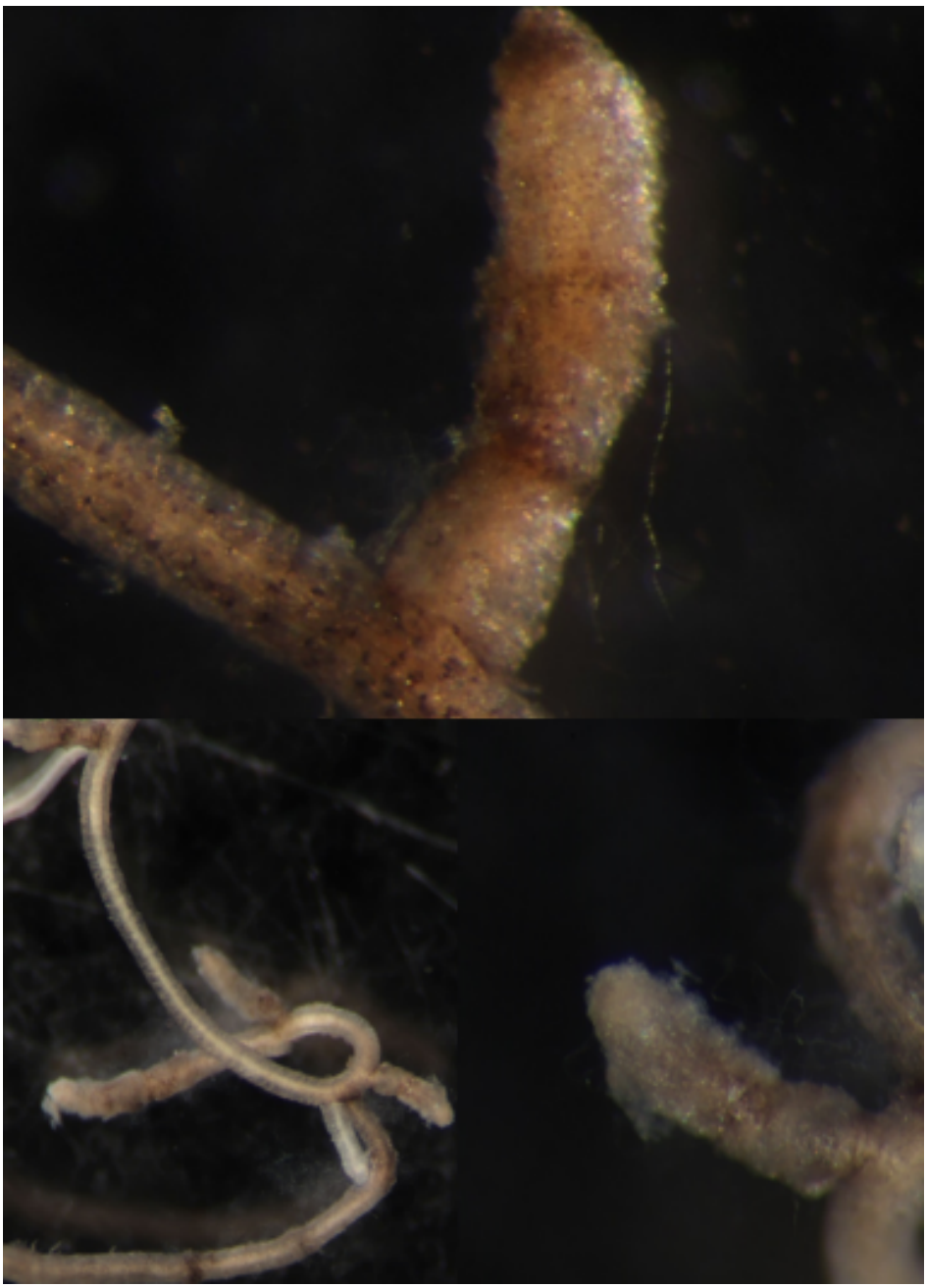

Figure 3.11: Ectomycorrhizal root tips of *Boletus fragrans* in *Cistus salviifolius* synthetized *in vitro* and observed under a stereomicroscope.

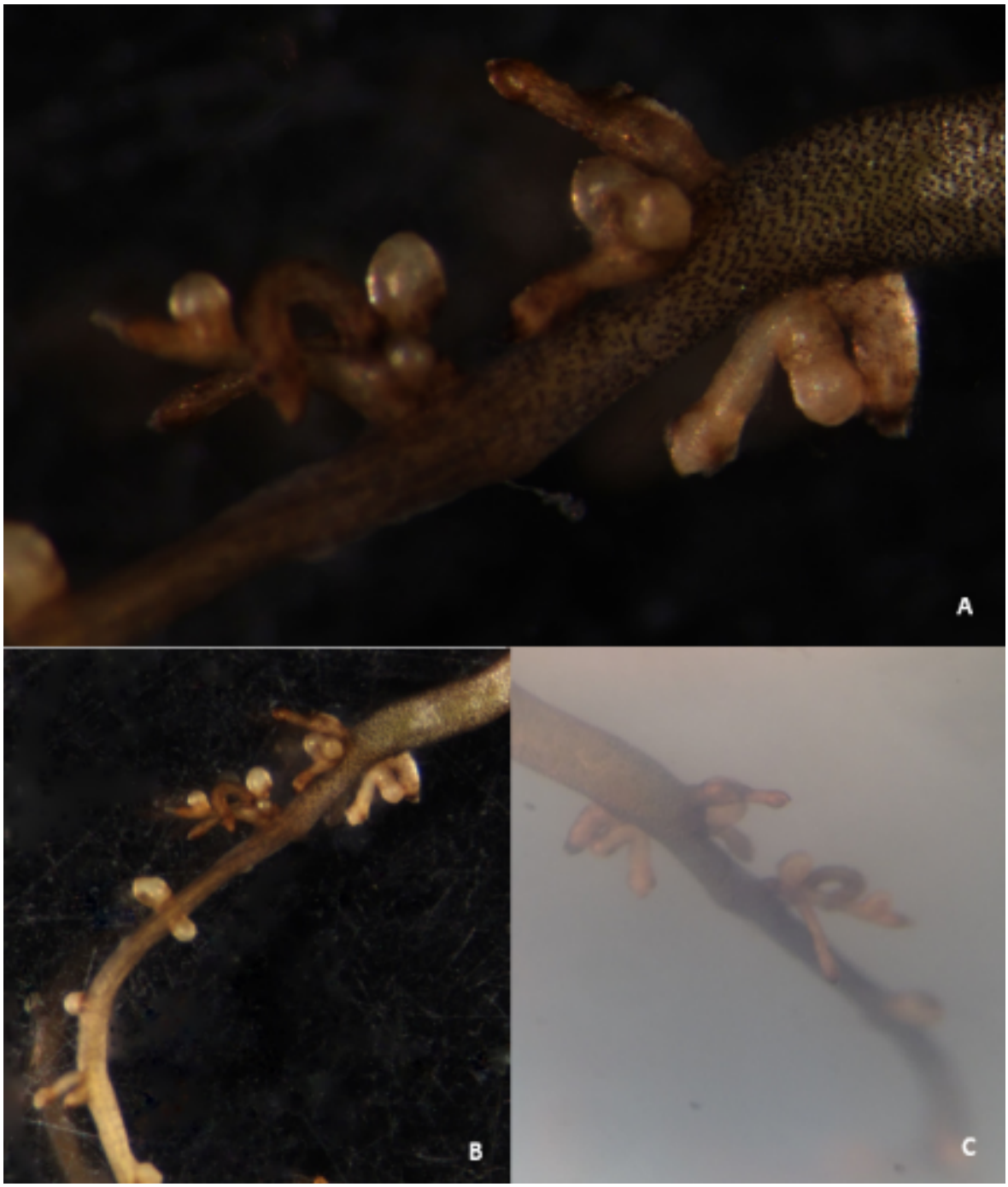

Figure 3.12: Ectomycorrhizal root tips of *Lactarius deliciosus* in *Cistus salviifolius* synthetized *in vitro* using the proposed method, observed through MS culture medium (C) and under a stereomicroscope (A, B).

Figure 3.13: Ectomycorrhizal root tips of *Lactarius deliciosus* in *Cistus ladanifer* synthetized *in vitro* using the proposed method (C), observed through MS culture medium (A, B).

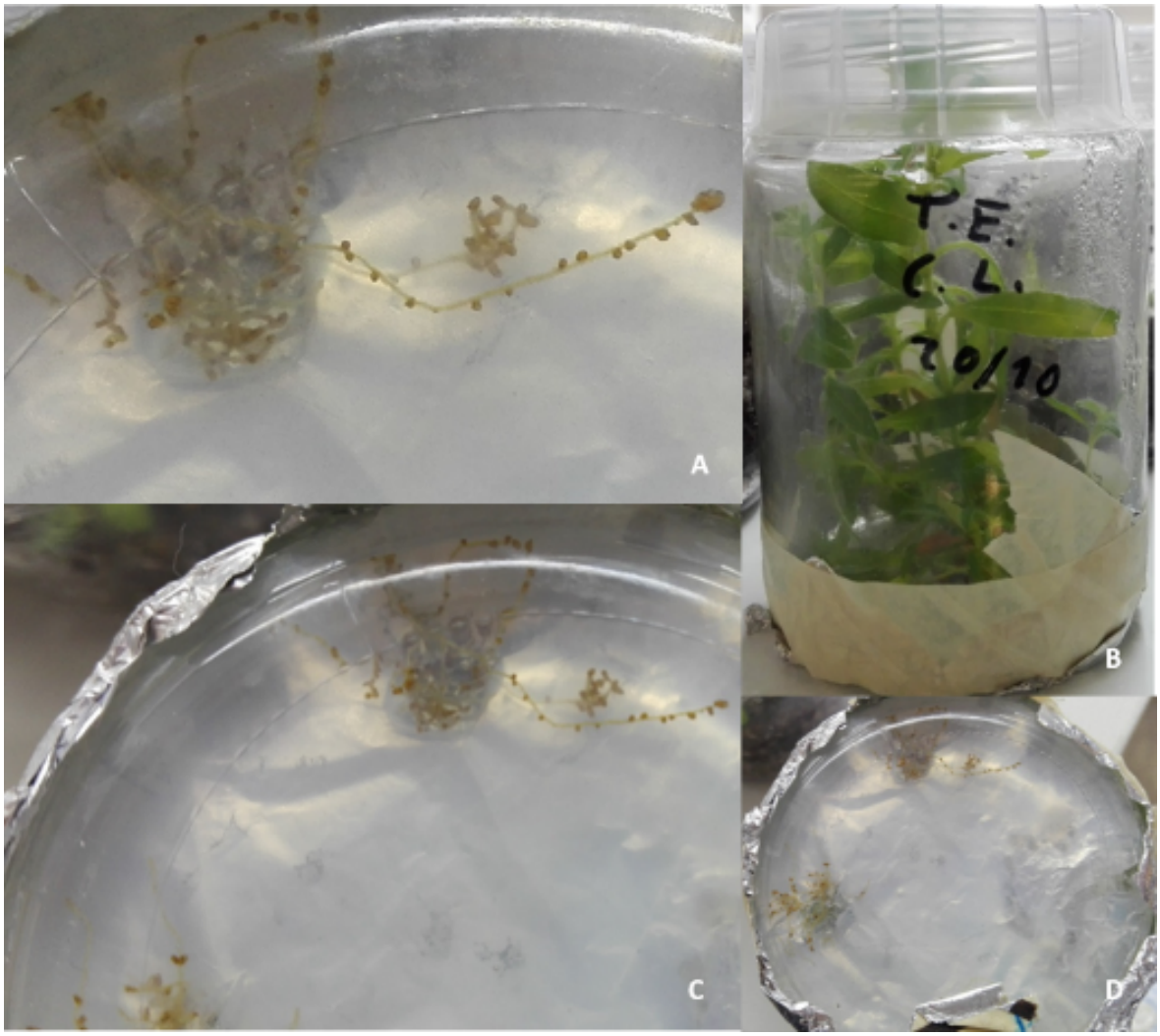

Figure 3.14: Ectomycorrhizal root tips of *Tricholoma equestre* in *Cistus ladanifer* synthetized *in vitro* using the proposed method (B), observed through MS culture medium (A, C, D).

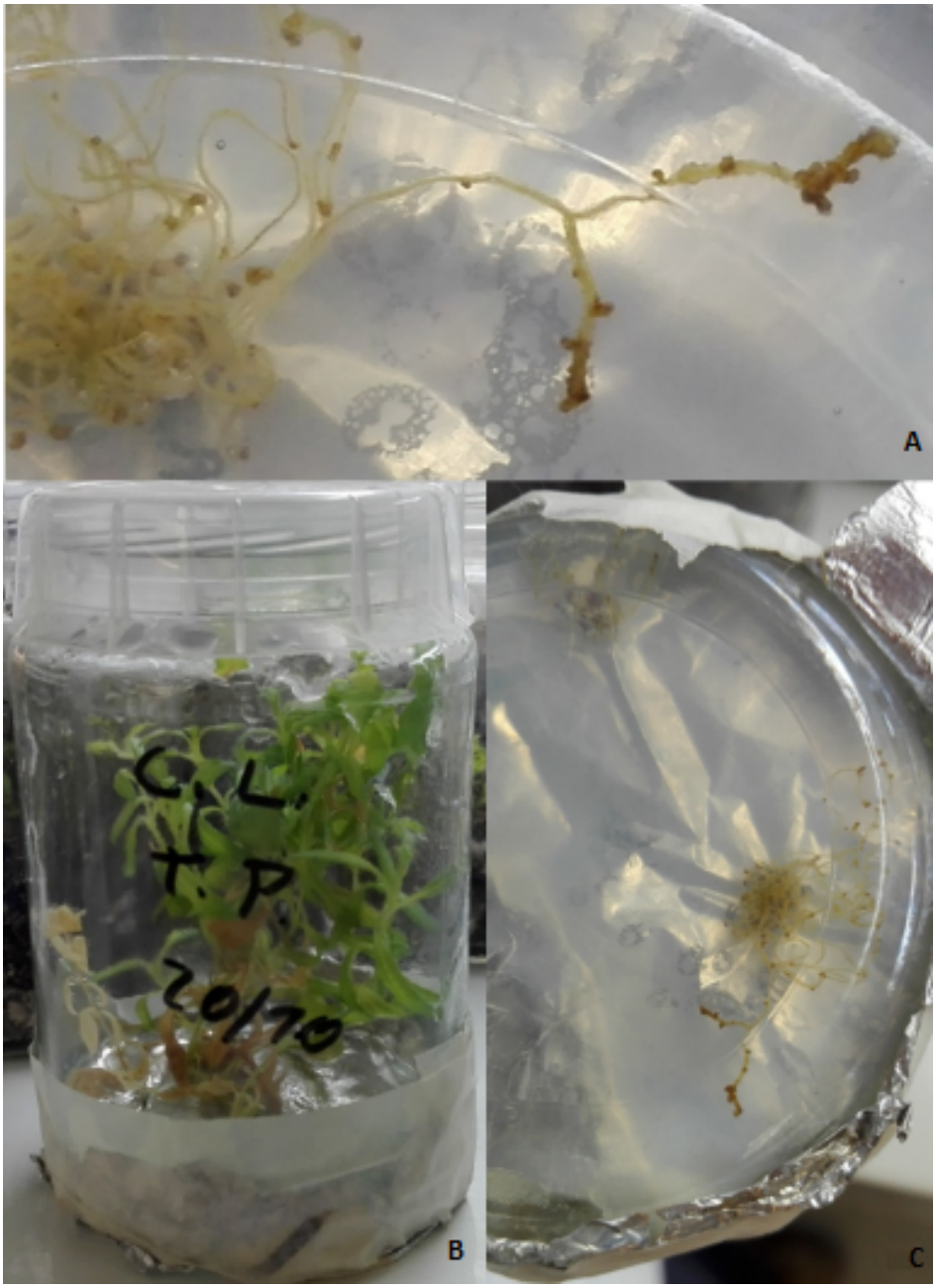

Figure 3.15: Ectomycorrhizal root tips of *Tricholoma portentosum* in *Cistus ladanifer* synthetized *in vitro* using the proposed method (B), observed through MS culture medium (A, C).

Table 3.8: Results of the combinations between three *Cistus* species and four edible ectomycorrhizal fungi. "+" successful establishment of ectomycorrhizas; "-" unsuccessful establishment of ectomycorrhizas.

Species	*C. psilosepalus*	*C. salviifolius*	C. ladanifer
B. fragans	+	+	–
L. deliciosus	+	+	+
T. equestre	+	–	+
T. portentosum	–	–	+

in our results, in particularly with *C. ladanifer* in which "Alcohol (96%)" treatment provided the highest germination rates along with "Distilled water at 100°C". Sodium hypochlorite (3%) was a better sterilization agent compared with Alcohol (96%) but even so, it was ineffective sterilizing *C. psilosepalus* and *C. salviifolius* seeds, with the aggravating factor that with a long time of exposure seeds became bleached and inviable, which could explain the low germination rates (when seeds were exposed to this agent). "Distilled water at 100°C" was the most efficient sterilization treatment, due probably to the fact that moist heat helped in fungal spores germination, reducing by other way their resistance to heat (Cenkowski et al. 2007). In addition, heat favours the germination of pyrophytic Cistaceae seeds (Alonso et al. 1992, Thanos et al. 1992, Ferrandis et al. 1999, Pérez-Fernández & Rodríguez-Echeverría 2003, Águeda et al. 2006), supporting the results that "Distilled water at 100°C" treatment enhanced *Cistus* spp. germination. Regarding the other combined treatments as "Distilled water at 100°C + Alcohol (96%)", "Distilled water at 100°C + Sodium Hypochlorite (3%)", "Distilled water at100°C + Alcohol (96%) + Sodium Hypochlorite (3%)" and "Alcohol (96%) + Sodium Hypochlorite (3%)", no conclusions can be taken with the present sampling and assays.

Regarding the optimization of plant growth conditions for mycorrhizal synthesis in *Cistus* spp., the containers had a clear effect on the development of these shrubs, which grow better in spacious flasks, probably due to the their rounded, upright or prostrate shrubby habit (Lenard

2008), depending on the species (http://www.cistuspage.org.uk), as well as their high growth rates (Patón et al. 1998, Oñate & Munné-Bosch 2008). Petri plates, as well as test tubes, were too small for *Cistus* spp. growth, and, after some time, plants have contacted with the containers walls with the consequence of plant tissues' rotting, being Petri plates more unsuitable than test tubes for these shrubs. However, our results are in contrary to the works of Águeda et al. (2006) and Zaretsky et al. (2006), which used, with positive results, test tubes to synthetize mycorrhizas in *Cistus* spp. Nevertheless, other studies regarding mycorrhizal synthesis in *Cistus* spp., used sterilized substrate in pots (Bustan et al. 2006, Zitouni-Haouar et al. 2014), which corroborates our hypothesis that wide containers are more suitable for these shrubs' habit. Concerning substrate optimization for plant growth, Vermiculite + Peat, in all proportions, had a strong negative effect on plant growth, while Perlite + Peat (1:1 and 2:1) and MS medium sustained healthy plants for a long time. This could probably be caused by the fact that crude vermiculite used, composed of flakes of hydrated phlogopite mica, along with peat and watering solution, created a very compact substrate in all proportions, while Perlite creates more aerated substrates, more suitable for *Cistus* spp. that inhabit manly in semi-arid areas (Arrington & Kubitzki 2003, Comandini et al. 2006) with siliceous and sandy soils (Demoly & Montserrat 1993) cit. (Águeda et al. 2006). Our results are in contrary with those of Águeda et al. (2008) that successfully obtained mycorrhizas in *Cistus* spp. using vermiculite + peat and with those of Bustan et al. (2006), which claimed that substrates with vermiculite, compared with substrates using perlite on their composition, were optimal for synthesis of mycorrhizas. This inconsistency could be due to the type of vermiculite used, which can be crude vermiculite that we used in our work, or exfoliated vermiculite, composed by concertina-shaped particles much more bulky that crude type and very widespread also. This different type of bulky vermiculite could have a distinct effect in substrate compaction.

The plant behaviour in MS medium was expected, because MS is quintessentially a plant culture medium that responds to plant nutritional requirements (Murashige & Skoog 1962) used also for mycelial cultures with very positive results (Maia & Yano-Melo 2001, Nasim et al. 2001, Idnurm 2010, Sanmee et al. 2010). Modified MS medium (with glucose) was also used for axenic synthesis of mycorrhizas between *Castanea sativa* and *Pisolithus tinctorius* (Martins 2008). The lack of exogenous glucose in any medium/substrate, allows to test the

real affinity between two possible partners (Duddridge 1986). Using MS agar medium (non-modified) for mycorrhizal synthesis is an ideal substrate for testing partners' compatibility and has also the advantage of an easy perception of when the establishment of the association occurs.

Regarding the *in vitro* synthesis, mycorrhizas were not obtained in several plant-fungus combinations, at least during the assay period, and probably due to a wide range of factors as plant death, fungi death, and the putative incompatibility between partners. All these factors needed to be evaluated in each case and new experiments should be done with the same and with different strains before concluding that partners are incompatible with each other. The only plant-fungus combination that intervenors seemed to be incompatible was *C. ladanifer* + *B. fragrans*, due to the fact that all the plant died in the presence of the fungus, in all replicates. A possible explanation, which deserves further investigation, is that in the absence of glucose the fungus became aggressive in the search for nutrients. The plant youth and stress condition could have contributed to plant death. This facultative necrotrophic fungal ability when exposed to stress have been referred by many authors (Brundrett 2004, Zeilinger et al. 2016). Another evidence of partner incompatibility is the production of phenolic compounds, which were observed on the brownish coloration of MS culture medium a week after fungal inoculation. This coloration could probably be due to the production of fungal exudates, as reported in "Chapter 2", or could be produced by plant roots in response to fungal invasion (Giovannetti & Lioi 1990) and observed on the dark/brownish coloration of the dead roots. Contrarily to our results, Águeda et al. (2008) achieved the association of three *Boletus* species with *Cistus ladanifer*, but didn't successfully achieved the association of *Boletus pinophilus* with this shrub species, demonstrating that congeneric species could have very distinct responses with the same species.

In the other failed plant-fungi combinations, in spite of that result, both plant and fungus were developing positively probably towards the establishment of the association. Also, very similar partnerships have been reported with *Tricholoma* spp. and *Cistus* spp. associated in natural conditions (Lavorato 1991, Vila & Llimona 2002, Comandini et al. 2006) and we expect that will be successfully achieved in the future after overcoming problems as contaminations and lack of plant material.

The successfully associations that, to our knowledge, have been reported for the first time in this work, support the plasticity of *Cistus* shrubs in establishing ectomycorrhizal associations with a wide range of fungal taxa as reported by Comandini et al. (2006). The ectomycorrhizal fungal species used in this work are considered "Broad host range" fungi (Molina et al. 1992) due to being able to establish mycorrhizal association with a wide number of plant species, belonging to diverse families, such as the association of *B. fragrans* with *Castanea* sp. (Rodríguez & de Ana Magán 1993), ectomycorrhizas of *L. deliciosus* with *Pinus* spp. (Guerin-Laguette et al. 2000, Díaz, Carrillo & Honrubia 2009) and the ectomycorrhizal association of *T. equestre* and *T. portentosum* in *Pinus densiflora*(Yamada et al. 2001, 2007). Although these plant - fungus combinations were not reported before, there are descriptions of the occurrence of *Lactarius deliciosus*, *Boletus* spp. and *Tricholoma* spp. sporocarps near *Cistus* spp. (Lavorato 1991, Zervakis et al. 2010, Vila & Llimona 2002) cit. (Comandini et al. 2006).

We found that mycorrhizal tips obtained by the proposed new method for mycorrhizal synthesis were very young and in early stages of development, which suggests that longer experiments are necessary in order to obtain mature ectomycorrhizas, suitable to morphological and anatomical characterization as well as evaluating the real potential of using *Cistus* spp. for sporocarp cultivation of these edible ectomycorrhizal species. It will be important to set up new experiments to compare plant-fungus combinations and ectomycorrhizas already obtained in agarized MS medium with other methods (as the control method with Perlite + Peat (1:1)) and that were not yet performed due to several external factors such as contaminations, low plant growth, lack of plant material and time restrictions.

Chapter 4

Morphologic characterization and Molecular Identification of Ectomycorrhizal root tips from *Cistus* spp. and *Pinus pinaster*

4.1 Introduction

The genus Mediterranean *Cistus* is represented by 12 species of shrubs in the Iberian Peninsula (Águeda et al. 2006, Alonso Ponce et al. 2011), 9 of which are native in Portugal (http://www.flora-on.pt) occupying 7% of the national forested area (Godinho-Ferreira et al. 2005). Being woody, evergreen and pyrophytic shrubs (Arrington & Kubitzki 2003, Comandini et al. 2006) all species of *Cistus* are early colonizers after a disturbance of fire or grazing, and so pioneer species of ecological successions (Correia 2002, Águeda et al. 2006, Comandini et al. 2006, Alonso Ponce et al. 2011). *Cistus* shrubs act also as a mycorrhizal fungal diversity and nutrients reservoir, having the ability to form ectomycorrhizas as well as arbuscular mycorrhizas (Simões et al. 2001, Smith & Read 2008) with many fungal species. Concerning ECM, these shrubs are known to establish ectomycorrhizal association with about 230 species of Ascomycota and Basidiomycota fungi, in which 35 are *Cistus*-specific mycobionts,

from Basidiomycota phylum and belonging nearly all to Russulaceae and Cortinariaceae families (Comandini et al. 2006). Due to all these features, *Cistus* shrubs have been considered potential candidates for the production of edible ectomycorrhizal sporocarps (Águeda et al. 2008).

There are approximately 25000 species of fungi able to establish ectomycorrhizal associations (Tedersoo et al. 2012, Tedersoo & Smith 2013, Rincón et al. 2015). These fungi have a fundamental role in ecosystems dynamics, helping in maintenance of their plant partners' fitness by assisting them in nutrient and water uptake (Smith & Read 2008, Itoo & Reshi 2014) as in many other aspects, as described in "Chapter 2". When establishing the association with fine roots, ectomycorrhizal fungi form typical structures, namely mantle (or sheath), Hartig net and extraradical mycelium (divided into rhizomorphs and emanating hyphae) (Smith & Read 2008), as detailed in "Chapter 3". The difference of ectomycorrhizal morphotypes from the other types of mycorrhizas, which makes their detection easy, lies in sheath's presence and thickness, which modifies root morphology, and in plant taxonomy (Brundrett 2004, Smith & Read 2008, Tedersoo et al. 2010). Ectomycorrhizal morphotypes are different between taxonomic groups and the analysis of their structural (anatomy) and morphological features, by a technique called morphotyping (Agerer 1989), can be used to taxonomically identify ectomycorrhizal fungi (Suz et al. 2008). Besides being a time-consuming technique to identify ectomycorrhizal fungal taxa, morphotyping had several issues as fungal subjective morphology that both could be equivalent among different taxa, or could vary among the same taxon with different plant partners, environments and morphotype age (Egger 1995, Menkis et al. 2005, Pestaña Nieto & Santolamazza Carbone 2009). In addition to that, the number of species that are still to identify is still very high (Nylund et al. 1995, Pestaña Nieto & Santolamazza Carbone 2009, Rincón et al. 2015), existing a wide range of morphologies unknown being impossible to proceed with a precise taxonomic identification in those cases. Classifying ectomycorrhizal root tips into "ad hoc morphological groups" (Dickie & Reich 2005) allows to make a morphological classification not so exhaustive than morphotyping (Agerer 1989), being a very good sorting method before further analysis, as the molecular approach (Dickie & Reich 2005, Walbert et al. 2010).

Several methodologies have been used to survey ectomycorrhizal fungal communities. The

identification and counting of sporocarps is an indirect and not a very accurate approach, due to the fact that many fungi do not fructify above ground as for example many dominant species from Ascomycota, Corticiaceae and Thelephoraceae taxa (Gardes & Bruns 1993, Gehring et al. 1998, Dahlberg 2001, Pestaña Nieto & Santolamazza Carbone 2009). In addition, sporocarp survey do not precisely correlates fungal sporocarps with the plant partners, not reflecting with precision the natural associations and fungal community structure (Horton & Bruns 2001, Comandini et al. 2006, Pestaña Nieto & Santolamazza Carbone 2009).

Molecular techniques as amplification of Barcoding regions through Polymerase chain reaction (PCR) technique allowed more accurate fungal diversity surveys by identification of fungal taxa present in roots and soil. DNA Barcoding regions were, for the first time, used and described in 2003 as DNA short sequences capable of distinguish different taxa (Hebert, Cywinska, Ball & DeWaard 2003) and are nowadays defined as sequences of 500-800bp (base pair) standard length used to identify all eukaryotic species amplified with primers that can be used in a wide range of taxa (Schoch et al. 2012). Barcoding sequences should be constant and unique for each species (Hebert, Cywinska, Ball & DeWaard 2003, Hebert, Ratnasingham & DeWaard 2003, Schoch et al. 2012). Their interspecific variability should be higher than intraspecific and barcode *loci* ought to be the same for all kingdoms (Schoch et al. 2012). Cytochrome c oxidase subunit 1 (CO1) is the *locus* accepted for all organisms since 2005 (Schindel & Miller 2005), but due the inconsistent results of CO1 region in fungi, nuclear rDNA internal transcribed spacer (ITS) region has been used since many years ago and was proposed as the Barcoding marker in fungi (Seifert 2009, Schoch et al. 2012). ITS region comprises two non-coding and variable sequences (called introns) located between three highly conserved coding-regions (called exons) and varying among 600-800bp length. In addition to length, ITS region has more features that made it an ideal barcode marker as revealing interspecific variation combined with the fact of having low intraspecific variation (Gardes & Bruns 1993, Horton 2002, Ragonezi et al. 2013). ITS primers, taking advantage of the conserved coding-regions to amplify the sequences of variable introns, were designed for the first time to successfully amplify in a wide range of organisms as animals and plants (White et al. 1990), being a problem when working with mycorrhizal symbiosis, where both plant and fungi DNA are present. The ITS1F-ITS4 primer pair that combines the amplification

of fungal DNA from Ascomycota and Basidiomycota taxa with a very faint amplification of plant DNA (Gardes & Bruns 1993), has been used in many works of ectomycorrhizal fungi barcoding in roots (Chen & Cairney 2002, Dickie & Reich 2005, Genney et al. 2006, Walbert et al. 2010), being not always necessary a secondary barcode region for species identification (Seifert 2009). Novel techniques as Next Generation Sequencing (NGS) can be used to make the most accurate assessment of soil fungal diversity on roots and soil by amplification of barcoding regions of samples with diverse fungal DNA at the same time, but such analysis do not allow to relate fungal morphology along with taxa (Rincón et al. 2015). Combining morphological characterization of ectomycorrhizal root tips sorted by "ad hoc morphological groups" (Dickie & Reich 2005) with a molecular approach by amplification of ITS region from individual morphological types enables to have root tips' morphology along with an accurate identification (Horton & Bruns 2001).

Only a few works have related ectomycorrhizal root tip morphology with an accurate taxonomic identification in *Cistus* spp. (Águeda et al. 2006, 2008), existing although some diversity surveys that do not report root tips' morphology (Buscardo et al. 2012). There are, to our concern, only eight ectomycorrhizal morphological types described in *Cistus* spp., highlighting the lack of knowledge of ectomycorrhizal root tips' morphology in *Cistus* spp. The existing morphological types described are: *Tuber melanosporum* + *Cistus incanus* (Fontana & Giovannetti 1978, Fusconi 1983, Wenkart et al. 2001, Bustan et al. 2006), *Laccaria laccata* + *Cistus ladanifer* (Torres et al. 1995), *Boletus rhodoxanthus* + *Cistus ladanifer* (Hahn 2001), *Lactarius tesquorum* + *Cistus* sp. (Nuytinck et al. 2004), *Boletus edulis* + *Cistus ladanifer* (Águeda et al. 2006), *Boletus aereus* + *Cistus ladanifer* (Águeda et al. 2008), *Boletus edulis* + *Cistus albidus* (Águeda et al. 2008), *Boletus reticulatus* + *Cistus albidus* (Águeda et al. 2008).

Pinus pinaster, commonly called "maritime pine" is a species distributed along the western Mediterranean basin (Campelo et al. 2015), habiting mainly, but not only, in acid and silicon costal soils (http://www.flora-on.pt) and it is economically very important for paper and wood industries (Berthier 2001). Maritime pine is one of the most important forest species in Portugal (Pereira 2002, Campelo et al. 2015), covering 27.3% of national forested area (Fig 4.1), being the most extensively distributed forest species (Godinho-Ferreira et al.

2005). This tree species had the ability to resist drought and high levels of salinity, allowing it to inhabit coastal habitats (Berthier 2001) and have been therefore cultivated in Portugal since ancient times to stabilize dunes Campelo et al. (2015). One feature responsible for the ability of maritime pine to successfully inhabit and colonize harsh habitats is its ability to form mycorrhizas with a wide range of fungal symbionts (Pera & Alvarez 1995, Pestaña Nieto & Santolamazza Carbone 2009). All Pinaceae are reported as establishing strictly ectomycorrhizal associations, although there are some evidences and observations of vesicular-arbuscular mycorrhizas in *Pinus* sp. and in other Pinaceae (Horton et al. 1998). Some ectomycorrhizal diversity surveys have been done until nowadays in *Pinus* spp. through molecular approach identifying individual ectomycorrhizal root tips (Pestaña Nieto & Santolamazza Carbone 2009, Buscardo et al. 2010, Cox et al. 2010, Walbert et al. 2010, Buscardo et al. 2011, 2012, Jarvis et al. 2013) and identifying soil and ectomycorrhizal fungi in roots by NGS techniques (Rincón et al. 2015), but very few relating ectomycorrhizal morphology with DNA barcoding (Pestaña Nieto & Santolamazza Carbone 2009, Walbert et al. 2010).

It is known that *Pinus pinaster* shares its ectomycorrhizal fungal partners with shrubs in pinewood understory, namely with *Cistus* spp. (Buscardo et al. 2012). This exchange of fungal symbionts is very important to maintain fungal inoculum in soil after a tree's disturbance as cutting, grazing and fire (Torres et al. 1995, Díez 1998, Águeda et al. 2006, Comandini et al. 2006). In a biotechnological perspective, this sharing could be very positive when is desired to have a pinewood producing specific edible ectomycorrhizal sporocarps due to the fact that inoculated *Cistus* spp. planted near old pines could act as a natural inoculum source.

"Perímetro Florestal Dunas de Cantanhede" is a forested area dominated by *Pinus pinaster* with *Cistus* spp. and *Halimium* sp. understory located on Portugal west coast (Campelo et al. 2015, Carvalho et al. 2015) known for its macromycetes richness, in particularly ectomycorrhizal fungi (Gonalves et al. unpublished data), being a suitable site for studying of ectomycorrhizal fungi communities.

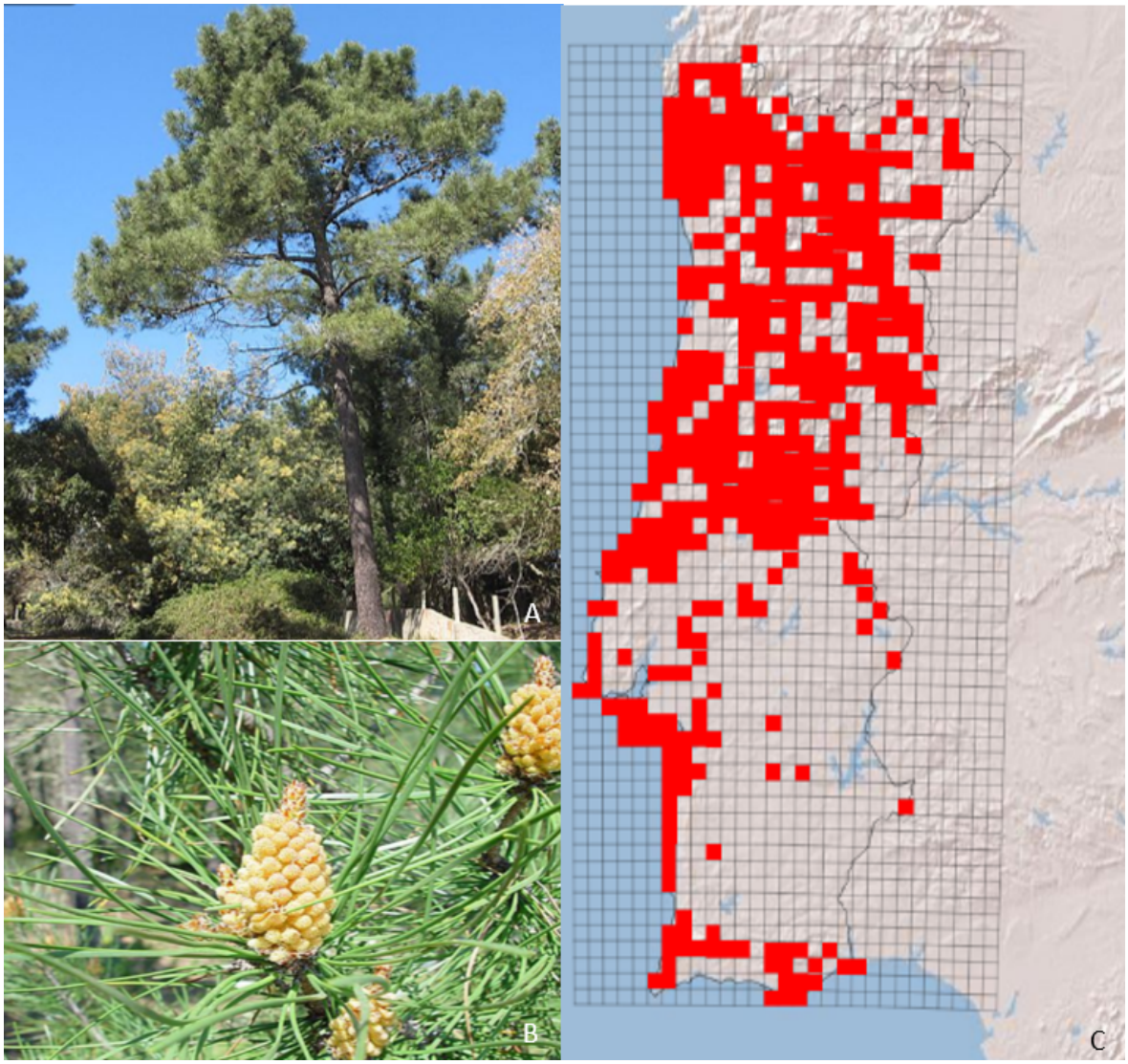

Figure 4.1: *P. pinaster* shoot system (A), flower (B) and distribution (C) (http://www.flora-on.pt).

4.2 Objectives

The aim of this work is to make a diversity survey in "Perímetro Florestal Dunas de Cantanhede" of ectomycorrhizal fungi associated with *Cistus* spp. and *Pinus pinaster* by sorting ectomycorrhizal root tips into "ad hoc morphological groups" and further molecular identification by DNA barcoding using ITS region; to record morphological appearance of ectomycorrhizal root tips of each fungal taxon associated with each plant partner; to briefly evaluate the extension of fungal partners sharing network between *Cistus* and *Pinus pinaster*; and finally try to assess the biotechnological potential of *Cistus* spp. for edible ectomycorrhizal sporocarps production and as inoculum source in pinewoods.

4.3 Materials and Methods

4.3.1 Study site

The study site was located at Cantanhede (40º21'35"N, 8º"49'10"W; Cantanhede, Coimbra, Portugal) and is called "Perímetro Florestal Dunas de Cantanhede" on Portugal west coast, represented in Figure 4.2. The area is dominated by *Pinus pinaster* trees with an average age of 45 years (Carvalho et al. 2015), with *Acacia longifolia* (invasive species), *Cistus psilosepalus*, *Cistus salviifolius*, *Halimium halimifolium* and *Corema album* as dominant understory. The silicon sandy soil is acidic with low water-holding capacity and the climate is typically Mediterranean with oceanic influence. The average annual temperatures and precipitation are 16.2ºC and 953mm, respectively (Campelo et al. 2015).

4.3.2 Root sampling and analysis

A square $10mx10m$ was marked within the study site in an area with the requirement of having at least 3 individuals of each studied species *Pinus pinaster*, *Cistus salviifolius* and *Cistus psilosepalus* (Fig. 4.3). Three individuals (n=3) for each species were randomly marked with plastic tags. Root samples were collected in mid-November using the methodology described by Pestaña Nieto & Santolamazza Carbone (2009) that consists in digging around the tree or shrub, in all four cardinal points, using a trowel and following the root

Figure 4.2: Study site surrounded by a red circle where roots were collected, called "Perímetro Florestal Dunas de Cantanhede" (40º21'35"N, 8º"49'10"W; Cantanhede, Coimbra, Portugal) (https://www.google.pt/maps).

Figure 4.3: Square ($10mx10m$) with marked *Pinus pinaster*, *Cistus psilosepalus* and *Cistus salviifolius* (arrows).

system from the base of each plant to a maximum depth of 10-20 cm (Fig. 4.4). Roots were collected based on the higher amount of fine secondary roots surrounded by soil clods, representative of higher amount of ectomycorrhizal root tips, and placed carefully along with soil clods into plastic bags for further laboratory analysis. Soil was separated from samples using a sieve. Roots were weighed and subsequently washed and stored at 4°C. Only 80g of ectomycorrhizal root tips of each individual were analysed under a stereomicroscope and sorted into "ad hoc morphological groups" (Dickie & Reich 2005) based on shape, ramifications, texture, colour and presence of emanating hyphae or rhizomorphs (Agerer 1989). Ectomycorrhizal root tips from each morphological group were photographed using a Leica®stereomicroscope. A subsample of each morphological group was placed in individual Eppendorf tubes soaked in alcohol (96%) and stored at -20°C. Some dubious root tips were cleared with KOH (10%) at 90°C for 1 hour, washed with distilled water and acidified with HCL (1%) overnight, and were sorted after microscopic observation of the mantle (Ingleby et al. 1990).

4.3.3 Molecular and data analysis

Ectomycorrhizal root tips were cleaned and 1 mm^2 of each root tip were used to DNA extraction. DNA was extracting using a REDExtract-N-Amp^{TM} (SIGMA-ALDRICH®Company) modified protocol adding 20μL of Extract solution to ectomycorrhizal tips and submitting it to 94°C for 10 min, 60°C for 13 min and 10°C for 15 min. In addition, an equal volume

Figure 4.4: Method for sampling of ectomycorrhizal roots as described by Pestaña Nieto & Santolamazza Carbone (2009).

of Dilution solution was added. The genomic extract was diluted 1:20 and quality of samples were assessed using NanoDrop^{TM} spectrophotometer before Polymerase Chain Reaction (PCR). PCR was performed using primer pair ITS1F/ITS4 and JumpStart^{TM} Taq DNA Polymerase without $MgCl_2$ (SIGMA-ALDRICH®Company) that was added subsequently according to manufacturers instructions. Only 1μL was used as DNA template. The termocycling parameters used were the following: 1 step of 94°C for 5 min, 33 cycles of 94°C for 45s ,57°C for 35s and 72°C for 35s, and 1 step of 72°C for 10 min. Amplifications were validated with an Electrophoresis using 2% agaroses gel. Single banded samples were further sequenced by the modified Sanger method performed by StapVita®, Portugal. Acquired sequences were edited using Geneious®software, sequences quality (HQ) was assessed and low quality sequences were discarded. A Basic Local Alignment Search Tool (BLAST) was performed in National Center for Biotechnology Information (NCBI) database to confirm species taxonomic identification. Species identification was performed using only 97% of sequence similarity. Sequences that were identified as the same taxon were assembled, using

Geneious®software (Biomatters). The maximum identity (%) was calculated and sequences with 97% of similarity were considered the same species. The Diagram of ectomycorrhizal fungal partners sharing between the assessed plant species was constructed using Fusion Tables application (http://www.tables.googlelabs.com), while Venn diagram was created using online application from Bioinformatics & Evolutionary Genomics online platform of University of Gent, Belgium (http://www.bioinformatics.psb.ugent.be).

4.4 Results

4.4.1 Fungal species identification

After the first screening, the number of "ad hoc morphological groups" sorted per plant species were: *C. salviifolius* 122, *C. psilosepalus* 110 and *P. pinaster* 62. A total of 105 different ectomycorrhizal morphological types were selected after a second screening (more exhaustive). After the amplification, the direct sequencing of 61 ectomycorrhizal samples (single banded) revealed the presence of 20 different fungal taxa, being 12 Basidiomycota while the other 8 taxa were Ascomycota. In 20,8% of the sequences, it was not possible to get an accurate taxonomic identification (genus/species), identifying only subphylum Pezizomycotina in three samples, and order Helotiales in two samples, which belong also to Pezizomycotina subphylum, all samples from Ascomycota phylum. These unidentified species were considered as ectomycorrhizal fungi. Fungal species *Oidiodendron* sp. *Oidiodendron maius* and *Fusarium oxysporum* were found in ectomycorrhizal root tips and were considered as non-ECM root inhabiting fungi, corresponding to 12,5% of sequences. Thus only 17 different fungal taxa were considered forming 21 different morphological groups, as can be observed in Figures 4.5–4.8. A number of 8 different species were identified, corresponding to 37% of sequences, while 10 sequences were only identified up to genus level, representing 41,7% of sequences. More details about fungal species identification are presented in Table 4.1. Moreover, was also identified, through microscopic appearance (mantle anatomy), *Cenococcum geophilum* associated with *C. salviifolius* (Fig. 4.9). However, it was not possible to sequence this fungus DNA due to external problems to the present work.

Table 4.1: Ectomycorrhizal fungal taxa identified in each partner plant species.

Sample code	Query cover	max.identity	Taxon	Reference NCBI code	Sequences origin
CS1.9	99%	93%	*Russula* sp.1	KF359616.1	*C. salviifolius*
CS1.15	98%	96%	*Tomentella* sp.	FJ897229.1	*C. salviifolius*
CS1.41	100%	99%	Pezizomycotina 1	EU232106.1	*C. salviifolius*
CS2.3	100%	95%	*Tylospora* sp.	AB456677.1	*C. salviifolius*
CS2.14	100%	98%	*Russula laricina*	KF850405.1	*C. salviifolius*
CS2.25	99%	99%	Helotiales	FN565262.1	*C. salviifolius*
CS2.29	100%	98%	*Sebacina* sp.	KM403035.1	*C. salviifolius*
CS2.30	100%	99%	*Fusarium oxysporum*	KU872840.1	*C. salviifolius*
CS2.31	98%	99%	*Archaeorhizomyces borealis*	NR_126144.2	*C. salviifolius*
CS3.2	98%	97%	*Tomentellopsis zygodesmoides*	KP814159.1	*C. salviifolius*
CS3.16	99%	97%	*Cortinarius subfloccopus*	JQ746615.1	*C. salviifolius*
CP1.20	100%	98%	*Sebacina vermifera*	JQ711843.1	*C. psilosepalus*
CP3.1	93%	86%	*Russula* sp.2	KF359616.1	*C. psilosepalus*
CP3.15	98%	99%	*Tomentella* sp.	FJ897229.1	*C. psilosepalus*
CP3.24	100%	99%	Pezizomycotina 1	EU232106.1	*C. psilosepalus*
PP2.4	99%	98%	*Rhizopogon roseolus*	KF990475.1	*P. pinaster*
PP2.11	97%	93%	*Russula* sp.3	KT933999.1	*P. pinaster*
PP2.12	99%	93%	*Russula* sp.1	KF359616.1	*P. pinaster*
PP2.16	97%	96%	*Russula* sp.4	KT933999.1	*P. pinaster*
PP2.20	99%	99%	*Oidiodendron maius*	KF359579.1	*P. pinaster*
PP2.33	100%	95%	Pezizomycotina 2	EU232106.1	*P. pinaster*
PP2.34	100%	96%	*Oidiodendron* sp.	JQ272359.1	*P. pinaster*
PP2.36	98%	98%	*Archaeorhizomyces borealis*	NR_126144.2	*P. pinaster*
PP3.1	99%	98%	Helotiales	FN565262.1	*P. pinaster*

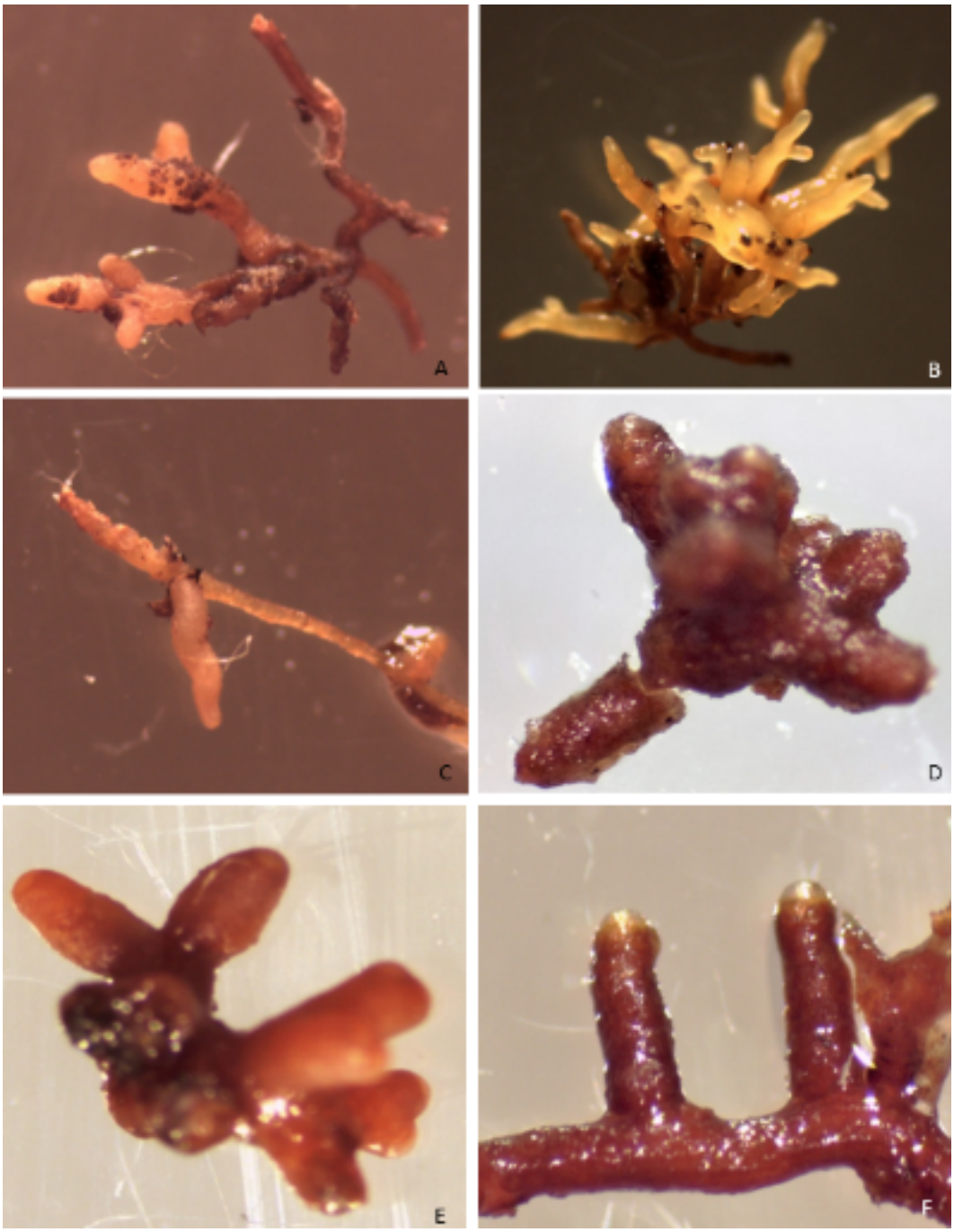

Figure 4.5: Ectomycorrhizal root tips of *Cistus salviifolius*. (A) *Russula* sp. (CS1.9); (B) *Tomentella* sp. (CS1.15); (C) Pezizomycotina1 (CS1.41); (D) *Tylospora* sp. (CS2.3); (E) *Russula laricina* (CS2.14); (F) Helotiales (CS2.25).

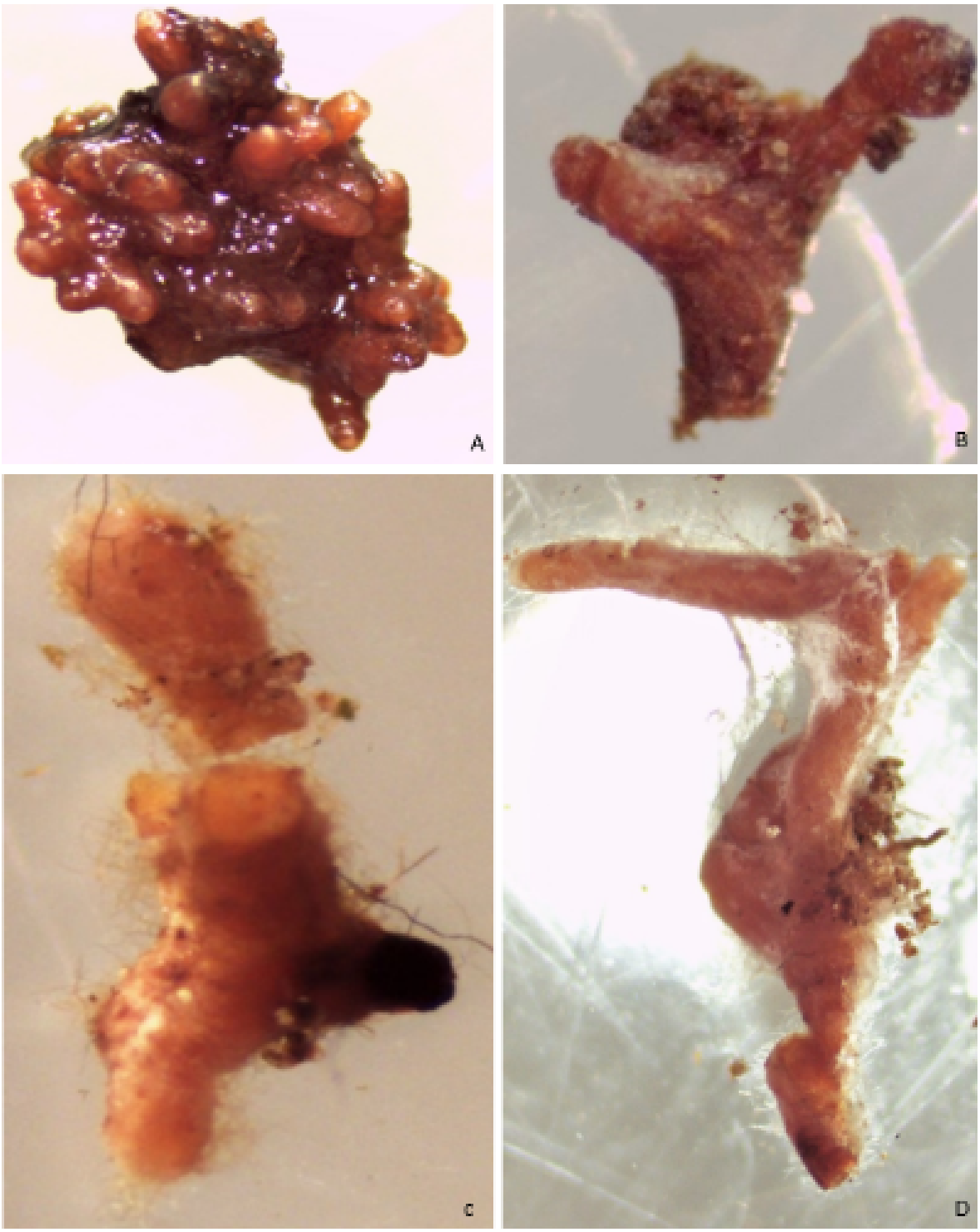

Figure 4.6: Ectomycorrhizal root tips of *Cistus salviifolius*. (A) *Sebacina* sp.(CS2.29); (B) *Archaeorhizomyces borealis* (CS2.31); (C) *Tomentellopsis zygodesmoides* (CS3.2); (D) *Cortinarius subfloccopus* (CS3.16).

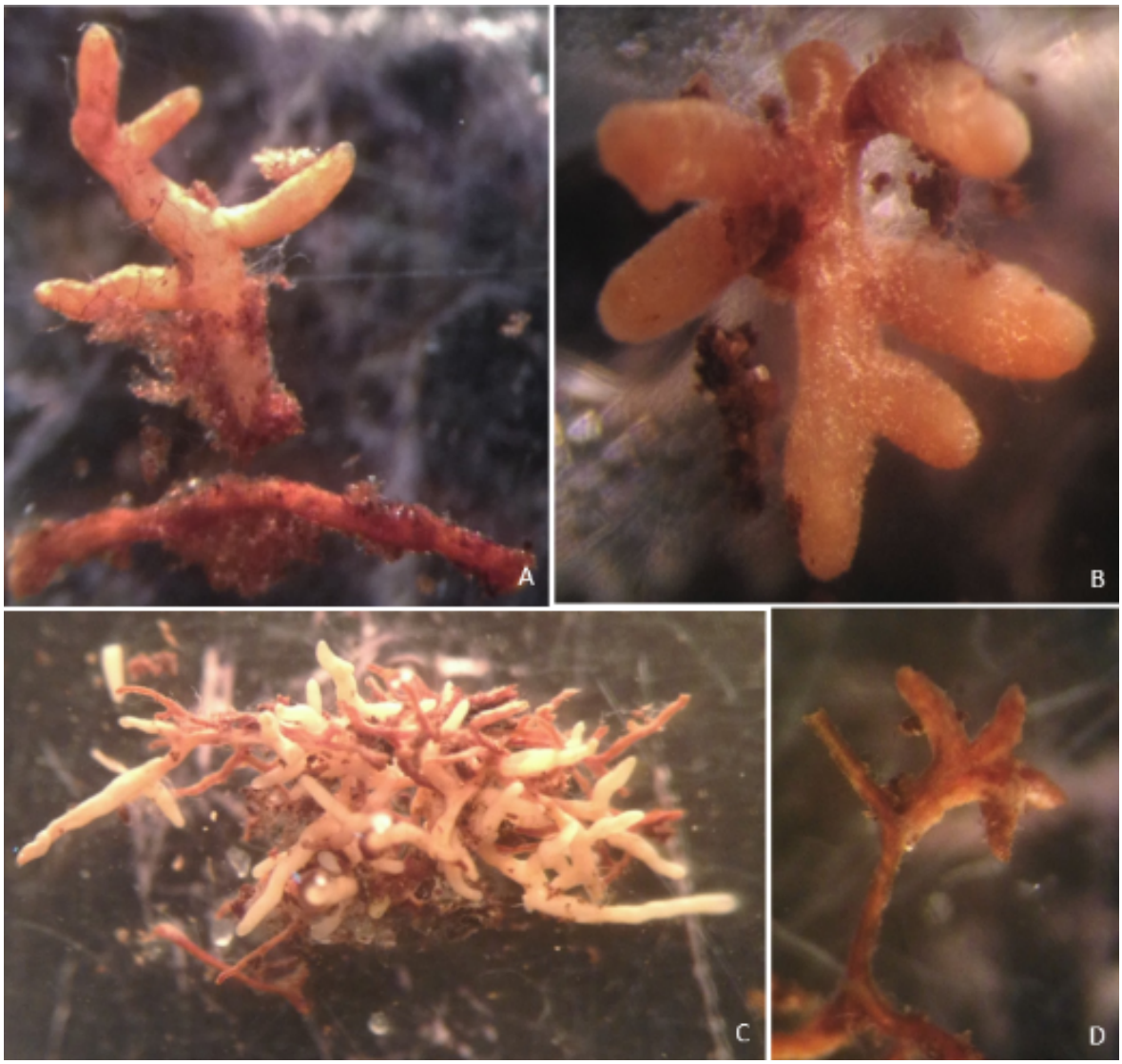

Figure 4.7: Ectomycorrhizal root tips of *Cistus psilosepalus*. (A) *Sebacina vermifera* (CP1.20); (B) *Russula* sp.2 (CP3.1); (C) *Tomentella* sp. (CP3.15); (D) Pezizomycotina 1. (CP3.24).

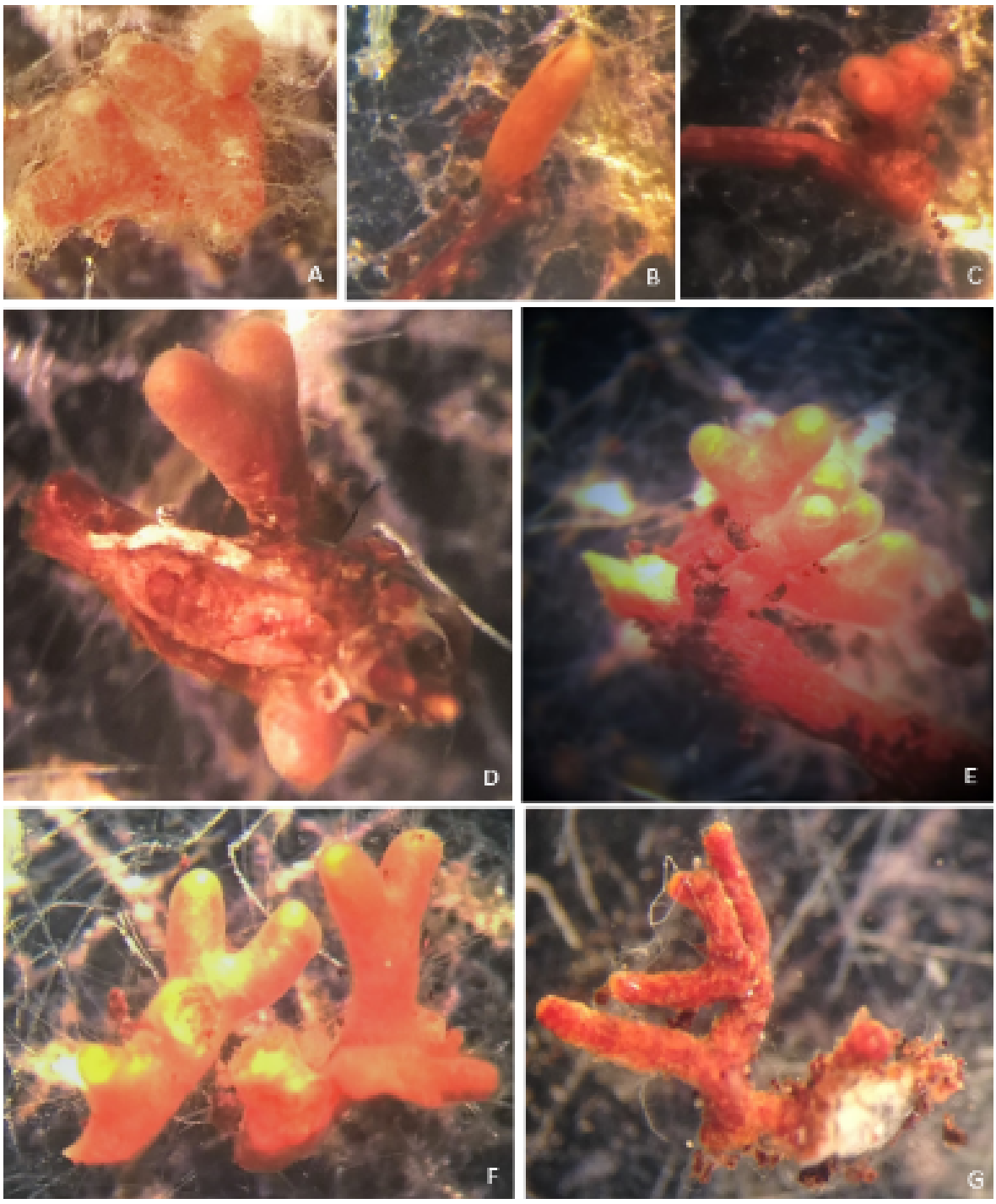

Figure 4.8: Ectomycorrhizal root tips of *Pinus pinaster*. (A) *Rhizopogon roseolus* (PP2.4); (B) *Russula* sp.3 (PP2.11); (C) *Russula* sp.1 (PP2.12); (D) *Russula* sp.4 (PP2.16); (E) Pezizomycotina 2 (PP2.33); (F) *Archaeorhizomyces borealis* (PP2.36); (G) Helotiales (PP3.1).

Figure 4.9: *Cenococcum geophilum* associated with *Cistus salviifolius* (Identified through microscopic appearance, as mantle anatomy).

4.4.2 Ectomycorrhizal fungal partner's sharing between hosts

All non-ECM root inhabiting fungal species were discarded for the ectomycorrhizal partner's sharing analysis. *Cistus salviifolius* was the species with more fungal partner's diversity, followed by *Pinus pinaster*. In addition, *C. salviifolius* was recorded also the species that shared fungal partners with all the other plant species assessed. All plant species share fungal partners with the other species, with the exception of *Pinus pinaster* + *Cistus psilosepalus*, which did not share any fungal symbiont. *Cistus salviifolius* + *Pinus pinaster* share 18,8% of ectomycorrhizal species among themselves, while *Cistus salviifolius* + *Cistus psilosepalus* share 12,5% of fungal species (Fig. 4.10). Fungal species *Tomentella* sp. and Peziziomycotina 1 were the sharing taxa between *C. psilosepalus* along with *C. salviifolius*, while Helotiales, *Archaeorhizomyces borealis* and *Russula* sp. 1 were the shared taxa between *C. salviifolius* and *P. pinaster*. More details can be seen in Fig. 4.11.

4.5 Discussion

Through this method, of DNA extraction and sequencing from individual ectomycorrhizal root tips in order to taxonomically identify the fungus associated with selected plants species, we can conclude that this showed to be a very good method to relate morphology of ectomycorrhizal root tips with an accurate fungal identification, as reported by Pestaña Nieto & Santolamazza Carbone (2009). However, regarding fungal diversity surveys, this method was poor comparing with other recent molecular tools such as NGS (Next Generation Sequencing), which allows to accurately identify fungal communities in roots and soil (Rincón et al. 2015), but is more precise than sporocarps surveys due to the fact that many fungi do not produce above ground or obvious sporocarps (Gehring et al. 1998, Dahlberg 2001, Pestaña Nieto & Santolamazza Carbone 2009) and also that the appearance of sporocarps do not guarantee their association with a specific plant species (Horton & Bruns 2001, Comandini et al. 2006, Pestaña Nieto & Santolamazza Carbone 2009). It is important to highlight the limited sampling of the present work, which do not represent reliably the diversity associated with these plants. However, in spite of the reduced sampling it was found a considerable taxa associated with the assessed plants, demonstrating the potential of *Cistus* spp. (and *P.*

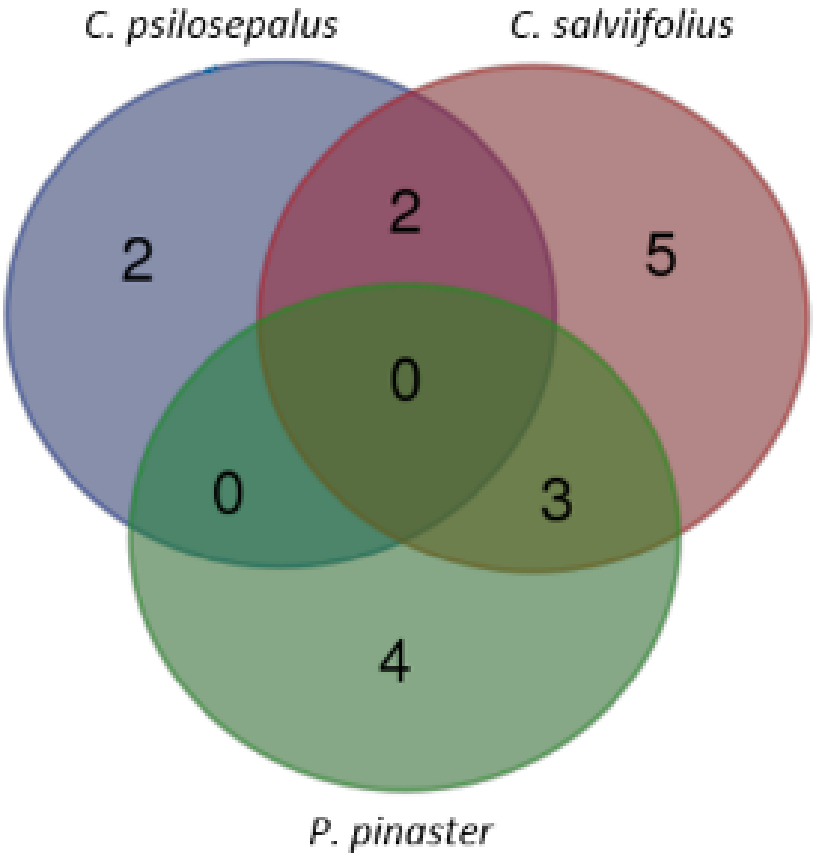

Figure 4.10: Number of ectomycorrhizal fungal partners shared and non-shared between *C. psilosepalus*, *C. salviifolius* and *P. pinaster*.

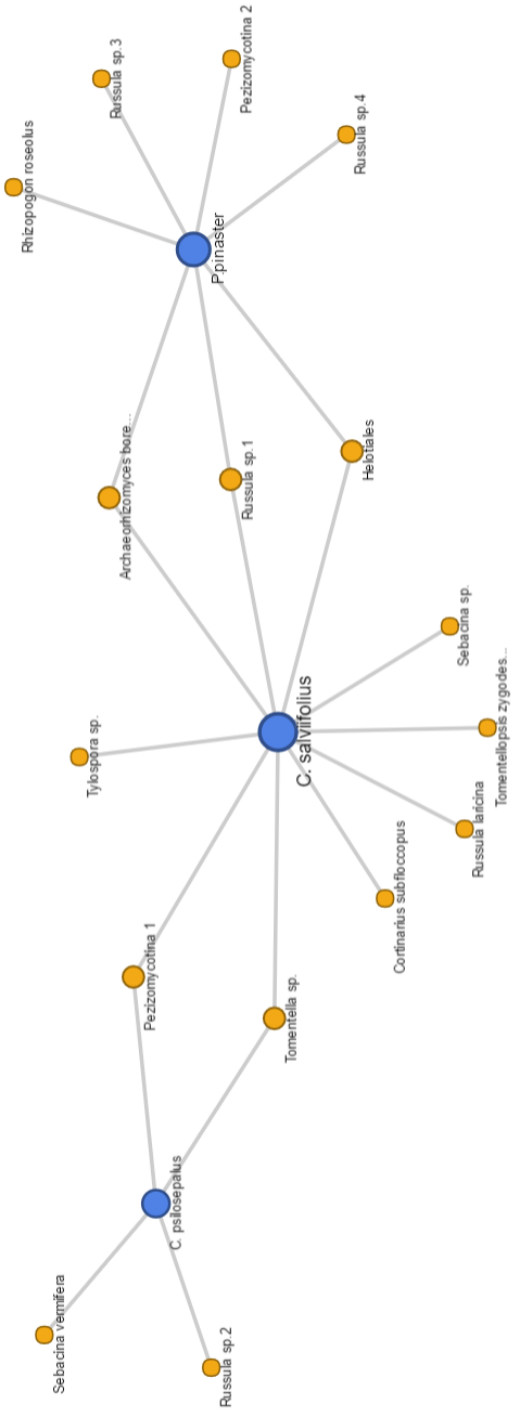

Figure 4.11: Network of species of ectomycorrhizal fungal partners (shared and non-shared) between *C. psilosepalus*, *C. salviifolius* and *P. pinaster*.

pinaster) as a diversity reservoir (Smith & Read 2008).

It was not possible to identify all sorted ectomycorrhizal tips due to the lack of sequences' quality as well as double banded samples which could not be sequenced. That could incorrectly lead to false interpretations as assuming that species as *C. psilosepalus* had lower fungal diversity compared with the other plant species, while the amount of "ad hoc morphological groups" sorted in *C. psilosepalus* and *C. salviifolius* was almost the same, 110 and 122 respectively. This could happen due to external problems to the present work as unwanted amplifications of plant or contaminant fungal DNA, which is possible to happen with this primer pair (ITS1F/ITS4) that could amplify ITS region of some species of plants (Gardes & Bruns 1993). Because of this, we would not state further conclusions about the amount of different mycorrhizal species found in *Cistus* spp. and *Pinus pinaster*, representing only a sample of the real fungal diversity. To made the assessment of the almost real fungal richness associated with these plants, should be performed a more exhaustive sampling in different seasons and, perhaps, using different molecular tools, as reported by Rincón et al. (2015).

Nevertheless, species of the families Russulaceae, Cortinariaceae and Thelephoraceae found associated with *Cistus* in the present work are in accordance with several previous reports as the study of Comandini et al. (2006), which reports Russulaceae and Cortinariaceae families as the most frequent fungal partners associated with these shrubs, and in the study from Pestaña Nieto & Santolamazza Carbone (2009) that reported Thelephoraceae as one of the most abundant ectomycorrhizal families in soil. The work of Buscardo et al. (2012) reported also species from Russulaceae, Cortinareaceae and Thelephoraceae families found in ectomycorrhizal root tips of *Cistus* spp. Furthermore, in the present work it is reported for the first time, to our knowledge, the first morphology record of the species *Archaeorhizomyces borealis* associated with *Cistus salviifolius*, which is a species that was described for the first time in 2014 (Menkis et al. 2014) in *Pinus* sp. The species *Oidiodendron maius* found in *Pinus pinaster*, which was categorized as non-ECM root inhabiting fungal species, is a saprobic species and mycorrhizal in Ericaceous roots (Rice & Currah 2006). It was also found this fungal species in ectomycorrhizal root tips of *Pinus pinaster* in the study of Buscardo et al. (2011). As this species is reported as a facultative mycorrhizal species with an unclear lifestyle, more studies should be performed in order to assess its real relationship with *Pinus*

sp. as well as its lifestyle. It is also important to highlight the identification (morphologic and anatomic) of *C. geophilum* in *Cistus salviifolius*, as it would be expected, being this fungus one of dominant and frequent ectomycorrhizal taxon (Jany et al. 2002). Moreover, Buscardo et al. (2012) had reported also its appearance in *Cistus ladanifer*, proving the ability of this species to establish ectomycorrhizas with *Cistus* sp.

Regarding morphology, this was the first time, to our concern, that these ectomycorrhizal roots tips' morphological aspect (created by these fungal species) was recorded in *Cistus* shrubs, which will be very important for future works with ectomycorrhizas in *Cistus* sp. It is very clear, in the present work, a distinct difference between the fungal morphology of the same fungus in different plant partners, e.g. *Archaeorhizomyces borealis*, Helotiales, *Sebacina vermifera* and Peziziomycotina 1, as well as similar morphologies of different fungi in the same plant species, e.g. *Tomentella* sp. + *Sebacina vermifera* in *C. psilosepalus* and *Russula* spp. + Pezizomycotina 2 + *Archaeorhizomyces borealis* in *P. pinaster*, which leads to many problems of taxonomic identification based only in morphology (Egger 1995, Menkis et al. 2005, Pestaña Nieto & Santolamazza Carbone 2009), justifying the use of molecular tools to assess the taxonomic identification of ectomycorrhizal fungi responsible for each root tip (Gardes & Bruns 1993).

An objective and solid evaluation about ectomycorrhizal fungal sharing between assessed plant partners is not possible to made, although, was proved that *Cistus* shrubs shared fungal partners among them as well as share also with *Pinus pinaster*. Similar results have been obtained by Buscardo et al. (2012) that found similar species in *Cistus* sp. and in *Pinus pinaster* in different habitats, and found also associated with *Cistus ladanifer* species that we found in our work associated with *P. pinaster*, demonstrating that *Cistus* can share much more fungal partners with *Pinus* compared with the diversity discovered in the present work. Moreover, our study proved also that *Cistus* can be used as a natural inoculum source in pinewoods when planted near old pines.

Regarding biotechnological potential of *Cistus* spp. for edible ectomycorrhizal sporocarps production, these shrubs demonstrate to be associated with species of Basidiomycota sporocarp producers as *Cortinarius* sp. and *Russula* sp. This genus (*Russula*) comprise several species that produce edible sporocarps as *Russula laricina* (http://www.first-nature.com),

which we found associated with *Cistus salviifolius*. In the works of Comandini et al. (2006) and Buscardo et al. (2012) it was also demonstrated the associations of *Cistus* with some edible ectomycorrhizal fungi. Altogether, these results clearly demonstrate the potential of *Cistus* shrubs for stablishing ectomycorrhizal associations with edible fungi in natural conditions, and probably the potential of sustaining fungus sporocarps either. However, more studies are needed to assess the real potential of *Cistus* shrubs to profitably produce edible sporocarps.

Chapter 5

Final Remarks

5.1 Saprobic and ectomycorrhizal fungal requirements

Saprobic fungi had generally higher growth rates than ectomycorrhizal fungi, as was expected due to their lifestyles and genetic basis, being easier to saprobes to get nutrients from the culture media. The ectomycorrhizal fungi studied demonstrated to be sensitive to high temperatures, not growing at 30°C ±1, contrarily to saprotrophs. The temperature that allowed most of fungal species studied to have the highest growth rates was 24°C ±1, being the best temperature when producing spawn for cultivation of saprobic fungi and fungal inoculum for mycorrhizal synthesis. The only exception was *Pleurotus ostreatus* that had its maximal growth at 30°C ±1. The lowest temperature (4°C ±1) revealed to be useful when attempting to maintain cultures for long periods, lowering fungal metabolism as well as their growth rates. In spite of different fungal lifestyles having different nutritional requirements, a comprehensive culture medium with various forms (regarding complexity) of organic nutrients, as Potato Dextrose Agar (PDA) medium, proved to fulfil fungal requirements of all lifestyles. On the other hand, a specific culture medium as modified Melin-Norkrans (MNM) medium proved also to be a suitable medium for some ectomycorrhizal species as *Boletus fragans*.

In spite of the evidence of fungal growth in MS medium, all fungi showed a low growth response in it, demonstrating that were not in optimal conditions. Thus this medium may be advantageous for mycorrhizal synthesis protocols urging the fungus to establish the partnership with plant roots.

5.2 Novel method in ectomycorrhizal synthesis in *Cistus* spp.

A mycorrhizal synthesis method was created meeting both plant and fungal requirements, resulting mainly from the optimization of studied plants culture conditions. Plantlets were axenically placed in flasks with non-modified Murashige and Skoog (MS) medium and inoculated with ectomycorrhizal fungus set on a previously opened ditch in the agar. Both fungus and roots were protected against light by a sterile aluminium disk. This technique showed to be very useful in testing partners compatibility as well as in monitoring mycorrhizal development. However, more tests and comparisons with other methods are needed in order to better understand its strengths and limitations.

5.3 The potential of *Cistus* shrubs for cultivation of edible ectomycorrhizal sporocarps

Cistus shrubs demonstrated having a wide plasticity concerning the diversity of their fungal partners due to successfully establishing ectomycorrhizas in almost all assessed plant-fungus combinations as well as to the great diversity of fungal taxa found associated with these shrubs in nature. These shrubs demonstrated also their ability of association with edible ectomycorrhizal fungi in both mycorrhizal synthesis essays and in natural conditions. The potential of using *Cistus* shrubs to produce edible ectomycorrhizal mushrooms still needs to be assessed with further experiments, as field essays with mycorrhized shrubs.

5.4 Ectomycorrhizal fungal network and biotechnological application of *Pinus pinaster* and *Cistus* spp. relationships

It is important to highlight the wide diversity of ectomycorrhizal fungi found associated with *Cistus* and *Pinus pinaster*, in spite of the limited sampling. Thus, the found fungal community was probably a small sample of the real diversity, which could be highlighted with a greater sampling combined with different techniques. Our study proved the sharing of fungal symbionts between *Cistus* spp. and *Pinus pinaster* although, both plants are known to be capable of establishing ectomycorrhizal associations with edible fungi. However, very

few different species were found, in our work, shared with *P. pinaster* and *Cistus* spp., due probably to the limited sampling. Nevertheless, it is known their ability of sharing ectomycorrhizal species, including edible ones. This demonstrates once more that this sharing was inadvertently underestimated in the present work. Therefore, our study suggested that these relationships and networks could be applied using mycorrhized shrubs with edible fungi as inoculum source in pinewoods. This could lead to cultivation edible ectomycorrhizal mushrooms in natural pine forests, using these shrubs as vector of the intended fungi.

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