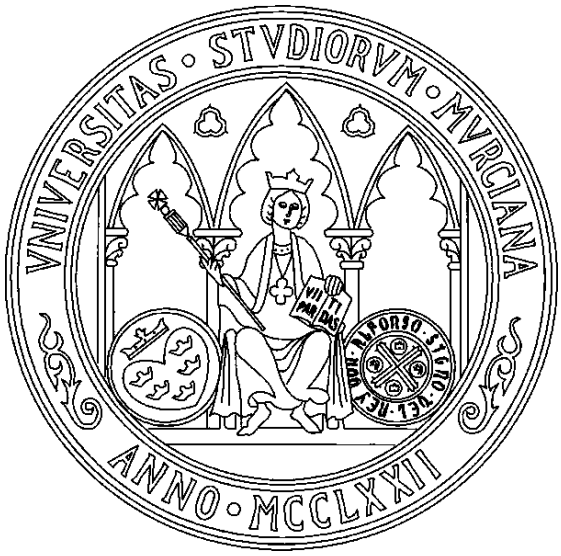

UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Boletus edulis and *Cistus ladanifer*: characterization of its ectomycorrhizae, *in vitro* synthesis, and realised niche.

Boletus edulis y *Cistus ladanifer*: caracterización de sus ectomicorrizas, síntesis *in vitro* y área potencial.

Dª. Beatriz Águeda Hernández

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Boletus edulis AND *Cistus ladanifer*: CHARACTERIZATION OF ITS ECTOMYCORRHIZAE, *in vitro* SYNTHESIS, AND REALISED NICHE

tesis doctoral

BEATRIZ ÁGUEDA HERNÁNDEZ

Memoria presentada para la obtención del grado de Doctor por la Universidad de Murcia:

Dra. Luz Marina Fernández Toirán
Directora, Universidad de Valladolid
Dra. Asunción Morte Gómez
Tutora, Universidad de Murcia

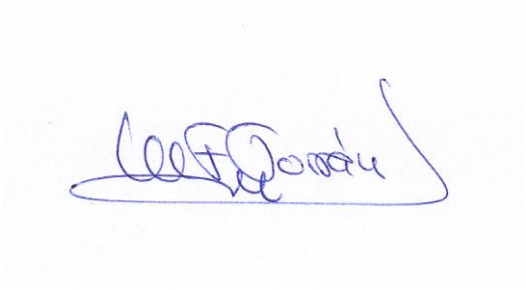
2014

UNIVERSIDAD DE
MURCIA

Dª. Luz Marina Fernández Toirán, Profesora Contratada Doctora de la Universidad de Valladolid, como Directora, y Dª. Asunción Morte Gómez, Profesora Titular de la Universidad de Murcia, como Tutora, AUTORIZAN:

La presentación de la Tesis Doctoral titulada: '*Boletus edulis* and *Cistus ladanifer*: characterization of its ectomycorrhizae, *in vitro* synthesis, and realised niche', realizada por Dª Beatriz Águeda Hernández, bajo nuestra inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 31 de julio de 2014



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Not everything that can be counted counts,
and not everything that counts can be counted.

Albert Einstein

Le petit prince, alors, ne put contenir son admiration:
-Que vous êtes belle!
-N´est-ce pas, répondit doucement la fleur.
Et je suis née meme temps que le soleil...
Le petit prince devina bien qu´elle n´était pas
trop modeste, mais elle était si émouvante!
-C´est l´heure, je crois, du petit déjeuner, avait-elle bientôt
ajouté, auriez-vous la bonté de penser à moi…

Antoine de Saint-Exupéry
Le Petit Prince

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AGRADECIMIENTOS

'El sistema científico nacional agoniza ante la mirada impasible de nuestros dirigentes. Los recursos económicos, materiales y humanos son cada vez menores porque la inversión en investigación es cada día más limitada. Por un lado, el presupuesto global languidece, las convocatorias e ingresos se retrasan y se dotan de menos recursos a los grupos exigiendo los mismos objetivos científicos. Por otro lado, estos recursos dependen cada vez más de un modelo de cofinanciación que es engañoso, ya que permite que el dinero presupuestado no llegue a ejecutarse si los centros y grupos sobre los que recae la misma, no cuentan con recursos suficientes para hacer frente a dichos gastos. Además, los requisitos para acceder a dichas ayudas son cada vez menos realistas y menos consecuentes con la situación del sistema de I+D+i.'

Descapitalizando la ciencia.
Asamblea General de Ciencia. Investigadores y trabajadores del CSIC, Universidades, OPIs y Spin-off.
Contra el desmantelamiento del sistema público de investigación.
29 mayo 2014

En los últimos tres años he tenido que escribir tres veces los agradecimientos de un trabajo académico. Los tres años han comenzado con palabras en defensa de la ciencia. Seguimos igual. No es cierto, empeoramos. Perdemos la esperanza en nuestro trabajo. Vivimos en un momento social en que el cambio debe ser hacia un modelo productivo basado en el conocimiento, pero a la vez se dan pasos que provocan un daño irremediable a corto y largo plazo a la infraestructura científica y a su capital humano en España. Me gustaría superar totalmente el cierre del centro de investigación dónde llevaba trabajando 15 años sin otra explicación que la necesidad de recortar gastos, pero me sigue pesando. Continuar en la investigación no es fácil.

No es fácil, pero no se llega solo a ningún sitio. Muchas personas me han ayudado y este es un buen lugar y un buen momento para darles a todos las gracias.

Esta Tesis Doctoral nunca podría haberse realizado sin el apoyo constante de mi directora, Marina Fernández-Toirán. El valor del trabajo diario, la crítica constructiva, el ir siempre hacia delante, la sensación de que nunca dejará de creer en mí,… Es una suerte que sea mi maestra.

Tampoco hubiese sido posible sin Asun Morte, mi tutora de la Universidad de Murcia, que me ha facilitado todos los trámites académicos y cuya disponibilidad y ayuda han sido claves en los procesos finales (que no han sido sencillos) de esta tesis.

A lo largo de estos años ha sido clave el apoyo que he recibido de la comunidad científica y del mundo forestal. También el que he recibido de mis compañeros en Valonsadero y ahora en la Universidad de Valladolid. Me gustaría personificarlo en Reinhard Agerer, Teresa Ágreda, Jorge Aldea, Rafa Alonso, Juan Barriuso, Óscar Cisneros, Ana de Miguel, Raquel de Pedro, Hermi de la Varga, Loli García, Ana Hernández, Jorge Herrero, Sara Hortal, Javier Ligos, Pedro Medrano, Pilar Modrego, Randy Molina, Txemi Olano, Pedro Ortega, Xavier Parladé, Joan Pera, Santiago Reyna, Fernando Rubio, Sergio Sánchez, Francesco Tagliaferro, Antonio Turrientes y Alessandra Zambonelli. Nunca dejaré de agradecer sus conversaciones, visitas, llamadas y correos, su protección y su ánimo.

No me gustaría olvidar aquí a mis profesores. Siempre me acompañarán en la huella que sus conocimientos, sus enseñanzas y sus experiencias han dejado en mi. Entre ellos, Madre Rosario que me enseñó a leer, la señorita Julita en el colegio, Inés González Doncel y Pedro M. Díaz Fernández ya en la universidad… Tampoco a los que contribuyeron a la experiencia educativa más importante que he tenido: el Grupo Scout Doce Linajes 276, a mis scouters, a mis compañeros, a mis lobatos y rovers, a mis alumnos en los cursos y, sobre todo, a todos aquellos con los que tuve la suerte de compartir mis propias aventuras como scouter y coordinadora. Ver otras realidades ha supuesto aumentar mis conocimientos y la superficie de mis dudas, pasar un tiempo durante este último año en Haití con la ONGD Cives Mundi, gracias a Teresa Vicente, Joaquín Alcalde, Luciano Jiménez y Vicky Tortosa, ha sido clave para no olvidarme de la suerte que tengo de ser mujer en el primer mundo, de poder dedicarme a la ciencia, lo más apasionante del mundo.

Son los últimos, pero no los menos importantes. Mis padres, mi hermano y el resto de mi familia, que siempre están y han estado conmigo. Mis amigos y amigas, especialmente Ra y A, con los que comparto lo importante de la vida. JM, que me lleva de su mano a otros caminos. Ellos son la red social que no deja que me caiga.

Aún sigo creyendo que puedo cambiar el mundo, dejarlo mejor de como lo encontré. Creo firmemente que la investigación es una de las bases para conseguir un planeta más justo. Estudiar las micorrizas es invertir en el futuro.

ABSTRACT

The *Boletus edulis* species complex includes four species: *Boletus aereus, Boletus edulis, Boletus pinophilus*, and *Boletus reticulatus*, that have the great economic importance all around the world for their edibility. Cistaceae are plants belonging to primary succession stages of tree stands, that are ecologically important species because they may act as a reservoir of ectomycorrhizal fungi inoculum after a forest disturbance. *Boletus edulis* sporocarps are regularly observed in certain regions of Central Spain, which have recurrent fires and are dominated exclusively by *Cistus ladanifer*. In many of these areas they appear not to be harvested, and constitute an underappreciated and underexploited resource. Its productive association opens a new way of sustainable rural development. To describe its structures, to check the viability of the association under laboratory conditions and also its realized niche and climatic suitability are previous steps that will allow us to arise a new way to exploiding it.

To provide a detailed description based on standard morphological and anatomical characters of the ectomycorrhizae of *Boletus edulis* on *Cistus ladanifer*, field ectomycorrhizae were sampled. This mycorrhizae has traits typical of Boletales. The identification of the fungal symbiont was confirmed by ITS rDNA sequence comparison between mycorrhizae and sporocarps. The fact that this fungus is able to fruit when associated to unusual host plants, may be seen as a dispersion strategy to assure genetic variation, favouring the maintenance of soil inoculum reservoria for later successional stages.

To test the ability of the *Boletus edulis* species complex to form ectomycorrhizae with *Cistus* sp. under controlled conditions as well as provide detailed anatomical descriptions of the formed ectomycorrhizae, ectomycorrhizas of *Boletus aereus, Boletus edulis,* and *Boletus reticulatus* were synthesized *in vitro* with *Cistus albidus* and *Cistus ladanifer*. The formed ectomycorrhizae were very similar, with typical traits of Boletales, similar to those formed by the same fungal species with other hosts and in the wild.

To define the realized niche of the ectomycorrhizal association where *Boletus edulis* produces sporocarps associated with *Cistus ladanifer* in peninsular Spain, species distribution models based on climatic variables and corrected under lithological criteria were developed. The climatic niche is mesothermal, Mediterranean and humid. Soils are strongly acid, with loam texture, low in organic matter and in an oligotrophic mull form. The optimal of this association occupies 1,785 km^2, 16.3 % of the potential area.

Controlled mycorrhization and outplanting of inoculated seedlings might be a feasible and promising way to exploit this symbiosis providing economic benefits. To accomplish this, further research is needed to determine the appropriate inoculation methods with compatible strains, the persistence of ectomycorrhizae on outplanted, inoculated seedlings, and the factors triggering sporocarps production.

RESUMEN

El grupo *Boletus edulis* incluye cuatro especies que poseen una gran importancia económica en todo el mundo por su valor gastronómico: *Boletus aereus, Boletus edulis*, *Boletus pinophilus* y *Boletus reticulatus.* Las especies de la familia Cistaceae son plantas características de las primeras etapas sucesionales de los bosques, ecológicamente importantes por su papel como reservorio de inóculo de hongos ectomicorrícicos ante perturbaciones ecológicas. Los carpóforos del grupo *Boletus edulis* son recolectados de forma habitual en algunas regiones de la España Central, en zonas de matorral en las que se producen fuegos recurrentes y que están dominadas de forma exclusiva por *Cistus ladanifer*. Estas setas constituyen un recurso poco apreciado y poco explotado, pero la asociación entre estas dos especies abre una nueva vía de desarrollo rural sostenible. Describir sus estructuras, comprobar la viabilidad de la asociación en condiciones de laboratorio y calcular su área potencial son pasos previos que permitirán desarrollar su explotación.

Se estudian ectomicorrizas naturales de *Boletus edulis* y *Cistus ladanifer* con el objetivo de realizar la descripción detallada de sus caracteres anato-morfológicos. La ectomicorriza presenta los rasgos típicos de los Boletales. La identificación del simbionte fúngico se confirmó comparando las secuencias de la región ITS del rDNA de las micorrizas y los carpóforos. El hecho de que este hongo sea capaz de fructificar en asociación con plantas hospedantes no habituales, puede ser una estrategia de dispersión para asegurar así la variación genética de la especie, favoreciendo el mantenimiento del reservorio de inóculo para etapas posteriores de sucesión del bosque.

Se sintetizan *in vitro* ectomicorrizas de *Boletus aereus, Boletus edulis* y *Boletus reticulatus* con *Cistus albidus* y *Cistus ladanifer,* para comprobar la capacidad micorrícica de las especies ensayadas bajo condiciones controladas y realizar así la descripción anato-morfológica detallada de estas estructuras. Las micorrizas formadas son todas muy similares, con rasgos típicos de los Boletales; similares a los que presentan estas mismas especies de hongos con otros hospedantes y a las que se encuentran de forma natural.

Para definir el área potencial de la asociación ectomicorrícica en la que *Boletus edulis* produce carpóforos junto con *Cistus ladanifer* en la España peninsular se utilizaron modelos de distribución de especies basados en variables climáticas con corrección litológica. El nicho climático es mesotérmico, Mediterráneo y húmedo; los suelos, fuertemente ácidos, con textura de limosa, pobres en materia orgánica y con humus oligotrófico. El área climáticamente óptima de esta asociación ocupa 1.785 km^2, el 16,3 % de su área potencial.

La micorrización controlada y la instalación de plántulas inoculadas puede ser una forma viable y prometedora para explotar con beneficios económicos este tipo de simbiosis. Para conseguirlo, es necesario realizar más trabajos para determinar métodos de inoculación apropiados con cepas compatibles, la persistencia de las ectomicorrizas en las plantas micorrizadas instaladas en campo, y los factores que desencadenan la producción de carpóforos.

1. INTRODUCTION.

The word mycorrhiza has its origin in the Greek words fungus (*mukés*) and root (*rhiza*), and it defines its symbiotic association. The plant obtains water and minerals from the fungus, and the fungus obtains photosynthetic products and vitamins from the plant (Allen 1991). There is the possibility that one fungus forms mycorrhizae with more than one species of plant and it is usual that different fungi form mycorrhizae with the same plant species (Brundrett 2002; Simard et al. 2012).

Mycorrhizae are divided into several groups according to their morphology. There are seven important mycorrhizal types (Smith and Read 2008): arbuscular mycorrhizae, ectomycorrhizae, ectendomycorrhizae, arbutoid mycorrhizae, monotropoid mycorrhizae, ericoid mycorrhizae, and orchid mycorrhizae. Depending on whether they have penetration or not (respectively) of the fungi in the root cortex cells, two main groups could be established: endomycorrhizae and ectomycorrhizae. Ectomycorrhiza is always formed by septate fungi, Ascomycota and Basidiomycota, and occasionally by Glomeromycota. They cover the root, forming a fungal mantle, and the intracellular space, forming the Hartig net in gymnosperms and angiosperms.

About 95 % of the trees in temperate areas (mainly Pinaceae, Fagaceae, Dipterocarpaceae, and Caesalpinoidaceae families) form ectomycorrhizae with a number of Ascomycota and Basidiomycota species, including edible fungi with a high commercial value such as *Tuber* sp., *Boletus* sp., and *Lactarius* sp. (Smith and Read 2008).

Fungal structures formed in those associations (mantle, cystidia, extraradical mycelium, and rhizomorphs) increase the root exploration area in the soil and allow both the tree and the fungi to thrive (Agerer 1995).

The study of ectomycorrhizal anatomy and morphology has always been limited by it requiring access to the microscopic structures in the soil. Although mycorrhizae were discovered by Albert B. Frank in 1885 (Frank 2005), their detailed description did not begin until the 1980s. Since the beginning of the twenty-first century, molecular tools that can detect and measure the presence of a fungus in the soil have significantly increased available knowledge on fungal identification and dynamics. Nowadays, there is abundant data about ectomycorrhizal fungal communities in a forest, but there is little knowledge about how these organisms interact with each other, simply because there is scarce knowledge about their structures and about how they live together in the roots.

Mycorrhizae are classic examples to explain the mutualistic interaction between two different organisms in nature: a plant and a fungus. Both species establish a permanent relationship, they live together in symbiosis. Although the line between parasitism and mutualism is fine and negative interactions between plant and fungus can occur with changing environmental conditions, the relationship is generally positive for both symbionts (Allen 1991).

1.1. The huge diversity of ectomycorrhizal fungi in the forest ecosystems and its relevance.

There are more than 3,500 land plant species that have the ability to form mycorrhizae (Wang and Qiu 2006). A relatively small number are exclusively ectomycorrhizal (probably around 3 % of seed plants) (Meyer 1973), but their global importance is greatly increased by their disproportionate occupancy of terrestrial land surfaces and their economic value as the main producers of timber. Smith and Read (2008) and Wang and Qiu (2006) counted the plant genera reported to contain at least one species of ectomycorrhizae. Compiled in Table 1.1, those authors reflect that there are 195 terrestrial plant genera belonging to 65 families which could form ectomycorrhizae, including the ones that form conifer forests, deciduous forests and sclerophyll forests, three of the main forest types on Earth.

Over 5,000 fungal species form ectomycorrhizae (Agerer 2006; Rinaldi et al. 2008; Tedersoo et al. 2010) (Table 1.2). Within Basidiomycota exclusively Hymenomycetes and within Ascomycota exclusively Ascomycetes contribute to this type of symbiosis. Pezizales are mostly responsible for ascomycetous ectomycorrhizae, with their hypogeous derivates, whereas Boletales, Gomphales, Thelephorales, Amanitaceae, Cantharellaceae, Cortinariaceae, Russulaceae, and Tricholomataceae form ectomycorrhizal relationships within Hymenomycetes (Agerer 2006).

Table 1.1. Plant genera with at least one species forming ectomycorrhizae. Compiled from Wang and Qiu (2006) and Smith and Read (2008).

Division	Family	Genus	ECM	AM	EEM	ORM	ABM	ERM	Remarks
Bryophyta	Aneuraceae	*Cryptothallus* Malmb.							Mycoheterotrophy (via ECM)
	Arnelliaceae	*Southbya* Spruce							Fungal association with Basidiomycetes
	Calypogeiaceae	*Calypogeia* Raddi							Fungal association with Ascomycetes
	Cephaloziaceae	*Cephalozia* (Dumort.) Dumort.							Fungal association with Ascomycetes
		Nowellia F.Stevens							Fungal association with Ascomycetes
		Odontoschisma (Dumort.) Dumort.							Fungal association with Ascomycetes
	Cephaloziellaceae	*Cephaloziella* (Spruce) Schiffn.							Fungal association with Ascomycetes
	Jungermanniaceae	*Barbilophozia* Loeske							Fungal association with Ascomycetes and Basidiomycetes
		Lophozia (Dumort.) Dumort.							Fungal association with Basidiomycetes
		Nardia Gray							Fungal association with Basidiomycetes
	Lepidoziaceae	*Kurzia* Martens							Fungal association with Ascomycetes
		Lepidozia (Dumort.) Dumort.							Fungal association with Ascomycetes
Pteridophyta	Dryopteridaceae	*Dryopteris* Adans.	ECM	AM					
Spermatophyta: Gymnospermae	Pinaceae	*Abies* Mill.	ECM						
		Cathaya Chun et Kuang	ECM						
		Cedrus Trew	ECM	AM					
		Keteleeria Carrière	ECM						
		Larix Mill.	ECM						
		Picea D.Don ex Loudon	ECM		EEM				
		Pinus L.	ECM	AM	EEM				
		Pseudolarix Gordon	ECM						
		Pseudotsuga Carrière	ECM	AM	EEM				
		Tsuga (Endl.) Carrière	ECM	AM					
	Gnetaceae	*Gnetum* L.	ECM	AM					
	Araucariaceae	*Wollemia* W.G.Jones, K.D.Hill et J.M.Allen	ECM						
	Cupressaceae	*Cupressus* L.	ECM	AM					
		Juniperus L.	ECM	AM					
Spermatophyta: Angiospermae	Orchidiaceae	*Cephalanthera* Rich.	ECM			ORM			Mycoheterotrophy (via ECM)
		Corallorhiza Gagnebin							Mycoheterotrophy (via ECM)
		Epipactis Zinn	ECM			ORM			
		Hexalectris Raf.							Mycoheterotrophy (via ECM)
		Neottia Guett.	ECM			ORM			
	Cyperaceae	*Kobresia* Willd.	ECM	AM					
	Poaceae	*Festuca* L.	ECM	AM					
	Ranunculaceae	*Clematis* L.	ECM	AM					
	Polygonaceae	*Coccoloba* P.Browne	ECM	AM					
		Polygonum L.	ECM	AM					
	Caryophyllaceae	*Silene* L.	ECM	AM					
	Nyctaginaceae	*Neea* Ruiz et Pav.	ECM	AM					
		Pisonia L.	ECM	AM					
		Torrubia Vell.	ECM	AM					
	Ericaceae	*Arbutus* L.	ECM				ABM		
		Arctostaphylos Adans.	ECM	AM	EEM		ABM	ERM	
		Chimaphila Pursh	ECM						
		Gaultheria L.	ECM						
		Kalmia L.	ECM						
		Ledum L.	ECM						
		Leucothoë D.Don	ECM						
		Rhododendron L.	ECM						
		Vaccinium L.	ECM						
	Rubiaceae	*Rubia* L.	ECM	AM					
	Oleaceae	*Fraxinus* L.	ECM	AM					
	Bignoniaceae	*Jacaranda* Juss.	ECM						
	Aquifoliaceae	*Ilex* L.	ECM	AM					
	Campanulaceae	*Lobelia* L.	ECM	AM					
	Goodeniaceae	*Brunonia* Sm. ex R.Br.	ECM						
		Calogyne R.Br.	ECM	AM					
		Dampiera R.Br.	ECM	AM					
		Goodenia Sm.	ECM	AM					
	Asteraceae	*Angianthus* J.C.Wendl.	ECM	AM					
		Helichrysum Mill.	ECM	AM					
		Helipterum DC.	ECM	AM					
		Homogyne Cass.	ECM	AM					
		Mycelis Cass. in F.Cuvier	ECM	AM					
		Podolepis Labill.	ECM	AM					
		Waitzia J.C.Wendl.	ECM	AM					
	Caprifoliaceae	*Sambucus* L.	ECM	AM					
	Grossulariaceae	*Ribes* L.	ECM	AM					

Table 1.1. Plant genera with at least one species forming ectomycorrhizae. Compiled from Wang and Qiu (2006) and Smith and Read (2008). (continued).

Division	Family	Genus	ECM	AM	EEM	ORM	ABM	ERM	Remarks
Spermatophyta: Angiospermae	Myrtaceae	*Angophora* Cav.	ECM	AM					
		Baeckea L.	ECM						
		Callistemon R.Br. in Flinders	ECM	AM					
		Campomanesia Ruiz et Pav.	ECM	AM					
		Eucalyptus L'Hér.	ECM	AM					
		Kunzea Rchb.	ECM						
		Leptospermum J.R.Forst. et G.Forst.	ECM	AM					
		Melaleuca L.	ECM	AM					
		Tristania R.Br. ex W.T.Aiton	ECM	AM					
	Melastomataceae	*Graffenrieda* A.DC.	ECM	AM					
	Euphorbiaceae	*Amperea* A.Juss.	ECM						
		Poranthera Rudge	ECM						
		Uapaca Baill.	ECM	AM					
	Salicaceae	*Populus* L.	ECM	AM					
		Salix L.	ECM	AM					
	Cunoniaceae	*Ceratopetalum* Sm.	ECM	AM					
	Polygalaceae	*Comesperma* Labill.	ECM	AM					
		Polygala L.	ECM	AM					
	Mimosaceae	*Acacia* Mill.	ECM	AM					
	Fabaceae	*Aphanocalyx* Oliv.	ECM						
		Calliandra Benth.	ECM	AM					
		Didelotia Baill.	ECM	AM					
		Dillwynia Sm.	ECM	AM					
		Gleditsia L.	ECM	AM					
		Intsia Thouars	ECM	AM					
		Lonchocarpus Kunth in Humb.	ECM	AM					
		Microberlinia A.Chev.	ECM						
		Paraberlinia Pellegr.	ECM						
		Pultenaea Sm.	ECM	AM					
		Robinia L.	ECM	AM					
		Tetraberlinia (Harms) Hauman	ECM	AM					
		Toubaouate Aubrév. et Pellegr.	ECM						
		Vicia L.	ECM	AM					
	Caesalpiniaceae	*Afzelia* Sm.	ECM	AM					
		Aldina Endl.	ECM						
		Anthonotha P. Beauv.	ECM	AM					
		Bauhinia L.	ECM						
		Brachystegia Benth. in Benth. et Hook.f.	ECM						
		Cassia L.	ECM						
		Eperua Aubl.	ECM						
		Gilbertiodendron J.Léonard	ECM	AM					
		Julbernardia Pellegr.	ECM	AM					
		Monopetalanthus Harms in Engl. et Prantl	ECM						
		Paramacrolobium J.Léonard	ECM						
		Swartzia Schreb.	ECM						
	Rosaceae	*Adenostoma* Hook. et Arn.	ECM	AM					
		Chamaebatia Benth.	ECM						
		Cercocarpus Kunth in Humb.	ECM						
		Crataegus L.	ECM	AM					
		Dryas L.	ECM	AM					
		Malus Mill.	ECM	AM					
		Prunus L.	ECM	AM					
		Pyrus L.	ECM	AM					
		Rosa L.	ECM	AM					
		Rubus L.	ECM	AM					
		Sorbus L.	ECM	AM					
	Rhamnaceae	*Cryptandra* Sm.	ECM						
		Frangula Mill.	ECM	AM					
		Pomaderris Labill.	ECM						
		Rhamnus L.	ECM						
		Spyridium Fenzl in Endl. et al.	ECM						
		Trymalium Fenzl in Endl. et al.	ECM						
	Ulmaceae	*Celtis* L.	ECM						
		Ulmus L.	ECM	AM					
	Fagaceae	*Castanea* Mill.	ECM	AM					
		Castanopsis (D.Don) Spach	ECM	AM					
		Fagus L.	ECM	AM					
		Lithocarpus Blume	ECM	AM					
		Nothofagus Blume	ECM	AM					
		Pasania (Miq.) Oerst.	ECM	AM					
		Quercus L.	ECM	AM					
		Trigonobalanus Forman	ECM	AM					

Table 1.1. Plant genera with at least one species forming ectomycorrhizae. Compiled from Wang and Qiu (2006) and Smith and Read (2008). (continued).

Division	Family	Genus	ECM	AM	EEM	ORM	ABM	ERM	Remarks
Spermatophyta: Angiospermae	Juglandaceae	*Carya* Nutt.	ECM	AM					
		Engelhardia Lesch. ex Blume	ECM						
		Juglans L.	ECM	AM					
		Pterocarya Kunth	ECM						
	Betulaceae	*Alnus* Mill.	ECM	AM	EEM				
		Betula L.	ECM	AM	EEM				
		Carpinus L.	ECM	AM					
		Corylus L.	ECM	AM					
		Ostrya Scop.	ECM	AM					
		Ostryopsis Decne.	ECM	AM					
	Casuarinaceae	*Allocasuarina* L.A.S.Johnson	ECM	AM					
		Casuarina L.	ECM	AM					
	Cistaceae	*Cistus* L.	ECM	AM					
		Helianthemum Mill.	ECM	AM	EEM				
		Tuberaria (Dunal) Spach	ECM						
	Dipterocarpaceae	*Anisoptera* Korth. in Temminck	ECM	AM					
		Balanocarpus Bedd.	ECM	AM					
		Catylelobium Sm.	ECM	AM					
		Dipterocarpus C.F.Gaertn.	ECM	AM					
		Dryobalanops C.F.Gaertn.	ECM	AM					
		Hopea Roxb.	ECM	AM					
		Monotes A.DC.	ECM	AM					
		Shorea Roxb. ex C.F.Gaertn.	ECM	AM					
		Valica L.A.S.Johnson	ECM	AM					
	Tiliaceae	*Tilia* L.	ECM	AM					
	Aceraceae	*Acer* L.	ECM	AM					
	Compositae	*Lactuca* L.	ECM	AM					
	Elaegnaceae	*Shepherdia* Nutt.	ECM						
	Epacridaceae	*Astroloma* R.Br.	ECM						
	Gentinaceae	*Bartonia* Muhl. ex Willd.	ECM						
	Hammamelidaceae	*Parrotia* C.A.Mey.	ECM						
	Myricaceae	*Comptonia* L'Hér. ex Aiton	ECM						
		Myrica L.	ECM						
	Papilionoideae	*Brachysema* R.Br. in W.T.Aiton	ECM			ORM			
		Chorizema Labill.	ECM						
		Daviesia Sm.	ECM						
		Eutaxia R.Br. ex W.T.Aiton	ECM						
		Gompholobium Sm.	ECM	AM					
		Hardenbergia Benth. in Endl. et al.	ECM	AM					
		Jacksonia R.Br. ex Sm. in Rees	ECM						
		Kennedia Vent.	ECM						
		Mirbelia Sm.	ECM	AM					
		Oxylobium Andrews	ECM	AM					
		Platylobium Sm.	ECM						
		Pultenaea Sm.	ECM						
		Viminaria Sm.	ECM	AM					
	Platanaceae	*Platanus* L.	ECM	AM					
	Sapindaceae	*Allophylus* L.	ECM						
		Nephelium L.	ECM						
	Sapotaceae	*Glycoxylon* Ducke	ECM						
	Sterculiaceae	*Lasiopetalum* Sm.	ECM	AM					
		Thomasia J.Gay	ECM						
	Stylidiaceae	*Stylidium* Sw. ex Willd.	ECM	AM					
	Thymeliaceae	*Pimelia* Link	ECM	AM					
	Vitaceae	*Vitis* L.	ECM	AM					

Mycorrhizal types by Smith and Read (2008): ECM, ectomycorrhizae; AM, arbuscular mycorrhizae; EEM, ectendomycorrhizae; ORM, orchid mycorrhizae; ABM, arbutoid mycorrhizae; ERM, ericoid mycorrhizae.

Table 1.2. Fungus genera with at least one species forming ectomycorrhizae. Compiled from Agerer (2006), Rinaldi et al. (2008) and Tedersoo et al. (2010).

Division	Family	Genus
Zygomycota	Endogonaceae	*Densospora* McGee
		Endogone Link
		Sclerogone Warcup
Ascomycota	Gloniaceae	*Cenococcum* Moug. & Fr.
	Chaetosphariaceae	*Chloridium* Link
	Discinaceae	*Gymnohydnotrya* B.C. Zhang & Minter
		Gyromitra Fr.
	Elaphomycetaceae	*Elaphomyces* Nees
		Pseudotulostoma O.K. Mill. & T.W. Henkel
	Helotiaceae	*Hymenoscyphus* Gray
	Herpotrichiellaceae	*Phialophora* Medlar
	Helvellaceae	*Balsamia* Vittad.
		Barssia Gilkey
		Cazia Trappe
		Fischerula Mattir.
		Helvella L.
		Hydnotria Fr.
		Leucangium Quél.
		Underwoodia Peck
		Picoa Vittad.
		Wynnella Boud.
	Pezizaceae	*Amylascus* Trappe
		Chromelosporium Corda
		Galactinia (Cooke) Boud.
		Hydnobolites Tul. & C. Tul.
		Hydnoplicata Gilkey
		Hydnotryopsis Gilkey
		Marcelleina Brumm., Korf & Rifai
		Muciturbo P.H.B. Talbot
		Mycoclelandia Trappe & G.W. Beaton
		Pachyphloeus Tul. & C. Tul.
		Peziza L.
		Ruhlandiella Henn.
		Sarcosphaera Auersw.
		Scabropezia Dissing & Pfister
		Sphaerozone Zobel
		Tirmania Chatin
	Pyronemataceae	*Genabea* Tul. & C. Tul.
		Genea Vittad.
		Geopora Harkn.
		Gilkeya M.E. Sm., Trappe & Rizzo
		Glischroderma Fuckel
		Humaria Fuckel
		Hydnocystis Tul. & C. Tul.
		Meliniomyces Hambl. & Sigler
		Nothojafnea Rifai
		Otidea (Pers.) Bonord.
		Phaeangium Pat.
		Pulvinula Boud.
		Sphaerosoma Klotzsch
		Sphaerosporella (Svrček) Svrček & Kubička
		Sowerbyella Nannf.
		Tarzetta (Cooke) Lambotte
		Trichophaea Boud.
		Wilcoxina Chin S. Yang & Korf
	Terfeziaceae	*Cazia* Trappe
		Delastria Tul. & C. Tul.
		Loculotuber Trappe, Parladé & I.F. Alvarez
		Terfezia (Tul. & C. Tul.) Tul. & C. Tul.
	Tuberaceae	*Choiromyces* Vittad.
		Dingleya Trappe
		Labyrinthomyces Boedijn
		Paradoxa Mattir.
		Reddellomyces Trappe, Castellano & Malajczuk
		Tuber P. Micheli ex F.H. Wigg.
Basidiomycota	Agaricaceae	*Gymnogaster* J.W. Cribb
		Riessiella Jülich
		Timgrovea Bougher & Castellano
	Albatrellaceae	*Albatrellus* Gray
		Polyporoletus Snell
		Scutiger Paulet
Basidiomycota	Amanitaceae	*Amanita* Pers.
		Amarrendia Bougher & T. Lebel
		Torrendia Bres.
	Atheliaceae	*Amphinema* P. Karst.
		Byssocorticium Bondartsev & Singer
		Byssoporia M.J. Larsen & Zak
		Piloderma Jülich
		Tylospora Donk
	Bankeraceae	*Bankera* Coker & Beers ex Pouzar
		Boletopsis Fayod
		Hydnellum P. Karst.
		Phellodon P. Karst.
		Sarcodon Quél. ex P. Karst.
	Bolbitiaceae	*Descomyces* Bougher & Castellano
		Descolea Singer
	Boletaceae	*Afroboletus* Pegler & T.W.K. Young
		Aureoboletus Pouzar
		Austroboletus (Corner) Wolfe
		Boletellus Murrill
		Boletus L.
		Boletochaete Singer
		Bothia Halling, T.J. Baroni & Manfr. Binder
		Chamonixia Rolland
		Fistulinella Henn.
		Gastroboletus Lohwag
		Gastroleccinum Thiers
		Gastrotylopilus T.H. Li & Watling
		Heimioporus E. Horak
		Leccinellum Bresinsky & Manfr. Binder
		Leccinum Gray
		Paxillogaster E. Horak
		Phylloboletellus Singer
		Phylloporus Quél.
		Porphyrellus E.-J. Gilbert
		Pseudoboletus Šutara
		Pulveroboletus Murrill
		Retiboletus Manfr. Binder & Bresinsky
		Rhodactina Pegler & T.W.K. Young
		Royoungia Castellano, Trappe & Malajczuk
		Setogyroporus Heinem. & Rammeloo
		Sinoboletus M. Zang
		Strobilomyces Berk.
		Tubosaeta E. Horak
		Tylopilus P. Karst.
		Veloporphyrellus L.D. Gómez & Singer
		Xanthoconium Singer
		Xerocomus Quél.
	Cantharellaceae	*Cantharellus* Adans. ex Fr.
		Craterellus Pers.
		Pterygellus Corner
	Ceratobasidiaceae	*Ceratobasidium* D.P. Rogers
	Chondrogastraceae	*Chondrogaster* Maire
	Clavulinaceae	*Clavulina* J. Schröt.
		Membranomyces Jülich
	Cortinariaceae	*Anamika* K.A.Thomas, Peintner, M.M.Moser & Manim.
		Cortinarius (Pers.) Gray
		Cuphocybe R. Heim
		Dermocybe (Fr.) Wünsche
		Destuntzia Fogel & Trappe
		Gigasperma E. Horak
		Hebeloma (Fr.) P. Kumm.
		Inocybe (Fr.) Fr.
		Mackintoshia Pacioni & Sharp
		Mycoamaranthus Castellano, Trappe & Malajczuk
		Naucoria (Fr.) P. Kumm.
		Rozites P. Karst.
		Setchelliogaster Pouzar
		Stephanopus M.M. Moser & E. Horak
		Thaxterogaster Singer
	Cribbeaceae	*Mycolevis* A.H. Sm.
	Dyplocystidiaceae	*Dyplocystis* Berk. & M.A. Curtis
		Tremellogaster E. Fisch.

Table 1.2. Fungus genera with at least one species forming ectomycorrhizae. Compiled from Agerer (2006), Rinaldi et al. (2008) and Tedersoo et al. (2010). (continued).

Division	Family	Genus
Basidiomycota	Entolomataceae	*Entoloma* (Fr.) P. Kumm.
		Rhodogaster E. Horak
		Richoniella Costantin & L.M. Dufour
	Gallaceaceae	*Gallacea* Lloyd
		Hallingea Castellano
	Gomphaceae	*Clavariadelphus* Donk
		Gomphus Pers.
		Turbinellus Earle
	Gomphidiaceae	*Brauniellula* A.H. Sm. & Singer
		Chroogomphus (Singer) O.K. Mill.
		Cystogomphus Singer
		Gomphidius Fr.
		Gomphogaster O.K. Mill.
	Gyroporaceae	*Gyroporus* Quél.
		Rubinoboletus Pilát & Dermek
	Hydnaceae	*Hydnum* L.
	Hydnangiaceae	*Durianella* Desjardin, A.W. Wilson & Manfr. Binder
		Hydnangium Wallr.
		Laccaria Berk. & Broome
		Maccangia Wallr.
		Podohydnangium G.W. Beaton, Pegler & T.W.K. Young
	Hygrophoraceae	*Camarophyllus* (Fr.) P. Kumm.
		Hygrophorus Fr.
	Hymenochaetaceae	*Coltricia* Gray
		Coltriciella Murrill
		Hymenogaster Vittad.
		Quadrispora Bougher & Castellano
	Hysterangiaceae	*Aroramyces* Castellano & Verbeken
		Hysterangium Vittad.
	Inocybaceae	*Auritella* Matheny & Bougher
	Leucogastraceae	*Leucogaster* R. Hesse
		Leucophleps Harkn.
	Melanogastraceae	*Alpova* C.W. Dodge
		Corditubera Henn.
		Hoehnelogaster Lohwag
		Melanogaster Corda
	Mesophelliaceae	*Andebbia* Trappe, Castellano & Amar.
		Castoreum Cooke & Massee
		Gummiglobus Trappe, Castellano & Amar.
		Gummivena Trappe & Bougher
		Malajczukia Trappe & Castellano
		Mesophellia Berk.
		Nothocastoreum G.W. Beaton
	Octavianiaceae	*Octaviania* Vittad.
	Paxillaceae	*Austrogaster* Singer
		Austropaxillus Bresinsky & Jarosch
		Gymnopaxillus E. Horak
		Gyrodon Opat.
		Paragyrodon (Singer) Singer
		Paxillus Fr.
Basidiomycota	Polyporaceae	*Sistotrema* Pers.
	Ramariaceae	*Austrogautieria* E.L. Stewart & Trappe
		Gautieria Vittad.
		Ramaria Fr. ex Bonord.
	Rhizopogonaceae	*Rhizopogon* Fr.
		Fevansia Trappe & Castellano
		Rhopalogaster J.R. Johnst.
	Russulaceae	*Arcangeliella* Cavara
		Cystangium Singer & A.H. Sm.
		Elasmomyces Cavara
		Gastrolactarius R. Heim ex J.M. Vidal
		Gymnomyces Massee & Rodway
		Lactarius Pers.
		Macowanites Kalchbr.
		Martellia Mattir.
		Multifurca Buyck & V. Hofstetter
		Russula Pers.
		Zelleromyces Singer & A.H. Sm.
	Sebacinaceae	*Sebacina* Tul. & C. Tul.
		Tremellodendron G.F. Atk.
		Tremelloscypha D.A. Reid
	Sclerodermataceae	*Astraeus* Morgan
		Calostoma Desv.
		Chlorogaster Læssøe & Jalink
		Horakiella Castellano & Trappe
		Pisolithus Alb. & Schwein.
		Scleroderma Pers.
	Stephanosporaceae	*Mayamontana* Castellano, Trappe & Lodge
	Strophariaceae	*Alnicola* Künher
	Suillaceae	*Boletinus* Kalchbr.
		Gastrosuillus Thiers
		Psiloboletinus Singer
		Suillus Gray
	Thelephoraceae	*Polyozellus* Murrill
		Pseudotomentella Svrček
		Thelephora Ehrh. ex Willd.
		Tomentella Pers. ex Pat.
		Tomentellopsis Hjortstam
	Tricholomataceae	*Catathelasma* Lovejoy
		Lyophyllum P. Karst.
		Tricholoma (Fr.) Staude
	Truncocolumellaceae	*Truncocolumella* Zeller
	Tulasnellaceae	*Tulasnella* J. Schröt.

Wild edible fungi are an important socioeconomic resource in many regions of the world. More than 2,000 fungi are known to produce edible sporocarps (Boa 2004). Over the last decade, the market value, consumer demand, and interest in managing forests for non-timber products have increased (Pilz et al. 1999; Díaz-Balteiro et al. 2003). This resource not only is a food source but also could be an important income

generator in rural forest areas if used properly (Boa 2004; Barroetaveña et al. 2008). In addition, edible mushrooms also represent the basis of multiple products made by manufacturers, including medicine (Table 1.3), and are the source of a new wave of tourism resulting from recreational programs linked to nature.

Table 1.3. Ectomycorrhizal species used as food and medicine all over the world, extracted from Boa (2004).

Ectomycorrhizal genus	Number of edible species	Number of medicinal species
Amanita	83	7
Boletus	72	7
Cantharellus	42	3
Cortinarius	50	10
Laccaria	14	4
Lactarius	94	7
Leccinum	26	-
Morchella	18	5
Ramaria	44	5
Russula	128	25
Suillus	27	2
Terfezia	7	-
Tricholoma	52	17
Tuber	18	-

Nowadays, the commercial value of forest fungi may equal or even surpass that of timber, especially in the Mediterranean area (Arnolds 1995; Alexander et al. 2002; De Román and Boa 2006; Reyna 2012); therefore, fungi have become strategic in the conservation and management of forest systems. Where mushroom picking is a significant forest resource, it should be included in forest management and planning (Palahí et al. 2009). Paradoxically, only in the last 10 years has this resource begun to be integrated within forest planning, most of the time sporadically (Martínez de Aragón et al. 2007). The lack of information and the low predictability of harvesting sporocarps may be partly the cause of this absence.

Sporocarp formation of these fungi is linked to habitat characteristics and climate conditions, but this data alone does not explain all the trends of fungal fruiting and dynamics. Factors influencing sporocarp formation are not yet apparent. Sporocarp formation is probably the most complicated stage in the life cycle of fungi. This situation is even more complex for ectomycorrhizal fungi, which require symbiotic association with a host plant. Fungal and host genes, environmental and physiological conditions, and nutritional state of mycelium and the host trigger sporocarp formation in ectomycorrhizal fungi, but the process is not fully understood to date (Murat et al. 2008).

Different factors are known to influence the sporocarp formation of fungi. Several studies have shown that fructification is linked to habitat characteristics (Alonso Ponce et al. 2011) and climate conditions, mainly soil moisture and temperature (Salerni et al. 2002; Martínez de Aragón et al. 2007; Barroetaveña et al. 2008; Bonet et al. 2010; Pinna et al. 2010). However, the same studies stated that this data alone does not explain all the trends in the dynamics of fungal fruiting. Host conditions also obviously affect this process (Kües and Martin 2011; Ortega-Martínez et al. 2011). In early successional or disturbed habitats, nutrients are primarly abiotic, ecosystems tend to have high entropy levels, and symbioses, primarly mycorrhizae, are not well developed; but later successional habitats have a high degree of symbiosis, organically bound nutrients, and low entropy, suggesting that mycorrhizae are important in the successional process and ecosystem development (Allen 1991).

The ‘universal’ latitudinal gradient of diversity that characterizes the distribution of richness of most terrestrial and marine macro-organisms has a

unimodal form in ectomycorrhizal fungi (Tedersoo et al. 2012). There is lower ectomycorrhizal fungal richness in tropical ecosystems, failing to conform to the 'universal' pattern. Tedersoo and Nara (2010) propose three explanations for this unique fact. Firstly, the strictly temperate ectomycorrhizal fungal lineages probably evolved at higher latitudes with the Pinaceae hosts, but may be inferior competitors in tropical conditions. Secondly, when both soil and roots are regarded as habitats for ectomycorrhizal fungi, the lower diversity and abundance of these habitats may account for the lower ectomycorrhizal fungal diversity in the tropics. Thirdly, there is the effect of resource availability and fragmentation of the suitable host distribution.

1.2. Ectomycorrhizae structures.

The development of an ectomycorrhiza begins with contact between the fungus and the roots. Establishment of the symbiosis must be under the control of the genes of both partners (Smith and Read 2008). Until the symbiotic function starts, there are five stages of development (Malajczuk et al. 1990):

1. *preinfection*, characterized by hyphal contact with the root;
2. *symbiotic initiation*, characterized by fungal attachment to the epidermis;
3. *fungal colonization*, with hyphal penetration between epidermal cells and the formation of the initial mantle layers;
4. *symbiotic differentiation*, in which the Hartig net proliferates and there is a rapid buildup of mantle hyphae;

5. and *symbiotic function*, meaning the end of Hartig net growth and the development of a consistent mantle, tightly pressed against the epidermal cells.

1.2.1. The Hartig net and the mantle.

The Hartig net formation is the first step in the beginning of ectomycorrhizal relationships and indicates the existence of a true ectomycorrhizal association. This is the contact zone between the symbionts, where the interchange of nutrients between fungi and host plant is produced. The structure is usually formed from the inner part of the root to the outer. Fungal hyphae penetrate between the epidermal cells, which become progressively more radially enlarged from the apex back (Massicotte et al. 1986) (Figure 1.1).

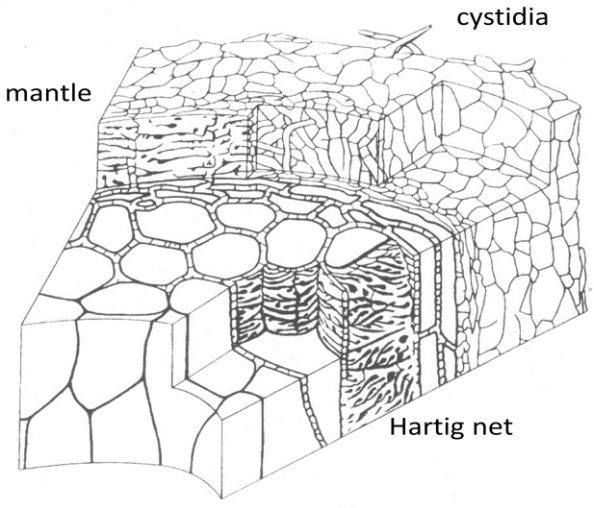


Figure 1.1. Basic structure of an ectomycorrhizae, extracted from Smith and Read (2008).

Although the study of the anatomy of ectomycorrhizae began with the study of root longitudinal and cross sections, nowadays, they are considered as minor features (Agerer 2006). Their only differences are regarding host genera, based on the depth of the penetration. In the majority of angiosperms, penetration is confined to the epidermal layer, forming an 'epidermal' Hartig net and with the hyphae wholly or partially encircling the epidermal root cells (Godbout and Fortin 1983; Agerer and Rambold 2004-2013). In the gymnosperms, by contrast, the Hartig net typically penetrates beyond the epidermis to enclose several layers of cortical cells, sometimes even extending to the endodermis (Agerer and Rambold 2004-2013).

Whereas the Hartig net forms the most extensive interface between fungus and plant, its biomass in most ectomycorrhizae is relatively small compared to that of the overlying mantle (Smith and Read 2008). The ectomycorrhizal fungal mantle protects the Hartig net in order to make the association between fungus and plant as stable as possible, improving the way of life for both partners. The mantle is a significant concentration of biomass for the fungus and it could also be used as a resistant structure.

Usually, the mantle can be divided into three layers in plane view: inner, middle, and outer mantle layers (Agerer and Rambold 2004-2013). The inner and outer mantle layers are always present in the ectomycorrhizae, while middle mantle layers may or may not present or there may be more than one, depending on its structure. The inner mantle layer is in full contact with the root surface, and it is usually thinner than the rest of the layers.

There are two basic types of mantles (Agerer 1991): plectenchymatous mantles, in which the hyphae can be recognized individually, and pseudoparenchymatous mantles, in which the individual hyphae cannot be distinguished because they have been enlarged and have lost their original form, thus resembling a true parenchyma. Pseudoparenchymatous mantles appear more advanced than plectenchymatous ones in a structural and evolutionary sense (Agerer 1995).

Mantle types are described in detail by Agerer (1987-2012, 1991, 1995, 2006) and Agerer and Rambold (2004-2013) and divided into 16 types (Figure 1.2): nine plectenchymatous and seven pseudoparenchymatous. Laticifers, the latex-containing, long, thick, scarcely branched hyphae can occur in all of the mantle types (Agerer 2006). Pseudoparenchymatous mantles prevent the formation of emanating elements, perhaps due to the great cell dimensions of the outer mantle layer (Agerer 2006). They are seemingly destined to have close contact with the soil particles surrounding them and usually increase in size due to the hydrophilicity of their cells.

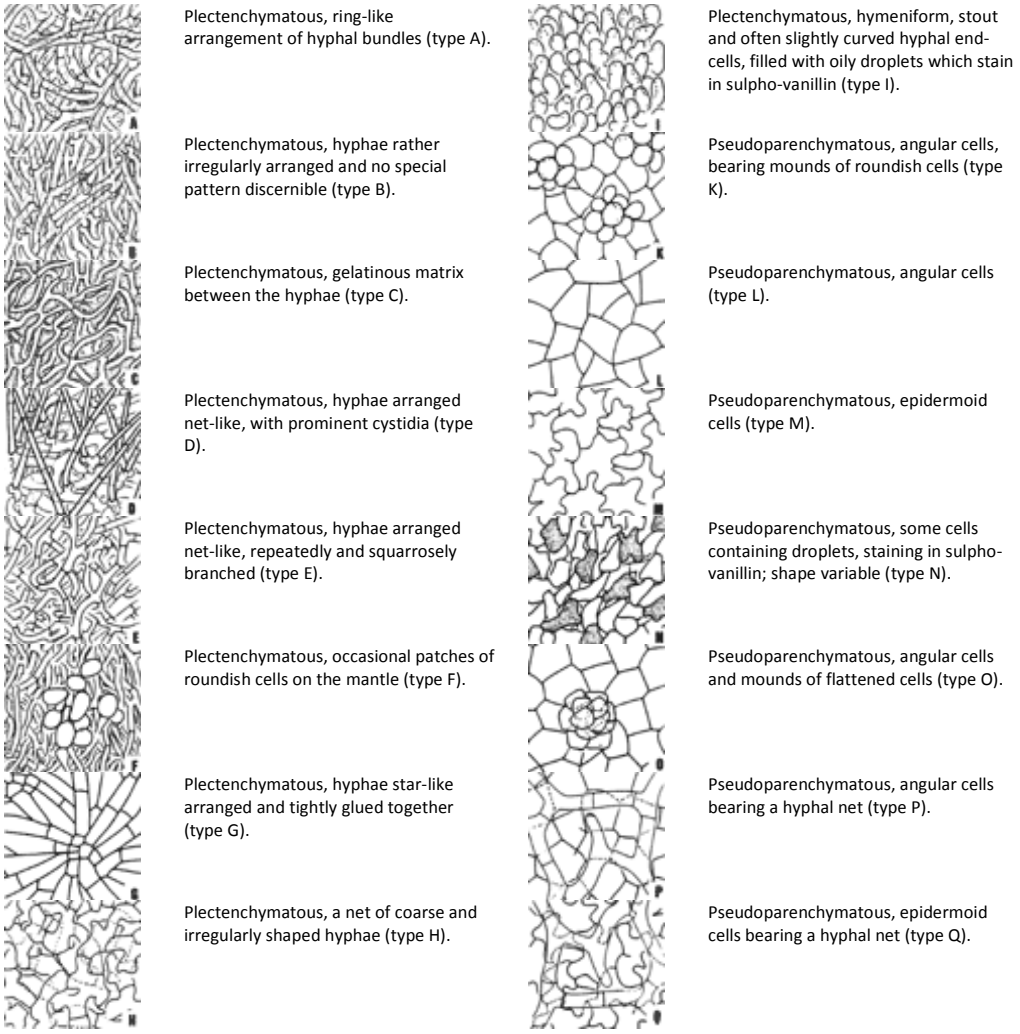


Figure 1.2. Ectomycorrhizal mantle types, extracted from Agerer and Rambold (2004-2013).

1.2.2. The emanating elements.

Extraradical mycelium, cystidia, and rhizomorphs are emanating elements from the ectomycorrhizal mantle. These three elements may all be represented together in an ectomycorrhizal type, alternatively only one or two elements may be present without the second or third, or the case may be that none are represented at all. It all depends on fungal colonization strategies, physiological aspects, and competition abilities.

Emanating hyphae can have various shapes in the same ectomycorrhizal type (Agerer 1991). It can also form anastomoses, have simple septa or clamps, a rough surface, or even crystals.

Cystidia occur not only on the cap skin, gills, and stipe of sporocarps but also on the ectomycorrhizae (Agerer 2006). Although they are not very common, the 15 types compiled by Agerer (1991) and Agerer and Rambold (2004-2013) are distinctive for some fungal groups. The structures seem to be specialized in short-distance nutrition processes, increasing the mantle's field of influence.

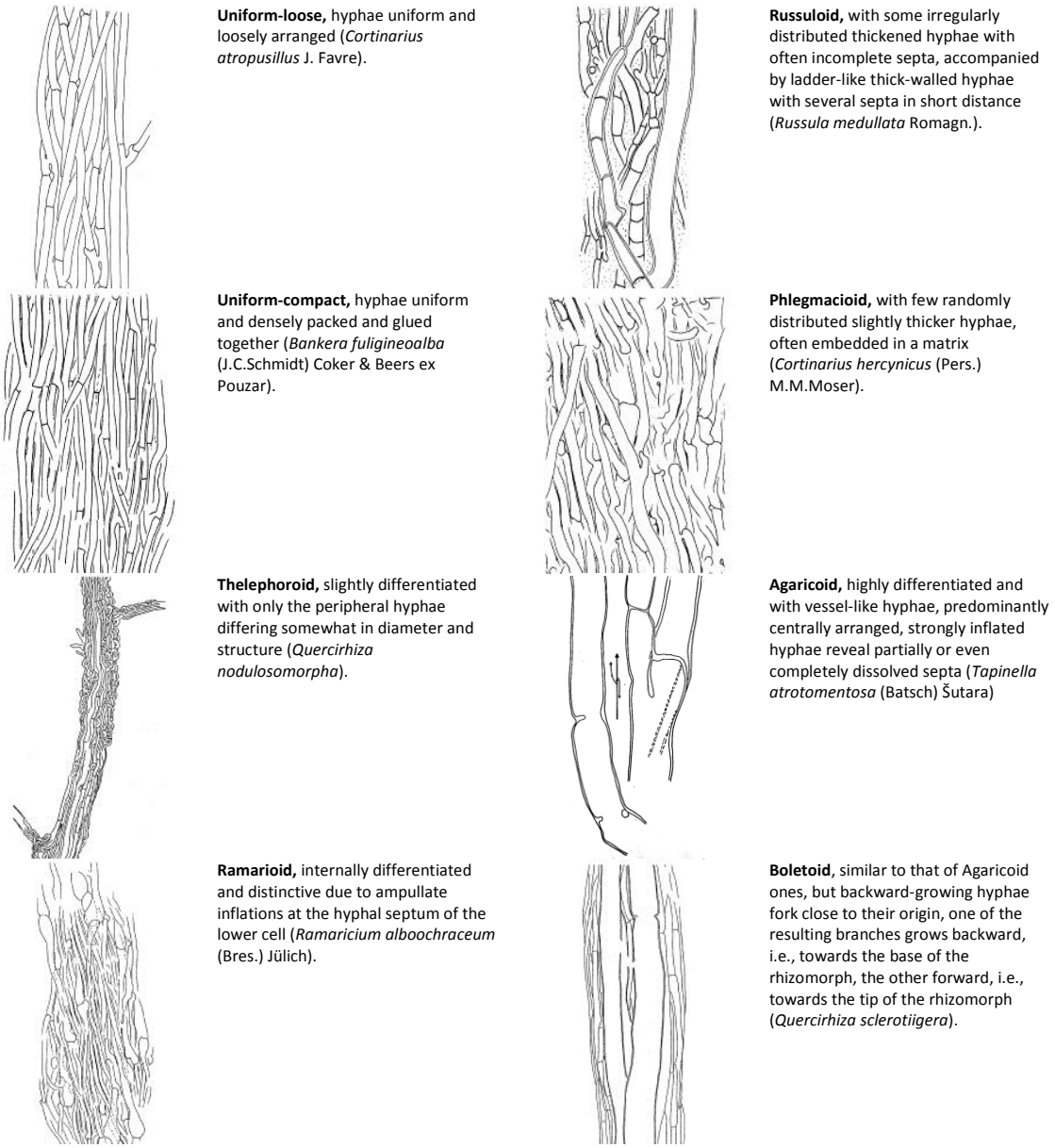
Rhizomorphs are multi-hyphal linear aggregates (Agerer 1999), divided into eight types (Agerer 1999; Agerer and Iosifidou 2004) by their structure. Their function is directly related to nutrient uptake and transport and they are capable of exploring large distances from the mantle.

Acquisition and transport of water and nutrients are performed exclusively by fungal mycelium in the soil, especially by the most distal parts of rhizomorphal hyphae, while the mantle is in fact an outwardly sealed compartment solely involved with storage and exchange between symbionts (Agerer 2001).

Rhizomorphs are hyphae connected by various mechanisms and growing more or less parallel and more or less closely together over a greater distance (Agerer 1987-2012), forming multi-hyphal linear aggregates (Agerer 1999). Based on structural, ontogenic and functional similarities between all multi-hyphal linear aggregates, Cairney et al. (1991) recommend that the term rhizomorph be used to describe all those structures, irrespective of their internal organization and their ontogeny (Agerer and Iosifidou 2004). The term rhizomorph appears to be the first used to describe this type of structure and also highlights their root-like morphology. A comprehensive typology of rhizomorph structures was first published in 1991 by Agerer, who extended it to include ontogenetical aspects in 1999. Eight rhizomorph types could be distinguished (Agerer 1999, 2006; Agerer and Iosifidou 2004) (Figure 1.3): *uniform-loose*, composed of normal vegetative hyphae; *uniform-compact*, that possess uniformly shaped densely agglutinated hyphae; *thelephoroid*, with slightly differentiated hyphae; *ramarioid*, internally differentiated; *russuloid*, which have irregularly distributed thickened hyphae with often incomplete septa; *phlegmacioid*, with a few randomly distributed slightly thicker hyphae often embedded in a matrix; *agaricoid*, highly differentiated with vessel-like hyphae; and *boletoid*, also highly differentiated and with vessel-like hyphae but ramified as split type.

Rhizomorphs in the Gomphales, ramarioid type with oleocanthocystidia and/or oleoacanthohyphae, thin-walled and with irregular globular yellowish cells (Agerer and Iosifidou 2004; Agerer 2006), seem to be strongly influenced by the functional demands of the symbiosis because it enables the fungi to extract water from soil particles to an extremely high degree (Agerer 2006).

Boletales are characterized by the long-distance exploration-type (Agerer 2001) and boletoid rhizomorphs (Agerer 2006), which are defined by three essential characters: (1) runner hyphae that grow very fast and with few ramifications; (2) the formation of split-type hyphal ramifications to facilitate forward and backward transport in rhizomorphs; and (3) the increase of hyphal diameters with a contemporary dissolution of their septa to reduce the transport resistance of solutions (Raidl 1997; Agerer 1999). These rhizomorphs are usually hydrophobic, thus preventing leakage when water is transported from distal regions to the proximal sink (Unestam and Sun 1995; Agerer 2006).



Uniform-loose, hyphae uniform and loosely arranged (*Cortinarius atropusillus* J. Favre).

Russuloid, with some irregularly distributed thickened hyphae with often incomplete septa, accompanied by ladder-like thick-walled hyphae with several septa in short distance (*Russula medullata* Romagn.).

Uniform-compact, hyphae uniform and densely packed and glued together (*Bankera fuligineoalba* (J.C.Schmidt) Coker & Beers ex Pouzar).

Phlegmacioid, with few randomly distributed slightly thicker hyphae, often embedded in a matrix (*Cortinarius hercynicus* (Pers.) M.M.Moser).

Thelephoroid, slightly differentiated with only the peripheral hyphae differing somewhat in diameter and structure (*Quercirhiza nodulosomorpha*).

Agaricoid, highly differentiated and with vessel-like hyphae, predominantly centrally arranged, strongly inflated hyphae reveal partially or even completely dissolved septa (*Tapinella atrotomentosa* (Batsch) Šutara)

Ramarioid, internally differentiated and distinctive due to ampullate inflations at the hyphal septum of the lower cell (*Ramaricium alboochraceum* (Bres.) Jülich).

Boletoid, similar to that of Agaricoid ones, but backward-growing hyphae fork close to their origin, one of the resulting branches grows backward, i.e., towards the base of the rhizomorph, the other forward, i.e., towards the tip of the rhizomorph (*Quercirhiza sclerotiigera*).

Figure 1.3. Rhizomorph types, extracted from Agerer (1999, 2006).

1.2.3. The exploration types.

There are three structures that ectomycorrhizae use to expand root systems: extraradical mycelia, cystidia, and rhizomorphs. Every structure that emanates from the ectomycorrhizae should perform a role mainly concerning the process of nutrition and protection against pathogens.

Due to the amount, range, and differentiation of the mycelia structures emanating from the hyphal mantle into the soil, i.e., the extraradical mycelia, several functional groups, so-called exploration types, can be distinguished (Agerer 2001). The exploration types cover ectomycorrhizae with almost smooth mantles (*contact exploration type*), ectomycorrhizae with distinct emanating hyphae and rather limited growth into the surrounding soil (*short-distance exploration type*), and a variety of ectomycorrhizae with rhizomorphs grouped according to rhizomorph range and internal organization. The *medium-distance exploration type* has rather far-reaching rhizomorphs that are internally either undifferentiated or have internal hyphae of an enlarged diameter in certain fungal species. The highest differentiated exploration type, the *long-distance exploration type*, features very long rhizomorphs with internal vessel-like transport hyphae (Agerer 2001, 2006). Long-distance exploration types are more prevalent in areas of low root density while short-distance exploration types are more common in areas of high root density (Peay et al. 2011). Long-distance exploration type fungi are also able to colonize root plants at long distances, and continue to increase their biomass by tapping into multiple plants in an area, with this pattern congruent with the idea that mycelia networking is most advantageous to

high-biomass structures, like the rhizomorphs related with this exploration type (Simard et al. 2012).

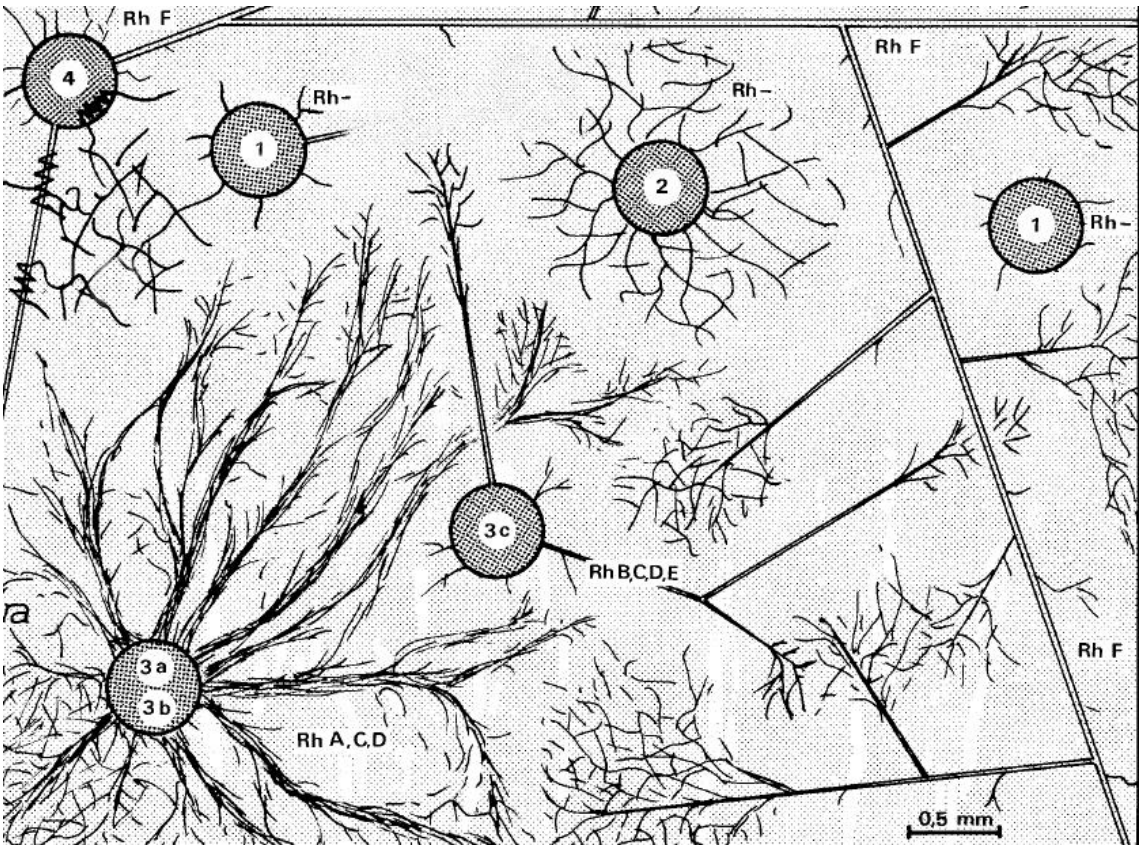


Figure 1.4. Schematic drawings of different exploration strategies, represented by cross-sections of ectomycorrhizae and the extramatrical mycelium. 1 Contact exploration type; 2 short-distance exploration type; 3a, b medium-distance fringe exploration type and medium-distance mat exploration type; 3c medium-distance smooth exploration type; 4 long-distance exploration type. All figures are to scale (Rh rhizomorph, – rhizomorph lacking, A-F organization types of rhizomorphs according to Agerer 1987-2012. Extracted from Agerer (2001).

The anatomy and architecture of extraradical mycelial systems may determine the potential for nutrient capture (Smith and Read 2008). The extraradical mycelia of ectomycorrhizal fungi enormously increase the space plant roots occupy for water and nutrient gain (Cairney and Burke 1986; Read 1992; Leake et al. 2004; Smith and Read 2008), but the costs for ectomycorrhizae are substantial (Rygiewicz and Andersen

1994; Weigt et al. 2011). Therefore, the exploration types raise two different issues in the functioning of the mutualistic symbiosis: (1) the benefit for the plant as expressed by the range of soil occupation by the mycelium to explore and exploit the soil and finally transport water and nutrients to the roots and (2) the costs for the tree which are mainly expressed by the carbohydrates that have to be invested by the tree to support its fungal partner (Weigt et al. 2012).

While the study of exploration types requires morphological observation of mycorrhizae, extraradical mycelial study is much more difficult to carry out using observational techniques. The methods to study the structure and function of extraradical mycorrhizal mycelia such as biochemical and DNA-based markers, in vitro and in soil observation and root-free hyphal compartmentalisation were reviewed by Leake et al. in 2004. The distribution and dynamics of extraradical fungal mycelia in the soil were poorly understood until appropriate methods for their study were developed (Anderson and Cairney 2004). The development of techniques based on direct nucleic acid extraction coupled with polymerase chain reaction (PCR) amplification have provided new insights into the ecology of these soil fungi (Guidot et al. 2002, 2003). Real-time PCR allowed for the relative or absolute quantification of fungal biomass and compared to other quantification techniques such as total hyphal length or biochemical markers, provides a species-specific measure for mycelial biomass estimations (Landeweert et al. 2003). This technique has been adapted for monitoring plant pathogenic fungi (Hietala et al. 2003; Gachon and Saindrenan 2004) and mycorrhizal fungi (Schubert et al. 2003; Kennedy et al. 2007; Parladé et al. 2007; van der Linde et al. 2009).

Nowadays, specific markers for real-time PCR have been developed for some edible ectomycorrizal species: *Lactarius deliciosus* (L.) Gray (Parladé et al. 2007; Hortal et al. 2008, 2009), *Boletus edulis* Bull. (De la Varga et al. 2012, 2013), *Tuber aestivum* Vittad. (Gryndler et al. 2013), *Tuber melanosporum* Vittad. (Zampieri et al. 2012; Parladé et al. 2013), and *Tuber magnatum* Picco (Iotti et al. 2012).

Several studies have tried to relate the amount of soil mycelia to the number of ectomycorrhizae and the sporocarp production for *Boletus edulis* (De la Varga et al. 2012, 2013); *Boletus* sp., *Cortinarius* sp., and *Tomentella* sp. (Kjøller 2006); *Lactarius deliciosus* (Parladé et al. 2007; De la Varga et al. 2013); *Cortinarius* sp. and *Russula* sp. (Peter et al. 2001); *Clavulina cristata* (Holmsk.) J. Schröt., *Lactarius subdulcis* (Pers.) Gray, and *Xerocomus pruinatus* (Fr. & Hök) Quél. (Rineau et al. 2010); and *Tuber melanosporum* (Suz et al. 2008). However, no conclusive results have been obtained to date. The sporocarp production of edible species depends on both the host tree preference and the ecological environment: fungal communities, climate, soil and tree development, among other factors (Salerni et al. 2002; Laganà et al. 2003; Martínez de Aragón et al. 2007; Barroetaveña et al. 2008; Bonet et al. 2010; Pinna et al. 2010; Buée et al. 2011; Ortega-Martínez et al. 2011; Savoie and Largeteau 2011; Martínez-Peña et al. 2012). Egli et al. (2010) showed an increase in fruit body production after thinning, relating the production of sporocarps with the previous annual tree growth. Similarly, Bonet et al. (2012) found an increase in the sporocarp production of *Lactarius deliciosus* after thinning and related it to changes in soil fertility.

The apparent uncoupling between above- and belowground fungal components has been observed in ectomycorrhizal species (Gardes and Bruns 1996; Hynes et al.

2010; De la Varga et al. 2012, 2013). It has been demonstrated that the belowground mycelial system of *Suillus grevillei* (Klotzsch) Singer (Zhou et al. 2001), *Tricholoma matsutake* (S. Ito and S. Imai) Singer (Lian et al. 2006), *Hydnellum peckii* Banker, and *Phellodon tomentosus* (L.) Banker (van der Linde et al. 2009) is not always centered on the sporocarps and there was no quantitative relationship between the belowground abundance of mycelia and the number or distribution of sporocarps. Suz et al. (2008) compared non-productive and productive trees in a *Tuber melanosporum* orchard, finding apparently higher quantities of mycelia in soil samples taken around nonproductive trees. Peintner et al. (2007), studying the soil fungal communities in a *Castanea sativa* Mill. forest, demonstrated that the overlap between above and belowground fungal communities was very low. In their study, *Boletus* mycelia, compared with other soil fungi, were rare and scattered, whereas their sporocarps were the dominant in the mushroom production of that forest. *Tuber magnatum* mycorrhizae are scarce or absent even where their sporocarps are found (Bertini et al. 2005; Murat et al. 2005), but Zampieri et al. (2010) have shown that *Tuber magnatum* mycelium is widely distributed in the soil of truffle grounds.

1.3. Ectomycorrhizae patch dynamics in the roots.

The distribution and interaction of ectomycorrhizae in the root system are conditioned by many factors. Some of them are related with the root morphology of the host tree, and also with ectomycorrhizal morphology, but abiotic factors, such as soil properties, also have an influence.

Ectomycorrhizal communities are impressively diverse, even in stands dominated by a single plant species (Horton and Bruns 2001). Buée et al. (2009) found around 1,000 fungal molecular operational taxonomic units in 4 g of forest soil, in which ectomycorrhizal fungi represented more than 50 % of the 30 most abundant genera. Fungi initially colonize isolated points along a root system and proliferate locally through vegetative production, so their distribution is clustered, and there are extreme changes in species presence and abundance by a few centimeters (Taylor 2002; Lilleskov et al. 2004; Kennedy et al. 2009).

There is a strong spatial variation, but there is also variation due to seasonal factors. *Boletus edulis* and *Lactarius deliciosus* present changes in its extraradical mycelium characterized by seasonal variability, with a clear increase in the amounts of biomass during the coldest months of the year and with variability strongly dependent on the weather (De la Varga et al. 2013). For both species, the minimum mycelium quantity was detected before or at the same time as the fructification period, which could indicate an allocation of resources to produce sporocarps.

Fungi are organisms that compete among themselves when resources are limited. Mycorrhizal fungi compete for two general classes of resources: host-derived carbon and soil- or detritus-derived mineral nutrients, both types of resources are

arrayed in space (Bruns 1995). The creation of additional habitats for ectomycorrhizal fungi is related to small-scale natural disturbances in the roots and in the soil. There are three key abiotic factors that determine the presence of ectomycorrhizal fungi in the soil: temperature, pH and nitrogen (Erland and Taylor 2002). There is also another factor to take into account: competitive interactions between fungi may significantly influence temporal patterns of the ectomycorrhizal community structure (Kennedy et al. 2011b).

As was stated by Bruns (1995), there are four ways in which ectomycorrhizal competitors could coexist in a small homogeneous host environment: niche partitioning, disturbance-related patch dynamics, density-dependent mortality, and competitive networks. Kennedy et al. (2009), Kennedy (2010), and Peay et al. (2011) tried to explain the coexistence among ectomycorrhizal competitors assuming that space is a limiting resource, and that vacant space is re-colonized by the first-available recruit and there are strong priority effects in space occupancy. These authors affirm that it could be a 'lottery' for root space driven by widespread spore dispersal and rapid colonization. Spore dispersal would be related to sexual species reproduction, as the species invest in forming sporocarps, while the rapid colonization would be related to extraradical mycelium growth. Differences in colonization and competitive abilities may facilitate species co-existence in ectomycorrhizal fungal communities (Peay et al. 2007).

1.4. Developing general predictive models for mycorrhizal fungal communities.

Our understanding of distribution and community organization of ectomycorrhizal fungal communities on the roots is in its infancy. Although some regional models of ectomycorrhizae, sporocarp production, and environmental relationships have been developed over recent years (Bergemann and Largent 2000; Barroetaveña et al. 2008; Wolfe et al. 2010; Alonso Ponce et al. 2010a, 2011), sporocarps represent a biased subsample of the belowground community (Gardes and Bruns 1996). However, the transcendence of sporocarps as non-timber forest products justifies the development of sporocarp-based distribution models, particularly for edible ectomycorrhizal fungi.

Nevertheless, relatively little is known about how mycorrhizal fungi interact with large-scale environmental processes (Peay et al. 2010; Bingham and Simard 2012; Simard et al. 2012). Through the years, autoecological features of hosts have usually been accepted to be the same for the fungal associate. Nonetheless, this statement does not appear to be entirely true, at least from the sporocarp formation perspective, even in fungi with ecological host specificity (Hirose et al. 2010). For example, *Boletus edulis* sporocarps are usually collected in *Pinus sylvestris* L. stands in Spain but never in France (Alonso Ponce et al. 2011), and *Boletus aereus* Bull. sporocarps are collected in drier, warmer locations than *Boletus edulis* for the same host plant (Oria-de-Rueda et al. 2008).

Moreover, patterns of fungal distribution are governed by several factors. Structural differences between sporocarps determine dispersal ability, though most

spores only travel very short distances from their point of origin (Peay et al. 2010). Ectomycorrhizal fungi possess some saprotrophic capacity, particularly if there is some selection pressure maintaining it, such as the occasional loss of connection with a living host plant (Koide et al. 2008).

The traditional view of the nature of species assemblages, derived from plant ecology, has lead us to focus on predicting individual species distribution models rather than whole communities (Lilleskov and Parrent 2007). They also predict current distribution in relation to static environmental variables, assuming equilibrium conditions. This assumption may not be valid when modeling fungal communities in a changing environment. One of the next challenges for mycorrhizal ecologists will be to develop dynamic community models to define baseline species distribution data in the context of rapid global change (Lilleskov and Parrent 2007).

1.5. Diversity of ectomycorrhizal types in the root systems.

While there are genetic and physiological barriers to certain plant–fungus associations (Molina and Trappe 1982b), host specificity of ectomycorrhizal fungi does not appear to be absolute (Águeda et al. 2006; Dickie 2007). Thus, the host preference of mycorrhizal fungi reflects a realized, rather than a fundamental, niche.

Host receptivity and host range of mycorrhizal fungi will clearly limit fungal colonization from the limited propagule banks in primary succession systems. The plant community structure will also influence the identity of fungal taxa residing in the

propagule bank and those fungi that establish and sustain colonization in a host root system (Jumpponen and Egerton-Warburton 2005).

As was stated by Horton and Bruns in 2001, the most abundant and frequent taxa on ectomycorrhizal roots in conifer communities in both Europe and North America, and other angiosperm forests, are members of Russulaceae, Thelephoraceae, and non-thelephoroid resupinates. Later studies by De Román and De Miguel (2005), Genney et al. (2006), Buée et al. (2009), Águeda et al. (2010), Pickles et al. (2010), Pritsch et al. (2010), Kennedy et al. (2011a), García-Barreda and Reyna (2012), Põlme et al. (2013), and Roy et al. (2013) have confirmed this fact.

Regardless of the host and condition, ectomycorrhizal fungal communities usually include the presence of *Cenococcum geophylum* Fr. (the one notable exception to the rule of niche differentiation) across soil profiles at all stages of stand development, at every distance from forest edges and in every season of the year (Dickie 2007; Ishida et al. 2007). This Ascomycetous, belonging to short-distance exploration type, could be one of the species specialized in primary nutrition processes due to its sclerotia being able to resist the worst of conditions until it establishes symbiosis with a host plant.

There are few differences found in comparing the ectomycorrhizae associated with coniferous forests to those with deciduous forests. Besides *Cenococcum geophylum*, ectomycorrhizal fungal communities in coniferous forests are dominated by Russuloid and Thelephoroid groups, and also by Amanitaceae, Boletaceae, Clavulinaceae, *Cortinarius* sp., *Inocybe* sp., and *Piloderma* sp. (Izzo et al. 2005; Koide et al. 2005; Genney et al. 2006; Pickles et al. 2010; Auèina et al. 2011). In those fungi, the

dominance of medium-distance exploration types (Thelephoroid, Russuloid, Clavulinaceae, and *Cortinarius* sp.) could be remarked upon, combined with short-distance types (*Inocybe* sp. and *Piloderma* sp.) and long-distance types (Boletaceae and Amanitaceae).

Deciduous mixed forest in France is dominated by Sebacinales, *Lactarius* spp., *Scleroderma* sp., *Russula* spp., *Inocybe* sp., *Cortinarius* sp., *Amanita* sp., *Pseudotomentella* sp., and *Boletus* sp. (Buée et al. 2009), with this community very similar to that belonging to coniferous forests.

In angiosperm Mediterranean forests, ectomycorrhizal fungal communities seem to be dominated by Thelephoroid, Pezizales, Boletales, and *Pisolithus* sp. (De Román and De Miguel 2005; Águeda et al. 2010; Hynes et al. 2010; Benucci et al. 2011; García-Barreda and Reyna 2012). The presence of long-distance and short-distance exploration types is more relevant, with medium-distance exploration types in a secondary role. Differences could be more related to abiotic factors than to host preference. Long-distance exploration types could be more resistant to the harsh conditions of a Mediterranean climate, and short-distance types need to invest less energy in developing their structures.

Recent studies about the ectomycorrhizal fungal communities in alder forests on a local scale (Pritsch et al. 2010), regional scale (Kennedy et al. 2011a; Roy et al. 2013), and global scale (Põlme et al. 2013) show that they are not primarily rich, regardless of whether sampling intensity. The special characteristics of the *Alnus* spp. forests habitats seem to be the ones that make its ectomycorrhizal diversity low, contrasting to the rest of the stands (Horton et al. 2013). Russuloid and other short-

distance exploration types dominate this community, with a very low rate of long-distance exploration types.

1.6. Edible ectomycorrhizal mushrooms.

Wild edible mushrooms sporocarps have been historically used as food and medicines by many different cultures along the world. Nowadays, the human being profits a total of 2,327 species in more than 80 countries, but only a little portion of them are trading (Boa 2004).

One of the most valuable species is *Boletus edulis*. *Boletus edulis* species complex (*Boletus aereus*, *Boletus edulis, Boletus pinophilus* Pilát & Dermek, and *Boletus reticulatus* Schaeff.) has the major importance due to its edibility (Singer 1986; Hall et al. 1998), and they are widely consumed. Those species are only harvested from the wild (Cannon and Kirk 2007) and no controlled production has been done to date. Total annual worldwide consumption of *Boletus edulis* species complex is between 20,000 and 100,000 tons (Hall et al. 1998). Important markets include North America, France, Italy, and Germany (Hall et al. 1998).

In the last years, there is a change trend and mushrooms harvesting is considered nowadays one of the driving forces of the sustainable development in areas with low incomes, providing a chance of their inhabitants to increase their incomes (Wang and Hall 2004). Mushroom harvesting also helps to set up population in rural environments, seriously affected by depopulation in the last decades, promoting activities that generate economic and social incomes.

In the autonomous community of Castilla y León in Spain, mushroom trading has reached a high development degree. Its forests have the great suitability for wild edible mushrooms production and exploitation, and produce the most worldwide valuable species. The estimated mushrooms annual production in Castilla y León forests worth approximately 80 million Euros, reaching triple in years with good harvests (Martínez-Peña et al. 2011). *Boletus edulis* production in Castilla y León is almost 1,600 tons, worth 1.5 million Euros (Ortega-Martínez et al. 2011).

At present, mushrooms harvesting gets involved to the 54 % of the rural population of this region, 567,715 pickers. The 14 % of those mushrooms are sold, so, in a year with good harvest, it is estimated that 17,543 tons could be trading, generating direct incomes worth 65 million Euros per year to the pickers (Aldea et al. 2012).

The main wild edible mushrooms species harvested in Castilla y León for trading and personal consumption are *Boletus edulis* species complex, *Lactarius deliciosus* species complex (*Lactarius deliciosus*, *Lactarius sanguifluus* (Paulet) Fr., and *Lactarius semisanguifluus* R. Heim & Leclair), and *Pleurotus eryngii* (DC.) Quél. Amongst the rural inhabitants, 35 % of them pick *Lactarius deliciosus* species complex, 29 % *Boletus edulis* species complex, and 25 % *Pleurotus eryngii* (Aldea et al. 2012).

The important trade in multiple products based on edible mushrooms have inspired a new sort of tourism known as mycotourism. In Castilla y León, 54 % of the rural lodgings have mycotourist guests, that mainly come from País Vasco, Cataluña, Madrid, Comunidad Valenciana, Aragón, and Navarra regions (Aldea et al. 2012).

Edible wild ectomycorrhizal mushroom production has decreased over the years (Wang and Hall 2004). Climate change, habitat degradation, and overexploitation seem to be some of the reasons of this reduction (Boa 2004). The development of controlled inoculation techniques in order to establish mushrooms producer plantations could be a sustainable way to take advantage of those trading without affecting conservation of the native fungal populations.

Edible mycorrhizal mushrooms can only be grown in specialised plantations on the roots of suitable trees and shrubs and then only with difficulty. There are some scarce examples of success between the Ascomycetes over the world, like *Terfezia claveryi* Chatin (Morte et al. 2012), *Tuber melanosporum*, and *Tuber aestivum* (Chevalier and Sourzat 2012). Even though there are some achieves with Basidiomycetes species like *Lactarius deliciosus* (Guerin-Laguette et al. 2000; Parladé et al. 2004), its cultivation as a feasible shift it is far away.

Keeping in mind that the availability of the majority of the edible ectomycorrhizal mushrooms come from the wild, it is worth to establish forest management guidelines in order to maintain and/or increase sporocarps production, maintaining at the same time fungal species diversity (Ortega-Martínez and Martínez-Peña 2008; Egli et al. 2010; Martínez-Peña et al. 2012).

Although there are a lot of unknown variables around the mechanisms that trigger sporocarps production, estimation of the sporocarps production and fungal diversity in the Spanish forests is an outstanding line of research. There are notable works by the Spanish research groups in order to clear the relationship between the sporocarps production and the biotic and abiotic factors (Fernández-Toirán et al. 2006;

Martínez de Aragón et al. 2007; Alonso Ponce et al. 2011; Martínez-Peña et al. 2012; Ágreda et al. 2014; Bonet et al. 2014; de Miguel et al. 2014).

1.7. *Boletus* genus.

The genus *Boletus* belongs to the family Boletaceae, order Boletales, division Basidiomycota. It is formed by 300 species with epigeous fructification (Muñoz 2005; Agerer 2006).

All the species belonging to this genus are ectomycorrhizal and form fleshy sporocarps that have the hymenium formed by a spongy mass of downward-pointing tubes, curved or sharp, lightish yellow, white, or green. Hymenium is easy removable from the rest of the sporocarp. Their cuticle is usually brown, without volva remains, smooth and matte. The stipe has varied shapes, sometimes it could be more fleshy than the cap, and it is usually reticulated. Spores are white to whitish-yellow, greenish in some species; spindled to elliptic, smooth, guttulated, with thick walls. Hyphae without clamps. Basidia with four spores.

Boletus is a widely represented genus in both, Northern and Southern Hemispheres, living in tropical and mid-latitude forests. A large number of plants are suitable hosts: Fagales-Fagaceae (*Castanea, Castanopsis, Fagus, Lithocarpus, Quercus*), and Betulaceae (*Carpinus, Corylus, Betula, Ostrya, Populus*); Malvales-Malvaceae (*Tilia*), and Cistaceae (*Cistus*); Malpighiales-Salicaceae (*Salix*); Ericales-Ericaceae (*Arctostaphylos*); and Pinales-Pinaceae (*Abies, Keteleeria, Picea, Pinus, Tsuga*) (Molina and Trappe 1982b; Singer 1986; Mello et al. 2006; Águeda et al. 2008).

The species with the major socioeconomic importance in the *Boletus* genus are those belonging to the *Boletus edulis* species complex: *Boletus aereus, Boletus edulis, Boletus pinophilus,* and *Boletus reticulatus* (Figure 1.5). Morphologically, their sporocarps have thin tubes, whitish at the beginning, then yellowish and olive-green at the end, white trama that does not change when cut, and thick, fleshy, and reticulate stipe.

Figure 1.5. Sporocarps of the four species of the *Boletus edulis* species complex. a: *Boletus aereus*; b: *Boletus edulis*; c: *Boletus pinophilus*; d: *Boletus reticulatus*.

Boletus genus ectomycorrhizae are white to yellowish and they are characterized by its plectenchymatous mantle (type A, B, or C), and by its boletoid rhizomorphs, with nodes (Agerer 2006). Emanating hyphae are smooth or covered by crystals, without clamps (Agerer 2006). Cystidia lacking of with cystidia-like hyphal ends (Agerer 2006). They belong to the long distance exploration type and are hydrophobic (Agerer 2001).

Specifically, *Boletus edulis* ectomycorrhizae are hydrophobic, white-yellowish, with type F rhizomorphs (Agerer 2006) and three-layered plectenchymatous mantle forming ring structures in plan view. Other additional characters are the presence of short, inflated, smooth cells, cystidia-like in the surface of the rhizomorphs. External hyphae of rhizomorphs are slightly twisted. Hartig net penetrates one and a half row in the epidermal root cells. All hyphae without clamps. (Gronbach 1998).

As other ectomycorrhizal fungi, while *Boletus* sporocarps dominated the above ground fungal community when fructify, *Boletus* mycelia are rare in the soil and had a scattered distribution (Peintner et al. 2007). Although it is known that fructification is affected by habitat characteristics and climatic conditions (Bonet et al. 2004; Pinna et al. 2010), this process cannot be triggered without the presence of mycelium and ectomycorrhizae in the soil. Thanks to the progress of real-time PCR techniques, De la Varga et al. (2012) found that there is no correlation between the concentration of *Boletus edulis* mycelia and mycorrhizae and *Boletus edulis* sporocarps production in a *Pinus sylvestris* forest, whereas a statistically significant positive correlation was detected between the concentration of mycelia in the soil samples and the presence of ectomycorrhizae in these samples. Although the detection of ectomycorrhizae and

mycelium is feasible nowadays, no conclusions can be given on how this fungus completes its life cycle in time and space or on the population size (Mello 2012).

1.8. *Cistus* genus.

The family Cistaceae belongs to the order Violales, division Magnoliophyta, and has eight genus and about 200 species (Castroviejo et al. 1993). Those shrubs take up hugh areas in the Iberian Peninsula, mainly as members of the undergrowth in *Quercus ilex* L., *Quercus faginea* Willd., *Pinus* sp., and *Fagus sylvatica* L. forests. In particular, Cistaceae species are associated with the regressive phases of those forests, although sometimes become the main species in large areas. Mediterranean region is considered its diversification focus, almost for *Cistus* genus (Castroviejo et al. 1993), conveying a sense of its importance in this region. Cistaceae species are mostly pyrophytic: their germination is linked to high temperatures and therefore they directly depend on fires in Mediterranean ecosystems to reproduce (Alonso et al. 1992).

The genus *Cistus* is represented in the Iberian Peninsula by 12 shrub species, all belonging to pioneer communities growing readily in degraded areas (San Miguel et al. 2008). Its role in the recovering of the ecosystems after forest fires in Mediterranean areas is of the greatest importance. In fact, there is a relationship between the fire and the elimination of the dormancy that cause the cover of the seeds in *Cistus* genus (Pérez-Fernández and Rodríguez-Echeverría 2003).

Cistus ladanifer L. grows in the western Mediterranean, extending from Portugal and Morocco to the French Riviera and Algeria, and in southern and western Spain in zones with hot, dry summers, on siliceous soils over slate and granite (Demoly and Montserrat 1993). According to López Leiva et al. (2009) it occupies 350,000 ha over Castilla y León region.

Cistus sp. can form both ecto- and arbuscular mycorrhizae (Smith and Read 2008). More than 200 ectomycorrhizal fungal species belonging to 40 genera are reported to associate with *Cistus* (Comandini et al. 2006) (Table 1.4). Rockroses (*Cistus* and *Helianthemum* sp.) are ecologically important species because they may act as a reservoir of mycorrhizal fungi after a forest disturbance (Torres et al. 1995).

Table 1.4. Ectomycorrhizal genus associated with *Cistus* species, extracted from Comandini et al. (2006).

Ectomycorrhizal genus		Host
Ascomycota	*Elaphomyces*	*Cistus ladanifer* L.
	Balsamia	*Cistus albidus* L.
	Picoa	*Cistus albidus* L.
	Delastria	*Cistus albidus* L.
		Cistus ladanifer L.
		Cistus salvifolius L.
	Terfezia	*Cistus* sp.
		Cistus albidus L.
		Cistus ladanifer L.
		Cistus monspeliensis L.
		Cistus populifolius L.
		Cistus salvifolius L.
	Tirmania	*Cistus* sp.
	Genea	*Cistus albidus* L.
	Genabea	*Cistus albidus* L.
	Hydnocystis	*Cistus* sp.
		Cistus ladanifer L.
	Choiromyces	*Cistus ladanifer* L.
	Tuber	*Cistus* sp.
		Cistus albidus L.
		Cistus crispus L.
		Cistus incanus L.
		Cistus ladanifer L.
		Cistus laurifolius L.
		Cistus monspeliensis L.
		Cistus salvifolius L.
Basidiomycota	*Amanita*	*Cistus* sp.
		Cistus albidus L.
		Cistus ladanifer L.
		Cistus monspeliensis L.
		Cistus salvifolius L.
	Torrendia	*Cistus monspeliensis* L.
		Cistus salvifolius L.
	Cortinarius	*Cistus* sp.
		Cistus albidus L.
		Cistus crispus L.
		Cistus ladanifer L.
		Cistus laurifolius L.
		Cistus monspeliensis L.
		Cistus salvifolius L.
	Hebeloma	*Cistus* sp.
		Cistus albidus L.
		Cistus ladanifer L.
		Cistus laurifolius L.
		Cistus monspeliensis L.
		Cistus salvifolius L.
	Hymenogaster	*Cistus albidus* L.
	Inocybe	*Cistus* sp.
		Cistus albidus L.
		Cistus incanus L.
		Cistus ladanifer L.
		Cistus monspeliensis L.
		Cistus salvifolius L.
	Hygrophorus	*Cistus* sp.
		Cistus albidus L.
		Cistus ladanifer L.
		Cistus monspeliensis L.
	Laccaria	*Cistus* sp.
		Cistus albidus L.
		Cistus ladanifer L.
		Cistus monspeliensis L.
Basidiomycota	*Tricholoma*	*Cistus* sp.
		Cistus albidus L.
		Cistus monspeliensis L.
	Boletus	*Cistus* sp.
		Cistus albidus L.
		Cistus ladanifer L.
		Cistus monspeliensis L.
	Chalciporus	*Cistus* sp.
		Cistus albidus L.
		Cistus monspeliensis L.
		Cistus salvifolius L.
	Leccinum	*Cistus* sp.
		Cistus albidus L.
		Cistus ladanifer L.
		Cistus monspeliensis L.
		Cistus salvifolius L.
	Suillus	*Cistus* sp.
	Xerocomus	*Cistus* sp.
		Cistus albidus L.
		Cistus monspeliensis L.
	Melanogaster	*Cistus albidus* L.
		Cistus clusii Dun.
		Cistus ladanifer L.
		Cistus laurifolius L.
		Cistus monspeliensis L.
	Paxillus	*Cistus* sp.
		Cistus laurifolius L.
		Cistus monspeliensis L.
	Rhizopogon	*Cistus crispus* L.
		Cistus ladanifer L.
		Cistus monspeliensis L.
	Astraeus	*Cistus albidus* L.
		Cistus monspeliensis L.
	Pisolithus	*Cistus ladanifer* L.
		Cistus monspeliensis L.
	Scleroderma	*Cistus monspeliensis* L.
		Cistus salvifolius L.
	Wakefieldia	*Cistus albidus* L.
	Gymnomyces	*Cistus crispus* L.
		Cistus ladanifer L.
	Lactarius	*Cistus* sp.
		Cistus albidus L.
		Cistus incanus L.
		Cistus ladanifer L.
		Cistus monspeliensis L.
		Cistus salvifolius L.
	Russula	*Cistus* sp.
		Cistus albidus L.
		Cistus ladanifer L.
		Cistus monspeliensis L.
		Cistus salvifolius L.
	Hysterangium	*Cistus ladanifer* L.
		Cistus laurifolius L.
		Cistus monspeliensis L.
		Cistus populifolius L.
	Amphinema	*Cistus* sp.
	Thelephora	*Cistus albidus* L.
		Cistus monspeliensis L.
	Cantharellus	*Cistus* sp.
		Cistus albidus L.
		Cistus monspeliensis L.

2. OBJECTIVES.

We hypothesized that:

1. *Boletus edulis* Bull. is able to produce sporocarps associated with *Cistus ladanifer* L. forming ectomycorrhizae in the wild;
2. Species of the *Boletus edulis* species complex ought to be able to associate with rockroses under lab conditions forming similar structures as those in the wild;
3. This simbiotical association should have a defined realized niche determined by certain ecological abiotical conditions.

In order to clarify them, the particular aims of this doctoral dissertation are:

1. To provide a description and characterization of the ectomycorrhizae of *Boletus edulis* on *Cistus ladanifer*;
2. To test the ability of the *Boletus edulis* species complex to form ectomycorrhizas with *Cistus* sp. under controlled conditions as well as provide detailed anatomical descriptions of the formed ectomycorrhizae;
3. To define the realized niche of the ectomycorrhizal association where *Boletus edulis* produces sporocarps associated with *Cistus ladanifer* in peninsular Spain.

3. Characterization and identification of field ectomycorrhizae of *Boletus edulis* and *Cistus ladanifer*.

3.1. Introduction

Boletus L., especially the *Boletus edulis* species complex, is a cosmopolitan genus of ectomycorrhizal fungi widely represented in the warmer parts of the Northern Hemisphere. These species have a great economic importance for their edibility (Singer 1986; Hall et al. 1998). The genus comprises more than 1,000 species with epigeous fructification, inhabiting forests in tropical and mid latitudes, forming ectomycorrhizae mainly with trees and shrubs of Pinaceae, Fagaceae, and Betulaceae (Singer 1986).

The *Boletus edulis* species complex includes four species: *Boletus aereus* Bull., *Boletus edulis* Bull., *Boletus pinophilus* Pilát & Dermek, and *Boletus reticulatus* Schaeff. The identification of the fruiting bodies of these four species traditionally has been very difficult because it is based exclusively on a few, highly variable morphological characters. Recent studies showed that these four species can be successfully discriminated by an extensive analysis of the internal transcribed spacer of the nuclear rDNA region (Leonardi et al. 2005).

The plants of the Cistaceae family are fairly abundant in the Northerm Hemisphere and South America. The family has eight genera with almost 200 species (Muñoz and Navarro 1993). The *Cistus* L. genus is represented in the Iberian Peninsula by 12 shrub species, all belonging to primary succession stages of tree stands, growing readily in degraded areas. The Cistaceae species in general are pyrophytic. Their germination is related to high temperatures, and they are adapted to forest fires in Mediterranean forests (Alonso et al. 1992). *Cistus ladanifer* L. lives in the western

Mediterranean, from Portugal and Morocco to the French Riviera and Algeria, in zones with hot, dry summers, 0-1,500 m a.s.l., on silicon soil in the southern half of the Iberian Peninsula and on slate and granite in the western part (Demoly and Monserrat 1993).

All Cistaceae are ectomycorrhizal plants (Smith and Read 2008; Brundrett 2002), but available descriptions for *Cistus* ectomycorrhizal types are scarce. Only four morphotypes of ectomycorrhizae described in association with *Cistus* sp. have been found; they are *Boletus rhodoxanthus* (Krombh.) Kallenb. with *Cistus* cf. *ladanifer* (Hanh 2001), *Laccaria laccata* (Scop.) Fr. with *Cistus ladanifer* (Torres et al. 1995), *Lactarius tesquorum* Malençon with *Cistus* sp. (Nuytinck et al. 2004), and *Tuber nigrum* Allioni with *Cistus incanus* L. (Fontana and Giovanetti 1978-79; Fusconi 1983; Wenkart et al. 2001). Rockroses (*Cistus* and *Helianthemum* Mill.) are ecologically important species because they may act as a reservoir of mycorrhizal fungi inoculum after a forest disturbance (Torres et al. 1995; Díez 1998).

Previous references to Cistaceae associations with Boletales in Spain have been compiled in Table 3.1. No previous worldwide literature references have been found about the harvest of *Boletus edulis* sporocarps in pure stands of *Cistus* sp. The aim of this chapter is to provide a first description and characterization of the ectomycorrhizae of *Boletus edulis* on *Cistus ladanifer* collected in their natural habitat, as well as the molecular analyses of the fungal symbiont.

Table 3.1. Literature references of harvesting of Boletales sporocarps in stands with Cistaceae species in Spain.

Species	Host	Reference
Boletus aemilii Barbier	*Cistus* sp.	Llamas and Terrón 2003
Boletus aereus Bull.	*Cistus* sp.	Sánchez Rodríguez et al. 2004
Boletus aestivalis (Paulet) Fr.	*Cistus* sp.	Sánchez Rodríguez et al. 2004
Boletus corsicus Rolland	*Cistus ladanifer* L.	Oria de Rueda and Díez 2002
Boletus impolitus Fr.	*Cistus monspeliensis* L.	Pando 2000
Boletus queletii Schulz. var. *zugazae* Moreno	*Cistus ladanifer* L.	Moreno 1977
Boletus rhodoxanthus Kallenb.	*Cistus* cf. *ladanifer* L.	Hahn 2001
Chalciporus piperatus (Bull. ex Fr.) Bataille.	*Cistus ladanifer* L.	Pando 2000
Leccinum corsicum (Roll.) Sing.	*Cistus* sp.	Llamas and Terrón 2003
	Cistus sp.	Sánchez Rodríguez et al. 2004
	Cistus albidus L.	Moreno Arroyo et al. 1996
	Cistus ladanifer L.	Moreno Arroyo et al. 1996
		Pando 2000
	Cistus laurifolius L.	Oria de Rueda and Díez 2002
	Cistus monspeliensis L.	Moreno Arroyo et al. 1996
Leccinum hispanicum Moreno	*Cistus ladanifer* L.	Moreno 1977
Leccinum lepidum (Bouchet ex Essette)Quadr.	*Cistus ladanifer* L.	Pando 2000
Leccinum quercinum (Pilát) E.E. Green & Watling	*Cistus* sp.	Pando 2000
Paxillus rubicundulus Orton	*Cistus populifolius* L.	Pando 2000
Xerocomus chrysenteron (Bull.)Quél	*Cistus* sp.	Pando 2000
Xerocomus ichnusanus Alessio, Galli et Littini	*Cistus ladanifer* L.	Pando 2000

3.2. Material and methods.

The *Boletus edulis* sporocarps and ectomycorrhizae were collected in Nov 2004 in a single area of the province of Zamora, in the municipality of Riofrío (Castilla y León, Spain), UTM coord.: 29T0 735590, 4633695, about 872 m a.s.l., in loamy soil composed of slate and sandstone, pH 5.0. The harvested fungal specimens were collected in pure stands of 8-year-old *Cistus ladanifer* shrubs. No trees were present. Soil cores were collected from beneath the sporocarps and stored at 4 ºC for later analysis in the laboratory. The roots with ectomycorrhizae and the soil rhizomorphs were extracted

carefully with the aid of a stereomicroscope. The use of water was avoided because of the clay soil. To complete the cleaning, the excised roots and ectomycorhizae were placed in an ultrasonic bath with deionized water and some drops of Tween 20® detergent at 20 ºC for 15 min. Samples of the sporocarps, ectomycorrhizae and rhizomorphs were inmediatly frozen at -20 ºC for further molecular analyses. Dried sporocarps and ectomycorrhizae fixed in FAA (Verlhac et al. 1990) were stored as voucher specimens in the Dpto. Inv. Exp. For. Valonsadero with the codes: VALONSADERO - FUNGI 2081 and VALONSADERO - MYCORRHIZA 019 respectively.

The general methodology and terminology for characterizing the ectomycorrhizae follows Agerer (1987-2012, 1991) and Agerer and Rambold (2004-2013). For the observation of the mantle ectomycorrhizae were grated with the peeling technique (Agerer 1991). Mantle and rhizomorph preparations of fresh ectomycorrhizae were fixed on slides with lacto-glycerin for microscope observation. For longitudinal and cross sections (5-7 µm thick) ectomycorrhizae and rhizomorphs were embedded in liquid parafine, cut with a Microm HM 340E microtome and stained with hematoxilin-eosin.

Molecular characterization was carried out by sequencing fragments of the nuclear ribosomal DNA region of sporocarps, ectomycorrhizae, and rhizomorphs. DNA extraction from fungal tissue, ectomycorrhizae, and rhizomorphs was performed with the Qiagen ® DNeasy Plant Mini Kit. Amplifications of ITS rDNA sequences were carried out with an Applied Biosystems ® 9700 PCR machine using the universal primers ITS1 (5’ TCCGTAGGTGAACCTGCGG 3’) and ITS4 (5’ TCCTCCGCTTATTGATATGC 3’) (White et al. 1990) as well as the fungal specific ITS1F (5’

CTTGGTCATTTAGAGGAAGTAA 3') (Gardes and Bruns 1993) and the *Boletus* specific BED-4 (5' GTTTGTATACATTCTGGACATGCG 3') (Moor et al. 2002). Sequence alignments were performed with the BioEdit program version 5.0.9 (Hall 1999). Identification was carried out by comparing our sequences with the existing ones in the GenBank database.

3.3. Results.

3.3.1. Morphological characters.

Ectomycorrhizal system infrequently found, 1.5-1.8 mm long, monopodial-pinnate, ramifications of second order lacking or less developed, with 4 tips per 10 mm (Figure 3.1a). Main axes 0.2-0.3 mm diam. Unramified ends 0.4-0.6 mm long, 0.2-0.3 mm diam, sinuous, yellow whitish getting more yellow with age, whitish tip, not contrasting, inflated, club-shaped. Surface of unramified ends smooth and shiny, distinct, mantle not transparent and cortical cells not visible, rhizomorphs infrequent, emanating hyphae absent. Rhizomorphs, thin (0.25-0.1 mm) when originating directly from ectomycorrhizae, at a greater distance very thick, up to 2 mm diam, white near the mycorrhiza and at the base of the fruitbody and nearby, frequently ramified at restricted points, round in cross-section, surface smooth, connection to mantle kind distinct, origin location distal and proximal, joint angle to the mantle 30º, ramification common with an angle of 60º. Sclerotia not observed.

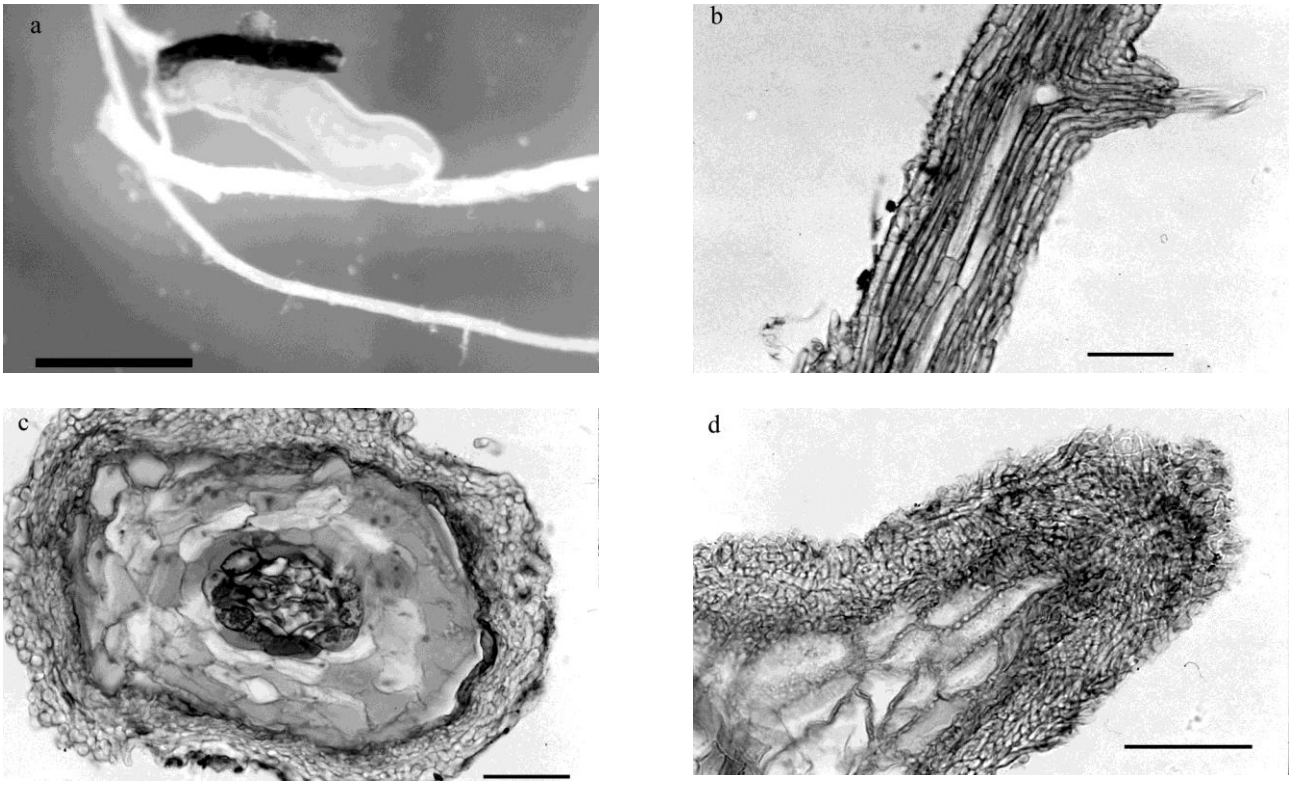


Figure 3.1. Anatomical and morphological characters of *Boletus edulis* ectomycorrhizae. (a) Morphological aspect of an ectomycorrhizae and rhizomorphs. Scale bar = 1 mm. (b) Longitudinal section of a rhizomorph. Scale bar = 25 µm. (c) Cross section of a mycorrhiza. Scale bar = 25 µm. (d) Longitudinal section of a mycorrhiza. Scale bar = 25 µm.

3.3.2. Anatomical characters of mantle in plan views.

Mantle plectenchymatous in all layers; all hyphae colourless, clamps lacking. Outer mantle layer (Figure 3.2a) with a net of branching hyphae in a regular ring-like arrangement, hyphae 4-5(8) µm diam, cells 22-23 µm long, colorless, matrix not observed, hyphae junctions angle 120º, septa as thick as walls, cells slightly inflated in middle portions, cell wall surface smooth. Middle mantle layer (Figure 3.2b) densely plectenchymatous, with distinct hyphal bundles forming ring-like patterns like the outer layers, hyphae colourless, 4-5 µm thick, cells 20 µm long, cell-walls smooth,

anastomoses not observed, matrix lacking. Inner mantle layer (Figure 3.2c) densely plectenchymatous, with broad streaks of parallel hyphae, colourless, 3-5 µm diam, cells 20-22 µm long. Tip with the same structural characteristics as in the older parts of mantle.

3.3.3. Anatomical characters of emanating elements.

Rhizomorphs: highly differentiated (type E according to Agerer 1987-2012), forming internal nodia and nodia at branching points, with hyphae empullate and conical young side-branches, presence of trumpet-like inflated hyphae, 10-11 µm thickness; central vessel-like hyphae present (Figure 3.2b), 7-8 µm diam, cells 70-85 µm length, 1-1,5 µm thick wall, ramification with one side-branch at septum; central nonvessel-like hyphae with septa as thick as the cell walls, 7 µm diam, cells 27 µm length; peripheral hyphae not specialized, 3-4 µm diam, cells 34 µm length, colourless, mostly smooth, but sometimes little granules on the cell wall present, slightly dotted surface, with the hyphae slightly twisted (Figure 3.1d) and some vesicles appear in the margin (Figure 3.1e) formed by empullated septa of the peripheral hyphae. Clamps lacking.

Emanating hyphae: lacking.

Cystidia: lacking.

Chlamydospores: not observed.

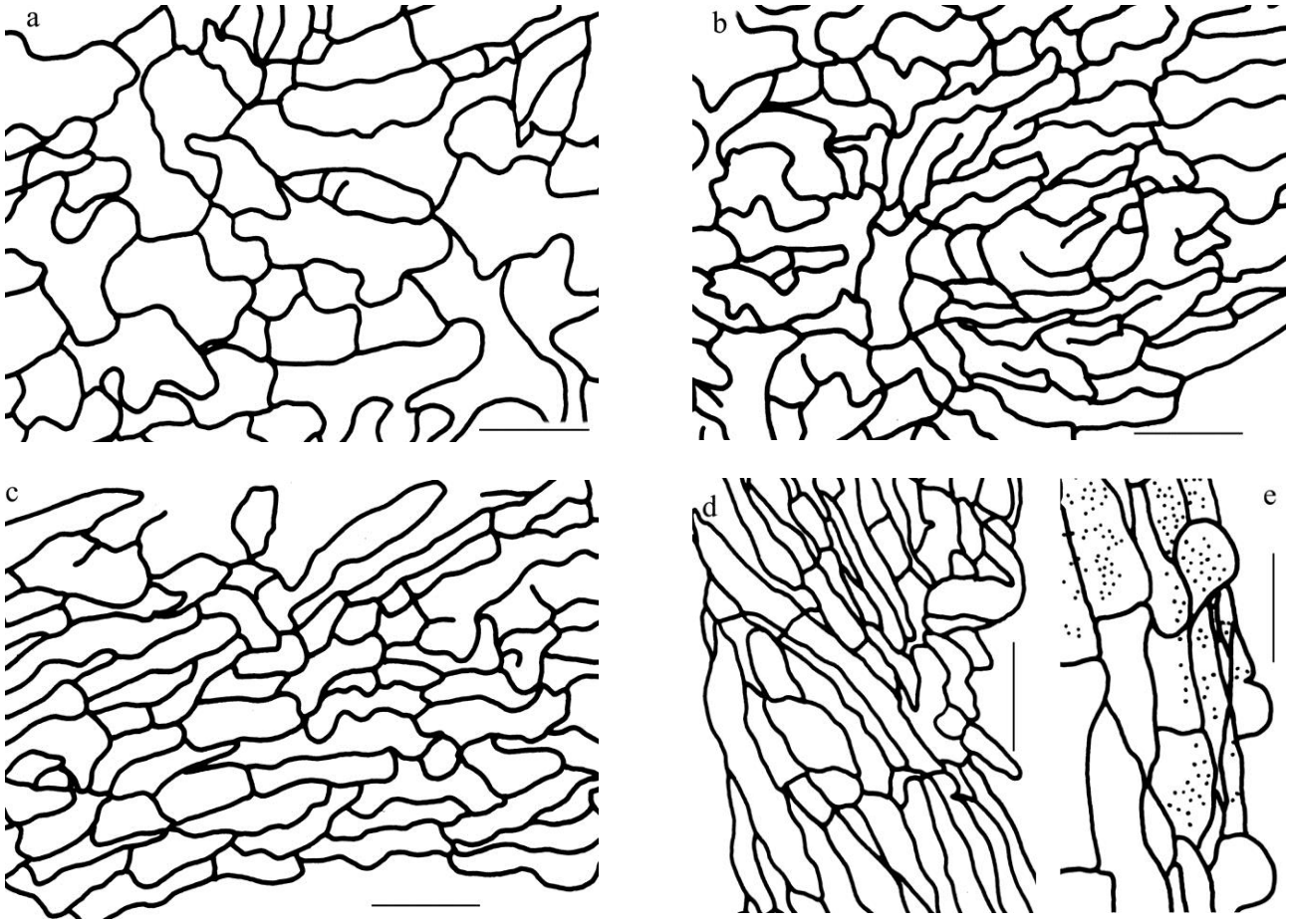


Figure 3.2. Anatomical characters of *Boletus edulis* ectomycorrhizae. (a) Outer mantle layer with a plectenchymatous structure formed by a loose net of hyphae. (b) Middle mantle layer with a plectenchymatous structure forming ring-like structures. (c) Inner mantle layer with a densely plectenchymatous structure. (d) Surface of rhizomorph, showing slightly twisted hyphae. (e) Vesicles on the margin of rhizomorph. Scale bars = 10 µm.

3.3.4. Anatomy of the mantle in longitudinal section.

Mantle 18-24 µm thick, 20-22 µm at ectomycorrhizal tip, three different layer discernable (Figure 3.1d), all of them plectenchymatous, outer mantle with few calyptra cell remains, hyphae 5-7 µm tangential length, 3-4 µm radial diam; middle mantle plectenchymatous, hyphae 7-8 µm tangential length, 2-3 µm radial diam; inner mantle plectenchymatous, hyphae 6-7 µm tangential length, 3-4 µm radial diam. Tannin cells absent. Cortical cells tangentially-oval to –elliptic or –cylindrical, and

obliquely oriented, 24-36 µm tangential length, 12-24 µm radial diam, CCt= 30 µm, CCq= 18 µm. Hartig net present in one or in one-half row of cortical cells, adjoining endodermis free of this, hyphal cells around cortical cells beaded, 2-3 µm thickness, two hyphal rows around cortical cells. Hartig net structure (in plan view) infrequently lobed, lobes without septa, 1.5-2 µm width.

3.3.5. Anatomy of the mantle in cross-section.

Different layers discernible in the mantle (Figure 3.1c). Outer mantle layer plectenchymatous, without calyptra cell remains, hyphae 12-14 µm tangential length, 7-9 µm radial diam. Middle mantle layer plectenchymatous, hyphae 13 µm tangential length, 4 µm radial diam. Inner mantle layer plectenchymatous, hyphae 7 µm tangential diameter, 4 µm radial diam. Cortical cells rectangular, 17-18 µm tangential length, 12-17 µm radial diam, CCt= 18 µm, CCq= 15 µm. Hartig net apparently 1 ½ rows deep, hyphal cells around cortical cells beaded, 2 µm thickness filling two rows around cortical cells.

3.3.6. Chemical reactions.

Brillant-cresyl-blue, dense blue; formol 40 %, only the mantle turns grey-greenish; Melzer's reagent, dextrinoid; ruthenium-red, pink-reddish; toluidin-blue, dense blue. The rest (acid fuchsin, anilin, etanol 70 %, $FeSO_4$, guaiac, KOH 10 %, lactic acid, phenole, phenole-anilin, sudan III, sulpho-vanillin and water) absent.

3.3.7. DNA-Analysis.

Sequences of the nuclear ribosomal DNA fragments were registered in the NCBI GenBank database with the following accesion numbers: DQ002921 for the sporocarp sequence, DQ002922 for the mycorrhiza sequence and DQ002923 for the rhizomorph sequence. ITS1/ITS4 amplifications were successful for the sporocarp samples but failed with mycorrhizas and rhizomorphs which were successfully amplified using the specific ITS1F/BED-4 primers pair. Alignments of the three structures had a 100 % coincidence in the ITS1 region. A search for highly similar sequences by the MegaBLAST procedure was performed to compare our complete sporocarp ITS1, 5.8S and ITS2 sequence with the GenBank ones. A 99-100 % identity with 13 *Boletus edulis* entries, two *Boletus aestivalis*, two *Boletus persoonii* Bon and one *Boletus venturii* Bon was found.

3.4. Discussion.

3.4.1. Ectomycorrhiza description and characterization.

Characterization of rhizomorph structures seems to be very important for distinguishing ectomycorrhizae in the Boletales (Brand 1989). After the revision of the previously-published *Boletus* genus ectomycorrhizae descriptions by Ceruti et al. (1983-84), Ceruti et al. (1987-88), Garrido (1988), Gronbach (1988), Agerer and Gronbach (1990), Franz and Acker (1995), Hahn (2001), Palfner (2001), and Agerer and Rambold (2004-2013) it can be concluded that the ectomycorrhizae of this genus are

characterized by the lack of hyphal clamps, the plectenchymatous mantle, and rhizomorphs with differenciated hyphae. The mantles of all of the *Boletus* ectomycorrhizae described are formed by three plectenchymatous layers of colourless hyphae forming ring-like structures (type A, Agerer 1991).

Hahn (2001) described vesicles in the margin of the rhizomorphs formed by *Boletus rodoxanthus* similar to those described in this study for *Boletus edulis*; however, whereas the vesicles of the latter species are smooth, the vesicles of the former species are covered with a dense layer of smooth crystals. Rhizomorphs of *Boletus loyo* Phillippi and *Boletus putidus* E. Horak (Palfner 2001) are similar to the *Boletus edulis* described here, but both present cystidia. The two descriptions of *Boletus reticulatus* (Ceruti et al. 1983-84; Garrido 1988) described the characters of the mantle exclusively. All the descriptions of *Boletus edulis* ectomycorrhizae (Ceruti et al. 1987-88; Garrido 1988; Gronbach 1988; Agerer and Gronbach 1990; Franz and Acker 1995; Palfner 2001; Agerer and Rambold 2004-2013) report smooth hyphae and differentiated rhizomorphs according to Agerer (1999). Although some of the hyphae of the ectomycorrhizae described in here are slightly dotted and the external hyphae of the rhizomorphs are slightly twisted, those characteristics could not be considered definitive.

Molecular characterization allowed the identification of the fungal symbiont present in mycorrhizas and rhizomorphs as *Boletus edulis*. GenBank sequence comparisons were mainly based on the data provided by Leonardi et al. (2005). Although the similarity between the sequence obtained in this work and a few other *Boletus* species or varieties was also high, all of them belong to the *Boletus edulis*

species complex. Because average nucleotide diversity inside the *Boletus edulis* species is low compared to other species of the complex (Leonardi 2005), the full coincidence of the fragment amplified from mycorrhizae and rhizomorphs of the sporocarp turned out to be informative for confirming the identity of the fungal partner. On the other hand, the lack of success in the PCR amplification from ectomycorrhizae and rhizomorphs when using the universal primers ITS1 and ITS4 indicates that specific primers for PCR amplification can be necessary when working with field, nonaseptic material.

3.4.2. Ecological and practical implications.

Boletus edulis species complex is associated with a wide range of host trees. *Boletus edulis* and *Boletus pinophilus* sporocarps are found in temperate conifer and broadleaf forests, whereas *Boletus aereus* and *Boletus reticulatus* sporocarps are more termophilic and are usually found in broadleaf and conifer forests (Alessio 1985).

There are few references of mycorrhizal associations of *Boletus edulis* with shrubs. Manavella (2004) harvested sporocarps of this species in the Italian Alps, at 2,500 m a.s.l., with presence of *Juniperus communis* L. subsp. *alpina* (Suter) Čelak. and *Arctostaphylos uva-ursi* (L.) Spreng. Both shrubs can form ericoid and vesiculo-arbuscular mycorrhizae, whereas the former forms also ectomycorrhizae (Harley and Harley 1987). Molina and Trappe (1982a) reported members of the Boletales forming arbutoid mycorrhizae with ericaceous shrubs and ectomycorrhizae with coniferous trees.

The extent by which plants benefit from a symbiosis with mycorrhizal fungi varies depending on identity of the plant and the fungus, the physiological state of the plant, and environmental conditions (van der Heijden and Sanders 2002). Allen (1991) stated that some plants may form symbiosis with certain fungi depending on the ecological conditions. Molina et al. (1992) proposed the concept of ecological specificity that is the influence of biotic and abiotic factors on the ability of plants to form functional mycorrhizae with particular fungi in natural soils. Also Brundrett (2002) suggested that mycorrhizal fungi have a very limited capacity for distinguishing the roots of different plant species, so plants primarily would regulate specificity.

Boletus edulis is one of the species which seems to follow this pattern, being able to produce sporocarps in association with unusual host plants such as *Cistus ladanifer*, a pioneer early stage shrub, when species of Fagales or Pinaceae are absent. This situation would favour the maintenance of soil inoculum reservoria for successional stages. Also the fact that *Boletus edulis* is able to fruit when associated to 8-year-old rockroses may be seen as a dispersion strategy to assure genetic variation (Horton and Bruns 2001).

Studies on wild sporocarp production of edible *Boletus* have been carried out in different environmental situations (Rondet and Leprince 2001; Martínez 2003; Salerni and Perini 2004). Controlled cultivation and mycorrhizal synthesis studies with *Boletus* are relatively abundant (Pantidou 1961, 1962, 1964; Tozzi et al. 1980-81; Molina and Trappe 1982b; Poitou et al. 1982; Ceruti et al. 1983, 1985; Poitou and Mamoun 1984; Zucherelli 1988; Meotto and Pellegrino 1989). The association with *Cistus ladanifer* reported here, together with the early sporocarp production, offers an alternative

economic resource for developing countries and for marginal and inland areas with low incomes. The only attempts to produce edible sporocarps have been done with *Helianthemum* inoculated with *Terfezia* (Morte et al. 2004). Nursery-controlled inoculations designed to establish short-term production plots could be seen as a feasible and promising way to exploit this peculiar symbiosis.

4. Mycorrhizal synthesis between *Boletus edulis* species complex and rockroses (*Cistus* sp.).

4.1. Introduction.

Boletus Fr. is a cosmopolitan genus of ectomycorrhizal fungi widely represented in the temperate zones of Northern and Southern Hemispheres. The genus comprises more than 1,000 species with epigeous fructification, inhabiting forests in tropical and mid latitudes. *Boletus* forms ectomycorrhizae with a large number of suitable hosts: Fagales – Fagaceae (*Castanea* Mill., *Castanopsis* (D.Don) Spach, *Fagus* L., *Lithocarpus* Blume, *Quercus* L.) and Betulaceae (*Betula* L., *Carpinus* L., *Corylus* L., *Ostrya* Scop.); Malvales – Malvaceae (*Tilia* L.) and Cistaceae (*Cistus* L.); Malpighiales – Salicaceae (*Populus* L., *Salix* L.); Ericales – Ericaceae (*Arctostaphylos* Adans); and Pinales – Pinaceae (*Abies* Mill., *Keteleeria* Carrière, *Picea* D.Don ex Loudon, *Pinus* L., *Tsuga* (Endl.) Carrière) (Olivier et al. 1997, Águeda et al. 2006, Mello et al. 2006).

The *Boletus edulis* species complex (*Boletus edulis* Bull. *sensu stricto*, *Boletus aereus* Bull., *Boletus pinophilus* Pilát & Dermek, and *Boletus reticulatus* Schaeff.) has great economic importance for its edibility (Singer 1986, Hall et al. 1998) being *Boletus edulis* a major commercial mushroom consumed worldwide. This fungal species is collected exclusively from the wild (Cannon and Kirk 2007) and no controlled production has been done to date.

Edible mycorrhizal mushrooms are not only a gourmet food but also a source of income for collectors (Wang and Hall 2004). Total annual worldwide consumption of *Boletus edulis* complex is between 20,000 and 100,000 tons (Hall et al. 1998). Important markets include North America, France, Italy and Germany (Hall et al. 1998). The estimated annual production of *Boletus edulis* species complex from the

autonomous community of Castilla y León in Spain, is 8,500 tons, worth approximately 38 million Euros (Martínez-Peña et al. 2006-2008).

In some regions of Central Spain (Zamora, León, and Salamanca provinces) with abundant fires and dominated exclusively by *Cistus ladanifer* L.*, Boletus edulis* sporocarps are regularly observed. Águeda et al. (2006) described field ectomycorrhizas formed by these organisms and sequenced them for taxonomic verification. This fact is important since *Cistus* species occur in degraded areas where economic resources are scarce to maintain human population. The family Cistaceae, with eight genera and almost 200 species (Muñoz and Navarro 1993) is distributed primarily in the temperate areas of Europe and the Mediterranean basin, but is also found in North and South America (López González 2001). The genus *Cistus* is represented in the Iberian Peninsula by 12 shrub species, all occurring during primary succession of tree stands. Cistaceae species are pyrophytic in general, and their germination is benefited by high temperatures where they are adapted to fires in Mediterranean forests (Alonso et al. 1992). *Cistus* species often form pure stands in vast areas heavily subjected to fire and/or grazing because of their ability as early colonizers after disturbance.

Cistus can form both ecto- and arbuscular mycorrhizas (Smith and Read 2008). More than 200 fungal ectomycorrhizal species belonging to 40 genera are reported to associate with *Cistus* (Puppi and Tartaglini 1991, Comandini et al. 2006). Rockroses (*Cistus* and *Helianthemum* sp.) are ecologically important species because they may act as a reservoir of mycorrhizal fungi after a forest disturbance (Torres et al. 1995, Díez 1998).

Among the edible mycorrhizal mushrooms, only *Tuber melanosporum* Vittad. and *T. uncinatum* Chatin have been cultivated commercially (Wang and Hall 2004).

Also, some success has been achieved with *Lactarius deliciosus* (L.) Gray, *Lyophyllum shimeji* (Kawam.) Hongo, *Tuber borchii* Vittad., and *Rhizopogon roseolus* (Corda) Th. Fr. (Wang and Hall 2004). Few attempts to produce edible sporocarps using a Cistaceae host have been reported, most of them involving *Tuber melanosporum* and *Cistus* sp. (Chevalier et al. 1975, Fontana and Giovanetti 1978, Giovaneti and Fontana 1982, Díez et al. 1994, Wenkart et al. 2001, Roth-Bejerano et al. 2003), or *Terfezia claveryi* Chatin and *Helianthemum* sp. (Morte et al. 2004).

Ectomycorrhizal synthesis experiments are useful to determine fungus-plant host compatibility and for morphological and physiological research (Giomaro et al. 2005). Here, this technique has been used to test the ability of the *Boletus edulis* species complex to form ectomycorrhizas with *Cistus* sp. under controlled conditions as well as provide detailed anatomical descriptions of the formed ectomycorrhizas. This research is part of a project aimed at promoting shrub inoculations for the production of edible mycorrhizal fungi (Martínez-Peña et al. 2007).

4.2. Materials and methods.

4.2.1. Fungal isolates.

Fungal isolates of *Boletus aereus, Boletus edulis, Boletus reticulatus,* and *Boletus pinophilus* were obtained from sporocarps collected in northern Spain (Table 4.1). Isolations were made by explants from sporocarp tissues plated on modified Melin-Norkrans agar culture medium (MMN) (Marx 1969) or biotin-aneurin-folic acid agar culture medium (BAF) (Oort 1981) and maintained by transferring to fresh media every 3 months.

Table 4.1. Fungal isolates used in the ectomycorrhizal synthesis.

Species	Site (region)	Host	Strain code	GenBank code
Boletus aereus	Osor (Cat)	*Castanea sativa* Mill.	393 IRTA	EU554663
Boletus edulis	Aránser (Cat)	*Pinus uncinata* Ramond ex DC	375 IRTA	EU554664
Boletus reticulatus	Ocenilla (CyL)	*Quercus pyrenaica* Willd.	1054 DIEFV	EU554661
Boletus pinophilus	La Póveda (CyL)	*Pinus sylvestris* L.	1082 DIEFV	EU554662

Cat: Cataluña; CyL: Castilla y León

The identification of the fungal species was confirmed by molecular analysis of the internal transcribed spacer (ITS) of the nuclear rDNA region (Leonardi et al. 2005).

Amplicons obtained with the ITS1/ITS4B primers (Gardes and Bruns 1993) from each fungal isolate were purified and sequenced in both directions. The ITS sequences obtained were compared with sequences deposited in GenBank to confirm their taxonomic identification, and submitted to the GenBank databases under accession numbers reported in Table 4.1.

4.2.2. Pure culture synthesis procedures.

Cistus albidus L. and *Cistus ladanifer* seeds were rinsed in 90 ºC water for 30 min, maintained in 30 % sodium hypochlorite for 10 min, and washed in sterile distilled water. Disinfected seeds were placed on BAF agar Petri dishes and stratified for 2-3 weeks at 4 ºC. Plates were then placed at room temperature for seed germination (20-23 ºC). After two weeks of incubation, seeds showing contamination were discarded.

Aseptically germinated seedlings (radicle 1-2 cm long) were transferred into ectomycorrhizas synthesis tubes (Molina 1979) filled with a sterilized mixture of 10 ml peat, 110 ml vermiculite and 60 ml BAF nutrient solution modified reducing the glucose to 20 g/l. The synthesis tubes were inoculated with 10 ml of a mycelium culture of either *Boletus aereus, Boletus edulis, Boletus reticulatus*, and *Boletus pinophilus* grown in BAF liquid medium. All the fungi were tested with *Cistus albidus* and *Cistus ladanifer* with four replicates for each fungus-host combination. Synthesis tubes with the inoculated seedlings were grown for 4-5 months at 20-25 ºC under fluorescent lights (150 µmol s^{-1} m^{-2} [400-700 nm], 16 h/day). At the end of the growing period, seedlings were removed from the synthesis tubes and root systems washed and examined for ectomycorrhizal formation.

4.2.3. Morphological description of ectomycorrhizae.

Ectomycorrhizal roots and rhizomorphs were carefully extracted with the aid of a stereomicroscope, fixed in FAA (Agerer 1986) and stored as voucher specimens in the Dpto. Inv. Exp. For. Valonsadero (Soria, Spain). The general methodology and terminology for characterizing the ectomycorrhizas follows Agerer (1987-2012, 1991) and Agerer and Rambold (2004-2013). For the observation of the mantle, the ectomycorrhizae were grated with the peeling technique (Agerer 1991). Mantle and rhizomorph preparations of fresh ectomycorrhizas were fixed on slides with lactic acid for microscope observation.

4.3. Results.

Boletus aereus, Boletus edulis, and *Boletus reticulatus* formed ectomycorrhizae with *Cistus ladanifer* and *Cistus albidus*, whereas *Boletus pinophilus* did not form ectomycorrhizae with either *Cistus* host (Table 4.2).

Table 4.2. Formation of ectomycorrhizae in each host-fungus combination. Four replicates were tested for each combination. The crosses indicates the number of replicates in which ectomycorrhizal formation was detected. -: no mycorrhizal formation in any replicate.

Fungal species	Strain code	Plant species	
		Cistus albidus	*Cistus ladanifer*
Boletus aereus	393 IRTA	++++	++++
Boletus edulis	375 IRTA	++	++++
Boletus reticulatus	1054 DIEFV	++++	++
Boletus pinophilus	1082 DIEFV	-	-

Mycorrhizal structures of each fungal species were identical in both *Cistus* species as it has been described for other host-fungus combinations (Agerer and Rambold 2004-2013). Consequently, only one complete description is given for each fungal species indicating the host plant taken into account in each case. Herbarium codes for the described mycorrhizae were: VALONSADERO – MYCORRHIZA 049 for *Boletus aereus* and *Cistus ladanifer* (from strain 393 IRTA), VALONSADERO – MYCORRHIZA 047 for *Boletus edulis* and *Cistus albidus* (from strain 375 IRTA), and VALONSADERO – MYCORRHIZA 045 for *Boletus reticulatus* and *Cistus albidus* ectomycorrhizae (from strain 1054 Valonsadero).

4.3.1. *Boletus aereus + Cistus ladanifer.*

Mantle type: plectenchymatous, colourless, clamps lacking, outer mantle layer with ring-like arrangement of hyphal bundles, Type A (Figure 4.1b); middle mantle layer hyphae arrangement without pattern; inner mantle layer hyphae arrangement with broad streaks of parallel hyphae. Tip with the same structural characteristics as in the older parts of mantle.

Rhizomorphs: up to 15 mm, highly differentiated, boletoid, with vessel-like hyphae with partially or even completely dissolved septa centrally arranged; forming nodia at branching points, clamps absent, colourless, without short inflated cells. Peripheral hyphae similar to mantle cystidia, colourless, smooth (Figure 4.1c).

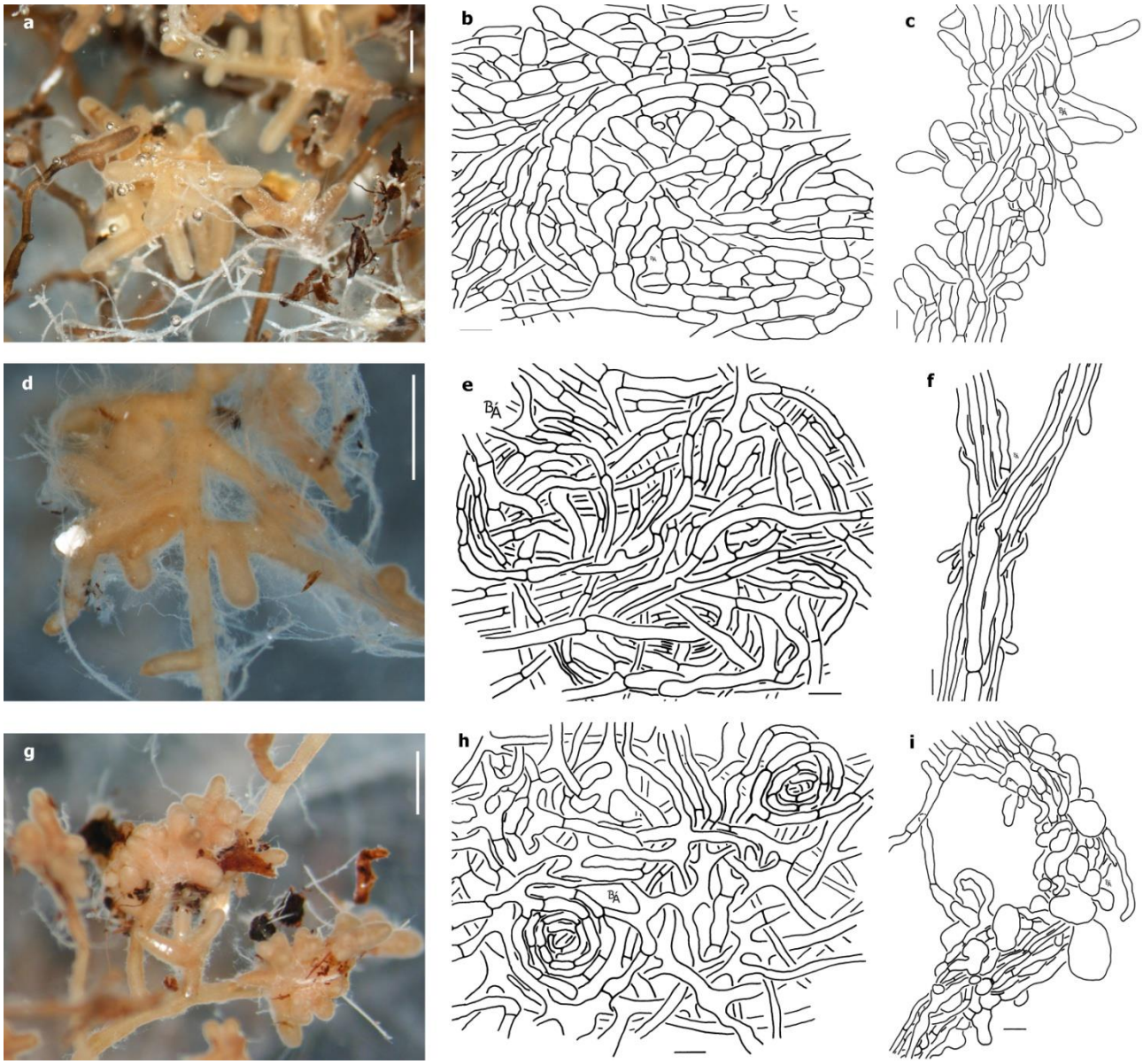


Figure 4.1. Morphological and anatomical characters of the ectomycorrhizas obtained in pure culture synthesis. (a) Ectomycorrhizas and rhizomorphs of *Boletus aereus* and *Cistus ladanifer*. Bar = 10 mm. (b) Outer mantle layer of *Boletus aereus* and *Cistus ladanifer*. Bar = 10 µm. (c) Surface of rhizomorph with cystidia of *Boletus aereus* and *Cistus ladanifer*. Bar = 10 µm. (d) Ectomycorrhizas and rhizomorphs of *Boletus edulis* and *Cistus albidus*. Bar = 10 mm. (e) Outer mantle layer of *Boletus edulis* and *Cistus albidus*. Bar = 10 µm. (f) Rhizomorph with vessel-like hyphae of *Boletus edulis* and *Cistus albidus*. Bar = 10 µm. (g) Ectomycorrhizas and rhizomorphs of *Boletus reticulatus* and *Cistus albidus*. Bar = 10 mm. (h) Middle mantle layer of *Boletus reticulatus* and *Cistus albidus*. Bar = 10 µm. (i) Surface of rhizomorph with cystidia of *Boletus reticulatus* and *Cistus albidus*. Bars = 10 µm.

Cystidia: awl-shaped, bristle-like, present on outer mantle layer and on rhizomorphs; ramification presence-position absent or proximal, monopodial or bifurcate; branches ramification absent; septa: present, simple, septa number 2-3; surface smooth.

Emanating hyphae: clamps lacking, straight, colourless; ramification approximately 90º, adjacent to septum, one side-branch at septum; cells even or slightly constricted, smooth.

Exploration type: long distance.

Hydrophobic.

Morphological characters: ectomycorrhizal system solitary, monopodial-pyramidal or irregularly pinnate; unramified ends straight, not inflated, cylindrical, yellowish; mantle surface smooth and glistening, loosely woolly or forming rings (reticulate) (Figure 4.1a). Emanating hyphae present, infrequent, not specifically distributed. Rhizomorphs round or roundish, white, frequently ramified at restricted points, connection to mantle kind distinct, origin location proximal, surface smooth or hairy.

4.3.2. *Boletus edulis + Cistus albidus.*

Mantle type: plectenchymatous, colourless, clamps lacking; outer mantle layer ring-like arrangement of hyphal bundles, Type A (Figure 4.1e); middle mantle layer hyphae arrangement ring-like; inner mantle layer hyphae arrangement with broad streaks of parallel hyphae. Tip with the same structural characteristics as in the older parts of mantle.

Rhizomorphs: highly differentiated, boletoid, with vessel-like hyphae with partially or even completely dissolved septa centrally arrangend; forming nodia at branching points, clamps absent, colourless, without short inflated cells (Figure 4.1f).

Cystidia: lacking.

Emanating hyphae: clamps lacking, wavy to slightly tortuous, colourless; ramification Y-shaped, adjacent to septum, one side-branch at septum; cells slightly constricted, smooth.

Exploration type: long distance.

Hydrophobic.

Morphological characters: ectomycorrhizal system monopodial-pyramidal or irregularly pinnate, dichotomous-like; unramified ends straight, not inflated, cylindrical, white to yellowish getting more yellow with age; mantle surface shiny and silvery, loosely woolly (Figure 4.1d). Emanating hyphae present, abundant, not specifically distributed. Rhizomorphs round or roundish, white, frequently ramified at restricted points, connection to mantle kind distinct, surface smooth or woolly.

4.3.3. *Boletus reticulatus + Cistus albidus.*

Mantle type: plectenchymatous, colourless, clamps lacking; outer mantle layer with hyphae rather irregularly arranged with no special pattern discernable (Type B); middle mantle layer hyphae arrangement without pattern or ring-like (Figure 4.1h); inner mantle layer hyphae arrangement ring-like or with broad streaks of parallel hyphae. Tip with the same structural characteristics as in the older parts of mantle.

Rhizomorphs: highly differentiated, boletoid, with vessel-like hyphae with partially or even completely dissolved septa centrally arranged; forming nodia at branching points, clamps absent, colourless, without short inflated cells, clamps absent. Peripheral hyphae not specialized or roundish, inflated, as short cells, smooth (Figure 4.1i).

Cystidia: two different types, type 1 present on outer mantle layer and on rhizomorphs and type 2 present only on rhizomorphs. Type 1 thin-walled, slightly tapering, often rather similar to ends of normal hyphae, ramification presence-position absent or proximal, monopodial or bifurcate, branches ramification absent, septa present, simple, septa number 1-3, surface smooth. Type 2 globular, ramification presence-position absent, septa absent, cell wall colour similar to mantle cells, surface smooth.

Emanating hyphae: clamps lacking, wavy or irregularly inflated or even beaded, colourless; ramification acute or Y-shaped, adjacent to septum, one side-branch at septum; cells even or slightly constricted, smooth.

Exploration type: long distance.

Hydrophobic.

Morphological characters: ectomycorrhizal system: solitary, monopodial-pinnate or coralloid; unramified ends straight or bent, not inflated, cylindrical, white to yellowish getting more yellow with age; mantle surface shiny and smooth, silvery in some zones, loosely grainy or warty (Figure 4.1g). Emanating hyphae present, infrequent, not specifically distributed. Rhizomorphs round or roundish, white, frequently ramified at restricted points; connection to mantle kind distinct, surface: smooth.

4.4. Discussion.

Of the eight tested combinations, only *Boletus pinophilus* failed to form ectomycorrhizae with either host, despite the extensive growth of the fungus in the substrate and the adequate root development of both *Cistus* species. Sporocarps of *Boletus aereus, Boletus edulis,* and *Boletus reticulatus* are found associated with Cistaceae plants in the Mediterranean region (Oria de Rueda 2007), while sporocarps of *Boletus pinophilus* are associated to Pinaceae or Fagaceae species. The incompatibility of *Boletus pinophilus* and *Cistus* spp. must be confirmed or rejected by testing a wider range of fungal strains.

Over the last 40 years many researchers have synthesized ectomycorrhizae of the *Boletus edulis* species complex on various hosts. Froidevaux and Amiet (1975) synthesized *Boletus edulis* and *Pinus mugo* Turra ectomycorrhizas; Tozzi et al. (1980)

obtained ectomycorrhizae of *Boletus edulis* and *Quercus pubescens* Willd.; Molina and Trappe (1982a,b) synthesized ectomycorrhizae between *Boletus edulis* and eight hosts in the genera *Arbutus, Arctostaphylos, Larix, Picea, Pinus,* and *Tsuga*; Ceruti et al. (1983-84) obtained *Boletus aereus* and *Quercus pubescens* ectomycorrhizae; Ceruti et al. (1985) obtained mycorrhizae of *Boletus aereus* and *Castanea sativa* Mill.; Poitou et al. (1982) synthesized ectomycorrhizae in pure culture between *Pinus radiata* D. Don and *Boletus edulis* and *Boletus aereus*; and Duñabeitia et al. (1996) obtained *Boletus pinophilus* ectomycorrhizae by inoculating *Pinus radiata* seedlings with a spore suspension of 10^6-10^7 spores per plant.

Seedlings inoculated with *Boletus* species have been outplanted in attempts to promote fruiting of the valuable edible sporocarps. Olivier et al. (1997) describes plantations of *Castanea sativa* and *Pinus uncinata* Ramond ex DC. inoculated with *Boletus edulis* and *Boletus aereus*. Meotto et al. (1999) outplanted *Castanea sativa* seedlings inoculated with mycelial cultures of *Boletus edulis*. Unfortunately, sporocarp production has not been reported from either study.

Description and identification of ectomycorrhizae have evolved greatly following the systematic studies by Agerer (1986, 1987-2012, 1991) and molecular characterization based on DNA analysis (Gardes and Bruns 1993). Although there are some descriptions for *Boletus* mycorrhizas, most are not precise (De Román et al. 2005). The ectomycorrhizae of the three *Boletus* species obtained here are very similar and fit well with the characters described in Agerer (2006) for this genus: plectenchymatous mantles from the types A, B or C, boletoid rhizomorphs with nodes and with or without short inflated cells, emanating hyphae smooth or covered by

crystals, clamps lacking, cystidia lacking or with cystidia-like hyphal ends, and white to yellowish hydrophobic ectomycorrhizas. All, *Boletus aereus, Boletus edulis,* and *Boletus reticulatus*, form white monopodial-pinnate ectomycorrhizas with three-layered plectenchymatous mantle on plan view and boletoid rhizomorphs. *Boletus aereus* forms monopodial-pyramidal ectomycorrhizas with ring-like outer mantle layer, and emanating cystidia formed by two or three short cells on the outer mantle layer and rhizomorphs. *Boletus edulis* has ring-like outer and middle mantle layers, without cystidia and rhizomorphs also without cystidia or globular cells. *Boletus reticulatus* has ring-like or with broad streaks of parallel hyphae inner mantle layer, emanating cystidia on the outer mantle layer and globular and awl-shaped cystidia on rhizomorphs.

The cystidia of *Boletus aereus* and *Boletus reticulatus* ectomycorrhizae are similar to those present on the hymenia of some *Boletus* species, like *Boletus reticulatus, Boletus edulis* or *Boletus regius* Krombh., appearing as fusiform or globoid structures (Muñoz 2005). However, it can not be discounted that the formation of these elements, as well as the emanating hyphae on *Boletus edulis*, could be influenced by the experimental conditions of the synthesis tubes.

The abundance and size of rhizomorphs found in this study, especially for *Boletus aereus*, conform to those found in the field. In natural conditions, the mycelium of *Boletus* is concentrated as rhizomorphs with a high degree of spatial heterogeneity. However, the type of soil could determine the spread of exploratory elements of the *Boletus* ectomycorrhizae, such as cystidia, rhizomorphs and emanating

hyphae, as demonstrated for *Lactarius deliciosus* mycorrhizal seedlings (Hortal et al. 2008).

There are no previously reported descriptions of *Boletus aereus* ectomycorrhizae. All previous descriptions of *Boletus edulis* ectomycorrhizae (Ceruti et al. 1987-88, Garrido 1988, Gronbach 1988, Agerer and Gronbach 1990, Franz and Acker 1995, Palfner 2001, Agerer and Rambold 2004-2013, Águeda et al. 2006) report characters that fit with those we described here. Ceruti et al. (1983-84), Ceruti et al. (1985), and Garrido (1988) only described the characters of mantle on cross-sections of *Boletus reticulatus*, so comparison with our study is not possible.

Considering that fungus is the main factor for determining the anatomical structures of ectomycorrhizae, those are identical for the same fungal species regardless of the host. Although morphological characters are mainly driven by the plant genus, some fungi can control, at least partially, the final form (Agerer and Rambold 2004-2013). Both aspects are true for the three described *Boletus edulis* complex ectomycorrhizae, which show the same structures when associated to *Cistus* as compared to other Pinaceae and angiosperm hosts.

The natural sporocarp production of *Boletus edulis* in association with *Cistus ladanifer* (Águeda et al. 2006) offers an alternative economic resource for marginal and inland areas with low incomes. Controlled mycorrhization with *Boletus edulis* on *Cistus* and outplanting of inoculated seedlings might be a feasible and promising way to exploit this symbiosis providing economic benefits. To accomplish this, further research is needed to determine the appropriate inoculation methods with compatible

Boletus strains, the persistence of *Boletus* ectomycorrhizae on outplanted, inoculated seedlings, and the factors inducing sporocarp production.

5. Rockroses and *Boletus edulis* ectomycorrhizal association: realized niche and climatic suitability in Spain.

5.1. Introduction.

Edible ectomycorrhizal mushrooms, like other valuable non-timber products, comprise a high-value resource in forest areas, often outstripping timber in Mediterranean regions (Bonet et al. 2010), with significant recreational, ecological and social added benefits. In fact, over 800 fungal species are consumed in more than 80 countries (Boa 2004), 174 of which are ectomycorrhizal fungi (Wang et al. 2001), offering an alternative economic resource for marginal and inland areas with low incomes.

In the Northern Hemisphere, particularly in Europe, the *Boletus edulis* species complex (*Boletus edulis* Bull. *sensu stricto*, *Boletus aereus* Bull., *Boletus pinophilus* Pilát & Dermek, and *Boletus reticulatus* Schaeff.) are of great economic importance (Boa 2004); *Boletus edulis* is a major commercial mushroom consumed worldwide. These fungal species are collected exclusively from the wild (Cannon and Kirk 2007). *Boletus edulis* is mainly associated with a large number of trees and shrubs of Fagales, Malvales, Malpighiales, Ericales, and Pinales (Águeda et al. 2008). Nevertheless, some references to the association between Boletales and Cistaceae can be found (Águeda et al. 2006). *Boletus edulis* sporocarps are regularly observed in certain regions of Central Spain (in the provinces of Zamora, León and Salamanca) which have recurrent fires and are dominated exclusively by *Cistus ladanifer* L. In many of these areas they appear not to be harvested, and given the dominance of cistaceous scrublands, they constitute an underappreciated and underexploited resource (Oria de Rueda et al. 2008).

The genus *Cistus* L. is represented in the Iberian Peninsula by 12 shrub species, all belonging to pioneer communities growing readily in degraded areas (San Miguel et al. 2008). Cistaceae species are mostly pyrophytic: their germination is linked to high temperatures and therefore they directly depend on fires in Mediterranean ecosystems to reproduce (Alonso et al. 1992). More specifically, *Cistus ladanifer* grows in the western Mediterranean, extending from Portugal and Morocco to the French Riviera and Algeria, and in southern and western Spain in zones with hot, dry summers, on siliceous soils over slate and granite (Demoly and Montserrat 1993). *Cistus* can form both ecto- and arbuscular mycorrhizas (Smith and Read 2008). More than 200 ectomycorrhizal fungal species belonging to 40 genera are reported to associate with *Cistus* (Comandini et al. 2006). Rockroses (*Cistus* and *Helianthemum* sp.) are ecologically important species because they may act as a reservoir of mycorrhizal fungi after a forest disturbance (Torres et al. 1995).

Mycorrhizal associations have a genetic as well as an environmental basis (Allen 1991). Ectomycorrhizal relationships among fungi and plants hinge on the species, the physiological status of the plant and the environmental conditions (van der Heijden and Sanders 2002). Actually, some plants are able to form mycorrhizas with certain fungi in particular ecological conditions, but not in others (Allen 1991), therefore the assessment of the ecological features where these relationships occur is especially relevant. Abiotic factors, such as light, temperature, humidity and nutrient availability, exert a decisive influence on sporocarp formation (Erland and Taylor 2002; Barroetaveña et al. 2008; Murat et al. 2008).

Understanding of the controls on mycorrhizal fungal species distribution is still in a nascent phase, especially when compared with that of more mature fields of plant and animal community ecology and biogeography (Lilleskov and Parrent 2007). It is essential to provide environmental practitioners with well-documented studies, based on ecological niche modelling, regarding the suitability of natural resources in economically-depressed areas where vast *Cistus ladanifer* shrublands prevail. Excellent tools to use are the so-called *species distribution models* (SDM) (Guisan and Zimmermann 2000; Guisan and Thuiller 2005), widely used over the last two decades in several fields of ecology such as conservation (e.g. Falcucci et al. 2009), adaptation to climate change (e.g. Hijmans and Graham 2006), invasions (e.g. Broennimann and Guisan 2008) and reforestation (e.g. Rubio and Sánchez Palomares 2006), also recently applied to predict fungal distributions (Wollan et al. 2008; Wolfe et al. 2010). Therefore, the objectives of this work are to characterize the realized niche where the *Boletus edulis* and *Cistus ladanifer* ectomycorrhizal association produces sporocarps and to predict the territory climatically and lithologically suitable for fruiting in peninsular Spain.

5.2. Materials and methods.

5.2.1. Study area.

The unique area where *Boletus edulis* sporocarps are known to be collected in association with rockroses is the western part of Castilla y León region. To predict the amount of territory suitable for the association between *Boletus edulis* and *Cistus ladanifer,* surveys were carried out on all polygons of the Spanish Forest Map in the region of Castilla y León (Pérez Ortiz and Esteban 2008; López Leiva et al. 2009) where tree canopy cover was less than 10 % and *Cistus ladanifer* was the main shrub species. The preliminary study area therefore comprised 1,118 polygons totalling almost 72,000 ha. To refine the search, a form was sent to local mycological societies and forest service offices to obtain particular locations where *Boletus edulis* was known to be harvested in *Cistus ladanifer* shrublands. As a result, the study area was reduced to ca. 45,000 ha.

5.2.2. Data.

Throughout the autumns of 2006-2009, 19 sites were ascertained to produce *Boletus edulis* sporocarps in pure *Cistus ladanifer* shrublands (Figure 5.2a). No other *Cistus* species was present in the area. To accept a site for sampling, no potential host tree individuals (mature or juvenile) could be found in the surroundings (at least in 30 m). Only one sample was collected in every homogeneous area visited. Close areas were regarded as non-homogeneous when there was a significant change in abiotic (aspect, slope, bed rock, etc.) or biotic (species composition of the scrubland, age or

size of the *Cistus ladanifer* individuals, etc.) conditions. Coordinates of the site were fixed to 1 m and a top soil sample to 20 cm was collected, at the exact point where the sporocarp was found. This sample was used to perform soil laboratory analysis and subsequent edaphic parameters (Table 5.1). Sporocarp identity was confirmed in the laboratory using the keys of Moser (1986) and Muñoz (2005), and deposited as voucher specimens at JCYL-FUNGI herbarium in CIF Valonsadero - Junta de Castilla y León (Soria, Spain). Their herbarium codes are detailed in Table 5.3, and every sample can also be checked at www.gbif.es.

The models for climatic estimation developed by Sánchez Palomares et al. (1999), which are functions of altitude, geographical position and hydrographical basin, were used to calculate 15 climatic parameters as shown in Table 5.1.

Table 5.1. Parameters, acronyms and laboratory methods used for the characterization of the niche where the *Boletus edulis-Cistus ladanifer* ectomycorrhizal association produces sporocarps.

CLIMATIC			
Pluviometric			
MAP	Mean annual precipitation (mm)	SMP	Summer precipitation (mm).
WTP	Winter precipitation (mm).	ATP	Autumn precipitation (mm).
SPP	Spring precipitation (mm).		
Thermometric			
MAT	Mean annual temperature (ºC)	ATT	Autumn mean temperature (ºC).
SMT	Summer mean temperature (ºC).	PET	Potential evapotranspiration (mm) (Thornthwaite 1948).
Hydric regime			
SUP	Annual moisture surplus (mm) (Thornthwaite and Mather 1957).	LDR	Length of drought (months) (Walter and Lieth 1960).
DEF	Annual moisture deficit (mm) (Thornthwaite and Mather 1957).	IDR	Intensity of drought (%) (Walter and Lieth 1960).
MI	Moisture index (%) (Thornthwaite and Mather 1957).	VERN	Vernet index (Vernet and Vernet 1966).
EDAPHIC (20cm topsoil)			
Physical properties			
FE	Fine earth (%).	PER	Permeability (Gandullo 2000).
SAND	Sand (%) (pipette method).	ME	Moisture equivalent (Sánchez Palomares and Blanco 1985).
SILT	Silt (%) (pipette method).	WHC	Water holding capacity (Gandullo 2000).
CLAY	Clay (%) (pipette method).		
Chemical properties			
PHW	pH in water.	NA	Sodium (ppm) (AAS)[a].
PHK	pH in KCl.	K	Potassium (ppm) (AAS)[a].
OM	Organic matter (%) (Walkley-Black procedure).	CA	Calcium (ppm) (AAS)[a].
N	Nitrogen (%) (Kjeldahl method).	MG	Magnesium (ppm) (AAS)[a].
CN	Carbon-nitrogen ratio.	CEC	Cation exchange capacity (meq/100 g) (ammonium acetate method).
P	Phosphorus (ppm) (Olsen method).	BS	Base saturation (%).

[a] AAS: atomic absorption spectrometry

5.2.3. Niche definition and distribution model calibration.

Using the values of the parameters listed in Table 5.1, the niche of *Boletus edulis-Cistus ladanifer* association was defined following the method proposed by Alonso Ponce et al. (2010b). After a minor modification (Alonso Ponce et al. 2010a), this method also allowed prediction of the distribution of the association, based on an additive potentiality index (API). This index ranges from 0 to 1 and is based on the ecological field theory. It uses the Mahalanobis distance to compute similarities between points in the environmental space, which has proved to be superior in this

kind of model (Farber and Kadmon 2003). Moreover, the algorithm permits assignment of different power (*penetration*) to create the ecological field around each observation depending on their situation in the points swarm, which in turn allows dealing with outliers efficiently. This algorithm belongs to the so-called envelope models group, such as Bioclim (Busby 1991), Domain (Carpenter et al. 1993) or Lives (Li and Hilbert 2008), but shows three main advantages: (i) it copes with correlations among environmental variables; (ii) it does not assign suitability for all environmental combinations within the boundaries of the envelope; and (iii) it takes advantage of outliers instead of precluding them.

We also used Maxent to produce a second model for comparison. Maxent (Phillips et al. 2006) is a widely-used species distribution modelling technique that has demonstrated superiority to other algorithms (Elith et al. 2006) even with small sample sizes (Hernández et al. 2006). The same input data and validation procedures have been used in both modelling methods, as explained next.

Only four out of the 15 climatic variables (Table 5.2) were used to calibrate the model: summer precipitation (SMP), autumn precipitation (ATP), autumn mean temperature (ATT) and intensity of drought (IDR), with a pixel resolution of 500 m. Three reasons support this severe shrinkage. On the one hand, the sample size (19) does not permit more than four predictors, as the ratio observations/predictors should never fall far below five in multivariate analysis (Hair et al. 1999). Secondly, it is widely accepted that the selection of environmental predictors for species distribution models must be based on biological and ecological knowledge (Guisan et al. 2006; Austin, 2007). Thus, parameter selection has been based on literature regarding

abiotic environmental variables triggering sporocarp formation for ectomycorrhizal fungi in general (Erland and Taylor 2002), for basidiomycetes (Vogt et al. 1992), for *Suillus luteus* (Barroetaveña et al. 2008) and for *Boletus edulis* and *Tuber melanosporum* (Murat et al. 2008). Lastly, edaphic variables have been ruled out as no high-resolution raster sets for such properties are currently available for peninsular Spain. This liability has been partially overcome by using lithological information (IGME 2006) to preclude calcareous zones from the predicted suitable area.

Table 5.2. Parameters and acronyms used for calibrating the predicted territory where the *Boletus edulis*–*Cistus ladanifer* ectomycorrhizal association produces sporocarps.

SMP	Summer precipitation (mm).	ATP	Autumn precipitation (mm).
ATT	Autumn mean temperature (°C).	IDR	Intensity of drought (%) (Walter and Lieth 1960).

5.2.4. Distribution model validation.

Data were split in two sets, 70 % for calibration and 30 % for validation (Fielding and Bell 1997). This procedure was repeated 50 times. Initially, the presence/expected curve was used, as proposed by Boyce et al. (2002)and subsequently modified by Hirzel et al. (2006) (hereafter Boyce-Hirzel index) to ascertain the lower threshold for the suitability index (API or logistic entropy in Maxent) to predict a point as presence. This index partitions through a 'moving window' the range of the suitability index. In every window, the predicted frequency of evaluation points and the expected frequency are computed. The former is the proportion of validation points predicted as presences in the range of the index in each window, while the latter is the frequency expected from a random distribution, i.e. the

relative area covered by the range of the index in each window. Next, the predicted-to-expected ratio is calculated. Thus, the higher the ratio, the more different the suitability prediction from a prediction by chance. Furthermore, as confidence intervals can be estimated, when this interval does not include the 1-horizontal line and lies above it, it can be concluded that the model is significantly better than a random model.

Once the lower threshold for the suitability index has been fixed, any cell with a predicted value of suitability below it will be referred to as unsuitable. Moreover, the shape of the presence/expected curve provides a practical rule to choose different classes of habitat suitability and their boundaries, by identifying crisp changes in the slope or in the confidence interval around the presence/expected curve (Hirzel et al. 2006). The latter confidence interval can also be used to rate the robustness of the model: the narrower the interval, the more robust it is. Furthermore, as this interval changes along the curve, different parts of the model can be more accurate than others (Hirzel et al. 2006). In addition, a robust version of the Wilcoxon-Mann-Whitney test (Mee 1990; García Pérez 2005; R Development Core Team 2009) has been used to compare the predicted distributions of the suitability indexes for calibration and evaluation sets, as a robust model should not produce significantly different predicted distributions for the two datasets.

Finally, the prediction ability of the model has been measured through the sensitivity, i.e. the percentage of actual presences predicted as presences by the model, calculated both for calibration and validation sets, as well as for the pooled set.

5.3. Results.

5.3.1. Realized niche.

Climatic and edaphic variables in each of the 19 sampled sites are summarized in Table 5.3. The characterization of the climatic niche (Tables 5.4 and 5.5) revealed that most of the sampled locations could be classified as mesothermal, Mediterranean, and humid. 32 % of the sites were moist subhumid, though annual precipitation was not meager at 681 mm and most of the sites underwent a long dry season: length of drought (LDR) mean was 2.6 months, and LDR lower threshold was 1.4 months. Nevertheless, the intensity of drought (IDR) was weak (IDR mean, 10.4 %; IDR upper threshold, 13.4 %) since the moderate summer mean temperature of below 20 ºC offset the scarce summer precipitation, which was hardly 70 mm.

Table 5.3. Climatic and edaphic variables of the 19 sampled sites. Acronyms in Table 5.1.

ID	X UTM	Y UTM	HERB CODE	CISTUS AGE	MAP	WTP	SPP	SMP	ATP	MAT	SMT	ATT	PET	SUP	DEF	MI	LDR	IDR	VERN
1	226792	4496581	2253	7	747	254	206	61	226	10.9	19.1	11.5	661	405	319	32.4	2.534	9.2	-11.37
2	189803	4705835	2275	15	1159	448	288	119	304	11.3	17.7	12.2	667	697	205	86.0	1.417	1.5	-6.04
3	235498	4617605	2254	15	676	236	185	70	185	11.3	19.5	11.9	676	335	334	19.8	2.599	10.3	-9.58
4	241890	4614644	2246	13	629	217	173	68	172	11.4	19.7	12.1	679	295	345	12.9	2.712	12.3	-9.71
5	245723	4612979	2245	11	599	206	165	66	163	11.5	19.8	12.2	683	269	353	8.4	2.794	13.9	-9.81
6	251321	4605672	2410	18	542	183	150	62	147	11.8	20.1	12.4	691	220	369	-0.2	2.987	18.2	-10.14
7	242202	4614738	2408	10	631	218	173	68	172	11.4	19.7	12.0	679	296	344	13.2	2.705	12.2	-9.68
8	232293	4621396	2405	16	680	238	186	70	185	11.4	19.6	12.0	678	337	335	20.0	2.610	10.4	-9.68
9	242750	4625886	2403	14	672	233	184	72	183	11.1	19.3	11.8	670	331	329	19.9	2.552	9.9	-9.09
10	238047	4634927	2394	9	703	246	192	74	191	11.1	19.2	11.7	668	356	322	24.4	2.481	8.8	-8.94
11	239237	4635495	2391	13	697	243	190	74	190	11.1	19.2	11.7	668	351	322	23.6	2.489	9.0	-8.93
12	239347	4630620	2393	9	702	245	192	74	192	11.0	19.2	11.7	667	356	321	24.5	2.476	8.8	-8.93
13	211263	4619570	2962	9	791	284	216	75	216	11.5	19.7	12.1	682	429	320	34.8	2.446	7.8	-9.82
14	216221	4642606	2986	12	832	299	226	80	227	11.0	19.1	11.6	667	464	298	42.8	2.265	6.2	-8.81
15	245651	4630074	2993	20	686	237	187	73	187	10.9	19.1	11.6	665	343	322	22.5	2.482	9.1	-8.76
16	248308	4620836	2988	12	602	206	165	67	163	11.4	19.7	12.1	680	271	349	9.0	2.759	13.4	-9.50
18	240194	4671914	3013	15	776	272	209	82	212	10.6	18.5	11.2	652	415	292	36.8	2.217	6.0	-7.85
19	185119	4714287	3063	15	1081	419	267	113	283	12.3	18.6	13.1	695	611	224	68.6	1.585	2.2	-6.56
20	213689	4715862	3045	11	1341	492	344	146	359	9.5	16.0	10.8	618	876	152	127.0	0.069	0.0	-4.07

ID	FE	SAND	SILT	CLAY	PER	ME	WHC	PHW	PHK	OM	N	CN	P	NA	K	CA	MG	CEC	BS
1	49.0	44.5	49.6	5.9	5.0	26.3	43.7	4.76	3.74	5.52	0.18	18.2	8.8	6.3	70.2	89.4	16.0	17.3	4.5
2	66.5	50.9	37.0	12.2	5.0	25.6	21.0	4.94	3.92	5.34	0.48	6.5	16.3	8.7	117.9	498.5	95.0	18.4	19.6
3	60.7	52.0	43.8	4.3	4.0	20.3	50.0	4.89	3.91	2.38	0.12	11.6	3.2	5.4	51.3	75.0	12.9	7.8	8.1
4	76.6	37.5	54.3	8.2	3.0	24.1	68.7	5.55	4.34	2.00	0.11	10.2	9.1	5.9	92.2	287.2	64.1	11.3	19.7
5	53.3	44.0	47.7	8.3	4.0	21.6	45.0	5.21	3.95	1.25	0.08	8.8	9.6	5.7	61.0	103.2	31.9	8.1	11.8
6	31.3	41.8	46.5	11.7	5.0	27.0	23.6	4.53	3.83	4.67	0.22	12.5	5.9	18.3	364.0	681.9	195.0	17.7	33.9
7	78.2	35.8	53.5	10.6	3.0	24.7	68.8	5.19	4.16	1.81	0.09	11.7	2.5	12.6	81.9	217.0	60.0	7.0	26.4
8	73.2	26.4	61.7	11.8	3.0	29.5	72.7	5.45	4.38	3.63	0.12	17.1	5.1	11.2	90.5	436.6	76.7	13.4	23.0
9	65.2	32.4	55.9	11.7	4.0	25.7	60.7	5.57	4.41	1.68	0.09	10.6	4.5	10.5	67.2	366.3	63.6	8.8	29.1
10	65.3	60.5	31.1	8.5	5.0	18.4	46.0	4.70	4.15	1.90	0.09	11.9	5.7	7.9	60.7	104.4	14.3	8.4	9.9
11	59.1	44.4	42.2	13.4	5.0	24.1	54.7	5.01	3.99	2.58	0.11	14.2	14.8	8.9	46.7	194.7	34.5	16.0	8.8
12	85.8	41.8	44.4	13.8	4.0	25.2	77.0	4.93	4.02	2.92	0.11	15.4	6.4	13.3	58.8	96.0	17.2	11.4	7.3
13	85.4	22.1	64.2	13.8	3.0	29.3	79.0	4.86	3.96	2.25	0.13	10.1	3.7	7.5	104.6	268.1	68.0	13.1	12.2
14	86.3	62.9	26.9	10.2	5.0	20.0	75.5	5.13	4.19	3.50	0.14	14.0	4.5	8.2	51.4	67.7	14.6	12.3	4.6
15	72.0	51.6	38.9	9.5	4.0	21.7	58.4	4.78	3.89	2.70	0.09	17.1	16.4	7.0	55.5	236.9	34.8	11.0	10.1
16	82.1	46.5	44.7	8.8	4.0	22.8	65.4	5.22	4.26	2.62	0.14	11.2	3.4	5.3	85.9	298.9	48.5	8.5	17.0
18	61.6	37.6	44.3	18.1	4.0	28.7	60.6	5.00	4.12	4.28	0.15	16.3	17.0	6.8	62.3	354.1	68.5	15.3	10.8
19	50.4	38.7	42.2	19.1	5.0	30.5	22.6	5.11	3.92	5.9	0.2	14.2	4.7	11.5	105.7	456.9	59.0	23.5	13.1
20	50.6	46.1	43.9	10.0	5.0	23.7	35.5	5.03	4.09	3.2	0.2	9.7	4.6	6.3	120.4	169.8	29.5	10.9	13.1

Table 5.4. Climatic and edaphic classifications of the realized niche where the *Boletus edulis*–*Cistus ladanifer* ectomycorrhizal association produces sporocarps, based on the values of some of the climatic and edaphic elaborate parameters.

Parameter	Class	No	%
Climate			
PET	Mesothermal	19	100.0
MI	Perhumid	1	5.3
	Humid	11	57.9
	Moist subhumid	6	31.6
	Dry subhumid	1	5.3
VERN	Mediterranean	19	100.0
Soil			
Texture	Loam	10	52.6
	Silt loam	5	26.3
	Sandy loam	4	21.1
OM	Very poor	5	26.3
	Poor	11	57.9
	Regular	3	15.8
Humus	Eutrophic mull	2	10.5
	Oligotrophic mull	12	63.2
	Moder	5	26.3
PHW	Moderately acid	2	10.5
	Strongly acid	16	84.2
	Very strongly acid	1	5.3

Table 5.5. Parametric climatic variables of the realized niche where the *Boletus edulis*-*Cistus ladanifer* ectomycorrhizal association produces sporocarps: 0.2-trimmed mean (M), standard deviation (SD_C), coefficient of variation of the central range (CV_C); standard deviation (SD_F), coefficient of variation of the full range (CV_F); upper and lower thresholds (UT and LT); upper and lower compensation thresholds (UCT and LCT); and upper and lower limits (UL and LL).

	LL	LCT	LT	M	UT	UCT	UL	SD_C	CV_C	SD_F	CV_F
MAP	542	542	599	681	1081	832	1341	77	11.3	208	27.1
WTP	183	183	206	238	419	299	492	31	12.9	86	31.5
SPP	150	150	165	186	267	226	344	20	10.8	48	23.3
SMP	61	62	62	72	113	82	146	5	7.3	22	27.7
ATP	147	147	163	186	283	227	359	21	11.4	54	25.7
MAT	9.5	10.6	10.6	11.2	11.5	11.8	12.3	0.3	2.7	0.6	4.9
SMT	16.0	18.5	17.7	19.4	19.7	20.1	20.1	0.4	2.0	0.9	4.9
ATT	10.8	11.2	11.2	11.9	12.2	12.4	13.1	0.3	2.5	0.5	4.1
PET	618	652	652	674	683	691	695	10	1.4	16	2.5
SUP	220	220	269	338	611	464	876	64	19.0	162	40.3
DEF	152	292	205	330	349	369	369	20	6.1	55	18.0
MI	-0.2	-0.2	8.4	20.8	68.6	42.8	127.0	11.4	54.9	30.7	93.1
LDR	0.07	2.22	1.42	2.57	2.76	2.99	2.99	0.20	7.8	0.67	28.6
IDR	0.0	6.0	1.5	10.4	13.4	18.2	18.2	3.18	30.5	4.44	49.9
VERN	-11.37	-10.14	-10.14	-9.28	-6.56	-7.85	-4.07	0.59	6.4	1.67	18.9

Our results showed that soils where *Boletus edulis-Cistus ladanifer* association produces sporocarps (Tables 5.4 and 5.6) could be regarded as strongly acid, with loam to sandy loam texture, permeable, poor in organic matter, predominantly in an oligotrophic mull form, moderately poor in nitrogen and poor in P, K, Mg, and Ca. Especially noteworthy was the very narrow textural range, hardly 2.6 % of the textural triangle was occupied by the analyzed sample and permeability was high: in a scale from 1 to 5 it always measured above 3.

Table 5.6. Parametric edaphic variables of the realized niche where *Boletus edulis-Cistus ladanifer* ectomycorrhizal association produces sporocarps: 0.2-trimmed mean (M), standard deviation (SD_C), coefficient of variation of the central range (CV_C); standard deviation (SD_F); coefficient of variation of the full range (CV_F); upper and lower thresholds (UT and LT); upper and lower compensation thresholds (UCT and LCT); and upper and lower limits (UL and LL).

	LL	LCT	LT	M	UT	UCT	UL	SD_C	CV_C	SD_F	CV_F
FE	31.3	31.3	49.0	64.1	85.4	86.3	86.3	15.3	23.9	14.9	22.7
SAND	22.1	26.4	26.4	46.7	52.0	62.9	62.9	9.6	20.6	10.3	23.9
SILT	26.9	26.9	31.1	42.9	55.9	61.7	64.2	8.8	20.6	9.4	20.4
CLAY	4.3	4.3	5.9	10.4	13.8	18.1	18.1	4.0	38.3	3.7	33.2
PER	3.0	3.0	3.0	4.4	5.0	5.0	5.0	0.8	18.3	0.8	18.7
ME	18.4	18.4	20.0	24.0	28.7	29.5	29.5	3.6	15.0	3.4	13.8
WHC	21.0	21.0	23.6	52.1	75.5	77.0	79.0	19.5	37.4	18.6	34.4
PHW	4.53	4.53	4.70	4.94	5.45	5.45	5.57	0.27	5.5	0.3	5.5
PHK	3.74	3.74	3.83	4.01	4.34	4.38	4.41	0.18	4.6	0.2	4.7
OM	1.25	1.25	1.68	3.46	4.67	5.52	5.52	1.46	42.3	1.4	43.8
N	0.08	0.08	0.09	0.16	0.18	0.48	0.48	0.10	61.8	0.1	60.0
CN	6.5	6.5	8.8	13.6	17.1	18.2	18.2	3.4	25.1	3.2	24.9
P	2.5	3.2	3.2	9.0	16.3	17.0	17.0	4.9	54.3	4.9	63.5
NA	5.3	5.4	5.4	9.0	12.6	18.3	18.3	3.7	40.9	3.4	38.5
K	46.7	51.3	51.3	94.9	104.6	364.0	364.0	77.7	81.9	69.8	75.9
CA	67.7	67.7	75.0	249.4	436.6	681.9	681.9	189.2	75.8	169.6	64.4
MG	12.9	12.9	14.3	52.5	76.7	195.0	195.0	47.4	90.4	42.3	80.0
CEC	7.0	7.8	7.8	12.8	17.3	18.4	18.4	4.7	36.6	4.4	35.0
BS	4.5	4.5	4.6	13.1	26.4	33.9	33.9	8.5	64.8	8.4	56.1

Furthermore, climatic parameters exhibited a lower variation expressed by the coefficient of variation in their central range (12.5 %) but significantly ($p \leq 0.05$) different from that of the full range (26.7 %) (Figure 5.1), both in the whole climatic

parameters set and the pluviometric and thermometric; this was not true in the case of those regarding the hydric regime. Conversely, we did not find any difference in the coefficient of variation of the edaphic parameters concerning the full range or the central range. Nevertheless, variation of the edaphic parameters in the central range (39.9 %) was significantly higher ($p \leq 0.05$) than that of the climatic central range. The lowest variation was found in the thermometric parameters set (2.2 %, central range; 4.1 %, full range), among the climatic ones, and in the parameters relating to physical properties, among the edaphic (24.9 %, central range; 23.9 %, full range).

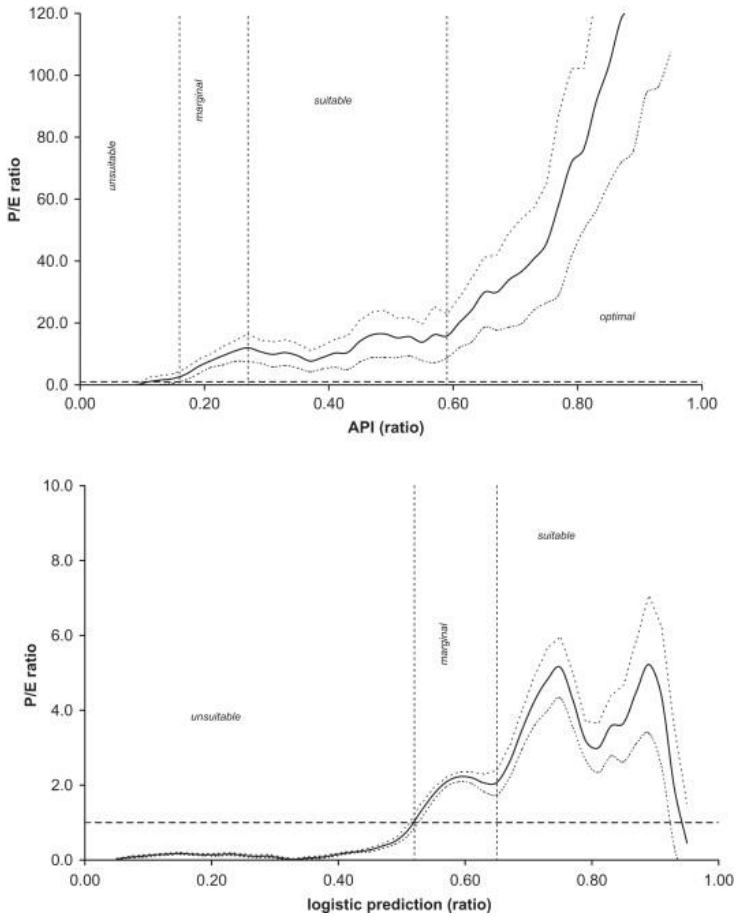


Figure 5.1. Validation for *Boletus edulis-Cistus ladanifer* association suitability model (top: API; bottom: Maxent). Predicted/expected P/E curve computed by a moving window of width 0.1. Continous line, mean; dotted lines, 95 % confidence interval; horizontal dashed line, P/E ratio = 1.

5.3.2. Distribution model.

Evaluation of the presence/expected curve enabled determination of the performance and the boundaries of three habitat suitability classes (Table 5.7 and Figure 5.2) for both indexes (API and Maxent): marginal, suitable and optimal. The shape of the curve for Maxent showed poor behavior of the model, mainly at high values of the suitability index, and advised against defining an optimal class. In fact, the mean maximum logistic prediction for the sampling points was 0.5979, that is very far from the highest value (0.8614) in the whole prediction area. This meant that presences fell in medium or low suitability classes, which was not desirable in any case. Furthermore, the highest value of the presence/expected ratio for Maxent was 5.2, dramatically lower than 259.3 for API.

Table 5.7. Habitat suitability class boundaries and potentially occupied area of the realized niche where the *Boletus edulis-Cistus ladanifer* ectomycorrhizal association produces sporocarps.

Class	Suitability lower boundary	Km^2	%
	API		
Marginal	0.0927	4283	39.2
Suitable	0.1565	4689	44.5
Optimal	0.3420	1785	16.3
	MAXENT		
Marginal	0.4568	20,508	41.2
Suitable	0.5711	29,226	58.8

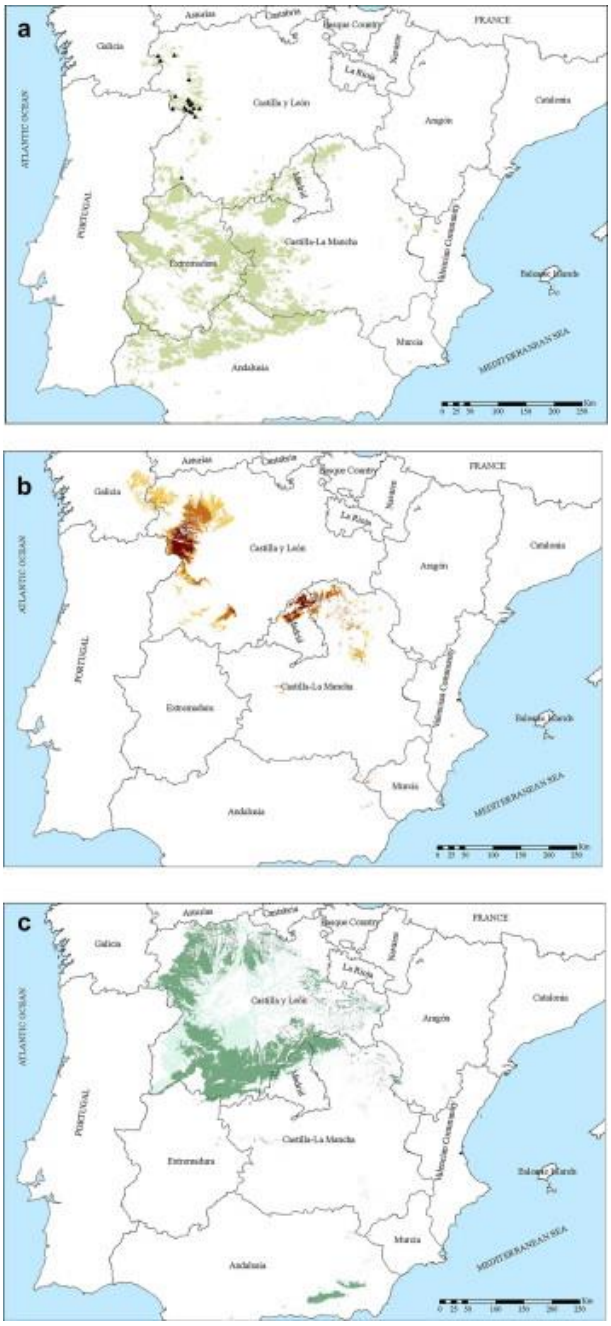


Fig 5.2. (a) Distribution of *Cistus ladanifer* scrublands in Spain (Pérez Ortiz and Esteban 2008). ▲: sample points. (b) and (c) *Boletus edulis-Cistus ladanifer* ectomycorrhizal association suitability model in Spain (b: API; c: Maxent). Three suitability classes are depicted (see Table 5.6): marginal (light), suitable (dark), optimal (very dark).

Validation results demonstrated a greater robustness of our model than Maxent. Despite the small size of the sample, the mean values for sensitivity reached

almost 100 % for the calibration and more than 90 % for the pooled sets (Table 5.8), and exceeded 73 % for the validation set. Furthermore, only 6 % of the 50 replications showed significant differences ($p \leq 0.05$) between the predicted API distribution for the calibration and the validation sets (Wilcoxon-Mann-Whitney robust test, Table 5.8), and the 95 %-confidence interval for the mean of the Mee's statistic (*m*) included the critical value of 0.5. Finally, the 95 %-confidence interval for the predicted/expected curve stayed over the critical threshold (P/E = 1) and was mainly constant along the suitable and optimal classes (Figure 5.1), while in the marginal region the interval scarcely bordered on such limit between two points (API = 0.182 and 0.241).

Table 5.8. Validation of the suitability model for the realized niche where the *Boletus edulis-Cistus ladanifer* ectomycorrhizal association produces sporocarps. Left: mean, standard deviation (SD), minimum and maximum of the sensitivity for calibration, validation and pooled sets (50 repetitions), as calculated for predicted suitability thresholds of 0.0882 for API and 0.4568 for Maxent (resulting from the Boyce-Hirzel index for presence-only data). Right: Wilcoxon-Mann-Whitney robust test (Mee 1990) for comparing the predicted suitability indexes distributions of calibration and validation sets (50 repetitions). M, Mee statistic; lci and uci, lower and upper confidence interval (p = 0.95) form; % sign, percentage of repetitions showing significant differences between both distributions.

Sensitivity (%)				Wilcoxon–Mann–Whitney robust test			
N = 50	Calibration set	Validation set	Pooled set	*N* = 50	M	lci	uci
API							
Mean	99.7	73.3	91.4	Mean	0.406	0.179	0.686
SD	1.5	19.9	6.6	SD	0.137	0.092	0.112
Min	92.3	33.3	73.7	Min	0.141	0.035	0.427
Max	100.0	100.0	100.0	Max	0.667	0.394	0.874
				% sign	6.0		
MAXENT							
Mean	83.9	85.2	84.5	Mean	0.433	0.198	0.709
SD	4.7	13.3	2.2	SD	0.166	0.112	0.135
Min	78.6	60.0	84.2	Min	0.077	0.017	0.288
Max	92.9	100.0	100.0	Max	0.756	0.482	0.912
				% sign	8.0		

Figures 5.2b and 5.2c show the territory predicted as potential for *Boletus edulis-Cistus ladanifer* association in any of the three classes and both modelling techniques. Maxent predicted a much wider distribution area (almost 50,000 km^2) than API (scarcely 11,000 km^2), mainly in the Castilla y León region. In this region, Maxent seemed to be largely overestimating the suitable area. Two other major differences were the lack of predicted area in Galicia and the northwestern edge of Castilla y León as well as the significant high-suitability region in the south-east Spain (Andalusia) according to the Maxent model.

Finally, according to API, only 16.3 % of the potential area was considered to be optimal, representing about 1,785 km^2, and suitable class rose above 4,600 km^2.

5.4. Discussion

A thorough survey of *Cistus ladanifer* scrublands has permitted definition of the main features of the climatic and edaphic habitat where the *Boletus edulis-Cistus ladanifer* association produces sporocarps, not only qualitatively but also quantitatively. The 4 yr duration of the survey can be considered long enough for evaluating sporocarps presence/absence in an area, more so since weather conditions varied between the sampling years, and that when *Boletus edulis* produces sporocarps, it does abundantly. Moreover, the territory predicted as suitable for the *Boletus edulis-Cistus ladanifer* association in peninsular Spain has been modelled based on climatic variables and corrected under lithological criteria, showing great potential mainly in the Castilla y León region.

Boletus edulis is a fungus with worldwide distribution. It usually appears in acidic soils in the warmer parts of the temperate zones (Singer 1986; Muñoz 2005), but the characteristics of its realized niche relevant to production of sporocarps are not detailed at any length in the literature. In general, climatic characteristics agree with those reported in this work, but results presented here suggest that soils where the *Boletus edulis-Cistus ladanifer* association produces sporocarps are slightly better than those on which *Cistus ladanifer* grows in Spain (San Miguel et al. 2008). Thus, soils where *Cistus ladanifer* is associated with *Boletus edulis* are richer in organic matter and have mull humus type rather than mor.

Particular abiotic factors that influence the triggering of *Boletus edulis* sporocarp formation remain unclear (Murat et al. 2008). Sporocarp formation for *Suillus luteus* is related to a high soil water content and a high content of organic matter (Barroetaveña et al. 2008), with this process negatively related to duff cover, total duff depth and canopy cover on a local scale. It is plausible that abiotic variables leading *Suillus luteus* into sporocarp formation could be similar for *Boletus edulis*, as both species are members of Boletales order and because their structures are similar. Conversely, in our regional-scale study, climatic variables, especially temperature, are found to be critical for the *Boletus edulis-Cistus ladanifer* association, as their ranges are significantly narrower than the edaphic ones, with the exception of texture, which is expected to also control *Boletus edulis* sporocarp formation in the study area.

Although sporocarps represent a biased subsample of the below-ground community of ectomycorrhizal fungi (Gardes and Bruns 1996), the transcendence of sporocarps as non-timber forest products justifies the development of sporocarp-

based distribution models, particularly for edible ectomycorrhizal fungi. In fact, some regional models of ectomycorrhizal sporocarp-environment relationships have been developed in the last years (Bergemann and Largent 2000; Barroetaveña etal. 2008; Alonso Ponce et al. 2010a; Wolfe et al. 2010). Nevertheless, relatively little is known about how mycorrhizal fungi interact with large-scale environmental processes across most of the world (Peay et al. 2010). Through the years, autecological features of hosts have usually been accepted to be the same for the fungal associate. Nonetheless, this statement does not appear to be entirely true, at least from the sporocarp formation perspective, even in fungi with ecological host specificity (Hirose et al. 2010). For example, *Boletus edulis* sporocarps are usually collected in *Pinus sylvestris* L. stands in Spain but never in France (Rondet and Leprince 2001), and *Boletus aereus* sporocarps are collected in drier, warmer locations than *Boletus edulis*, for the same host plant (Oria de Rueda et al. 2008). In our surveys we did find one location with *Boletus aereus*, whose climatic characteristics significantly differ from the ranges for *Boletus edulis* presented here (results not shown). Thus, different fungi seem to have different niches for the same plant associate.

Moreover, patterns of fungal distribution are governed by several factors. Structural differences between sporocarps determine dispersal ability, though most spores travel only very short distances from their point of origin (Peay et al. 2010). Ectomycorrhizal fungi possess some saprotrophic capacity, particularly if there is some selection pressure maintaining it, such as the occasional loss of connection with a living host plant (Koide et al. 2008). Actually, seven of our locations were previously agricultural lands while the rest were forests affected by recurrent fires, both

situations implying that *Boletus edulis* must have survived for a long time by either growing saprotrophically or forming resistant structures. This period can be substantially reduced if the species associates to *Cistus ladanifer* and forms sporocarps, assuring its future with sexual propagules. In addition, *Cistus ladanifer* can act as a refuge plant and serve as fungal inoculum for regenerating tree species following disturbances (Wiensczyk et al. 2002). Thus, identifying zones where *Boletus edulis-Cistus ladanifer* association is expected to be viable is doubly important: from an economical point of view, *Cistus ladanifer* can provide high *Boletus edulis* sporocarp production as soon as 3 yr after seed germination (Oria de Rueda et al. 2008), and from an ecological perspective, *Boletus edulis-Cistus ladanifer* association contributes to maintain and extend the source of inoculum after fire, log harvesting or land abandonment.

Our model has shown an adequate robustness notwithstanding the small number of presence locations, as the validation procedures have upheld, and its performance was demonstrated to be superior in this case to the widely-used algorithm Maxent. Though Maxent needs only-presence data, it also uses background information to generate pseudoabsences. This fact, along with the small size of our sample, may have reduced the performance of Maxent, even though it has in other studies produced better results than other techniques with small datasets (Hernández et al. 2006). Our algorithm was not designed specifically to deal with small sample sizes but with presence-only data, high-correlated variables and outliers. Nevertheless, the latter are specially influential in short datasets, which can explain its better accuracy. Thus, when managing samples with few records for fungal species,

particular care should be taken to identify possible outliers, wrong-georeferenced registers, and algorithms that do not need absence or pseudoabsence data should be employed. This is especially significant when utilizing online databases and herbaria records, as the information about localization and host plant is frequently insufficient to date.

Multiple lines of evidence endorse our confidence in the model. Firstly, an early version of our model permitted us to spot two new locations in the autumn of 2009 (no. 19 and 20, see Table 5.3) as well as a third one which unfortunately was burnt. Secondly, it does not predict the presence of *Boletus edulis* in the large pure *Cistus ladanifer* scrublands of Extremadura, Andalucia or in the Ávila province (south of the Castilla y León region), where no *Boletus edulis* sporocarp harvesting has been identified in rockroses thus far. Finally, the lithological correction assures that most unsuitable soils have been precluded from the predicted area. Given that texture can be a constraining factor in *Boletus edulis-Cistus ladanifer* association distribution, a finer adjustment could be addressed in the future when a model of soil properties for the whole studied territory is available.

The future refinement of this model, by adding locations with and without the presence of *Boletus edulis* in the soil, will allow us to improve the knowledge about the influence of abiotic factors on the triggering of sporocarp production, a feature of vital importance in the life cycle of ectomycorrhizal fungi which is still insufficiently known. For this purpose, a more accurate distribution map of *Cistus ladanifer* should be elaborated, in order to exclude polygons where tree hosts or other *Cistus* species are present. Finally, this work contributes to the understanding of the large-scale spatial

distributional ranges for fungal species, providing foresters with a reliable tool for managing the large *Cistus ladanifer* scrublands in western Spain.

6. Conclusions.

6.1. Characterization and identification of field ectomycorrhizae of *Boletus edulis* and *Cistus ladanifer*.

1. *Boletus edulis* is able to produce ectomycorrhizae and sporocarps in association with unusual host plants such as *Cistus ladanifer*, a pioneer early stage shrub. This situation would favour the maintenance of soil inoculum reservoria for successional stages. Also these fact may be seen as a dispersion strategy to assure genetic variation.
2. *Boletus edulis* and *Cistus ladanifer* ectomycorrhiza has traits typical of Boletales: whitish with three differentiated plectenchymatous layers in the mantle in plan view forming ring-like structures and rhizomorphs with highly differentiated hyphae. The inflated, smooth cystidia-like clavate end-cells on the surface of the rhizomorphs and their slightly twisted external hyphae are additional characterizing features. The Hartig net occupies one and a half rows of cortical cells, partly reaching the endodermis. Not all hyphae have clamps. The identification of the fungal symbiont as *Boletus edulis* was confirmed by ITS rDNA sequence comparison between mycorrhizae and sporocarps.

6.2. Mycorrhizal synthesis between *Boletus edulis* species complex and rockroses (*Cistus* sp.).

3. Ectomycorrhizae of *Boletus aereus, Boletus edulis,* and *Boletus reticulatus* were synthesized *in vitro* with *Cistus albidus* and *Cistus ladanifer* using synthesis tubes filled with a mixture of sterilized peat-vermiculite and nutrient solution. Nevertheless, no *Boletus pinophilus* ectomycorrhizae where formed.

4. The formed ectomycorrhizae were very similar, with white monopodial-pinnate morphology, a three-layered plectenchymatous mantle on plan view, and boletoid rhizomorphs. They present similar character with those formed by the same fungal species with other hosts, and also with those formed in the wild.
5. Controlled mycorrhization and outplanting of inoculated seedlings might be a feasible and promising way to exploit this symbiosis providing economic benefits. To accomplish this, further research is needed to determine the appropriate inoculation methods with compatible strains, the persistence of ectomycorrhizae on outplanted, inoculated seedlings, and the factors triggering sporocarps production.

6.3. Rockroses and *Boletus edulis* ectomycorrhizal association: realized niche and climatic suitability in Spain.

6. The climatic niche of *Boletus edulis* and *Cistus ladanifer* could be considered as mesothermal, Mediterranean and humid.
7. Soils are strongly acid, with loam texture, low in organic matter, and in an oligotrophic mull form.
8. Fungal spatial distribution models are reliable tools for managing these scrublands in western Spain. According to the presence/expected curve, 16.3 % of the potential area, 1,785 km^2, is considered to be optimal for *Boletus edulis* and *Cistus ladanifer* ectomycorrhizal association and most of the suitable territory is within the Castilla y León region.

9. Although some regional models of ectomycorrhizae, sporocarp production and environment relationships have been developed over the last few years, our understanding of the distribution and community organization of ectomycorrhizal fungal communities on the roots is still in its infancy.

7. Conclusiones.

7.1. Caracterización e identificación de las micorrizas en campo de *Boletus edulis* y *Cistus ladanifer*.

1. *Boletus edulis* es capaz de producir ectomicorrizas y carpóforos en asociación con plantas hospedantes no habituales, como *Cistus ladanifer*, un arbusto típico de etapas primarias de sucesión. Esta situación favorecería el mantenimiento del reservorio de inóculo del hongo en el suelo para etapas de sucesión posteriores. Este hecho puede ser visto como una estrategia de dispersión para asegurar la variación genética de la especie.
2. La ectomicorriza formada entre *Boletus edulis* y *Cistus ladanifer* tiene los rasgos típicos de los Boletales: blanquecina, con el manto formado por tres capas plectenquimatosas con estructuras en forma de anillo y rizomorfos con hifas diferenciadas. Las células de la superficie de los rizomorfos ampulosas, lisas, parecidas a cistidios, con terminación clavada; y la disposición de sus hifas, ligeramente retorcidas, son otros carateres típicos adicionales. La red de Hartig ocupa una fila y media de las células corticales, alcanzando parcialmente la endodermis. Ninguna hifa tiene fíbulas. La identificación del simbionte fúngico como *Boletus edulis* se confirmó mediante la comparación de las secuencias de la región ITS del rDNA de las micorrizas y los carpóforos.

7.2. Síntesis micorrícica de las species del grupo *Boletus edulis* y *Cistus* sp.

3. Se sintetizaron *in vitro* micorrizas de *Boletus aereus, Boletus edulis* y *Boletus reticulatus* con *Cistus albidus* y *Cistus ladanifer* utilizando tubos de síntesis rellenos con una mezcla de turba-vermiculita esterilizada y solución de nutrientes. Sin embargo, no se consiguió la formación de micorrizas de *Boletus pinophilus* con ninguna de las dos especies hospedantes ensayadas.
4. Las micorrizas formadas son todas muy similares, blancas, ramificadas de forma monopodial-pinnada, con manto compuesto de tres capas plectenquimatosas y rizomorfos boletoides. Sus caracteres son similares a los que presentan estas mismas especies de hongos con otros hospedantes y con aquellas recolectadas en campo.
5. La micorrización controlada y la instalación de plántulas inoculadas puede ser una forma viable y prometedora para explotar este tipo de simbiosis con beneficios económicos. Para conseguirlo, es necesario realizar más investigación de cara a determinar métodos de inoculación apropiados con cepas compatibles, la persistencia de las ectomicorrizas en las plantas micorrizadas instaladas en campo, y los factores que desencadenan la producción de carpóforos.

7.3. La asociación ectomicorrícica entre las jaras y *Boletus edulis*: área potencial y aptitud climática en España.

6. El nicho climático de *Boletus edulis* y *Cistus ladanifer* puede ser caracterizado como mesotérmico, Mediterráneo y húmedo.
7. Los suelos son fuertemente ácidos, con textura limosa, pobres en materia orgánica y con humus oligotrófico.

8. Los modelos de distribución de especies para hongos son herramientas fiables de cara a la gestión de estas áreas arbustivas del oeste español. De acuerdo con la curva de presencia predicha / presencia esperada, el 16,3 % del área potencial, 1.785 km^2, son considerados climáticamente óptimos para la asociación ectomicorrícica entre *Boletus edulis* y *Cistus ladanifer*, situándose la mayor parte de este territorio en la región de Castilla y León.
9. Aunque algunos modelos regionales de producción de carpóforos ectomicorrícicos y de relaciones ambientales han sido desarrollados en los últimos tiempos, el conocimiento de la distribución y la organización de la comunidad de hongos ectomicorrícicos en las raíces se encuentra todavía en sus inicios.

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