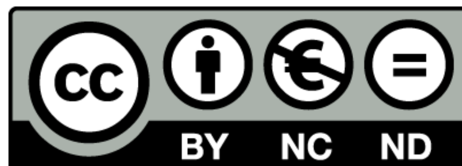
Universitat de Barcelona

Traceability of the mycorrhizal symbiosis in the controlled production of edible mushrooms

Traçabilitat de la simbiosi micorízica en la producció controlada de fongs comestibles

Herminia De la Varga Pastor



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TRACEABILITY OF THE MYCORRHIZAL SYMBIOSIS ON THE CONTROLLED PRODUCTION OF EDIBLE FUNGI

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Herminia De la Varga Pastor

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Traçabilitat de la simbiosi micorízica en la producció controlada de fongs comestibles

Memòria presentada per Herminia De la Varga Pastor per optar al grau de Doctora per la Universitat de Barcelona

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Agraïments

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Contents

AGRAÏMENTS 3

CONTENTS 5

List of Figures 10
List of Tables 12

GENERAL INTRODUCTION 15

1. Edible mushrooms and their importance 17
2. Species studied in this thesis 20
2.1. *Boletus edulis* 20
2.2. *Lactarius deliciosus* 21
2.3. *Rhizopogon roseolus* 22
2.4. *Tuber melanosporum* 22
3. Molecular techniques used 26
3.1. Conventional PCR 26
3.2. Real-time PCR 28
3.3. Simple sequence repeat (SSR) analysis 31

PURPOSE AND GENERAL OBJECTIVES 34

CHAPTER 1 37

Production of *Boletus edulis* inoculated plants in pure cultures and in nursery conditions

1. Introduction 39
2. Materials and Methods 44
2.1. Synthesis of mycorrhizas in pure culture conditions 44
2.1.1. Preparation of host plant seedlings under axenic conditions 44
2.1.2. Isolation and maintenance of *B. edulis* pure cultures 45
2.1.3. Synthesis of mycorrhizas in pure culture conditions 47
2.2. Synthesis of mycorrhizas in nursery conditions 47
2.2.1. Acclimatization of *in vitro* mycorrhizal plants 47
2.2.2. Controlled inoculations of *Cistus* spp. plants with species of the *B. edulis* complex for the production of mycorrhizal plants in nursery conditions. 50

2.3. Viability of *B. edulis* complex cultures stored in water at 4°C 52
3. Results 53
3.1. Synthesis of mycorrhizas in pure culture 53
3.2. Production of mycorrhizal plants in nursery conditions 57
3.2.1. Acclimatization of *in vitro* mycorrhizal plants 57
3.2.1.1. Acclimatization of plants inoculated under pure culture synthesis conditions 57
3.2.1.2. Acclimatization of plants inoculated under pure culture synthesis conditions in two different substrates 57
3.2.2. Controlled inoculations of *Cistus s*pp. plants with species of the *B. edulis* complex for theproduction of mycorrhizal plants in nursery conditions. 58
3.2.2.1. Inoculations with spores suspension 58
3.2.2.2. Inoculations with mycelial inoculum 58
3.3. Viability of *B. edulis* complex cultures stored in water at 4°C 60
4. Discussion 62

CHAPTER 2 69

Use of molecular techniques for the traceability of ectomycorrhizal fungi: Seasonal dynamics of *Boletus edulis* and *Lactarius deliciosus* mycelium in pine forests of central Spain

1. Introduction 71
2. Materials and Methods 74
2.1. Design of *B. edulis* probes and primers for their mycelium detection. 74
2.1.1. Design of primers and probes 74
2.1.2. DNA extractions 74
2.1.3. Construction of standard curves 74
2.1.4. Quantification of extraradical soil mycelium of standards and samples by real-time PCR 75
2.2. Validation method by comparison of conventional PCR and quantitative real time PCR. 76
2.2.1. Study site 76
2.2.2. *Boletus edulis* soil mycelium quantification 78
2.2.3. Quantification of ectomycorrhizas 78
2.3. Seasonal dynamics of *Boletus edulis* and *Lactarius deliciosus* extraradical mycelium in pine forests of central Spain. 79
2.3.1. Study site 79
2.3.2. Soil sampling 81

2.3.3. Soil DNA extraction 81
2.3.4. Quantification of extraradical soil mycelium by real-time PCR 81
2.4. Data analyses 82
3. Results 83
3.1. Validation by comparison of conventional PCR and quantitative real time PCR. 83
3.2. Seasonal dynamics of *Boletus edulis* and *Lactarius deliciosus* extraradical mycelium in pine forests of central Spain. 86
3.3. Quantification and dynamics of *Boletus edulis* and *Lactarius deliciosus* ectomycorrhizas. 91
3.4. Relation of extraradical mycelium and ectomycorrhiza of *Boletus edulis* and *Lactarius deliciosus*. 94
4. Discussion 94

CHAPTER 3 103

Traceability of ectomycorrhizal fungi in plantations: persistence and quantification of *Rhizopogon roseolus* and *Tuber melanosporum* extraradical soil mycelium in de novo and old plantations

1. Introduction 105
2. Materials and Methods 108
2.1. Design of *Tuber melanosporum* probes and primers for their mycelium detection. 108
2.1.1. Design of primers and probes 108
2.1.2. DNA extractions 109
2.1.3. Oligonucleotides test by real-time PCR 109
2.2. Traceability of different ectomycorrhizal fungi in monitored plantations 109
2.2.1. Study sites 109
2.2.1.1. *Rhizopogon roseolus* x *Pinus pinea* plantation in Cabrils 110
2.2.1.2. *Tuber melanosporum* x *Quercus ilex* plantation in Cercs (Berga) 110
2.2.1.3. Quantification of extraradical mycelium of *Tuber melanosporum* in soils from mature truffle orchards in Navarra (northern Spain) 115
2.2.3. Inoculated plants 117
2.2.4. Soil sampling 118
2.2.5. Tracking colonization by *R. roseolus* and *T. melanosporum* 119
2.2.5.1. Radical colonization (mycorrhizas) 119
2.2.5.2. DNA extraction and soil mycelium quantification by real-time PCR 119
2.3. Data analyses 121

3. Results **121**
3.1. Design of *T. melanosporum* probes and primers for mycelium detection and quantification. 121
3.2. Traceability of different ectomycorrhizal fungi in monitored *de novo* plantations. 123
3.2.1. *Rhizopogon roseolus* x *P. pinea* plantations in Cabrils 123
3.2.1.1. Radical colonization: mycorrhiza 123
3.2.1.2. Extraradical colonization: soil mycelium 124
3.2.1.3. Extraradical colonization: rhizospheric soil mycelium 127
3.2.1.4. Tree growth 127
3.2.1.5. Correlations between soil mycelium and climatic data. 128
3.2.2. *Tuber melanosporum x Q. ilex* plantation in Cercs 129
3.2.2.1. Radical colonization: mycorrhiza 129
3.2.2.2. Extraradical colonization: soil mycelium 129
3.2.2.3. Plant growth 130
3.2.2.4. Correlations between mycorrhizas, mycelium and climatic data. 130
3.2.3. Quantification of extraradical mycelium of *Tuber melanosporum* in soils from truffle orchards in Navarra (northern Spain) 131
4. Discussion **133**
4.1. Design of *T. melanosporum* probes and primers for their mycelium detection. 133
4.2. Traceability of different ectomycorrhizal fungi in monitored plantations. 135
4.3. Quantification of extraradical mycelium of *Tuber melanosporum* in soils from truffle orchards in Navarra (northern Spain) 137

CHAPTER 4 **141**

Spatial genetic structure of *Tuber melanosporum* in a productive orchard determined by mating type genes and microsatellites analysis

1. Introduction **143**
2. Materials and Methods **146**
2.1. Study site 146
2.2. DNA extractions and molecular analysis: Genotyping of ectomycorrhizas, ascocarps and soil DNA 148
3. Results **151**
3.1. Spatial distribution of Mating types 151
3.2. Genets spatial distribution 152
4. Discussion **157**

4.1. Distribution of *T. melanosporum* strains of different mating types 157
4.2. Spatial genetic structure of *T. melanosporum* in the studied orchard 158

CONCLUSIONS 161

Chapter 1 164
Chapter 2 164
Chapter 3 165
Chapter 4 166

RESUM 167

ANNEX 190

Scientific Publications 198
Conference participations and contributions 199

REFERENCES 200

List of Figures

Figure I. 1. *Main natural distribution areas for Tuber melanosporum.* ____ 23

Figure I. 2. *Life cycle of Tuber melanosporum.* ____ 25

Figure I. 3. *Example of agarose gel with different amplified samples and DNA marker.* ____ 28

Figure I. 4. *Standard curve construction with the amplification curves of a dilution bank samples with known amounts of DNA.* ____ 29

Figure I. 5. *Example of the result for the SSR screening for three samples given by a Beckman CEQ 8000 Genetic Analyzer.* ____ 33

Figure 1.1. *Detail of the acclimatization process of the in vitromycorrhizal plants to nursery conditions in peat-vermiculite (1:1; v:v) containers.* ____ 48

Figure 1.2. *Plants acclimated to nursery conditions after their mycorrhization in pure culture conditions using two diferents substrates (peat-vermiculite 1:1; and 1:10; v:v).* ____ 49

Figure 1.3. *Seedlings of Pinus pinaster, Cistus ladanifer and Cistus albidus at the moment of inoculation with spore suspension (left) and some months after (right).* ____ 50

Figure 1.4. *A) Mycelial inoculums produced in presence (left) or not (right) of host plant used to inoculate Cistus seedlings. B) Cistus albidus seedling inoculated with Be 375 mycelium inoculum. C) C. ladanifer and C. albidus inoculated with mycelial inoculums.* ____ 52

Figure 1.5. *Glass tubes containing distilled water with mycelium disks stored in darkness at 4°C; Petri dishes of six different strains growing on BAF medium after 24 months of cold storage (15 days old colonies).* ____ 53

Figure 1.6. *Images from different mycorrhizas and other structures of diferent Boletus – plant combinations.* ____ 54

Figure 1.7. *Boletus edulis 2057 x Pinus pinaster mycorrhizas transferred into the synthesis growth substrate used for the synthesis of mycorrhiza in pure culture (peat-vermiculite 1:10; v:v) and results of the PCR with specific B. edulis primers (Mello et al. 2006) after electrophoresis on Agarose (2%) gel. C+: positive control, DNA extraction from B. edulis pure culture mycelium.* ____ 57

Figure 1.8. *Evolution of the total percentage of isolates mantaining their 100% of growth viability after the different time transfers to fresh BAF agar medium, for each species (top image) and the total (lower image).* ____ 61

Figure 1.9. *Lactarius deliciosus sporocarps grown in nursery containers.* ____ 66

Figure 2.1. *One of the fenced areas selected in Pinar Grande for the quantification of B. edulis soil mycelium.* ____ 77

Figure 2.2. *Mean precipitation (mm) and mean temperature (^{o}C) from September 2009 to august 2011 (12 months) in Pinar Grande and from September 2010 to September 2011 (13 months) in Pinares Llanos de Almazán.* ____ 80

Figure 2.3. *Standard curve obtained for mycelium quantification of Boletus edulis in Pinar Grande (A) and Lactarius deliciosus in Pinares Llanos de Almazán (B) in soil by real-time TaqMan PCR.* ____ 86

Figure 2.4. *Mycelium dynamics and sporocarp production for Boletus edulis (A) and Lactarius deliciosus (B) over 12 months.* ____ 88

Figure 2.5. *Detail of two L. deliciosus sporocarps in the forest* ____ 90

Figure 2.6. *Mycorrhiza dynamics for Boletus edulis (A) and L. deliciosus (B) over 12 months.* ____ 92

Figure 2.7. *Means and Tukey's HSD test (α=0,05) for the amount of A) B. edulis and B) L. deliciosus mycorrhizal roots in soil (250 cm^{3}) in each plot.* ____ 93

Figure 2.8. *Correlation between the number of B. edulis ectomycorrhizal rootlets in 250 cm^3 of soil and the concentration of B. edulis soil mycelium (mg/g) per soil sample (α=0.05). A) From September to November 2008, 3 months; and B) from September 2009 to August 2010, 12 months.* 94

Figure 3.1. *Detail of the Quercus ilex x T. melanosporum plantation after six months of its establishment.* 111

Figure 3.2. *Plan of the P. pinea x R. roseolus plantation established in Cabrils in 2009.* 112

Figure 3.3. *Plan of the P. pinea x R. roseolusplantation established in Cabrils in 2010.* 113

Figure 3.4. *Plan of the Q. ilex x T. melanosporum plantation established in Cercs (Berguedà) in 2010. Non-inoculated plants (red) were randomly distributed.* 114

Figure 3.5. *Mean precipitation (mm) and mean temperature (^{o}C) from April 2009 to December 2011 in Cabrils and from April 2010 to November 2011 in Cercs.* 116

Figure 3.6. *Detail of one Q. ilex in Cercs and example of the sampling methodology in one single plant.* 118

Figure 3.7. *Comparison of the standard curves obtained for the quantification of T. melanosporum mycelium by real-time PCR using UPL (open diamonds; above) and TaqMan® (black squares; below) combined with immature (left) and mature (right) ascocarps.* 122

Figure 3.8. *Standard curves used for the quantification of extraradical mycelium. A) Rhizopogon roseolus mycelium in Cabrils soil samples. B) Tuber melanosporum mycelium in Cercs soil samples.* 123

Figure 3.9. *Differences of mycelium biomass in Cabrils 2 plantation for the sampling years (in autumn 2010 and 2011), the plantation spacing (1x1 m or 2x2 m) and inoculation (R. roseolus and non-inoculated – control).* 127

Figure 3.10. *Tree growth for the volume of P. pinea in Cabrils 1 (cm^3). Differences (α=0.05) were found between inoculated and control plants in both years (2009 and 2010), but not differences on growth rates.* 128

Figure 3.11. *Differences on the total mycorrhization percentage plants at the establishment – Spring (t=0) after 6 months - Autumn (t=6), 12 months - Spring (t=12) and 18 months - Autumn (t=18). Bars show the standard deviation. Letters show the differences established by the Tukey test.* 129

Figure 3.12. *A) Evolution of the T. melanosporum mycelium quantities detected in soil after 6, 12 and 18 months of the plantation establishment. Bars show the standard deviation. B) Correlation between the % of T. melanosporum ectomycorrhizas per plant and the concentration of T. melanosporum soil mycelium (mg/g) (log transformed) from the soil collected under these plants (r= 0.7814; p = 0.0129; α= 0.05; N=9).* 130

Figure 3.13. *Standard curves used for mycelium quantification of T. melanosporum by real-time PCR in the two sampling sites (Tierra Estella and Valdorba). Curves were generated by plotting the Ct values of DNA extracts obtained from known amounts of the target fungi added to soil of each sampling site.* 132

Figure 3.14. *Biomass of extraradical soil mycelium of Tuber melanosporum from eight soils of two productive areas of Navarre (northern Spain).* 132

Figure 4. 1. *The two C. avellana (F10 and F11) and the small oak (E10) selected from de Rollainville orchard.* 147

Figure 4. 2. *Sampling of root tips (A) and soil samples (B) under two hazels (F10 and F11 - circles) and one small oak (E10 - square) in the spring of 2011 in the Rollainville orchards.* 147

Figure 4. 3. *The area marked with a dot line represents the hazels brulé. Examples of the selection of T. melanosporum ectomycorrhizas from root pieces.* 148

Figure 4. 4. *a) Multiplex PCR amplification of DNA from ascocarps with mating type specific primers for MAT(+) and MAT(-). b) Example of PCR amplification of ectomycorrhizas DNA with T. melanosporum specific primers ITS4LNG-ITSML.* ________ *149*
Figure 4. 5. *Examples of the results for the SSR screening for three samples given by the Beckman CEQ 8000 Genetic Analyzer.* ________ *150*
Figure 4. 6. *Tuber melanosporum mating type identification of the maternal tissue of ascocarps (A), ectomycorrhizas (B) and soil samples (C).* ________ *155*
Figure 4. 7. *Tuber melanosporum genet distribution in the Rollainville orchard.* ________ *156*

List of Tables

Table 1.1. *Published works in which Boletus mycorrhizal plants were produced.* ________ *39*
Table 1.2. *Boletus edulis complex isolates used in the ectomycorrhizal synthesis and obtained from sporocarps collected in different forest stands of northern Spain.* ________ *46*
Table 1.3. *List of mycorrhizal plants transferred to nursery conditions to test if substrate type influences in the maintenance of mycorrhizas of the B. edulis complex. *This combinations could not be tested in Synthesis substrate as the seedlings grown on that substrate died.* ________ *49*
Table 1.4. *Results of the synthesis of B. edulis complex mycorrhizas in pure culture.* ________ *54*
Table 1.5. *Results of the synthesis of B. edulis complex mycorrhizas in pure culture.* ________ *56*
Table 1.6. *Inoculations with Boletus spp. spore suspensions. B. edulis (A) : Origin Alto Abejera; B. edulis (P): origin Pinar Grande.* ________ *58*
Table 1.7. *Inoculations with Boletus spp. mycelium (B. reticulatus 1053, B. edulis 375 and B. aereus 393). Laccaria bicolor used as a positive control. Inoculum type: (1) mycelium grown in peat:vermiculite; (2) mycelium grown in peat:vermiculite in presence of the respective host plant (P. pinaster or C. albidus).* ________ *59*
Table 1.8. *Analysis of variance for the viability of Boletus spp. isolates by the species and the storage time in water at 4°C.* ________ *60*

Table 2.1. *Top soil properties measured in the sampling plots in Pinar Grande.* ________ *76*
Table 2.2. *Top soil (0-20 cm) properties measured in the four sampling plots* ________ *79*
Table 2.3. *Mean production of Boletus edulis sporocarps (kg/ha) and Lactarius deliciosus sporocarps (kg/ha) between 1995 and 2007 and 1997 and 2009; and in the harvesting season 2009-2010 and 2010-2011 respectively.* ________ *80*
Table 2.4. *Detection of B. edulis mycelia in soil samples by real-time PCR (RT-PCR) in mg mycelium/g soil and conventional PCR using specific primers.* ________ *84*
Table 2.5. *Analysis of variance for the concentration of B. edulis mycelium in soil samples (mg of mycelium / g of soil) (Test of Brown-Forsythe: Prob > F = 0.2063).* ________ *85*
Table 2.6. *Number of mycorrhizal short roots per 250 cm^3 of soil and soil mycelial concentration (mg mycelium / g soil) obtained in the different plots and sampling dates.* ________ *85*
Table 2.7. *Analysis of Variance of the number of mycorrhizal short roots (log transformed) per volume of soil (250 cm^3). (Test Brown-Forsythe: Prob > F = 0.9047).* ________ *85*
Table 2.8. *Analysis of variance of the concentration of mycelium of Boletus edulis in the soil samples of Pinar Grande* ________ *87*
Table 2.9. *Means and Tukey HSD test (α=0,05) for the amount of Boletus edulis mycelium in soil. Dates not sharing same letter are significantly different* ________ *87*

Table 2.10. *Analysis of variance of the concentration of mycelium of Lactarius deliciosus in the soil samples of Pinares Llanos.* ____ *89*
Table 2.11. *Mean differences analysis by Tukey test (α= 0.05) of the concentration of mycelium of Lactarius deliciosus in the soil samples of Pinares Llanos for Plots and Dates. Levels not sharing same letter are significantly different.* ____ *90*
Table 2.12. *Correlations between mycelium biomass (mg mycelium/g soil) and climate variables (Spearman correlation coefficient, significance level ≤ 0.05).* ____ *91*
Table 2.13. *Correlations between Lactarius deliciosus sporocarps production (kg/ha) and climate variables.* ____ *91*
Table 2.14. *Analysis of variance of the number of Boletus edulis ectomycorrhiza in the soil samples of Pinar Grande.* ____ *93*
Table 2.15. *Means and Tukey's HSD test (α=0,05) for the amount of B. edulis ectomycorrhiza in soil (250 cm^3).* ____ *93*

Table 3.1. *Top soil (0-20 cm) properties measured in Cabrils.* ____ *110*
Table 3.2. *Top soil (0-20 cm) properties measured in Cercs plantation.* ____ *111*
Table 3.3. *Average sporocarp production data of the plots (provided by the owners). The production in the three Valdorba plots is considered together for the three host species.* ____ *115*
Table 3.4. *Soil characteristics of the different sampled plots. The three plots in the Valdorba site are considered together in the soil analysis. n.d.: not determined* ____ *116*
Table 3.5. *Establishment and sampling dates in Cabrils and Cercs plantations.* ____ *118*
Table 3.6. *Analysis of variance for the R. roseolus mycorrhizas percentage in Cabrils 2 after 6 months in field, by spacing (1x1/2x2) and inoculation (yes/no) of the trees.* ____ *124*
Table 3.7. *Analysis of variance for the R. roseolus mycelium quantities in Cabrils 1 by sampling date, spacing (1x1/2x2) and inoculation (yes/no) of the trees.* ____ *125*
Table 3.8. *Analysis of variance for the R. roseolus mycelium quantities in Cabrils 2 by sampling year (2010/2011), spacing (1x1/2x2) and inoculation (yes/no) of the trees.* ____ *126*
Table 3.9. *Correlations between mycelium biomass and climate variables.* ____ *128*
Table 3.10. *Analysis of variance for the mycorrhization % after 18 months in field, by time.* ____ *129*
Table 3.11. *Correlations between mycorrhizal percentage and climate variables.* ____ *131*

Table 4. 1. *SSR markers used for the ascocarps and ectomycorrhiza genotyping.* ____ *151*
Table 4. 2. *Characterization of the genotypes detected in ascocarps and ectomycorrhizas.* ____ *153*
Table 4. 3. *Comparison of the genotypes (on ascocarps and ectomycorrhizas) and the mating type detected for each type of sample (ascocarps, ectomycorrhiza and soils).* ____ *153*
Table 4. 4. *Characterization of the genotypes detected in the ectomycorrhizas.* ____ *154*

Table A.1. *Results of the synthesis of B. edulis complex mycorrhizas in pure culture.* ____ *192*
Table A.2. *Raw data of the number mycelium disks of Boletus spp. and R. roseolus strains that regrew in BAF medium after different storage times at 4°C in sterile water.* ____ *193*
Table A.3. *Table A.4. Raw data of T. melanosporum quantification in Navarra* ____ *196*

I

General Introduction

Introduction

1. Edible mushrooms and their importance

Edible mushrooms are an important forest resource which contributes significantly to socioeconomic and environmental value of a territory (Boa 2004). Due to the gradual change in land use taking place in several areas, such as the Mediterranean (mainly caused by the abandonment of unproductive areas and agroforestry), alternative productions as ectomycorrhizal edible fungi can be a way to maintain rural and forest landscape, trying to achieve a balance between ecological and economic needs of agroforestry systems.

Most edible wild fungi are associated with the roots of trees forming a symbiosis called ectomycorrhiza, which plays a crucial role in maintaining the balance of the ecosystem (Smith and Read 2008). Basically, the fungus has the role of providing nutrients and water from the soil to the plant through a mycelial network that expands through the soil and connects the host plants roots with several fungal species and individuals, increasing the volume of soil explored by the root system of the plant (Selosse et al. 2006; Smith and Read 2008). The plant provides the fungus products of photosynthesis translocated to the roots. Due to the complexity of this symbiosis ectomycorrhizal fungi are difficult to cultivate. Many of these species do not grow or have difficulties to be grown on the known culture media (Brundrett et al. 1996). In this context, some of the research that has been carried out and exposed in this work aims to improve the methodology and technology for the controlled production of edible ectomycorrhizal fungi.

One half of the worldwide exported edible mushrooms are ectomycorrhizal fungi and more than a thousand of ectomycorrhizal mushrooms species are currently eaten in the world (Sitta and Davoli 2012). Ectomycorrhizal associations are dominant among the major forest trees in the Mediterranean, temperate, boreal and some tropical regions, involving the families Pinaceae, Nothofagaceae, Myrtaceae and Dipterocarpaceae, making ectomycorrhizal fungi ecologically relevant (Smith and Read 2008). There are several dozen species of edible wild mushrooms that can be harvested in Europe and which have economic importance like: *Amanita caesarea* (Scop.: Fr.) Grév., *Boletus edulis* Bull.: Fr., *Lactarius deliciousus* L.: Fr., *Cantharellus cibarius* Fr., *Hydnum repandum* L. ex Fr., *Tricholoma portentosuum* (Fr.) Quélet, *Morchella* spp., *Cantharellus tuberiformis* Fr. etc. The

harvesting and marketing of these mushrooms is clearly increasing, providing an important socio-economic value. Currently, the way to obtain most of these mushrooms depends exclusively on their collection from the wild. This fact has increased the exploitation of this resource and in some places it has began to be a danger for the sustainability of this activity and the maintenance of biodiversity in forest ecosystems. Due to habitat degradation and the overexploitation of fungal resources, harvesting of edible ectomycorrhizal fungi has declined during the past years (Wang and Hall 2004). Moreover, the high variability in the natural production of edible fungi is a serious problem for the development of economic sectors related to the production of stable resources, especially gastronomy and mycological tourism.

For these reasons, it is important the development of a mycosilviculture leading to maintain and increase the production of sporocarps, preserving at the same time the conservation and diversity of the fungal species (Ortega-Martínez and Martínez-Peña 2008; Egli et al. 2010). Some crop and silvicultural practices, like clearance, pruning, thinning, fertilization or summer irrigation, can enhance fungus production. Furthermore, for economical and environmental reasons, controlled mycorrhization with ectomycorrhizal edible fungi and the establishment of productive plantations with nursery inoculated plants is an interesting alternative to complement the harvesting in natural areas, especially for the most prized and consumed species (Hall et al. 2003; Boa 2004; Hall et al. 2005; Karwa et al. 2011; Voces et al. 2012; Wang et al. 2012). Cultivated truffles are among the most important mushroom crops in Europe, which include different species, being *Tuber melanosporum* the most important (Mosquera-Losada et al. 2008). In the Mediterranean area, beside truffles, other fungal species producing considerable economic benefits are also *B. edulis*, *B. pinophilus* Pilát and Dermek and *L. deliciosus*. Particularlly, in Spain the annual income from the commercialization of wild edible fungi goes beyond that from other forest resources (cork, chestnuts, acorns, resin and also wood in areas with limited tree growth) (Álvarez et al. 2000). To carry out the experiments detailed in this thesis four species of edible ectomycorrhizal fungi were selected: *Boletus edulis, Lactarius deliciosus, Tuber melanosporum* and *Rhizopogon roseolus.* Which are among the most prized edible ectomycorrhizal fungi worldwide (Wang et al. 2002). The last species is widely appreciated in the far eastern countries (East Asia), a potential market, where it is considered a delicacy.

The traceability of the mycorrhizal symbiosis is necessary to achieve sustainable production of edible mushrooms. To get to that point we need methods enabling to control and to monitor the species concerned, either in established and controlled plantations, or in areas where there is a natural production of these fungi. In other words, we need a broad knowledge of the fungal species to exploit, both in natural and modified environments (either in laboratory or in plantations). We need to know well its ecology, phenology and life cycle, to trace the fungus in all phases (mycelium, ectomycorrhizas, fruiting...) as well as the pattern of fruit body production of each species.

For a sustainable mushroom production we need techniques to detect the fungus without disturbing the ecosystem. The sporocarps production of edible fungi in productive plantations established with mycorrhizal plants can take a long time. For this reason, it is important to establish methodologies designed to monitor the persistence of the inoculated fungus, at different vegetative stages of the symbiosis, to determine sporocarp production possibilities. In the same way, the study of factors that influence the persistence of the symbiosis requires having the proper tools to characterize and quantify the fungus to be grown. The morphological characterization of ectomycorrhizas has been used both for taxonomic and ecological purposes (Agerer 2006), but there are many situations in which morphological and anatomical characters are not conclusive for the fungus identification. Molecular techniques based on the analysis of DNA using the technique of *Polymerase Chain Reaction* (PCR) have complemented the morphological studies, opening new possibilities to explore the cryptic phases of the symbiosis (mycorrhizas and extraradical mycelium) increasing the knowledge of their ecophysiological role in the soil. These techniques have been used for the identification of mycorrhizas (Gardes and Bruns 1993; Horton 2002; Mello et al. 2006) and fungal communities (Anderson and Cairney 2004; Martin et al. 2007). One of the critical steps in monitoring the fungal persistence is the detection of the extraradical mycelium. This is the most metabolically active phase of the symbiosis (Smith and Read 2008) but also the most unknown due to the lack of methodologies for their study. Previous studies on the detection of soil mycelium were based on biochemical methods, particularly the detection of ergosterol in soil, but in recent years molecular techniques have allowed more specific studies (Dickie et al. 2002; Landeweert et al. 2003; Landeweert et al. 2005; Suz et al. 2006; Suz et al. 2008; Courty et al. 2010; Pickles et al. 2010; Douhan et al. 2011) even have been used for the specific quantification of extraradical mycelium in soil by using the real-time PCR technique (Landeweert et al.2003; Raidl et al. 2005;

Kennedy et al. 2007; Parladé et al. 2007; Hortal et al. 2008; Suz et al. 2008; Parladé et al. 2009; Zampieri et al. 2012).

The present thesis is divided in four chapters representing the different phases in the processes to produce ectomycorrhizal mushrooms: from the culture of fungal species, the production of mycorrhizal plants, the plantation establishment, and monitoring fungal species in plantations and in the wild.

2. Species studied in this thesis

Throughout the experiments carried out in the thesis, four edible ectomycorrhizal species were selected to work with: *Boletus edulis*, *Lactarius deliciosus*, *Rhizopogon roseolus* and *Tuber melanosporum*. These species are among the most prized edible ectomycorrhizal fungi (Wang et al. 2002). In the following lines a short description of them is provided:

2.1. *Boletus edulis*

Boletus edulis Bull. (1972) is a basidiomycete fungus, characterized by a cosmopolitan distribution, concentrated in cool-temperate to subtropical regions (Hall et al. 1998). It is found naturally in the northern hemisphere, and introduced in some places in the southern hemisphere (southern Africa, Australia and New Zealand). In Europe *B. edulis* distribution extends from northern Scandinavia to Greece, Italy and Spain, arriving to Morocco (Hall et al. 1998). It is widely known in Europe and it is subjected to a strong commercial exploitation in some areas due to its prized gastronomical value. In English it is commonly named king bolete or porcini, in Spanish it is called hongo, boletus or boleto, and in Catalonia it is commonly known as cep, similarly at the name given in France, cêpe.

It is found frequently in association with a large number of trees and shrubs of the families: Fagaceae (*Castanea, Castanopsis, Fagus, Lithocarpus, Quercus*), Betulaceae (*Carpinus, Corylus, Betula, Ostrya, Populus*), Malvaceae (*Tilia*), Salicaceae (*Salix*), Ericaceae (*Arctostaphylos*) and Pinaceae (*Abies, Keteleeria, Picea, Pinus, Tsuga*) (Olivier et al. 1997; Águeda et al. 2008), but it has been also reported the association between Boletales and some Cistaceae (Lavorato 1991; Vila and Llimona 2002; Águeda et al. 2006). Actually boletes are only collected from the wild (Cannon and Kirk 2007) and by now no controlled production has been achieved. In Spain the amount of boletes harvested annually ranges from

2,000 to 20,000 tm depending on the year (Oria-de-Rueda et al. 2008), most of them collected from forests dominated by *Pinus* species.

There exist several similar *Boletus* species that can be considered as the *Boletus edulis* complex (*B. edulis* Bull. *sensu stricto, Boletus aereus* Bull., *Boletus pinophilus* Pilát & Dermek, and *Boletus reticulatus* Schaeff.). Molecular phylogenetic analyses have proven these three are all distinctive and separate species (Mello et al. 2006).

B. edulis sporocarps are characterized by a large brown cap, 7–30 cm, convex in shape when young and flattens with age. The under surface of the cap is made of thin tubes. This pore surface is whitish when young, but ages to a greenish-yellow. The stout stipe, or stem, is white or yellowish in colour, 8–25 cm tall and 10 cm thick, and partially covered with a raised network pattern, or reticulations.

2.2. *Lactarius deliciosus*

Lactarius deliciosus (L.) Gray is a basidiomycete from the Russulales family. It is an appreciate mushroom collected and commercialized in many countries, especially in the Mediterranean region of Europe and North-Africa, but also in Asia (Boa 2004) where is a widely desired edible and supposes an important economic income. In Spain, this mushroom is probably the most abundantly marketed species and the price paid to collectors ranges from 1 to 12 € /kg, being even higher when mushrooms are sold to wholesale markets and to retailers (Voces et al. 2012; De Román and Boa 2006). Usually one can buy it in the market from 15 to 25 €/kg.

It is commonly known in English as Saffron milk cap mushroom (Wang and Hall 2004), as rovelló in Catalonia and níscalo or robellón in Spanish.

Lactarius deliciosus sporocarp has a convex to vase shaped carrot orange cap, rolled when young, 4 to 14 cm (1.5–7 in) across, often with darker orange lines forming concentric circles. The cap is sticky and viscid when wet. It has crowded decurrent gills and a squat orange stipe 3 to 8 cm long and 1 to 2 cm thick. It stains a deep green color when handled and, when fresh, the mushroom exudes orange latex.

It is a heliophilous species typical of sunny mountains, but it is found in a wide range of conditions in conifer forests, in young stands (Fernández-Toirán et al. 2006) but also in oldest ones (Bonet et al. 2004). It appears as fruiting bodies early in autumn (or late summer) from September to middle December. It forms

ectomycorrhizas with several species from the family Pinaceae (Hutchison 1999)and is mainly collected from the wild. This species can be easily isolated and adequately formulated for nursery inoculations (Parladé et al. 2004), and it has been shown to produce edible sporocarps from inoculated plants (Wang and Hall 2004).

2.3. *Rhizopogon roseolus*

Rhizopogon roseolus (Corda) Th. Fr. (1909) is a hypogeous basidiomycete. The genus *Rhizopogon* has more than 150 species, but only *R. roseolus* has commercial value (Wang et al. 2012), however Boa (2004) listed also two other eaten species (*R. luteolus* Krombh. and *R. piceus* Berk. & M.A. Curtis). It is widely appreciated in Japan, where it is known as shoro, and it is considered a delicacy. Although it has been eaten in Japan since more than 200 years ago, it is rare now and that is why it is cultivated in forest plantations since the 80s (Wang et al. 2012; Wang et al. 2002). In Catalan it is known as fetjó, fetjó rosat o patata de bosc, and in Spanish it is named criadilla de monte, criadilla rosada or criadilla de pinar. In Spain it is considered an edible fungus but with poor quality, only when young, so it is not a marketable mushroom as it is in Japan or China.

R. roseolus sporocarps are small, between 1,5 and 5 cm in diameter. They have a tuberculate form, similar to a small potato, roundish but irregular. Attached on the lower half it usually have white to yellowish abundant mycelial cords or rhizomorphs. It has a thin peridium and its colour can vary from white or yellowish cream in youth to a finally tesselate-areolate surface covered with appressed, white to yellowish or dark brown-reddish fibrils. The gleba, at first, is bright yellow then darkening from the center to pale olive brown to yellowish brown.

This species is symbiotic with several coniferous species, especially with those from the genus *Pinus*. They are commonly collected in autumn, and sometimes in spring, forming groups of sporocarps. Nursery plants mycorrhizal with *R. roseolus* are easy to produce by inoculations with spore suspension (Parladé et al. 1996).

2.4. *Tuber melanosporum*

Tuber melanosporum Vittad. (1981) is an ectomycorrhizal ascomycete fungi characterized by the production of hypogeous fruitbodies, truffles, with high economic and social value (Boa 2004; Mello et al. 2006) and are the most appreciated truffles of the commercialized species in Spain and France (Reyna 2000). Black truffle mycorrhizal plants production was introduced in France, in the

1970s by the INRA and IPLA (Reyna-Domenech et al. 2008) and now constitute the most extended ectomycorrhizal edible species worldwide (Hall et al. 2005). Black truffles are also some of the most expensive edible mushrooms. For the last season 2012-2013, in the Spanish markets, the mean price for the black truffle was about 550 €/Kg, and between 500 and 1200 €/Kg in France (data extracted from la *Llotja de Contractació i Mercat en origen de Vic*, the local market fixing truffle prices in Spain).

This species is symbiotic with several oak species, hazelnut trees and other broad-leaved trees (but also conifers and some shrubs (*Cistus* spp.))(Ceruti et al. 2003). Their natural distribution area comprises Mediterranean calcareous areas in Spain, France, and Italy, but they have been successfully introduced in a range of countries, especially in the southern hemisphere by planting inoculated seedlings (Wang and Hall 2004).

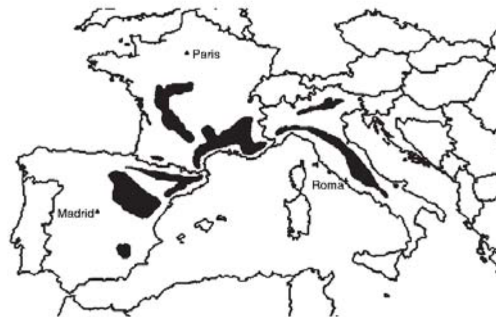


Figure I. 1. Main natural distribution areas for *Tuber melanosporum*. Figure taken from Reyna-Domenech et al. (2008)

It has been recently discovered that *T. melanosporum* is characterized by a prevalently haploid and heterothallic life cycle (Figure I.1) controlled by two alternate mating types (*MAT (+)* and *MAT (-)*) (Martin et al. 2010; Rubini et al. 2011b; Kües and Martin 2011). Its mycorrhiza are formed from homokaryotic haploid mycelia and, from them, diffuse extraradical mycelium spreads from the mantle into the surrounding soil (in late spring). That is when this mycelium (from one mating type), mate the opposite mating type in soil mycelium and sexual reproduction happens, and after that ascogonia formation starts. Ascocarps develop in late spring and enlarge in autumn and winter (taking 6 to 9 months), and mature in January or February of the following year. The vegetative tissues of truffles (gleba) are of the same mating type of the ectomycorrhiza found at the

nearby host tree roots. Consequently, the opposite mating type must be living free, as extraradical mycelium, in the soil. But ascospores are multinucleate with haploid nuclei. Both mating types are found within asci across the spores (1:1 relation) confirming a life cycle with an obligate outcrossing reproductive mode (Rubini et al. 2011b).Vegetative hyphae are plurinucleate and contain only one type of parental information, one mating type (Paolocci et al. 2006).

Truffle productive regions are located in areas with Mediterranean to temperate climate. The mean annual temperature is between 8.6 and 14.8°C, being the mean temperature of the warmest month between 16.5–23°C, and the mean temperature of the coldest month between 1–8°C.The mean annual rainfall is 450–900 mm. They grow in well-aerated and well-drained calcareous soils, with pH 7.5–8.5 (Callot 1999).

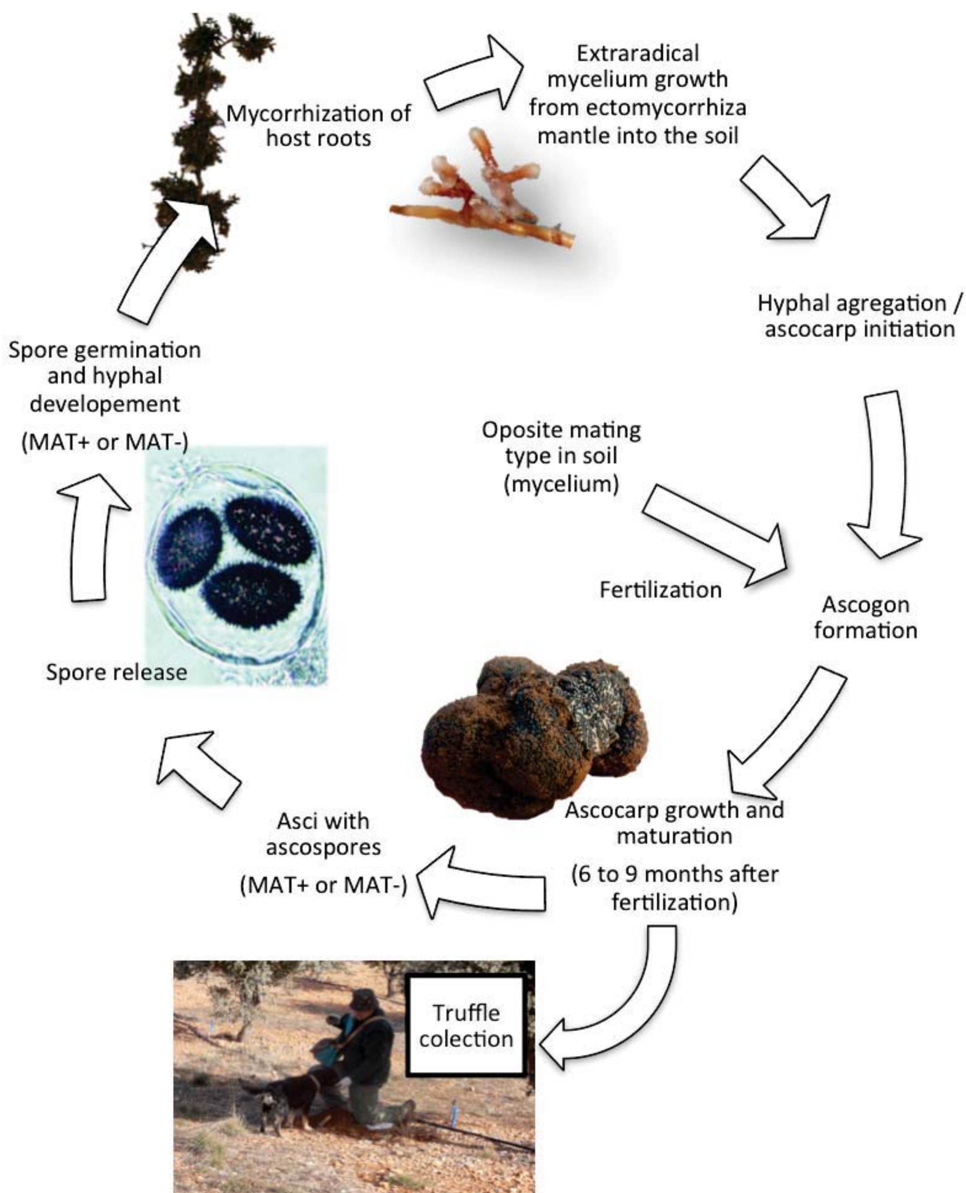


Figure I. 2. Life cycle of *Tuber melanosporum*. Sexual reproduction is controlled by a single mating type locus with two alternate forms *MAT(+*) and *MAT(−*). The vegetative tissues of truffles (gleba) are formed as a result of a fertilization process (when compatible mycelia – *MAT(+)* and *MAT(-)* – mate and develop an ascocarp) are of the same mating type of the ectomycorrhiza found at the nearby host tree roots. Consequently, the opposite mating type must be living free, as extraradical mycelium, in the soil. Both mating types are found within asci across the spores (1:1 relation). Vegetative hyphae are plurinucleate and contain only one mating type.

3. Molecular techniques used

Soil mycelium is the more cryptic structure of the fungal-plant symbiosis, but, from an ecophysiological point of view, it is also the most important because its role in the water and nutrients uptake from soil, as well as its role in the expansion of the fungal colonization and the production of reproductive structures. The PCR-based techniques allow us to identify and quantify the extraradical mycelium of the mycorrhizal species. These techniques open up a new way to study the presence and the spatial distribution of the fungal mycelium in the soil.

3.1. Conventional PCR

Polymerase chain reaction (PCR) is a biochemical technology, used in molecular biology, which allows amplifying a specific region of target DNA. This technique enables to produce millions of copies of a specific DNA sequence in approximately two hours. The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses (Carr and Moore 2012). A basic PCR set up requires several components and reagents. These components include:

- DNA template extracted from soil samples, mycelium pure cultures, ectomycorrhizas or sporocarps.

- Two primers that are complementary to the 3' ends of each of the sense and anti-sense strand of the DNA target. These primers can be more or less specific (they can amplify, for example, for fungal DNA, for basidiomycetes, or be species-specific).

- Taq DNA polymerase. A Polymerase enzyme that synthesizes DNA molecules from their nucleotide building blocks. DNA polymerase can add free nucleotides only to the 3' end of the template DNA strand. This results in elongation of the newly forming strand in a 5’-3’ direction. Taq polymerase was isolated from *Thermobius aquaticus* and it is characterized by being heat stable and that is why it is normally used in the PCR.

- Deoxynucleoside triphosphates (dNTPs, nucleotides containing triphosphate groups), the building blocks from which the DNA polymerase will synthesize the new DNA strands.

- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- Divalent cation magnesium ions and monovalent cation potassium ions.

The PCR is commonly carried out in a reaction volume of 10–200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler, which heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction.

PCR consists of a series of 20-40 repeated temperature changes (cycles), with each cycle commonly consisting of three discrete temperature steps. The temperatures used and the lengths of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers (Rychlik et al. 1990). The steps of PCR reactions comprise:

1. Denaturation step: the reaction is heated to 94–98 °C. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, resulting on single-stranded DNA molecules.

2. Annealing step: The temperature is lowered to 50–65 °C allowing annealing of the primers to the single-stranded DNA template. The polymerase binds to the primer-template hybrid and begins DNA formation.

3. Extension step: The temperature at this step depends on the DNA Taq polymerase used, but commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified.

4. Final elongation: This single step is performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

In the thesis conventional PCR reactions were done in a reaction volume of 25-50 µl, PCR reactions and were performed in a GeneAmp® 9700 thermocycler (Applied Biosystems, CA, USA) usually with an initial denaturation step at 95°C for 5 min, 35

cycles at 94°C for 45 s, annealing between 50 and 65°C (depending on the primer pairs used) for 45 s, extension at 72°C for 1 min, and a final elongation step at 72°C for 7 min. The exact thermal profile is described in each Materials and Methods section when PCR reactions were performed.

To check the amplification of the desired DNA fragment, agarose gel electrophoresis is employed for size separation of the PCR products. The size of PCR products is determined by comparison with a molecular weight marker (DNA ladder), which contains DNA fragments of known size, run on the gel alongside the PCR products.

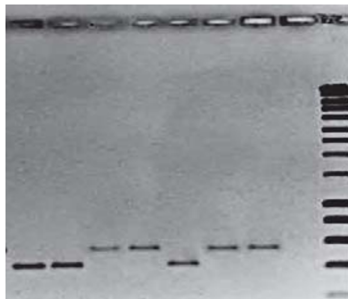


Figure I. 3. Example of agarose gel with different amplified samples and DNA marker.

3.2. Real-time PCR

The real-time PCR quantifies the number of target DNA copies formed during the PCR reaction. Their advantages are that it is accurate, reproducible and avoids post-PCR processes (Heid et al. 1996; Schena et al. 2004). The PCR product is detected as fluorescent molecules that hybridize with DNA target, generally, throughout a specific probe. The fluorescence emissions are equivalent to the number of DNA copies generated, these fluorescence data are stored in a computer, generating at the end of the process, a sigmoid DNA amplification curve for each sample.

One important difference between real time PCR and conventional PCR is that the DNA quantification is done in the exponential phase of the amplification (assuming that the efficiency of the reaction should be constant). Determining the fluorescence threshold (the fluorescence increase that is correspondent with the PCR product increase) it is possible to quantify the DNA target from the samples that we analyze, based on a standard curve constructed with known amounts of the target DNA.

To design the standard curve to which we will refer the samples to be analyzed, we should perform a serial dilution bank of a known quantity of target DNA, which will be analyzed by real time PCR. The cycle number at which PCR product starts to be detected in the exponential phase of the amplification curve, which is named the threshold cycle (C_t), is recorded for each sample, and related to the amount of starting DNA. The standard curve is created with the C_t values of each dilution bank sample (plotting the C_t values with the logarithm of the amount of DNA from each sample) (Figure I. 4). We will interpolate the Ct values of the samples to be analyzed to this standard curve, thereby obtaining the amount of target DNA present in each sample. As each standard curve is related to one DNA target and depends on the PCR reaction conditions, one standard curve for each experiment should be performed. The standard curve obtained should satisfy the minimum standards of the curve (described by the slope, the R^2 and the efficiency) for the DNA quantification by real-time PCR described by Bustin (2004; 2009).

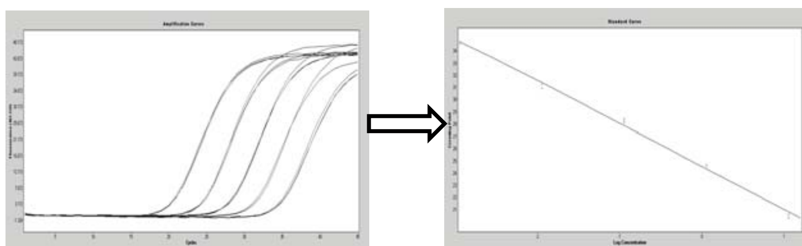


Figure I. 4. Standard curve construction with the amplification curves of a dilution bank samples with known amounts of DNA.

As said before, real time PCR involves the use of fluorescently labeled nucleic acid probes or primers, or DNA-binding fluorescent dyes such as SYBR® Green, to detect and quantify a PCR product at each cycle during the amplification. While DNA-binding dyes are easy to use, they do not differentiate between specific and nonspecific PCR products, and the accumulation of nonspecific PCR products could cause false-positive signals. Fluorescently labeled probes have the advantage that they recognize only specific PCR products (Heid et al. 1996). The main advantage of using probes over SYBR® Green in real-time PCR for fungal DNA quantification is their higher specificity (Schena et al. 2004). Moreover, DNA probes with conjugated minor groove binder (MGB®) form extremely stable duplexes with single- stranded DNA targets. Also, MGB® probes are shorter, have higher melting temperature (Tm), and are more specific than standard DNA probes, especially for single-base mismatches at elevated hybridization temperatures (Kutyavin et al.

2000a). This property is especially convenient when analyzing complex media as soils for specific quantification.

There exist different types of probes to be used in the reactions; in our assays we have mostly used specific primers in combination with specific TaqMan® hydrolysis probes, but tests with other hydrolysis probes types have been done, such as UPL (Universal Probe Library) probes or double-quenched probes (including a ZEN™ Internal Quencher, Integrated DNA Technologies Inc., San Jose, CA). Hydrolysis probes are short oligonucleotides complementary to a specific region of the DNA to be analyzed. TaqMan probes are designed to anneal within a DNA region amplified by a specific set of primers; they are characterized by being double labeled with a fluorophore at the 5´-end and a quencher at the 3´-end, the close proximity of them makes the quencher suppresses the fluorescence of the reporter dye. During the annealing step, the probe hybridizes to PCR product synthesized in previous amplification cycles. The resulting probe - target hybrid is a substrate for the 5´-3´ exonuclease activity of Taq DNA polymerase, which degrades the annealed probe (Schena et al. 2004) and liberates the fluorophore. The quencher is no longer suppressing the fluorophore and the fluorescence increases at a rate proportional to the amount of DNA template present.

UPL probes (Roche Applied Science) can be designed using the Universal ProbeLibrary (UPL) and the ProbeFinder software available on the online Assay Design Center (www.universalprobelibrary.com). The Universal ProbeLibrary includes 165 different probes, each made up of 8-9 nucleotides; this small size allows probe binding to several positions and DNA of different organism, obtaining specificity through the design of the pairs of primers accompanying the probe. They are hydrolysis probes dual-labeled with a reporter fluorophore (FAM) and a dark quencher dye. The Universal ProbeLibrary is either available as complete probe set organism specific sets for human, rat and mouse, but assays for other organisms, such as fungi, can be designed.

The method used for the Real-time PCR comprises:

1. Design or selection from literature of the specific primers and/or probe to be used.

2. Real-time PCR amplification

    a. Reactions in 20 µl containing 200nM Primers, 800nM probe, master mix (solution containing buffer, dNTPs and other

components formulated for real-time PCR, sometimes it includes passive reference dye, thermostable hot-start DNA polymerase, or others), ROX dye (optional passive reference dye) and deoionazed HPLC water

b. The thermal profile will be determined by the set of primers / probe and the reagents used.

c. The PCR system used for the amplification will be connected to a computer system in which the fluorescence emissions data, equivalent to the DNA copies generated, will be stored, resulting at the end of the process, a sigmoid DNA amplification curve for each sample.

d. Results collection and data processing to calculate quantities.

3.3. Simple sequence repeat (SSR) analysis

Simple sequence repeat (SSR) or microsatellites consist on tandemly repeated DNA sequences formed by one to six nucleotides abundant in prokaryotic and eukaryotic genomes (Karaoglu et al. 2005; Sharma et al. 2007). They are present in both coding and noncoding regions and are usually characterized by a high length polymorphism degree (Tóth et al. 2000). SSRs are very powerful genetic markers due to their high variability, and they have been used for genome mapping, forensic DNA studies, for population genetics and also for conservation and management of biological resources (Jarne and Lagoda 1996; Zane et al. 2002).

Traditionally, microsatellite loci have been isolated from partial genomic libraries (selected for small insert size) of the species of interest, screening several thousands of clones through colony hybridization with repeat- containing probes (Rassmann et al. 1991; Zane et al. 2002). Nowadays, with the sequencing technologies and bioinformatics the screening of SSR markers is easier and results are better.

The fact is that these codominant markers are highly reproducible, easy to score and offer the potential to be fungus-specific. Therefore, it is possible to use a set of SSR markers to distinguish genets and describe fungal populations (Kretzer et al. 2004; Jany et al. 2006).

For *Tuber melanosporum* there exist several works done with SSR markers, but with the sequenced genome of the black truffle (Martin et al. 2010; Murat et al. 2011) it was possible to characterize the SSR pattern in the black truffle genome and to identify new SSR polymorphic markers for population genetics (Murat et al. 2011). In Chapter 4 some of those SSR markers identified by Murat et al. (2011) were used to see if different individuals or genets differ in the number of repetition of the SSR analyzed. This is done by the amplification (by PCR) of the DNA region in which the SSR is located.

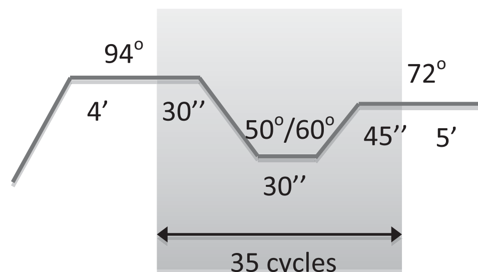
The method used for the SSR analysis comprises:

1. DNA extraction

2. PCR amplification with the primer pairs corresponding to the selected SSR markers described in Murat et al (2011). The forward primers are modified with a fluorophore that can be of three different dyes that would enable the SSR markers detection and identification in the Genetic Analyzer.

    a. PCR reactions in 10 µl reaction mixture: 1 µl reaction buffer 10x (Sigma), 200 µM dNTP, 1.5 mM $MgCl_2$, 10 pmol of each primer and 10 pmol of DNA target.

    b. Thermal profile:



3. Genotyping by separation capillary array (sequencer): the SSRs are detected by fluorescence emissions read by the sequencer.

    a. Reaction: 20 µl Sample Loading Solution (SLS, Beckman Coulter, Villepinte, France), 1 µl of specific size marker (DNA Size Standard kit – 600 (Beckman Coulter, Villepinte, France)) and 1 µl of the marked PCR product (diluted if necessary).

    b. Migration done at 6KV for 80 minutes.

4. Determination of size fragment: This part is done automatically with the software provided by the analyzer used but a manual verification of the size determined by the program, deletion of picks coming from contaminations according to their size and the detection of heterozygotes (if necessary) of each migration should be done.

5. Collection of the sizes of each PCR product for each SSR marker in a data sheet.

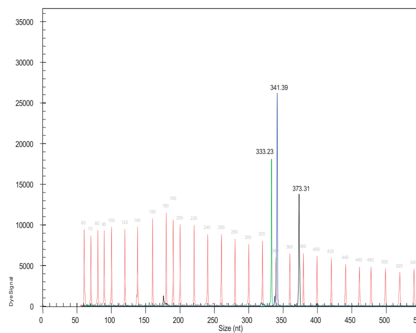


Figure I. 5. Example of the result for the SSR screening for three samples given by a Beckman CEQ 8000 Genetic Analyzer. The blue, green and black peaks correspond to the amplification for 3 different pair primers marked with the three different dyes (D2, D3 and D4. The reed ones correspond to the size marker (DNA Size Standard kit – 600bp) used as reference.

Purpose and General Objectives

The final goal of the research on the traceability of the mycorrhizal symbiosis of edible mushrooms is to monitor the factors influencing fungal persistence leading to a sustainable production of edible sporocarps. To achieve at these sustainable production of edible mushrooms we have two different ways:

- The establishment of controlled plantations.
- The management of natural productive areas.

In both situations it is necessary to have a good knowledge of the biology of the species wanted to be produced and collected (Wang and Hall 2004; Karwa et al. 2011; Savoie and Largeteau 2011; Wang et al. 2012). The generated new technologies improve the methods to detect and quantify the mycelium of the targeted fungal species in the soil. Moreover, the developed techniques do not disturb the host plants and the environment where ectomycorrhizal mushrooms grow. Molecular technologies as the real-time PCR can be used to detect one single species from a soil sample, simplifying the process of field sampling. Increasing the knowledge about ecological and life patterns of these fungi would allow to construct models and develop cultural procedures to increase mushrooms crops.

The final objective of this work is to produce the knowledge necessary to reach a sustainable production of different edible ectomycorrhizal fungal species, with a high commercial interest, through controlled mycorrhization of bushes and forest tree species and forest management. Doing that in a way that we could get an ecological benefit for the ecosystem and also for our society, as fungi have an important economical and socio – cultural value (gastronomical and medical) in some important areas of our planet.

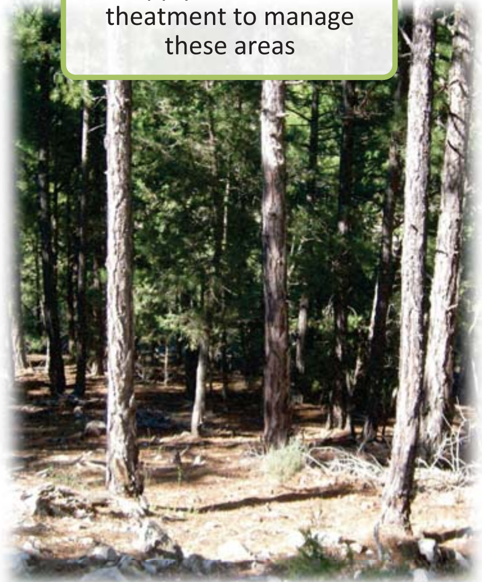
As a resume, to have a sustainable mushrooms production we need techniques to detect the fungus without disturbing the natural ongoing of the ecosystem. So, depending on which species we are looking for to increase the production or to collect we can:

1. Establishment and management of man-made plantations

- Selection and conservation of fungi in pure cultures
- Synthesis of mycorrhiza in controlled conditions
- Production of mycorrhizal seedlings
- Plantation Establishment
- Use molecular tecniques for the fungus traceability
- Apply silvicultural theatment to manage these areas

2. Management of natural productive areas

- Identify natural productive areas
- Use molecular tecniques for their traceability
- Apply silvicultural theatment to manage these areas



The specific **objectives** of the thesis were:

1. Synthesis of mycorrhizas and production of mycorrhizal seedlings with fungal isolates of the *Boletus edulis* complex. Development of methodologies that allow the production of nursery plants mycorrhizal with this fungal species and the establishment of plantations for the controlled production of *Boletus* spp. mushrooms, comparable to those established with *Tuber melanosporum* or *Lactarius deliciosus.*

2. Development of molecular techniques for the study of the ectomycorrhizal fungi designing specific primers and probes to detect and quantify the soil mycelium.

3. Implementation of the molecular techniques (real-time PCR, conventional PCR, genotyping using microsatellites markers and mating type genes primers) in monitored plantations and in natural productive areas in order to trace the studied fungal species: *Boletus edulis*, *Lactarius deliciosus*, *Tuber melanosporum* and *Rhizopogon roseolus*.

4. Identify potential correlations between the dynamics of soil mycelium quantity, abundance of mycorrhizas and mushrooms production.

1

Production of *Boletus edulis* inoculated plants in pure cultures and in nursery conditions

Chapter 1

Production of *Boletus edulis* inoculated plants in pure cultures and in nursery conditions

1. Introduction

The *Boletus edulis* complex is formed by: *B. edulis* Bull. *sensu stricto, B. aereus* Bull., *B. pinophilus* Pilát & Dermek, and *B. reticulatus* Schaeff., and comprise some of the most appreciated ectomycorrhizal edible mushrooms with an annual world market around one billion Euros (Mello 2012). In Spain the amount of boletes harvested annually varies from 2,000 to 20,000 Tm depending on the year (Oria-de-Rueda et al. 2008). As it was described in the general introduction, *Boletus* species are only collected from the wild (Cannon and Kirk 2007), most of them from forest dominated of *Pinus*, *Castanea* or *Quercus* species.

Many attempts to inoculate these ectomycorrhizal fungi on various hosts have been done to date (Table 1.1). In the first attempts on producing plants mycorrhizal with *Boletus* spp., those were obtained by pure culture methods and no outplanting experiments were made. From approximately the 90s, seedlings inoculated with *Boletus* species have been outplanted in attempts to promote fruiting of the valuable edible sporocarps (Olivier et al. 1997; Meotto et al. 1999; Chung et al. 2007). Unfortunately, any fruiting body production has been reported from these studies and, in most cases, when inoculated seedlings were outplanted *B. edulis* mycorrhizas were replaced by other fungi. Moreover, none of those studies provided any molecular identification of the inoculum used (mycelium or spores suspension) or the mycorrhiza obtained, since molecular techniques were not implemented and then it was not common to use them. Only in a work done by Águeda et al. (2008), where *B. aereus*, *B. edulis* and *B. reticulatus* ectomycorrhizas were successfully synthesized, molecular identification was provided. More recently Wu et al. (2012) co-inoculated *B. edulis* with the mycorrhiza helper bacterium *Bacillus cereus* in *Pinus thunbergii* seedlings under nursery conditions in China, obtaining positive results, and opening new perspectives on the cultivation of this fungus.

Table 1.1. (*Next page*) Published works in which *Boletus* mycorrhizal plants were produced.

Author(s)	Year	Fungal species	Host tree	Culture method	Inoculum	Reference
Froidevaux & Amiet	1975	*B. edulis*	*Pinus mugo*	pure cultured plants	mycelium	*Frodievaux and Aimet (1975)*
Tozzi et al.	1980	*B. edulis*	*Quercus pubescens*	pure cultured plants	mycelium	*Tozzi et al. (1980)*
Molina and Trappe	1982	*B. edulis*	*Arbutus sp.*	pure cultured plants	mycelium	*Molina and Trappe (1982a-1982b)*
		B. edulis	*Arctostaphylos sp.*		mycelium	
		B. edulis	*Larix sp.*		mycelium	
		B. edulis	*Picea sp.*		mycelium	
		B. edulis	*Pinus sp.*		mycelium	
		B. edulis	*Tsuga sp.*		mycelium	
Ceruti et al.	1983-1984	*B. aereus*	*Q. pubescens*	pure cultured plants	mycelium	*Ceruti et al. (1983-1984)*
	1985	*B. aereus*	*Castanea sativa*		mycelium	
	1987-1988	*B. edulis*	*P. sylvestris*		mycelium	*Ceruti et al. (1987-1988)*
	1987-1988	*B. edulis*	*Picea excelsia*		mycelium	
Poitou et al.	1982	*B. edulis*	*P. radiata*	pure cultured plants	mycelium	*Poitou et al. (1982)*
		B. aereus	*P. radiata*		mycelium	
	1984	*B. edulis*	*P. uncinata*		mycelium	*Poitou et al. (1984)*
		B. aereus	*P. uncinata*		mycelium	
Zuccherelli	1988	*B. edulis*	*Q. cerris, Q. rubra and C. Sativa*	nursery plants + plantation	mycelium	*Zuccherelli (1988)*
Bawadikji	1993	*B. edulis*	*P. sylvestris*	pure cultured plants	mycelium	*Bawadikji (1993)*
Duñabeitia et al.	1996	*B. pinophilus*	*P. radiata*	nursery plants	spores	*Duñabeitia et al. (1996)*
Olivier et al.	1997	*B. edulis*	*P. uncinata and C. sativa*	nursery plants + plantation	mycelium	*Olivier et al. (1997)*
		B. reticulatus	*P. uncinata and C. sativa*		mycelium	
Meotto et al.	1999	*B. edulis*	*C. sativa*	nursery plants + plantation	mycelium	*Meotto et al. (1999)*
Chung et al.	2007	*B. edulis*	*P. radiata*	nursery plants + plantation	spores (comercial)	*Chung et al. (2007)*
		B. pinophilus	*P. radiata*		spores (comercial)	
Águeda et al.	2008	*B. edulis*	*Cistus albidus and C. ladanifer*	pure cultured plants	mycelium	*Águeda et al (2008)*
		B. aereus	*Cistus albidus and C. ladanifer*		mycelium	
		B. reticulatus	*Cistus albidus and C. ladanifer*		mycelium	
Fu et al.	2009	*B. edulis*	*P. Massoniana*	pure cultured plants	mycelium	*Fu et al (2009)*
Wu et al.	2012	*B. edulis*	*P. Thunbergii*	nursery plants	mycelium + helper bacteria	*Wu et al. (2012)*

In the wild, *B. edulis* is found frequently in association with a large number of broadleaved trees and shrubs of the families Fagaceae (*Castanea, Castanopsis, Fagus, Lithocarpus, Quercus*), Betulaceae (*Carpinus, Corylus, Betula, Ostrya, Populus*), Malvaceae (*Tilia*), Salicaceae (*Salix*), Ericaceae (*Arctostaphylos*) and Pinaceae (*Abies, Keteleeria, Picea, Pinus, Tsuga*) (Olivier et al. 1997; Águeda et al. 2008).It has been also detailed the association between Boletales and some Cistaceae (Lavorato 1991; Vila and Llimona 2002; Águeda et al. 2006).The association between boletes and *Cistus* spp. have been regularly observed in certain regions of central and northwest Spain (in Castilla y León) and also in the northeast (in Catalunya). Some of these locations are dominated exclusively by *Cistus ladanifer* and *C. monpeliensis or C. albidus* respectively, being in many of these areas an underappreciated and underexploited resource (Oria-de-Rueda et al. 2008). Águeda et al. (2008) described the mycorrhiza obtained after the combination of different strains of *Boletus* spp. with Cistaceae (*C. albidus*, *C. ladanifer* and *C. laurifolius*), in our experiments we have followed the same guidelines used in that work by increasing both, the host species and the strains of the fungus, according to the different host combinations found in the wild, with special emphasis on Cistaceae.

We also have tried to establish different methods to obtain *Boletus* mycorrhizal plants in nursery conditions. With other ectomycorrhizal species, to produce nursery mycorrhizal plants, inoculations are made with spores (*T. melanosporum* or *R. roseolus* from sporocarps mixed with water) (Chevalier and Frochot 1997; Parladé et al. 1999) or with mycelium suspensions (*L. deliciosus*)(Guerin-Laguette et al. 2000; Parladé et al. 2004). These techniques were used also with *B. edulis* to determine if these were suitable methods to produce mycorrhizal plants in nursery conditions.

The selection and preservation of fungal isolates is a crucial stage for the controlled inoculation of plants. In the ectomycorrhizal laboratory of IRTA (Centre de Cabrils), the pure culture isolates are maintained in agarified biotin-aneurin-folic acid (BAF) medium (Oort 1981) in Petri dishes or in slants in screw-cap Pyrex glass tubes (13x100 mm), at 4°C with transfers every 2-3 months onto fresh medium. Cultures are incubated at 25°C until regrowth. In order to reduce these so frequent periodic transfers, new storage and maintenance techniques need to be evaluated. Due to the diversity within the different fungi, to ensure their viability and the physiological, morphological and genetic characteristics of the cultures over time, different methods of cultivation and preservation are required. The

choice of a maintenance method depends on the species of concern, the aim of its preservation and the facilities available. These storage methods can be divided in short-term and long-term preservation. The first ones are the most used methods for ectomycorrhizal culture collections, which involves the maintenance by serial transfers: fungal mycelium from an active growing culture is transferred to Petri dishes or test screw cap tubes (wrapped with Parafilm to avoid drying) filled with fresh agar medium (BAF, MMN or other mediums). Cultures are grown at 25°C and once the culture is established, kept at 4°C or room temperature. This method have several disadvantages: cultures must be examined periodically to check for any contamination or desiccation. Moreover, morphology, physiology and genetics of a cultured fungus may change over time and over several transfers (Hung and Molina 1986) even the ability to infect a host can be lost after repeated transfers(Marx and Daniel 1976; Marx 1980; Nakasone et al. 2004).

The long-term preservation methods include the sclerotization (preserving sclerotia or other long-term survival propagules or structures at 3 – 5°C) useful for various myxomycetes; drying (used for cultures that produce resting structures as spores) using silica gel, glass beads and/or soil as substrate; freezing methods with or without cryoprotectants, liquid nitrogen; lyophilization(for many spore-forming fungi producing large number of spores 10 µm or less in diameter); oil overlay (for mycelial or nonsporulating cultures that are not amenable to freezing or lyophilization and with no slow growth rates) and immersion in sterile distilled water (Nakasone et al. 2004).

The preservation in sterile water has been used successfully for the storage of oomycetes (Clark and Dick 1974), basidiomycetes (Richter and Bruhn 1989; Croan et al. 1999), mycorrhizal fungi (Richter 2008; Parladé et al. 2011), ascomycetes (Johnson and Martin 1992), hyphomycetes (Ellis 1979), plant pathogenic fungi (Burdsall and Dorworth 1994), aerobic actinomycetes, human pathogens and yeast(McGinnis et al. 1974; Qiangqiang et al. 1998; Borman et al. 2006),doing periodic verifications of their viability and replacing the collection with new colonies. The advantages are that less transfers to new medium need to be done (the periods of regrowthing are longer), sterile distilled water is cheaper than any other culture medium, and the time for preparing it is also reduced.

Previous works to evaluate sterile water as a storage medium for ectomycorrhizal fungi have been reported. Heninonen-Tanski (1990) stored 24 strains of different ectomycorrhizal fungi associated with pines in different media and concluded that sterile water, as a preserving medium for ectomycorrhizal fungi from agar culture

plugs, was the best method tested, recommending their storage at 4°C, but room temperature was also satisfactory. From the fungal strains tested, 23 of the 24 stored in water for 3 months at 4°C gave clear growth when transferred to fresh medium. Richter and Bruhn (1989; 2008) tested the viability of several saprotrophic and mycorrhizal basidiomycete cultures after 2 – 4 years and 20 years of cold storage in sterile water and saw that this storage method was good for maintaining some of the mycorrhizal genus, as *Laccaria*, but mycorrhizal fungus species demonstrated lower viability than saprotrophic fungi. Also Parladé et al. (2011) tested the tolerance to cold water storage of *Lactarius deliciosus* isolates. They saw that the survival, after 28 months, was isolate dependent. Whereas growth when transferred to BAF agar medium was obtained from most of the isolates after 12 months of cold storage in sterile water, only 10 out of 29 isolates showed unaffected growth after 28 months of storage.

To test the survival of species of the *Boletus edulis* complex to cold storage in sterile distilled water, 38 isolates were selected. Also 7 isolates of *Rhizopogon roseolus* were included in the assays as positive controls, because of their facility to regrowth after the transfer to new culture medium.

The **objectives** of this experimental part were:

1. The *in vitro* synthesis of mycorrhizas of *B. edulis* species complex with different host plants.

2. To establish methods for the production of plants mycorrhizal with species of the *B. edulis* complex in nursery conditions, as a first step towards the controlled production of boletes.

3. To establish conservation methods that lead to maintain the viability of the strains isolated in pure culture, reducing the time in which these isolates need to be transferred onto new fresh medium.

2. Materials and Methods

2.1. Synthesis of mycorrhiza in pure culture conditions

2.1.1. Preparation of host plant seedlings under axenic conditions

Different host plants: *Pinus pinaster* Ait., *Cistus albidus* L., *C. ladanifer* L., *C. laurifolius* L., *C. monspeliensis* L., *Castanea sativa* Mill., *Quercus ilex* L. and *Betula pendula* Roth*,* were selected to be inoculated with the species of the *B. edulis* complex.

Pinus pinaster was chosen as a model plant over other pine species because of its good growth and root formation under pure culture synthesis conditions. *P. pinaster* seedlings were obtained from seeds (lot A11300299 Llevant Region, collection date 1999 from the Seed Bank of the Generalitat Valenciana, Spain). Seeds were firstly washed in running tap water, and disinfected by immersing them into a 30% H_2O_2 solution for 30min. After that, a second washing with sterile distilled water was done, in order to remove any remaining H_2O_2solution. Once the seeds were disinfected, they were placed on Petri dishes with agar BAF medium (10 seeds per dish). Seeded plates were kept at 10°C for twenty days followed by incubation at 25°C until germination.

Cistus albidus (origin Mediterranean littoral, collection date 2000; and origin Sistema Central, Spain, collection date 2010), *C. ladanifer* (origin Sistema Central, Spain; collection date 2006), *C. laurifolius* (origin Sistema Central, Spain; collection date 2006) and *C. monspeliensis* (origin Sistema Central, Spain; collection date 2006), were the rockrose species selected for the inoculations with *Boletus* spp. In all the cases *Cistus* spp. seedlings were obtained from commercial seeds (provided by Corporación Semillas Zulueta, Navarra, Spain). First a pregerminative treatment was performed consisting in bringing seeds (water immersed) to 90 °C. After that, the disinfection process began by immersing the seeds into a 30% sodium hypochlorite solution for 10minutes, followed by a rinse with sterile distilled water. Fifty disinfected seeds per plate were placed in BAF agar medium Petri dishes, stratified at 4°C for twenty days, and incubated at 25°C until germination. Seeds showing bacterial or fungal contaminations were discarded.

Some tests were done with some broad-leaved tree species, which could be found associated with *B. edulis* in nature. The selected hosts trees were *Quercus ilex*, a common species in the Mediterranean forests; *Betula pendula* and chestnut (*Castanea sativa*) as Boletes are commonly recollected in forests were these tree

species are dominant. *Quercus ilex* seedlings were obtained from seeds collected in Cabrils, Barcelona, Spain. The outer cover of the seed was removed, leaving the inner cover protecting the cotyledons. Surface disinfection was performed by immersion in a 70% ethanol solution for 2 minutes, followed by immersion in a 30% sodium hypochlorite solution (commercial bleach) with 2 – 3 drops of Tween 80 for 25 minutes, and three consecutive rinses (5 minutes each one)in sterile distilled water. After disinfection the embryo axes were dissected with a scalpel removing the cotyledons (but leaving 4 – 5 mm of cotyledons linked to the cotyledonary petioles). These axes were then cultivated in culture tubes (20 x 160 mm) with 30 ml MS (Murashigue and Skoog 1962)culture medium (2.63 g/l Murashige and Skoog – nitrate concentration half reduced –, 0.1 mg/l BAP, 30 g/l Sucrose, DifcoAgar (0,7%), 1 ml/l streptomycin and Ph of 5.6 – 5.7) at 25°C in darkness for 12-14 days, followed by photoperiodic light (16 h light / 8 h dark) in controlled growth chamber until germination.

The *C. sativa* seedlings were obtained from commercial seeds (origin Galicia, Spain). Seeds were surface disinfected using the same method previously described for *Q. ilex* but using a 30% hydrogen peroxide solution, for 10 minutes, instead of the sodium hypochlorite solution (as tests done with both disinfection methods showed better results).

Betula pendula plantlets were obtained from *in vitro* somatic embryogenesis of seedlings resulting from in vitro germination, growth in plastic pots with MS medium (Murashigue and Skoog 1962) and maintained in controlled growth chamber with photoperiodic light (16 h light) at 25°C until having the desired growth.

2.1.2. Isolation and maintenance of *B. edulis* pure cultures

The cultures of the *B. edulis* species complex (*B. edulis sensu stricto, B. aereus*, *B. pinophilus*, and *B. reticulatus*) were obtained from sporocarps collected in different forest stands of northern Spain (table 1.2). Tissue explants obtained from sporocarps were plated on Petri dishes filled with modified Melin–Norkrans agar medium (MMN) (Marx 1969) or biotin-aneurin-folic acid agar medium (BAF) (Oort 1981). Plates were incubated at 25°C. Once the isolates were actively growing, and free of contaminants, they were kept in BAF agar medium at 4°C and maintained by transferring onto fresh medium every 3 months. The taxonomical identification of the fungal isolates was confirmed by sequencing the internal transcribed spacer (ITS) of the nuclear rDNA region(Leonardi et al. 2005), and comparing to the sequences available in the GenBank data base.

Fungal species	Strain	Collection Date	Collection Place	Host Plant	GenBank Acc. Num
Boletus aereus	Ba 315	**2000**	La Bisbal, Catalunya	*Quercus suber*	
	Ba 316	**1999**	La Bisbal, Catalunya	*Quercus suber*	KC261822
	Ba 317	**1999**	La Bisbal, Catalunya	*Quercus suber*	KC261821
	Ba 370	**2006**	Breda, Catalunya	*Castanea sativa*	EU554663
	Ba 393	**2006**	Osor, Catalunya	*Quercus suber*	KC261824
	Br 1050	**2006**	Ocenilla, Castilla y León	*Q. pyrenaica*	KC261838
	Ba 1051	**2006**	Ocenilla, Castilla y León	*Q. pyrenaica*	KC261823
	Ba 1058	**2006**	Ocenilla, Castilla y León	*Q. pyrenaica*	KC261825
Boletus reticulatus	Br 386	**2006**	Osor, Catalunya	*Castanea sativa*	KC261834
	Br 388	**2006**	Osor, Catalunya	*Castanea sativa*	KC261836
	Br 1053	**2006**	Ocenilla, Castilla y León	*Q. pyrenaica*	KC261835
	Br 1054	**2006**	Ocenilla, Castilla y León	*Q. pyrenaica*	EU554661
	Br 1055	**2006**	Ocenilla, Castilla y León	*Q. pyrenaica*	KC261839
	Br 1056	**2006**	Ocenilla, Castilla y León	*Q. pyrenaica*	KC261837
Boletus edulis	Be 369	**2006**	Zamora, Castilla y León	*Cistus ladanifer*	KC261819
	Be 375	**2006**	Arànser, Catalunya	*Pinus uncinata*	EU554664
	Be 391	**2006**	Zamora, Castilla y León	*Cistus ladanifer*	KC261820
	Be 392	**2006**	Garmo, Euskadi	*Pinus radiata*	KC750223
	Be 409	**2009**	Zamora, Castilla y León	*Cistus ladanifer*	KC750224
	Be 410	**2009**	Zamora, Castilla y León	*Cistus ladanifer*	KC750225
	Be 411	**2009**	Zamora, Castilla y León	*Cistus ladanifer*	KC750231
	Be 412	**2009**	Zamora, Castilla y León	*Cistus ladanifer*	KC750226
	Be 1098	**2007**	Molinos Razón, Castilla y León	*Pinus sylvestris*	
	Be 2017	**2008**	P. Grande, Castilla y León	*Pinus sylvestris*	HM579924
	Be 2022	**2008**	P. Grande, Castilla y León	*Pinus sylvestris*	HM579929
	Be 2037	**2008**	Arenillas, Castilla y León	*Cistus ladanifer*	HM579925
	Be 2044	**2008**	P. Grande, Castilla y León	*Pinus sylvestris*	HM579930
	Be 2057	**2008**	Zamora, Castilla y León	*Cistus ladanifer*	HM579926
	Be 2059	**2008**	Zamora, Castilla y León	*Cistus ladanifer*	KC750227
	Be 2063	**2008**	Carmona, Castilla y León	*Cistus ladanifer*	HM579928
	Be 2065	**2008**	Carmoba, Castilla y León	*Cistus ladanifer*	HM579927
	Be 2070	**2008**	León, Castilla y León	*Cistus ladanifer*	HM579923
	Be 2074	**2009**	Molinos Duero, Castilla y León	*Q. pirenaica*	KC750229
Boletus pinophilus	Bp 1042	**2006**	P. Grande, Castilla y León	*Pinus sylvestris*	KC261828
	Bp 1045	**2006**	P. Grande, Castilla y León	*Pinus sylvestris*	KC261830
	Bp 1099	**2007**	Molinos Razón, Castilla y León	*Pinus sylvestris*	KC261831
	Bp 2000	**2007**	Molinos Razón, Castilla y León	*Pinus sylvestris*	KC261832
	Bp 2007	**2008**	La Póveda, Castilla y León	*Pinus sylvestris*	KC750232
	Bp 2008	**2008**	La Póveda, Castilla y León	*Pinus sylvestris*	KC750233
	Bp 2010	**2008**	La Póveda, Castilla y León	*Pinus sylvestris*	KC261833

Table 1.2. *Boletus edulis* complex isolates used in the ectomycorrhizal synthesis and obtained from sporocarps collected in different forest stands of northern Spain.

2.1.3. Synthesis of mycorrhizas in pure culture conditions

Pure culture synthesis tubes (40 mm in diameter and 300mm in height, with glass stopper) (Molina 1979) were filed with 110 cm^3 sieved grade 3 vermiculite (Asfaltex, Barcelona, Spain), 10 cm^3sieved Floratorf torbe (Floragard, Oldenburg, Germany) and 60 ml liquid BAF medium (modified with 15 g glucose/l). The filed tubes were autoclaved for 40 minutes at 120°C.

Seeds germinated under axenic conditions, with a formed radicule (1 – 3 cm long) were individually transferred into the synthesis tubes in sterile conditions. Tubes were then inoculated with 10 ml of a mycelium culture of *B. aereus*, *B. edulis, B. reticulatus* or *B. pinophilus* grown in BAF liquid medium. Synthesis tubes seeded and inoculated were distributed in a completely randomized design in a growth chamber and grown for 4 – 5 months under controlled conditions (25±3°C,and a 135 µmol/s/m^2 16 h/day photoperiod provided by fluorescent lights)keeping the radical part in darkness. At the end of the growing period, after 4 – 5 months, seedlings were removed from the synthesis tubes and root systems washed and examined for ectomycorrhizal formation under a dissection microscope to evaluate the presence of ectomycorrhizas. Ectomycorrhizas were visually identified and described using a stereomicroscope.

2.2. Synthesis of mycorrhizas in nursery conditions

2.2.1. Acclimatization of *in vitro* mycorrhizal plants

2.2.1.1. Acclimatization of plants inoculated under pure culture synthesis conditions

Cistus albidus and *C. ladanifer* seedlings were inoculated with *B. edulis* (Be369 strain) mycelium following the technique formerly described for the synthesis of mycorrhizas in pure culture conditions. The inoculations were started between the end of April and the beginning of May 2009. Synthesis tubes were checked periodically under a stereomicroscope. Once the first mycorrhizas were established in the root system, plants were transferred to 250 cm^3 Forest-Pot containers in plant-growth substrate (peat-vermiculite 1:1; v:v) and put in acclimatization boxes (figure1.1) to adequate the plants to greenhouse conditions, in which seedlings were acclimatized to ambient light and temperature for 2 weeks by a progressive opening of the acclimatization box. Once the plants were adapted to the greenhouse conditions, they were taken out of the box and maintained with regular irrigation and fertilized every 2 weeks with 10 ml per seedling of a 20-7-19

(N-P-K) fertilizer (1.8 g/l) solution supplemented with micronutrients preparation of Fetrilon (0.12 g/l) and Hortrilon (0.28 g/l). After 6 months of growth in nursery conditions, the radical system of each plant was examined under the stereomicroscope, and the ectomycorrhizal roots were counted.

Figure 1.1. Detail of the acclimatization process of the *in vitro* mycorrhizal plants to nursery conditions in peat-vermiculite (1:1; v:v) containers.

2.2.1.2. Acclimatization of plants inoculated under pure culture synthesis conditions in two different substrates

As no positive results were obtained with the acclimatization of *Cistus* mycorrhizal plants, new experiments were done increasing the number and combinations of plant and fungal species, and modifying the methodology used for the acclimatization of mycorrhizal plants.

In order to test if substrate type influences in the maintenance of mycorrhizas of the *B. edulis* complex, some of the mycorrhizal plants produced in pure culture synthesis during January and February 2010 were selected to test the acclimatization of mycorrhizal *in vitro* plants. Once the establishment of the first mycorrhizas in the root system was confirmed, the seedlings were transferred, in May and June 2010, to 1 l. plastic pots to proceed with the acclimatization to nursery conditions. Two substrates were used: (1) half of the plants were transferred to containers with the growth substrate normally used in nursery conditions, peat-vermiculite, 1:1 (v:v), and (2) the other half to the substrate used for the production of mycorrhizal plants in axenic conditions, peat-vermiculite, 1:11 (v:v). The objective was to determine whether mycorrhizas formed during the synthesis in pure culture were maintained in nursery conditions and if this fact is influenced by the substrate.

The transferred plants where acclimatized as it is shown in figure 1.2, by using a plastic bag (acrylic polypropylene) for covering each plant during the first two weeks. The seedlings were gradually adapted to nursery conditions by progressively introducing perforations in the polythene bags and subsequently

removing the polythene bags after 15 days. After that, plants were maintained in nursery conditions for six months, fertilized every 2 weeks with 10 ml per seedling of a solution of a 20-7-19 (N-P-K) fertilizer (1.8 g/l) supplemented with a micronutrients preparation Fetrilon (0.12 g/l) and Hortrilon (0.28 g/l). After this period of growth, radical systems were examined under the stereomicroscope. The plant fungus combinations tested are shown in table 1.3.

The ectomycorrhizas formed in the examined plants were collected and stored at -20°C until their use for molecular analysis. DNA was extracted from mycorrhizal root tips using DNeasy Plant Mini kit (Qiagen Ltd, Crawley, UK), and amplified by PCR using *B. edulis* specific primers (Mello et al. 2006).

Figure 1.2. Plants acclimated to nursery conditions after their mycorrhization in pure culture conditions using two diferents substrates (peat-vermiculite 1:1; and 1:10; v:v).

Standard substrate		Synthesis substrate	
Plant species	Fungal strain	Plant species	Fungal strain
B. pendula	Ba 317	*B. pendula*	Ba 317
B. pendula	Be 2022	*B. pendula*	Be 2022
P. pinaster	Be 410	*P. pinaster*	Be 410
P. pinaster	Be 412	*P. pinaster*	Be 412
P. pinaster	Be 2017	*P. pinaster*	Be 2017
P. pinaster	Be 2037	*P. pinaster*	Be 2037
P. pinaster	Be 2057	*P. pinaster*	Be 2057
P. pinaster	Be 2059	*P. pinaster*	Be 2059
P. pinaster	Be 2063	*P. pinaster*	Be 2063
C. monspeliensis	Ba 393	*C. monspeliensis*	Ba 393
C. monspeliensis	Be 2044	*C. monspeliensis*	Be 2044
C. monspeliensis	Ba 1038*		
C. monspeliensis	Be 2070*		
Q. ilex	Ba 1050*		

Table 1.3. List of mycorrhizal plants transferred to nursery conditions to test if substrate type influences in the maintenance of mycorrhizas of the *B. edulis* complex. *This combinations could not be tested in Synthesis substrate as the seedlings grown on that substrate died.

2.2.2. Controlled inoculations of *Cistus* spp. plants with species of the *B. edulis* complex for the production of mycorrhizal plants in nursery conditions.

2.2.2.1. Inoculations with spore suspensions

At the end of April 2009 *C. albidus*, *C. ladanife*r and *P. pinaster* seedlings (250 per species) were grown on 250 cm^3 containers with disinfected (120°C, 60 min) plant-growth substrate composed by peat (Floratorf)-vermiculite(1:1;v:v). Plants were inoculated with spore suspensions of *Boletus* spp. from three different sporocarps with different origins: *Pinus pinaster* seedlings were inoculated with spores obtained from a sporocarp of *B. edulis* collected in Pinar Grande (Soria, Spain) under *P. pinaster*; *C. albidus* and *C. ladanifer* were inoculated with spores of *B. edulis* from a sporocarp collected in Alto Abejera (Zamora, Spain) and spores of *B. reticulatus* from Zamora (Spain), both collected in rockrose formations.

Spore suspensions were prepared by homogenizing sporocarps in water using a laboratory blender. Suspensions obtained were filtered through a sieve (0.1 mm diameter). For each fungus, initial spore concentration was measured with an hematocytometer. Each seedling was inoculated with 10 ml of the corresponding spore suspension receiving a concentration of: 1.86 x 10^8*B. edulis* spores per *P. pinaster* plant, 1.095 x 10^8 *B. edulis* spores per *Cistus* spp. plant, and 1.63 x 10^8 *B. reticulatus* spores per *Cistus* spp. plant. At the same time control non-inoculated plants were prepared.

Figure 1.3. Seedlings of *Pinus pinaster*, *Cistus ladanifer* and *Cistus albidus* at the moment of inoculation with spore suspension (left) and some months after (right).

Seedlings were grown in a greenhouse under regular irrigation and fertilized every 2 weeks with 10 ml per seedling of a 20-7-19 (N-P-K) fertilizer (1.8 g/l) solution supplemented with micronutrients preparation: Fetrilon (0.12 g/l) and Hortrilon

(0.28 g/l). After 7 months of growth plants were measured (stem diameter and height of the plant) and their roots were examined under a dissection microscope to determine their mycorrhizal status. The mycorrhizal roots observed were counted, identified and annotated. Some mycorrhizal tips, representative of each morphological type observed, were stored at -20°C to be used for molecular analysis.

2.2.2.2. Inoculations with mycelial inoculum

Mycelium of different strains (Be 375 of *B. edulis*, Ba 393 of *B. aereus*, Br 1053 of *B. reticulatus* and the strain S238N of *Laccaria bicolor* as positive control) were grown in solid enriched substrate (vermiculite 1100 ml, peat 100 ml and modified BAF medium – ½ of glucose concentration), for two months at 25°C in 2000 ml Pyrex glass bottles. Fungal strains used for mycelial inoculum production were selected according to the results obtained in pure synthesis experiments. Two types of mycelial inoculum were produced for each strain. The first type was the conventional method, consisting of mycelium grown in enriched substrate (1100 ml vermiculite, 100 ml peat and modified BAF medium). The second inoculum type consisted of mycelium grown in the same enriched substrate in presence of host plants (a combination of *P. pinaster* and *C. albidus*) (Figure 1.4 A). Including host plants while mycelium grows could provide a higher mycelial density in the inoculum. These seedlings were germinated in pure culture conditions, as explained in the previous section, and transferred to glass bottles (in axenic conditions) while incubating the inoculum. The fungal inoculums were mixed with the plant-growth substrate (peat-vermiculite; 1:1; v:v), before seeding, in a proportion of 1:10 (v:v; inoculum :substrate). Fifty plants were prepared for each of the combinations (plant, fungal strain and type of inoculum – produced in presence or not of host plant). Inoculations were performed at the beginning of May 2009. Seedlings were grown in a greenhouse and fertilized every 2 weeks with 10 ml per seedling of a solution of a 20-7-19 (N-P-K) fertilizer (1.8 g/l) supplemented with the micronutrients preparation: Fetrilon (0.12 g/l) and Hortrilon (0.28 g/l).

After 7 months of growth plants were measured (trunk diameter and height of the plant) and their root systems examined under a dissection microscope. The mycorrhizal roots observed were counted, identified and annotated. Some representative mycorrhizal tips were stored at -20°C to be used for molecular analysis and identification.

Figure 1.4. A) Mycelial inoculums produced in presence (left) or not (right) of host plant used to inoculate Cistus seedlings. B) *Cistus albidus* seedling inoculated with Be 375 mycelium inoculum. C) *C. ladanifer* and *C. albidus* inoculated with mycelial inoculums.

DNA of the stored mycorrhizas was extracted using the DNAeasy Plant Mini Kit (Qiagen, Hilden, Germany) for their molecular analysis. PCR amplification of the internal transcribed spacer (ITS) region was performed with the specific *Boletus edulis* complex primers (Mello et al. 2006)and also with the universal primers ITS1 and ITS4 (White et al. 1990). The DNA amplified with universal primers was purified with the High Pure PCR Product Purification (Roche) and sent for sequencing to Serveis Cientificotècnis de la Universitat de Barcelona.

2.3. Viability of *B. edulis* complex cultures stored in water at 4°C

Thirty mycelium disks (4 mm diameter) were taken with a cork borer from 30 day – old cultures (grown on BAF agar Petri dishes at 25°C)of the selected isolates (9 isolates of *Rhizopogon roseolus*, as control cultures, and 38 isolates of different species of the *B. edulis* complex). *R. roseolus* isolates were coming from individuals collected under pine stands and isolated on BAF medium following the same protocol described previously for *B. edulis* isolates. Mycelium disks were introduced in screw-cap 52 × 23 mm glass tubes containing 10 ml of sterile distilled water (Figure 1.5). Two tubes per strain, with 15 mycelium disks each one, were prepared and the vials were maintained in darkness at 4°C. At the same time, 5 of the disks of each strain were placed into Petri dishes with BAF agar medium in order to obtain data of the initial state of growth capability for the isolates at the initial time (July 15th, 2010). In all strains a normal growth of the 5 disks was observed except for the strains Be412 (growth of one single disk), Bp2007 (growth of two disks) and Bp2010 (growth of three discs).

Fungal viability was determined after 3, 6, 12 and 24 months by culturing five disks from each isolate onto Petri dishes with BAF agar medium. The growth of the isolates was monitored over 20 – 30 days (because some of the strains have a slow growing rate) and the number of discs grown per strain was annotated for each storage period.

Figure 1.5. Glass tubes containing distilled water with mycelium disks stored in darkness at 4°C; Petri dishes of six different strains growing on BAF medium after 24 months of cold storage (15 days old colonies).

3. Results

3.1. Synthesis of mycorrhizas in pure culture

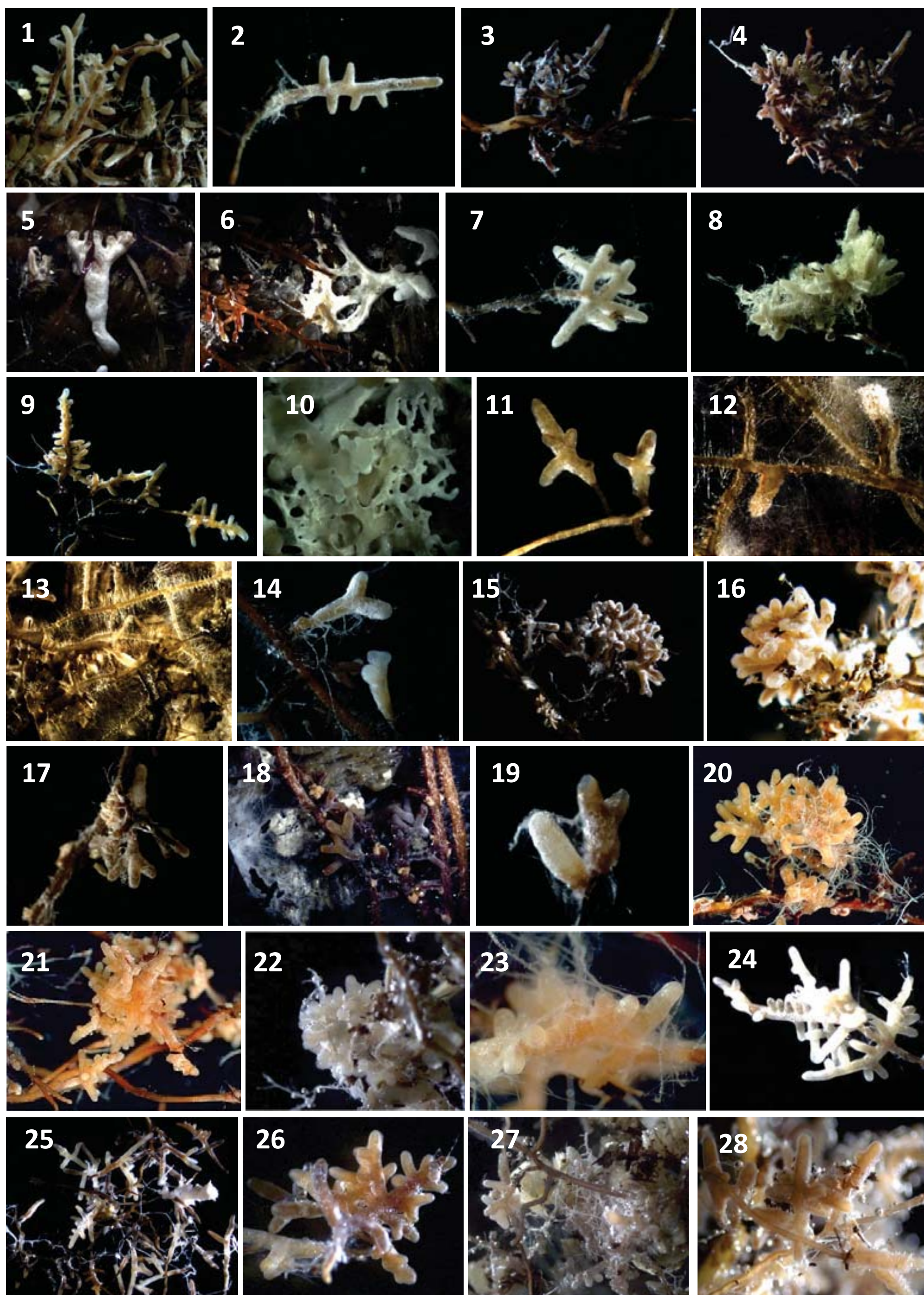
The results of pure culture synthesis are shown in tables 1.4 and 1.5. The species *B. aereus*, *B. edulis*, *B. reticulatus* and *B. pinophilus* formed ectomycorrhizas the vegetal species tested, showing differences between isolates. In general isolates of *Boletus* spp. grow well in pure culture conditions, having an extensive growth in the substrate. The mantle of the ectomycorrhizas was white-pinky to yellow, very similar in all plant species. The ectomycorrhizas formed in Cistus, *C. sativa* and *B. pendula* are smaller and thinner than the ones in *P. pinaster*. In *Cistus* spp. and broad-leaved species the ectomycorrhizas appear as solitary elements, monopodial-pyramidal or irregularly pinnate or coralloid. In *P. pinaster* the ectomycorrhizas were characterized by a dichotomous branching, long and straight, usually forming coralloid structures (Figure 1.6). Emanating hyphae were frequent (in general) and not specifically distributed. Rhizomorphs were also frequent, round or roundish, white, frequently ramified, with smooth surface, and connection to mantle kind distinct. Structures similar to primordia were usually found in the substrate (images 5, 6 and 10 in figure 1.6), formed by mycelial aggregates, white to beige or light pink in color, with very dense texture, similar to the stipe of carpophores, more or less tubular and ramified, with emanating hyphae and rhizomorphs. As it is shown in tables 1.4 and 1.5 not all the possible combinations were performed, mainly with *C. sativa*, *B. pendula* and *Q. ilex* combinations, due to the difficulties in obtaining axenic seedlings. (All data are shown in table A.1 in the Annex).

Combinations with no positive results are not shown in tables. These tested combinations were: Ba370 x *C. sativa*; Ba370 x *B. pendual;,* Be412 x *B. pendula*; Be2059 x *B. pendula*; Ba1051 x *Q. ilex*; Ba317 x *P. pinaster*; Ba317 x all *Cistus* spp.; Bp 1042 and Bp 1045 x *P. pinaster*, *C. albidus*, *C. ladanifer* and *C. monspeliensis*; and Bp2010 x P pinaster.

Fungal species	Strain	*Castanea sativa*	*Betula pendula*	*Quercus ilex*
Boletus aereus	Ba 317	-	++	++
Boletus aereus	Ba 393	++++		
Boletus aereus	Br 1050			+
Boletus aereus	Ba 1058			+
Boletus edulis	Be 369			+
Boletus edulis	Be 375			+
Boletus edulis	Be 392	+	++	
Boletus edulis	Be 2022	-	+++	
Boletus edulis	Be 2065	-	++	
Boletus edulis	Be 2070	+++		-

Table 1.4. Results of the synthesis of *B. edulis* complex mycorrhizas in pure culture. – any mycorrhizal plant, + 25%, ++ 50%, +++ 75%, ++++ 100% of the mycorrhizal plants.

Figure 1.6. (*Next page*) Images from different mycorrhizas and other structures of diferent *Boletus* – plant combinations. Pictures done with digital camera AxioCam ICc3 in a Zeiss Stemi 2000C stereomicroscope (Carl Zeiss Inc., USA). 1 – 2: Ba 315 x *Cistus monspeliensis*; 3 – 4: Be 2063 x *C. monspeliensis*; 5 – 6: Ba 393 x *C. monspeliensis*; 7 – 8: Be 2044 x *C. monspeliensis*; 9 – 10: Ba 393 x *Castanea sativa*;11: Ba 2070 x *C. sativa*;12 –13: Be 2022 x *Betula pendula*; 14 – 15: Be409 x *Pinus pinaster*; 16 – 17: Be 412 x *P. pinaster*; 18: Be2063 x *P.pinaster*; 19: Be 2059 x P. *pinaster*; 20: Br 1054 x *P. pinaster*; 21: Br 1054 x *C. albidus*; 22: Bp 1099 x *C. albidus*; 23: Ba 393 x *C. albidus*; 24: Be 1098 x *C. albidus*; 25: Ba 1051 x *C. ladanifer*; 26: Br 386 X *C. ladanifer*; 27: Br 386 x *C. laurifolius*; Ba 316 x *C. laurifolius*.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Fungal species	Strain	*Pinus pinaster*	*Cistus albidus*	*Cistus ladanifer*	*Cistus laurifolius*	*Cistus monspeliensis*
Boletus aereus	Ba 315	-	+	+++	-	++
Boletus aereus	Ba 316	-	++++	+++	+	+
Boletus aereus	Ba 317	-	-	-	-	-
Boletus aereus	Ba 370	+	+++	+++		+++
Boletus aereus	Ba 393	-	++++	++++	+	+++
Boletus aereus	Br 1050	-	++	+		++
Boletus aereus	Ba 1051	-	+++	+++	-	+
Boletus aereus	Ba 1058	-	+	+		+
Boletus reticulatus	Br 386	-	++++	+++	++	+++
Boletus reticulatus	Br 388	++	++++	-		++
Boletus reticulatus	Br 1053	+++	+++	++	+	+
Boletus reticulatus	Br 1054	+	++++	-		-
Boletus reticuatus	Br 1055	-	++++	+++		+++
Boletus reticulatus	Br 1056	-	-	-		+
Boletus edulis	Be 369	-	++++	++++	++++	++++
Boletus edulis	Be 375	++++	++	++++	++	+++
Boletus edulis	Be 391	-	-	-		++
Boletus edulis	Be 392	++	++	-	-	-
Boletus edulis	Be 409	++				-
Boletus edulis	Be 410	++++				-
Boletus edulis	Be 411	++				-
Boletus edulis	Be 412	+++				-
Boletus edulis	Be 1098	+	+	+		
Boletus edulis	Be 2017	+++	+			
Boletus edulis	Be 2022		++			
Boletus edulis	Be 2037	++++	-			
Boletus edulis	Be 2044	-	-			++
Boletus edulis	Be 2057	++	++			
Boletus edulis	Be 2059	+++	+++			
Boletus edulis	Be 2063	+++				+
Boletus edulis	Be 2065		+			
Boletus edulis	Be 2070	-	-			+++
Boletus pinophilus	Bp 1042	-	-	-	-	
Boletus pinophilus	Bp 1045	-	-	-	-	
Boletus pinophilus	Bp 1099	++	++++	++++		
Boletus pinophilus	Bp 2007	++				
Boletus pinophilus	Bp 2008	+				
Boletus pinophilus	Bp 2010	-				

Table 1.5. Results of the synthesis of *B. edulis* complex mycorrhizas in pure culture. – any mycorrhizal plant, + 25%, ++ 50%, +++ 75%, ++++ 100% of the mycorrhizal plants.

3.2. Production of mycorrhizal plants in nursery conditions

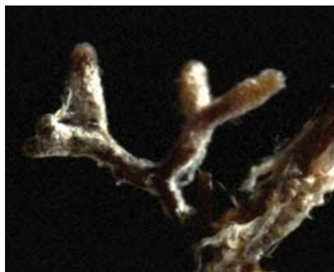
3.2.1. Acclimatization of *in vitro* mycorrhizal plants

3.2.1.1. Acclimatization of plants inoculated under pure culture synthesis conditions

The plants inoculated in pure culture synthesis conditions grew well in nursery after their acclimatization. Nevertheless six months after the transfer to nursery conditions, no mycorrhizas of *B. edulis* were detected in *C. albidus* and *C. ladanifer* plants, whereas mycorrhizas of other fungi were present in the roots. These mycorrhizas were analyzed by sequencing the ITS rDNA region, and the comparison with data available in GenBank showed that were compatible with *Tomentella ellisii.*

3.2.1.2. Acclimatization of plants inoculated under pure culture synthesis conditions in two different substrates

The acclimated plants using two different growth substrates were also adapted to nursery conditions, but the acclimatization was difficult with some *Betula pendula* that presented difficulties to survive. From the 34 plants transplanted 32 survived. According to the results obtained from the mycorrhizal root tips molecularly analyzed only one plant (*P. pinaster* x Be 2057) maintained the *Boletus* mycorrhizas (figure 1.7). It cannot be ensured that there are differences between the two substrates used, but the only plant maintaining the mycorrhizas after the acclimatization was transferred to substrate used for the synthesis of mycorrhiza in pure culture (peat-vermiculite 1:10; v:v).



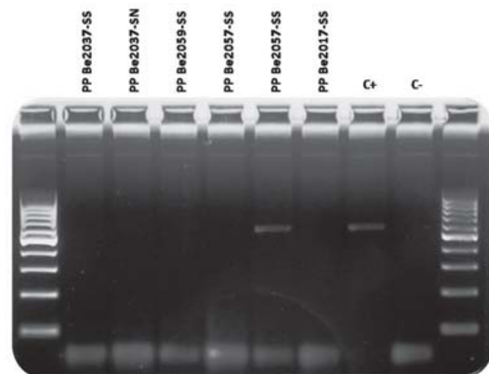


Figure 1.7. *Boletus edulis* 2057 x *Pinus pinaster* mycorrhizas transferred into the synthesis growth substrate used for the synthesis of mycorrhiza in pure culture (peat-vermiculite 1:10; v:v) and results of the PCR with specific B. edulis primers (Mello et al. 2006) after electrophoresis on Agarose (2%) gel. C+: positive control, DNA extraction from *B. edulis* pure culture mycelium.

3.2.2. Controlled inoculations of *Cistus* spp. plants with species of the *B. edulis* complex for the production of mycorrhizal plants in nursery conditions.

3.2.2.1. Inoculations with spores suspension

The results obtained in the inoculations with spore suspensions are shown in table 1.6. After 7 months growing in nursery conditions, plants were 21.52 (±5.87) cm high and had a diameter of 3.13 (±0.32) mm in average, without differences between treatments. No mycorrhizas of *Boletus* spp. were observed in any of the tested combinations. A 32.04% (±8.29) of mycorrhizas of other fungi were detected. According to the comparison of the sequences of their ITS rDNA regions, the mycorrhizas detected in all the inoculations (with spore suspension and with mycelium) belongs predominantly to fungal species of the families *Thelephoraceae* and *Pyronemateceae.*

Inoculations with *Boletus* spp. spore suspension (2009)			
Vegetal species	**Fungal species**	**% *Boletus* mycorrhizas**	**% other mycorrhizas**
Pinus pinaster	Control	0	29.41
	B. edulis (A)	0	32.66
	B. edulis (P)	0	29.97
	B. reticulatus	0	28.86
Cistus albidus	Control	0	31.72
	B. edulis (A)	0	46.38
	B. edulis (P)	0	36.62
	B. reticulatus	0	44.98
Cistus ladanifer	Control	0	30.87
	B. edulis (A)	0	21.61
	B. edulis (P)	0	19.34
	B. reticulatus	0	21.06

Table 1.6. Inoculations with *Boletus* spp. spore suspensions. *B. edulis* (A) : Origin Alto Abejera; *B. edulis* (P): origin Pinar Grande. %: mean percentage of mycorrhizal roots per plant.

3.2.2.2. Inoculations with mycelial inoculum

The results obtained with mycelial inoculum are shown in table 1.7. After 7 months growing in nursery conditions, plants were 10.13 (±1.83) cm high and had a diameter of 2.21 (±0.12) mm in average, without differences between treatments. No *Boletus* spp. mycorrhizas were observed in any combination. *Laccaria bicolor,* used as a positive control, produced mycorrhizal roots in inoculated plants, except of *C. ladanifer* when inoculated with mycelium grown by the standard method

(inoculum type 1). Mycorrhizas from other fungi were present in all plants. No differences between the mycelial inoculum production types (with or without the presence of the respective host plant) were detected for *Boletus* species. When analyzing data obtained for *L. bicolor*, differences in inoculum type were detected (p = 0.0082; α=0.05), having a high percentage of *L. bicolor* mycorrhizas when using mycelium grown in presence of host plant (inoculum type 2). The biggest difference was detected for *L. bicolor* and *C. ladanifer* combination, in which mycorrhizal plants were obtained just using mycelium grown in presence of host plant (the inoculum type 2).

Inoculations with *Boletus* spp. Mycelial inoculums (2009)				
Vegetal species	**Fungal species**	**Inoc. type**	**% mycorrhizas of the inoculated fungus**	**% other contaminant mycorrhizas**
Pinus pinaster	Control	No	0	38.25
	L. bicolor	1	17.44	13.10
	Br 1053	1	0	38.29
	Be 375	1	0	35.46
	Ba 393	1	0	39.25
	L. bicolor	2	24.75	32.99
	Br 1053	2	0	40.63
	Be 375	2	0	31.84
	Ba 393	2	0	33.19
Cistus albidus	Control	No	0	44.05
	L. bicolor	1	5.74	0.00
	Br 1053	1	0	47.67
	Be 375	1	0	48.72
	Ba 393	1	0	44.02
	L. bicolor	2	18.38	17.42
	Br 1053	2	0	38.13
	Be 375	2	0	35.74
	Ba 393	2	0	24.60
Cistus ladanifer	Control	No	0	23.68
	L. bicolor	1	0	19.04
	Br 1053	1	0	16.44
	Be 375	1	0	19.53
	Ba 393	1	0	15.76
	L. bicolor	2	21.20	9.91
	Br 1053	2	0	25.43
	Be 375	2	0	17.23
	Ba 393	2	0	9.10

Table 1.7. Inoculations with *Boletus* spp. mycelium (*B. reticulatus* 1053, *B. edulis* 375 and *B. aereus* 393). *Laccaria bicolor* used as a positive control. Inoculum type: (1) mycelium grown in peat:vermiculite; (2) mycelium grown in peat:vermiculite in presence of the respective host plant (*P. pinaster* or *C. albidus*). The % represents the mean percentage of mycorrhizal roots per plant.

3.3. Viability of *B. edulis* complex cultures stored in water at 4°C

After 24 months of storage at 4°C in sterile water, all isolates of *R. roseolus* maintained a 100% of growth viability. Between 96% and 100% of isolates belonging to *B. aereus*, *B. edulis* and *B. reticulatus* maintained a 100% of viability after 3 months of storage, while at 12 months the number of isolates of these three species that maintained the 100% of viability was around 70%. In the case of *B. pinophilus* the number of viable isolates decreased to approximately 40% at three months, maintaining this rate after 12 months of storage at 4°C in sterile water. After 24 months of cold storage in water, about 45% of *B. aereus* and *B. edulis* isolates were maintaining their growth capacity, just a 20% of the *B. pinophilus,* and none of the *B. reticulatus* isolates maintained their viability.

The statistical analysis for the viability of the different isolates, using as factors the species and the storage time, showed differences for species ($p < 0,0001$; $\alpha=0,05$) and storage time ($p < 0,0001$; $\alpha=0,05$). No interactions were detected. The Tukey's test to compare means separated *B. pinophilus* from the other *Boletus* species (*B. aereus*, *B. edulis* and *B. reticulatus*) (table 1.8); concerning the storage time the test provided three groups (figure1.8), highlighting the differentiation of the isolates viability after 24 months of cold storage in water.

Analysis of Variance for 'Viability after cold storage'

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob> F
Model	19	14.132572	0.743820	6.6349	<0.0001*
Error	170	19.058165	0.112107		
C. Total	189	33.190737			

Effect tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob> F
Species	4	3	2.8653055	8.5196	<0.0001*
Storage time	5	4	9.2131171	20.5454	<0.0001*
Species*S.time	20	12	1.8744240	1.3933	0.1732

Means Comparisons for all pair using Tukey-Kramer HSD

Level			Mean	Std error	N
B. aereus	A		80 %	6.05 %	40
B. edulis	A		81 %	4.01 %	85
B. reticulatus	A		75%	7.90 %	30
B. pinophilus		B	48%	8.04 %	35

Table 1.8. Analysis of variance for the viability of *Boletus* spp. isolates by the species and the storage time in water at 4°C. *Statistically significant differences ($\alpha=0,05$).Means are expressed as percentage (%) of isolates maintaining their 100% of growth viability after their transfer to fresh BAF agar medium.

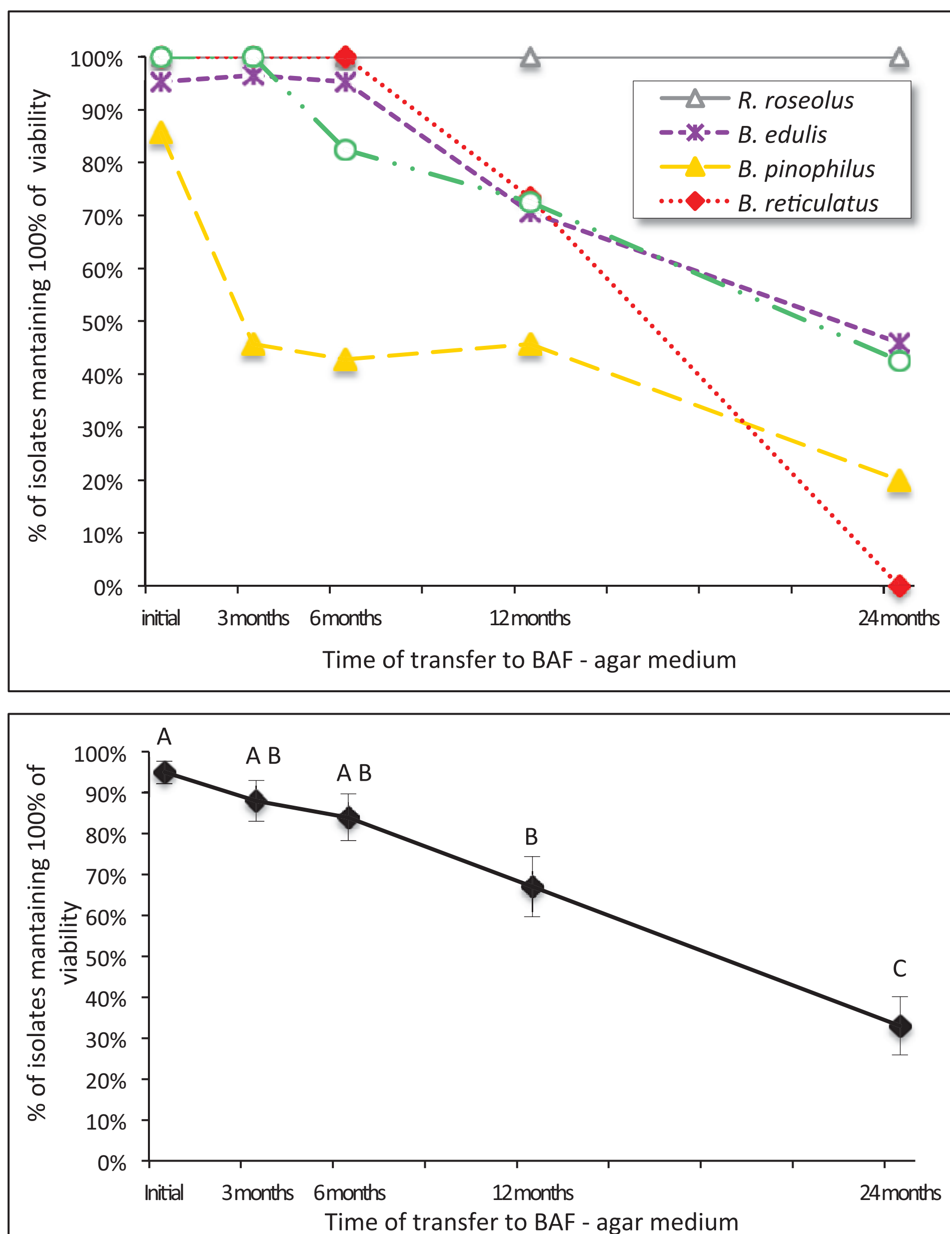


Figure 1.8. Evolution of the total percentage of isolates mantaining their 100% of growth viability after the different time transfers to fresh BAF agar medium, for each species (top image) and the total (lower image). Bars show the standard error (N=38). Letters indicate the Tukey's test results.

4. Discussion

The species of the *B. edulis* complex are among of the most appreciate edible ectomycorrhizal mushrooms and are sold all over the world due to their particular gastronomical characteristics. This is why *B. edulis* is also one of the ectomycorrhizal species that has been tried to be cultivated in controlled conditions. Many researchers have tried to produce mycorrhizal plants in order to obtain controlled productions with these fungi, but with scarce success to date. Our experience does not bring much additional information, as our attempts to obtain *Boletus* mycorrhizal plants in nursery conditions, applying mycelial inoculum or spore suspensions, were not successful. It is clear that these are difficult species to work with, but as we obtained well mycorrhizal plants in axenic conditions, the option to get them in nursery conditions shouldn't be so far.

After the different attempts done to obtain *Boletus* spp. mycorrhizal plants, with the available methods, it was only possible to produce inoculated plans in pure culture synthesis, were all the parameters are controlled and the conditions to force the symbiosis between the host plant and the fungus are the optimal. This forced conditions allowed also obtaining mycorrhizas in combinations between non-compatible species, as the mycorrhizas obtained in *P. pinaster* x *B. aereus* (only with one isolate), that shouldn't be considered to be necessarily found in nature. Looking at the results obtained (table 1.4 and 1.5), in general *Boletus* spp. grow well in pure culture conditions with the different host plants tested, but we should highlight the good results obtained with the combinations done with the different *Cistus* species seedlings. Tests with broad-leaved trees (*C. sativa*, *B. pendula* and *Q. ilex)* were not large in number of combinations, due to the difficulties in obtaining axenic seedlings, free from other contaminant fungi, and the problems encountered with seed germination or plants grown in these conditions. The procedures to obtain axenic seedlings were not entirely satisfactory (especially with the chestnuts). When stronger disinfection treatments were applied, germination and seed viability were drastically reduced. The results with *B. pendula* seedlings obtained by somatic embryogenesis were good, but the availability of these seedlings was limited. Because of these good results with *B. pendula* seedlings, test with *C. sativa* and *Q. ilex* plants obtained from *in vitro* somatic embryogenesis of seedlings resulting from in vitro germination were performed. However, when transferring the plants from the in vitro medium to the synthesis tubes, plants were not surviving, irrespectively if the fungal inoculum was introduced in the tube or not.

Nursery inoculations, either with mycelium or with spore suspensions, did not work for the *B. edulis* complex in our experiments. Some previous attempts without success have been reported in the literature (Hall et al. 1998; Wang and Hall 2004; Giomaro et al. 2005), as mentioned in the introduction (table 1.1). Nevertheless there has been reported also some success in producing Boletus mycorrhizal plants *in vitro* and in nursery conditions, although, as already mentioned in the introduction, molecular analysis to ensure the presence of *B. edulis* mycorrhizas were only used by Águeda et al. (2008). Duñabeitia et al. (1996) synthesized *B. pinophilus* ectomycorrhizas by inoculating *P. radiata* seedlings with a spore suspension of 10^7 – 10^8 spores per plant, using the same method and similar concentrations to those used in our experiments. In that work they do not give the mycorrhizal percentages obtained. In a more recent work, published by Chung et al. (2007), they inoculated with a spore suspensions of 10^6 spores per plant, obtaining plants with a 30% of mycorrhizas of *B. edulis* and *B. pinophilus*, also using the same methodology that we use for our inoculations. Moreover, in this work and some others, the inoculated seedlings were outplanted to field in natural conditions, and reported positive results (Meotto et al. 1999; Chung et al. 2007) , maintaining Boletus ectomycorrhizas two or six years after outplanting, with mycorrhizal percentages between 17 and 11% respectively. However fruiting body production has never been reported (Olivier et al. 1997; Meotto et al. 1999; Wang and Hall 2004; Mello 2012).

In our attempts on producing *B. edulis* mycorrhizal plants we saw that Boletus mycorrhizas were quickly replaced by other ectomycorrhizal fungi best adapted to nursery conditions (*Thelephoraceae* and *Pyronemateceae)*, especially for those acclimated plants in which Boletus mycorrhizas synthesized *in vitro* disappeared. This fact was also seen by Meotto et al. (1999) when outplanting their porcini colonized plants produced in greenhouse conditions. The competition on colonizing the root by other fungi is an important factor interfering in the mycorrhization process. Species of *Thelephoraceae* or *Pyronematavea* competing with *B. edulis* complex species have a higher growth rate and are able to colonize rapidly the new fine roots available, probably displacing the Boletus and leading the fungus to disappear. This factor is difficult to control because the contaminants detected are very common in nurseries.

According to our results, one technique that needs to be improved is the acclimatization of *Boletus* mycorrhizal plants produced under axenic condition, but this method may be difficult to apply if we think on to produce a large number of

plants mycorrhizal with *Boletus* because of the high economic and time cost that entails.

In a recent work done in China (Wu et al. 2012) *B. edulis* was co-inoculated with the mycorrhiza helper bacterium *Bacillus cereus* in *Pinus thunbergii* seedlings, under nursery conditions. They saw that the helper bacteria increased the mycorrhizal establishment in their plants. Other works stated that helper bacteria, such as *Bacillus*, can promote the formation of ectomycorrhizal roots (Garbaye 1994; Poole et al. 2001; Bending et al. 2002; Deveau et al. 2007; Kataoka and Futai 2009; Aspray et al. 2013). Wu et al. (2012) used *B. edulis* mycelium to inoculate plants in nursery conditions, and after 9 months, they obtained great mycorrhizal rates (42 % for *B. edulis* inoculations and 56% and 62% for *B. edulis* + *B. cereus* inoculations). However any molecular identification was provided, and according to our results and other works, we cannot discard that all those mycorrhizas could be formed by other ectomycorrhizal fungi with a similar morphological appearence. Anyway, they obtained interesting results, opening new perspectives on the cultivation of *B. edulis*. Other authors reported the need of a third organism to produce a stable system, Hall and colleagues (Hall et al. 2003; 2005; Wang and Hall 2004) reported that Boletus needs another organism to maintain the stability of its mycorrhizas, particularly the coexistence of *B. edulis* with *Amanita excelsior* or *A. muscaria,* in other works the association of *B. edulis* and other fungal species were not found (Peintner et al. 2007), but suggested that probably other fungal or bacterial species in the soil must be able to fulfill the role of this other organism needed.

Differences in mycorrhization success between different strains, within the same fungal species, prove that the selection of isolates is a crucial step to produce mycorrhizal plants under controlled inoculations. It is possible the identification of ectomycorrhizal fungi at species level by analyzing the internal transcribed spacer (ITS) regions in the ribosomal genes (rDNA) (Bruns et al. 1991; Horton and Bruns 2001; Gomes et al. 2002; Horton 2002; Bridge et al. 2005). However, intraspecific differences in the ITS rDNA region show limited variability (Nilsson et al. 2008) which restrict the detection at isolate level making necessary the use of sequencing or high resolution techniques for DNA analysis to distinguish strain differences. Such ITS intra and inter-specific variability within the different species of the *B. edulis* complex was analyzed by Leonardi el at. (2005).

Differences in isolates are not only seen in mycorrhization capability, they also present different growing rates and characteristics when growing in pure cultures.

Some of them, as seen in the images, form aggregates when growing in synthesis tubes. Some authors have observed these formations before in *B. edulis* (Díaz et al. 2009) and *B. reticulatus* (Olivier et al. 1997). They tend to call them primordia, describing them as a structure that could precede the fruiting body formation (Yamanaka et al. 2000). The formation of Boletus sporocarps in pure culture has been only reported two times, firstly in 1962 by Karpinski, who obtained *B. edulis* basidioma, and then by Yamanaka et al. in 2000, who describes the formation of *B. reticulatus* young fruit body in pure culture. Both agree that the Boletus cultures tested loose or decrease their capability to form fruit bodies one year after the culture.

As seen before, the 39 isolates tested for the synthesis of mycorrhizas in pure culture showed differences in root colonization. The intraspecific variation in the colonization and grown ability of different ectomycorrhizal fungi in *P. pinaster* pure cultures was previously reported by Pera and Álvarez (1995) and also more recently by Parladé et al. (Parladé et al. 2011). In this study they showed the intraspecific variability of 25 *Lactarius deliciosus* isolates when colonizing *P. pinaster* seedlings in pure culture conditions, showing also that tolerance to cold storage in water was also isolate dependent for this species. *L. deliciosus*, in contrast to *B. edulis*, can be easily inoculated (using mycelial inoculum) in nursery conditions to produce mycorrhizal plants (Guerin-Laguette et al. 2000; Parladé et al. 2004). Nowadays plants commercially inoculated, with *L. deliciosus* are sold by different nursery suppliers, and orchards have been established from nursery inoculated plants with good sporocarp productions (Wang et al. 2001; Wang and Hall 2004; Hall et al. 2005), even obtaining fruit bodies in nursery containers (Figure 1.9). The differences in *L. deliciosus* isolates was also observed in nursery inoculated plants and also after being outplanted in the open field (Hortal et al. 2009). Other edible ectomycorrhizal mushrooms can be inoculated in nursery seedlings, as *T. melanosporum* and *R. roseolus*, obtaining good mycorrhization percentages with spore suspension inoculum (chapter 3). Both species are successfully cultivated in different parts of the world (Wang and Hall 2004) and also *R. roseolus* produces fruit bodies in nursery containers.

More and more edible ectomycorrhizal mushrooms are successfully cultivated (different species of truffles – *T. melanosporum*, *T. aestivum*, and *T. borchii, T. formosum* –, *Terfezia laveryi*, *L. deliciosus, L. hutsudake, Lyophyllum shimeji*, *R. roseolus, Tricholoma matsutake and Suillus granulatus* (Wang and Hall 2004; Wang et al. 2012)), and some progress towards *B. edulis* cultivation was pointed out by

Guerin-Laguete et al. (2011). Nevertheless there is still much to do in the production of *B. edulis* mycorrhizal plants and the controlled production of *B. edulis* sporocarps.

Nowadays, the new sequencing technologies are opening new perspectives trough understanding biology and ecology of ectomycorrhizal fungi (Martin et al. 2011). The *B. edulis* genome has been just sequenced and preliminary data are available in The Genome Portal of the Department of Energy Joint Genome Institut (JGI) (Grigoriev et al. 2012), http://genome.jgi.doe.gov/Boled1/Boled1.home.html. Probably this will allow the identification of key genetic trails involved in the symbiosis process and the sporocarp formation, probably resulting, in a near future, in the development of tools for the *B. edulis* cultivation and management.

Figure 1.9. *Lactarius deliciosus* sporocarps grown in nursery containers. A) *L. deliciosus* sporocarp grown in nursery containers with 2 years old *P. pinaster*at IRTA nursery (unpublished data). B) Image available in Robin Pépinières web site (www.robinpepinieres.com) of *L. deliciosus* fructification on one year old *P. nigra* seedling.

The preservation of ectomycorrhizal fungal strains and isolates collections in the laboratory is also important for their in-depth study and for the maintenance and availability of inoculum. One of the problems added to the difficulties of some fungi to grown as pure culture, as *B. edulis* (due to their slow growth), is that characteristics of fungi such as pathogenesis, virulence, growth and colonization ability are known to change over time when mycelium is continually subcultured in agar (Hung and Molina 1986). It is necessary to find methods in which both the viability and the stability of living cells are ensured during the preservation period. An alternative to agar mediums is the maintenance of fungal isolates in distilled sterile water, stored at 4°C.

According to the results obtained, distilled water is a good method to maintain *Boletus* isolates up to a maximum of 12 months. It is a suitable method for the

storage of all tested species except for *B. pinophilus* isolates (their viability is reduced more than a half just three months after storage).In previous works it was stated that mycorrhizal fungi present less viability rates to this type of conservation method than saprophytic fungi, (Richter 2008). Basidiomycota showed higher variability in their regrowth capability when compared with Ascomycota and Deuteromycota after 20 months storage in cold water (at 4 or 18 degrees (Smith et al.(1994)). Richter (2008) tested 9 isolates of the genus Boletus, and only one of them survived after 2 – 4 years (Richter and Bruhn 1989) and after 20 years (Richter 2008). They conclude that the mycorrhizal genera *Boletus*was unsuited for this type of conservation method when used for long-term storage (more than 2 years), but according to our results it is a good storage method up to one year, at least for some species of the genera.

Principal advantages of cold conservation, compared to periodical transfers to fresh BAF agar medium in Petri dishes, are that it is an economic, simple and effective method; save working time and also the storage space is reduced. On the other hand, some of the isolates do not have good regrowth response (it is convenient to reactivate the strain with several discs to ensure their growth), so, although more research need to be done, we can say that tolerance to cold storage is strain dependent, as it was seen for *L. deliciosus* (Parladé et al. 2011); after 6 – 12 months of storage some strains seem to need more time to reactivate their growth, but once they start, their activity is normal. This fact has also been reported by Camelini et al. (2011) for *Agaricus subrufescens*, showing a decreased rate of growth after cold storage compared to two other conservation methods.

In conclusion, storage in cold sterile water is a simple and effective method for the storage and conservation of *B. edulis* species complex and *R. roseolus*, taken into account the strain, especially for *Boletus* species. This selection of isolates is also a crucial step for controlled inoculations to produce mycorrhizal plants, as seen with the different success between different strains within the same species in pure culture inoculations. The *in vitro* inoculation is a good method for the synthesis of *B. edulis* mycorrhizal plants, but the methodology to obtain nursery mycorrhizal plants needs more research. One possibility that needs to be improved is the acclimatization of mycorrhizal roots from in vitro-produced plants to the nursery environment.

Use of molecular techniques for the traceability of ectomycorrhizal fungi: Seasonal dynamics of *Boletus edulis* and *Lactarius deliciosus* mycelium in pine forests of central Spain

2

Publications product of this chapter:

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De la Varga H, Águeda B, Martínez-Peña F, Parladé J, Pera J (2012) Quantification of extraradical soil mycelium and ectomycorrhizas of Boletus edulis in a Scots pine forest with variable sporocarp productivity. Mycorrhiza 22: 59-68.

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Chapter 2

Use of molecular techniques for the traceability of ectomycorrhizal fungi: Seasonal dynamics of *Boletus edulis* and *Lactarius deliciosus* mycelium in pine forests of central Spain

1. Introduction

The development of methods for controlled inoculation of plants aimed at establishing productive plantations could be a sustainable way to exploit the collection and marketing of edible mushrooms without affecting the conservation of natural populations (Wang and Hall 2004). Different species of truffles (*Tuber melanosporum* Vittad., *Tuber aestivum* Vittad., and *Tuber borchii* Vittad.) have been cultivated commercially around the world (Chevalier and Frochot 1997; Bonet et al. 2009), and some success has been achieved with other species , such as *Lactarius deliciosus* (L.) Gray (Guerin-Laguette et al. 2000; Parladé et al. 2004), *Lyophyllum shimeji* (Kawam.) Hongo or *Rhizopogon roseolus* (Corda) Th. Fr. *(Wang et al. 2001; Wang and Hall 2004)*. For a large number of fungal species, including *B. edulis*, and due to the difficulties encountered in growing and producing inoculated plants (as seen in the previous chapter), harvesting in natural forests is the only way of obtaining this resource (Wang and Hall 2004; Cannon and Kirk 2007). Since the availability of the majority of edible species depends, almost exclusively, on their natural and unpredictable fructification, it is important to establish forest management guidelines leading to maintain and increase the production of sporocarps, preserving at the same time the conservation and diversity of the fungal species (Ortega-Martínez and Martínez-Peña 2008; Egli et al. 2010). To achieve this goal it is required to use, adapt or develop mechanisms that enable to trace the fungi in all their stages (mycelium, mycorrhizas and fruiting bodies), particularly as extraradical soil mycelium.

The sporocarp production of edible species depends on both the host tree preference and the ecological environment (fungal communities, climate, soil, tree development) (Laganà et al. 2002; Salerni et al. 2002; Bonet et al. 2004; Martínez de Aragón et al. 2007b; Barroetaveña et al. 2008; Bonet et al. 2010; Pinna et al. 2010; Savoie and Largeteau 2011; Buée et al. 2011; Ortega-Martínez et al. 2011; Martínez-Peña et al. 2012). Egli et al. (2010) showed an increase of fruit body

productions 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 *L. deliciosus* after thinning and related it to changes in soil fertility. Whereas the same studies stated that these data alone do not explain all the trends in the dynamics of fungal fruiting. The appearance of mushrooms is obviously linked to the presence of mycelium in the soil. It can be hypothesized that the concentration of soil hyphae and/or ectomycorrhizas could also be related to the amount of sporocarps produced and several studies have tried to relate these three elements (Peter et al. 2001; Kjøller 2006; Parladé et al. 2007; Suz et al. 2008; Rineau et al. 2010; De la Varga et al. 2012). However, no conclusive results have been obtained to date.

Mycelium of ectomycorrhizal fungi growing in the soil may represent 30–80% of the fungal biomass (Wallander et al. 2001; Högberg and Högberg 2002) and creates an extensive and dynamic mycelial network, which plays a key role in the nutrient uptake by plants and in the reciprocal transfer of carbon and nutrients between plants of the same ecosystem (Read 1992; Simard et al. 2002). Little is known about the annual dynamics of the mycelium of ectomycorrhizal fungi in forests soils, but its knowledge is winning importance for its role on the carbon cycle (Cairney 2012), the mobilization of soil nutrients (Kjøller 2006), and the assemblages of different components of the ecosystem (plants, fungi, microfauna, and microorganisms) (Fitter and Garbaye 1994; Courty et al. 2010).

The distribution of extraradical mycelium in the soil is still poorly understood due to the difficulty of adapting appropriate methods for its study (Anderson and Cairney 2004). The development of techniques based on direct nucleic acid extractions coupled with polymerase chain reaction amplifications (PCR) has provided new insights into the ecology of these soil fungi (Guidot et al. 2002). The real-time PCR 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; van der Linde et al. 2009). This technique allowed for the relative or absolute quantification of fungal biomass and compared to other quantification techniques, as total hyphal length or biochemical markers, provides a species-specific measure for mycelial biomass estimations (Landeweert et al. 2003; Parladé et al. 2007; Hortal et al. 2009; De la Varga et al. 2012; Parladé et al. 2013).

Boletus edulis and *L. deliciosus* are two valued wild edible fungi, gastronomically appreciated and sold in local markets of many countries, particularly in Europe.

These mushrooms are collected from the wild and, in some geographic areas, its trade has become an important economic income, as valuable nonwood forest products (Boa 2004). *B. edulis* can be found in association with a large number of trees and shrubs of Fagales, Malvales, Malpighiales, Ericales and Pinales (Águeda et al. 2008). No controlled production has been done to date, and harvesting in natural forests is the only way of obtaining this resource (Wang and Hall 2004; Cannon and Kirk 2007). *L. deliciosus* generally associates with species from the family Pinaceae (Hutchison 1999) and is mainly collected from the wild. This species can be easily isolated and adequately formulated for nursery inoculations (Parladé et al. 2004), and it has been shown to produce edible sporocarps from inoculated plants (Wang and Hall 2004).

The **objectives** of this chapter were:

1. To design specific oligonucleotides for the detection and quantification of *B. edulis* extraradical mycelium in a Scots pine forest soil by real-time TaqMan PCR;
2. To validate the real-time PCR technique for the detection of *B. edulis* mycelium in soil by comparing conventional and real-time PCR;
3. To determine the concentration of soil mycelium and the abundance of mycorrhizal short roots during the fruiting season in different forest plots representing a gradient of expected mushroom productivity;
4. To study the seasonal belowground dynamics of extraradical mycelium of *Boletus edulis* and *Lactarius deliciosus* in two different pine forests along a period of 12 months.

2. Materials and Methods

2.1. Design of *B. edulis* probes and primers for their mycelium detection.

2.1.1. Design of primers and probes

The design of new species - specific primers and TaqMan® probes for the detection of *B. edulis* mycelium in soil samples, was performed with the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA), based in the variability of the rDNA internal transcribed spacer (ITS) region detected in the alignments among different sequences of *B. edulis*.

Specific *B. edulis* primers, RVS-Bedu (TGCACAGGTGGATAAGGAAACTAG), FWD-Bedu (CTGTCGCCGGCAACGT), and TaqMan® probe STQBedu (6FAM-CCCTTTCTCTT TCGTGGAACCTCCCC-TMR) (Table A.3 in the Annex) amplify a 75 bp fragment. A search for highly similar sequences (megablast) was performed in the GenBank database to test the specificity of the designed oligonucleotides. The oligonucleotides combinations for *B. edulis* detection and quantification were tested *in silico* by blasting them against the NCBI GenBank database to check for specificity. Further in vivo specificity tests were carried out with DNA extracted from four forest soils of areas unsuitable for *B. edulis* and DNA from pure cultures or sporocarps of *Amanita caesarea, A. ponderosa, B. aereus, B. pinophilus, B. reticulatus, Laccaria bicolor, Lactarius deliciosus, Pisolithus tinctorius, Rhizopogon roseolus, Russula integra* and *T. melanosporum*.

2.1.2. DNA extractions

Soil DNA extractions were performed with the PowerSoil™ DNA Isolation Kit (MoBio laboratories Inc., Carlsbad, CA, USA) from 0.25 g of soil per sample according to manufacturer’s instructions. Five extractions of each soil sample were processed in order to avoid the variability that entails working with a heterogeneous medium as the soil. A total of 375 samples were processed. The extracted DNA was stored at −20°C. DNA extractions from pure cultures were performed with the Dneasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France) following the manufacturer’s instructions. The extracted DNA was stored at −20°C.

2.1.3. Construction of standard curves

Standard curves for mycelium quantification by real-time PCR were generated using known amounts of mycelium from active growing colonies of *B. edulis* (one

month old). From these amounts serial dilutions are analyzed by real time PCR and the Ct values obtained for each dilution were plotted against the corresponding initial amount of mycelium to generate the standard curve. Thus, we obtain a direct correspondence between the DNA extracted from known amounts of mycelium and the Ct values.

A total of 570 mg of *B. edulis* mycelium grown on a cellophane sheet on agarified biotin-aneurin-folic acid (BAF) medium (Oort 1981) were added to 10 ml of distilled autoclaved water and fragmented in a Polytron (Kinematika GmbH, Kriens—Lucern, Switzerland) at medium speed for 5 s. An aliquot of 100 µl of the mycelial suspension was mixed with 0.5 g of a soil collected in Pinar Grande. The soil was previously checked for the absence of *B. edulis* DNA by conventional PCR with universal (ITS1F/ITS4) and specific primers (Bedu1F/Bedu2R). Soil with added mycelium was left to dry to constant weight at air temperature (20–25°C). DNA was extracted from the soil with the added mycelium, and control soil sample without added mycelium, using PowerSoil™ DNA Isolation Kit according to manufacturer's instructions. Tenfold serial dillutions from the extract were prepared until 10^{-6}, obtaining serial concentrations of: 11.40000, 1.14000, 0.11400, 0.01140, 0.00114, 0.00011, and 0.00001 mg of mycelium/g of soil.

2.1.4. Quantification of extraradical soil mycelium of standards and samples by real-time PCR

Boletus edulis DNA extracts were amplified by real-time PCR in a LightCycler® 480 real-time PCR system (Roche), using the kit LightCycler ®480 Probes Master (Roche) according to the manufacturer's instructions for a final reaction volume of 20 µl. The reaction mix contained 10 µl of 2×conc. LightCycler® 480 Probes Master PCR mix buffer, 1 µl of waterLightCycler®480 Probes Master PCR-grade, 1.6 µl of each primer, and 0.8 µl of probe. Primers and probe working concentrations were 800 and 100 nM, respectively. The analyses were performed on 96-well plates (Light- Cycler® 480 Multiwell plate 96). PCR conditions were 10 min at 95°C, 45 cycles at 95°C for 15 and 50 s at 60°C, and a final step of 30 s at 40°C. LightCycler® data were processed with the LightCycler ® 480 Software Version 1.5.

For each reaction, 5µL of DNA extracted from soil samples were added as a template, which was previously measured with a Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Three replicates of each sample were included in the analysis, as well as a negative control (using de-ionized water instead of DNA template) and the standards (three

replicates of each serial dilution) in each plate. Absolute quantification of mycelium biomass of *B. edulis* in each soil sample was determined by interpolation of the Ct value in the standard curve, and the results (microgram target DNA in 20 µl PCR mixture) were converted to milligrams of mycelium per gram of soil.

2.2. Validation method by comparison of conventional PCR and quantitative real time PCR.

2.2.1. Study site

The study site was located in Pinar Grande (Soria, Spain), a homogeneous *Pinus sylvestris* L. stand that covers a 12,533 ha area situated in the Sistema Ibérico mountain range, in the inner northeast zone of the Iberian Peninsula. The altitude ranges between 1,097 and 1,543 m above sea level with dominating west and east orientations. Soils are acidic brown earths or alluvial with marked acid pH (4–5), sandy loam to sandy texture, a low holding capacity, and low fertility. Soil properties are detailed in Table 2.1.

Average annual rainfall is 865 mm/year, 69 mm/year falling in July and August, and 132 mm/year in September and October. Medium annual temperature is 8.8°C, with July being the warmest month (17.4°C). The frost period begins in November and ends in April, with frequent frosts in late spring and early autumn. Scots pines forest management is characterized by a rotation age of 100 years and a regeneration period of 20 years. The forest is managed by clearcutting with soil movement and sowing.

From 1995 to 2008, the autumnal average production of *B. edulis* in Pinar Grande was 30.3 kg/ha, being the most productive stands those belonging to the age class comprised between 31 and 70 years, with a 69% of the total production (Ortega-Martínez et al. 2011; Martínez-Peña et al. 2012).

Top soil (0-20 cm) properties in Pinar Grande													
Plot	Sand (%)	Silt (%)	Clay (%)	Coarse elements (%)	pH (H_2O)	Organic matter (%)	N (%)	K (ppm)	Mg (ppm)	Ca (ppm)	Fe (ppm)	P (ppm)	C/N
9A	84	11	5	0.1	4.2	4.3	0.13	85	18	103	6650	6.55	19
4C	56	32	12	6.6	4.7	3.4	0.11	78	35	417	4780	2.21	18
6D	43	45	12	0.1	4.5	7.2	0.20	76	70	920	4160	2.89	20
7B	54	31	15	0.3	4.7	5.6	0.27	95	93	520	8014	13.97	12

Table 2.1. Top soil properties measured in the sampling plots in Pinar Grande.

For the present study, 18 permanent fenced areas established in Pinar Grande since 1995 for quantifying autumnal sporocarps production (Ortega-Martínez et al. 2011; Martínez-Peña et al. 2012) were used. These areas, performed by stand age class according to the forest management plan (with trees ranging between 16 and 70 years old), are divided in six 5x5 m plots each one. One plot in five areas was selected for soil sampling, so that ectomycorrhizas and soil were sampled in five 5 x 5 m plots selected by their average production of sporocarps between 1995 and 2007 to represent a gradient between minimum and maximum productivity (Table 2.3 and Figure A1 in the Annex). Soil samples in each plot were taken three times in 2008: in mid-September, mid-October, and mid-November (before, during, and after the fruiting season peak, respectively). At each sampling time, 5 soil samples were extracted in each plot using a cylindrical (2 cm radius, 20cm deep – 250 cm^3) soil borer (Taylor 2002). Samples were taken next to the angles and in the center of the squares plots with a minimum distance of 30 cm apart from any tree trunk.

Soil samples were let to dry at air temperature (20–25°C) to constant weight and then sieved and homogenized. Ten grams of soil were separated for molecular analysis and the remaining was frozen at −20°C and stored until ectomycorrhizal identification and quantification.

Figure 2.1. One of the fenced areas selected in Pinar Grande for the quantification of *B. edulis* soil mycelium.

2.2.2. *Boletus edulis* soil mycelium quantification

A total of 375 samples were processed for their DNA extraction, as detailed on page 6 (DNA extractions). Five extractions of each soil sample were performed, in order to avoid the variability that entails working with a heterogeneous medium as the soil. The extracted DNA was stored at −20°C.

In order to validate the detection of *B. edulis* mycelium by real-time PCR technique conventional PCRs were performed, for comparison purposes, using the specific primer pair Bedu1F (ATGGAGGAGTCAAGGCTGTC) Bedu2R (TAGATTAGAAG CGATTCACT) developed by Mello et al. (2006); only on the first extraction made in each combination of date and sampling point in each plot, a total of 75 samples were processed. Amplifications were obtained by PCR reactions containing 2 µl (from a 10 µM stock) of each primer, 25 µl of 2x PCR Solution Premix Taq™ (TaKaRa Ex Taq™ Version; TAKARA BIO INC., Japan), 2 µl of DNA template (corresponding to 20–40 ng of DNA) and HPLC water (Scharlau-Chemie, Barcelona, Spain) to a final volume of 50 µl. PCR reactions were performed in a GeneAmp® 9700 thermocycler (Applied Biosystems, CA, USA) with an initial denaturation step at 95°C for 5 min, 35 cycles at 94°C for 45 s, annealing at 60° C for 45 s, extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. Soil DNA extracts were also amplified by real-time PCR, as detailed in page 9 for *B. edulis* DNA extracts. The standard curve used is described in page 11.

2.2.3. Quantification of ectomycorrhizas

Each Pinar Grande soil sample was gently washed with tap water for extracting root tips. Under the stereomicroscope, *B. edulis* ectomycorrhizal root tips were identified according to its morphology (Águeda et al. 2008) and counted. Ectomycorrhizal root tips representative of *B. edulis* morphotype, obtained from each plot and sampling date, were separated and frozen. Molecular confirmation was carried out by amplification of the rDNA ITS region by PCR reactions using the universal primers for fungi ITS1F and ITS4 (Gardes and Bruns 1993). The PCR products were purified with a Roche® High Pure PCR Product Purification Kit (Roche Applied Science, IN, USA) and sequenced with both primers using a 3730 DNA Analyzer (Applied Biosystems, CA, USA). The forward and reverse sequences obtained were aligned (BioEdit Sequence Alignment Editor v. 7.0.9.0.) and identified by selecting the closest matches of a megablast searching in the GenBank database. Sequences were deposited in the GeneBank database with the accession numbers JF728991 to JF729002 and JF739384.

Samples collected in Pinares Llanos, were processed as explained before, but ectomycorrhizal root tips of *L. deliciosus* morphotype were only identified in 8 of the 12 months sampled, from each plot and sampling point (from September 2010 to January 2011 and from April to June 2011). Molecular confirmation was also carried out by amplification of the ITS region, as for *B. edulis*.

2.3. Seasonal dynamics of *Boletus edulis* and *Lactarius deliciosus* extraradical mycelium in pine forests of central Spain.

2.3.1. Study site

The study sites were located in the Iberian System mountain range, in the inner north-east zone of the Iberian Peninsula: the annual dynamics of *B. edulis* extraradical mycelium was performed in four of the plots described before and used for the validation of the method (section 2, a) in tins chapter page 7). In the last three production seasons (2008 to 2010) the mean average productions in the studied area was 9.23 kg/ha.

The study site for the quantification of *L. deliciosus* mycelium was located in Pinares Llanos, a homogeneous *Pinus pinaster* Ait. stand located in Almazán (Soria, Spain), covering an area of 10,994 ha. The altitude ranges from 1,000 to 1,200 m above sea level. Soils are regosols and arenosols on a predominance of tertiary and quaternary materials, with slightly acid pH (5-6), sandy texture and low fertility. Soil properties are detailed in Table 2.2. The average annual rainfall is between 500 and 700 mm/year (93 mm/year in summer and 164 mm/year in autumn). The medium average temperature is 11.8 ºC. Trees are harvested every 80 years, with a regeneration period of 10 years. The management of the forest uses a rotation of 80 years and a regeneration period of 20 years. Actually, the management is based on thinning by clear cutting with soil movement and sowing.

Top soil (0-20 cm) properties in Pinares Llanos													
Plot	Sand (%)	Silt (%)	Clay (%)	Coarse elements (%)	pH (H_2O)	Organic matter (%)	N (%)	K (ppm)	Mg (ppm)	Ca (ppm)	Fe (ppm)	P (ppm)	C/N
Pp10	82	14	4	6.9	6	1	0	66	25	375	1791	1.92	25
Pp6	83	12	5	2.0	6.2	1.4	0.1	104	51	501	2223	2.33	23
Pp8	82	14	4	13.5	6.4	0.6	0.1	59	37	409	1987	5.13	8
Pp5	84	11	5	9.9	5.1	1.3	0	55	41	310	2341	0.63	33

Table 2.2. Top soil (0-20 cm) properties measured in the four sampling plots

From 1997 to 2011, the average production of *L. deliciosus* in Pinares Llanos was 6.08 kg/ha. In the last three production seasons (2009 to 2011), the mean average productions in the studied plots was 4.87 kg/ha (Table 2.3), being the most productive stands those belonging to the age class comprised between 11 and 20 years old (Ágreda 2012).

Boletus edulis (kg/ha)			*Lactarius deliciosus* (kg/ha)		
Plot	**1995/2007**	**2009 /2010**	**Plot**	**1997/2009**	**2010/2011**
9A	38.49	6.4	Pp10	4.14	0
4C	16.47	48.9	Pp6	21.98	9.4
6D	10.97	66.9	Pp8	0	0
7B	0	3.3	Pp5	4.40	5.5

Table 2.3. Mean production of *Boletus edulis* sporocarps (kg/ha) and *Lactarius deliciosus* sporocarps (kg/ha) between 1995 and 2007 and 1997 and 2009 (used to select the plots to represent a gradient between minimum and maximum productivity); and in the harvesting season 2009-2010 and 2010-2011 respectively.

Climatic data were obtained from nearby measurement stations in each study site. The parameters used were those available in each weather station (Figure 2.2). We have missing data in Pinar Grande for the temperature from February to April (three months) and humidity for the months of December 2009 and from February to April 2010 (four months) due to problems in the measurement station. An estimation of the temperatures of the months with missing data was done, using temperatures from another nearby measurement station.

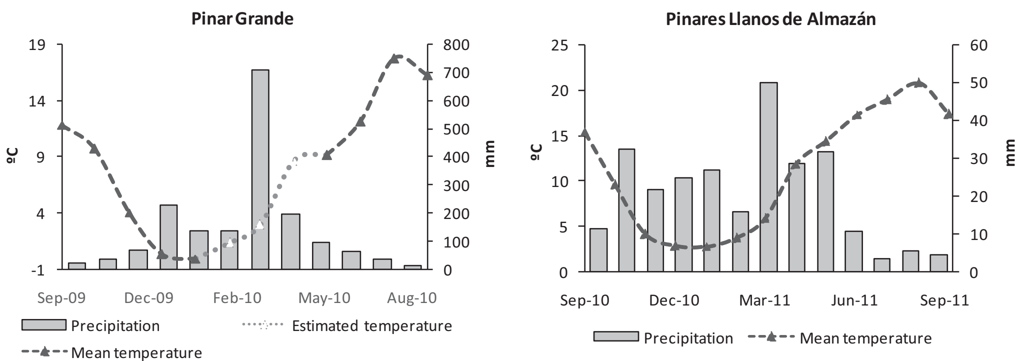

Figure 2.2. Mean precipitation (mm) and mean temperature (^{o}C) from September 2009 to august 2011 (12 months) in Pinar Grande and from September 2010 to September 2011 (13 months) in Pinares Llanos de Almazán.

2.3.2. Soil sampling

In Pinar Grande one plot in each area was selected for soil sampling. Soil was sampled monthly for one year in each plot, and at each sampling time, five soil samples were randomly extracted, with a minimum distance of 30 cm apart from any tree trunk, using a cylindrical (2 cm radius, 20 cm deep —250 cm3) soil borer (Taylor 2002). A total of 240 soil samples were processed (five sample points in each of the four plots every month) from September 2009 to August 2010.

In Pinares Llanos, permanent fenced plots established since 1997 for quantifying autumnal sporocarps production (Fernández-Toirán et al. 2006) were used. These areas, performed by stand age class according to the forest management plan, cover 150 m^2 (5 x 30 m) each. Soils were sampled in four plots selected by their average production of sporocarps between 1995 and 2009 to represent a gradient between minimum and maximum productivity (Table 2). Five soil samples were taken monthly along a random transect in each plot, harvesting the samples at a minimum distance of 30 cm apart from any tree trunk, with the same cylindrical soil borer used in Pinar Grande. Five samples per plot were harvested for 12 months (from September 2010 to September 2011, no sampling on August 2011), having a total number of 240 samples processed. Soil samples of both study sites were let to dry at air temperature (20-25 ºC) to constant weight and then sieved (1 mm mesh diameter) and homogenized. Ten grams of each soil sample was separated and stored at -20 ºC for molecular analysis.

2.3.3. Soil DNA extraction

DNA extractions were performed with the PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) from 0.25 g of soil per sample according to manufacturer’s instructions. A total of 480 samples (240 of each study site) were processed. The extracted DNA was stored at -20°C until use.

2.3.4. Quantification of extraradical soil mycelium by real-time PCR

Real–time PCRs were performed using the specific probes and primers designed for *B. edulis* (section 1, a in page 5) and by Parladé et al. (2007) for *L. deliciosus* (complete oligonucleotides in Table A.3 in the Annex).

Two standard curves for mycelium quantification were generated, one for each location/species, using known amounts of mycelium from active growing colonies of *B. edulis* and *L. deliciosus* as described by Parladé et al. (2007), Hortal et al.

(2008), and De la Varga et al. (2012). For the *B. edulis* mycelium quantification the standard curve used was the same described before for the validation of the method (figure 2.3). The standard curve for the detection and quantification of *L. deliciosus* mycelium in Almazán was generated as for *B. edulis* (section 1, c in page 6), but starting from 1200 mg of *L. deliciosus* grown on a cellophane sheet on BAF medium, and mixed with 0.5 g of a soil collected in Pinares Llanos. The soil was previously checked for the absence of *L. deliciosus* DNA by conventional PCR with universal (ITS1F/ITS4) and specific fungal primers ITS1F/LdITS2R for *L. deliciosus* (Hortal et al. 2006). Tenfold serial dilutions from the extract were prepared until 10^{-6}, obtaining serial concentrations of 24.0000, 2.4000, 0.2400, 0.0240, 0.00240, 0.000240, and 0.000024 mg of mycelium/g of soil for *L. deliciosus.*

Boletus edulis DNA extracts were amplified by real-time PCR as described in page 9; and *L. deliciosus* DNA extracts were amplified by real-time PCR in a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), with the Premix Ex Taq™ (Perfect Real Time) (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions for a final reaction volume of 20 µl with a concentration of 800 nM of each primer and 200 nM of probe. Five microliters of DNA extracted from soil samples was added as a template for each reaction. The analyses were performed on 48-well plates (MicroAmp® Fast Optical 48-Well Reaction Plate). Three replicates of each sample were included in the analysis, as well as a negative control (using deionized water instead of DNA template) and the standards (three replicates of each serial dilution) in each plate. Real-time PCR cycling conditions were 30 s at 95 °C, 40 cycles at 95 °C for 5 s, and 34 s at 60 °C.

LightCycler® data were processed with the LightCycler® 480 Software v1.5; StepOne™ data were processed with the StepOne™ Software v2.2.1. The results (µg target DNA in 20 µl PCR mixture) were converted to milligrams mycelium per gram soil. Ct values (cycle number at which the fluorescence emission exceeds a fixed threshold established in the exponential phase of the amplification curve) for each concentration were plotted against the logarithm of the corresponding amount of mycelium to generate the standard curve. Absolute quantification of mycelium biomass of *B. edulis* and *L. deliciosus* in each soil sample was determined by interpolation of the Ct value in the standard curve.

2.4. Data analyses

Statistical significance of the differences in the number of mycorrhizal tips per volume of soil and soil mycelial concentration (mg mycelium/g soil) obtained in the

different plots and sampling dates were determined by a multifactorial analysis of variance. Differences between means were analyzed by Tukey's honestly significant difference (HDS) test ($\alpha = 0.05$). The correlations between soil mycelium biomass, abundance of mycorrhizas, plot productivity and climatic parameters were determined by Pearson's bivariate correlation analysis. The correlations between sporocarps production with climatic data and soil mycelium concentration, for the months of the fructification period, were also done by Pearson's bivariate correlation analysis. Data were transformed when necessary in order to meet the assumptions of parametric statistical tests. Statistical analyses were performed with JMP®, version 7 (SAS Institute Inc., Cary, NC, USA; 1989-2007).

3. Results

3.1. Validation by comparison of conventional PCR and quantitative real time PCR.

Conventional PCR using specific primers allowed detecting the presence of *B. edulis* mycelium in 19 of the 75 soil samples. Real-time TaqMan PCR, using the specific primers and probe designed, allowed to detect *B. edulis* in 30 additional extractions, giving a total of 49 positive samples. The lowest limit of detection using conventional PCR, with the specific primers Bedu1F and Bedu2R, was calculated to be around 0.039 mg of mycelium per gram of soil, according to the quantification by real-time PCR (Table 2.4). The results obtained by conventional PCR were not consistent when the concentration of soil mycelium ranged between 0.039 and 0.321 mg/g soil, finding either positive or negative amplifications in the given range. Samples with soil mycelial concentrations higher than 0.321 mg/g resulted always in positive amplification by conventional PCR with specific primers. Real-time PCR, using the oligonucleotides and conditions established in the present work, detected concentrations as low as 0.001 mg of mycelium per gram of soil.

The concentration of *B. edulis* mycelium in the soil (mg mycelium/g soil) did not differ significantly between plots (Table 2.5), irrespective of their average productivity in terms of kg of sporocarps of *B. edulis* collected during the preceding 10 years. The correlation between the concentration of soil mycelium and the productivity of the plots reported between 1995 and 2007 was not significant ($F=0.595$, $p=0.443$). Also, there were no significant differences in soil mycelial

concentration over time, from September to November, within the fructification period (Tables 2.6). The number of mycorrhizal short roots per soil volume showed significant differences between the plots and the three sampling times. No interactions between the two parameters were detected (Table 2.7). The number of mycorrhizal tips varied significantly over time, detecting the highest number of mycorrhizas per soil volume in September (79.8 mycorrhizas/250 cm^3 of soil), being significantly reduced in October and November (Table 2.6). No significant correlation between the number of mycorrhizas and the productivity of the plot (kg of *B. edulis*/ha year) was detected (F=0.255, p=0.615).

Plot	Sampling point	September		October		November	
		RT-PCR	*PCR*	*RT-PCR*	*PCR*	*RT-PCR*	*PCR*
9A	a	0.310	+	0.105	+	0.347	+
9A	b	0.000	-	0.000	-	0.000	-
9A	c	0.001	-	0.067	-	0.033	-
9A	d	0.026	-	0.000	-	0.006	-
9A	e	0.000	-	0.000	-	0.000	-
4C	a	0.149	+	0.077	-	0.039	+
4C	b	0.007	-	0.004	-	0.002	-
4C	c	0.001	-	0.000	-	0.000	-
4C	d	0.001	-	0.000	-	0.000	-
4C	e	0.000	-	0.000	-	0.000	-
6D	a	0.136	-	0.089	-	0.085	+
6D	b	0.000	-	0.000	-	0.000	-
6D	c	0.671	+	0.018	-	0.091	+
6D	d	0.139	+	0.070	+	0.032	-
6D	e	0.000	-	0.027	-	0.000	-
5A	a	0.951	+	0.592	+	0.853	+
5A	b	0.321	-	0.137	-	0.299	-
5A	c	0.387	+	0.088	+	0.041	+
5A	d	0.000	-	0.000	-	0.000	-
5A	e	0.138	+	0.019	-	0.049	+
7B	a	0.001	-	0.001	-	0.004	-
7B	b	0.016	-	0.150	-	0.119	+
7B	c	0.000	-	1.799	+	0.000	-
7B	d	0.004	-	0.005	-	0.001	-
7B	e	0.000	-	0.000	-	0.001	-

Table 2.4. Detection of *B. edulis* mycelia in soil samples by real-time PCR (RT-PCR) in mg mycelium/g soil and conventional PCR using specific primers. Positive (+) or negative (-) amplifications were determined by the presence or absence of a 750 bp band in a 2% agarose gel electrophoresis. Both conventional and real-time PCR were performed using the first extraction made in each of the five sampling points per plot.

Analysis of Variance for ‘*B. edulis* Mycelium quantity’					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**	**Prob> F**
Model	14	1.173	0.084	1.166	0.3245
Error	60	4.312	0.072		
C. Total	74	5.484			
Effect tests					
Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob> F**	
Plot	4	0.519	1.805	0.1397	
Date	2	0.039	0.270	0.7643	
Plot*Date	8	0.615	1.070	0.3962	

Table 2.5. Analysis of variance for the concentration of *B. edulis* mycelium in soil samples (mg of mycelium / g of soil) (Test of Brown-Forsythe: Prob > F = 0.2063).

Plot	**nº of mycorrhizas**			**mg mycelium / g of soil**	
9A	14.87		B	0.085	A
4C	42.07	A	B	0.019	A
6D	97.73	A		0.258	A
5A	23.93	A	B	0.058	A
7A	14.33		B	0.140	A
Date					
September	79.84	A		0.129	A
October	27.44		B	0.128	A
November	8.48		B	0.080	A

Table 2.6. Number of mycorrhizal short roots per 250 cm^3 of soil and soil mycelial concentration (mg mycelium / g soil) obtained in the different plots and sampling dates. Values within each analysis sharing the same letter are not different by Tukey’s HSD test ($\alpha = 0.050$)

Analysis of Variance for ‘*B. edulis* ectomycorrhizas’					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**	**Prob> F**
Model	14	128.471	9.176	3.195	0.0009*
Error	60	172.334	2.872		
C. Total	74	300.805			
Effect tests					
Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob> F**	
Plot	4	47.500	4.134	0.0050*	
Date	2	63.589	11.070	< 0.0001*	
Plot*Date	8	17.382	0.756	0.6491	

Table 2.7. Analysis of Variance of the number of mycorrhizal short roots (log transformed) per volume of soil (250 cm^3). (Test Brown-Forsythe: Prob > F = 0.9047).

3.2. Seasonal dynamics of *Boletus edulis* and *Lactarius deliciosus* extraradical mycelium in pine forests of central Spain.

The standard curves for the quantification of extraradical mycelium of *B. edulis* and *L. deliciosus* are shown in figure 2.3. Both curves satisfy the requirements for real-time PCR in terms of efficiency (R^2 = 0.99 and efficiencies of 98 % and 103.14 %, respectively). Using the oligonucleotides and conditions established in the present work, we detected concentrations as low as 0.001 mg *B. edulis* mycelium per gram soil, and 0.002 mg *L. deliciosus* mycelium per gram of soil. From the total of the 480 soil samples analyzed, 345 showed positive amplification (152 for *B. edulis* samples and 193 for *L. deliciosus* samples).

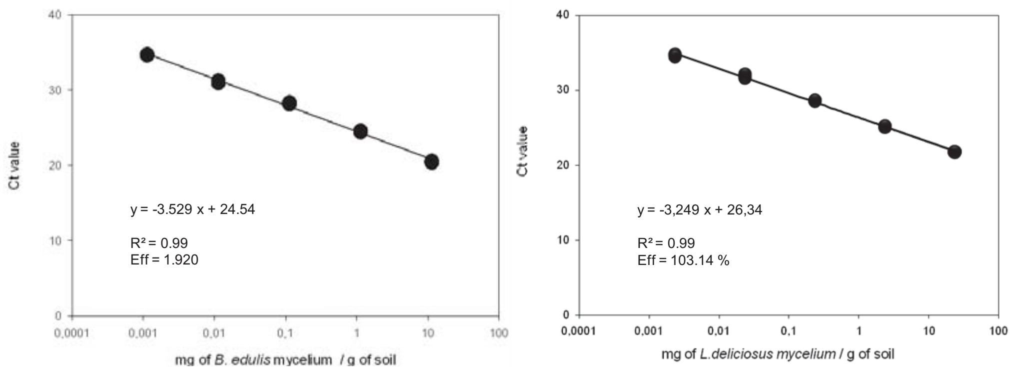

Figure 2.3. Standard curve obtained for mycelium quantification of *Boletus edulis* in Pinar Grande (A) and *Lactarius deliciosus* in Pinares Llanos de Almazán (B) in soil by real-time TaqMan PCR. The curves were generated by plotting the obtained Ct values against the logarithm of known amounts of mycelium added to soil.

The seasonal dynamics of the mycelium amount (milligrams mycelium per gram of soil) for *B. edulis* and *L. deliciosus* are shown in figure 2.4. For *B. edulis* the maximum amount of soil mycelium was detected between January and April, with a peak in February 2010 (an average of 0.16 mg mycelium/g soil). We found the opposite situation in October 2009 (an average of 0.017 mg of *B. edulis* mycelium/g soil). A second peak of slight increase of mycelial concentration was detected in summer (June – August).

The concentration of *B. edulis* mycelium in the soil (milligrams mycelium per gram of soil) (Table 2.8) showed no significant differences between plots with different sporocarp production, but there was a significant difference over time. The mean highest amount of *B. edulis* mycelium was detected in the samples taken in February 2010 (0.157 mg mycelium/g soil) and the minimum mean was detected in October 2009 (0.017 mg mycelium/g soil) (Table 2.9). Tukey's test separate months

into three groups, from which we must highlight the significantly different group formed by the samples taken from January to April, when we detected the highest amount of *B. edulis* mycelium in the soil. No interactions between the two factors (plot and date) were detected (Table 2.8).

Analysis of Variance of the '*B. edulis* mycelium concentration'					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**	**Prob > F**
Model	47	3114.5732	66.2675	2.0459	0.0004*
Error	192	6218.8565	32.3899		
C. Total	239	9333.4297			

Effect Tests					
Source	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Plot	3	3	148.7975	1.5313	0.2077
Date	11	11	1976.4767	5.5474	<.0001*
Plot*Date	33	33	989.2990	0.9256	0.5885

Table 2.8. Analysis of variance of the concentration of mycelium of *Boletus edulis* in the soil samples of Pinar Grande

Dates	**mg mycelium / g soil**				**Std err**	***N***
Sep-09	A			0.0307	0.0129	20
Oct-09	A			0.0170	0.0072	20
Nov-09	A	B		0.0201	0.0045	20
Dec-09	A			0.0241	0.0144	20
Jan-10	A	B	C	0.1058	0.0681	20
Feb-10			C	0.1576	0.0665	20
Mar-10	A	B	C	0.1492	0.0693	20
Apr-10		B	C	0.1102	0.0533	20
May-10	A	B		0.0362	0.0199	20
Jun-10	A	B		0.0611	0.0353	20
Jul-10	A			0.0352	0.0223	20
Aug-10	A	B		0.0748	0.0363	20

Table 2.9. Means and Tukey HSD test (α=0.05) for the amount of *Boletus edulis* mycelium in soil. Dates not sharing same letter are significantly different

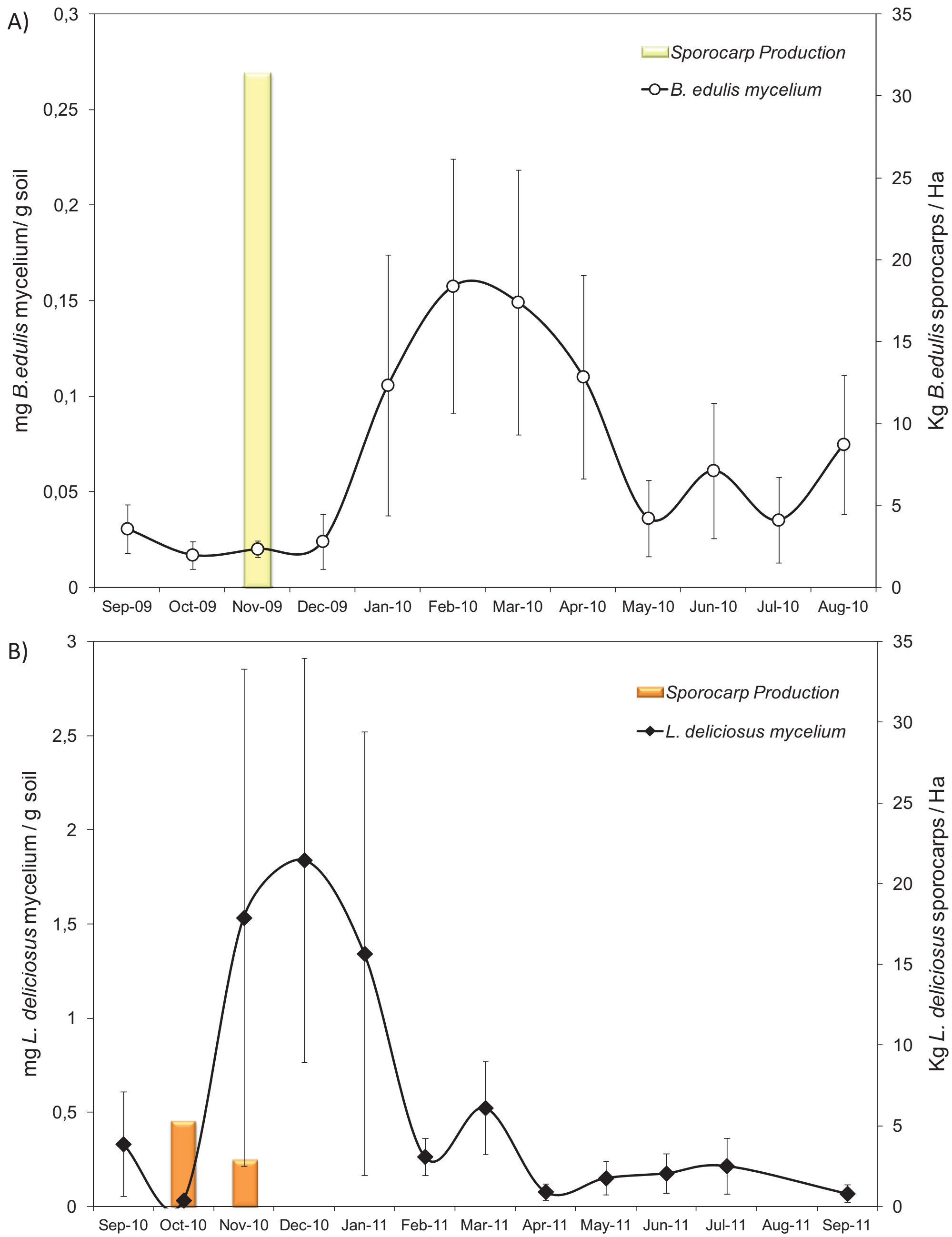

Figure 2.4. Mycelium dynamics and sporocarp production for *Boletus edulis* (A) and *Lactarius deliciosus* (B) over 12 months. Mean amounts of mycelium quantities are expressed as mg of mycelium/g soil (left axis) and sporocarp production as Kg/Ha (right axis). The bars show the standard error for each mean (N=20 for each month).

For *L. deliciosus*, the pattern of mycelium concentration is similar to that of *B. edulis*, but the production peaks are displaced 2 months (Figure 2.4). The period of maximum production of mycelia runs from November to January, with a peak in December (1.84 mg mycelium/g soil) and the minimum mycelium average in October (0.033 mg mycelium/g soil). There is also a second peak, less pronounced, in March. The statistical analyses (Table 2.10) show a significant difference between the plots where the samples were taken and also over time (with a clear differentiation of December, when we detected the maximum amount of *L. deliciosus* mycelia). Tukey's test divides the plots sampled into two groups: Pp-10 with Pp-6 and Pp-5 with Pp-8 (Table 2.11).

For *B. edulis* from Pinar Grande, correlations of mycelium quantities with climatic data were tested. The analysis (Table 2.12) showed a positive correlation between the mycelium quantities of *B. edulis* in soil (mg mycelium/g soil) and the precipitation amounts (mm). No correlations were found with other parameters as relative humidity (%) neither with monthly temperature means (^{o}C). A negative correlation was found between the mycelium quantity and the mean temperature of the previous month. For data obtained from *L. deliciosus* in Pinares Llanos, the correlations of mycelium quantities with climatic parameters (Table 2.13) showed negative correlations between the mycelium quantity (mg mycelium/g soil) and the (1) monthly temperature means (^{o}C) and (2) the radiation (MJ/m^{2}). Positive correlations between the mycelium and the average monthly relative humidity (%) were detected. No significant correlations were found with precipitation (mm) data.

Analysis of Variance					
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	47	2901.1027	61.7256	1.6713	0.0086*
Error	191	7054.1664	36.9328		
C. Total	238	9955.2692			
Effect Tests					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Date	11	11	914.3761	2.2507	0.0135*
Plot	3	3	678.6271	6.1249	0.0005*
Date*Plot	33	33	1325.8865	1.0879	0.3517

Table 2.10. Analysis of variance of the concentration of mycelium of *Lactarius deliciosus* in the soil samples of Pinares Llanos.

The sporocarp production of *B. edulis* and *L. deliciosus* are shown in Table 2.4, respectively. No correlations between sporocarp production and soil mycelium

concentration were found. No correlations were found between the *B. edulis* sporocarp production and weather parameters. The correlations between *L. deliciosus* sporocarp production and climatic parameters for the fructification period (Table 2.13) showed a positive correlation between production and (1) precipitation (mm) and (2) maximum relative humidity (%). Negative correlations of sporocarp production with the maximum and minimum temperatures (°C) were detected.

Figure 2.5. Detail of two *L. deliciosus* sporocarps in the forest

			mg mycelium / g soil	Std err	*N*
Plots					
Pp-8	A		0.2798	0.1202	60
Pp-5	A	B	0.4981	0.3411	60
Pp-6		B	0.7563	0.4059	60
Pp-10		B	0.6326	0.4495	60
Dates					
Sep-10	A	B	0.3321	0.2788	20
Oct-10	A	B	0.0332	0.0138	20
Nov-10	A	B	1.5339	1.3196	20
Dec-10		B	1.8400	1.0738	20
Jan-11	A	B	1.3431	1.1780	20
Feb-11	A	B	0.2652	0.0980	20
Mar-11	A	B	0.5242	0.2467	20
Apr-11	A	B	0.0789	0.0436	20
May-11	A	B	0.1514	0.0869	20
Jun-11	A	B	0.1767	0.1060	20
Jul-11	A	B	0.2156	0.1474	20
Sep-11	A		0.0690	0.0465	20

Table 2.11. Mean differences analysis by Tukey test (α= 0.05) of the concentration of mycelium of *Lactarius deliciosus* in the soil samples of Pinares Llanos for Plots and Dates. Levels not sharing same letter are significantly different.

Variables correlated	Species	R	Signif.
Mycelium biomass - Mean temperature (°C)	*B. edulis*	- 0,0152	0,8398
Mycelium biomass - Mean temp. prev. month (°C)	*B. edulis*	- 0.4226	0.0028*
Mycelium biomass - Relative Humidity (%)	*B. edulis*	- 0.0284	0.7217
Mycelium biomass - Radiation (MJ/m2)	*B. edulis*	–	–
Mycelium biomass - Monthly Precipitation (mm)	*B. edulis*	0.1627	0.0157*
Mycelium biomass - Mean temperature (°C)	*L. deliciosus*	- 0.1616	0.0124*
Mycelium biomass - Mean temp. prev. month (°C)	*L. deliciosus*	- 0.1145	0.0773
Mycelium biomass - Radiation (MJ/m2)	*L. deliciosus*	- 0.1710	0.0450*
Mycelium biomass - Relative Humidity (%)	*L. deliciosus*	0.1527	0.0182*
Mycelium biomass - Monthly Precipitation (mm)	*L. deliciosus*	- 0.0919	0.1568

– No data available

* Significant correlation level (p ≤ 0.05)

Table 2.12. Correlations between mycelium biomass (mg mycelium/g soil) and climate variables (Spearman correlation coefficient, significance level ≤ 0.05). The climatic data used were from the parameters available in the weather station of each study site. *R*: Correlation coefficient; *Signif*.: Signification probability

Variables correlated	Species	*R*	*Signif.*
Production - Mean temperature (°C)	*L. deliciosus*	- 0.2223	0.9812
Production - Mean Max. temperature (°C)	*L. deliciosus*	- 0.3158	0.0043*
Production – Mean Min. temperature (°C)	*L. deliciosus*	- 0.2438	0.0293*
Production - Radiation (MJ/m2)	*L. deliciosus*	- 0.2025	0.8743
Production - Relative Humidity (%)	*L. deliciosus*	- 0.2539	0.7500
Production – Max. Relative Humidity (%)	*L. deliciosus*	0.3635	0.0009*
Production - Monthly Precipitation (mm)	*L. deliciosus*	0.3958	0.0003*

* Significant correlation level (p ≤ 0.05)

Table 2.13. Correlations between *Lactarius deliciosus* sporocarps production (kg/ha) and climate variables (Spearman correlation coefficient, significance level ≤ 0.05). Data from months corresponding to the fructification period of *L. deliciosus* (September-October-November-December). The climatic data used were those available from the weather station. *R*: Correlation coefficient; *Signif*.: Signification probability

3.3. Quantification and dynamics of *Boletus edulis* and *Lactarius deliciosus* ectomycorrhizas.

The results of the quantification of *B. edulis* and *L. deliciosus* ectomycorrhiza are shown in Figure 2.6. For *L. deliciosus* ectomycorrhizas were counted from samples of 8 months (September, October, November, December, January, Mars – only the Pp-5 samples – May, June and July).

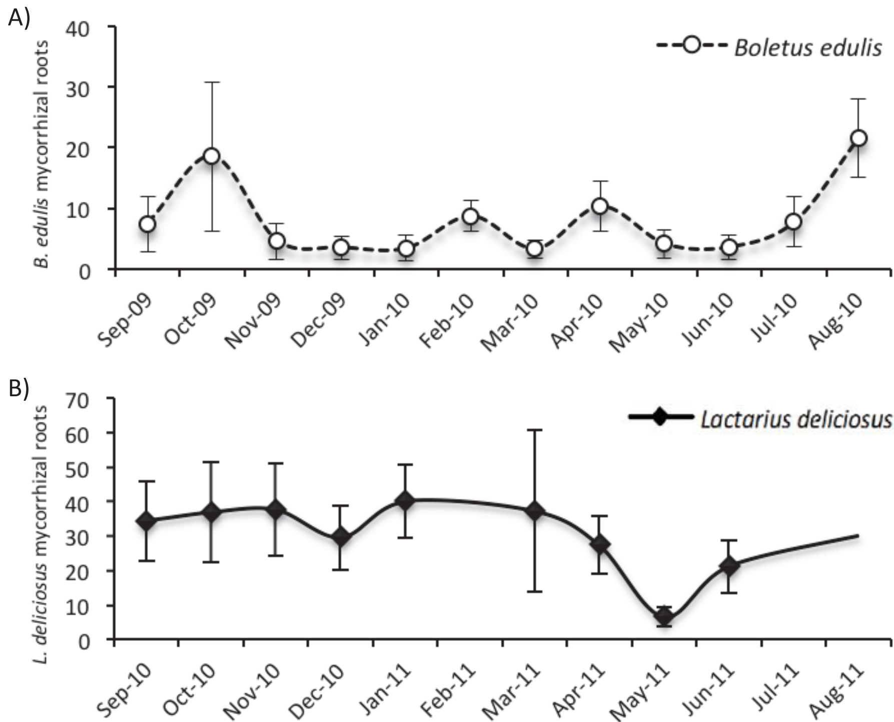

Figure 2.6. Mycorrhiza dynamics for *Boletus edulis* (A) and *L. deliciosus* (B) over 12 months. Missing data for February, July and August 2011. Mean number of mycorrhizas is expressed as mycorrhizal roots/250 cm^3 soil. Bars show the standard error for each mean (N=20 for each month, N=5 for March 2011).

The analysis of variances for the Pinar Grande samples showed significant differences along time and between the four plots. No interaction between factors was detected (Table 2.14). The highest number of *B. edulis* mycorrhiza was detected in October 2009 (244 mycorrhizas in 250 cm^3 of soil), and the highest mean in August 2010 (21.65 mycorrhizas in 250 cm^3 of soil); the minimum mean was the one for March 2010 (3.3 mycorrhizas in 250 cm^3 of soil). Tukey's test separates months in three groups and divides plots into 2 groups (Figure 2.7), in which the ones that have more mycorrhizal roots are the ones that had more sporocarp production on that season.

For the *L. deliciosus* mycorrhizas collected in Pinares Llanos, the analysis of variances only showed significant differences between the four plots ($p = 0.0272$; $\alpha=0.05$), no differences along time were detected ($P = 0.4426$; $\alpha=0.05$). The highest number of *L. deliciosus* mycorrhiza was detected in October 2010 (236) and the minimum in May 2011 (3.3). Tukey's test separates months in three groups (Table 2.15) and divides plots into 2 groups (Figure 2.7).

Analysis of Variance					
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	47	365.1685	7.76954	1.4668	0.0384*
Error	192	1017.0015	5.29688		
C. Total	239	1382.1700			
Effect Tests					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Date	11	11	117.66763	2.0195	0.0285*
Plot	3	3	116.33889	7.3212	0.0001*
Date*Plot	33	33	131.16198	0.7504	0.8353

Table 2.14. Analysis of variance of the number of *B. edulis* ectomycorrhiza in the soil samples of Pinar Grande.

Dates	nº of mycorrhizas				Std err	N
Sep-09			C	7.45	4.5731	20
Oct-09		B	C	18.6	12.3539	20
Nov-09			C	4.65	2.9747	20
Dec-09			C	3.55	1.9132	20
Jan-10			C	3.4	2.2236	20
Feb-10	A	B		8.7	2.6439	20
Mar-10			C	3.3	1.5063	20
Apr-10	A	B	C	10.3	4.1834	20
May-10			C	4.1	2.3462	20
Jun-10			C	3.6	2.0538	20
Jul-10		B	C	7.75	4.2388	20
Aug-10	A			21.65	6.4890	20

Table 2.15. Means and Tukey's HSD test (α=0.05) for the amount of *B. edulis* ectomycorrhizas in soil (250 cm^3). Dates not connected by same letter are significantly different.

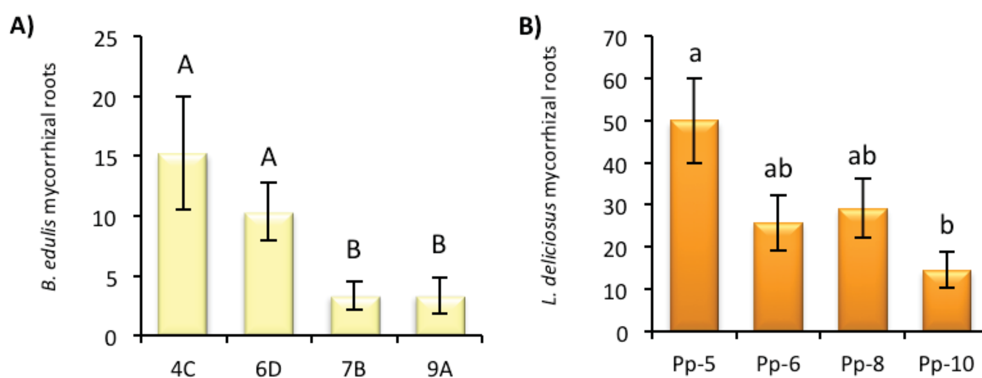

Figure 2.7. Means and Tukey's HSD test (α=0.05) for the amount of A) *B. edulis* and B) *L. deliciosus* mycorrhizal roots in soil (250 cm3) in each plot. Plots not connected by same letter are significantly different.

3.4. Relation of extraradical mycelium and ectomycorrhiza of *Boletus edulis* and *Lactarius deliciosus*.

A statistically significant positive correlation was detected between the concentration of mycelia of *B. edulis* in the soil samples and the amount of short roots mycorrhizal with *B. edulis,* in these samples, in autumn 2008 ($R = 0.2290$; $p = 0.0481$) and along the 12 months analyzed, from September 2009 to August 2010 ($R = 0.287$; $p = 0.0305$) (Figure 2.8). No correlation was detected between *L. deliciosus* number of mycorrhizal roots and mycelium amounts.

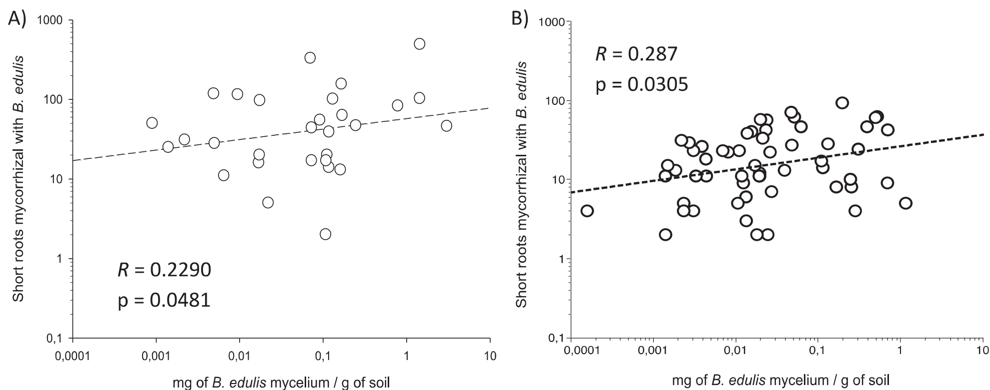

Figure 2.8. Correlation between the number of *B. edulis* ectomycorrhizal rootlets in 250 cm^3 of soil and the concentration of *B. edulis* soil mycelium (mg/g) per soil sample (α=0.05). A) From September to November 2008, 3 months; and B) from September 2009 to August 2010, 12 months.

4. Discussion

DNA quantification by real-time PCR using specific primers and probes provides the estimation of extraradical mycelium biomass for individual species in soil. Specific primers and probe for *B. edulis* (Fwd-Bedu, Rvs-Bedu, and STQBedu), have been designed in the rDNA ITS1 region. The internal transcribed spacer region of ribosomal DNA has been widely used because different specific primers have been designed to identify *B. edulis* (Moor et al. 2002; Mello et al. 2006; Bin et al. 2008) and several group-specific primer sets exist (Gardes and Bruns 1993; Martin and Rygiewicz 2005). Large quantities of sequences are available for this gene region on a wide range of fungal species, making easier to design specific and efficient primers for real-time PCR. Nevertheless, ITS regions of the ribosomal DNA gene are multicopy regions of the genome (Gardes and Bruns 1993), known to be different

in number between species (Debaud et al. 1999). Since copy numbers of ITS genes on the genome vary between fungal species, it would be most reliable to use a gene with a known and constant number of copies for quantification purposes. In our studies, we quantified the presence of two single species, *B. edulis* and *L. deliciosus*, and the number of copies of the ITS region is assumed to be constant within a species (Cassidy et al. 1984; Raidl et al. 2005).

Real-time TaqMan PCR allowed the quantification of the fungal mycelium, and compared to conventional PCR, increased the detection of mycelium of *B. edulis* in soil samples in a 158%. Real-time PCR detected amounts as low as 0.0011 mg of *B. edulis* mycelium per gram of soil in the samples taken in the *P. sylvestris* forest of Pinar Grande. The conditions of both PCR reactions (conventional and real-time) are different and this affects the efficiency of the reaction resulting in a greater effectiveness of real-time PCR in order to detect the presence of the target fungus DNA when small quantities are present in the sample.

The size of the fragments amplified by the specific primers designed to real-time PCR was 75 bp, approximately 6.8 times shorter than the fragment amplified by conventional PCR using the primers Bedu1F and Bedu2R. Due to the size of the fragment amplified, real-time PCR might be less sensitive than conventional PCR to DNA fragmentation that might occur during processing of samples, and it could contribute to improve the effectiveness of real-time PCR. But, at the same time, it must be taken into account that it would be possible that we were detecting the presence of DNA extracted from mycelium already dead, introducing an overestimation in the quantification of the active mycelium. Using a vital stain, it is possible to determine whether living and/or dead fungal hyphae are being quantified, but this distinction is not possible when analyzing fungal DNA extracted from soil (Landeweert et al. 2003). On the other hand, Lindahl et al. (2010) found that dead mycorrhizal mycelium in the soil is rapidly degraded by opportunistic saprotrophic microorganisms. Also, Parladé et al. (2007) showed that the extraction and quantification of DNA from mycelium growing in a Petri dish with nutrient medium is dramatically reduced with aging of the colony. A long persistence of dead mycelium, or their DNA, in a medium as a forest soil is unlikely, and DNA recovered in the extraction procedure is more likely to belong to actively growing mycelium.

DNA quantification by real-time PCR using specific primers and probes provides the estimation of extraradical mycelium biomass for individual species in soil. Using this technique, it has been possible to do a first approach to the dynamics of the

extraradical mycelium of *B. edulis* during the autumn period, and to evaluate the dynamics of the extraradical soil mycelium of *B. edulis* and *L. deliciosus* for a whole year.

Soil DNA concentration is considerably heterogeneous. The application of molecular techniques based in DNA analyses in environmental samples strongly depends on the availability of reliable DNA extraction protocols for different types of soils (Feinstein et al. 2009). In our experiments, we used a commercial kit (PowerSoil™ DNA Isolation Kit) specific for soil DNA extraction, that has been reported as efficient to reduce de coextraction of PCR inhibitory substances (Dineen et al. 2010). We focused on only one fungal species on each experiment (*B. edulis* and *L. deliciosus*) thus, great biases due to differences in efficiency during the extraction protocol were not expected. It is accepted that standard PCR amplifications were inhibited when DNA concentrations exceeded 1 µg/ml in the reaction mixture. DNA extracted in each soil sample was spectrophotometricaly quantified (A 260/280) rending a value of 1.92 in average. Total DNA extracted from a gram dry weight of soil ranged from 71.6 to 409.2 µg/g, and the mean concentration of total DNA in the real-time PCR reaction mixture was 0.12 µg/ml (±0.07 SD).

Guidot et al. (2002) found between 10 and 0.07 ng/gsoil of *Hebeloma cylindrosporum* Romagn. DNA in the vicinity of fruit bodies. Increasing the number of soil replicates extracted per treatment and testing each DNA extract in triplicate would possibly reduce the observed fluctuations (Landeweert et al. 2003). In the *B. edulis* experiment done to adapt the technique, five sampling points per plot were used and DNA extractions were repeated five times per sample. Nevertheless, a high variability was still detected among mycelial concentrations. To minimize plot variability, the sampling design of ectomycorrhizal fungal mycelia could be optimized in future experiments collecting larger or pooled soil core samples (Guidot et al. 2002; Ranjard et al. 2003; Kang and Mills 2006). A further bias factor when quantifying mycelium by molecular techniques based in DNA analysis is the potential presence of both spores and mycelium in a single sample, which are likely to be co-extracted during DNA extraction process. The possibility of detecting basidiospores-derived DNA instead of mycelial DNA is a potential problem when using molecular methods on soil extracts (Guidot et al. 2002). Some authors tried to avoid the extraction and amplification of spores by collecting soil samples either before (Landeweert et al. 2005) or after (Dickie et al. 2002) the sporocarp production season at the field sites. Nevertheless, the persistence of fungal spores

in soil is not well-known and could introduce a bias when considering soil samples, in particular during the fruiting season. In our experiment to validate the method, the samples were taken in 2008 during the fruiting season; but during all the period of sampling in the studied plots, only one sporocarp of *B.edulis* was recorded in plot 4C. Consequently, a great influence due to the presence of dispersed spores was not expected.

Boletus edulis and *L. deliciosus* reveal a mycelium dynamics characterized by a high seasonal variability with marked peaks. A seasonal pattern of the mycelium biomass has been reported in other works based on ergosterol and phospholipid fatty acids analysis of ectomycorrhizal mycelium colonization of in-growth mesh bags placed in soils of coniferous and deciduous forests (Wallander et al. 2001; Hagerberg and Wallander 2002; Nilsson et al. 2007; Wallander et al. 2011). These studies reported a high variability in mycelium biomass between locations and years, which could be due to climate variations, as suggested by Majdi et al.(2008).

When comparing the results obtained for the quantification of *B. edulis* mycelium in autumn 2009 with the ones from 2008, we have to highlight that the amounts detected in 2008 were 10 times higher than the ones detected in 2009 (average in autumn 2008, 0.125; average in autumn 2009, 0.015). However, the behavior of the different plots is similar (as in 2008, no differences between plots or time were observed for the 3 months) and the decrease of mycelium during those months is also detected 1 year after. The main difference between those years was that, in 2008, no sporocarp production was detected, whereas in 2009, there was sporocarp production in November.

Both fungal species, *L. deliciosus* and *B. edulis,* have a different exploration type of extraradical mycelium, as proposed by Agerer (2001; 2006). The first species belongs to the contact or medium-distance smooth subtype having their mycelia concentrated as hyphae emanating from the ectomycorrhizas and slightly differentiated rhizomorphs, and the second species belongs to the long-distance type having their mycelia concentrated as very thick, differentiated rhizomorphs. The anatomical features of those structures suggest a different spatial distribution in the soil and a different behavior in response to the environmental changes, which could explain that *L. deliciosus* mycelium biomass measured is 10 times higher than *B. edulis* mycelium biomass. The manner in which a determined species extends in the soil and the differentiation of their extraradical mycelium and rhizomorphs in ectomycorrhizal fungi are extremely important ecological factors for its own behavior and also for its host's behavior (Agerer 2001).

However, in our study, both species showed a similar distribution pattern over time, with a clear increase on the amounts of soil mycelium biomass during the coldest months of the year.

Growth and biomass of mycelium in soil are strongly affected by edaphic and climate factors such as nutrient status, temperature, and moisture (Nilsson and Wallander 2003; Sims et al. 2007; Wallander et al. 2011; Cairney 2012). In our study, the temperature significantly affected the mycelial biomass of both fungi, with an inverse relationship. It has not been possible to collect data of soil temperature, but using the data from a nearby measurement station, we have calculated that soil temperatures at 10 cm depth are, on average, 1.55 °C higher than the atmospheric temperatures. Temperature affects *B. edulis* in a more delayed way than *L. deliciosus,* which can be explained by the slower growth of the mycelium of the former species observed in pure cultures in the laboratory (data not shown). Other climate factors affecting the extraradical mycelium are relative humidity, radiation, and precipitation, parameters that are closely related and together may act as regulators of the mycelium production in both species. According to our results, relative humidity is related with the variation of mycelium of *L. deliciosus.* In the case of *B. edulis,* relative humidity was not statistically related with mycelium biomass, but its effect can be underestimated because of missing data of 4 months (three of them when the mycelial amounts are higher), and although for *B. edulis* radiation was not included in the analysis (no data available), this factor is strongly related with temperature and a similar response to this factor would be expected.

For both species, we found the minimum mycelium quantity before or at the same time as the fructification period, which could indicate an allocation of resources to produce the sporocarps. After these fructification periods, we found the maximums of soil mycelium. In these periods, the temperatures are the lowest (minimums of −14 °C in Pinar Grande and −12 °C in Pinares Llanos), and it is when the photosynthetic and respiratory activity of the host trees are lowered, with a reduction of the absorption of water and nutrients by the radical system. Mycorrhizal fungi depend on photosynthetically fixed carbon produced by their associated trees, which may drive fungal growth (Egli 2011). Anderson and Cairney (2007) stated that extraradical mycelium grows from ectomycorrhizal root tips to forage for mineral nutrients and seek new root tips for colonization. If the fungi receive less inputs from the plant (when temperatures are the lowest), extraradical soil mycelium would need to forage for new resources and would grow to increase

their absorption surface. This hypothesis is also consistent with the decrease of mycelium biomass in temperate months (spring and early summer) observed in this work, when the photosynthetic and respiratory activities of host trees are higher. The germination of the spores just after the sporocarp production season could also be related with this fact.

In our results, we did not find any correlation between sporocarps production and mycelial biomass for any species, neither in the first analysis done in Pinar Grande, for the autumn period of 2008, the *B. edulis* productivity of the plots, in terms of mean sporocarp production, was not correlated either with the soil mycelium concentration estimated by real-time PCR or with the abundance of ectomycorrhizas counted in soil samples. The sporocarp production was neither correlated with any climatic factor for *B. edulis.* This mismatch of the belowground and aboveground fungal biomass has been reported for *B. edulis* (Peintner et al. 2007) and other ectomycorrhizal species (van der Linde et al. 2009; Hynes et al. 2010). 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 around sporocarps and there was no quantitative relationship between the belowground abundance of mycelium and the number or distribution of sporocarps. Suz et al. (2008) compared nonproductive and productive trees in a *T. melanosporum* orchard, finding apparently higher quantities of mycelium in soil samples taken around nonproductive trees. Peintner et al. (2007), studying the soil fungal communities in a *Castanea sativa* 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. The lack of correlations between the parameters analyzed indicates that other factors would be involved in sporocarp production. Egli et al. (2010) found that fruit body production was influenced by the previous annual tree growth, supporting the hypothesis that ectomycorrhizal fruit body production must be linked with the growth of the associated host trees. Also, Bonet et al. (2012) found a relationship between sporocarp production of *L. deliciosus,* forest thinning, and precipitation in August and September. In our results, we also found a correlation between sporocarp production of *L. deliciosus* and rainfall occurring during the same months of the fructification period. Similarly, Salerni et al. (2002) found that rainfall was the main influence on fruiting in the most important fruiting period

(autumn). We have also seen that the sporocarp production of *L. deliciosus* has an inverse relationship with maximum and minimum temperatures, but no significant relationship was found with the mean monthly temperature. The correlation of mushrooms production with rainfall, moisture, and temperature has been also observed in several works (Straatsma et al. 2001; Salerni et al. 2002; Kauserud et al. 2008; Martínez-Peña 2009; Büntgen et al. 2012). For both species, sporocarp production was related with the plot age class; young plots being the most productive (16–30 years old in Pinar Grande and 11–20 years old in Pinares Llanos). Many studies showed that silvicultural management as thinning favors strongly growing trees with high photosynthetic capacity, which tend to produce more ectomycorrhizal fruiting bodies than poorly growing trees (Egli et al. 2010; Savoie and Largeteau 2011). The number of *B. edulis* sporocarps increased in the medium-thinning treatments (Salerni and Perini 2004). Other factors as soil moisture and temperature have influence in the fructification of *B. edulis* (Savoie and Largeteau 2011).

A correlation between precipitation and mycelium quantity was found for *B. edulis*, but not for *L. deliciosus.* A significant relationship was observed between relative humidity and soil mycelium quantity of *L. deliciosus*, which agrees with the results of several works that ensure that one of the crucial factors for the ectomycorrhizal fungi is soil moisture, as well as temperature and precipitation (Martínez de Aragón et al. 2007a; Kauserud et al. 2008; Bonet et al. 2010; Büntgen et al. 2012). Also, an excess of water can stimulate the development of the mycelium at the expense of fungal fructification (Pinna et al. 2010), which can explain the results found for *B. edulis* in our work. Similarly, Treseder et al. (2010) determined that the slow turnover of fungal hyphae observed during the dry season in Californian grassland was due to drought.

Negative correlations between mycelium quantity and solar radiation and temperature were found for *L. deliciosus.* This may be due to decreased soil water availability as a consequence of high solar exposure and temperatures affecting transpiration and soil water evaporation in summer. On the contrary, low temperatures and a lower solar exposure make water available for the fungi in winter. These results agree with Bonet et al. (2012) who found that water availability was a crucial factor in relation to sporocarp production, ectomycorrhiza, and mushroom richness in forests.

No differences in the amount of mycelium of *B. edulis* were found between the different plots. In the case of *L. deliciosus*, we found statistically significant

differences between plots, but they were not related with either of the descriptive parameters measured (age class and sporocarp production).

The presence of *B. edulis* mycorrhizal roots decreased clearly with time along the fruiting season, in autumn 2008 the highest concentration was detected in the samples taken at September, and dropped to almost one third in October and nearly to a tenth in November. In 2009 this situation was moved one month, the highest number of mycorrhizas was found in October 2009 and it decreased significantly on the following months. The mycorrhizal dynamics for *B. edulis* and *L. deliciosus* differed considerably. While *L. deliciosus* mycorrhizas number seems to be constant (with the available data, 8 months out of 12, no differences along time were detected), for *B. edulis* the number of mycorrhizas was different along time, and, in our experiments, a statistically significant positive correlation was established between the concentration of *B. edulis* soil mycelium quantified by real-time PCR, and the number of *B. edulis* ectomycorrhizal tips counted in the same soil samples. Although in this work no relation was detected, extraradical soil mycelium of *L. deliciosus* was also positively correlated with the percentage of mycorrhizal short roots in other experiments and fungus–plant combinations (Parladé et al. 2007).

For *B. edulis* we also found that those plots with a higher number of mycorrhizas were the same that had maximum sporocarp production, but no relation between mycorrhizas and sporocarp production was detected for *L. deliciosus*. The relationships between spatial distribution of mycorrhizas and sporocarps differed among fungal species; in some cases, an overlap of spatial distribution has been observed while in other cases sporocarps occurred where no mycorrhizas were detected (Gardes and Bruns 1996; Kikuchi and Futai 2003). Ectomycorrhizas in the soil would be patchily distributed, partly attributable to underlying patterns of root distribution, and the position of these patches changes over time (Pickles et al. 2010). The spatial distribution of mycorrhizas and extraradical mycelium might not exactly match. Mycelium may proliferate in nutrient-rich patches independently of the location of mycorrhizas (Lilleskov and Bruns 2003). The obtained results could open the possibility of using quantification of soil mycelium by real-time PCR as a good indicator for root colonization in field conditions, especially when a nondestructive sampling or less time consuming analysis were required.

Primers and TaqMan® probe designed in this work are suitable for the specific detection and quantification of *B. edulis* mycelium in a forest soil by real-time PCR. *B. edulis* and *L. deliciosus* extraradical mycelium behave similarly with annual

dynamics marked by strong seasonality, but their ectomycorrhizas do not follow the same pattern. Longer studies during 2 or 3 years would be needed to confirm these patterns. These dynamics are strongly dependent on the climatic conditions, and although rainfall is important, it is not the only climatic factor that influences the production of mycelial biomass for these species, mean temperature and solar radiation are also relevant. Some of these parameters can be modified by human action by thinning practices inducing modification of sunlight penetration and watering trying to improve the production of these fungi. Soil moisture will be a parameter to be considered in future studies on the dynamics of soil mycelial biomass of ectomycorrhizal fungi.

3

Traceability of ectomycorrhizal edible fungi in plantations: persistence and quantification of *Rhizopogon roseolus* and *Tuber melanosporum* extraradical soil mycelium in *de novo* and old plantations

Publication product of this chapter:

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Chapter 3

Traceability of ectomycorrhizal fungi in plantations: persistence and quantification of *Rhizopogon roseolus* and *Tuber melanosporum* extraradical soil mycelium in *de novo* and old plantations

1. Introduction

Truffles are edible ectomycorrhizal fungi producing hypogeous fruit bodies with high economic and social value (Boa 2004; Mello et al. 2006). Nowadays, truffles are the most cultivated ectomycorrhizal mushrooms, existing a wide distribution and diffusion of truffle culture around the world (Hall et al. 2003; Iotti et al. 2012). Black truffles, *Tuber melanosporum* Vittad., are symbiotic with several oak species and hazelnut trees (Ceruti et al. 2003) and are the most appreciated of the commercialized species in Spain and France (Reyna 2000). Their natural distribution area comprises Mediterranean calcareous areas in Spain, France, and Italy, but they have been successfully introduced in a range of countries by planting inoculated seedlings (Wang and Hall 2004). Truffle culture provides economic returns in a sustainable way, considering the conservation of natural areas and the social benefits derived from maintaining rural populations in marginal areas for agriculture (Reyna 2007). The agronomic requirements for the establishment of truffle orchards are well reported (Verlhac et al. 1990) and it is estimated that most of the commercialized European truffles come from plantations however official and reliable production data are not available (Reyna 2007).

Long-term studies on cultural factors as irrigation, fertilization, and weed control have been carried out by several authors (Mamoun and Oliver 1997; Ricard et al. 2003; Bonet et al. 2006; Olivera et al. 2011). Given the long delay (around 10 years) between the establishment of the plantation and the formation of sporocarps (Shaw et al. 1996), short- and medium-term control of the survival and persistence of the fungal symbiont in plantations have to be evaluated by the assessment of vegetative structures as the ectomycorrhizas (Kagan-Zur et al. 2001; Olivera et al. 2011; Reyna and De Miguel 2012). Molecular techniques based on

the amplification of the ITS rDNA region allowed for the accurate identification of ectomycorrhizas belonging to species phylogenetically close to *Tuber* (Henrion et al. 1994; Paolocci et al. 1995; Mello et al. 1996; 1997; 1999). The advances in the knowledge of the life cycle and reproductive biology of the two most economically important truffle species worldwide, *T. magnatum* and *T. melanosporum* (Rubini et al. 2005; Paolocci et al. 2006; Riccioni et al. 2008), as well as the recent sequencing of the *T. melanosporum* genome (Martin et al. 2010) have displayed new horizons in truffle research. From this information, the genes involved in the sexual compatibility processes (MAT) have been identified (Rubini et al. 2011b) allowing a greater understanding of the reproductive mechanisms of the black truffle. Also, this research is crucial to improving the management strategies of plantations aimed at increasing the controlled production of this edible fungus. The detection of extraradical mycelium of the target fungal species in the soil is a sound complement to sporocarp production and ectomycorrhiza sampling for tracking the fungal persistence along the whole biological cycle. Specific amplification of soil DNA by PCR has been applied to detect mycelium of different *Tuber* species (Suz et al. 2006; Zampieri et al. 2010; Gryndler et al. 2011). Quantitative detection of *T. melanosporum* DNA in soil was carried out by Suz et al. (2008) and Zampieri et al. (2012) using real-time PCR with SYBR® Green dye for nucleic acid stain to compare the quantity of soil mycelium in productive vs. nonproductive truffle orchards. As it was seen in the previous chapter, more specific real-time PCR detection can be obtained using Taqman® probes, which are able to discriminate a single nucleotide mismatch. In the previous chapter and in the introduction it has been explained how this technique can be used for the detection and quantification of extraradical mycelium and how it has been adapted to detect different ectomycorrhizal species. Also, this technique allowed to show seasonal patterns in the distribution of extraradical mycelium of *B. edulis* and *L. deliciosus* in a forest soil and to study the correlations between extraradical mycelium and mycorrhizas.

When establishing a *de novo* truffle plantation with mycorrhizal plants, one crucial factor is the persistence of the fungus in tree roots and its expansion through the soil. The persistence of the fungus in the plantation can be evaluated by examining the mycorrhization status of the plants. To avoid destructive methods, *T. melanosporum* extraradical soil mycelium can be detected and quantified using molecular techniques, as real-time PCR both in newly established plantations and in mature orchards. In this assay we designed specific real time PCR primers and Taqman® probe for *T. melanosporum*, in order to detect and quantify the

mycelium both in established and natural truffle grounds, providing a non-destructive and less invasive method to determine the presence of the fungus in the plantation.

Two *Rhizopogon roseolus* plantations were also monitored since its establishment, as a model to determine the possible quantitative relationships between fruiting bodies production and the abundance of vegetative fungal structures (mycorrhizas and extraradical mycelium). *R. roseolus* is an ectomycorrhizal fungi frequently associated with natural or planted pines worldwide (Molina et al. 1999; Grubisha et al. 2002). It has been frequently used in nursery soils to favor success of pine plantations, as it provides beneficial conditions for the development of pine seedlings (Martín-Pinto et al. 2006). Mycorrhization of pine seedlings by *Rhizopogon* spp. can be achieved using either mycelial or spores inoculum (Parladé et al. 1996; 1999; 2004a; Rincón et al. 2001; Hortal et al. 2008). The production of mycorrhizal plants from spores inoculum is very efficient and commonly used when producing seedlings on large scales (Wang et al. 2012). *R. roseolus* fruits easily, and it is widely eaten in Asia (although in Europe it is not an appreciated edible mushroom), for these reasons, plantations to obtain sporocarps with commercial purposes, have been planted since the 80s in Asia and since late 90s in New Zeland (Wang et al. 2012). The TaqMan® real-time PCR technique to determine the quantity of *R. roseolus* extraradical soil mycelium has been adapted from Hortal et al. (2008), and from Kennedy et al. (2007) for *R. occidentalis* and *R. salebrosus*.

After adapting the technique for the detection of *T. melanosporum* and *R. roseolus* mycelium in soil, it was tested to be used in natural conditions, either in plantations or in the wild. In this study, we have detected and quantified the extraradical mycelium of *T. melanosporum* in a new plantation and in seven mature orchards (around 20 years old) and one natural site producing sporocarps in Navarre (northern Spain). Similarly, two plantations of *Rhizopogon*-inoculated stone pines (*Pinus pinea*) were established. The results on quantification of extraradical mycelium of *T. melanosporum* in mature orchards are discussed in relation with site productivity, cultural practices, and traceability of the fungal persistence in the soil.

The **objectives** of the present chapter were:

1. To design specific TaqMan® probe and two primers for the detection and quantification of *T. melanosporum* extraradical mycelium in soil by real-time PCR.

2. To quantify, by real-time PCR, the extraradical soil mycelium of *T. melanosporum* in a *de novo Quercus ilex* x *T. melanosporum* plantation, and the *R. roseolus* mycelium in two *de novo* pine plantations. In both cases plantations were monitored since the field establishment of inoculated seedlings. The probe and primers for *R. roseolus* were previously designed by Hortal et al. (2008).

3. To detect and quantify the extraradical mycelium of *T. melanosporum* by real-time PCR in mature orchards and natural sites in Navarra.

2. Materials and Methods

2.1. Design of *Tuber melanosporum* probes and primers for their mycelium detection.

2.1.1. Design of primers and probes

The design of new species - specific primers and TaqMan probes for the detection *T. melanosporum* mycelium in soil samples, was performed with the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA), based in the variability of the rDNA internal transcribed spacer (ITS) region detected in the alignments among different sequences of *T. melanosporum* available in the Genbank. Two different types of probes (and their corresponding primers) were designed, in order to compare them and select the one that best fits our needs. The designed probes were a TaqMan® - MGB (Applied Biosystems), designed according to the above explanation, and a UPL probe (Roche Applied science), designed using the Universal ProbeLibrary (UPL) and the ProbeFinder software (www.universalprobelibrary.com).

All the oligonucleotides combinations were tested *in silico* by blasting them against the NCBI GenBank database to check for specificity. Further *in vivo* specificity tests were carried out with DNA extracted from four forest soils of areas unsuitable for

T. melanosporum; and DNA from pure cultures or sporocarps of *Amanita caesarea, A. ponderosa, Boletus aereus, B. edulis, B. reticulatus, Laccaria bicolor, Lactarius deliciosus, Pisolithus tinctorius, R. roseolus, Russula integra, T. brumale*, and *T. indicum*.

2.1.2. DNA extractions

T. melanosporum ascocarps, ectomycorrhizas and pure cultures DNA extractions were performed with the Dneasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France) following the manufacturer's instructions. Soil DNA extractions were performed with the PowerSoil™ DNA Isolation Kit (MoBio laboratories Inc., Carlsbad, CA, USA) from 0.25 g of soil per sample according to manufacturer's instructions.

2.1.3. Oligonucleotides test by real-time PCR

To test the designed probes and primers and obtain the standard curves, four real-time PCR assays were performed, two for each type of ascocarp (mature or immature) and type of probe - primers set (TaqMan or UPL), using the dilutions for the standard curves and a negative (deoionized water) and positive (*T. melanosporum* DNA extractions from ascocarp, ectomycorrhiza and from soil containing *T. melanosporum*) controls in all of them.

To test and decide the oligonucleotides to be used, standard curves for mycelium quantification by real-time PCR were generated using known amounts of *T. melanosporum* ascocarps, using a similar method as the one explained in Chapter 2. In the case of *T. melanosporum,* DNA of two, mature and immature, black truffles was extracted from 10 mg (dry weight), previously dried in a continuous airflow stove at 30°C for 24 hours, until constant weight. With the DNA extracts two tenfold serial dilutions were performed, from 1:10 to 1:100000, and were used for the construction of the standard curves.

DNA extracts were amplified by real-time PCR in a StepOne™ Real-Time PCR System (Applied Biosystems; Foster City, CA, USA), as described for *L. deliciosus* quantification (in Chapter 2, section 2.3.4).

2.2. Traceability of different ectomycorrhizal fungi in monitored plantations

2.2.1. Study sites

Three de novo plantations were established in 2009 and 2010 in different locations. Two *R. roseolus x P. pinea* plantations were established in Cabrils

(Maresme, Barcelona, Spain), and one *T. melanosporum x Quercus ilex plantation* near Cercs (Berguedà, Barcelona, Spain). Seven mature truffle orchards and one natural site in Navarra (Spain) were also monitored.

2.2.1.1. *Rhizopogon roseolus* x *Pinus pinea* plantation in Cabrils

Two plantation were established in 2009 (Cabrils 1) and 2010 (Cabrils 2) in Cabrils (Maresme, Barcelona, Spain) with the plants described in the following section (2.2.3 page 117). These plantations were located in flat lands, at 82 m altitude, at 41° 31' N and 2°22' W. The annual mean temperature is 16.4^{o}C, the total annual precipitation is about 685.3 mm, the annual mean relative humidity is 68.78%, the annual mean daily solar radiation is 15.51 w/m^{2} and the total annual reference evapotranspiration is about 975.50 mm/year (average data for the three years sampled since the establishment of the plantations – 2009, 2010 and 2011-). The soil is characterized by a sandy loam texture with a moderately alkaline soil and a low content of organic matter (Table 3.1).

Top Cabrils soil properties									
Coarse elements (dS/m)	pH (H2O)	Organic matter (%)	N (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)	Na (ppm)	P (ppm)	CEC (meq/100g)
0.15	8.1	1.09	6	51	107	2107	43	28	6.2

Table 3.1. Top soil (0-20 cm) properties measured in Cabrils.

Plantations were designed with two different spacing densities (2x2 m and 1x1 m) in experimental units of 9 plants (inoculated and non-inoculated) with four replicates per treatment randomly distributed (figure 3.2 and 3.3). The distribution of treatments was the same for both plantations.

All the plants were measured in both height and diameter of the trunk, at the moment of establishment of the plantation and after one year. Also all the collected plants, for the evaluation of their ectomycorrhizal status, were measured (height and diameter). With this measures the volume (V) for each plant was calculated according to the formula $V = 1/3\ Basal\ area \times height$, as a growth measurement.

2.2.1.2. *Tuber melanosporum* x *Quercus ilex* plantation in Cercs (Berga)

This plantation was established in 2010 near Cercs (Berguedà, Barcelona, Spain) with the plants described in the following section (2.2.3 page 117). The plantation

is located in a flat land, at 1200 m altitude. The annual mean temperature is 12.4°C, the total annual precipitation is about 704.2 mm, the annual mean relative humidity is 75.63 %, the annual mean daily solar radiation is 16.62 w/m^2 and the total annual reference evapotranspiration is about 816.6 mm/year (average data for the two years sampled since the establishment of the plantations –2010 and 2011-). The soil is characterized by a clay loam texture with a moderately alkaline soil and a moderately high content of organic matter (Table 3.2).

Top soil properties									
Coarse elements (dS/m)	pH (H_2O)	Organic matter (%)	N (mg/kg)	K (mg/kg)	Mg (mg/kg)	Ca (mg/kg)	Na (mg/kg)	P (mg/kg)	Active limestone (%)
0.34	8.0	8.57	15	265	223	10168	47	6	<3

Table 3.2. Top soil (0-20 cm) properties measured in Cercs plantation.

The plantation was designed occupying an area of 2500 m^2 (0.25 Ha) in order to obtain a final spacing between trees of 5 x 5 m. In the central part of the plantation trees were planted in 2.5 x 2.5 m spacing, in order to be sampled without altering the final plantation. Interspersed among these trees 14 non-inoculated plants (control) were randomly planted (Figure 3.4). Plants were protected with plastic tubes. (Figure 3.1)

All collected plants, for the evaluation of their ectomycorrhizal status, were measured (height and diameter). With this measures the volume (V) for each plant was calculated according to the formula $V = 1/3\ Basal\ area \times height$, as a growth measurement.

Figure 3.1. Detail of the *Quercus ilex* x *T. melanosporum* plantation after six months of its establishment.

Figure 3.2. Plan of the *P. pinea* x *R. roseolus* plantation established in Cabrils in 2009. The combined spacing and type of plant (inoculated - orange/control - green) were randomly distributed.

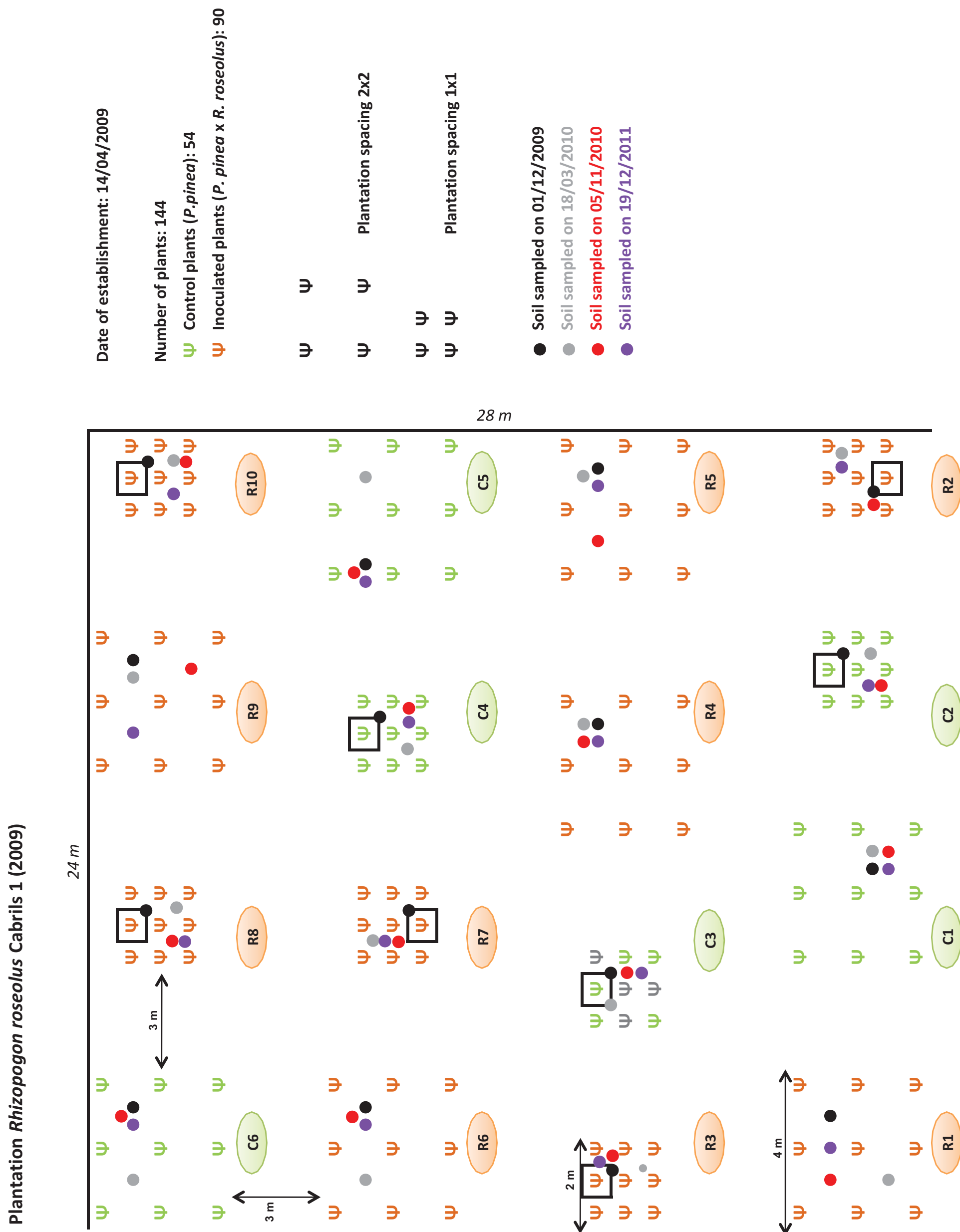


Figure 3.3. Plan of the *P. pinea* x *R. roseolus* plantation established in Cabrils in 2010. The combined spacing and type of plant (inoculated - orange/control - green) were randomly distributed.

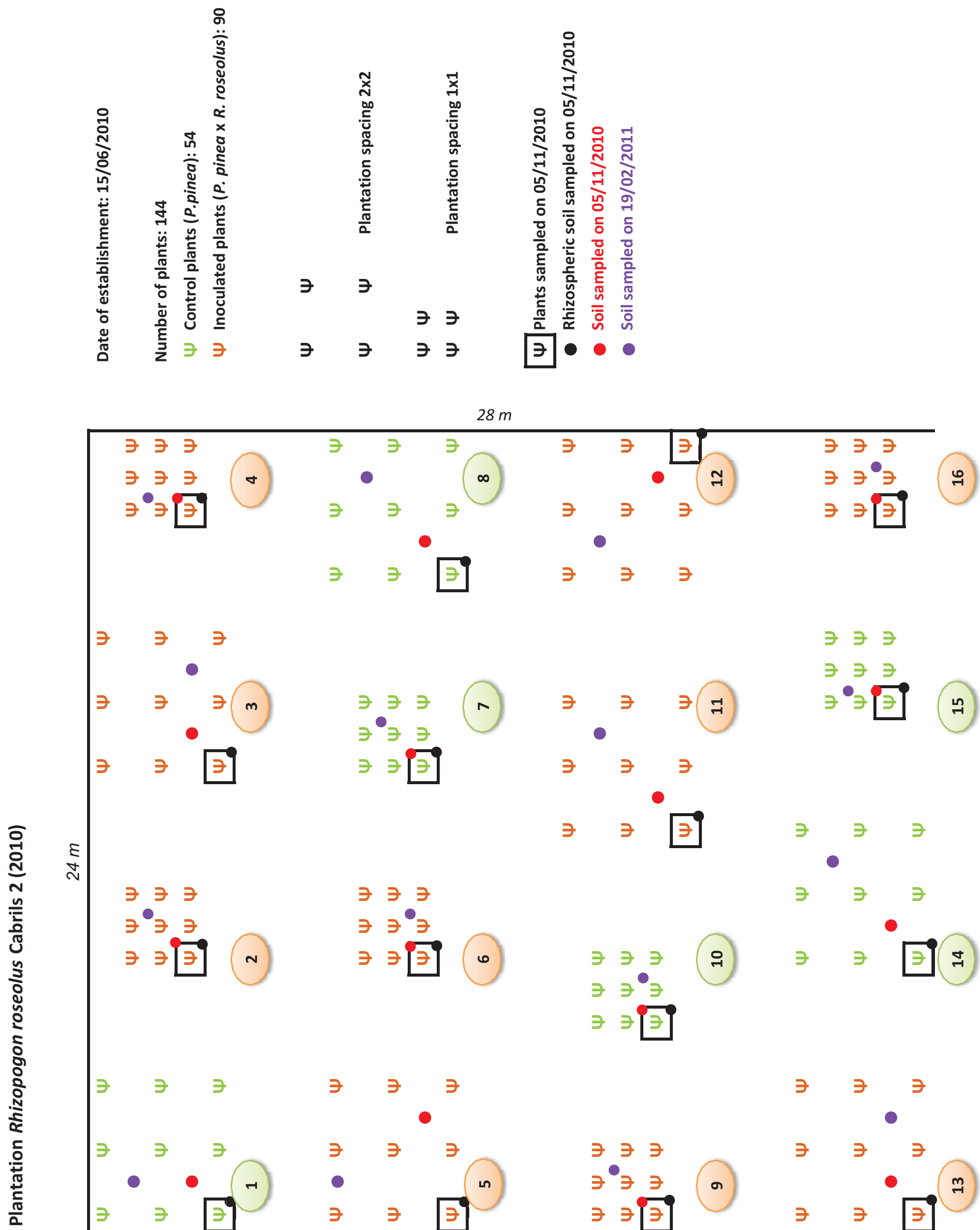
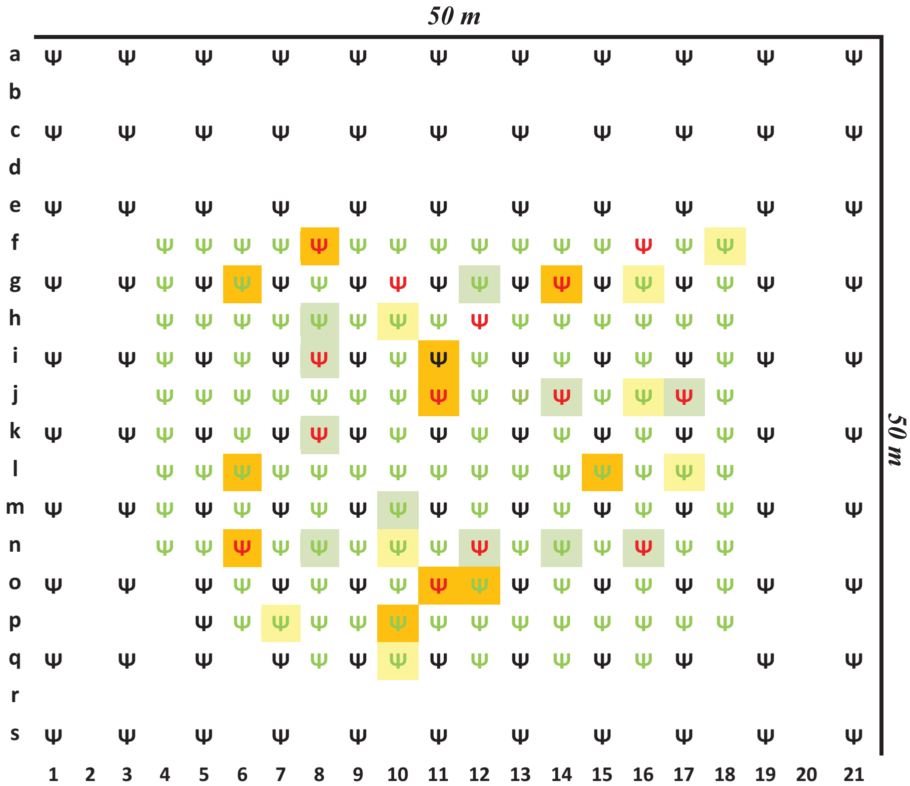


Figure 3.4. Plan of the *Q. ilex* x *T. melanosporum* plantation established in Cercs (Berguedà) in 2010. Non-inoculated plants (red) were randomly distributed.

Quercus ilex x *T. melanosporum* plantation in Cercs (Berga)

Area: 2500 m^2 (1/4 Ha) Establishment date: 29/04/2010

Initial number of plants: 258



Ψ Plants remaining in the final plantation (5x5 m) (110)

Ψ Plants to be sampled

Ψ Non-inoculated plants – Controls (14)

Sampled on 20/10/2010

Sampled on 15/04/2011

Sampled on 08/11/2011

2.2.1.3. Quantification of extraradical mycelium of *Tuber melanosporum* in soils from mature truffle orchards in Navarra (northern Spain)

A total of seven truffle orchards and one natural truffle ground were sampled in two sites, Tierra Estella and Valdorba, located in the truffle productive areas of Navarre (northern Spain). The orchards were established in areas producing natural *T. melanosporum* sporocarps (Sáez and De Miguel 1995). The four Tierra Estella orchards are composed of 75–80 evergreen oaks each (*Quercus ilex* subsp. *ballota* (Desf.) Samp.) established in contiguous sites. This area is at 625 m above sea level and the annual mean precipitation and temperature are 744 mm and 12.2 °C, respectively. One of the orchards was irrigated during July and August every 15 days to reach 60 and 80 mm of total precipitation per month, respectively, to avoid the estimated water deficit. On average, the amount of water provided was 25 mm every 15 days during July and August. The remaining orchards received no extra watering.

The natural truffle ground was located in a natural evergreen oak forest next to the Tierra Estella orchards. This area is a well-preserved mesic supramediterranean forest at 680 m above sea level, dominated by evergreen oaks and shrubs (*Spiraeo obovatae–Querceto rotundifoliae* sigmetum Rivas Godoy ex Loidi & F. Prieto 1986) (Loidi and Bascones 1995).The three orchards in the Valdorba site were established with three tree species: evergreen oak (74 trees), Portuguese oak (*Q. faginea* Lam.) (36 trees), and hazelnut (*Corylus avellana* L.) (108 trees). This area is at 580 m above sea level. The annual mean precipitation and temperature of the area are 773.4 mm and 12.4 °C, respectively. No irrigation was done in this site.

Mean truffle production and soil characteristics data of the sampling plots are summarized in Tables 3.3 and 3.4, respectively.

		Year of	Truffle production		
Location(plots)	Type of plot(s)	plantation	*T. mel*	*T. aes*	*T. bru*
Estella (1)	Productive watered	1990	3	1	1
Estella (1)	Productive *T. brumale*	1992	1	0	3
Estella (1)	Non Productive	1990	0	0	1
Estella (1)	Productive non watered	1990	3	1	1
Estella (1)	Evergreen oak forest	-	3	1	0
Valdorba (3)	Productive non watered	1993	2	1	0

0= no production, 1 = <5 kg/ha/year, 2 = 5–10 kg/ha/year, 3 = >10 kg/ha/year

Table 3.3. Average sporocarp production data of the plots (provided by the owners). The production in the three Valdorba plots is considered together for the three host species: *T. mel*: *Tuber melanosporum; T. aes: T. aestivum; T. bru: T. brumale*

Location (Plots)	Top soil properties Type of plot	pH (H_2O)	pH KCL	Texture	Organic Mater (g/kg)	CaO(g /kg)	Ca(g/kg)	Limestone (g/kg) Total	Active	CEC (cmol+/kg)
Estella (1)	Productive watered	8.2	8.0	Loam	0.114	9.9	9.675	811	155	9.7
Estella (1)	Productive *T. brumale*	8.3	8.0	Loam silt	0.130	10.7	10.386	680	160	14.4
Estella (1)	Non productive	8.3	8.0	Loam	0.129	10.3	9.857	742	164	9.0
Estella (1)	Productive non watered	8.4	8.0	Loam	0.102	11.4	10.000	759	171	10.6
Estella (1)	Evergreen oak forest	8.3	8.2	*n.d.*	0.086	11.0	10.219	718	159	10.2
Valdorba (3)	Productive non watered	8.1	7.6	Loam	0.111	11.3	10.224	150	43	13.3

Table 3.4. Soil characteristics of the different sampled plots. The three plots in the Valdorba site are considered together in the soil analysis. *n.d.*: not determined

2.2.2. Weather data

Weather data were obtained from automatic weather stations (Shema) located in Cabrils and in Guardiola de Berguedà, from the Agrometeorological data base of the Servei Meteorològic de Catalunya (Generalitat de Catalunya). The data used were: monthly means temperature (°C), the total monthly precipitation (mm/month), the monthly means relative humidity (%), the monthly means of the daily solar radiation (w/m^2) and the total monthly reference evapotranspiration (ETo) (mm/month) for the years 2009, 2010 and 2011, for Cabrils (figure 3.5), and 2010 and 2011 for Guardiola de Berguedà. The ETo data of the year 2009 were only available for the months November, October and December due to technical problems in the measurement station.

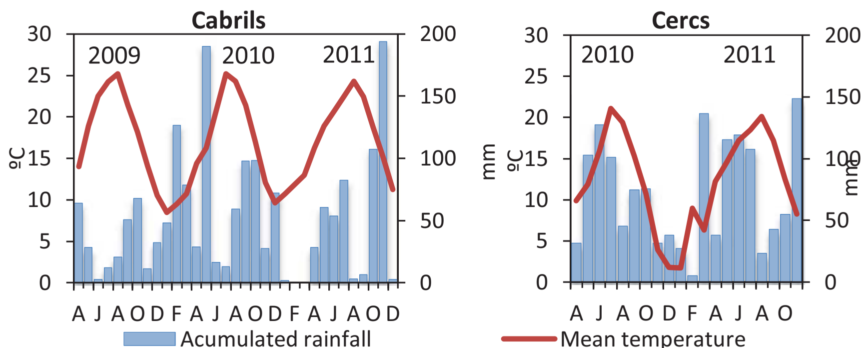


Figure 3.5. Mean precipitation (mm) and mean temperature (°C) from April 2009 to December 2011 in Cabrils and from April 2010 to November 2011 in Cercs.

2.2.3. Inoculated plants

For the Cabrils plantations, the inoculum of *R. roseolus* was obtained from sporocarps collected under a *P. pinea* plantation located near the place of establishment of the new plantations, where the species fruits naturally. The sporocarps, collected in autumn 2007, were dried (40 ºC overnight) and kept at ambient temperature (20-25°C). In spring 2008 and 2009 dried sporocarps were used to prepare a suspension with water by mixing all with a laboratory Waring blender at reduced velocity in order to release the spores (Parladé et al. 1996; Rincon et al. 2001). The *R. roseolus* spores concentration in the slurry was calculated using a Neubeuer counting chamber, and the inoculum density was adjusted by diluting the spore suspension up to the desired concentration.

A total of 200 seedlings of *P. pinea* were growing in a 300 cm^3 Forest-Pot containers in plant-growth substrate (peat, -vermiculite 1:1; v:v). The seeds were sown in April 2007 and 2008, two *P. pinea* seeds per cell (batch 1581 origin ES06 littoral Catalonia, Ministerio de MedioAmbiente D. G. Biodiversidad) previously immersed for 24 hour in water and kept at 4°C until sowing. After germination a single plant per container was left. One month latter seedlings were inoculated with a 10^7 spores/plant dose injected in the rhizosphere zone. The same amount of non-inoculated (control) plants was produced. The seedlings were maintained in a nursery with plastic roof, covered with a mesh until their transfer to field, watered daily and fertilized every 15 days as detailed in Chapter 1. The mean mycorrhization percentage was about 38.02% (mean of 10 plants randomly selected from the inoculated plants described above).

For the Cercs plantation, *Q. ilex* seedlings inoculated with *T. melanosporum* were supplied by the INOTRUF S.L. company (Sarrión, Teruel, Spain). The *Q. ilex* plants were produced from seeds (origin La Alcarria y Serranía de Cuenca), cultivated in 450 cm^3 individual full-pots for two years and inoculated with spores of *T. melanosporum*. The mean mycorrhization percentage was about 29.97 % (± 17.03), obtained from the root screening of 10 plants randomly selected on each batch.

Non-inoculated (control) plants were produced at the same time, in spring 2008, *Q. ilex* seedlings were grown in 300 cm^3 Forest –Pot containers in plant-growth substrate (peat-vermiculite 1:1; v:v). The seeds were sowed in spring 2008, two *Q. ilex* seeds per cell (origin littoral Catalonia, Maresme), after germination a single plant per container was left. The seedlings were maintained in a nursery with

plastic roof, covered with a mesh until their outplanting to field in April 2010, under the same conditions described for the *P. pinea* x *R. roseolus* seedlings.

2.2.4. Soil sampling

In Cabrils, soil samplings were carried out in autumn and spring, and in *T. melanosporum* plantation after 6, 12 and 18 months of its establishment (20/10/210 – 15/04/2010 and 08/11/2011) (Table 3.5), using a cylindrical (2 cm radius, 20 cm deep - 250 cm^3) soil borer (Taylor 2002).

Plantation	Establishment	Sampling dates			
Cabrils 1	14/04/2009	01/12/2009	18/03/2010	05/11/2010	19/12/2011
Cabrils 2	16/06/2010	05/11/2010	19/12/2011		
Cercs	19/04/2010	20/10/2010	15/04/2010	08/11/2011	

Table 3.5. Establishment and sampling dates in Cabrils and Cercs plantations.

At each sampling time one soil sample, from the center of four plants, was extracted in each experimental unit (figures 3.2 and 3.3). For the plantation Cabrils 2 soil from the rhizosphere of the extracted plants in November 2010 was also collected by extracting cylinders at 5 cm from the plant. A total of 112 soil samples were processed (16 sample points in each plantation every sampling date and 16 rhizosphere soil samples from Cabrils 2).

In Cercs, as it is shown in figure 3.6, at each sampling time (table 3.5), 4 soil samples were extracted at 5 cm of the trunk of each extracted plant, and mixed together in a plastic bag. A total of 27 soil samples were processed. Soil samples were let to dry at air temperature (20-25ºC) to constant weight and then sieved (1mm mesh diameter) and homogenized. Soil sample were stored in plastic bags at -20ºC until DNA extraction for molecular analysis.

Figure 3.6. Detail of one *Q. ilex* in Cercs and example of the sampling methodology in one single plant.

In Navarra orchards, soil sampling was carried out in April 2011 by taking soil cylinders with a soil borer within the 'burnt' area or *brulé* (the vegetation-devoid zone around the host plant as a result of the interaction with the fungus (Streiblová et al. 2012)). Four areas of 100 m^2 (with nine trees each) were randomly delimited in each plot. Five soil cylinders of 200 ml volume (3.2 cm diameter and 20 cm deep) were taken at 1 m from the trunk of five trees in each area. A total of 160 soil cylinders (corresponding to 160 trees, 20 for each plot) were processed.

Soil samples were classified in plastic bags and kept at −20 °C until processing. Before DNA extraction, each soil sample was air-dried at room temperature and then sieved through 1-mm mesh to eliminate stones and roots.

2.2.5. Tracking colonization by *R. roseolus* and *T. melanosporum*

2.2.5.1. Radical colonization (mycorrhizas)

In Cabrils, one plant per experimental unit was extracted from each plantation in the first sampling date (01/12/2009 in Cabrils 1 and 5/11/2010 in Cabrils 2). In Cercs, on each sampling time, several plants were randomly extracted (as indicated in figure 3.5). In order to evaluate their ectomycorrhizal status, plants were harvested using a shovel, preserving the radical system, and stored in plastic bags at 4°C, no more than one day, until their analysis.

Each plant was measured and their root system evaluated under a dissecting microscope to determine the percentage of *R. roseolus* or *T. melanosporum* mycorrhizas. These percentages were determined by counting the number of mycorrhizal and non-mycorrhizal tips after washing the roots with tap water. The presence of other ectomycorrhizal fungi in the roots was also quantified and determined. Mycorrhizas identity was verified by molecular tools when necessary (PCR with specific primers and or sequencing of the PCR product of the ITS region).

2.2.5.2. DNA extraction and soil mycelium quantification by real-time PCR

Total DNA was extracted from 0.25 g of each of the 299 soil samples (112 in Cabrils plantations, 27 in Cercs plantation and 160 in Navarra orchards) with the kit Power Soil® (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's protocol. The extracted DNA was stored at -20°C until use.

The quantification of the extraradical soil mycelium was determined by analysis of the soil samples by real-time PCR using the primers and the TaqMan-MGB® probe designed by Hortal et al. (2008) for *R. roseolus* and the primers and TaqMan-MGB® probe designed and explained before for *T. melanosporum*. (Complete oligonucleotide sequences on Table A.3 in the Annex).

Standard curves, one for each location, for the mycelium quantification were generated by mixing known amounts of the target fungi with soil collected near the sampling sites, but free of the fungi to be quantified, as described by Parladé et al. (2007), Hortal et al. (2008), De la Varga et al. (2012) and Parladé et al. (2013).

For *R. roseolus* analysis, the standard curve was performed as explained for *B. edulis* (Chapter 2) but starting from 1200 mg of pure culture mycelium and using soil collected in Cabrils, from a ground next to where the plantations were established. The soil was previously checked for the absence of *R. roseolus* by conventional PCR with universal primers ITS1F/ITS4 (White et al. 1990; Gardes and Bruns 1993). Tenfold serial dilution from the extract (soil plus mycelium) were prepared until 10^{-6}, obtaining serial concentrations of: 24, 2.4000, 0.2400, 0.0240, 0.00240, 0.000240 and 0.000024 mg of *R. roseolus* mycelium/g of soil.

Three standard curves were generated for the *T. melanosporum* soil mycelium quantification: one for Cercs and two for Navarra samples (one for each site, Tierra Estella and Valdorba). 0.01 g of a fresh immature sporocarp of *T. melanosporum* were mixed with 0.24 g of a soil core taken from a cereal field adjacent to each of the study areas and free of the target fungi, resulting on serial concentrations of: 40, 4, 0.4, 0.04 and 0.004 mg of *T. melanosporum* mycelium/g of soil. The amount of extracted DNA was measured with a Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). For *T. melanosporum*, fresh immature sporocarps were chosen over mycelium because of the difficulties in culturing this fungal species in pure culture in the laboratory (Hall et al. 2001; Iotti et al. 2002).

Specific quantification of soil mycelium was carried out with a StepOne™ Real-Time PCR System machine provided with the StepOne software v. 2.2 (Life Technologies, Carlsbad, CA). DNA samples and standards were prepared for real-time PCR as described for *L. deliciosus* quantification (in Chapter 2, Materials and Methods, section 3, d). Absolute quantification of mycelium biomass of *R. roseolus* and *T. melanosporum* for each soil sample was determined by interpolation of their Ct value in the corresponding standard curve.

2.3. Data analyses

Statistical significance of the differences in the number of mycorrhizal tips per volume of soil and soil mycelial concentration (mg mycelium/g soil) were determined by a multifactorial analysis of variance for Cabrils data. Differenced in volume plants, differences in the number of mycorrhizal tips per volume of soil and soil mycelial concentration in Cercs and in Navarra data were determined by a one-way ANOVA. Homogeneity of variances and normality of data were tested by Bartlett's test (Snedecor and Cochran 1989). Differences between means were analyzed by Tukey's HDS test ($p=0.05$) or Student's t-test ($\alpha=0.05$). The correlations between soil mycelium biomass, abundance of mycorrhizas, plot productivity and climatic parameters were determined by Pearson's Bivariate Correlation analysis. Data were transformed to $\log_{10} + 0.001$, when necessary, in order to meet the assumptions of parametric statistical tests. Statistical analyses were performed with JMP®8.0.1 (SAS Institute Inc., Cary, NC, 1989-2009).

The description of the assays characteristics and experimental conditions followed the MIQE Guidelines (Bustin et al. 2009) to provide essential information required for consistency of the quantitative real-time (qPCR) protocols.

3. Results

3.1. Design of *T. melanosporum* probes and primers for mycelium detection and quantification.

The result of the online UPL real time PCR assay construction selected the probe #93 and designed the left primer TmelPF (GGTTATAAGACCTGGATCAGTCACA) and the right primer TmelPF (CATCTAGGATGGGGGTCCTT), resulting in a 67 bp amplicon. The designed TaqMan® probe STQTmel (6FAM-TTCCACAGGTTA AGTGAC) and primers Rvs-Tmel (TCCCACAGGTGCCAGCAT) and Fwd-Tmel (TCTCTGCGTATCACTCCATGTTG) for the detection of *T. melanosporum* amplify a 61 bp. The last oligonucleotides combination were deposited in GenBank (probe unique identifier (PUID) number: 15262663).

The TaqMan® probe, tested *in silico* as a short input sequence in the NCBI Blast® procedure, showed full specificity (100 % coverage and identity) only for *T. melanosporum* sequences. Other *Tuber* species, phylogenetically close as *T. indicum* and *T. brumale* showed 77 % and 72 %, respectively, of the query

coverage. The *in vivo* tests, done by real-time PCR amplification, only gave positive results with *T. melanosporum* samples (soil, ascocarp and ectomycorrhiza samples). Negative samples, including DNA extractions from soils of cereal fields adjacent to the experimental plots, forest soils of areas unsuitable for *T. melanosporum*, and DNA from pure cultures or sporocarps of other fungal species, showed also no amplification.

The standard curves obtained with mature ascocarps for both type of probes were similar, showing UPL probe curve lower efficiency rates (Figure 3.7). With the immature T. melanosporum ascocarp the results were comparable, but the amount of template DNA added to the reaction needed to be increased 5 times. The control samples gave the same results in all tests and the efficiency of the curves were within the standard acceptance values, but having the TaqMan® curves a better efficiency, R^2 and slope values (very close to the optimal ones; R^2=1 and slope=-3.32).

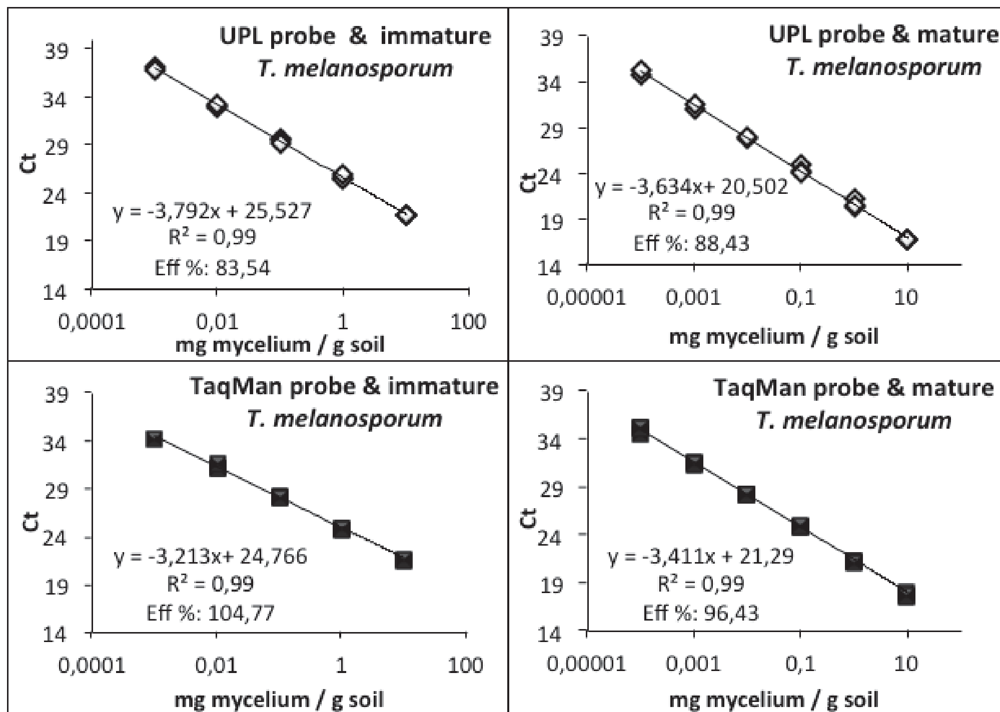


Figure 3.7. Comparison of the standard curves obtained for the quantification of *T. melanosporum* mycelium by real-time PCR using UPL (open diamonds; above) and TaqMan® (black squares; below) combined with immature (left) and mature (right) ascocarps.

3.2. Traceability of different ectomycorrhizal fungi in monitored *de novo* plantations.

The standard curves obtained for the quantification of *R. roseolus* and *T. melanosporum* soil mycelium in Cabrils and Cercs plantations are shown in figure 3.8, satisfying both the minimum standards for the DNA quantification by real-time PCR (Bustin 2004).

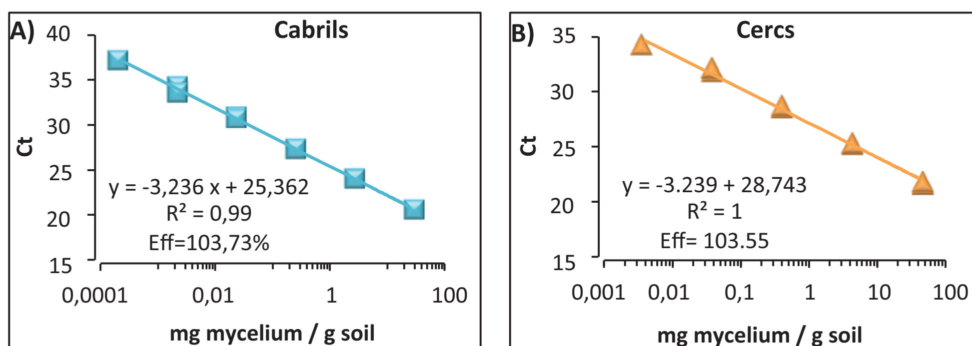


Figure 3.8. Standard curves used for the quantification of extraradical mycelium. A) *Rhizopogon roseolus* mycelium in Cabrils soil samples. B) *Tuber melanosporum* mycelium in Cercs soil samples.

3.2.1. *Rhizopogon roseolus* x *P. pinea* plantations in Cabrils

3.2.1.1. Radical colonization: mycorrhiza

After 8 months (autumn 2009), in **Cabrils 1**, *R. roseolus* mycorrhizas were present in all the inoculated plants analyzed and their percentage of mycorrhizal roots (mean of the collected plants) was about 52.23 % (± 11.45) for *R. roseolus* ectomycorrhizas (a 14% more than the initial mycorrhization rate, but not statistically significant), and 10.18 % for other fungi ectomycorrhizas. In non-inoculated plants (controls) no *R. roseolus* mycorrhizas were present but a 3.87 % of roots mycorrhizal with other ectomycorrhizal fungi were detected.

In **Cabrils 2** plantation, in autumn 2010 (after 6 months of field growth) radical colonization was detected in inoculated and non-inoculated plants. *R. roseolus* was present in the radical system of the inoculated plants, except of one, and their mycorrhization percentage was about 48.58% (± 23.38) for *R. roseolus* mycorrhizas (a 10% more than the initial mycorrhization rate, but not statistically significant), and about 5.95 % for other ectomycorrhizal fungi. In the control pines three of the six plants evaluated showed *R. roseolus* mycorrhizal roots, representing a 9.98 % of mycorrhization, and a 19.39 % were mycorrhizal with other ectomycorrhizal fungi.

The statistical analysis for the mycorrhizal percentage in 2010 (6 months in field) showed differences (p = 0.0478; α=0.05) for inoculated and non-inoculated plants (p= 0.0106; α=0.05), having the inoculated plants a higher percentage, but no differences for the spacing were detected (p= 0.4384;α=0.05) (table 3.6). No factor interactions were detected.

Analysis of Variance for 'mycorrization (%)' in Cabrils 2					
	DF	**Sum of Squares**	**Mean Square**	**F Ratio**	**Prob> F**
Model	3	5313.244	1771,08	3.6500	0,0478*
Error	11	5337.483	485.23		
C. Total	14	10650.727			
Effect tests					
Source	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob> F**
Spacing	1	1	0.6693	0.0014	0.9710
Inoculation	1	1	4577.9235	9.4346	0.0106*
Spacing*Inoculation	1	1	313.6280	0.6464	0.4384
Means Comparisons for all pair using Student's T test					
Level		**Mean**	**Std error**	**N**	
Control	B	9.9844	6.1813	6	
R. roseolus	A	48.5835	7.3939	10	

Table 3.6. Analysis of variance for the *R. roseolus* mycorrhizas percentage in Cabrils 2 after 6 months in field, by spacing (1x1/2x2) and inoculation (yes/no) of the trees. *Differences statistically significant (α=0.05).

3.2.1.2. Extraradical colonization: soil mycelium

Statistical analysis revealed that both plantations presented differences in soil mycelium densities (p<0.0001; α=0.05). In Cabrils 2 mycelium amounts detected were higher than in Cabrils 1 (after 6, and 18 months after plantation establishment). Each plantation was analyzed separately to determine if the spacing between plants is a differential factor for the expansion of the extraradical mycelium in the soil.

At the establishment time of both plantations, no mycelium of *R. roseolus* was detected in the soil, after eight months (in the first sampling date) in **Cabrils 1** extraradical mycelium was detected in three experimental units, two of them composed with non-inoculated plants (C2-0.00036 and C3-0.00052 mg mycelium / g soil) and the other one with plants inoculated with *R. roseolus* (R2-0.00072 mg mycelium / g soil). In spring 2010 (one year after plantation establishment) the fungus was only detected in the experimental units composed by inoculated

plants, and the quantities detected were smaller than in autumn samplings (2009, 2010 and 2011). In autumn 2010 and autumn 2011 *R. roseolus* extraradical mycelium was present in all the experimental units formed by inoculated plants, except of one, and in three control plants in 2010 and two control plants in 2011. The fungus was extended both in soil adjacent to the experimental units composed of *R. roseolus* inoculated plants, as in those of non-inoculated ones. Multifactorial ANOVA (Table 3.7) showed that soil mycelium amounts were different between samples (p = 0.0039; α=0.05), and no interactions were found between factors. A significant difference was detected between sampling dates. There was an increase of extraradical mycelium for the last sampling date, after 32 months (December 2012), being statistically different from the rest (p = 0.0005; α=0.05). No significant differences were found in the *R. roseolus* soil mycelium between inoculated and control plants in Cabrils 1, neither for the spacing between plants inside the experimental units, although smaller spacing experimental units seems to have more extraradical soil mycelium than de 2x2 m spacing ones. No significant correlation was found between *R. roseolus* mycorrhizas and extraradical soil mycelium.

Analysis of Variance for 'Mycelium quantity' in Cabrils 1

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob> F
Model	15	46.099038	3.07327	2.7620	0.0039*
Error	48	53.409840	1.11270		
C. Total	63	99.508878			

Effect tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob> F
Date	3	3	23.529151	7.0486	0.0005*
Spacing	1	1	1.750889	1.5735	0.2158
Date*spacing	3	3	3.074361	0.9210	0.4379
Inoculation	1	1	2.653931	2.3851	0.1291
Date*Inoculation	3	3	5.369805	1.6086	0.1997
Spacing*Inoculation	1	1	0.281441	0.2529	0.6173
Date*Spacing*Inoculation	3	3	0.314912	0.0943	0.9628

Means Comparisons for all pair using Tukey-Kramer HSD

Level			Mean	Std error	N
01/12/2009		B	0.00010	5.63e-5	16
18/03/2010		B	0.00046	0.00027	16
5/11/2010		B	0.00375	0.00288	16
19/12/2011	A		0.62856	0.46628	16

Table 3.7. Analysis of variance for the *R. roseolus* mycelium quantities in Cabrils 1 by sampling date, spacing (1x1/2x2) and inoculation (yes/no) of the trees. *differences statistically significant (α=0,050). Mean are showed in mg mycelium / g soil. Levels not sharing same letter are significantly different (α=0.05).

In **Cabrils 2**, established in 2010, the *R. roseolus* mycelium was also extended all over the plantation. After six months of the establishment, the fungus was present in the roots of all the inoculated plants and also in 4 of the six non-inoculated plants analyzed. At this time extraradical *R. roseolus* soil mycelium was present in all experimental units, except in one composed by non-inoculated plants (also absent of *R. roseolus* mycorrhizas).

The statistical analysis showed significant differences in the quantities of *R. roseolus* soil mycelium (p = 0.0337; α=0.05) along the time, being higher in 2011, after 18 months of the plantation establishment (p = 0.0086; α=0.05) and also for the plantation spacing (p = 0.0154; α=0.05), being higher in those experimental units with 1x1 spacing (table 3.7). No significant differences between inoculated and non-inoculated plants were found, but the extraradical mycelium quantities detected were higher in soils with inoculated plants and lowest spacing (1x1 m), (figure 3.9). No factor interactions were detected.

Analysis of Variance for 'Mycelium quantity' in Cabrils 2					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**	**Prob> F**
Model	7	15.287079	2.18387	2.6782	0.0337*
Error	24	19.570047	0.81542		
C. Total	31	34.857127			
Effect tests					
Source	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob> F**
Year (2010/2011)	1	1	6.6715200	8.1817	0.0086*
Inoculated (yes/no)	1	1	0.0013505	0.0017	0.9679
Year*Inoculated	1	1	0.0970696	0.1190	0.7331
spacing (1x1 m / 2x2 m)	1	1	5.5535482	6.8107	0.0154*
Year*spacing	1	1	0.3217865	0.3946	0.5358
Inoculated*spacing	1	1	0.3834822	0.4703	0.4994
Year*Inoculated*spacing	1	1	0.7328528	0.8987	0.3526

Table 3.8. Analysis of variance for the *R. roseolus* mycelium quantities in Cabrils 2 by sampling year (2010/2011), spacing (1x1/2x2) and inoculation (yes/no) of the trees. *Differences statistically significant (α=0.05). Mean are shown in mg mycelium / g soil. Levels not sharing same letter are significantly different (α=0.05).

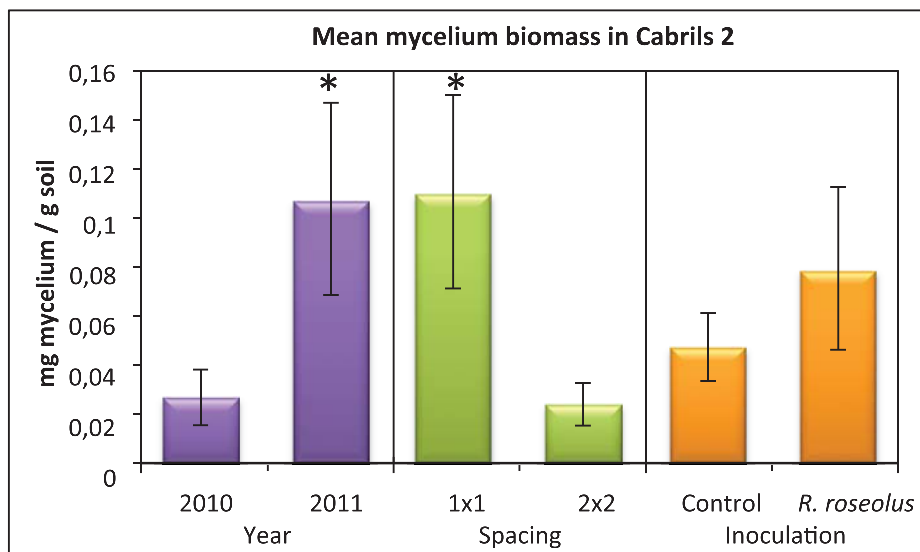


Figure 3.9. Differences of mycelium biomass in Cabrils 2 plantation for the sampling years (in autumn 2010 and 2011), the plantation spacing (1x1 m or 2x2 m) and inoculation (*R. roseolus* and non-inoculated – control). Means are shown in mg *R. roseolus* mycelium / g of soil. *Statistically significant differences (α=0,050). N for each year and spacing level=16; N for Control plants = 12; N for *R. roseolus* inoculated plants=20.

3.2.1.3. Extraradical colonization: rhizospheric soil mycelium

In the rhizospheric soil analyzed in **Cabrils 2**, *R. roseolus* mycelium quantities were higher than in the other soil samples ($p < 0.0001$; α=0.05). *R. roseolus* mycelium was detected in inoculated and non-inoculated plants, not detecting statistically significant differences between the initial inoculation of the trees (inoculated or control) or their spacing (p=0.1205; α=0.05).

In **Cabrils 2**, the mycorrhizal percentages were positively correlated with the rhizospheric mycelium (r=0.6938; p = 0.0059; α=0.05), but not correlated with the extraradical soil mycelium.

3.2.1.4. Tree growth

In Cabrils 1, at the moment of the plantation establishment the volume of the *P. pinea* was significantly different between control and inoculated plants (p =0.0486; α=0.05). These differences remained after one year of growth in the field (p=0.0275; α=0.05), being inoculated plants higher in volume (figure 3.10).

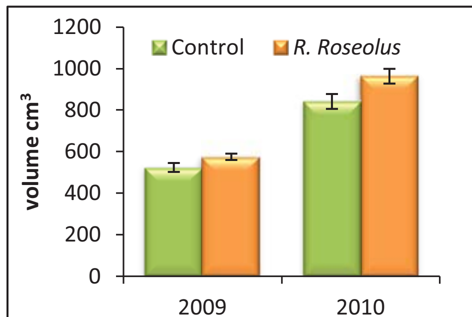


Figure 3.10. Tree growth for the volume of *P. pinea* in Cabrils 1 (cm^3). Differences (α=0.05) were found between inoculated and control plants in both years (2009 and 2010), but not differences on growth rates.

Growing rates were not significantly different after one year (p=0.2485; α=0.05), being the inoculated plants little bigger in volume. In Cabrils 2 the situation was the same, no differences were found between growing rates of inoculated and control plants, neither after six months of the plantation establishment (p=0.1587;α=0.05) when inoculated plants were 486.34 (±120.46) cm^3 and control plants were 595.50 (±174.18) cm^3.

3.2.1.5. Correlations between soil mycelium and climatic data.

Correlation between climate conditions and extraradical mycelium was detected. *R. roseolus* extraradical soil mycelium was positively correlated with monthly precipitation (mm) and also with the monthly average of the daily global solar radiation (W/m2). The reference evapotranspiration was negatively correlated with the amount of extraradical soil mycelium; also a negative correlation was detected with the mean monthly temperature (°C). No correlation was found with the mean monthly relative humidity (%) (Table 3.9).

Variable correlated	Correlation coefficient (R)	Signification prob.
Mycelium biomass - Monthly Precipitation (mm)	0.5499	<0.0001*
Mycelium biomass - Mean temperature (ºC)	-0.4221	<0.0001*
Mycelium biomass - Relative Humidity (%)	0.1921	0,0607
Mycelium biomass - Radiation (W/m2)	0.5203	<0.0001*
Mycelium biomass - ETo (mm)	-0.3955	<0.0001*

* Significant correlation (level ≤ 0,05)

Table 3.9. Correlations between mycelium biomass (mg mycelium/g soil) and climate variables (Spearman correlation coefficient, significance level ≤ 0.05). The climatic data used were from the parameters available in the weather station in Cabrils, next to the plantations.

3.2.2. *Tuber melanosporum x Q. ilex* plantation in Cercs

3.2.2.1. Radical colonization: mycorrhiza

One year after the establishment of the plantation, the root colonization of *Q. ilex* plants by *T. melanosporum* was higher than at the initial time. At that moment, no colonization was detected in the radical systems of the control plants. Differences in time were detected in the total mycorrhization percentage after 18 months (table 3.10). Tukey's test showed that the highest mycorrhization of the plants was detected in spring 2011(figure 3.11).

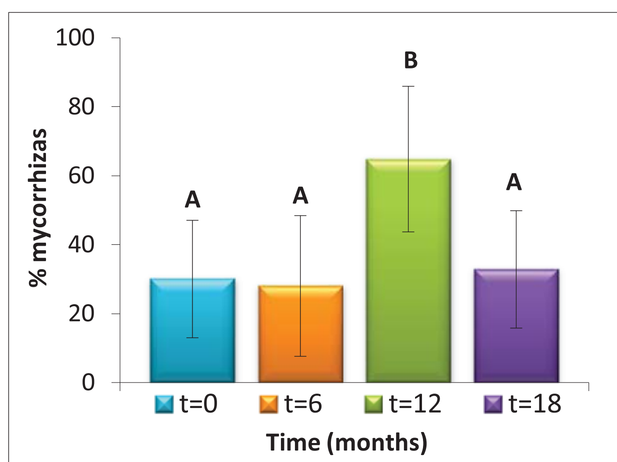


Figure 3.11. Differences on the total mycorrhization percentage plants at the establishment – Spring (t=0) after 6 months - Autumn (t=6), 12 months - Spring (t=12) and 18 months - Autumn (t=18). Bars show the standard deviation. Letters show the differences established by the Tukey test.

Analysis of variance of 'mycorrhization (%)' by time					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**	**Prob> F**
Months	3	8070.142	2690.05	7.8421	0.0003*
Error	42	14407.020	3343.02		
C. Total	45	22477.162			

Table 3.10. Analysis of variance for the mycorrhization percentage after 18 months in field, by time. *Differences statistically significant (α=0.05).

3.2.2.2. Extraradical colonization: soil mycelium

Tuber melanosporum extraradical soil mycelium was detected from the first sampling (6 months), and although there was an increase of mycelium along time (figure 3.12, A), the amounts were not statistically different ($p = 0.52$; $\alpha=0.05$).

Positive correlation of mycorrhizas and extraradical soil mycelium (r= 0.7814; p=0.0129; α= 0.05; N=9) (figure 3.12, B) was detected in spring 2011, after 12 months of the plantation establishment, when the number of mycorrhizas per plant was the highest. There was no statistically significant correlation when all samples were analyzed together (r=0.2502; p=0.2082; α=0.05), neither analyzing the autumn 2011 data (r=0.1343; p = 0.5952; α=0.05).

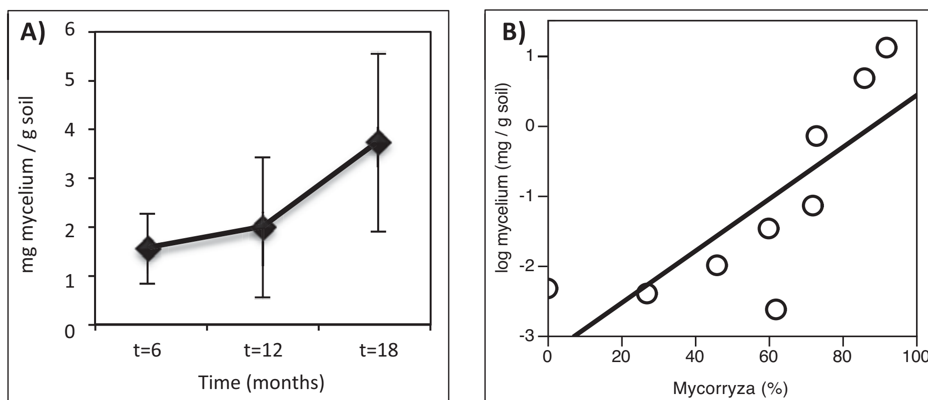


Figure 3.12. A) Evolution of the *T. melanosporum* mycelium quantities detected in soil after 6, 12 and 18 months of the plantation establishment. Bars show the standard deviation. B) Correlation between the % of *T. melanosporum* ectomycorrhizas per plant and the concentration of *T. melanosporum* soil mycelium (mg/g) (log transformed) from the soil collected under these plants (r= 0.7814; p = 0.0129; α= 0.05; N=9).

3.2.2.3. Plant growth

The growth of *Q. ilex* plantlets was significantly different between control and inoculated plants 12 months after the establishment of the plantation (p-value=0.0066; α=0.05). Mycorrhizal plants were significantly greater in volume (382.9 (±74.1) cm^3 in average for mycorrhizal plants and 53.7 (±40.4) cm^3 for control plants).

3.2.2.4. Correlations between mycorrhizas, mycelium and climatic data.

No correlations between soil mycelium biomass and climate conditions were detected. Using the pairwise correlations coefficient, correlation between weather and mycorrhizal percentage was detected. *T. melanosporum* mycorrhizal percentage was positively correlated with mean and maximum temperatures (^oC), the monthly average of the daily global solar radiation (W/m2) and the mean evapotranspiration rate (mm) of the current month. No correlations were found

with precipitation from the current month, but a significant positive relation was found with the precipitation of the previous month (Table 3.11).

Variable correlated	Correlation coefficient (R)	Signification prob.
Mycorrhizas (%) - Mean Temperature (°C)	0.4207	0.0289*
Mycorrhizas (%) - Max. Temperature (°C)	0.3935	0.0423*
Mycorrhizas (%) - Min. Temperature (°C)	-0.3139	0.1108
Mycorrhizas (%) - Radiation (W/m2)	0.4134	0.0321*
Mycorrhizas (%) - Monthly Precipitation (mm)	-0.3492	0.0742
Mycorrhizas (%) - Precipitation previous month (mm)	0.4794	0.0114*
Mycorrhizas (%) - Relative Humidity (%)	0.2109	0.2910
Mycorrhizas (%) - ETo (mm)	0.3899	0.0444*

* Significant correlation (level ≤ 0,05)

Table 3.11. Correlations between mycorrhizal percentage and climate variables (Spearman correlation coefficient, significance level ≤ 0.05). The climatic data used were from the parameters available in the local weather station.

3.2.3. Quantification of extraradical mycelium of *T. melanosporum* in soils from truffle orchards in Navarra (northern Spain)

Amplification of *T. melanosporum* DNA from the soil was obtained in 131 out of the 160 processed samples. Standard curves for DNA quantification in both soils were very similar (Figure 3.13) indicating that no different PCR inhibition effects occurred due to the soil composition. The detection limit of the technique, based on the maximum DNA dilution giving acceptable fluorescence signal within the standard curve, was 1.48 µg of mycelium/g of soil (corresponding to tenfold dilutions up to 10^{-4} of the DNA extracted from the initial mixture of sporocarp and soil). This is equivalent to 0.04 ng of DNA/µL considering that the amount of DNA obtained in the extraction process from 1 mg of the immature *T. melanosporum* sporocarp used for the standards was 172 ng.

The extraradical mycelium biomass detected in the soil from the natural truffle ground in the Tierra Estella area was significantly greater ($p < 0.001$) than that detected in any of the other orchards (Figure 3.14). Soil from productive, non-irrigated orchards contained significantly more extraradical mycelium ($p < 0.001$) than the rest of orchards in the Tierra Estella site (productive watered, nonproductive, or producer of *T. brumale*). The lowest amount of extraradical

mycelium of *T. melanosporum* was detected in the plot producing *T. brumale,* in which sporadic fruiting of *T. melanosporum* was also recorded (Table 3.5).

No significant differences in soil mycelium biomass were detected between the orchards established with the three different host tree species in the Valdorba site ($p = 0.155$) (Figure 3.14). The comparison of soil mycelium biomass between non-irrigated evergreen oak orchards in both sites showed significantly more mycelium biomass ($p < 0.001$) in the Tierra Estella than in the Valdorba site.

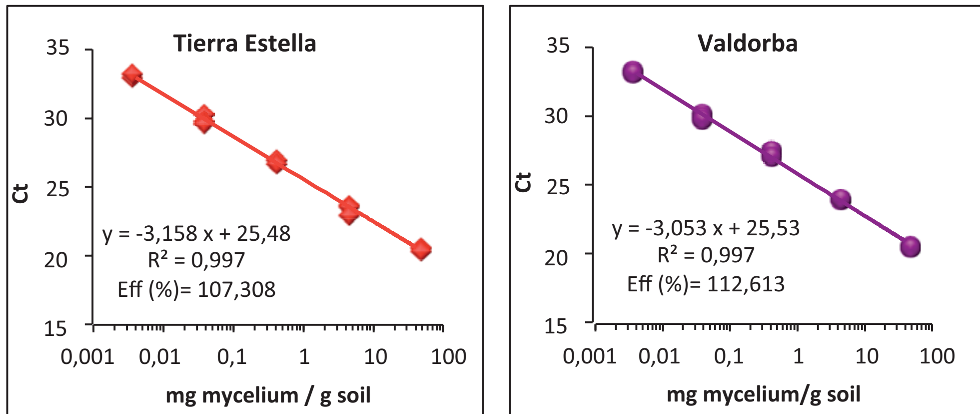


Figure 3.13. Standard curves used for mycelium quantification of *T. melanosporum* by real-time PCR in the two sampling sites (Tierra Estella and Valdorba). Curves were generated by plotting the Ct values of DNA extracts obtained from known amounts of the target fungi added to soil of each sampling site.

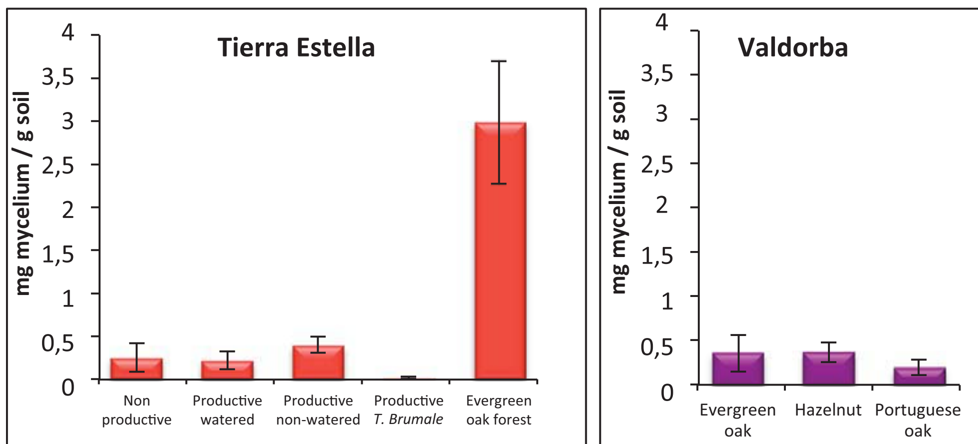


Figure 3.14. Biomass of extraradical soil mycelium of *Tuber melanosporum* from eight soils of two productive areas of Navarre (northern Spain). Bars represent standard errors of each mean (N=20 for each soil)

4. Discussion

4.1. Design of *T. melanosporum* probes and primers for their mycelium detection.

Specific primers and probe for *T. melanosporum* were designed to be used in real-time PCR. The size of the fragments amplified was 61 and 67 bp for *T. melanosporum* TaqMan® and UPL oligonucleotides respectively. The oligonucleotides (Fwd-Tmel, Rvs-Tmel, STQTmel and Fwd-Tmel PF, Rvs-Tmel PF, UPL #93) used in this work, have been designed in the rDNA ITS1 region. The internal transcribed spacer region of ribosomal DNA has been widely used because different specific primers have been designed to identify *T. melanosporum* (Paolocci et al. 1999; Séjalon-Delmas et al. 2000; Douet et al. 2004; Bonito 2009) and several group-specific primer sets exist (Gardes and Bruns 1993; Martin and Rygiewicz 2005). Large quantities of sequences are available for this gene region on a wide range of fungal species, making easier to design specific and efficient primers for real-time PCR. Nevertheless, ITS regions of the ribosomal DNA gene are multicopy regions of the genome (Gardes and Bruns 1993), known to be different in the number of copies between species (Debaud et al. 1999). Since copy numbers of ITS genes on the genome vary between fungal species, it would be most reliable to use a gene with a known and constant number of copies for quantification purposes. In our studies, we quantified the presence of two single species, *R. roseolus* and *T. melanosporum*, and the number of copies of the ITS region is assumed to be constant within a species (Cassidy et al. 1984; Raidl et al. 2005).

In relation to the two types of oligonucleotides designed (UPL and TaqMan® probes), it has been found that both types of probes gave good results and amplify *T. melanosporum* DNA. In both situations, the standard curves obtained satisfy the requirements in terms of efficiency (Bustin 2004), but TaqMan® probes gave better efficiency results, being the curves closer to the optimal values (R^2=1 and slope=-3.32), but otherwise UPL probes are cheaper and can be considered for works with a huge number of samples. In our works, as we pretend to detect accurately very small quantities of soil mycelium, we have selected the TaqMan® probes.

In order to obtain the standard curves for fungal biomass quantification in soil samples we usually use known quantities of mycelium grown on pure cultures, but because of the difficulties in culturing *T. melanosporum* on agar media (Hall et al. 2001; Iotti et al. 2002) fungal tissues from an immature ascocarp were used in this

case. Fresh immature sporocarps were chosen over mature ones due to the better amplifications obtained with these, and also because their tissue should be more similar to extraradical mycelium than the mature ones.

This study is the first attempt to quantify extraradical mycelium of *T. melanosporum* in the soil using Taqman® probes and also the first time that *T. melanosporum* mycelium is monitored since the initial establishment of an orchard. The main advantage of using probes over SYBR Green® in real-time PCR for fungal DNA quantification is their higher specificity (Schena et al. 2004). Moreover, DNA probes with conjugated minor groove binder (MGB®) form extremely stable duplexes with single- stranded DNA targets. Also, MGB® probes are shorter, have higher melting temperature (Tm), and are more specific than standard DNA probes, especially for single-base mismatches at elevated hybridization temperatures (Kutyavin et al. 2000b). This property is especially convenient when analyzing complex media as soils for specific quantification.

Molecular techniques to detect *T. melanosporum* from soil DNA have been adapted by designing specific primers for conventional PCR (Suz et al. 2006) or for real-time PCR using SYBR Green® (Suz et al. 2008; Zampieri et al. 2012). Suz et al. (2006; 2008) found greater quantities of mycelium in nonproductive orchards than in productive ones, although the difference was not significant. On the other hand, Zampieri et al. (2012) found that the amount of *T. melanosporum* DNA in productive soils was statistically higher than that in nonproductive soils. In our study, the range of mycelial biomass detected in the truffle orchards (0.2– 0.4 mg mycelium/g soil) using Taqman-MGB® probes was similar to the data reported by Suz et al. (2008) using SYBR Green® in productive and nonproductive 10-year-old orchards (0.15–0.4 mg mycelium/g soil). The sensitivity of the real-time technique allowed for a detection limit of 0.00148 mg mycelium/g soil, ten times lower than the limit of 0.0114 mg mycelium/g soil (corresponding to 3.6 ng DNA/g soil) calculated by Suz et al. (2006) using conventional PCR. With the *R. roseolus* oligonucleotides the real-time detected a minimum of 0.00011 mg mycelium per gram of soil in the Cabrils plantations. In previous studies using Taqman® real-time PCR, similar detection limits were obtained for *L. deliciosus* (0.0024 mg mycelium/g soil) (Parladé et al. 2007; Hortal et al. 2008) and *B. edulis* (0.0011 mg mycelium/g soil) (Chapter 2). However, to make the results comparable, it will be necessary to standardize the methodologies adapted for real-time PCR experiments, especially the characteristics of the fungal material used for the standards and the units of the quantification results. The use of immature fresh sporocarps to elaborate the

standards may be an alternative for fungi which do not grow well in agar media as *Tuber* species (Suz et al. 2008). However, the equivalence of these determinations for absolute quantifications of mycelium belonging to dikariontic and haploid fungal species in the field remain to be further investigated.

4.2. Traceability of different ectomycorrhizal fungi in monitored plantations.

In the new plantations established with *R. roseolus* and *T. melanosporum* mycorrhizal plants, it was possible to detect and quantify the extraradical mycelium from the target fungi since the beginning, just some months after planting the mycorrhizal plants. It was the first time that extraradical mycelium of *T. melanosporum* was monitored since the establishment of an orchard. In both sites, at the end of the samplings, the studied fungus, expanded through the soil plantation.

For *R. roseolus* seedlings an increase in number of 10% of *R. roseolus* ectomycorrhizas was detected in both plantations, and in Cabrils 2 also a half of the control plants analyzed became with a 10% of the roots colonized by this fungus. This could be explained because Cabrils plantations were established in a place where natural inoculum exists (Hortal et al. 2008). During the experiment, no sporocarps were observed in any plantation, neither under nearby adult pines. Previous analysis of the soil showed that both plots were free of *R. roseolus* mycelium and Cabrils 2, in which non-inoculated plants were infected by *R. roseolus*, is the farthest from the adult pines from where *R. roseolus* sporocarps had been seen and collected. Certainty, in these plantations, we saw that concerning mycelium amounts detected, both were different. In the one established later mycelium colonized all the plantation and also non-inoculated plants. Probably there was a certain amount of already existing propagules that were not initially detected. In Cabrils 2 plantation we also could establish a positive relation between *R. roseolus* mycorrhizas and mycelium quantities, as Hortal et al. (2008) did before, but just for those soil samples taken near the host plant.

Analyzing mycelium biomass, as expected, there was more extraradical mycelium in those experimental units where trees were only 1x1 m spaced, but just significant for Cabrils 2 and for the samples taken in 2011 in Cabrils 1. In both plantations there was an increase in mycelium biomass in 2011 (samples taken in autumn), which made that differences through time were statistically detectable. Analyzing climate data it was seen that it was in that period (autumn 2011) when the accumulated precipitation was higher than in the other years, being the rainfall

data double than in 2009 and 2010. Probably these increase in water availability produced a faster growth of the mycelium than in previous samplings. In chapter 2 it was seen how mycelium dynamics of *B. edulis* and *L. delicious* were strongly dependent on climatic conditions. With data analyzed in these experiment it can be seen that *T. melanosporum* mycorrhizas seem to be more affected by climate variations than soil mycelium, but for *R. roseolus* the situation is the opposite, as correlation with climate variations were only detected for extraradical mycelium. In both situations, fungi are affected especially by precipitation, but also by temperature, radiation and evapotranspiration, as it was also seen for *B. edulis* and *L. deliciosus*. Other works have also found that *T. melanosporum* mycorrhizas are strongly affected by water (Mamoun and Olivier 1990; Bonet et al. 2006; Reyna-Domenech et al. 2008) and solar radiation(Reyna-Domenech et al. 2008; Garcia-Barreda and Reyna 2012). Silvicultural treatments that influence these conditions are recommended, when plantation ages, to increase their mycorrhizas and also truffle production (Reyna-Domenech et al. 2008).

In the *T. melanosporum* plantation, although the initial mycorrhization percentage was not so high, after one year of field growth it increased being above the double (reaching a 92 % in some plants in spring 2011). We found the highest number of mycorrhizas per plant in April. Bonet el at. (2001), after following mycorrhizal *T. melanosporum* percentage along one year, found that in April there were the maximum numbers of mycorrhizas suggesting that month to be the best to observe mycorrhization. It was also in that sampling time when we found that the number of mycorrhizas and the extraradical mycelium amount were positively correlated. Bonet et al. (2006) also evaluated the *T. melanosporum* mycorrhizas development of inoculated *Q. ilex* seedlings in a plantation after 18 months in the field (to evaluate the effects of water deficit, weed control and fertilization), and also saw that *T. melanosporum* was not easily displaced in this establishment phase of the plantation. Their results showed an increase of 10% in *T. melanosporum* mycorrhizas, similar to the results obtained in our plantation at the same time, after 18 months, although the time when seedlings were outplanted and sampled were different (winter and summer for them and spring and autumn for us). Anyway, the fungus persisted in the root system of the seedlings, probably due to that soil and climatic conditions meet the ecological parameters observed for truffle producing areas (Reyna-Domenech et al. 2008). Perhaps the initial mycorrhization percentage may not be so important if ecological conditions are suitable for the fungus.

4.3. Quantification of extraradical mycelium of *Tuber melanosporum* in soils from truffle orchards in Navarra (northern Spain)

It was found a significantly greater amount of mycelium biomass (up to ten times higher) in the natural truffle ground compared to the man-made truffle orchards in the same area. This difference is surprising, since truffle orchards are especially cultured to favor truffle persistence. Since the methodology used evaluates soil fungal DNA from both mycelium and spores, it cannot be discarded that dissemination of propagules is more effective in natural forests due to the major dispersal of fungal spores by animals (Maser et al. 2008).

No clear relationship has been established between extraradical mycelium and sporocarp formation. Suz et al. (2006) found more mycelium biomass in nonproductive plots vs. productive ones, although differences were not significant, whereas Zampieri et al. (2012) detected significantly more *T. melanosporum* DNA in productive plots. Both studies performed the sampling in April (as in the present study, just after the period of truffle production). Suz et al. (2006)hypothesized that the allocation of resources for sporocarp formation could have diminished the extraradical mycelium in the soil. On the other hand, Murat et al. (2005) and Zampieri et al. (2010) analyzed the presence of ectomycorrhizas and extraradical mycelium of *T. magnatum* in natural truffle grounds and found no relationship with the production of sporocarps. However, these studies have been carried out with punctual samplings at one particular time. In Chapter 2 it was seen that quantitative evaluation of extraradical mycelium of *B. edulis* and *L. deliciosus* in natural pine forests with a gradient of sporocarp productivity showed significant variation between sampling times in the soil colonization by extraradical mycelium. Again, no relationship was found between extraradical mycelium and sporocarp productivity in *B. edulis* productive plots, but vegetative structures (ectomycorrhizas and extraradical mycelium) were positively correlated. This correlation was also seen when analyzing the new *T. melanosporum* and *R. roseolus* plantations studied in this chapter.

In the Tierra Estella site, it was found significantly more mycelium biomass in nonirrigated plots than in irrigated ones. Olivera et al. (2011) and Bonet et al. (2006)showed an increase of *T. melanosporum* mycorrhizas in seedlings with moderate watering (50 % of the water deficit in summer) compared to either plants with a high level of irrigation or nonirrigated. Also, a high irrigation schedule may affect competition leading to a replacement of *T. melanosporum* with other ectomycorrhizal fungi (Mamoun and Olivier 1990). In this study, plots producing

mainly *T. brumale* showed the lowest amount of *T. melanosporum* mycelium but maintaining a low sporocarp production. The coexistence of both species was studied by Mamoun and Olivier (1993), who reported that *T. brumale* protects the roots from the establishment of other ectomycorrhizal symbionts and favors the maintenance of *T. melanosporum* under conditions of high water content.

Nonproductive orchards showed significantly less mycelium biomass than productive ones in nonirrigated plots in the Tierra Estella site. Suz et al. (2008) suggested that the differences in mycelium biomass may be related to the overall stage of fungal development. Since mycelium of *T. melanosporum* has been detected in nonproductive plots, possibly the amount was not sufficient yet to shift from vegetative growth to fruit body production. However, this factor itself cannot explain the sporadic fruiting of *T. melanosporum* in the plots producing *T. brumale* (with the lowest amount of *T. melanosporum* extraradical mycelium).

The available annotated *T. melanosporum* genome (Martin et al. 2010) offers novel insights into key biological functions driving sporocarp formation. Recent data on *T. magnatum* and *T. melanosporum* indicate a prevalently haploid and heterothallic life cycle for these truffles (as explained in the introduction and in figure I.1), similar to that described for heterothallic filamentous ascomycetes, and controlled by two alternate mating types (Kües and Martin 2011; Rubini et al. 2011b). Recently, Zampieri et al. (2012) detected mating type genes for *T. melanosporum* under productive trees and generally not under unproductive trees even though *T. melanosporum* was detected. In all three situations, the mating type genes were detected by conventional PCR when more than 0.3 ng of *T. melanosporum* DNA was present. This finding is important, since real-time PCR may increase the detection limit of the single locus MAT genes to relate their presence with the production of fruiting bodies.

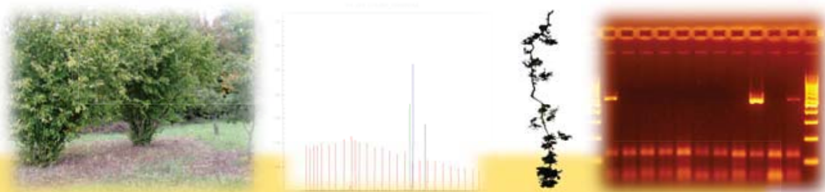
Mycelium concentration in the Valdorba site was not significantly different between the three host tree species. Average sporocarp production in the Valdorba site was lower than in Tierra Estella and the concentration of mycelium in the soil was also significantly lower when comparing the evergreen oak orchards in both sites. Soil analysis revealed a higher amount of active carbonate in the Tierra Estella site than in Valdorba. Active carbonate (calcium carbonate extractable with ammonium oxalate) has been related to greater burnt areas (produced by the concentration or mycelium) and to a higher sporocarp productivity (García-Montero et al. 2007).

In conclusion, plantations established *de novo* with *T. melanosporum* and *R. roseolus* confirm that both species behave different, but that the establishment of the mycorrhizal trees is critical. We saw that both fungi persisted in the radical system of all plants. The first stage of a plantation, when the fungus spreads, is highly influenced by the seedlings, local climate, soil conditions and also planting distances. The studied fungi behave different in soil, as our results suggest that *R. roseolus* spreads out faster through soil than *T. melanosporum*, but both showed positive correlation between vegetative structure (ectomycorrhiza and extraradical mycelium), but only when samples are collected near the host plant. These fungal species are modulated by weather conditions, *R. roseolus* mycelium is correlated with climates conditions, but for *T. melanosporum* only mycorrhizas seem to correlated with climate variations.

TaqMan ® real-time PCR technique is a good method for the traceability of *R. roseolus* and *T. melanosporum* without disturbing host trees, allowing us to detect the persistence and expansion of the fungus through a plantation from its establishment. For both fungal species soil mycelium could be quantified from 6 months after establishing the orchards and it was increasing with time. Quantitative results are useful to evaluate the fungal response to cultural treatments and to understand the dynamics of *T. melanosporum* mycelium in the soil, especially in mature plantations where root sampling for the evaluation of ectomycorrhizas is difficult. Further studies to complete and compare the annual cycles of soil mycelium biomass under different ecological or cultural conditions could elucidate their relationship with the onset of truffle production.

Spatial genetic structure of *Tuber melanosporum* in a productive orchard determined by mating type genes and microsatellites analysis

4



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Chapter 4

Spatial genetic structure of *Tuber melanosporum* in a productive orchard determined by mating type genes and microsatellites analysis

1. Introduction

Truffles are among the most appreciated edible ectomycorrhizal mushrooms, and much research has been done through this species, mainly *T. melanosporum*, elucidating about how to produce their well-appreciated hypogeous fruit bodies. The presence of *T. melanosporum* mycelium in soil samples from an orchard, or the quantity of the detected mycelium, does not determine the productivity of truffles in the orchard (chapter 2; Suz et al. 2008; Zampieri et al. 2012). This fact could be explained by the characteristic life cycle of truffle (figure I.2 in the general introduction).

Ectomycorrhizal fungi are filamentous fungi that, as many others, are able to reproduce both sexually (through the production of meiospores) and asexually (through mycelial growth). Sexual reproduction is important for the evolution and adaptation of species since it induces recombination eliminating deleterious mutations and producing better progeny. However, compared to asexual reproduction, sexual reproduction has several disadvantages: transmission of transposable elements, diseases and energy costs (Otto 2009; Zemach et al. 2010). In filamentous fungi, in addition to the widespread potential for asexual (vegetative) reproduction, two main types of sexual reproduction can be found: homothallism and heterothallism. Homothallic fungi are self-fertile, and the heterothallic species need the presence of a partner of the opposite mating type to reproduce and are therefore self-sterile (Murtagh et al. 2000; Paoletti et al. 2007; Kronstad 2007). The understanding of the ability to promote sexual reproduction are among the most important topics concerning edible mycorrhizal fungi that have high economic values, such as *Cantharellus* spp., *Tuber* spp., *Boletus* spp. and *Tricholoma matsutake* (Wang and Hall 2004). The balance between vegetative and sexual dissemination of *Tuber* spp. is poorly understood, although this information is critical for enhancing fructification in truffle orchards.

As it was explained in the description of the species (in the General Introduction), true truffles belong to the ascomycete genus *Tuber,* which is estimated to comprise more than 200 symbiotic species (Bonito et al. 2010). Some of these species produce edible fruiting bodies with distinctive bouquet. To complete their life cycle these fungi establish symbiosis with roots of trees and shrubs by promoting the formation of ectomycorrhizas. The production of truffles has dramatically decreased during the last century as a result of rural desertification, a reduction in truffle ground area and climate changes, among other causes (Büntgen et al. 2012). In order to preserve and increase truffle production, their life cycle, reproductive mode and ecology need to be elucidated. The difficulty in cultivating them on synthetic media, as well as the impossibility of mating them under controlled conditions, have done that the life cycle of Tuber spp. could not yet been reproduced in vitro (Rubini et al. 2007). However, population genetics analyses coupled to methodological progresses in the isolation and typing of single mycorrhizal root tips, asci and ascospores, have clarified the basic aspects of the life cycle and reproductive biology of the two most economically important truffle species worldwide: *T. magnatum* and *T. melanosporum* (Rubini et al. 2005; Paolocci et al. 2006; Riccioni et al. 2008). Evidence of recombination events coupled with the analysis of the mating type (*MAT*) locus (the genomic region that governs the fungal sexual cycle (Fraser & Heitman, 2003)) has revealed that *T. melanosporum* is a heterothallic species (Riccioni et al. 2008; Martin et al. 2010; Rubini et al. 2011a; 2011b). The *MAT* locus is a unique region of the fungal genome that governs the establishment of cell-type identity and orchestrates the sexual cycle (Fraser and Heitman 2003). All known heterothallic ascomycetes have a single *MAT* locus with two alternative genes (*MAT1-1-1* and *MAT1-2-1)* (Metzenberg and Glass 1990; Debuchy et al. 2010). In heterothallic fungi, same clone mating is prevented because only haploid cells that carry different alleles at the mating type locus/ loci can fuse (Murtagh et al. 2000; Kronstad 2007; Paoletti et al. 2007; Billiard et al. 2012). It has been evidenced that truffle ectomycorrhizas are structures made up of single homokaryotic tissue of uniparental origin. So it was suggested that the haploid mycelium forming the mycorrhizas serves as the maternal partner in the cross, which must have come into contact with an opposite mating type strain acting as the paternal partner (Rubini et al. 2011a). However, the paternal reproductive structure in *Tuber* spp. has not yet been identified, as this structure is undetectable at maturity (Paolocci et al. 2006; Rubini et al. 2011b). Therefore, these findings raise the question of whether the spatial distribution of strains with different mating type represents a limiting factor for mating and thus for fructification. To this end, recently, Rubini et al. (2011a) reported a biased

distribution of the two mating types in artificially inoculated seedlings, as well as in host plants from a natural *T. melanosporum* site. Similarly, Zampieri et al. (2012) identified only one mating type in the soil harvested from under a single tree, and Linde and Selmes (2012) found that only half of the investigated trees in Australian truffle orchards harbored ectomycorrhizas of both mating types in their roots.

The genetic structure of *T. melanosporum* populations at a small scale has not been adequately investigated. Therefore, in the present study, the spatial distribution of *T. melanosporum* strains on a man-made truffle ground was mapped. To this end, a study was carried out by finely mapped sampling strategy in a productive *T. melanosporum* orchard located in northern France (Rollainville). Genetic profiles of ectomycorrhiza and fruit bodies, using simple sequence repeat (SSR) markers and mating type genes, have been investigated. Also it was mapped the spatial distribution of both mating types genes in *T. melanosporum* soil mycelium under two productive trees.

The **objectives** were to:

1. Test if there is a mating type dependent host plant colonization pattern by truffle strains.
2. Characterize at a small-scale level the spatial distribution of *T. melanosporum* genets.

2. Materials and Methods

2.1. Study site

The orchard studied is situated in Rollainville in the Western part of the Vosges range, France, on a limestone plateau from the Jurassic era (latitude 48° 18’ 42’’, longitude 5° 44’ 13’’; elevation 360 m; annual rainfall 941 mm with a maximum in July; mean annual temperature 9.5°C). The soil is a brown calcarisol with a silty clay texture, highly alkaline (water pH 7.97), rich in organic matter (9.4%) and has a limestone content of 8.8%. This soil is poor in available phosphorus and moderate in available potassium and magnesium. It is a free draining soil highly granular and aerated.

The truffle orchard was established in 1991 on a site previously used for cereal cultivation by planting *Corylus avellana* trees artificially inoculated with *T. melanosporum* (Naudet Pépinières). The first truffles were harvested in November 2005. Each tree was identified by a letter and a number and its truffle production assessed for five years.

In 2010, two trees, F10 and F11 (Figure 4.1), were selected for their high truffle production. A grid of 1m x 1m squares was set up with camping pickets and light cord (Figure 4.2). During the 2010–2011 period of truffle production (winter, from October to February), all the ascocarps produced beneath these two trees were located using a well-trained dog. The ascocarps were carefully retrieved from the soil using a small spade and precisely mapped on the grid to within 5 cm.

In the spring of 2011 (April and May), 42 root samples and 48 soil cores were collected in the first 10 cm of soil and mapped using the same grid used to position the ascocarps (Figure 4.2A and B). Tree fine roots were carefully retrieved from the soil and washed in water under a dissecting microscope and a light microscope to test for the presence of *T. melanosporum* ectomycorrhizas, that were identified by morphotyping on the basis of color, mantle shape and surface texture (Zambonelli et al. 1993; Rauscher et al. 1995). From each root sample, five single *T. melanosporum* ectomycorrhizas were selected along a root piece, mapping the single ectomycorrhiza selected as shown in figure 4.3. Samples were stored at 20°C for molecular analysis. Finally, all plant debris was discarded from the soil, and soil samples were kept at 20°C.

Figure 4. 1. The two *C. avellana* (F10 and F11) and the small oak (E10) selected from de Rollainville orchard.

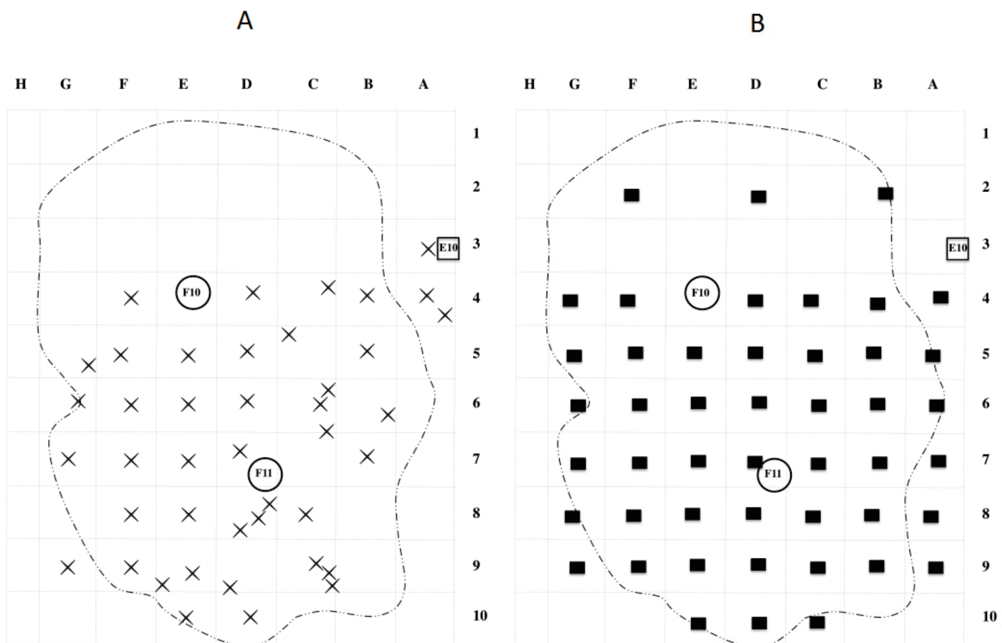

Figure 4. 2. Sampling of root tips (A) and soil samples (B) under two hazels (F10 and F11 - circles) and one small oak (E10 - square) in the spring of 2011 in the Rollainville orchards. The area marked with a dot line represents the hazels *brulé*.

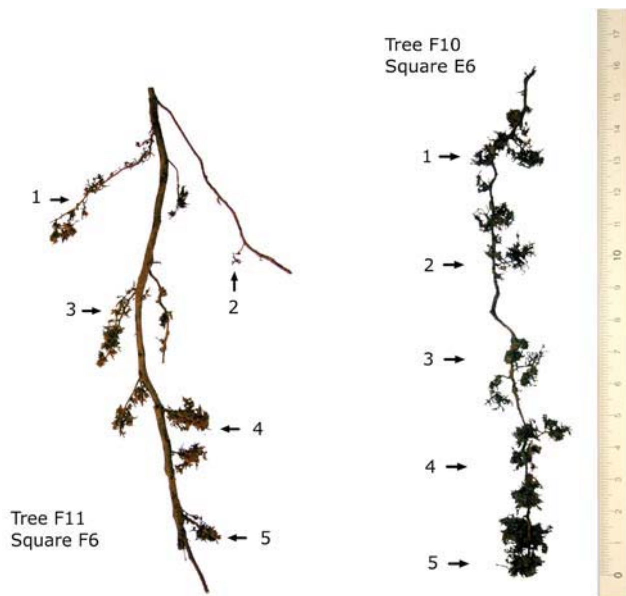

Figure 4. 3. Examples of the selection of *T. melanosporum* ectomycorrhizas from root pieces. Each unique tip was stored in individual microcentrifuge tubes for DNA extractions.

2.2. DNA extractions and molecular analysis: Genotyping of ectomycorrhizas, ascocarps and soil DNA

Genomic DNA was isolated from single mycorrhizal root tips and ascocarps using a Dneasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France) following the manufacturer's instructions. The identity of *T. melanosporum* ectomycorrhizas and ascocarps was confirmed by molecular analysis using species-specific internal transcribed spacer (ITS) primers ITS4LNG and ITSML (Rubini et al. 1998; Paolocci et al. 1999). Soil DNA was extracted using the Fast DNA Spin kit for soil (MP Biomedicals, Illkirch, France), according to Luis et al. (2004).

The presence of fungal species in soil samples was tested by amplifying soil DNA with the fungal universal primers ITS1f/ITS4 as described by Zampieri et al.(2010). Whereas the presence of *T. melanosporum* structures was assessed by PCR amplification with *T. melanosporum* specific ITS primers described earlier. Analyses of the mating type of *T. melanosporum* ectomycorrhizas and ascocarps were carried out using either the *MAT1-2-1* or the *MAT1-1-1*genes specific primers following PCR conditions previously described by Rubini et al. (2011b). Hereafter, the *MAT1-2-1* and *MAT1-1-1* are called *MAT*(+) and *MAT*(-), respectively. For the free-living soil mycelia, two independent PCR reactions with both *MAT* primers pairs were carried out. Each reaction was done with the same conditions as the

multiplex reaction described by Rubini et al. (2011b): PCR reactions were performed in a 25 µl reaction mixture containing 2.5 µl of 10x reaction buffer 10x (Sigma), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 10 nM of each primer, 1 U of Taq polymerase (SIGMA), 16.55 µl of deoionized sterile water and 5-20 ng of DNA. The following cycling parameters were used for the *MAT* loci: 4 min at 94°C followed by 30 cycles of denaturation (35 for soil sample analysis) at 94°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 1min and a final extension of 5 min at 72°C. PCR bands were separated by agarose (1%) gel electrophoresis in the presence of ethidium bromide and then photographed under a UV transilluminator (examples in figure 4.4)

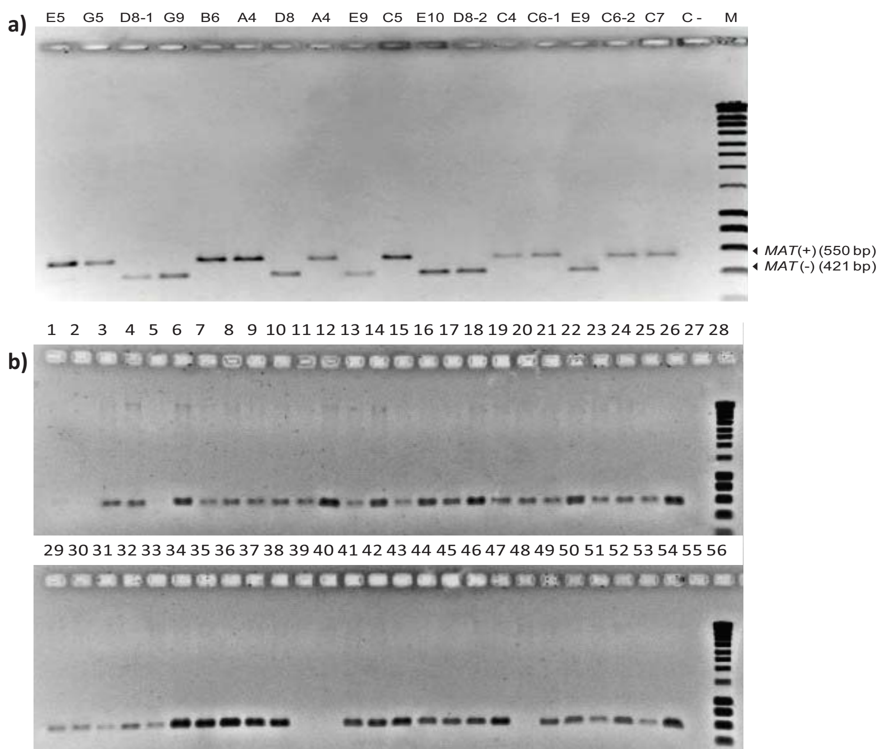

Figure 4. 4. a) Multiplex PCR amplification of DNA from ascocarps with mating type specific primers for *MAT*(+) and *MAT*(-). b) Example of PCR amplification of ectomycorrhizas DNA with *T. melanosporum* specific primers ITS4LNG-ITSML. Numbers 1 to 25 and 29 to 53 are ectomycorrhizas. 26 and 54 are positive controls; 27 and 55 are negative controls (water); 28 and 56 are the marker. Marker: 1 kb DNA Ladder. Sizes (bp): 200, 400, 600, 800, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, 10000.

Simple sequence repeat (SSR) genotyping of *T. melanosporum* ectomycorrhizas and ascocarps was performed using primer pairs relative to ten SSR markers (Table 4.1), previously selected for their polymorphism among truffle ascocarps of different geographical origin (Murat et al. 2011). PCR conditions and genotyping of ascocarps

and ECMs were realized as described in Murat et al. (2011).For the analysis of SSR, PCR reactions were performed in a 10 µl reaction mixture containing 1 µl reaction buffer 10x (Sigma), 200 µM dNTP, 1.5 mM $MgCl_2$, 10 pmol of each primer and 10 pmol of DNA target. The forward primers were modified, adding a fluorophore with different dyes, D2, D3 or D4 (Sigma, Milan, Italy), allowing a better detection and differentiation of them in further analysis. For the analysis of the ectomycorrhizas 3 root tips where used for each sampled plot. The thermal profile adopted to amplify SSR loci is that reported by Murat et al. (2011): 4 min at 94°C followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C (60°C for Tm112 and Tm1) for 30 s, extension at 72°C for 45 s and a final extension of 5 min at 72°C.

All PCR reactions were carried out In a GeneAmp® 9700 PCR system (Applied Biosystems, Foster City, CA, USA). All PCR experiments included negative controls (no DNA template). PCR products for the SSR markers were analyzed in a genome lab separation capillary array 33-75B (Beckman Coulter, Villepinte, France), using a Beckman CEQ 8000 Genetic Analyser (Beckman Coulter, Villepinte, France). Each reaction contained 20 µl of Sample Loading Solution (SLS, Beckman Coulter, Villepinte, France), 1 µl of specific size marker (DNA Size Standard kit – 600 (Beckman Coulter, Villepinte, France)) diluted five times and 1 µl of the marked PCR product diluted 5, 10 or 100 times (depending on the sample). PCR products were migrated at 6KV for 80 minutes. The size of the fragments was determined automatically by the program provided with the Beckman CEQ 8000 Genetic Analyzer (Figure 4.5). After a manual revision and correction of each migration, the sizes of each PCR product for each SSR marker were collected in an Excel sheet.

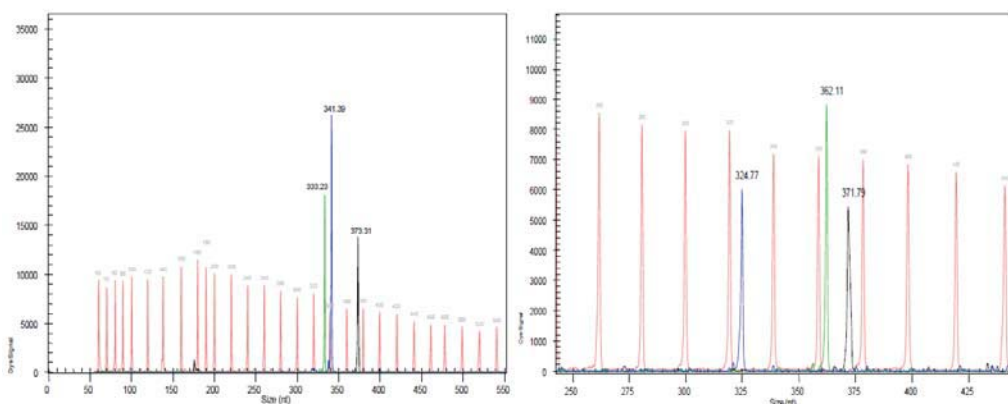

Figure 4. 5. Examples of the results for the SSR screening for three samples given by the Beckman CEQ 8000 Genetic Analyzer. Blue, green and black peaks correspond to the amplification for the primers Tm75, Tm22 and Tm269 respectively. The reeds ones correspond to the size marker (600bp) used as reference.

SSR	Motif	Fwd Primer	Rvs Primer	Loc	Polim	Na	Size (bp)
Tm112	(TAT)19	GGAGGCTCGAG CTTTCATCGG	CTGCGTACCAG GCACAACATC	In	No	1	172
Tm16	(ATA)12	GTGATATCGTTC GGGTTGTC	GGGCGAAAAAC ACAGGAG	IG	Si	2	253-256
Tm2	(TAT)15	ATCTTCGCTGCT TGATACAT	GGGGTAATGCA GGTCTACGA	IG	Si	2	164-207
Tm241	(TAA)17	CCCTTTAGCTTT CCTATCTATC	TGGTGGATGTT GTTGCACTC	IG	Si	2	225-228
Tm98	(TAT)15	CTCCCCAATCAT TCAATCACTCC	GGATTCGGGGT AATATAGTTG	IG	Si	2	248-269
Tm1	(ATTG)18	CAACTAGCAACC GCAGAACA	TGCACGGTTCA GCTATAACAC	IG	Si	2	330-338
Tm75	(GAAA)14	CCGCTGTCATAC GGGACTACTAA	CTGCAAGATCG TCATCCGAGAC	IG	Si	2	304-344
Tm9	(ATCA)12	GCAAAATAACC GAATGCTGA	TGTCCAATGGG TGAGGGTAG	IG	Si	2	327-339
Tm22	(CCTCAT)17	ATTCCTTCTGCC GTCGTTG	ACCATGGCACCT GTATTTCC	IG	Si	4	324-360
Tm269	(TGTTGC)15	TGTCGATCTCCA CCTTCATTT	CAAAGAAGCCC CTCACCTTC	IG	No	1	374

Table 4. 1. SSR markers used for the ascocarps and ectomycorrhiza genotyping. SSR – Name according to Murat et al. (2011); Loc – Localization in genome: In – Intron, IG –Intergenic region; Polim – Polymorphism; Na – Number of alleles observed.

3. Results

3.1. Spatial distribution of Mating types

During the harvesting season in 2010 – 2011, seventeen *T. melanosporum* ascocarps were sampled under trees F10 and F11. The fruiting bodies production was located in two parts of the *brulés*, one corresponding to the lines 4 to 7 and the second to the lines 8 to 10. The maternal tissue (gleba) of the ascocarps harvested along the transect, corresponding to lines 4 to 7, always carried the *MAT(+)* locus whereas that of the ascocarps from lines 8 to 10 always had the *MAT(-)* locus (Figure 4.6A and 4.7).

A total of 42 root samples were collected and 205 *T. melanosporum* ectomycorrhizas covering the two productive areas and some non-productive parts of the *brulés* were identified. *T. melanosporum* ectomycorrhizas were missing from only two root samples located in squares G6 and G7 (Figure 4.6B). One hundred and eight out of 117 *T. melanosporum* ectomycorrhizas selected were successfully genotyped with *MAT*-specific primers. For ectomycorrhizas harvested in square G5, D7 and one sample in A4 no amplifications with *MAT* primers were obtained. For

each *T. melanosporum* ectomycorrhiza, only one *MAT*-specific band was obtained and, as for the maternal tissue of the ascocarps, a clear spatial segregation between mycelia of different mating types was observed (Figure 4.6B).

Forty-eight soil samples were harvested to cover all of the *brulés* area and analyzed for the mating type spatial distribution. At least one gene was amplified in 45 of the soil samples. Mycelia harboring the *MAT*(+) idiomorph were identified in almost all soil samples, even next to sites in which the mycorrhizas and the gleba of the collected ascocarps were formed by mycelia harboring *MAT*(-) (Figure 4.6C). By contrast, the presence of only *MAT*(-) in soils was confined to squares E8 and F8 (Figure 4.6C).

3.2. Genets spatial distribution

Two of the SSRs tested were monomorphic (Tm16 and Tm269) when analyzed on ascocarps and ectomycorrhizas. The remaining eight SSRs identified two to four alleles (Table 4.2). According to the multilocus profiling obtained by merging the information from the SSR and the *MAT*-based screening, seven strains or multilocus genotypes, two with the *MAT*(-) and five with the *MAT*(+) idiomorph, were identified among the maternal tissue of the 17 harvested truffles (Table 4.3). Genotyping of 36 *T. melanosporum* ectomycorrhizas harvested in squares A3, B4, D4, F4, B5, D6, C9, F9 and D10 failed. Consequently, these ectomycorrhiza samples were discarded from future analyses. The multilocus genotyping was successfully realized on 81 ectomycorrhizas and a total of nine genets were identified, three with the *MAT*(-) and the remaining with *MAT*(+) (Table 4.4).

The root tips harvested at the same position shared the same multilocus genotype with the exception of one sample in D8 where two multilocus genotypes, but both with the same mating type *MAT*(-), were found (Figure 4.7 and Table 4.3). Mycelium forming mycorrhizas and maternal tissue of the ascocarps shared the same genotype when harvested at the same location, with the exception of one ascocarp in square E9 (Figure 4.7 and table 4.3). The multilocus genotypes of three mycelia forming mycorrhizal were not recorded in the maternal tissue of the ascocarps whereas the multilocus genotype exhibited by the maternal tissue of a single ascocarp was not recorded among the sampled ectomycorrhizas.

According to these results, it was possible to estimate the maximum size for two of the three *T. melanosporum* *MAT*(-) genets to be around 8 m^2 and 6 m^2, whereas the largest size among the eight *MAT*(+) genets was smaller at 2.5 m^2 (Figure 4.7).

Genotype	MAT	SSR markers (Bp) Tm16	Tm241	Tm2	Tm98	Tm112	Tm9	Tm1	Tm75	Tm22	Tm269
A	+	172	253	207	225	248	338	344	327	324	374
B	-	172	256	207	225	248	330	304	327	360	374
C	+	172	253	164	225	248	330	344	339	342	374
D	+	172	256	164	228	269	330	344	339	330	374
E	+	172	253	164	225	248	330	304	339	342	374
F	+	172	256	207	225	248	330	344	339	330	374
G	-	172	253	207	225	248	330	304	327	360	374
H	+	172	253	207	225	248	330	344	339	342	374
I	-	172	256	164	225	248	330	304	327	360	374
J	+	172	256	210	225	248	330	344	339	360	374
Alleles		1	2	3	2	2	2	2	2	4	1

Table 4. 2. Characterization of the genotypes detected in ascocarps and ectomycorrhizas. The allele length is detailed (base pairs – Bp) for each SSR marker and the mating type (MAT) for each genotype.

	Ascocarps		Ectomycorrhiza		Soil samples
Plot	**MAT**	**Genotype**	**MAT**	**Genotype**	**MAT**
A4	+	D	+	D	+
A4	+	E	+	D	+
B6	+	C	+	C	+
C4	+	F	+	F	+
C5	+	F	+	F	+
C6	+	C	+	C	+
C6	+	C	+	H	+
C7	+	C	+	C	+
D8	-	B	-	G	- / +
D8	-	G	-	G/B	- / +
D8	-	G	-	G	- / +
E5	+	A	+	A	+
E9	-	B	-	G	- / +
E9	-	G	-	G	- / +
E10	-	G	-	G	- / +
G5	+	A	-	-	-
G9	-	B	-	B	- / +

Table 4. 3. Comparison of the genotypes (on ascocarps and ectomycorrhizas) and the mating type detected for each type of sample (ascocarps, ectomycorrhiza and soils). The localization on the gird is also shown (plot).

		T. mel		MAT		SSR analysis											
Plot	Tree	PCR	Ne	PCR	Ne	Tm16	Tm241	Tm2	Tm98	Tm112	Tm9	Tm1	Tm75	Tm22	Tm269	Ne	Genotype
A4	F10	+	5	+	5	172	256	164	228	269	330	344	339	330	374	3	D
B4	F10	+	5	+	5	-	-	-	-	-	-	-	-	-	-	3	
B5	F10	-	5	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd		
B6	F10	+	5	+	5	172	253	164	225	248	330	344	339	342	374	3	C
B7	F10/F11	+	5	+	5	172	253	164	225	248	330	344	339	342	374	3	C
C4	F10	+	5	+	5	172	256	207	225	248	330	344	339	330	374	3	F
C5	F10	+	5	+	5	172	256	207	225	248	330	344	339	330	374	3	F
C6-1	F10	+	5	+	5	172	253	164	225	248	330	344	339	342	374	3	C
C6-2	F10	+	5	+	5	172	253	207	225	248	330	344	339	342	374	3	H
C7	F10/F11	+	5	+	5	172	253	164	225	248	330	344	339	342	374	3	C
C8	F11	+	5	-	5	172	256	207	225	248	330	304	327	360	374	3	B
C9	F11	+	5	-	5	172	256	207	225	248	330	304	327	360	374	3	B
D4	F10	-	5	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd		
D5	F10	+	5	+	5	172	256	210	225	248	330	344	339	360	374	3	J
D6	F10	+	5	+	5	-	-	-	-	-	-	-	-	-	-	3	
D7	F11	-	5	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd		
D8-1	F11	+	5	-	5	172	253	207	225	248	330	304	327	360	374	3	G
D8-2	F11	+	5	-	5	172	256/253	207	225	248	330	304	327	360	374	3	G / B
D8-3	F11	+	5	-	5	172	253	207	225	248	330	304	327	360	374	3	G
D9	F11	+	5	-	5	172	256	207	225	248	330	304	327	360	374	3	B
D10	F11	+	5	-	5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3	
E5	F10	+	5	+	5	172	253	207	225	248	338	344	327	324	374	3	A
E6	F10/F11	+	5	-	5	172	256	164	225	248	330	304	327	360	374	3	I
E7	F11	+	5	-	5	172	-	207	225	248	330	304	327	360	374	3	G
E8	F11	+	5	-	5	172	253	207	225	248	330	304	327	360	374	3	G
E9-3	F11	+	5	-	5	172	253	207	225	248	330	304	327	360	374	3	G
E9-4	F11	+	5	-	5	172	253	207	225	248	330	304	327	360	374	3	G
E10	F11	+	5	-	5	172	253	207	225	248	330	304	327	360	374	3	G
F4	F10	+	5	+	5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3	
F5	F10	+	5	+	5	172	253	207	225	248	338	344	327	324	374	3	A
F6	F10/F11	+	5	-	5	172	253	207	225	248	330	304	327	360	374	3	G
F7	F10/F11	+	5	-	5	173	254	208	226	249	331	305	328	361	375	3	G
F8	F11	+	5	-	5	172	253	207	225	248	330	304	327	360	374	3	G
F9	F11	+	5	-	5	-	-	-	-	-	-	-	-	-	-	3	
G5	F11	-	5	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd		
G9	F11	+	5	-	5	172	256	207	225	254	330	304	327	360	374	3	B

Table 4. 4. Characterization of the different genotypes detected in the ectomycorrhizas. Plot – Position in the gird; Tree – Tree origin of the roots; T.mel – Results of the PCR done with specific primers; PCR – Result of the PCR +: positive amplification (band) and -: negative amplification (no band); Ne – Number of ectomycorrhiza analyzed; MAT – results of the PCR done with specific *MAT* primers +: *MAT(+)* and -: *MAT(-);*SSR analysis – The allele length is detailed (base pairs – Bp) for each SSR marker; na – not analyzed. and the mating type (*MAT*) for each genotype

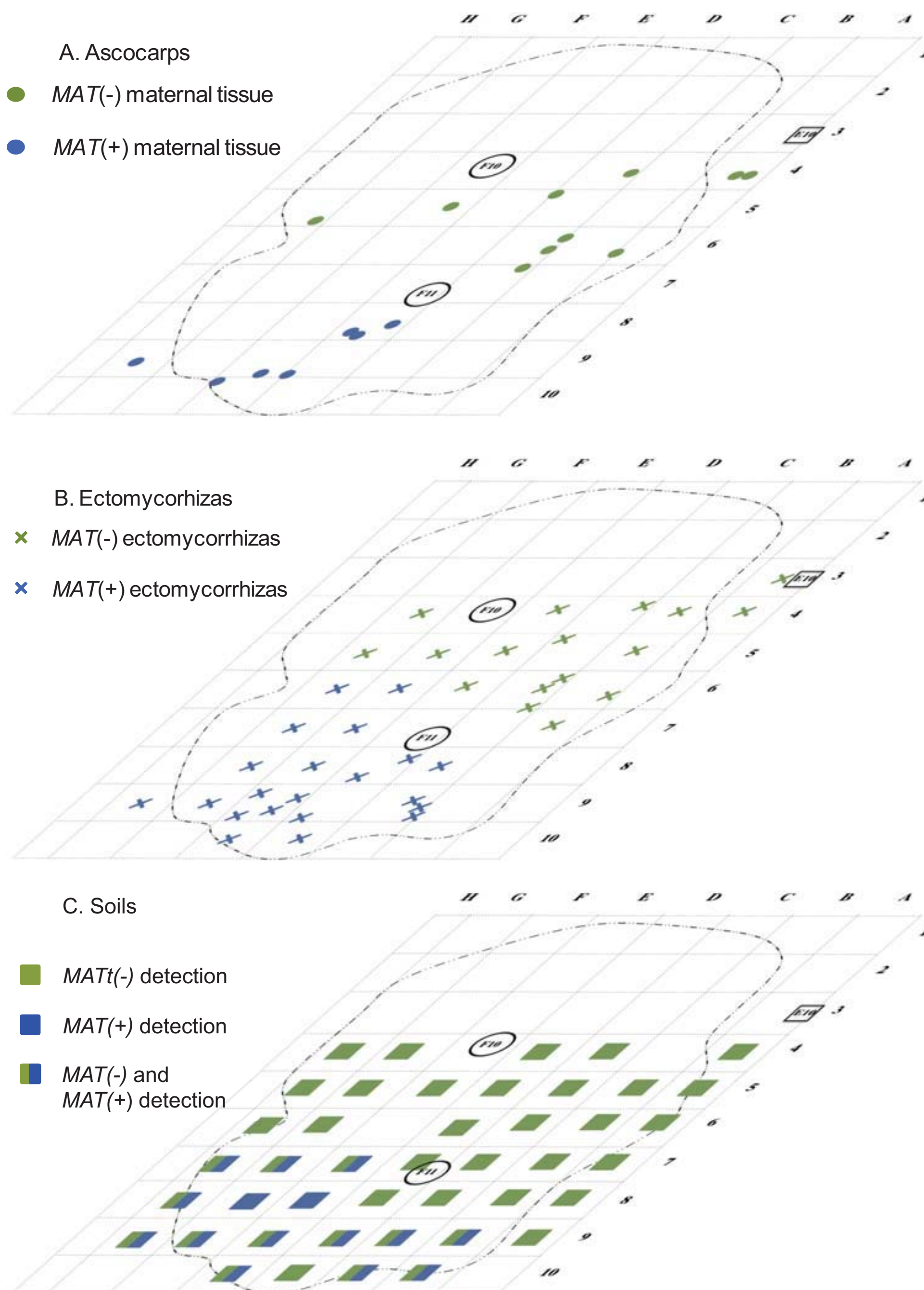

Figure 4. 6. *Tuber melanosporum* mating type identification of the maternal tissue of ascocarps (A), ectomycorrhizas (B) and soil samples (C) harvested in the Rollainville orchard.

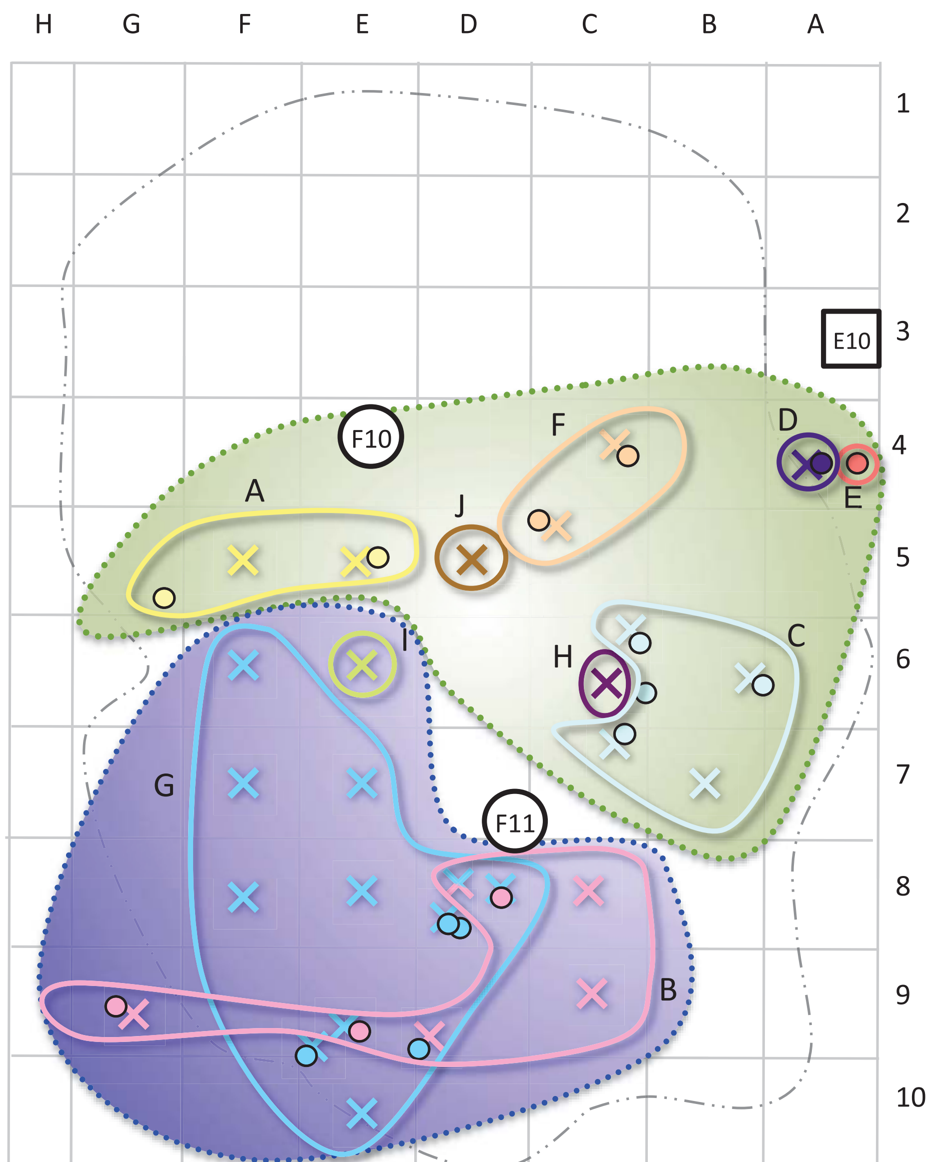

Figure 4. 7. *Tuber melanosporum* genet distribution in the Rollainville orchard. The *green section contains genets* with *MAT*(-) and genets with *MAT*(+) in blue section of the figure. The identification was realized on the maternal tissue of ascocarps (circle) and ectomycorrhizas (cross). The samples belonging to the same genets are identified with the same color and the putative position of the genets is indicated by a line of the same color of the genet. Each genet is identified by a letter which correspond with genotypes in tables 4.3 and 4.4.

4. Discussion

Thanks to a fine scale survey and genotyping of ectomycorrhizas from the *T. Melanosporum* orchard, a pronounced spatial genetic structure in T. melanosporum population in the orchard was identified. It could be seen that the distributional pattern of *T. melanosporum* genotypes on its hosts was nonrandom, as indicated by the mating type locus. These data coupled to the screening of soil mycelium and ascocarps provide us basic information on *T. melanosporum* vegetative and reproductive patterns.

4.1. Distribution of *T. melanosporum* strains of different mating types

Very few studies have investigated the *in situ* distribution of filamentous ascomycetes according to their mating type. Zaffarano et al. (2011) studied heterothallic endophytes belonging to the *Phialocephala fortinii sensu lato* – *Acephala applanata* species complex. They did not identify any patchy distribution, as both mating types of these endophytes were found in the same locations. On the contrary, the *MAT*-based screening of ectomycorrhizas from the rootlets of nursery inoculated plants growing in pots demonstrated that the coexistence of both mating types on the same radical system is a possible but temporary condition, because one mating type tends to outcompete the other over time (Rubini et al. 2011a), as previous analyses carried out on a natural truffle stand have shown that each single plant hosts a single *T. melanosporum* strain (Rubini et al. 2011a). With the results obtained after the extensive screening based on the mating type genes and SSR loci of ectomycorrhizas, it is shown that the presence of different strains on a single host plant in open field condition is also possible, sharing them the same mating type.

In the studied site large soil patches (up to 15m^2) were detected, where all of the *T. melanosporum* mycorrhizas displayed the same mating type. Nevertheless, plants from these sites harbored different genets, suggesting a nonrandom distribution of *T. melanosporum* strains of opposite mating type. Furthermore, in a recent work Linde and Selmes (2012) reported that 50% of plants screened from three *T. melanosporum* orchards in Australia harbored ectomycorrhizas of the same mating type, as well as a skewed frequency of the two mating types in these sites. The presence of a single mating type has primarily been observed on older plants from either natural or cultivated sites, rather than on nursery seedlings (Rubini et al. 2011a; Linde and Selmes 2012; present study). This nonrandom distribution of genotypes could reflect competition between genets of different mating types.

Anyway, this hypothesis needs to be corroborated with future investigations to evaluate whether mating-type-linked genes mediate this phenomenon.

4.2. Spatial genetic structure of *T. melanosporum* in the studied orchard

Both sexual and vegetative propagation are important to form and determine fungi populations. Ectomycorrhizal populations result in a trade-off between asexual, vegetative extension of mycelium in soil and recruitment of dispersed meiotic spores (Douhan et al. 2011). Studying the genetic structure of populations can provide information on the ecological strategies of a given species. In the studied orchard, 9 genets were identified at root level, and the patch of mating type *MAT*(+) comprised six genotypes while the patch of mating type *MAT*(-) only three. This is in agreement with Bertault et al.(2001) and Riccioni et al.(2008) who identified several genotypes among the ascocarps harvested under single trees from natural sites. According to our results, *T. melanosporum* genets were estimated to cover few m^2 (with a maximum of 2,5 m^2 in *MAT(+)* region and up to 8 m^2 in *MAT(-))*. The mean size of these genets was similar to that observed for early successional or pioneer fungi, such as *Laccaria bicolor* (maximum size = 3.3 m^2; Selosse et al., 1999) and *Hebeloma cylindrosporum* (maximum size = 7 m^2; Gryta et al., 2000). Douhan et al. (2011) reported that the genet size depends more on competition and the time elapsed since the last disturbance, than on other factors such as forest age. Interestingly, *T. melanosporum* fructifications are always found in open plantations and, when the plantation or the forest is aged and closed, the production of fructification decline or stops. It has also seen that the presence of competing fungi in closed canopies depresses *T. melanosporum* fructification (Hall et al. 2007; Garcia-Barreda and Reyna 2012). The management of truffle orchards (e.g. tree pruning, tillage, grass cutting and searching for truffles with dogs) leads to the creation of open woodlands. The disturbance resulting from the truffle orchard management might thus favor the persistence of different, small *T. melanosporum* genets under the trees, regardless of their age.

In this study, the genetic diversity of ascocarps, ectomycorrhizas and soil samples was compared. A fairly good correlation was observed between the genetic structure of ascocarps and ectomycorrhizas, as six out of the ten genets were shared between the two structures. Such a correlation between ectomycorrhizas and fruiting bodies was already observed previously in a natural *T. melanosporum* site (Rubini et al. 2011a) and in other ectomycorrhizal species such as *Hebeloma cylindrosporum* (Guidot et al. 2001), *Suillus grevillei* (Zhou et al. 2001), *S. pictus* (Hirose et al. 2004) and *Tricholoma matsutake* (Lian et al. 2006). But for *Laccaria*

amethystina, Hortal et al. (2012) found only nine genets in both forms out of the 332 and 265 genets identified as fruiting bodies and mycorrhizas, respectively.

In this work, the presence of *T. melanosporum* was also analyzed in soil samples. We have been able to identify the mating type of the *T. melanosporum* mycelium in most of the soil samples. It is important to highlight that in addition to *MAT*(-), *MAT*(+) soil mycelium have been detected next to *MAT*(-) ectomycorrhizas. These results could be explained as considering *T. melanosporum* soil mycelium more dynamic and with a higher turnover than mycorrhizas.

With the obtained results, it is easy to question how sexual reproduction can occur and how strains of opposite mating type can meet each other in truffle orchards, like the one studied, where large patches of ectomycorrhizas of the same mating type are present. Following the theory, these large patches are destined to be infertile unless a sexual partner arrives. In *Daldinia loculata*, a non ectomycorrhizal heterothallic ascomycete, the two sexual partners can often be quite distant, but this fungus differentiates micro-conidia that are dispersed by wind over long distances (Guidot et al. 2003). Truffles live and fruit underground and production of mitotic conidia has been reported only for *Tuber borchii* (Urban et al. 2004).

The maternal tissue of the ascocarps harvested almost always showed the identical multilocus genotypes of the nearby ectomycorrhizas, which matches with the theory that the mycorrhizal strains behave as the maternal partner in the cross (Rubini et al. 2011a). In the orchard, the productive field patch hosting *MAT*(-) ectomycorrhizas is surrounded by soil in which both *MAT*(-) and *MAT*(+) mycelium was present, whereas no *MAT*(-) soil mycelium was detected around the patch of *MAT*(+) ectomycorrhizas, although this was a productive site too.

If *T. melanosporum* was supposed to produce mitotic conidia, environmental factors such as wind and water might contribute to disseminate them, as in other fungal species (e.g. for *Coniothyrium minitans,* (Yang et al. 2009)). On the other hand, the sexual partner can also be naturally brought near root-resident strains by soil microfauna or by other animals (Kendrick 1985; Lehmkuhl et al. 2004; Kataržytė and Kutorga 2011)that are attracted by truffle volatiles (Pacioni et al. 1991; Hochberg et al. 2003; Hohmann and Huckschlag 2005; Splivallo et al. 2011). Finally, humans can be an important vector for fungal dissemination as we can spread truffle spores or any detached cells via agricultural machinery and soil practices in truffle grounds (Agrios 2005). However, the dispersal of these elements as well as the presence of mycelia of opposite mating type in the soil are necessary but not

sufficient conditions to ensure mating with root colonizing strains. With the perfection of the real-time PCR technique to quantify the presence of both mating types in truffle grounds it would be possible to detect if there exist any threshold or ratio needed to ensure this mating between strains. The technique should be improved, but the detection of *MAT* genes and the relative quantity from one and the other will be able to be analyzed in soil samples. This will be useful to compare productive and nonproductive truffle grounds and to try to understand why a productive orchard can became unproductive.

In conclusion, this study shows that several genets can be found on the same host plant colonized by *T. melanosporum* and, more importantly, that this distributional pattern appears to be linked to the mating type locus. This finding calls for further studies to understand how mating genes drive phenomena of intraspecific competition in this species. Indeed, long before this truffle mating strategy was discovered, many truffle growers were inoculating soil with mature ascocarps, although this is not the case for the truffle orchard analyzed here. Whether inoculating soil each year with spores will be sufficient to promote production remains to be elucidated, but any cultivation practice aimed at supplying the sexual partners to root resident strains should promote truffle mating and production.

Conclusions

Conclusions

The mycorrhization of plants with species of the *B. edulis* complex has been achieved under pure culture synthesis conditions. The production of *B. edulis* mycorrhizal plants under nursery conditions needs still more research to determine an appropriate procedure.

Progress of new technologies in molecular biology (real-time PCR, sequencing, etc.) and the availability of fungal genomes have led to the development, implementation and use of techniques for the traceability of edible ectomycorrhizal fungi. Through the thesis it has been demonstrated that the real-time PCR technique, with the design of specific oligonucleotides for the detection and quantification of extraradical mycelium in soil, allows us to determine the concentration of soil mycelium in forests and plantations, increasing detection limits over conventional PCR. Specific primers and probes have been designed for *B. edulis* and *T. melanosporum* for real-time PCR amplification, allowing the traceability of these fungal species in the controlled production of edible mycorrhizal mushrooms. No direct relationships were found between soil mycelium amounts and sporocarp production for any of the studied fungal species, but it was possible to obtain positive correlations between vegetative structures (ectomycorrhiza and extraradical mycelium) for *B. edulis*, *R. roseolus* and *T. melanosporum*, when samples were collected near the host plant, in the zone influenced by the rhizosphere. The results obtained open the possibility of using quantification of soil mycelium by real-time PCR as a good indicator for root colonization in field conditions (in natural areas or in manmade orchards), especially when a nondestructive sampling or less time consuming analysis were required.

Further and longer studies to complete and compare the seasonal dynamics of soil mycelium biomass under different ecological or cultural conditions could elucidate their relationship with the onset of sporocarp production.

The extraradical mycelial amounts of *B. edulis*, *L. deliciosus* and *R. rosolus* and *T. melanosporum* mycorrhizas were correlated with climate parameters as temperature, rainfall, solar radiation, relative humidity and evapotranspiration.

Moreover, it has been possible to map the distribution of different *T. melanosporum* genotypes trough the traceability of ascocarps, ectomycorrhizas and soil samples.

Specific conclusions of each chapter are listed below:

Chapter 1

1. The *in vitro* inoculation of axenically grown plants is a sound method for the synthesis of mycorrhizas with species of the *B. edulis* complex.
2. The selection of appropriate isolates is a crucial step for controlled inoculations to produce mycorrhizal plants, as seen with the different success between strains of the same species in pure culture inoculations.
3. The production of *Boletus* spp. mycorrhizal plants in nursery conditions could be achieved by acclimation of *in vitro*-produced mycorrhizal plants. This methodology needs more research to be improved. Current methods for the production and application of fungal inoculum on containerized seedlings have not worked with the *B. edulis* species complex.
4. The preservation of mycelium in cold sterile water is a simple and effective method for the storage and conservation of most of the isolates of *B. edulis* species complex (except for *B. pinophilus*)

Chapter 2

5. The primers and the TaqMan® probe designed in this thesis are suitable for the specific detection and quantification of *B. edulis* mycelium in a forest soil by real-time PCR.
6. *Boletus edulis* and *L. deliciosus* extraradical mycelium behave similarly with annual dynamics characterized by a high seasonality, with minimum amounts of soil mycelium before or at the same time of fructification period followed by marked maximums after fructification.
7. *Boletus edulis* and *L. deliciosus* ectomycorrhizas do not follow the same pattern as the extraradical mycelium. *Boletus edulis* mycorrhizas increased in the fructification period, whereas for *L. deliciosus* the number of mycorrhizas seems to be constant.

8. A positive correlation was established between the concentration of *B. edulis* soil mycelium quantified by real-time PCR, and the number of *B. edulis* ectomycorrhizal tips counted in the same soil samples.

9. Plots with the highest number of mycorrhizas of *B. edulis* had also the highest sporocarp production. This positive correlation between abundance of mycorrhizas and mushroom production was not found for *L. deliciosus*.

10. Mycelium biomass dynamics are strongly dependent on three climatic factors: rainfall, mean temperature and solar radiation.

Chapter 3

11. The primers and the TaqMan® probe designed for *T. melanosporum* are suitable for the specific detection and quantification of soil mycelium, by real-time PCR, in natural truffle grounds and in plantations.

12. TaqMan® real-time PCR technique is a good method for the traceability of *T. melanosporum* and *R. roseolus* without disturbing host trees, allowing us to detect the persistence of the fungus and to follow-up the expansion of the soil mycelium after the plantation establishment.

13. The first stages of plantations, when the fungus spreads, are highly influenced by local climate, soil conditions and planting distances.

14. Positive correlation between vegetative structures, ectomycorrhizas and extraradical mycelium, were observed for *T. melanosporum* and *R. roseolus* when samples were collected near the host plant, in the zone influenced by the rhizosphere.

15. These fungal species are differently affected by weather conditions: rainfall, temperature, solar radiation and evapotranspiration. *Rhizopogon roseolus* mycelium is more affected by climatic conditions than their ectomycorrhizas, contrary for *T. melanosporum* mycorrhizas seem to be more affected by climate variations than soil mycelium.

16. No direct quantitative relationship has been established between extraradical soil mycelium and sporocarp formation. Mycelium of *T. melanosporum* was detected in nonproductive plots and also in orchards producing *T. brumale*. Nevertheless, productive orchards showed higher

mycelium biomass than nonproductive ones. Mycelium concentration was not significantly different between areas with three different host tree species established in the same site.

17. The natural truffle ground had greater amount of mycelium biomass than the man-made truffle orchards in the same area.
18. Nonirrigated plots were containing more *T. melanosporum* mycelium biomass than irrigated ones.
19. Quantitative results are useful to evaluate the fungal response to cultural treatments and to understand the dynamics of *T. melanosporum* mycelium in the soil, especially in mature plantations where root sampling for the evaluation of ectomycorrhizas is difficult.

Chapter 4

20. The genetic structure and turnover of *T. melanosporum* mycelia are more dynamic in soil than that at the root level.
21. Several genets can be found on the same host plant colonized by *T. melanosporum* and this distributional pattern appears to be linked to the mating type locus.
22. Further studies are needed to understand how mating genes drive phenomena of intraspecific competition in this species.
23. Any cultivation practice aimed at supplying the sexual partners to root resident strains should promote truffle mating and production.

Resum

Resum de la tesi doctoral redactat en Català, llengua oficial de la UB, que proporciona una idea precisa del contingut de la tesi doctoral que es presenta.

Abstract of the doctoral thesis written in Catalan, the official UB language which provides an accurate idea of the content of the doctoral thesis presented.

Resum

Els fongs comestibles constitueixen un important recurs forestal que contribueix significativament al valor socioeconòmic i ambiental d'un territori (Boa 2004). Degut al progressiu canvi d'us del sòl que s'està produint a diverses àrees, com les mediterrànies (propiciat principalment per l'abandonament de zones agroforestals poc productives), produccions alternatives com els fongs ectomicorízics comestibles poden constituir un incentiu pel manteniment de zones rurals i forestals, així com el seu paisatge associat, assolint així un equilibri entre les necessitats ecològiques i econòmiques dels sistemes agroforestals.

La majoria de fongs silvestres comestibles s'associen amb les arrels dels arbres formant una simbiosi denominada ectomicoriza, que exerceix un paper crític en el manteniment de l'equilibri de l'ecosistema (Smith and Read 2008). Bàsicament, el fong té el paper de proporcionar nutrients i aigua del sòl a la planta a través de la xarxa de miceli que s'estén pel sol i que s'uneix a les arrels d'aquesta, incrementant el volum de sòl explorat pel sistema radical (Smith and Read 2008). La planta, proporciona al fong productes de la fotosíntesi translocats a l'arrel. Degut a la complexitat d'aquesta simbiosi el fongs ectomicorízics son difícils de cultivar. Moltes d'aquestes espècies no creixen o ho fan amb dificultat en els medis de cultiu coneguts (Brundrett et al. 1996). En aquest context, part de la investigació que s'ha dut a terme, i que s'exposa al Capítol 1, té com a objectiu la millora de la tecnologia relacionada amb la producció controlada de fongs ectomicorízics comestibles, com és el cas de *Boletus edulis*.

La recol·lecció i comercialització d'aquests bolets està en clar creixement, ja que proporciona un valor socioeconòmic cada vegada més important. Actualment, l'obtenció de la majoria d'aquests bolets (excepte la tòfona i en alguns indrets el rovelló) depèn exclusivament de la recol·lecció de la producció natural, la qual cosa ha fet augmentar l'explotació d'aquest recurs fins a començar a posar en perill la sostenibilitat d'aquesta activitat i el manteniment de la biodiversitat dels ecosistemes forestals. D'altra

banda, l'elevada variabilitat en la producció natural dels fongs comestibles és un problema seriós per al desenvolupament de sectors econòmics relacionats amb la producció estable del recurs, especialment la gastronomia, la restauració i el turisme micològic. Per aquestes raons econòmiques i mediambientals, la producció controlada de fongs ectomicorízics comestibles mitjançant la inoculació de plantes en viver i l' establiment de plantacions productives és una alternativa per complementar la recol·lecció en zones naturals, especialment per aquelles espècies més preuades i consumides(Hall et al. 2003; Boa 2004; Hall et al. 2005; Karwa et al. 2011; Voces et al. 2012; Wang et al. 2012). Per dur a terme els experiments realitzats al llarg d'aquesta tesi doctoral es van seleccionar quatre espècies de fongs ectomicorízics comestibles: *Boletus edulis, Lactarius deliciosus, Rhizopogon roseolus* i *Tuber melanosporum.* Les quals es troben entre els fongs micorízics comestibles més apreciats arreu del món (Wang et al. 2002).

L'objectiu de l'estudi i la traçabilitat de la simbiosi micorízica és arribar a una producció sostenible de fongs comestibles. Per arribar a aquest punt abans és necessari disposar de metodologies que ens permetin controlar i fer un seguiment de les espècies en qüestió, ja sigui en plantacions establertes i controlades, o en zones on hi hagi una producció natural d'aquests fongs. En altres paraules, és necessari un ampli coneixement del comportament de les espècies fúngiques que es volen explotar, tant en condicions naturals com en ambients modificats. Cal conèixer la seva ecologia, el seu comportament, el cicle vital, poder traçar totes les estructures del fong (miceli, ectomicorizes, esporocarps...) així com el patró de fructificació de cada espècie.

Per tal d'aconseguir una sostenibilitat en la producció de bolets comestibles podem optar, depenent de l'espècie, per l'establiment de plantacions controlades (com és el cas de la tòfona), o bé gestionar les zones naturals productives (boscos, arbredes, prats... depenent de l'espècie a explotar). En ambdues situacions, cal tenir un bon coneixement de la biologia i el comportament de l'espècie que es vulgui produir i recollir (Wang and Hall 2004; Karwa et al. 2011; Savoie and Largeteau 2011; van der Linde et al.

2012). Però, també és necessari generar noves tecnologies per millorar els mètodes de detecció dels fongs, sobretot en el sòl, que permetin assegurar que l'espècie en qüestió es troba al medi i que evoluciona de la manera esperada. És molt important que aquestes tècniques per traçar el desenvolupament dels fongs no alterin les plantes hoste ni l'ambient on aquests creixen, per tal de pertorbar al mínim la plantació o la zona de producció natural. Tecnologies moleculars com la PCR en temps real poden ser emprades per detectar una espècie en concret tant sols agafant una petita mostra de sòl, simplificant el procés de mostreig de camp. Les tècniques moleculars han esdevingut una eina important a tenir en compte, ja que no només poden ser útils per detectar i fer un seguiment dels fongs, sinó que també ens han de sevir per ampliar els coneixements de la biologia dels fongs ectomicorízics. Com més coneixements tinguem sobre la genètica, i l' ecologia d'aquests espècies fúngiques, més fàcils serà poder establir metodologies i models, que portin cap a un cultiu controlat d'aquests fongs sense destorbar, ni sobreexplotar, el medi en el que viuen.

L'objectiu final de les línies de treball en les que s'emmarca aquesta tesi és generar els coneixements necessaris perquè, a través de la micorizació controlada d'arbustos i espècies arbòries forestals, es pugui arribar a una producció sostenible de diferents espècies de fongs micorízics comestibles amb un alt interès comercial. Obtenint així un benefici per a l'ecosistema i també per a la nostra societat, ja que aquests fongs tenen un valor econòmic i socio-cultural important en gran part del planeta.

El **objectius** específics de la tesi son:

1. La síntesi de micorizes i la producció de planta micorizada amb aïllats fúngics del complex *Boletus edulis.* El desenvolupament de metodologies que permetin la producció de plantes de viver micorizades amb aquesta espècie fúngica i l'establiment de plantacions per a la producció controlada de *Boletus* spp., comparables a les establerts amb *Tuber melanosporum* o *Lactarius deliciosus.*

2. Desenvolupament de tècniques moleculars per a l'estudi dels fongs ectomicorízics. Disseny d’encebadors i sondes específiques per detectar i quantificar el miceli del sòl.

3. L'aplicació de les tècniques moleculars (PCR a temps real, PCR convencional, genotipat utilitzant marcadors microsatèl·lits i encebadors per determinar el tipus de compatibilitat) en les plantacions controlades i a les zones productives naturals per tal de traçar les espècies fúngiques estudiades: *Boletus edulis*, *Lactarius deliciosus*, *Tuber melanosporum* i *Rhizopogon roseolus*.

4. Identificar les possibles correlacions entre la dinàmica de les quantitats de miceli present al sòl, l’ abundància de micorizes i la producció de bolets.

La producció de carpòfors de fongs comestibles en plantacions productives establertes amb plantes micorizades pot comportar molt de temps. Per aquest motiu, és molt important establir metodologies dirigides al seguiment de la persistència del fong inoculat en les diferents fases vegetatives de la simbiosi per determinar les possibilitats de producció. De la mateixa manera, l’estudi dels factors que poden influir en la persistència de la simbiosi requereix disposar de les eines apropiades per caracteritzar i quantificar el fong que es vol cultivar. La caracterització morfològica d’ectomicorizes s’ha fet servir tant amb finalitats taxonòmiques com ecològiques (Agerer 2006), però existeixen molts casos en els que els caràcters anatòmics o morfològics no són concloents per la identificació del fong. Les tècniques moleculars, basades en l’anàlisi de l’ ADN mitjançant l’ús de la tècnica de la *Polymerase Chain Reaction* (PCR), han complementat els estudis morfològics, obrint noves possibilitats d’exploració de les fases críptiques de la simbiosi (les micorizes i el miceli extraradical) i incrementant el coneixement del seu paper ecofisiològic al sòl. Aquestes tècniques s’han aplicat a la identificació de micorrizes (Gardes and Bruns 1993; Horton 2002; Mello et al. 2006) i de comunitats (Anderson and Cairney 2004; Martin et al. 2007). Un dels passos crítics en el seguiment de

la persistència fúngica es poder detectar de forma fiable el miceli extraradical d'aquests fongs. Aquesta fase és la més activa metabòlicament de la simbiosi (Smith and Read 2008) però també la més desconeguda degut a la falta de metodologies per al seu estudi. La majoria del estudis previs sobre detecció de sistemes miceliars es van basar en mètodes bioquímics, principalment la detecció d'ergosterol a sòls, però en els últims anys les tècniques moleculars han permès estudis més específics (Dickie et al. 2002; Landeweert et al. 2003; Landeweert et al. 2005; Suz et al. 2006; Suz et al. 2008; Pickles et al. 2010; Douhan et al. 2011) fins i tot aplicant-se per la quantificació específica del miceli al sòl fent us de la PCR en temps real (Landeweert et al. 2003; Raidl et al. 2005; Kennedy et al. 2007; Parladé et al. 2007; Hortal et al. 2008; Suz et al. 2008; Parladé et al. 2009; Zampieri et al. 2012).

La tesi està estructurada en quatre capítols que representen diferents aproximacions per arribar a la producció de bolets ectomicorrízics. El primer pas per establir una plantació per obtenir bolets d'una determinada espècie, és la producció de plantes inoculades amb el fong per tal de poder ser trasplantades a camp. Al **Capítol 1 (Producció de plantes inoculades amb *Boletus edulis* en condicions de cultiu pur i de viver)**, es tracta aquest primer pas. El complex d'espècies que formen el grup *B. edulis* (*B. aereus, B. edulis, B. pinophilus* i *B. reticulatus*) formen part dels bolets més apreciats en la cuina. A Espanya anualment es recullen entre 200 i 20000 Tm de ceps, el que el converteix en un dels bolets ectomicorízics més importants a nivell comercial, arribant a moure un mercat de més d'un milió d'euros a nivell mundial. Aquests bolets només son recollits de la natura ja que encara no s'ha arribat a establir plantacions amb plantes inoculades. Degut a la seva importància diversos autors han intentat produir planta micorizada amb *B. edulis*, tant en cultiu pur, com en condicions de viver (taula 1.1), amb més o menys èxit. Per tal de produir planta micorizada amb *B. edulis*, es van seleccionar diferents hostes (*Cistus albidus, C. ladanifer, C. laurifolius, C. monspeliensis, P. pinaster, Quercus ilex, Betula pendula* i *Castanea sativa*) i diferents soques de les espècies que formen el grup *B. edulis* (*B. edulis*

sensu stricto, B. aereus, *B. pinophilus*, i *B. reticulatus*) i es van produir plantes micorizades en condicions axèniques obtenint bons resultats, sobretot amb les espècies de *Cistus* (taula 1.3 i figura 1.6). Algunes de les combinacions es van seleccionar per aclimatar les plantes obtingudes en cultiu pur a condicions d'hivernacle, mantenint les condicions que es fan servir per al creixement de planta en viver. La pràctica totalitat dels intents va donar resultats negatius, les micorizes de *Boletus* es van perdre als 6 mesos de ser aclimatades. Tant sols en una de les combinacions (*Pinus pinaster* x *Boletus edulis* Be2057) es van detectar micorizes de *B. edulis* en les plantes aclimatades. Per tal de produir planta micoritzada en condicions de viver, es van fer inoculacions amb miceli (crescut en substrat de torba i vermiculita en presència o no de plàntules de *P. pinaster* i *C. albidus*) i amb suspensions d'espores (obtingudes de carpòfors de diferents orígens), sobre pins i estepes, però en cap cas es van obtenir resultats positius.

Un pas clau en la producció de planta inoculada és la selecció de la soca a fer servir i el manteniment d'aquestes al laboratori. Un del problemes afegits a les dificultats de fer créixer fongs com *B. edulis* (amb un creixement molt lent) en cultius purs és que les característiques d'aquests (creixement i capacitat colonitzadora) canvien al llarg del temps després de continues transferències en medis basats en agar (Hung and Molina 1986). Per això és necessari trobar mètodes per emmagatzemar aquests fongs, en els que la viabilitat i l'estabilitat de les seves característiques es mantinguin durant el temps. Una alternativa als procediments basats en la transferència periòdica a medis d'agar és el manteniment d'aïllats en aigua destil·lada a baixa temperatura (Parladé et al. 2011). Per comprovar la viabilitat dels aïllats de boletus després d'ésser emmagatzemats en aigua a baixa temperatura, es van seleccionar 38 soques d'espècies del complex *B. edulis* i 9 de *R. roseolus* (com a control), de les que es van emmagatzemar, a 4°C, 30 discs de miceli en vials de vidre amb tap roscat que contenien 10 ml d'aigua destil·lada estèril. Per avaluar la viabilitat dels fongs, al cap de 3, 6, 12 i 24 mesos d'emmagatzemament, es van sembrar 5 discs de cada aïllat en plaques de medi BAF. Al final de l'assaig es va comprovar que tot els aïllats de *R. roseolus* mantenien un 100 % de viabilitat. Entre el 96% i el 100% dels aïllats de *B. aereus, B. edulis* i *B. reticulatus* mantenien el 100%

de viabilitat després de 3 mesos. Als 12 mesos d'emmagatzematge, el número d'aïllats d'aquestes espècies que mantenien un 100% de viabilitat es trobava al voltant d'un 70%. Per als aïllats de *B. pinophilus* la seva viabilitat va disminuir en tres mesos a un 40%, mantenint-se així fins als 12 mesos. Després de 24 mesos d'emmagatzematge en aigua estèril a 4°C al voltant del 45% d'aïllats de *B. aereus* i *B. edulis* mantenien la seva capacitat de creixement, mentre que tant sols ho feien un 20% dels de *B. pinophilus* i cap dels aïllats de *B. reticulatus* (Figure 1.8). En analitzar estadísticament les dades es va veure que existia una diferència entre les espècies i el temps d'emmagatzematge ($p < 0.0001$; $\alpha = 0.05$). El test de Tukey per comparar mitjanes va separar *B. pinophilus* de la resta d'espècies. En conclusió, l'emmagatzematge en aigua estèril a 4°C és un mètode senzill i eficaç per a l'emmagatzematge i la conservació de *R. roseolus*, per a les espècies del complex *B. edulis* resulta molt variable tenint en compte la soca, i poc aconsellable especialment per a les espècies de *B. pinophilus i B. reticulatus*.

La selecció dels aïllats és també un pas crucial per a les inoculacions controlades per produir plantes micorizades, com s'ha vist en el diferent èxit obtingut entre les diferents soques de la mateixa espècie en les inoculacions en cultiu pur (Taules 1.5, 1.6 i figura 1.6). La inoculació *in vitro* és un bon mètode per a la síntesi de les plantes micorizades amb, però la metodologia per a l'obtenció de plantes micorizades en viver necessita més investigació. Una possibilitat que necessita ser millorada és l'aclimatació de les plantes micorizades en condicions axèniques.

La producció de plantes micorizades amb *B. edulis* per establir plantacions controlades encara no és factible, i per tant, l'opció que ens queda per incrementar la obtenció d'aquests bolets és la gestió dels boscos que els produeixen de forma natural. La disponibilitat de la majoria dels fongs micorízics comestibles depèn, gairebé de forma exclusiva, de la seva fructificació natural (Boa 2004). Donada la poca predictibilitat d'aquesta fructificació, és important establir mètodes de maneig dels boscos per mantenir i incrementar la producció de bolets, preservant al mateix temps la conservació i diversitat de les espècies fúngiques (Ortega-Martínez and Martínez-Peña 2008; Egli et al. 2010). Per tal d'arribar a aquesta fita es

necessari fer us, adaptar o desenvolupar mecanismes que permetin l'estudi i la traçabilitat dels fongs en tots els seus estadis (miceli, micorizes i cossos fructífers), particularment en el cas del miceli extraradical en el sòl.

La formació d'esporocarps de *B. edulis* i de *L. deliciosus* està relacionada amb les característiques de l'hàbitat i les condicions climàtiques, però aquestes dades per si soles no expliquen el perquè de la fructificació d'aquests ni la dinàmica del fongs. Es podria hipotetitzar que la quantitat de miceli present al sòl podria estar relacionada amb la producció de carpòfors. El miceli dels fongs ectomicorízics que creixen al sòl representen entre un 30% i un 80% de la biomassa fúngica (Wallander et al. 2001; Högberg and Högberg 2002) i crea una extensa i dinàmica xarxa miceliar, que juga un paper clau en l'obtenció de nutrients de les espècies vegetals i en la transferència recíproca de carboni i nutrients entre plantes del mateix ecosistema (Read 1992; Simard et al. 2002). Es sap poc sobre la dinàmica anual del miceli dels fongs ectomicorízics al bosc, però el seu estudi està guanyant importància degut al seu rol en el cicle del carboni (Cairney 2012), la mobilització de nutrients del sòl (Kjøller 2006), i la unió dels diferents components de l'ecosistema (plantes, fongs, microfauna i microorganismes) (Fitter and Garbaye 1994; Courty et al. 2010). El principal problema és que la distribució del miceli extraradical al sòl és poc coneguda, degut a la dificultat d'adaptar mètodes apropiats per al seu estudi (Anderson and Cairney 2004). Per això l'objectiu primer del segon capítol d'aquesta tesi és el disseny d'oligonucleòtids específics per la detecció i quantificació de *B. edulis* per PCR quantitativa en temps real, així com determinar la concentració de miceli extraradical al sòl i l'abundància d'arrels micorizades en parcel·les d'un bosc, representant un gradient de productivitat.

En una primera part, al **Capítol 2** ***(Us de tècniques moleculars per la traçabilitat de fongs ectomicorízics: Dinàmica estacional del miceli de*** **Boletus edulis** ***i*** **Lactarius deliciosus** ***a boscos de pins del centre d'Espanya)*** es van provar els encebadors i la sonda dissenyats per la quantificació de *B. edulis* en mostres de sòl preses en un bosc de *P. sylvestris* de Sòria. Els resultats de la quantificació per PCR en temps real es van comparar amb els obtinguts després d'amplificar l'ADN per PCR convencional. Es van agafar

mostres de sòl (cinc cilindres de 250 cm^3 per parcel·la) mensualment, de setembre a novembre, a parcel·les permanentment vallades de Pinar Grande (Sòria, Espanya), un bosc de pi roig situat al nord del Sistema Ibèric. Els plots es van seleccionar establint un gradient de productivitat de carpòfors de *B. edulis*, de 0 a 38.5 Kg/ha i any, segons el pes mitjà d'esporocarps recollits durant els últims 10 anys. A cada mostra de sòl es van identificar i contar les arrels micorizades amb *B. edulis*, i es va extreure l'ADN total del sòl, fent servir el kit d'extracció PowerSoil™ DNA Isolation Kit. El miceli extraradical de *B. edulis* present al sòl es va quantificar per PCR en temps real fent servir els encebadors i la sonda TaqMan® específics dissenyats per aquest fi, i també va ser amplificat per PCR convencional fent servir encebadors específics dissenyats per Mello et al. (2006). En comparar els resultats obtinguts amb les dues tècniques es va comprovar que la PCR en temps real és més específica i detecta quantitats molt més petites de miceli de *B. edulis* al sòl (amb la PCR en temps real es va detectar *B. edulis* en 49 mostres de sòl, mentre que amb la PCR convencional només es va detectar en 19 de les 75 mostres analitzades en ambdós casos). El mínim detectat amb la PCR convencional es va estimar al voltant de 0.039 mg de miceli per gram de sòl (Table2.4), mentre que amb la PCR en temps real es va estimar en 0.001 mg de miceli per gram de sòl.

La concentració de miceli de *B. edulis* al sòl (mg de miceli / g de sòl) no va presentar diferències significatives al llarg dels tres mesos ($p=0.7643$), ni entre les diferents parcel·les ($p=0.1397$). El nombre d'arrels micorizades amb *B. edulis* per volum de sòl va ser significativament diferent en les parcel·les estudiades ($p =0.005$) així com al tres mesos mostrejats ($p <0.0001$). No es va trobar cap relació significativa ($p=0.615$) entre el número de micorizes i la productivitat de les parcel·les (Kg d'esporocarps de *B. edulis*/ ha i any). Però si es va trobar una correlació positiva ($p =0.0481$) entre la concentració de miceli de *B. edulis* i la presència de micorizes de *B. edulis* a les mostres de sòl. La productivitat de les parcel·les, esporocarps produïts durant els últims 10 anys, no va estar correlacionada ni amb la concentració de miceli al sòl ni amb la presencia o l'abundància d'ectomicorizes de *B. edulis*.

En l'últim apartat del capítol 2 es va estudiar la dinàmica anual del miceli extraradical i la producció d'esporocarps de *B. edulis* i *L. deliciosus*, a dos boscos de pins diferents (Pinar Grande i Pinares Llanos, respectivament), de Sòria. Es van agafar mostres de sòl (cinc per parcel·la) mensualment (entre setembre de 2009 i agost de 2010 a Pinar Grande i entre setembre de 2010 i setembre de 2011 a Pinares Llanos) a vuit parcel·les permanents (quatre per cada lloc). El miceli extraradical de *B. edulis* i *L. deliciosus* es va quantificar per PCR en temps real, amb l'ADN extret de les mostres de sòl, utilitzant els encebadors específics i la sonda TaqMan® dissenyades per *B. edulis* i les presents a la bibliografia per *L. deliciosus* (Hortal et al. 2006). Al mateix temps també es van identificar i contar les micorizes de *B. edulis* i *L. deliciosus* presents a les respectives mostres de sòl.

Les quantitats de miceli de *B. edulis* detectades al sòl no van ser significativament diferents entre les parcel·les, però si es van detectar diferències al llarg del temps, amb un màxim al febrer (0.1576 mg de miceli / g sòl) i un mínim a l'octubre (0.0170 mg de miceli / g sòl). Per *L. deliciosus* es van detectar diferències significatives entre les parcel·les i al llarg del temps. La quantitat més alta de miceli es va trobat al desembre (1.84 mg de miceli / g de sòl) i el mínim al febrer (0.0332 mg de miceli / g de sòl). Les quantitats de miceli de *B. edulis* es van correlacionar positivament amb la precipitació del mes en curs i negativament amb la temperatura mitjana del mes anterior. La biomassa miceliar de *L. deliciosus* es va correlacionar positivament amb la humitat relativa i negativament amb la temperatura mitjana i la radiació solar. No es va observar correlació significativa entre la productivitat de les parcel·les i la biomassa de miceli del sòl per a cap de les dues espècies. Es van detectar diferencies significatives entre el número de micorizes de *B. edulis* de Pinar Grande al llarg del temps i entre els diferents plots. El màxim de micorizes es va detectar a l'octubre de 2009 (244 micorizes en 250 cm^3 de sòl), i el mínim al març de 2010 (3.3 micorizes en 250 cm^3 de sòl). El test de Tukey va separar les parcel·les en dos grups, de manera que les que presentaven major número de micorizes de *B. edulis* també van tindre la major producció d'esporocarps de *B. edulis* en aquella temporada.

Per les micorizes de *L. deliciosus* recollides a Pinares Llanos, es van trobar diferencies significatives entre les parcel·les (p =0.0272). El màxim número de micorizes es va detectar a l'octubre de 2010 (236 en 250 cm^3 de sòl) i el mínim al maig de 2011 (3.3 en 250 cm^3 de sòl). El test de Tukey també va separar les parcel·les en dos grups, però no es va trobar cap relació ni amb la productivitat ni amb la biomassa miceliar d'aquestes.

No es van trobar correlacions significatives entre la producció d'esporocarps de *B. edulis* i els paràmetres meteorològics. La producció de bolets de *L. deliciosus* es va correlacionar positivament amb la precipitació i la humitat relativa, i negativament amb les temperatures màximes i mínimes. En aquest assaig també es va trobar una correlació positiva entre el número de micorizes de *B. edulis* i el miceli extraradical de les mostres de sòl, però aquesta relació no es va poder establir per a *L. deliciosus*.

En conclusió, els encebadors i la sonda TaqMan® dissenyats en aquest capítol serveixen per la detecció i quantificació del miceli de *B. edulis*, en sòls forestals, mitjançant la PCR en temps real. Ambdues espècies, *B. edulis* i *L. deliciosus* tenen una distribució similar al llarg del temps, presentant una dinàmica anual que es caracteritza per una variabilitat estacional, amb un clar augment de les quantitats de biomassa durant els mesos més freds de l'any, però les seves ectomicorizes no segueixen el mateix patró. Estudis més llargs, durant dos o tres anys, són necessaris per confirmar aquests patrons de miceli i de micorizes. Aquestes dinàmiques del miceli d'ambdues espècies són fortament dependents de les condicions climàtiques i, tot i que la pluja és important, no és l'únic factor climàtic que influencien la producció de biomassa miceliar per aquestes espècies. La temperatura mitjana i la radiació solar també son rellevants. El que és important és que alguns d'aquests paràmetres poden ser modificats per l'acció de l'home per mitjà de les pràctiques silviculturals que aplica en les masses arbrades; per exemple, amb tales controlades pot induir modificacions en la penetració de la llum influint en la fructificació d'aquests fongs.

Després de veure com es pot adaptar la tècnica de la PCR quantitativa en temps real per la detecció del miceli extraradical de fongs com *B. edulis* i *L. deliciosus*, tant per fer un seguiment d'aquests en condicions naturals, com

per a establir les relacions entre la concentració de miceli i la quantitat d‘ectomicorizes o les condicions climàtiques, es raonable pensar que aquesta tècnica també pot ser útil per fer el seguiment de fongs que actualment es cultiven en plantacions establertes amb plantes micorizades. Un exemple de fong que ja es pot obtenir a partir de plantacions és la tòfona negra (*Tuber melanosporum)*, que produeix cossos fructífers hipogeus amb un elevat valor socioeconòmic (Boa 2004; Mello et al. 2006). Avui en dia aquest és el fong ectomicorízics més àmpliament cultivat (Hall et al. 2003; Iotti et al. 2012). Viu en simbiosi amb diferents espècies de roures, alzines i avellaners i s’estima que la majoria de les tòfones europees comercialitzades provenen de plantacions (Reyna 2007). Existeixen molts estudis sobre les pràctiques agronòmiques per al seu cultiu (reg, fertilització, control de males herbes, etc.) (Mamoun and Oliver 1997; Ricard et al. 2003; Bonet et al. 2006; Olivera et al. 2011). Des del moment que s’estableix la plantació fins que s’obtenen les primeres tòfones transcorre un llarg període de temps (al voltant de 10 anys) (Shaw et al. 1996). Poder avaluar la supervivència i la persistència del simbiont fúngic en les plantacions a curt i mig termini, ofereix les dades necessàries per poder predir la continuïtat de la plantació. Normalment aquest control s’ha fet a través de l'avaluació de la presència i abundància d’ectomicorizes (Kagan-Zur et al. 2001; Olivera et al. 2011; Reyna and De Miguel 2012). La detecció del miceli extraradical de *T. melanosporum* pot ser un bon complement al seguiment de la producció d’ esporocarps i a l’avaluació de les ectomicorizes per al seguiment de la persistència del fongs al llarg de tot el cicle biològic. L’amplificació específica d’ AND d’aquest fong en sòl s’ha aplicat abans (Suz et al 2006;. Zampieri et al 2010; Gryndler et al 2011), així com la detecció quantitativa (Suz et al. 2008; Zampieri et al. 2012), però utilitzant la PCR en temps real amb SYBR® green, un mètode menys específic. Fent servir sondes Taqman®, la detecció del fong és més específica (explicat de forma detallada al capítol 2 i a la introducció), per aquesta raó, al **Capítol 3** ***(Traçabilitat de fongs ectomicorízics a plantacions: persistència i quantificació dl miceli extraradical de* Rhizopogon roseolus *i* Tuber melanosporum *a plantacions noves i velles)***, s’ha aplicat la PCR quantitativa en temps real per traçar fongs en

plantacions, tant des del moment inicial d'establir una plantació com en plantacions de tòfona madures.

En aquest capítol es van dissenyar encebadors i una sonda TaqMan® específica per *T. melanosporum*, per fer servir en PCR en temps real, per tal de detectar i quantificar el miceli present en el sòl, tant en zones naturals de producció de tòfona, com en plantacions establertes amb planta micorrizada en viver. Aquestes tècniques permeten establir un mètode no destructiu, i menys invasiu, per a determinar la presència del fong en plantacions. Els oligonucleòtids dissenyats es van provar en una nova plantació establerta amb planta micorizada amb *T. melanosporum*, i en dues plantacions establertes amb pins micorizats amb *R. roseolus*. La tècnica de PCR en temps real per determinar la quantitat de miceli extraradical de *R. roseolus* en mostres de sòl ja va ser adaptada amb anterioritat per Hortal et al. (2008). Quan s'estableix una nova plantació, amb l'objectiu de produir tòfones amb plantes micorizades, un factor crucial és la persistència del fong en les arrels dels arbres que la formen, així com que aquest s'expandeixi pel sòl de la plantació. Generalment, la forma de valorar aquesta persistència consistia quasi exclusivament en extreure algunes plantes, o extreure mostres d'arrels directament de terra, per avaluar l'estat de la micorizació. Per tal d'evitar mètodes destructius, com aquests dos, que poden alterar la plantació, podia ser útil fer us de tècniques moleculars basades en l'anàlisi de l'ADN, com la PCR quantitativa en temps real, que permet detectar i quantificar el miceli extraradical de *T. melanosporum* en mostres de sòl. Els objectius principals d'aquesta part de la tesi eren: El disseny d'encebadors i sondes TaqMan®específics per la detecció i quantificació del miceli extraradical de *T. melanosporum* en el sòl; provar els oligonucleòtids dissenyats en una plantació nova de *Quercus ilex* x *T. melanosporum*, des del seu establiment; i detectar i quantificar el miceli extraradical de *T. melanosporum*, per PCR en temps real, en plantacions madures i zones naturals productores de tòfona negra de Navarra.

Per tal d'arribar a aquest fi es van monitoritzar, tant el miceli extraradical com el percentatge de micorizació de les plantes, en una nova plantació de *Q. ilex* x *T. melanosporum*, des del seu establiment (a la primavera de 2010)

fins a 18 mesos després (tardor de 2011). La plantació es troba situada al Prepirineu oriental, al Berguedà, i ocupa una extensió de 0.25 Ha. Es van plantar 229 plantes de *Q. ilex* inoculades amb *T. melanosporum*. Intercalats entre aquests arbres es van plantar, distribuïts a l'atzar, 14 plantes no inoculades (controls). Es van fer mostrejos de plantes inoculades, plantes control i mostres de sòl (4 mostres de sòl a 5 cm del tronc de cada planta mostrejada) al cap de 6, 12 i 18 mesos després de l'establiment de la plantació. Es va fer el recompte del nombre de micorizes de *T. melanosporum* per cada planta. De les mostres de sòl es va extreure l' ADN, i es va quantificar el miceli extraradical de *T. melanosporum* mitjançant la PCR quantitativa en temps real, utilitzant els encebadors i la sonda TaqMan® específics dissenyats.

També es van analitzar dues noves plantacions de *R. roseolus,* establertes a Cabrils al 2009 i al 2010, amb pins micorizats amb aquest fong, fent servir els oligonucleòtids descrits a la bibliografia (Hortal et. 2008). Aquestes plantacions es van establir en un disseny de blocs al atzar, amb unitats experimentals formades per nou arbres micorizats amb *R. roseolus*, o controls no micorizats. Les unitats es van plantar seguint dos marcs de plantació diferents: un marc de plantació de 2x2 m i l'altre d'1x1 m (figures 3.3 i 3.4). Al cap de 6, 12 i 18 mesos (en el cas de la plantació Cabrils 1) i 6 i 12 mesos (per la plantació Cabrils 2) es van mostrejar plantes (control i inoculades) i sòls de cada unitat experimental. De les mostres de sòl es va extreure l'ADN i es va quantificar el miceli de *R. roseolus*. De les plantes mostrejades es va avaluar el seu sistema radicar i es van contar les micorizes de *R. roseolus* presents.

En ambdós casos es van utilitzar les dades meteorològiques disponibles en estacions automàtiques situades prop de les plantacions.

A la nova plantació de *T. melanosporum* es va poder detectar i quantificar el miceli extraradical de *T. melanosporum* des del primer mostreig (després de 6 mesos d'establir la plantació) i, tot i que es va veure un increment del miceli al sòl al llarg del temps, les quantitats de miceli al sòl no van ser significativament diferents al cap de 24 mesos. Es detectà una correlació positiva ($r=0.7814$; $p = 0.0129$) entre el número de micorizes i la

concentració de miceli extraradical a les mostres de sòl que es van agafar al cap d'un any d'establir la plantació (primavera de 2011). En aquest moment es detectava també el major número de micorizes per planta. En analitzar estadísticament el percentatge de micorizació observat a les plantes mostrejades es va comprovar que hi havia diferències significatives en el número de micorizes de *T. melanosporum* detectades al cap de 12 mesos del trasplantament a camp ($p = 0.0236$). Pel que fa a les condicions meteorològiques no es va trobar cap correlació significativa d'aquestes amb les quantitats de miceli detectades a les mostres de sòl, però si es va veure que el número de micorizes de *T. melanosporum* estava relacionat positivament amb les temperatures màximes i la temperatura mitjana mensual, amb la mitja mensual de la radiació solar, la mitja mensual de la taxa d'evapotranspiració, i amb la precipitació del més anterior.

D'acord amb els resultats obtinguts, es pot concloure que a aquesta plantació el fong va persistir al sistema radical de totes les plantes inoculades i que el miceli extraradical de *T. melanosporum* es va estendre al sòl i va incrementar amb el temps. Pel que fa a les micorizes es va veure que aquestes semblen estar més afectades per les variacions climàtiques que el miceli del sòl. La tècnica de la PCR en temps real, fent us de sondes TaqMan®, és un bon mètode per la traçabilitat de *T. melanosporum* sense pertorbar els arbres hostes, permetent així la detecció de la persistència i l'expansió del fong en una plantació tofonera des del moment del seu establiment.

A les plantacions de Cabrils, amb pins micorrizats amb *R. roseolus*,es va determinar que el fong era present a totes les plantes mostrejades i que, al cap d'un any va arribar a colonitzar plantes control de la plantació Cabrils 2. Pel que fa a la quantitat de miceli les dues plantacions van seguir desenvolupaments diferents, havent molt més miceli a la plantació establerta un any més tard. En aquesta, Cabrils 2, va ser possible establir una relació positiva entre les micorizes i les quantitats de miceli de *R. roseolus*, com havia estat descrit anteriorment per Hortal et al. (2008). Les quantitats de miceli detectades van ser majors en les unitats experimentals en que les plantes estaven més juntes (marc de plantació de 1x1m). A les

dues plantacions es va produir un increment en la biomassa de miceli al 2011 (mostres preses a la tardor), va ser en aquest període quan es va enregistrar la precipitació acumulada més elevada, sent les dades de pluja recollida gairebé el doble que en el 2009 i el 2010. Al analitzar les dades climatològiques no es van trobar relacions entre les micorizes de *R. roseolus* i els paràmetres climàtics, però si es va veure com la concentració de miceli en el sòl estava significativament relacionat amb la precipitació, la temperatura, la radiació i la taxa d'evapotranspiració.

Els encebadors i la sonda dissenyats per a detectar i quantificar el miceli de *T. melanosporum* es van utilitzar també en una zona natural de producció, concretament en set plantacions tofoneres (d'uns 20 anys) situades a Tierra Estella i Valdorba, a Navarra (al nord d'Espanya, dintre de l'àrea de distribució natural de la tòfona negra). A Tierra Estella es van analitzar quatre plantacions formades per 75-80 alzines (*Quercus ilex* subsp. *ballota* (Desf.) Samp.) diferenciades per haver una productora de *T. melanosporum* regada (per tal d'evitar el dèficit hídric estimat de l'estiu), una productora de *T. melanosporum* sense reg, una plantació productora de *T. brumale* i una plantació que actualment no produeix tòfones. A Valdorba es van analitzar tres plantacions formades per tres espècies arbòries diferents, totes elles productores de tòfona negra: una plantació establerta amb 74 *Q. ilex*, una altra amb 36 *Q. faginea* Lam. i la tercera composta per 108 *Corylus avellana* L.. Cap d'elles regada.

L'objectiu era veure si existien diferències en la quantitat de miceli present al sòl en relació amb la productivitat del lloc, les pràctiques culturals aplicades o l'espècie vegetal associada. Es van analitzar 160 mostres de sòl, en les que es va detectar amplificació d'ADN de *T. melanosporum* en un total de 131 mostres. El límit de detecció es va establir en 1.48 µg de miceli / g de sòl. Els resultats van mostrar que la biomassa de miceli extraradical detectada a les mostres de sòl procedents de la zona de producció natural de tòfona era significativament més elevada (fins a 10 vegades més gran), que el miceli detectat a qualsevol de les altres set plantacions analitzades ($p < 0.001$). Els sòls analitzats provinents de la plantació productiva no regada de Tierra Estella tenien significativament més miceli extraradical que la

resta de plantacions regades, la productora de *T. brumale* o la no productiva. Al comparar la biomassa de miceli extraradical de *T. melanosporum* de les plantacions de *Q. ilex* no regades de les dues localitats, es va veure que hi havia significativament ($p < 0.001$)més miceli a la plantació de Tierra Estella.

Dels resultats obtinguts es pot concloure que l'etapa inicial d'una plantació, quan el fong s'estén per aquesta, està molt influenciada per les plantes, el clima local, les condicions del sòl i el marc de plantació. Els fongs estudiats es comporten de forma diferent al sòl, els resultats suggereixen que *R. roseolus* s'estén a través del sòl més ràpidament que *T. melanosporum*, tots dos van mostrar una correlació positiva entre les seves estructures vegetatives (ectomicorizes i miceli extraradical), però només en les zones properes al sistema radical de la planta hoste. Com es va veure amb *R. roseolus*, on no es va trobar relació amb miceli detectat en mostres de sòl més distants dels arbres hostes.

En definitiva, la tècnica de PCR en temps real, fent us de sondes TaqMan®, és un bon mètode per a la traçabilitat dels fongs ectomicorízics sense afectar els arbres hostes, el que ens permet detectar la persistència i l'expansió del fong a través d'una plantació de planta micorrizada des del seu establiment. Aquest estudi és el primer intent de quantificar miceli extraradical de *T. melanosporum* en mostres de sòl utilitzant sondes TaqMan®. Els resultats quantitatius obtinguts són d'especial interès per avaluar la resposta del fong a tractaments de cultiu, com el reg, i per controlar la dinàmica del miceli extraradical de *T. melanosporum* al sòl, sobretot en plantacions madures on el mostreig d'arrels per a l'avaluació d'ectomicorizes pot ser molt difícil. Estudis addicionals per completar i comparar els cicles anuals de la biomassa de miceli de *T. melanosporum* present al sòl, en diferents condicions ecològiques o culturals, podrien aclarir la seva relació amb l' inici de la producció de les preuades tòfones.

Com es veu dels resultats obtinguts al Capítol 3, l'existència o no del fong en el sòl, o les quantitats en que es troba, no determinen, per si soles, la productivitat de tòfones en una plantació. És per això que, finalment, al

Capítol 4 **(Estructura genètica espaial de *Tuber melanosporum a una plantació productiva determinada per l'anàlisi dels gens del tipus de compatinbilitat i marcadors microsatèl·lits*)** s'ha estudiat l'estructura genètica de la població de *T. melanosporum* en una plantació, concretament sota dos arbres amb una producció elevada de tòfones, fent servir marcadors microsatèl·lits neutres i caracteritzant els tipus de compatibilitat i la seva distribució a la plantació.

L'estructura genètica de les poblacions de fongs ectomicorízics és el resultat tant de la seva reproducció sexual com de la propagació vegetativa. Al capítol 4 s'ha analitzat l'estructura genètica espacial de la població de *T. melanosporum*. L'estudi va tenir lloc en una plantació del nord-est de França, a Rollainville, de la qual es van seleccionar dos avellaners i un petit roure pel seu historial d'elevada producció de tòfones. Sota aquest arbres es van mostrejar ascocarps (hivern 2010-2011), micorizes i sòl (primavera de 2011), mapejant detalladament cada mostra (figura 4.2). De totes les mostres agafades es va aïllar l'ADN genòmic de cada micoriza i ascocarps fent servir el kit d'extracció DNAeasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France), seguint les instruccions del fabricant. Per les extraccions de l' ADN de les mostres de sòl es va fer servir el kit Fast DNA Spin kit for soil (MP Biomedicals, Illkirch, France), seguint les indicacions de Luis et al. (2004). Per totes les mostres es va verificar que es tractes o que hi hagués, en el cas dels sòls, *T. melanosporum* fent us de primers específics per l'espècie (ITS4LNG i ITSML) (Rubini et al. 1998; Paolocci et al. 1999). Per cada mostra es va determinar el tipus de compatibilitat (*MAT*), fent us dels primers descrits per Rubini et al. (2011b). A més a més, les mostres de micorizes i els ascocarps es van genotipar fent us de 10 marcadors microsatèl·lits o S*imple Sequence Repeat* (SSR) neutres, descrits prèviament per Murat et al. (2011).

Unint els resultats dels SSR i del tipus de compatibilitat es van obtenir set genotips multilocus o soques diferents presents entre les micorizes i els ascocarps (taula 4.2). Els perfils genètics dels ascocarps es van comparar amb els de les micorizes subjacents i es va veure que, exceptuant un cas, les micorizes i les tòfones recollides a un mateix punt compartien genotip

(taula 4.3 i figura 4.7). Es va observar un patró de distribució no aleatori de *T. melanosporum*, resultant en àrees de camp colonitzat per genets que comparteixen els mateixos tipus de compatibilitat. S'observà una clara divisió entre la zona dominada pel tipus de compatibilitat *MAT(-)* (on es van detectar set genets diferents), i la del *MAT(+),* on només es van caracteritzar dos genets, però que ocupaven un espai molt més gran que la resta.

En les mostres de sòl, va ser possible identificar el tipus de compatibilitat, del miceli de *T. melanosporum* detectat, en la majoria de les mostres. És important destacar que, el *MAT(-)* es va detectar gairebé a tots el sòls analitzats (figura 4.6), i el *MAT(+)* es va detectar també al voltant de micorizes amb un tipus de compatibilitat *MAT(-)*. L'àrea en la que es troben les micorizes amb *MAT(-)* està envoltat per sòl en el que trobem miceli amb els dos tipus de compatibilitat, *MAT (-)* i *MAT (+),* mentre que no es detectà miceli *MAT(-)* al sòl collit al voltant de l'àrea amb micorizes *MAT (+),* tot i que es tracta d'una zona molt productiva. Aquests resultats podrien explicar-se considerant que el miceli extraradical de *T. melanosporum* és més dinàmic i/o presenta una taxa de renovament més elevada que a les micorizes. Amb els resultats obtinguts, és fàcil preguntar-se com pot ocórrer la reproducció sexual i com les soques amb el tipus de compatibilitat oposat poden trobar-se en plantacions, com l' estudiada, on es troben grans extensions d'ectomicorizes amb un únic tipus de compatibilitat. Segons la teoria, aquestes zones estarien destinades a ser infèrtils, mentre no arribi una parella compatible. Probablement el tipus de compatibilitat que falta a algunes zones, pot ser portat a elles per microfauna del sòl, o per altres animals atrets pels volàtils de la tòfona (Kendrick 1985; Lehmkuhl et al. 2004; Kataržytė and Kutorga 2011; Pacioni et al. 1991; Hochberg et al. 2003; Hohmann and Huckschlag 2005; Splivallo et al. 2011). Els humans poden ser un vector important per la disseminació del fong, ja que es poden dispersar espores de tòfona o qualsevol cèl·lula aïllada a través de la maquinària agrícola i de les pràctiques que es fan als sòls de les plantacions tofoneres (Agrios 2005). No obstant això, la dispersió d'aquests elements, així com la presència de miceli del tipus d'aparellament oposat al sòl, són condicions necessàries però no suficients per a garantir l'aparellament amb les soques

que es troben formant les micorizes. Amb l'adaptació i el perfeccionament de la PCR en temps real per quantificar la presència dels dos tipus de compatibilitat, es podrà determinar si és necessària una determinada quantitat de miceli d'un tipus o de l'altre i establir una relació entre aquests.

En conclusió, aquest estudi demostra que en una mateixa planta hoste colonitzada per *T. melanosporum* podem trobar diversos genetes i, sobretot, que aquest patró de distribució sembla estar relacionat amb el locus que determina el tipus de compatibilitat. Aquest descobriment requereix més estudis per comprendre com els gens *MAT* determinen fenòmens de competència intraespecífica en aquesta espècie. De fet, molt abans de que es descobrís l'estratègia d'aparellament de la tòfona, molts conreadors de tòfones inoculaven el terra amb ascocarps madurs, tot i que aquesta pràctica no s'ha utilitzat mai a la plantació estudiada. Si la inoculació del sòl cada any amb espores és suficient per promoure la producció de tòfones de la plantació encara no s'ha aclarit, però qualsevol pràctica de cultiu destinada a proveir les parelles sexuals a les soques que es troben al sistema radical de les plantes hoste, hauria de servir per promoure l'aparellament de la tòfona i la seva reproducció.

Conclusions

La micorizació de plantes amb espècies del complex *B. edulis* s'ha aconseguit en condicions de cultiu pur. La producció de les plantes micorizades amb *B. edulis* en condicions de viver necessita encara més investigació per determinar el procediment adequat.

El progrés de les noves tecnologies en la biologia molecular (PCR en temps real, seqüenciació, etc) i la disponibilitat dels genoma d' alguns fongs han portat al desenvolupament, implementació i ús de tècniques per a la traçabilitat dels fongs micorízics comestibles. Al llarg de la tesi s'ha demostrat que la tècnica de PCR en temps real, amb el disseny d'oligonucleòtids específics per a la detecció i quantificació de miceli extraradical, ens permet determinar la concentració de miceli present al sòl en boscos i plantacions, augmentant els límits de detecció de la PCR

convencional. S’han dissenyats encebadors i sondes específics per a *B. edulis* i *T. melanosporum* per a l'amplificació per PCR en temps real, el que permet la traçabilitat d'aquestes espècies de fongs en la producció controlada de fongs micorízics comestibles. No s'ha trobat relació directa entre les quantitats de miceli del sòl i la producció d’esporocarps per cap de les espècies de fongs estudiats, però ha estat possible obtenir correlacions positives entre les estructures vegetatives (ectomicorizes i miceli extraradical) de *B. edulis*, *R. roseolus* i *T. melanosporum*, en mostres recollides a prop de la planta hoste, a la zona d'influència de la rizosfera. Els resultats obtinguts obren la possibilitat d'utilitzar la quantificació de miceli del sòl per PCR en temps real com un bon indicador per a la colonització de les arrels en condicions de camp (a les zones naturals o en plantacions establertes per l'home), especialment quan es requereix un mostreig no destructiu o anàlisis que portin menys temps.

Estudis addicionals i més llargs, per completar i comparar la dinàmica estacional de la biomassa del miceli del sòl en diferents condicions ecològiques o culturals, podrien aclarir la seva relació amb la producció de bolets.

Les quantitats de miceli extraradical de *B. edulis*, *L. deliciosus* i *R. rosolus* i també les micorizes de *T. melanosporum* es van correlacionar amb paràmetres climàtics com la temperatura, la precipitació, la radiació solar, la humitat relativa i l'evapotranspiració.

A més, ha estat possible determinar la distribució de diferents genotips de *T. melanosporum* a través de la traçabilitat d’ascocarps, ectomicorizes i mostres de sòl.

Annex A

Fungal species	Strain	*Pinus pinaster*	*Cistus albidus*	*C. ladanifer*	*C. laurifolius*	*C. monspeliensis*	*Castanea sativa*	*Betula pendula*	*Quercus ilex*
Boletus aereus	Ba 315	-	+	+++	-	++			
Boletus aereus	Ba 316	-	++++	+++	+	+			
Boletus aereus	Ba 317	-	-	-	-	-	-	++	++
Boletus aereus	Ba 370	+	+++	+++		+++	-	-	
Boletus aereus	Ba 393	-	++++	++++	+	+++	++++		
Boletus aereus	Ba 1050	-	++	+		++			+
Boletus aereus	Ba 1051	-	+++	+++	-	+			-
Boletus aereus	Ba 1058	-	+	+		+			+
Boletus reticulatus	Br 386	-	++++	+++	++	+++			
Boletus reticulatus	Br 388	++	++++	-		++			
Boletus reticulatus	Br 1053	+++	+++	++	+	+			
Boletus reticulatus	Br 1054	+	++++	-		-			
Boletus reticuatus	Br 1055	-	++++	+++		+++			
Boletus reticulatus	Br 1056	-	-	-		+			
Boletus edulis	Be 369	-	++++	++++	++++	++++			+
Boletus edulis	Be 375	++++	++	++++	++	+++			+
Boletus edulis	Be 391	-	-	-		++			
Boletus edulis	Be 392	++	++	-	-	-	+	++	
Boletus edulis	Be 409	++				-			
Boletus edulis	Be 410	++++				-			
Boletus edulis	Be 411	++				-			
Boletus edulis	Be 412	+++				-		-	
Boletus edulis	Be 1098	+	+	+					
Boletus edulis	Be 2017	+++	+						
Boletus edulis	Be 2022		++				-	+++	
Boletus edulis	Be 2037	++++	-						
Boletus edulis	Be 2044	-	-			++			
Boletus edulis	Be 2057	++	++						
Boletus edulis	Be 2059	+++	+++					-	
Boletus edulis	Be 2063	+++				+			
Boletus edulis	Be 2065		+				-	++	
Boletus edulis	Be 2070	-	-			+++	+++		-
Boletus edulis	Be 2074	-	-						
Boletus pinophilus	Bp 1042	-	-	-	-				
Boletus pinophilus	Bp 1045	-	-	-	-				
Boletus pinophilus	Bp 1099	++	++++	++++					
Boletus pinophilus	Bp 2007	++							
Boletus pinophilus	Bp 2008	+							
Boletus pinophilus	Bp 2010	-							

Table A.1. Results of the synthesis of *B. edulis* complex mycorrhizas in pure culture. - any mycorrhizal plant, + 25%, ++ 50%, +++ 75%, ++++ 100% of the mycorrhizal plants. (Chapter 1)

Fungal Species	Strain num.	Storage time at 4°C				
		Initial	3 months	6 months	12 months	24 months
Boletus aereus	315	5	5	3	5	4
	316	5	5	5	5	5
	317	5	5	5	0	0
	393	5	5	5	5	0
	370	5	5	5	4	3
	1050	5	5	0	0	0
	1051	5	5	5	5	5
	1058	5	5	5	5	0
Boletus edulis	369	5	5	5	5	5
	375	5	5	5	0	0
	391	5	5	5	5	5
	392	5	5	5	5	5
	409	5	5	5	5	5
	410	5	5	5	5	0
	411	5	5	5	0	0
	412	1	5	5	5	4
	2059	5	5	5	5	0
	2022	5	5	5	5	0
	2017	5	2	2	0	0
	2037	5	5	5	5	5
	2044	5	5	5	5	3
	2063	5	5	4	4	2
	2065	5	5	5	1	0
	2070	5	5	5	0	0
	2057	5	5	5	5	5
Boletus pinophilus	1042	5	5	5	5	0
	1045	5	5	5	5	2
	1099	5	0	0	1	0
	2000	5	5	0	0	0
	2007	2	0	0	0	0
	2010	3	0	0	5	5
	2008	5	1	5	0	0
Boletus reticulatus	386	5	5	5	0	0
	388	5	5	5	2	0
	1053	5	5	5	5	0
	1054	5	5	5	5	0
	1055	5	5	5	5	0
	1056	5	5	5	5	0
Rhizopogon roseolus	7	5	5	5	5	5
	169	5	5	5	5	5
	308	5	5	5	5	5
	319	5	5	5	5	5
	371	5	5	5	5	5
	374	5	5	5	5	5
	376	5	5	5	5	5
	407	5	5	5	5	5
	408	5	5	5	5	5

Table A.2. Raw data of the number mycelium disks of *Boletus* spp. and *R. roseolus* strains that regrew in BAF medium after different storage times at 4°C in sterile water. (Chapter 1)

Species	Primer/probe name	Primer / probe sequene	Fragment size (bp)	Reference
Boletus edulis	FWD – Bedu	5’– CTGTCGCCGGCAACGT– 3’	75	(De la Varga *et al.*, 2012)
	RVS – Bedu	5’– TGCACAGGTGGATAAGGAAACTAG– 3’		
	STQBedu	5’– 6FAM-CCCTTTCTCTTTCGTGGAACCTCCCC-TMR– 3’		
Tuber melanosporum	Fwd – Tmel	5’– TCTCTGCGTATCACTCCATGTTG– 3’	61	(Parladé *et al.* 2013)
	Rvs – Tmel	5’– TCCCACAGGTGCCAGCAT– 3’		
	STQTmel	5’– 6FAM-TTCCACAGGTTAAGTGAC– 3’		
	UPL #93	TCTGGTCC	67	-
	Fwd Tmel PF	5’– GGTTATAAGACCTGGATCAGTCACA– 3’		
	Rvs Tmel PF	5’– CATCTAGGATGGGGGTCCTT– 3’		
Rhizopogon roseolus	Fwd – Rro	5’– TCGACTTTGCGCGACAAG– 3’	60	(Hortal *et al.*, 2008)
	Rvs – Rro	5’– CATGCGCTTCAGCAAACG– 3’		
	STQRro	5’– FAM-ATCATTATCACGCCGAAAG-MGB– 3’		
Lactarius deliciosus	FWD-Ldel	5’–TTGACGCAAAAGTCGTGCAC– 3’	101	(Parladé *et al.*, 2007)
	RVS-Ldel	5’–ATCGGTTCGATCCCAAAAGG– 3’		
	STQLdel	5’–VIC-TCTCGCATAAAATCCA-MGB– 3’		

Table A.3. Oligonucleotides (primers and probes) designed and/or used in the thesis to quantify DNA by real-time PCR technique.

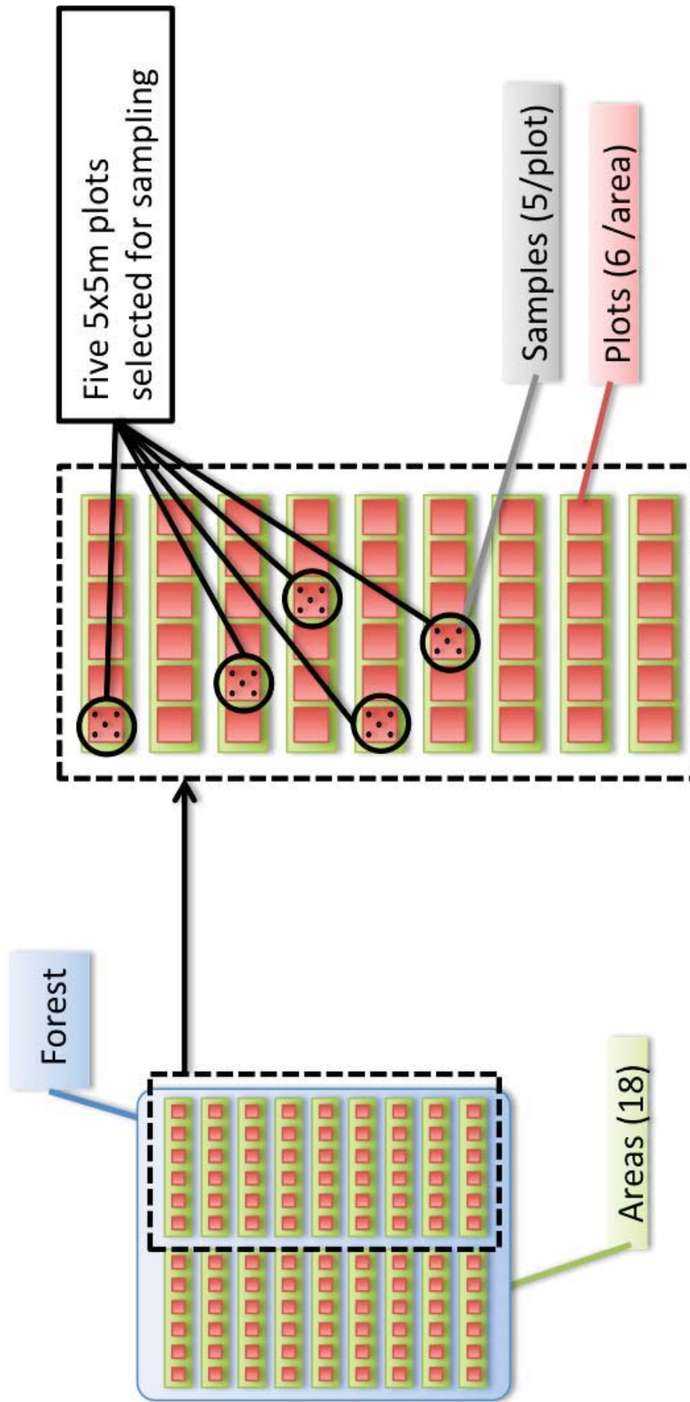

Figure A. 1. Scheme of the sampling method applied in Pinar Grande. In the stand, 18 areas were fenced since 1995. Each area was divided in 6 5x5m plots. % Plots of 6 different areas, selected by their sporocarp production (representing a gradient) were selected to analyze soil mycelium biomass. Five soil samples in each selected plot were taken for the analysis.

Table A.4. Raw data of *T. melanosporum* quantification in Navarra orchards (Chapter 3)

Sierra Estella - *T. melanosporum* natural ground				Sierra Estella - Non productive			
Sample Name	**Quantity Mean**	**Quantity SD**	**Averages**	**Sample Name**	**Quantity Mean**	**Quantity SD**	**Averages**
SV-I-1	5,623156	0,213924		NP-I-1	3,133730	0,132723	
SV-I-2	11,826007	0,382286		NP-I-2	0,344806	0,035265	
SV-I-3	8,187901	0,309227		NP-I-3	0,002196	0,000708	
SV-I-4	1,476931	0,102220		NP-I-4	0,001153	0,000114	
SV-I-5	0,017399	0,000011	5,426279	NP-I-5	0,001142	0,000036	0,696605
SV-II-1	0,428857	0,024613		NP-II-1	0,001655	0,000388	
SV-II-2	1,838016	0,028704		NP-II-2	0,001556	0,000012	
SV-II-3	6,878015	0,623175		NP-II-3	0,002060	0,001001	
SV-II-4	0,945499	0,061664		NP-II-4	0,005252	0,001519	
SV-II-5	1,021405	0,067867	2,222358	NP-II-5	0,127815	0,003001	0,027667
SV-III-1	2,344377	0,238591		NP-III-1	0,001711	0,000067	
SV-III-2	0,168572	0,015953		NP-III-2	0,000670	0,000020	
SV-III-3	2,054269	0,197887		NP-III-3	0,002419	0,000586	
SV-III-4	1,859547	0,041876		NP-III-4	0,001909	0,000101	
SV-III-5	0,612064	0,049035	1,407766	NP-III-5	0,001560	0,001165	0,001654
SV-IV-1	0,001524	0,001179		NP-IV-1	0,001454	0,000174	
SV-IV-2	2,998389	0,350062		NP-IV-2	1,328258	0,218697	
SV-IV-3	0,670050	0,026054		NP-IV-3	0,007085	0,001652	
SV-IV-4	3,235128	0,222456		NP-IV-4	0,001631	0,000183	
SV-IV-5	5,671850	0,292873	2,515388	NP-IV-5	0,002081	0,000658	0,268102
	2,892948				**0,248507**		
Sierra Estella - Productive Tuber brumale				**Valdorba- hazelnut**			
Sample Name	**Quantity Mean**	**Quantity SD**	**Averages**	**Sample Name**	**Quantity Mean**	**Quantity SD**	**Averages**
Tb-I-1	0,012521	0,000171		A-I-2	0,408425	0,031101	
Tb-I-2	0,001690	0,000382		A-I-2	0		
Tb-I-3	0,001975	0,000640		A-I-3	0		
Tb-I-4	0,074773	0,009846		A-I-4	1,474788	0,125617	
Tb-I-5	0,012169	0,000170	0,020626	A-I-5	0,335492	0,019224	0,443741
Tb-II-1	0			A-II-1	0,005048	0,001178	
Tb-II-2	0			A-II-2	0,000000		
Tb-II-3	0,001437	0,000115		A-II-3	0,898150	0,014290	
Tb-II-4	0,006102	0,001727		A-II-4	0,114437	0,000962	
Tb-II-5	0,002146	0,000028	0,001937	A-II-5	0,045948	0,001022	0,212717
Tb-III-1	0,001425	0,000346		A-III-1	0,874056	0,040993	
Tb-III-2	0,001013	0,000190		A-III-2	0,769353	0,042459	
Tb-III-3	0,002306	0,000791		A-III-3	0,677747	0,029273	
Tb-III-4	0,001789	0,000500		A-III-4	0,057843	0,004573	
Tb-III-5	0,000818	0,000228	0,001470	A-III-5	0,005506	0,000516	0,476901
Tb-IV-1	0,278022	0,008949		A-IV-1	0		
Tb-IV-2	0,004674	0,000668		A-IV-2	1,422473	0,053978	
Tb-IV-3	0			A-IV-3	0,002658	0,002625	
Tb-IV-4	0			A-IV-4	0,001825	0,000368	
Tb-IV-5	0,020845	0,005996	0,060708	A-IV-5	0,000709	0,000085	0,285533
	0,021185				**0,354722**		

Sierra Estella - Productive non watered			
Sample Name	Quantity Mean	Quantity SD	Averages
PSR-I-1	0,055996	0,000124	
PSR-I-2	0,012139	0,003087	
PSR-I-3	0,748807	0,022189	
PSR-I-4	0,620464	0,031500	
PSR-I-5	0,004212	0,000518	0,288324
PSR-II-1	1,961492	0,103691	
PSR-II-2	0,326124	0,033083	
PSR-II-3	0,430727	0,074354	
PSR-II-4	0,281766	0,034382	
PSR-II-5	0,191160	0,040070	0,638254
PSR-III-1	0,442507	0,027479	
PSR-III-2	0,367642	0,021096	
PSR-III-3	0,247029	0,029495	
PSR-III-4	0,467523	0,006882	
PSR-III-5	0,434735	0,028589	0,391887
PSR-IV-1	0,396257	0,021489	
PSR-IV-2	0,009669	0,002001	
PSR-IV-3	0,451224	0,024158	
PSR-IV-4	0,418292	0,013325	
PSR-IV-5	0,005015	0,001424	0,256091
	0,393639		

Sierra Estella - Productive watered			
Sample Name	Quantity Mean	Quantity SD	Averages
TmR-I-1	0,015383	0,001362	
TmR-I-2	0,000527	0,000030	
TmR-I-3	0,000779	0,000066	
TmR-I-4	0,670178	0,125235	
TmR-I-5	1,389518	0,265466	0,415277
TmR-II-1	0,745248	0,034958	
TmR-II-2	0,033384	0,001022	
TmR-II-3	1,476279	0,147045	
TmR-II-4	0		
TmR-II-5	0,001393	0,000165	0,451261
TmR-III-1	0,003815	0,001179	
TmR-III-2	0		
TmR-III-3	0,003224	0,000257	
TmR-III-4	0,005895	0,000014	
TmR-III-5	0		0,002587
TmR-IV-1	0,001920	0,000012	
TmR-IV-2	0,000635	0,000116	
TmR-IV-3	0,001560	0,000404	
TmR-IV-4	0		
TmR-IV-5	0,001265	0,000353	0,001076
	0,217550		

Valdorba - evergreen oak			
Sample Name	Quantity Mean	Quantity SD	Averages
E-I-1	0		
E-I-2	0		
E-I-3	0		
E-I-4	0		
E-I-5	0		0
E-II-1	0,002610	0,000201	
E-II-2	0,005965	0,001500	
E-II-3	0,854453	0,108295	
E-II-4	0,000976	0,000230	
E-II-5	0,007191	0,001488	0,174239
E-III-1	0		
E-III-2	0,002384	0,000013	
E-III-3	0,974385	0,018779	
E-III-4	4,053215	0,123200	
E-III-5	0,039116	0,007707	1,013820
E-IV-1	0,003137	0,000569	
E-IV-2	0		
E-IV-3	0,963675	0,087642	
E-IV-4	0		
E-IV-5	0		0,193362
	0,345355		

Valdorba - Portuguese oak			
Sample Name	Quantity Mean	Quantity SD	Averages
R-I-1	0,002798	0,001813	
R-I-2	0,002080	0,000757	
R-I-3	0		
R-I-4	0		
R-I-5	0,002840	0,002114	0,001544
R-II-1	0		
R-II-2	0,002268	0,000849	
R-II-3	0		
R-II-4	0,001974	0,000447	
R-II-5	0		0,000848
R-III-1	0,244113	0,042521	
R-III-2	0		
R-III-3	0		
R-III-4	0,003196	0,000409	
R-III-5	0		0,049462
R-IV-1	0,956574	0,099147	
R-IV-2	0,277473	0,013882	
R-IV-3	0,046890	0,001185	
R-IV-4	1,162978	0,090076	
R-IV-5	1,120592	0,028909	0,712902
	0,191189		

Scientific Publications

Parladé J, Pera J, **De la Varga H**, Hortal S (2009) Tracking inoculated edible ectomycorrhizal fungi by Real-Time PCR. New Biotechnology 25: S375-S375. doi: 10.1016/j.nbt.2009.06.924

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Lactarius deliciosus and *Tuber melanosporum* images: Herminia De la Varga Pastor

TRACEABILITY OF THE MYCORRHIZAL SYMBIOSIS ON THE CONTROLLED PRODUCTION OF EDIBLE FUNGI

Traçabilitat de la simbiosi micorízica en la producció controlada de fongs comestibles

Herminia De la Varga Pastor

DOCTORAL THESIS 2013