UNIVERSITY OF LJUBLJANA
BIOTEHNICAL FACULTY
DEPARTMENT OF FORESTRY AND RENEWABLE
FOREST RESOURCES

Yasmine PIÑUELA SAMANIEGO

ECTOMYCORRHIZA DIVERSITY IN NATURAL *Tuber aestivum* Vittad.GROUNDS

B. Sc. THESIS

Academic study Programmes

Ljubljana, 2012

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PESTROST EKTOMIKORIZE NA NARAVNIH RASTIŠČIH GOMOLJIKE *Tuber aestivum* Vittad.

DIPLOMSKO DELO
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Commission for the Study and Student Affairs at Department of Forestry and Renewable Forest Resources BF approved the topic of this thesis on a meeting on June 1st. 2012 and appointed as supervisor prof. dr. Hojka Kraigher and dr. Tine Grebenc as co-supervisor.

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This thesis is the result of my own research work. I agree to publish this work in full text on the web site of the Digitalna knjižnica Biotehniške fakultete. I declare that the work that I submitted in electronic form is identical to the printed version.

Yasmine PIÑUELA SAMANIEGO

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An ectomycorrhiza constitutes a mutualistic relationship between the mycelium of a fungus and the roots of a host plant. Truffles are important commercial ectomycorrhizal fungi, naturally present and abundant in Slovenia. The knowledge about their distribution and presence of associated fungi is scarce for natural sites but well defined for several truffles plantations established abroad.The study is the first example of the ectomycorrhiza community study from natural truffle sites in Slovenia.We aimed to analyse the ectomycorrhiza communities of natural *Tuber aestivum* sites from under two common ectomycorrhizal plant partners: hornbeam and oak. We have applied morphological and molecular approach in ectomycorrhiza identification to reveal the community structures. *Tuber aestivum* was observed in most soil samples in natural stands. *Cenococum geophylum* was present in all. *Russula* spp. only occurred with oaks while *Tomentella* spp, were more common with hornbeam. In comparison with truffle plantation, the community was different with only several in common, such as *T. aestivum, Tarzetta* sp. and some *Tomentella* species.

KLJUČNA DOKUMENTACIJSKA INFORMACIJA

ŠD	Du1
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AI	

Ektomikoriza je mutualističen odnos med micelijem glive in drobnimi koreninam gostiteljske rastline. Gomoljike prestavljajo pomembno komercialno vrsto ektomikoriznih gliv, ki jo na naravnih rastiščih v Sloveniji najdemo pogosto in v velikem številu. Znanje o pojavljanju gomoljik in drugih gliv v njihovi združbi na naravnih rastiščih je manjše, za razliko od poznavanja združb v nasadih gomoljik v tujini. V študiji prikaujemo prvi primer analize združbe ektomikoriznih gliv na naravnih rastiščih poletne gomoljike (*Tuber aestivum*) v Sloveniji. Cilj naše študije je bil analizirati združbo ektomikoriznih gliv na naravnih rastiščih poletne gomoljike pri dvez različnih drevesnih partnerjih v simbiozi, pri hrastu in belem gabru. Za namen identifikacije gliv v združbi ektomikoriznih gliv na rastiščih smo uporabili tako anatomsko-morfološki kot molekularni pristop. Ektomikorizo poletne gomojlike smo našli v večini vzorcev tal, medtem ko je bila vrsta *Cenococcum geophilum* prisotna v vseh vzorcih. *Russula* spp. so se pojavljale le s hrastom, *Tometella* spp. pa pretežno z gabrom. Primerjava združb z združbami ektomikoriznih vrt v producirajočem nasadu poletne gomoljike v Italiji je pokazala, da se združbi bitsveno razlikujeta, saj so vrste, ki se pojavljajo v obeh primerih le polenta gomoljika, ter vrsta iz rodu *Terzetta* in *Tomentella.*

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ABBREVATIONS AND SYMBOLS

ECM	Ectomycorrhiza
EmH	Emananting hyphae
FYROM	The Former Yugoslav Republic of Macedonia
UK	United Kingdom
Rcf	Relative centrifugal force
FAO	Food and Agriculture Organization
Taq	Termophilic bacterium *Thermus aquaticus*
KOD	Hot Start DNA Polymerase from Thermococcus kodakaraensis
FAA	Formaldehyde/acetic acid/alcohol
Ss DNA	Single stranded DNA
nr ITS DNA	Nuclear ribosomal Internal Trasncribed Spacer
UV	Ultraviolet
DIC	Digital Image Correlation
dd H2O	Double distilled water

1 INTRODUCTION

1.1 ECTOMYCORRHIZA: FUNGAL AND PLANT PARTNERS

Mycorrhiza is a symbiotic organ formed by roots of higher plants and the mycelium of mycorrhizal fungi. In mycorrhiza, both partners benefit from the association (Smith and Read, 2008). The physiology of the host plant in mycorrhiza is altered resulting in nutrition and growth enhancements, better health and vigor. The fungal partner in mycorrhiza receives products of the photosynthesis (carbo substances / sugars) while plants are supplied with water and inorganic nutrients (Serrada, 2008).

Ectomycorrhiza (ECM) is a type of mycorrhiza where the fungal hyphae forms a mantle around the short side roots, embed cells of primary cortex but never penetrates root cells. The diagnostic structures found in mature ectomycorrhiza are:
- Hyphal mantle: which covers fine roots in the absorption zone and helps the exchange of substances between soils, the external mycelium and roots and can also accumulates storage substances.
- Harting net: a labyrinth (network) of hyphae in tight connection with primary cortex of fine roots.
-The hyphae: the extraradical mycelium formed by thin filaments which spread into the soil and connect fungi with the substrate. Among special forms of the extraradical mycelium are rhizomorphs, formed by a bound of hyphae whose main function is the long-distance transport of water and the nutrients between different parts of the mycelium (Agerer, 1991).

Ectomycorrhizal fungi belong to three main divisions of the Kingdom Fungi: the phylum *Glomeromycota*; the phylum *Ascomycota* (orders *Elaphomycetales*, *Pezizales*) and the phylum *Basidiomycota* (orders *Agaricales, Boletales, Cortinariales, Phallales, Russulales, Stereales*) (Pegler et al., 1993). ECM is widespread in temperate and boreal forests with host trees belonging to angiosperms and gymnosperms (Smith and Read, 2008). ECM can be also found in tropical and subtropical regions, in particular in symbiosis with leguminous species (Smith et al., 2011).

Majority of ectomycorrhizal fungi are regarded as `macro fungi´ producing macroscopic reproductive structures (fruiting bodies or sporocarps) either above ground or below ground. The latter are called “hypogeous fungi”.

Hypogeous ectomycorrhizal fungi develop their sporocarps in the substrate (soil, sand, decomposing organic material) to avoid the threat of desiccation of sporocarps encountered in a terrestrial habitat (Pegler et al., 1993). Contrary to epigeous fungi, the dissemination of the spores is directly in soil by the natural breakdown of the fruitbodies or sporocarps remain in the substrate where they are found by various vectors (animals) that eat sporocarps and disperse spores (Claridge and Trappe 2005; Frank et al., 2006). The sporocarps produced by most of hypogeous species are very simple, and the similar habitat has resulted in the same structure for the different species, either globose or irregular, with an inner, spore producing region (gleba) and protected by the outer layer which envelopes it (peridium) (Hawker, 2008).

Hypogeous fungi are frequently associated with trees forming the ectomycorrhiza. They are found adjacent to the tree roots. The mycelium and/or rhizomorphs can extend for several meters from host tree in the case of undisturbed soils. Often the host-tree is specific to the fungus species (Pegler et al., 1993). Among hypogeous fungi genera, the genus *Tuber* (*Pezizales*) is frequently found associated with gymnosperms and angiosperms. The most common families of boreal and temporal plants which form ECM are *Pinaceae*, *Betulaceae, Fagaceae* and *Myrtaceae.* The truffles formed by the phylum *Ascomycota*, in particular the genus *Tuber*, are called “true truffles”. On the other hand “false truffles” are formed by a range of fungi genera mainly from *Basidiomycota*, the group which includes the most of the mushroom-forming fungal species. (Trappe et al., 2009).

Pezizalean (the true truffle) ECM possesses a relatively thin pseudoparenchymatous mantle, with few to abundant emanating hyphae, thick, stout and thick-walled (EmH), no rhizomorphs, no clamp connections, a well developed Hartig net. Cystidia are typical for several species (Agerer, 1991; Tedersoo et al., 2006).

As *Pezizales* preferentially inhabit mineral soils (Taylor and Bruns, 1999, cit. in Tedersoo et al., 2006) they are common in undisturbed or disturbed forests as pioneers species. There can be the dominant members in ECM fungal communities in early successional phases in forest ecosystems or after disturbances, especially *Tuber spp*., which dominate the roots in forest nurseries and clear cuts (Tedersoo et al. 2006).

1.2 DISTRIBUTION OF NATURAL TRUFFLE SITES IN EUROPE

The information on distribution of truffles in Europe is rather scarce. Among the comprehensive literature Pegler et al. (1993), Montecchi et al. (2000) and Ceruti et al. (2003) published reliable data on the natural distribution of hypogeous fungi (including true truffles) in Europe. They have described 178 hypogeous species. These monographic publications lists up to 32 *Tuber* spp. species for Europe. The regions with the greatest diversity are the Mediterranean and sub-Mediterranean zones of Europe (Iberian and Apenine Peninsulas and Southern France) (Ceruti et al., 2003).

Table 1: Distribution of truffle species from natural stands in the Balkan peninsula, Mediterranean basin and the rest of Europe.

Truffles species	**Localities in mid-west Balkan Peninsula**
Tuber rufum Pico.	Serbia, Montenegro, FYROM, Slovenia*
Tuber excavatum Vittad.	Very common in the whole Balkan Peninsula, Slovenia*
Tuber aestivum Vittad.	Serbia, Montenegro, Slovenia*, Croatia, FYROM (Grebenc, 2012)
Tuber brumale Vittad.	Serbia, Montenegro, Slovenia*, FYROM (Grebenc, 2012)
Tuber borchii Vittad.	Serbia (Avala mountain), Slovenia* , Hungary****
Tuber oligospermum (Tul.& C. Tul.)Trappe.	Hills of Western Serbia and Montenegro

continue

continue

Truffles species	**Localities in mid-west Balkan Peninsula**
Tuber mesentericum Vittad.	Serbia, Montenegro, Slovenia*
Tuber fulgens Quél.	Western and North-East of Serbia, Slovenia*
Tuber macrosporum Vittad.	Lowland of Balkan Peninsula, Serbia, Slovenia* and Hungary***
Tuber melanosporum Vittad.	Serbia and Slovenia*
Tuber foetidum Vittad.	Serbia, Hungary****
Tuber melanosporum Vittad.	Serbia and Slovenia*
Tuber magnatum Pico.	Lowland of Balkan Peninsula, Serbia**, Slovenia* and Hungary***
Tuber hiemalbum Chatin.	Slovenia*
Tuber puberulum Berk. & Broome	Slovenia*, Hungary****
Tuber nitidum Vittad.	Slovenia*

* Piltaver and Ratoša, 2006.
**Milenković et al., 1992; Glamočlija J. et al., 1997.
***Edvi et al., 2009.
****Halász et al. 2005
Rest of data: Milenković, 2009.

Truffles	**Localities in Mediterranean**	**Author/s who**

species	countries	mentioned it
Tuber borchii Vittad.	Italy, Spain, France, Portugal	Montecchi and Sarasini, 2000; Hall et al. 2007
Tuber oligospermum (Tul.& C. Tul.)Trappe.	Italy, Spain, Morocco	Montecchi and Sarasini, 2000; Hall et al. 2007
Tuber brumale Vittad.	Italy, France, Spain, Portugal	Montecchi and Sarasini, 2000; Hall et al. 2007
Tuber macrosporum Vittad.	Marche, Italy	Montecchi and Sarasini, 2000; Benucci et al. 2011
Tuber magnatum Pico.	Umbria, Italy (rarely). Northern and Central Italy and Istria in Croatia.	Milenković, 2009 Zambonelli et al., 1991; cit in Hall et al. 1998
Tuber aestivum Vittad.	Italy, France, Spain, Portugal, Croatia	Milenković, 2009; Hall et al., 2007; Chevalier and Frochot, 1997; cit in Benucci, 2011; Chevalier, 2009

Truffles species	Localities in North and Centre of Europe	Author/s who mentioned it
Tuber aestivum Vittad.	Ireland	Cullen et al., 2009
	United Kingdom	Milenković, 2009
	Austria	Pla et al., 2009
	Sweden	Lawrynovicz et al., 2009; Wedén, 2004
	Poland	continue

continue

Truffles species	**Localities in North and Centre of Europe**	**Author/s who mentioned it**
	Czech Republic	Gryndler et al., 2011
	Germany	Gross, 1975
Tuber borchii Vittad.	Czestochowa Upland, Poland	Lawrynowicz et al, 2009
Tuber puberulum Berk. & Broome.	France	Milenković M. 2009
	South Zealand, Denmark	Milenković, 2009
Tuber magnatum Pico.	Italy, Croatia	Rubini et al., 2005
	France	Zambonelli et al., 1991
	Switzerland	Lawrynowicz et al., 1993.

In Slovenia the past information on truffle species occurence is scarce. The mycology published record about truffles starts and finishes with the work of Giovanni Antonio Scopoli (Italian-Austrian physician and naturalist in Idrija, Slovenia) (Scopoli, 1771). After him, the information were absent both from general public and scientific audience because it is mostly provided by practical truffles hunters and there is no connection between them and the scientific audience. Piltaver and Ratoša made an inventory of all hypogeous fungi in Slovenia to complete the information (the species of truffles that they included are referenced in the table 1) (Piltaver and Ratoša, 2006).

Several localised information onf the presence of truffles in Slovenia is also available in the database generated by the Slovenian Mycological Society and Slovenian Forestry Institute on the occurrences of macromycetes in Slovenia (Jurc et al. 2005). It shows rather scarce representation of several species mainly as accidental collections. The public available databasedoes not reflect any organized survey (figure 1).

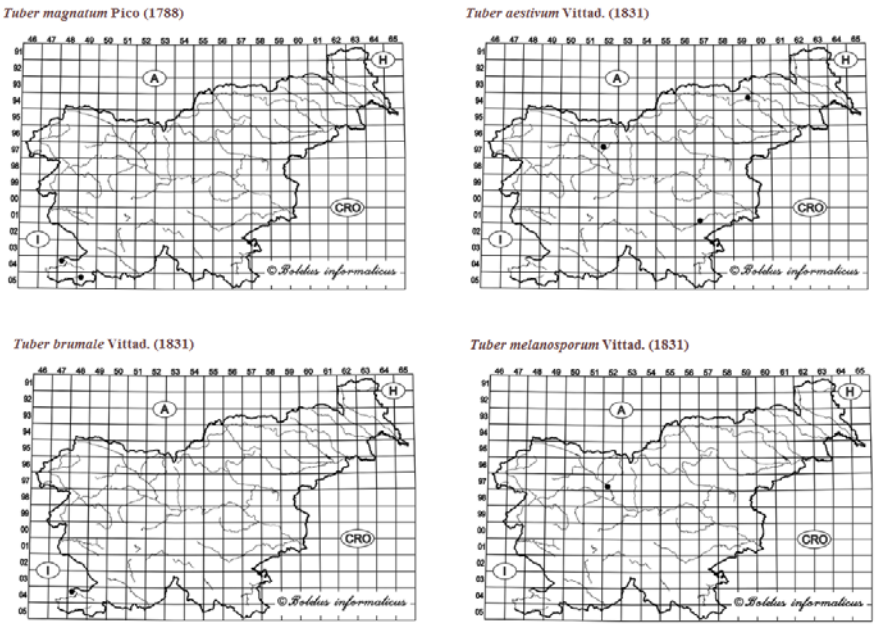

Figure 1: Distribution of the most important commercial truffles in Slovenia (Jurc et al., 2005).

1.3 PRODUCTIVE TRUFFLES ORCHARDS/PLANTATIONS IN EUROPE

Several truffle species are among best known ectomycorrhizal fungi for their cultivation in organized orchards. To cultivate truffles, seedlings have to be inoculated with truffles. Usually spores are used for inoculation (Chevalier and Frochot, 1997; cit in Benucci 2011) or rarely, mycelium from pure culture is applied (Sisti et al., 1998). Inoculated seedlings with established ectomycorrhiza are subsequently transplanted to a suitable field prepared or selected according to exact ecological (soil, climate, etc.) conditions of the specific host-fungus combination (Benucci et al., 2011).

The inoculation cultivation techniques are well developed for *Tuber melanosporum* (Bencivenga and Granetti, 1990, cit in Benucci et al., 2011), *Tuber borchii* (Zambonelli et al., 2002, cit in Benucci et al., 2011) and *Tuber aestivum* (Chevalier et al., 1997, cit in Benucci et al., 2011). It is not yet completely developed for *Tuber magnatum* despite several decades of intensive research by several research groups (Bertini et al., 2005, cit in Benucci et al., 2011). The main obstacle in *Tuber magnatum* inoculation and cultivation

are difficulties to obtain well mycorrhizes plants which are not infected with other ECM species.

Despite the invested efford, *Tuber aestivum* and *Tuber melanosporum* have been the only truffles which were cultivated widely and with considerable commercial success (Hall et al., 1998).

The production of some truffles species from natural stands (e.g. not organised plantations or intently managed productive forests) has declined in the last decades. In Europe the main reasons for the decline are overexploitation, destruction of natural habitat for agriculture purposes, etc. (Hall et al., 2003, cit in Benucci et al., 2011). The decline of natural production is the main reason for starting new and numerous orchards of truffles worldwide. Detailed published information on truffle orchards is difficult to obtain, yet the review disclosed the following countries to have established truffle orchards:

- Tuber aestivum: Italy, Spain, Sweden, Hungary, Austria, France (Chevalier, 2009), the UK (Thomas, 2009), Finland (Shamekh et al., 2009), Austria (Schrampf et al., 2009), Israel (Kagan-Zur et al., 2001), in Slovenia a few small-scale plantations were started by several individuals in the last decadesbut the success was minute (Grebenc et al., 2008).

- Tuber melanosporum: Italy (Donnini et al., 2009), France (Pla et al., 2009), Israel (Kagan-Zur et al., 2001), New Zealand (Hall et al., 2007), USA (Brunn, 2009).

- Tuber magnatum: Italy, New Zeland (Hall et al., 1998).

1.4 KEY FEATURES OF THE COMMERCIAL SPECIES *Tuber aestivum*

Tuber aestivum is the most widespread commercial truffle species in Europe. It was recorded in 26 out of 27 European countries (except Finland) as native species. In Europe and NorthAfrica, it is known from 10° of West longitude (Ireland) to 37° of East longitude and from 33° (Morocco) to 58° (Sweden) of North latitude (Chevalier, 2009). This wide distribution is explained with its broad tolerance (eurytopic) to soil parameters, climate and

precipitation and broad host range. An important character of the species is its tolerance to wide range of precipitaion quantities (Zoltán et al., 2009). In comparison to several other commercial truffle species, this species can also easily adapt to different climatic conditions and broader range of physical and chemical characteristics of soil. It grows in soils form sandy types to clayey or loamy textures, with only a minimum amount of lime (calcium carbonate) and high enough pH, it has ability to develop in soil rich in organic matter; nitrogen, phosphorus and/or potassium (Chevalier and Frochot, 1997; cit in Benucci , 2011).

For a regular production of sporocarps, the species requires sufficient rain in summer and not too low temperature in autumn. It can grow in oceanic, mediterranean, sub-mediterranean, semi continental, continental and in parts of subboreal climate. It is rare or absent in dry forest types and in the forest wetlands (Chevalier, 2009; Zoltan et al., 2009). In comparison to several other truffles (*Tuber melanosporum*) it grows in half-shaded places, without direct sunlight, to avoid the over drying of the soil, especially in summer season (Chevalier and Frochot, 1997; cit in Benucci 2011; Chevalier, 2009; Grebenc and Čater 2012).

Tuber aestivum has beed widely cultivated since 30 years over the Mediterranean Europe. Potential places for its cultivation are wide because it could be cultivated also in less suitable conditions such as acid soils with previous liming treatment. The climate is the most important limitation for its growth; the best fruitbodies to consume are the ones which grow in summer, for this reason, the Northern countries of Europe produce *Tuber aestivum* of presumably lower quality because the truffle is recollected in autumn (Chevalier, 2009).

Despite wide cultivation in Mediterranean, for other parts of Europe this practise is still in its beginning while the main supply of fresh truffles from these areas to the market come from the wild/natural growing populations (Pla et al., 2009).

2 HYPOTHESIS

In order to establish a successful truffle production, it is fundamental to study the conditions that promote truffle fructification through management of the natural truffles sites or cultivation (Benucci et al., 2011). The majority of information on truffle biology, ecology, distribution and associated ECM community originates from the Mediterranean area. From the ECM community point, the presence of other ECM species in truffles ground represents an important indicator of suitability of particular sites and plant-fungus host compatibility or, on the other hand, the non-*Tuber aestivum* ECM fungi can can represent a competitor for plant roots and nutrients (Hall et al., 2007). The identification and detection of the species in the ECM community is fundamental to assess the overall diversity and presence of potential competitors. Studies of ECM community from natural truffles sites in Slovenia wereup to date completely absentand the knowledge of the diversity of truffle environment is generally poor.

For these reasons we aimed to analyze the ECM communities in natural Tuber aestivum sites from Sub-Mediterranean climate. We have focused on common ECM partners of *Tuber aestivum*, namely *Ostrya carpinifolia* Scop. and *Quercus pubescens* L.We have performed the folloinw tasks:

1. - Analysis of the presence and distribution of *T.aestivum* ECM in soil samples of a standardized volume collected directly at the productive truffle site.
2. - Characterization of other (non-*Tuber aestivum*) ECM from the same soil samples.
3. - Morphoogical and molecular analysis of the ECM diversity and comparison of results with the previously published data from producing truffle plantations of the same or different plant-ECM fungus combination previously performed by Benucci et al. (2011).

Based on the proposed approaches we hypothesise that:

1. *Tuber aestivum* ECM is present on all sites where this species produces ripened sporocarps.
2. The species composition will differ among different tree partners analysed.
3. We expect differences in community structure among natural stands and previously analysed truffle plantation.

3 MATERIAL AND METHODS

3.1 SITE DESCRIPTION:

The study was performed in two natural *Tuber aestivum* stands, that were discovered in year 2007 and subsequently regularely checked for the production of this truffle species. Through the regular visits, the position and limis of the *Tuber aestivum* mycelium (based on the exact positions of sporocarps) were deducted. The two sites with regular production were Črni Kal and Rakitovec (table 2). Both sites are located in the SW Slovenia, 10 to 25km away from the Adriatic Sea and both with sub-Mediterranean climate conditions, as assessed from the climate data and general vegetation characteristics (Kutnar et al., 2002).

The rockbase at both locations is limestone or dolomite. Soils developed above the rockbase are lithic and rendzic leptosols, as well as cambisols. The surface shows and extensive microsite morphologic diversity resulting in pocket distribution of soils (Urbančič et al., 1999). The organic layers directly over the *Tuber aestivum* are shallow or absent, probably due to the years of truffle digging.

Table 2: Basic characteristics of Črni Kal and Rakitovec samplink sites
* (Atlas okolja, 2007).

Samples	1,2	3	11,12,13
Site - general name	Črni Kal (Koper)	Črni Kal (Koper)	Rakitovec
GPS - N	45_33'56,2"	45_33'50,2"	45_29'01,9"
GPS - E	13_52´22,9´´	13_52'24,1"	13_56'38,8"
Average corrected annual Precipitaion*(mm, +/- 100mm)	1150	1150	1550
Average snow cover (in days)*	<5	<5	5-20

Samples	1,2	3	11,12,13
Average anual air temperature* (in °C)	>12	>12	8-10
Woody species in site	*Quercus pubescens*	*Quercus pubescens*	*Ostrya carpinifolia*
Other wooden species	*Fraxinus ornus L., Juniperus communis L. Pyrus communis L.*	*Fraxinus ornus L., Ligustrum sp. Juniperus communis L.*	*Juniperus communis L.*

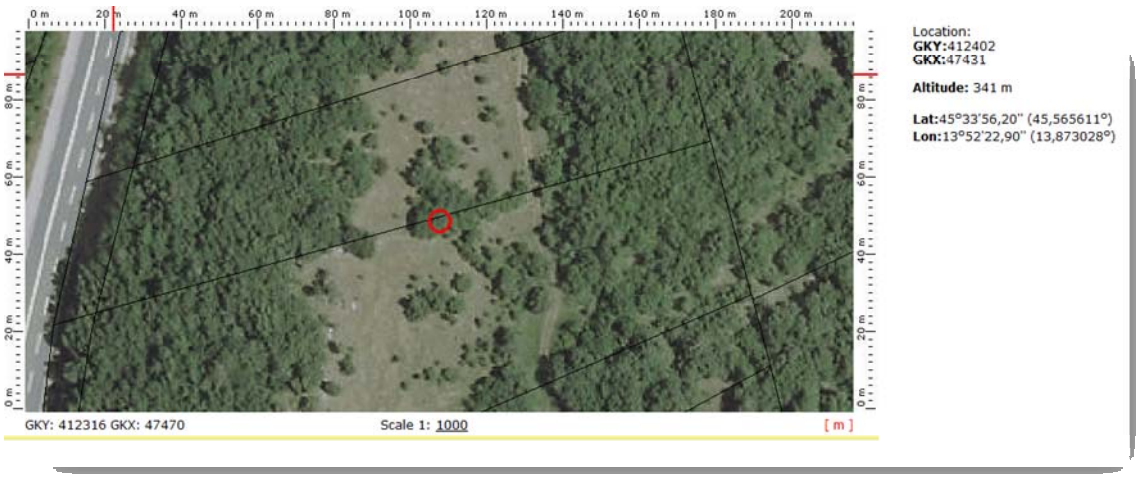

Figure 2: Areal photo of the Črni Kal sampling site, soil core locations 1 and 2. (Source:Brskanje. 2007)

Figure 3: Areal photo of the Črni Kal sampling site, soil core location 3.
(Source: Brskanje. 2007)

Figure 4: Areal photo of the Rakitovec sampling site, soil core locations 11-13.
(Source: Brskanje. 2007)

3.2 SOIL CORE SAMPLING

The soil samples were taken directly from the productive *Tuber aestivum* sites where the fungus was producing sporocarps at the time of the soil sampling, and based on the previous successful samplings (table 2). The productive sites were determined with the help of a trained truffle hunting dog (Labrador retriever) which pointed the exactly places of the ripened sporocarps.

The extraction of the representative and standardized soil sample was made with a metal soil core which was inserted into the soil down to 20cm deep. An equalamount of 274ml of soil was taken for all samples. To maintain the sample integrity all soil samples were kept in a plastic bag and stored in a refrigerator until it was analyzed. Three repetitions per site/plant partner were done.

Washing procedure:

To clean the roots and separate them from inorganic,dead organic material or soil particles, each soil sample was soaked in water for up to 24 hours before attempting to isolate all roots and ECM. To separate roots from other material a binocular with a daylight light quality, paintbrush and forceps of different sizes were used.

3.3 MORPHOLOGICAL AND ANATOMICAL METHODS

3.3.1 Differentiation of vital and non-vital ECM types:

First, the non-ECM roots were separated (e.g. grass roots, roots with arbuscular mycorrhiza appearance, etc.) from the ECM. This selection was based on a preliminary separation following the general morphological characteristics such as colour, size and basic anatomic features of root cross section, ramification and presence of emanating elements.
In the next step ECM roots pool was divided among living roots and dead or senescent roots. For this step we have used a binocular (Olympus SZT) with a help of forceps, scalpels and a paintbrush.

3.3.2 Characterization and identification:

The root tips were observed first under binocular and microscope slides under a light microscope (Olympus BH 2, © 2012 Olympus America Inc.) with a DIC (Nomarski interference contrast) system. As the next step, the identification of ECM was performed based on assessing of selected morphologicalECM characters (Fig. 5, 6) and comparison of the assessed traits with the available literature (Agerer, 1991; 1987-2008). Vital ECM root tips with the same morphological and anatomical features (Agerer, 1987-2008) were considered to be distinct morphotypes, and they were described briefly. Whenever possible, mantle and emanating elements (hyphae, rhizomorphs, cystidia) preparations were made from freshly isolated material. A compact mantle was slitted with a fine needle and the mantle peeled off. Care was taken that the adhering root cell fragments are not too thick. Each ECM type was photographed and selected characters crucial for identification were recorded. A representative sample of each ECM morphotype (if available) was stored in FAA.

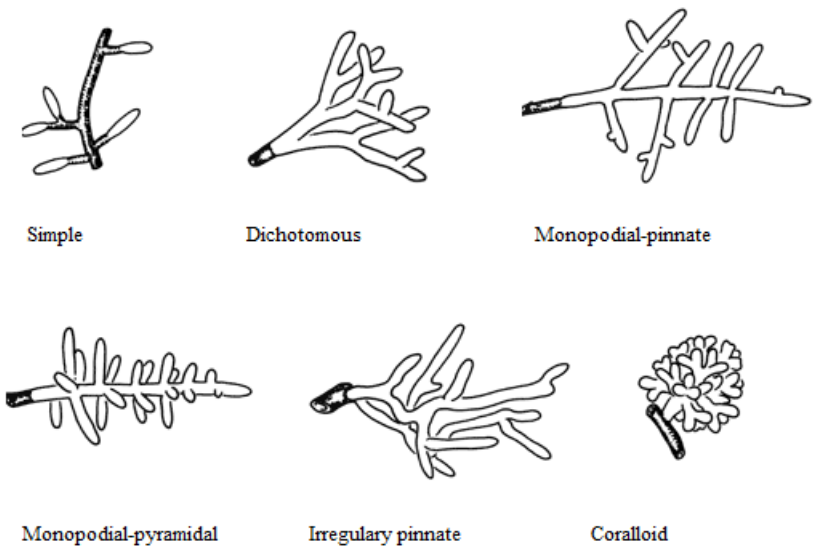

Figure 5: The key moprpgological ECM charater – ramification types of the mycorrhizal system. (Source: Deemy.2004-2012).

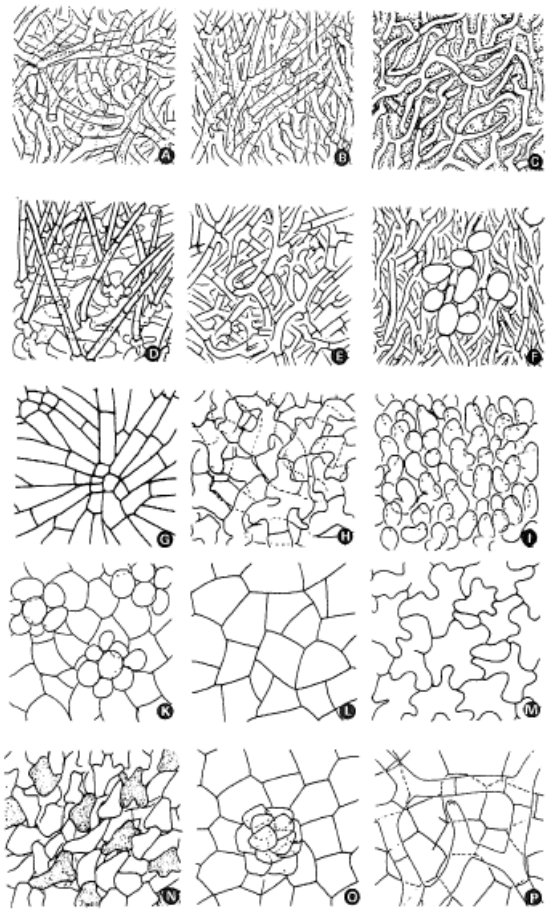

Figure 6: Schematic drawings of different mantle types in surface view; (A)-(I), plectenchymatous mantles; (K)-(P), pseudoparenchymatous mantles. (Agerer, 1991).

3.4 MOLECULAR METHODS

To obtain better supported identification, we combined morphological and anatomical identification approach with the molecular approaches for identification of fungi in ECM as the most economically and informative approach. The standardized PCR-ITS-rDNA-sequences composition molecular identification of ECM was applied (White et al., 1990; Grebenc and Kraigher. 2009).

3.4.1 DNA extraction

Five to ten ECM root tips from the same ramification system were carefully taken from each ECM morphotype pool for the DNA extraction. Only tips showing features of vital ECM (based on turgescence, colour, absence of saprophytic fungi when possible) were selected. To maintain the DNA sequence information, we have minimized the storage time of the samples to avoid the degradation of DNA. For this reason, the samples were kept refrigerated, to slow down the degradation. In morphotyping, the time involved in handling the roots in solution may cause problems, because the hyphal material may extends naturally around the surfaces, not necessarily belonging to the ECM under investigation (Kendall, 2006).

Prior to the extraction, each ECM root tip was cleaned of soil particles and stored in the DNA extraction buffer for no longer then one day. From each ECM type, a representative number of subsamples were used for DNA extraction.

DNA from the ECM was ectracted using Plant DNeasy® Mini Kit (QIAgen), a commercial kit for the total genomic DNA extraction from plant and fungal material.

The extraction procedure followed the producer's protocol (Dneasy Plant Mini kit. 2006):

I. Disruption the sample material with help of a mortar.

II. Addition of 4µl RNase A enzyme, vortex and incubate for 30 min at 65°C inverting the tube each 10 min during the incubation.

III. Adding 130µl Buffer AP2 and incubate on ice for 15 min. Mix and centrifuge for 5 min at 20 000 rcf.

IV. Pipette only the liquid, trying to avoid the soil particles on the bottom, and transfer it into 2ml collection tube. After that, centrifuge 2min at 20 000 rcf.

V. Add 1.5 volumes of Buffer AP3/E mixing by pipetting:

600µl sample x 1.5 = 900µl Buffer AP3/E

Total solution: 600µl + 900µl = 1500µl

VI. Transfer half of the mixture (750µl) into a DNeasy Mini spin column in a 2ml collection tube. Centrifuge 1min at 20 000 rcf. Remove flow-through.

Repeat the process with the remaining sample.

VII. Change the spin column into a new 2ml collection tube adding 500µl Buffer AW (Washing buffer) and centrifuge 2min at 20 000 rcf.

Remove the flow-through and repeat the process.

VIII. Transfer the spin column to a new 1.5 or 2 ml microcentrifuge tube.

Add 75 µl of Buffer AE, keep the sample in ambient temperature during 5 min and centrifuge 1min at 20 000 rcf.
Repeat the step adding 50µl instead of 75µl.

IX. Keep the solution in the fridge before the PCR reaction.

3.4.2 Amplification in PCR and control of the amplification

The amplification of the nr ITS DNA geneswas performed in a PCR (polymerase chain reaction). The target sequence for PCR analysis used were internal transcribed spacers 1 and 2 (ITS), 5.8 S RNA gene and flanking regions of 18S and 28S ribosomal genes. The whole region was amplifyed with ITS1 forward primer and ITS4 reverse primer, the most common primers used for Ascomycota (White et al., 1990; Gardes and Bruns, 1993).

The nucleotide sequence of the two primers was the following:

- ITS1f: 5´-CTTGGTCATTTAGAGGAAGTAA- 3´(Invitrogen ™; C8274 (B08))
- ITS4: 5´-TCCTCCGCTTATTGATATGC- 3´ (Invitrogen ™; D9582 (D01))

(Gardes and Bruns, 1993)

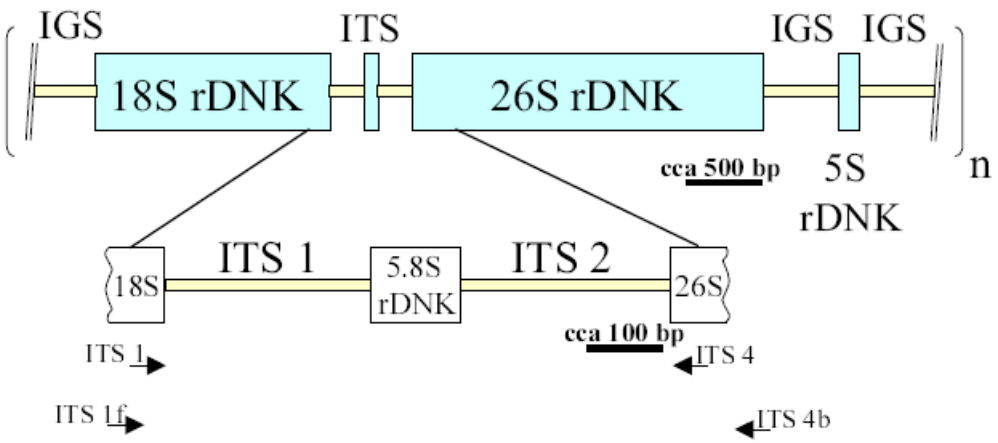

Figure 7: Selected sites for primers used in molecular identification of ECM based on ITS region in rDNA (Grebenc and Kraigher, 2006, modified from http://mollie.berkely.edu/~bruns/).

3.4.2.1 Basic elements of PCR reaction mixture

The PCR mixture and polymerase was selected based on the DNA amount and quality. The DNA quality was assesed with a preliminaring amplification using standard protocol used in the laboratory (Grebenc et al., 2009). Each reaction included a negative control (no DNA template) to test for the presence of DNA contamination of reaction mixtures or water.

For samples giving sufficient amplification after the standard protocol, the approach (a) was applied. If amplification was weak or not successful, a high quality polymerase was used instead folling the protocol (b). For each reaction we tried three different concentrations (using 1, 2, 5 µl of the extract) to finally choose the lowest amount of DNA solution which gave us the best results as the optimized reaction mix.

a) PCR was performed in 25 µl of PCR mix using the regular Taq polymerase (table 3).

Table 3: Basic elements of reaction mixture for PCR in good quality DNA using Taq-polymerase

Element	Amount	Company and catalog number
DNA extraction	1 µl	...
Primer	0,8 µl each	Invitrogen ™
10x PCR Gold Buffer	2,5 µl	Applied Biosystems® GeneAmp® (L00026-01)
Mg^{2+}	2,5 µl	© Roche Farma S.A. (58002032-01)
dNTPs	2,5 µl	Applied Biosystems® GeneAmp® dNTPs (N8080260)
Taq-polymerase	0,2 µl	AmpliTaq Gold® DNA Polymerase (L0112-01)
dd H_2O	13,7 µl *	Gibco® (1097-035)

*Depending of the amount of DNA. The amount of water is calculated as the difference between the sum of all the basic elements and the total value (25µl).

b) In case the high quality polymerase was required, the PCR was perofmed in 20 µl of PCR mix using the reaction compounds given in Table 4.KOD DNA polymerase demonstrates a 2- to 3-fold increase in PCR product formation compared to Taq. For this reason it is used when the quality of DNA is low (Benson et al., 2003).

Table 4: Basic compoundsof the reaction mixture for PCR using KOD polymerase.

Element	Amount	Company and catalog number
DNA extraction	1 µl	Company and catalog number
Primer	0,6 µl each	Invitrogen ™
10x PCR Gold Buffer	2 µl	Applied Biosystems® GeneAmp® (L00026-01)
Mg^{2+}	1,2 µl	© Roche Farma S.A. (58002032-01)
dNTPs	2 µl	Applied Biosystems® GeneAmp® dNTPs (N8080260)
KOD-polymerase	0,2 µl	Merck KGaA, Germany (71085)
dd H_2O	12,2 µl *	Gibco® (1097-035)

*Depending of the amount of DNA. The amount of water is calculated as the difference between the sum of all the basic elements and the total value (20 µl).

3.4.2.2 Temperature profile for the PCR cycles and steps leading to the amplification of DNA

PCR involves a number of cycles at different temperatures (figure 3). The cycle-sequencing perform both amplificatipon and sequence reaction in a thermal cycler (Gene Amp®; PCR System 9700; Applied Biosystems™).
The general sheme was used for all amplification and sequencing reactions in the study:

1.- Denaturation: 94°C. The primers access to the single stranded DNA because of the separation (denaturatione) of the paired strands.

2.- Annealing: cooled to 50/55/58°C depending on the polymerase and reaction volume. Primers select their complementary position.

3.- Elongation: heated to 72°C. Primer extension and final extension.

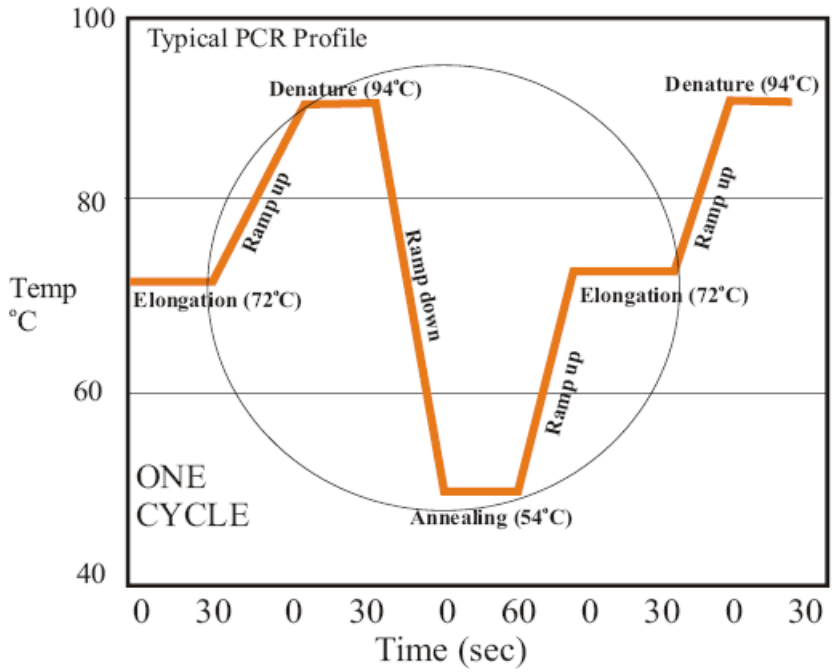

Figure 8: Schematic illustration of examplified PCR temperature profile with example reaction conditions. (Viljoen et al., 2005).

In the PCR with Taq-polymerse the followind programme was used:

- Denaturation: 94°C/5 min
- Amplification:

13x 95°C/35 sec., 55°C/55 sec., 72°C/45 sec.
13x 95°C/35 sec., 55°C/55 sec., 72°C/2 min.

9x 95°C/35 sec., 55°C/55 sec., 72°C/3 min.

- Final extensión: 72°C/10 min.
- Storage at 4°C

In the PCR with KOD-polymerse the following programme was used:

- Denaturation: 95°C/2 min.
- Amplification 95°C20 sec., 55°C/10 sec., 70°C/20 sec.
- Storage at 4°C

3.4.2.3 Electrophoresis

Each PCR and the purification steps were checked for a success by separating and visualisation of the DNA in agarose gel. DNA standards (O´Gene ruler™ 100 bp, Fermentas (#SM143)) and amplifyed DNA from PCR were mixed with 6X Orange Loading Dye Solution (Thermo Scientific (#R0631)) for loading the agarose gels. The agarose gel was prepared with 1,875g of agarose (Agarose, LE, Analytical Grade (Promega Corporation (0000015816)) diluted in 125ml of Buffer 0,5 TBE (Bio-Rad (161-0733)).
All electrophoreses were run in 0.5x TBE buffer. The electrophoresys run for 1.5h in 150V, except for DNA purification step, where the time was prolonged to up ti 2.5h for a better separation of DNA amplicons of similar size.

DNA was visualised by staining with ethidium bromide and illuminated in an UV trans-illuminator (GelDoc – BioRad). The gels were photographed with Gel-Doc (BioRad) sistems and analaysed and annotated in Quantity One software (BioRad).

3.4.3 DNA Purification by Centrifugation

After amplification and successful separation of amplifyed DNA in agarose gel, each DNA fragment was purified for the sequencing reaction. DNA was purified using Wizard® SV

Gel and PCR Clean-Up System (Promega Corporation) following the suggested protocol:

Gel Slice and PCR Product Preparation:

I. The DNA band from the gel after electrophoresis was excised and placed in a 1.5ml microcentrifuge tube.

II. Addition of 10 µl Membrane Binding Solution per 10 mg of gel slice. Vortex and incubate at 50-65°C until is dissolved.
Addition of equal volume of Membrane Binding Solution to the PCR reaction.
Insertting of a SV Minicolumn into Collection Tube.

III. Introducing the dissolved Gel Slice into the Minicolumn assembly and keep it at room temperature for 1 minute.

IV. Centrifuge at 16,000 x g for 1 minute.

V. Addition of a 700 µl of Membrane Wash Solution Repetition of the washing step with 500 µl and centrifuge for 5 minutes.

VI. Centrifuge collection tubes for 1 minute for column to dry Transfer of a minicolumn to a clean 1.5ml microcentrifuge tube.

VII. Addition of 50 µl of Nuclease-Free Water to the minicolumn, storage at ambient temperature for 1 minute and centrifuge at 16,000 x g for another minute.

VIII. Discardingminicolumn and storing its DNA at 4°C or - 20°C.

3.4.4 Sequencing, sequence analysis, comparison with available databases and identification of ECM

Sequencing was done by commercial company (Macrogen, S.Korea). Raw

electophrograms were analysed and read with Sequencher ™ 5.0. Build 7081(Gene Codes Corporation, USA).

Sequences were compared with public avalaible nucleotide databases (NCBI, UNITE). For this purpoose the following software was applied:

- In NCBI: Blast protocol, the MEGA BLAST (BLAST 2.2.26) software was applied. The complete sequence was blasted aginst complete nucleotide collection at GenBank (GenBank database 2012; Zhang et al., 2000).

- In UNITE: database BLAST-N 2.2.6 software was applied against the complete UNITE database (UNITE database 2012; Abarenkov et al., 2010).

Sequences were identified to the species level if the overall identity of the unknown sequence compared to any of databases was higher than 97%, for lower % if identity along the whole length of the sequence, the unknown sequence was identiyed to the genus or higher taxonomic unit level, depending on names of stored sequences given in databases.

4 RESULTS

4.1 TYPES OF ECM SPECIES

From 6 soil samples, we have separated and identified 31 different types of ectomycorrhize. Eight were identiyed to the species level, the rest remaing unidentiyfed or positioned in the genus.

4.1.1 *Tuber aestivum* Vittad.

Morphological characters
Ramification: Simple, monopodial-pinnate
Shape: Straight
Surface: Densely long-spiny
Size: 0,25-0,40 mm
Colour: Bright ochre, older parts brown

Anatomical characters
Emanating elements: Hyphae present, abundant; cystidia present,rhizomorphs absent
Emanating hyphae: Hyphae frequent and abundant over entire ECM system, septated, cell wall and intersepta thick, bright ochre, zig-zag shaped
Mantle type: Pseudoparenchymatous, mantles with angular cells (type L by Agerer 1987-2002), cell wall thick, uppermost hyphae bright orange to dark orange
Rhizomorphs: Absent
Cystidia: Awl-shaped, bristle-like (type A by Agerer R. 1987-2002)
Tree partners: *Quercus pubescens* and *Ostrya carpinifolia*

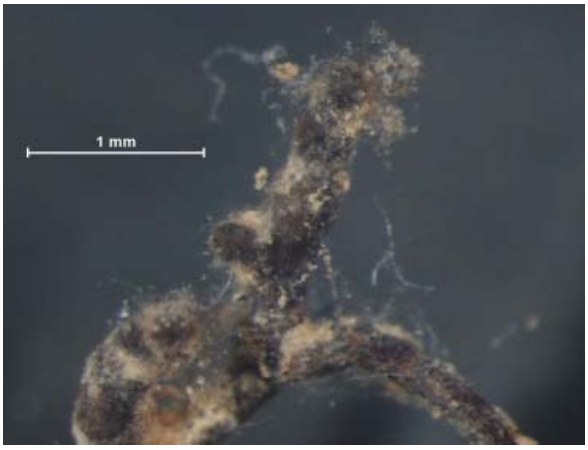

Figure 9: *Tuber aestivum* Vittad.

4.1.2 *Cenococcum geophilum* Fr.

Morphological characters
Ramification: Simple, monopodial-pinnate, monopodial-pyramidal
Shape: Straight
Surface: Densely grainy or warty or loosely
Size: Unknown
Colour: Black

Anatomical characters
Emanating elements: Hyphae infrequent, rhizomorphs absent
Emanating hyphae: Hyphae not visible
Mantle type: Pletenchytomatous mantle with hyphae in star-like arrangements which are tightly glued togheter (type G by Agerer 1987-2002), cell wall thin, membranaceously brownish
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Quercus pubescens* and *Ostrya carpinifolia*

Figure 10: *Cenococcum geophilum* Fr.

4.1.3 *Russula* sp. 1

Morphological characters

Ramification: Monopodial pinnate
Shape: Straight
Surface: Grainy, soil particles attached (infrequent)
Size: 0,5-1 mm
Colour: Orange brown

Anatomical characters
Emanating elements: Hyphae present,rhizomorphs absent
Emanating hyphae: Hyphae frequent and abundant over entire ECM system, septated, cell wall thin and intersepta thick, bright ochre-yellow, straight shape
Mantletype: Pseudoparenchymatous, mantle with angular cells (type L by Agerer 1987-2002) , thin cell wall, uppermost hyphae yellow-orange
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Quercus pubescens*

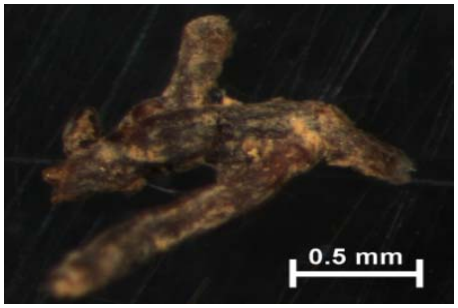

Figure 11: *Russula* spp.

4.1.4 *Byssocorticium atrovirens* (Fr.) Bondartsev & Singer

Morphological characters
Ramification: Simple, monopodial-pinnate
Shape: Straight to bent
Surface: Smooth
Size: Unknown
Colour: Dark brown to grey

Anatomical characters
Emanating elements: Hyphae present, frequent; rhizomorphs absent
Emanating hyphae: Hyphae no frequent, without septa, cell wall very thin almost non-existent, blue, straight shape with elbows
Mantle type: Plectenchymatous mantle with hyphaes rather irregularly arraged and no special pattern discernible (type by Agerer 1987-2002)
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Quercus pubescens*

4.1.5 *Tricholoma / Cortinarius* sp. 1

Morphological characters
Ramification: Simple, monopodial-pinnate
Shape: Sinuous to tortuous
Surface: Cottony, older parts shiny, substrate particles absent
Size: 0,20 mm
Colour: White, older parts brown

Anatomical characters
Emanating elements: Hyphae present, frequent; rhizomorphs observed
Emanating hyphae: Hyphae frequent over entire ECM system, not septated, cell wall thick, white colour, straight shape with curve ending
Mantle type: Pseudoparenchymatous, mantle with soft angular cells (type L by Agerer 1987-2002), thick cell wall, uppermost hyphae colorless with orange spots
Rhizomorphs: Present
Cystidia: Absent
Tree partners: *Quercus pubescens*

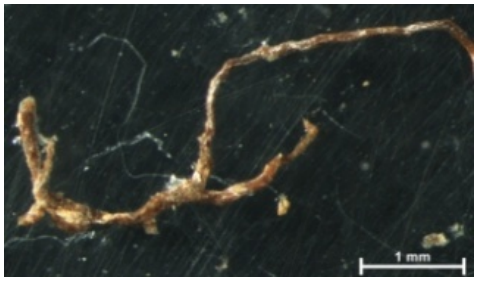

Figure 12: *Tricholoma / Cortinarius* sp. 1

4.1.6 *Tricholoma / Cortinarius* sp. 2

Morphological characters
Ramification: Simple, monopodial-pinnate
Shape: Bent
Surface: Shiny, soil particles infrequent
Size: 0,20 mm
Colour: Bright ochre
Anatomical characters
Emanating elements: Hyphae present, frequent; rhizomorphs not observed
Emanating hyphae: Hyphae no frequent
Mantle type: Pseudoparenchymatous, mantle with soft angular cells(type L by Agerer 1987-2002), uppermost hyphae orange colour
Rhizomorphs: Present
Cystidia: Absent
Tree partners: *Quercus pubescens*

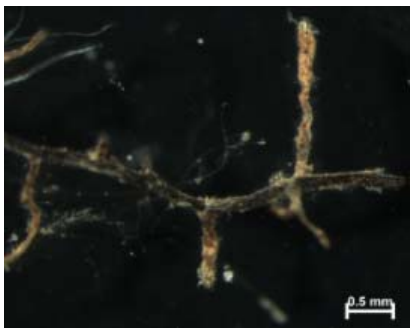

Figure 13: *Tricholoma / Cortinarius* spp.

4.1.7 *Tomentella* sp. 1

Morphological characters
Ramification: Monopodial pinnate
Shape: Straight
Surface: Shiny - spiny, soil particles attached (infrequent)
Size: 0,30-0,50 mm
Colour: Brown

Anatomical characters
Emanating elements: Hyphae present, infrequent;cystida present, frequent; rhizomorphs present
Emanating hyphae: Hyphae frequent over entire ECM system, septated, wall cell and intersepta thick, brown colour, straight shape
Mantle type: Pseudoparenchymatous mantle with angular cells(type L by Agerer 1987-2002), thick cell wall, uppermost hyphae brown with black spots
Rhizomorphs: Present
Cystidia: Present
Tree partners: *Quercus pubescens*

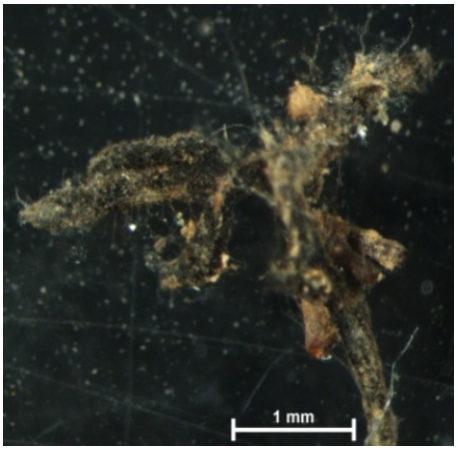

Figure 14: *Tomentella* spp.

4.1.8 *Tomentella subclavigera* Litsch.

Morphological characters
Ramification: Simple
Shape: Bent
Surface: Short spiny
Size: 1mm
Colour: Dark brown

Anatomical characters
Emanating elements: Hyphae infrequent
Emanating hyphae: Hyphae infrequent, without septa, cell wall and intersepta thin, colorless, not staright, long longitude
Mantle type: Plectenchymatous mantle hyphae arranged net-like, repeatedly and squarrosely branched (type E by Agerer 1987-2002), cell wall thick, orange-red colour
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

Figure 15: *Tomentella subclavigera* Litsch.

4.1.9 *Tomentella bryophila*(Pers.) Sacc.

Morphological characters
Ramification: Simple, monopodial-pinnate
Shape: Straight
Surface: Shiny, infrequent soil particles
Size: 0,25-0,30 mm
Colour: Black

Anatomical characters
Emanating elements: Hyphae/ cystidia infrequent, rhizomorphs absent
Emanating hyphae: Hyphae absent in the sample
Mantle type: Pseudoparenchymatous, mantle with soft angular cells (type L by Agerer 1987-2002), cell wall thick, brown or dark-brown colour
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

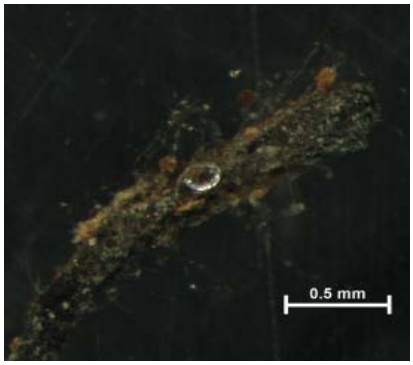

Figure 16: *Tomentella bryophila* (Pers.) Sacc.

4.1.10 *Russula odorata*Romagn.

Morphological characters
Ramification: Monopodial pinnate
Shape: Straight
Surface: Smooth
Size: 0-4 mm
Colour: Brown (light redbrown)

Anatomical characters
Emanating elements: Rhizomorphs absent, hyphae infrequent (not on older parts)
Emanating hyphae: Hyphae infrequent over entire ECM system, septated, cell wall slim, colorless.
Mantle type: Pseudoparenchymatous, some cells containing droplets, staining in sulpho-vanillin; shape variable (type N by Agerer 1987-2002), epidermoid cells bearing a hyphal net (type Qtype L by Agerer 1987-2002)
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Quercus pubescens*

4.1.11 *Russula luteoctata* Rea.

Morphological characters
Ramification: Monopodial-pinnate
Shape: Bent
Surface: Smooth

Size: 0,30-0,40 mm
Colour: Reddish
Anatomical characters
Emanating elements: Hyphae present, infrequent; rhizomorphs and cystidia not observed
Emanating hyphae: Hyphae infrequent, without septa, cell wall and intersepta thin, colorless, not staright
Mantle type: Plectenchymatous mantle hyphae arranged net-like, repeatedly and squarrosely branched (type E by Agerer 1987-2002), orange-red colour
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

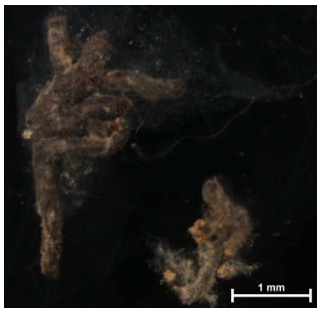

Figure 17: *Russula luteoctata* Rea

4.1.12 Unknown ECM from *Helotiales* 1

Morphological characters
Ramification: Monopodial pinnate
Shape: Bent to torturous
Surface: Shiny, smooth
Size: 0,20-0,45 mm
Colour: White, older parts black

Anatomical characters
Emanating elements: Hyphae and rhizomorphs present, frequent
Emanating hyphae: Hyphae frequent over entire ECM system, without septa, cell wall and intersepta thin, colourless, straight shape
Mantle type: Pseudoparenchymatous, mantle with angular cells(type L by Agerer 1987-2002), thick cell wall, uppermost hyphae colorless-yellow

Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Quercus pubescens*

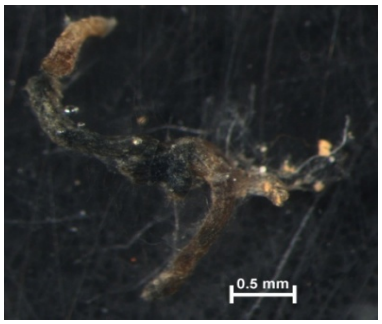

Figure 18: **Unknown ECM from*Helotiales*1.**

4.1.13 Unknown ECM from*Helotiales* 2

Morphological characters
Ramification: Monopodial pinnate
Shape: Straight to bent
Surface: Shiny with hyphae, short spiny
Size: 0,30-0,50 mm
Colour: Dark brown covers with white spiny, yellow tips

Anatomical characters
Emanating elements: Hyphae present, frequent; rhizomorphs and cystidia absent
Emanating hyphae: Hyphae frequent over entire ECM system, septated, short longitude, cell wall and intersepta thick, colorless and curve shape
Mantle type: Pseudoparenchymatous mantle with angular cells bearing a delicate hyphal net (type P by Agerer 1987-2002), cell walls thick, orange-red colour
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: Q*uercus pubescens*

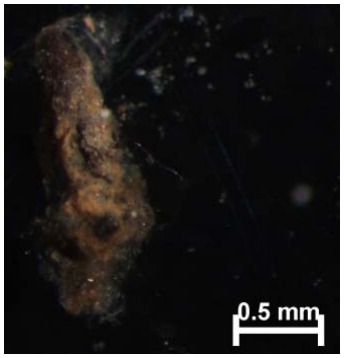

Figure 19: **Unknown ECM from*Helotiales*2**

4.1.14 Unknown ECM from *Helotiales* 3

Morphological characters
Ramification: Monopodial pinnate
Shape: Straight
Surface: Soil particles abundand, grainy

Anatomical characters
Size: 0,20-0,45 mm
Colour: Ochre-brown
Emanating elements: Hyphae present, infrequent; rhizomorphs and cystidia not observed
Emanating hyphae: Hyphae frequent over entire ECM system, septated, wall cell thin,intersepta thin, colourless, straight shape
Mantle type: Pletenchytomatous mantle with hypie in star-like arrangements which are tightly glued togheter (type G by Agerer 1987-2002), orange to red colour
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

Figure 20: **Unknown ECM from*Helotiales*3**.

4.1.15 Unknown ECM from *Pezizomycotina* 1

Morphological characters
Ramification: Monopodial pyramidal
Shape: Bent
Surface: Shiny, substrate particles present covering most of the ECM surface
Size: 0,25-0,35 mm
Colour: Ochre, older parts brown

Anatomical characters
Emanating elements: Hyphae present, infrequent; rhizomorphs present, infrequent
Emanating hyphae: Hyphae frequent over entire ECM system, without septa,cell wall thin, colorless-blue, not straight shape
Mantle type: Pseudoparenchymatous, mantle with angular cells(type L by Agerer 1987-2002), thick cell wall, uppermost hyphae orange with darker plots
Rhizomorphs: Present
Cystidia: Absent
Tree partners: *Quercus pubescens*

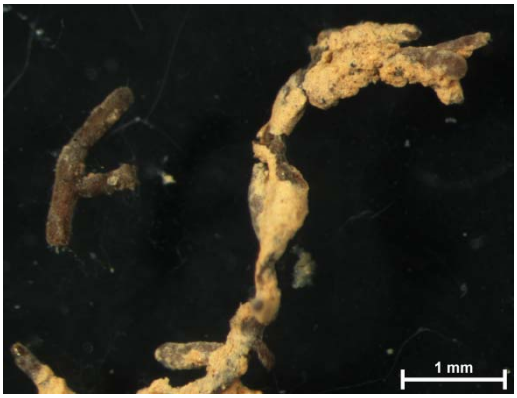

Figure 21: Unknown ECM from *Pezizomycotina* sp. 1

4.1.16 *Scleroderma areolatum* Ehrenb.

Morphological characters
Ramification: Simple, monopodial-pinnate
Shape: Bent
Surface: Silky
Size: 0,12-0,25 mm
Colour: White

Anatomical characters
Emanating elements: Hyphae abudand, rhizomorphs infrequent
Emanating hyphae: Hyphae frequent and abundant over entire ECM system, septated, cell wall thin, intersepta thick, colorless, staright shape
Mantle type: Plectenchymatous mantle hyphae arranged net-like, repeatedly and squarrosely branched (type E by Agerer 1987-2002), orange-red colour with darker spots
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

4.1.17 *Tarzetta* sp. 1

Morphological characters
Ramification: Monopodial pinnate
Shape: Straight to bent
Surface: Soil particles abundant

Size: 0,25-0,40 mm
Colour: Dark brown, ochre

Anatomical characters
Emanating elements: Hyphae, infrequent
Emanating hyphae: Hyphae frequent over entire ECM system, septated, cell wall thin, intersepta thin, colorless
Mantle type: Pseudoparenchymatous, mantle with epidermoid cells (type M by Agerer 1987-2002),cell wall thin, colourless
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

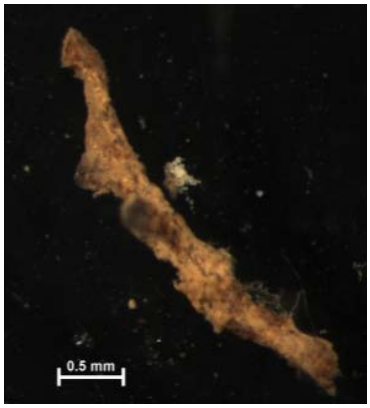

Figure 22: *Tarzetta* sp 1.

4.1.18 Unknown type of ECM 1 (type YPS 1.3, type YPS 2.8)

Morphological characters
Ramification: Simple or monopodial pinnate
Shape: Bent
Surface: Grainy surface
Size: 0,20-0,25 mm
Colour: Orange-ochre

Anatomical characters
Emanating elements: Hyphae present, frequent; rhizomorphs present, infrequent
Emanating hyphae: Hyphae frequent over entire ECM system, without septa, cell wall thin,

colourless, straight shape
Mantle type: Pseudoparenchymatous, mantle with soft angular cells (type L by Agerer 1987-2002), thin cell wall, uppermost hyphae colorless or bright orange, thick cell wall, uppermost hyphae colorless or bright orange, with some darker spots (corticall cells)
Rhizomorphs: Present
Cystidia: Absent
Tree partners: *Quercus pubescens*

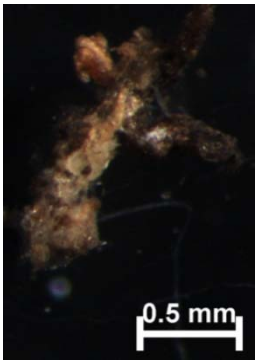

Figure 23: Type YPS 1.3

4.1.19 Unknown type of ECM 2 (type YPS 1.5)

Morphological characters
Ramification: Simple
Shape: Straight
Surface: Reticulate
Size: 0,20-0,25 mm
Colour: White-ochre, older parts carbonising

Anatomical characters
Emanating elements: Hyphae present, infrequent; rhizomorphs absent
Emanating hyphae: Hyphae frequent and abundant over entire ECM system, without septa,cell wall thick, colourless and straight shape
Mantle type: Pseudoparenchymatous mantle with epidermoid cells which have the appearance of cells of a leaf epidermis(type M by Agerer R. 1987-2002), thick cell wall, uppermost hyphae bright orange to dark orange
Rhizomorphs: Absent
Cystidia: Absent

Tree partners: *Quercus pubescens*

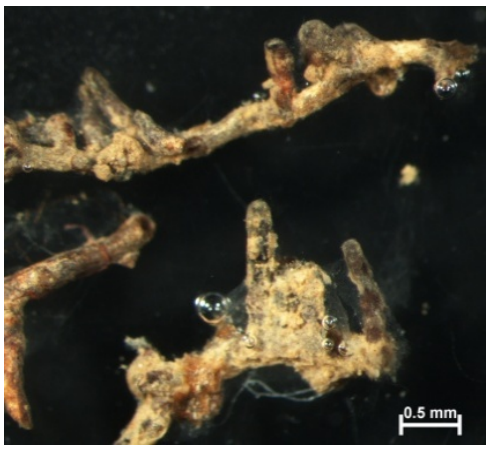

Figure 24: Type YPS 1.5

4.1.20 Unknown type of ECM 3 (type YPS 1.7, type YPS 1.9, type YPS 2.3)

Morphological characters
Ramification: Monopodial-monopodial pinnate
Shape: Straight to bent
Surface: Shiny, soil particles attached
Size: 0,25-0,40 mm
Colour: Ochre-brown

Anatomical characters
Emanating elements: Hyphae present, frequent; rhizomorphs absent
Emanating hyphae: Hyphae frequent over entire ECM system, without septa, cell wall and intersepta thin, colourless, straight shape
Mantle type: Pseudoparenchymatous, mantle with angular cells (type L by Agerer 1987-2002), thin cell wall, uppermost hyphae colourless-yellow with orange spots, soil particles attached to surface
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Quercus pubescens*

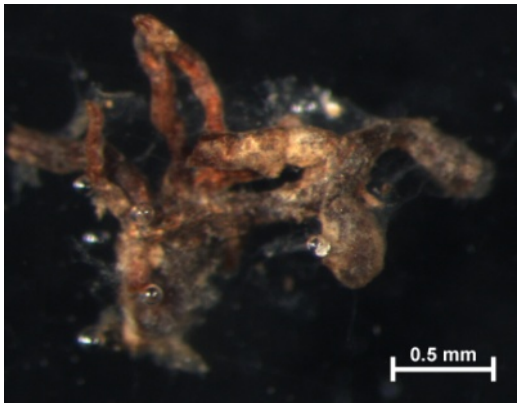

Figure 25: Type YPS 1.9

4.1.21 Unknown type of ECM 4 (type YPS 2.7)

Morphological characters
Ramification: Simple, monopodial-pinnate
Shape: Bent to sinuous
Surface: Shiny, soil particles frequent
Size: 0,25-0,35 mm
Colour: Yellow green, terminal tips ochre

Anatomical characters
Emanating elements: Hyphae present, infrequent; rhizomorphsobserved and cystidia not observed
Emanating hyphae: Hyphae infrequent, septated, wall cell thin and intersepta thick, colourless, straight shape with elbows
Mantle type: Pseudoparenchymatous, mantle with angular cells (type L by Agerer 1987-2002), thick cell wall, uppermost hyphae yellow-orange with black spots
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Quercus pubescens*

Figure 26: Type YPS 2.7

4.1.22 Unknown type of ECM 5 (Type YPS 3.2)

Morphological characters
Ramification: Monopodial pinnate
Shape: Straight to bent
Surface: Grainy; soil particles attached, infrequent
Size: 0,10-0,25 mm
Colour: Orange-brown

Anatomical characters
Emanating elements: Hyphae present, infrequent; rhizomorphs not observed
Emanating hyphae: Hyphae no frequent
Mantle type: Hyphae no frequent
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Quercus pubescens*

Figure 27: Type YPS 3.2

4.1.23 Unknown type of ECM 6 (type YPS 11.3)

Morphological characters
Ramification: Simple
Shape: Tortuous
Surface: Wolly
Size: Unknown
Colour: Brown

Anatomical characters
Emanating elements: Hyphae abudand, rhizomorphs infrequent
Emanating hyphae: Hyphae frequent and abundant over entire ECM system, septated, cell wall thin, intersepta thick, colorless, staright shape
Mantle type: Pseudoparenchymatous, mantle with angular cells (type L by Agerer 1987-2002), thick cell wall, uppermost hyphae yellow to colourless
Rhizomorphs: Undifferentiated rhizomorphs with rather smooth margins; the hyphae are compactly and are of uniform diameter
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

Figure 28: Type YPS 11.3

4.1.24 Unknown type of ECM 7 (type YPS 11.5)

Morphological characters
Ramification: Simple

Shape: Beaded
Surface: Reticular
Size: Unknown
Colour: Yellow

Anatomical characters
Emanating elements: Hyphae infrequent
Emanating hyphae: Hyphae frequent and abundant over entire ECM system, septated, cell wall thin, intersepta thin, brown, staright shape
Mantle type: Pseudoparenchymatous, mantle with angular cells(type L by Agerer R. 1987-2002), thick cell wall, uppermost hyphae colourless with orange spots
Rhizomorphs: Absent
Cystidia: Present
Tree partners: *Ostrya carpinifolia*

4.1.25 Unknown type of ECM 8 (type YPS 11.6)

Morphological characters
Ramification: Simple
Shape: Straight
Surface: Smooth to reticulate
Size: 1-2mm
Colour: Yellow, older parts carbonising

Anatomical characters
Emanating elements: Hyphae infrequent
Emanating hyphae: Hyphae infrequent , septated, cell wall thick, intersepta thin, colorless, not staright, long longitude
Mantle type: Transitional type between plectenchymatous and pseudoparenchymatous, irregulary shaped hyphae form a coarse net(type H by Agerer 1987-2002),thin cell wall, dark brown colour
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

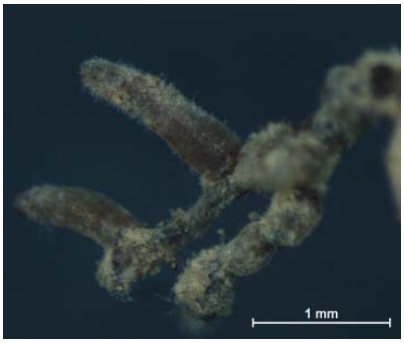

Figure 29: Type YPS 11.6

4.1.26 Unknown type of ECM 9 (Type YPS 11.7)

Morphological characters
Ramification: Monopodial pinnate
Shape: Straight
Surface:Wolly
Size: Unknown
Colour: Brown to black

Anatomical characters
Emanating elements: Hyphae/cystidia frequent, rhizomorphs present
Emanating hyphae: Hyphae infrequent , septated, cell wall thin, intersepta thin, colorless, not staright, long longitude
Mantle type: Plectenchymatous mantle hyphae arranged net-like, repeatedly and squarrosely branched(type E by Agerer 1987-2002), cell wall thick, orange-red colour
Rhizomorphs:Undifferentiated rhizomorphs with rather smooth margins; the hyphae are compactly and are of uniform diameter
Cystidia: Present
Tree partners: *Ostrya carpinifolia*

Figure 30: Type YPS 11.7

4.1.27 Unknown type of ECM 10 (Type YPS 12.4)

Morphological characters
Ramification: Monopodial - pinnate
Shape: Bent
Surface: Cottony
Size: 0,25-0,50 mm
Colour: Ochre

Anatomical characters
Emanating elements: Hyphae present, frequent; rhizomorphs and cystidia absent
Emanating hyphae: Hyphae frequent over entire ECM system, septated, short longitude, cell wall and intersepta thick, colorless and curve shape
Mantle type: Pseudoparenchymatous mantle with angular cells bearing a delicate hyphal net(type P by Agerer 1987-2002), cell walls thick, orange-red colour
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

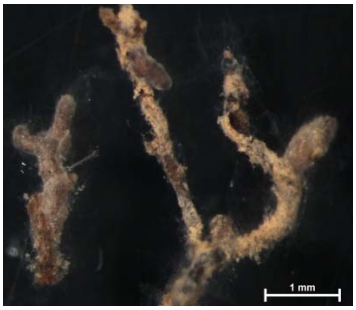

Figure 31: Type YPS 12.4

4.1.28 Unknown type of ECM 11 (type YPS 12.5)

Morphological characters
Ramification: Monopodial
Shape: Tortuous
Surface: Shiny
Size: 0,25-0,35 mm
Colour: White, older parts brown

Anatomical characters
Emanating elements: Hyphae present, frequent; rhizomorphs present, infrequent; cystidia absent
Emanating hyphae: Hyphae infrequent , without septa, colorless, short longitude
Mantle type: Plectenchymatous mantle hyphae arranged net-like, repeatedly and squarrosely branched (type E by Agerer 1987-2002), colourless with orange spots
Rhizomorphs: Differenciated rhyzomorphs with a central core of thick hyphae
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

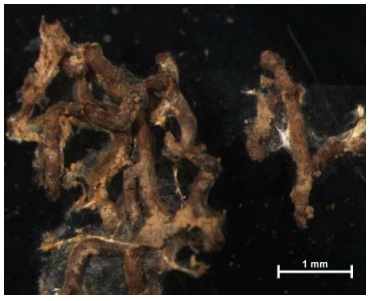

Figure 32: Type YPS 12.5

4.1.29 Unknown type of ECM 12 (type YPS 12.7)

Morphological characters
Ramification: Simple, monopodial-pinnate
Shape: Straight
Surface: Cottony
Size: 0,25-0,45 mm
Colour: Light brown

Anatomical characters
Emanating elements: Hyphae frequent and abundant over entire ECM system, septated, cell wall thin, intersepta thick, colorless, staright shape
Emanating hyphae: Pseudoparenchymatous, mantle with angular cells(type L by Agerer 1987-2002), thick cell wall, uppermost hyphae yellow to colourless
Mantle type: Undifferentiated rhizomorphs with rather smooth margins; the hyphae are compactly and are of uniform diameter
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

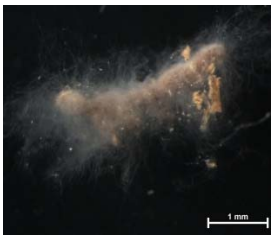

Figure 33: Type YPS 12.7

4.1.30 Unknown type of ECM 13 (type YPS 13.4)

Morphological characters
Ramification: Simple, monopodial-pinnate
Shape: Straigh
Surface: Cottony , grainy
Size: Unknown

Colour: Dark brown

Anatomical characters
Emanating elements: Hyphae present, frequent; rhizomorphs not observed; cystidia absent
Emanating hyphae: Not hyphae present in the sample
Mantle type: Transitional type between plectenchymatous and pseudoparenchymatous, irregulary shaped hyphae form a coarse net(type H by Agerer R. 1987-2002),cell wallsthick, orange to dark-orange colour
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

4.1.31 Unknown type of ECM 14 (type YPS 13.9)

Morphological characters
Ramification: Monopodial pinnate
Shape: Straight
Surface: Soil particles attached, frequent
Size: 0,30-0,40 mm
Colour: Ochre-brown

Anatomical characters
Emanating elements: Hyphae infrequent
Emanating hyphae: Hyphae frequent and abundant over entire ECM system, septated, cell wall thick, colorless, staright shape
Mantle type: Pseudoparenchymatous, mantle with soft angular cells(type L by Agerer 1987-2002), cell walls thick, red-orange colour
Rhizomorphs: Absent
Cystidia: Absent
Tree partners: *Ostrya carpinifolia*

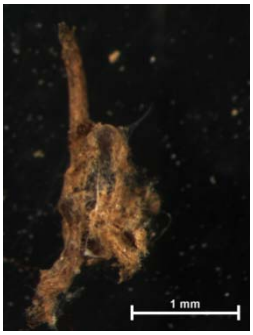

Figure 34: Type YPS 13.9

4.2 SEQUENCING RESULTS

Obtained sequences from analysed types of ECM were compared to available databases and firstly checked for potential amplification of saprotrophs. All sequences not corresponding to ECM genera (Rinaldi et al., 2008) were eliminated and only ectomycorrhizal were further identified (table 5).

Table 5: Identification and percentage of sequence similarity with best match(es) from GenBank and UNITE database after the BLAST analysis; the final (summary) identification and the soil sample where recorded are also given.

Type of ECM (nº folloing subchapters)	Best match in GenBank database after BLAST	Percentage of sequence similarity	Best match in UNITE database after BLAST	Percentage of sequence similarity	Final Identification	ECM sample code
4.1.1	*Tuber aestivum*	100%	…	…	*Tuber aestivum*	3.8
4.1.1	*Tuber aestivum*	96%	…	…	*Tuber aestivum*	13.5
4.1.1	*Tuber aestivum*	99%	…	…	*Tuber aestivum*	13.6
4.1.7	*Tomentella* spp.	99%	*Tomentella stuposa*	94%	*Tomentella* spp.	2.5, 3.5
4.1.8	Uncultured ECM (*Tomentella*)	99%	*Tomentella fuscocinera*	93%	*Tomentella subclavigera*	11.2
4.1.9	Uncultured *Tomentella*	96%	*Tomentella bryophila*	94%	*Tomentella bryophila*	13.11
4.1.9	Uncultured *Tomentella*	100%	*Tomentella spp.*	93%	*Tomentella spp.*	11.4
4.1.10	Uncultured ECM fungus	98%	*Russula odorata*	Not avalaible	*Russula odorata*	3.9
4.1.11	Uncultured ECM	92%	*Rusula luteotacta*	99%	*Rusula luteotacta*	12.6

continue

continue

Type of ECM (nº folloing subchapters)	Best match in GenBank database after BLAST	Percentage of sequence similarity	Best match in UNITE database after BLAST	Percentage of sequence similarity	Final Identification	ECM sample code
4.1.12	*Unknown ECM from Helotiales 1*	100%	…	…	*Unknown ECM from Helotiales 1*	1.8
4.1.13	Unknown ECM from *Helotiales* 2	96%	…	…	Unknown ECM from *Helotiales* 2	2.6
4.1.14	Unknown ECM from *Helotiales* 3	94%	…	…	Unknown ECM from *Helotiales* 3	12.3
4.1.15	Unknown ECM from *Pezizomycotina* 1	99%	…	…	Unknown ECM from *Pezizomycotina* 1	3.6
4.1.16	*Scleroderma areolatum*	99%	…	…	*Scleroderma areolatum*	13.10
4.1.17	Uncultured *Tarzetta*	1013	*Tarzetta*	99%	*Tarzetta* sp.	13.8

5 DISCUSSION

The study represents the first insight into the ectomycorrhizal community related to the natural *Tuber aestivum* ground in Slovenia. ECM community from truffles sites in Slovenia was completely unexplored and the knowledge of the diversity is rather scarce.With the selection of two common ECM partners of *T. aestivum* we have managed to obtain the representative set of associated ECM fungi analysed by the identification of ectomycorrhizae. *Tuber aestivum* ECM is well known from several ECM trees and shrubs (Agerer and Rambuuld, 2004-2011), this identification did not cause problems. Despite sampling of soil for ECM analysis in the vicinity of mature truffle sporocarps, the *Tuber aestivum* ECM was not discovered in all soil samples. This could be due to distribution of ECM and fungal mycelium in the soil, related to the pocket-like distribution of soil and large variaility in tis depth (Urbančič et al., 1999). The speceis composition among the two analysed plant ECM partners was expected, since *Ostrya carpinifoila* has more pioneer nature while *Quercus pubescens* usually grow in more stable forest stands (Culiberg, 1998). In terms of the differences of ECM diversity between the tree partners *Tricholoma/Cortinarius* type of ECM, *Tuber aestivum* and *Russula* spp. were more commonly present in ECM with *Q.pubescens*, which is in concordance with the succession stage of oak forests. Similar presence of ECM genera (except *Tuber* spp.) was also observed in stable old-grown forests such at Rajhenavski Rog secondary virgin forest (Grebenc et al., 2009). On the other hand with more pioneer *O. carpinifolia*, several species regarded as more early stage species were recorded, such as *Tomentella* spp. (Yorou et al., 2012) and *Scleroderma* spp. common in greenhouse experiments from young seedlings (Štraus et al., 2012). Still *T.aestivum, Tarzetta* spp., and some *Tomentella spp.* were found in common in both analysed places and plant partners.

Comparing the ECM community from productive *Tuber aestivum* sites, either natural or from truffle plantation, we have expect differences in community structure among natural stands plantation. We have focused on comparable *Tuber aestivum* plantaion from central Italy, where ECM community of 25 years old productive plantation was analysed by Benucci et al. (2011). Comparing to our study the study of Benucci et al. (2011) was

performed with *Corylus avellana L.* instead of oak. As expected, *T.aestivum* was found in every sample in plantation since it was introduced there with inoculated seedlings while in natural stand that was not the case. We explain this observation with lower and less abundand competition in plantations which are purposevely managed for the maintainance of the particular truffle species (Chevalier and Frochot, 1997; cit in Benucci 2011). The number of different types of ECM was comparable among natural stand and plantation regardless to the plant ECM partner at any of sites but the community composition was not the same. The number of *Tomentella* spp. and *Thelephoraceae* ECM was higher in plantation than in natural stands (Benucci et al., 2011); species, such as *Sebacina* spp., *Peziza michelii* (Boud.) Dennis, *Pseudomentella* spp., and different types of *Tuber* spp. (*T. rufum* Pico, *T. brumale* Vitt., *Tuber rapaeodorum* Tul. & C. Tul.) were not recorded from natural stand, whichindicate some crucial differences in the community structure drivers. And finally *Russula* spp. and *Cenococcum geophilum* Fr. were not recorded in plantation whereas they were a common species in natural stands, in particular with oaks. *C. geophilum*as a generalist was present in every sample from natural stands.

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