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Ectomycorrhizal community structure in a healthy and a *Phytophthora*-infected chestnut (*Castanea sativa* Mill.) stand in central Italy

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Abstract Ink disease caused by *Phytophthora cambivora* is a major disease of sweet chestnut (*Castanea sativa*). In two *C. sativa* stands in central Italy, one (Montesanti) that is infected with *P. cambivora* and the trees showing symptoms of ink disease and another healthy stand (Puzzella), the ectomycorrhizal (ECM) community structure was investigated. On the roots of the surviving trees of the diseased stand, 29 different ECM species were determined compared to 23 in the healthy stand. Eleven ECM species were common to both stands; however, a number of species were unique to one of the stands. *Cenococcum geophilum* was dominant at both sites, but the percentage colonisation was much higher at Montesanti (40.8%) compared to Puzzella (27.2%). There was a switch in species from *Russula vesca*, *Russula lepida* and *Russula azurea* at Puzzella to *Russula nigricans*, *R. lepida* and *Russula delica* at Montesanti. Both *R. vesca* and *R. azurea* were found only at the Puzzella site. At the diseased site, the ECMs formed had a smaller root tip diameter, and the ECM at the healthy site had more abundant extramatrical hyphae.

Keywords Ectomycorrhizas · Phytophthora · *Castanea sativa* · Ink disease

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Introduction

Sweet chestnut (*Castanea sativa* Mill.) has been an important multipurpose tree from the time when the ancient Greeks started with its active cultivation for fruit and timber, a practice that was subsequently propagated by the Romans throughout their Empire (Adua 1999; Zohary and Hopf 2000). At present, sweet chestnut cultivation continues to be a strong tradition in rural regions of countries like Italy, France, southern Switzerland, Spain, Portugal and Greece (Conedera et al. 2004). Here, the tree species has great rural economic value and plays an important agro-ecological role, among others, protection against fire and erosion, habitat for biodiversity and recreation. Recently, a high mortality in sweet chestnut stands caused by a dramatic resurgence of ink disease, one of the major chestnut diseases, has been reported (Bounous and Abreu 1998; Vannini and Vettraino 2001; Vettraino et al. 2001; Fleisch 2002). The disease is caused by the soil-borne Oomycetes *Phytophthora cambivora* (Petri) Buis and *Phytophthora cinnamomi* Rands and induces root and collar rot with dieback of branches, defoliation, gradual decline and death of the infected trees (Milburn and Gravatt 1932; Day 1938; Crandall et al. 1945). The predicted changes in climate in Italy, including general warming, summer drought, increased flood incidents and mild winters (CC 2007), are likely to further increase the impact of the pathogens involved, since the outlined conditions are known to favour development of *Phytophthora* through an increase in survival, dispersal and the number of reproduction cycles (Weste 1983). The model predictions of disease activity under climate change also indicate that *Phytophthora* pathogens that cause ink disease will become more virulent in their existing locations and are likely to spread

northwards and eastwards (Brasier and Scott 1994; Brasier 1999; Bergot et al. 2004).

Phytophthora disease results in severe leaf loss and subsequent death over a period of years (Crawford 1995). Crown defoliation has been shown to modify ectomycorrhizal (ECM) community structure (Kuikka et al. 2003) and increase the frequency of thin mantled ectomycorrhizal morphotypes (Saravesi et al. 2008). Alternatively, ECM fungi have been postulated as a biological option to prevent *Phytophthora* infection in new plantations and nursery stock. Numerous studies have highlighted the inhibitory ability of certain ECM fungal species, decreasing the virulence of *Phytophthora* in tree species like *Pinus echinata* Mill. and *Pinus taeda* L. (Marx 1973; Marx and Davey 1969a,b; Barham et al 1974), *Pinus patula* Schiede ex Schltdl. & Cham. (Marais and Kotze 1979) and *Eucalyptus marginata* Donn ex Sm. (Malajczuk 1988). A pot trial in which sweet chestnut was inoculated with four selected ECM fungi [*Laccaria laccata* (Scop.) Cooke, *Hebeloma* crustuliniforme (Bulliard) Quélet, *H. sinapizans* (Bulliard) Quélet, and *Paxillus involutus* (Batsch) Fries] also demonstrated this protective potential of ECM fungi against *P. cambivora* and *P. cinnamomi* infection (Branzanti et al. 1999).

Multiple mechanisms are hypothesised by which ECM fungi could confer protection against root pathogens, by providing a physical barrier to penetration, by secreting antibiotics inhibitory to pathogens, by utilising surplus carbohydrates, by favouring protective rhizosphere microorganisms and by inducing in the host, inhibitors to the pathogen (Zak 1964; Marx 1969a; 1969b; 1972). The relative importance of each mechanism varies based on the mycobiont species' characteristics. However, these mechanisms have been proposed mainly for single species of ECM fungi, whereas field grown trees often have complex communities. If ECM fungal communities in sweet chestnut stands are equally diverse as those seen with other tree species of temperate forests (e.g. Horton and Bruns 2001; Dahlberg 2001; Landeweert et al. 2003; Tedersoo et al. 2006; Gebhardt et al. 2007), the antagonistic functioning is likely to be very complex. Identification and quantification of ECM fungal species present on the roots of sweet chestnut in real ecosystems is therefore essential because it will allow an estimate of shifts in dominant taxa, with high functional relevance, and the uncommon species that make ECM fungal communities typically species rich.

In an ink-disease-plagued region of Italy, in the central Apennines, such an investigation was undertaken in two adjacent sweet chestnut stands that were similar in soil properties and site conditions. One stand had a high incidence of *Phytophthora* spp. in the soil, and the trees showed symptoms of decline, whereas the other stand showed no disease symptoms.

Materials and methods

Study sites

The study area is located 25 km to the southeast of Rieti, Italy, in the Central Apennines. Two *C. sativa* sites were investigated, one a communal orchard 'Puzzella', and the other an abandoned stand 'Montesanti'. The Puzella chestnut stand, (42°13.31′N, 13°3.11′E, 819 m above sea level, Mediterranean-wet climate), is an active communal orchard that is managed for the cultivation of nuts. It measures 2.8 ha, and although the precise age of the orchard is unknown, individual trees were estimated to range between 100 and 200 years old. The stand is stocked with about 100 large single stem trees per hectare and is positioned on a north facing slope of up to 30°. The large single-stem trees have an average diameter at breast height (DBH) of 82.4 cm and have been pruned regularly to produce crowns that result in optimal chestnut production (see Peraira-Lorenzo and Ramos Cabrer 2004). Apart from some mosses, no understory vegetation was observed, as it is systematically removed to facilitate nut collection. The stand at Montesanti (42°13.07′N, 13°3.01″E, 980 m above sea level, Mediterranean-wet climate) is a 3-ha former timber and chestnut orchard. It is positioned on a north-facing slope of up to 35°. The stand was established in the early 1920s and was abandoned after *P. cambivora* had infested it in the early 1990s, causing symptomatic trees and dieback. The presence of *P. cambivora* in this area was established in both soils and root tissue and was shown to be the dominant species (Vettraino et al. 2005). The stand is stocked at about 150 single-stem trees per hectare, of which the canopy dominants have an average DBH of 59.9 cm. The site is characterised by ample standing dead wood and consequently has many openings in its canopy, which has led to natural regeneration of sweet chestnut and the development of a grass-dominated understory. The younger, naturally regenerated trees were, however, more than 20 years old, and they were affected by *Phytophthora* infection as the dominant trees in the stand. The soil at both sites is Eutric Cambisol.

The loss of foliage due to *P. cambivora* at Montisanti was determined by advanced spectroscopic imaging (Vannini et al. 2005), and the presence of *P. cambivora* inoculum was also confirmed (Vettraino et al. 2005).

Soil and root sampling

A total of ten soil samples were collected from each site to assess belowground ECM species richness, composition, and root tip abundance. The sampling effort was split over two visits, one in November 2005 and one in September 2006. At the first visit, five dominant, *Phytophthora*-

infected chestnut trees were selected at Montesanti. Care was taken that selected trees had a relatively high number of leaves remaining in their disease-impacted crowns. This selection procedure was used to achieve the highest possible recovery of living fine root material, since numerous individuals were dying off or dead. One soil sample was taken, with a root auger soil-corer (8-cm diameter, 15-cm depth, Eikelkamp, The Netherlands), 2 m from the trunk base of each tree. All samples were taken upslope from the trees, again to improve the likelihood of retrieving a significant sample of chestnut roots, since both stands have quite steep slopes (up to 35°) and slope steepness is known to influence lateral root development (Chiatante et al. 2003). The same procedure was followed at Puzzella, although the health of the trees was not considered a factor in this study, since the trees were free of ink disease.

The first ten soil cores, sampled in 2005, were transferred to Tuscia University, at Viterbo, Italy, where they were cut lengthwise. One core half of each sample (375 ml soil) was submitted at the Dipartimento di Protezione delle Piante, Department of Plant Protection laboratory, where *Phytophthora* infestation rate was assessed by means of the *Rhododendron* leaf-baiting technique (Vettraino et al. 2001). The other core halves were transported on ice back to the laboratory at the School of the Environment and Natural Resources, Bangor University, where they were stored at 5°C for later examination of the mycorrhizal roots. The second set of ten soil cores, collected in 2006, were taken at 10-cm distance from the original core locations. Core samples were treated similarly; however, this time, instead of the *Phytophthora* analysis, one half of each core was oven-dried and passed through a 2-mm mesh screen and the pH, organic matter content and soil moisture content determined. Soil pH was determined in a 3:1 water/soil suspension. The mixture was shaken for 30 min then allowed to settle, and the pH of the supernatant was determined. Soil moisture was determined after drying at 70°C to constant weight, and on the dried samples, total organic matter was determined by loss on ignition after combustion in a muffle oven at 450°C for 16 h.

ECM root tip sorting

At the time of analysis, within 6 weeks of collection, soil samples (375 ml each) were placed in separate deep Petri dishes, filled with sterile water and soaked for several hours at 8°C. This facilitated the extraction of roots from the soil and helped to prevent root tips from breaking off during rinsing. Under a dissecting microscope at a magnification of 40 times, roots were determined to be living or dead based on the health of the stele. All live fine root parts were scanned on a flatbed scanner and the number of root tips, root length, and surface area determined using the analysis programme WinRhizo (ver. 2002.c; Regent Instruments, Quebec, Canada). Living roots were determined to be ECM based on the presence of a healthy fungal sheath or if they exhibited morphological signs, such as swelling, coupled with visual presence of hyphae when viewed under a dissecting microscope. All ECM root tips were sorted into broadly defined morphotypes based on morphological characteristics, including branching structure, shape and dimensions, mantle colour and texture and emanating hyphae and rhizomorphs based on described methods (Agerer 1987–1997). Care was taken to separate morphotypes when slight variations were found. The total number of root tips colonised by each of the ECM morphotypes was counted. From all morphotypes, samples were removed from the fine roots and stored in sterile distilled water at −80 C until DNA analysis. The fine roots were subsequently oven-dried (7 C) and weighed.

DNA extractions and PCR

For DNA extraction, one ECM root tip for robust ECM morphotypes and up to five ECM root tips for small and short ECM morphotypes were used. ECM fungal tissue samples were added to 1.5 ml centrifuge tubes and crushed with a micropestle (Eppendorf, UK) and suspended in 600 μl 2% cetyl trimethylammonium bromide buffer (1 M Tris–HCl, 5 M NaCl, 0.5 M EDTA, 0.2% 2-mercaptoethanol). Following a 60-min incubation at 60°C, samples were briefly vortexed with 600 μl chloroform/isoamyl alcohol (24:1) and centrifuged (13,000×*g*) for 15 min. The aqueous phase was removed to a new centrifuge tube. The DNA was precipitated with an equal volume of chilled isopropanol at −18°C. After 30 min, the precipitant was pelleted by a 30-min centrifugation (13,000×*g*). The supernatant was discarded, and the pellet was washed with 200 μl of 70% chilled ethanol. The pellet was allowed to dry for 15 min at room temperature before resuspension in 50–200 μl (depending on the size of the pellet) of sterile distilled water.

Polymerase chain reaction (PCR) was performed in a Techgene thermal cycler (FTGENE5D) in conditions previously described (Gardes and Bruns 1993) with 1 μl of the diluted nucleic acids in a 40 μl PCR reaction, containing 20 μl of RedTaq ReadyMix (Sigma-Aldrich Co., UK), 17 μl of sterile distilled water, and 1 μl of each of the two primers used in a particular reaction (1 μM concentration). Amplification of the internal transcribed spacer (ITS) region of the nuclear rDNA of all fungal samples was initially attempted with the fungal-specific ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) primer pair that targets both ascomycetes and basidiomycetes. With the use of the forward primer ITS1 (White et al. 1990) and the

reverse primer ITS4B (Gardes and Bruns 1993) another three primer combinations could be tried if the first primer pair gave a poor PCR result. The PCR products were purified with the Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI, USA).

Identification of fungi

Purified PCR products were sequenced at Macrogen Inc. (Seoul, Korea). The sequences obtained were compared with the sequences of known species in the UNITE database (Kõljalg et al. 2005) using Galaxy BLAST. When close matches were unavailable, a BLAST search was conducted in the National Centre of Biotechnology Information (NCBI) public sequence database. Based on sequence homology between acquired sequences and their closest matches with sequences from the above mentioned databases, three categories were used for taxonomic classification: >97%; species level identity, 95–97%; genus level identity, <95%; and family level identity (Table 1).

Calculations and statistical analyses

The numbers of root tips colonised by individual EMF species were determined for each root system using the molecular identification results. Relative abundance was calculated as the number of ECM root tips of individual species divided by the total number of ECM root tips for all species. Absolute frequency was calculated as the number of samples in which a species occurs divided by total number of samples. Relative frequency was calculated as the absolute frequency of individual species divided by the sum of absolute frequencies for all species. Importance values for individual ECM fungal species were calculated by summing their relative abundance and their relative frequency. Specific root tip density for individual ECM species was calculated as the absolute abundance of individual species on the roots in a soil core divided by the total surface area of the roots in that soil core. As the total number of root tips sampled varied at the study sites the method of rarefaction (Krebs 1989) was applied to allow for between-site comparison of species richness values using the online webpage rarefactor calculator (http://www2.biology.ualberta.ca/jbrzusto/rarefact.php). Species estimations were made with the programme ESTIMATES (Colwell 2006) using the Second-order Chao estimator of species richness (Chao 1987).

$$\widehat{S}_{\mathrm{Chao2}} = S_{\mathrm{obs}} + \frac{Q_1^2}{2Q_2}$$

S_{obs} the total number of species observed in all samples pooled

Q_1 the frequency of uniques

Q_2 the frequency of duplicates

To assess if soil properties, fine root parameters, and ECM tip density differed statistically between the two sites, Student's t tests (at p 0.05 level of acceptance) were performed in SigmaStat (SPSS Inc., Chicago, IL, USA) for Windows Version 3.0.1. Data were square root transformed if normality was not achieved.

ECM root morphology was analysed using one-way analysis of variance (ANOVA). Data were square root transformed if normality was not achieved. If normality was not achieved by arcsine, square-root or log-transformation significance was assessed with Kruskal–Wallis analysis (at p 0.05 level of acceptance) in SigmaStat.

Results

Fine root characteristics and fungal richness

Differences between means of soil property values measured in ten soil cores from each site were evaluated with a t test, and no significant differences were observed (Table 2). The pH at the infected site Montesanti was slightly lower and the organic matter content slightly higher than the healthy stand at Puzzella. Similarly, there were also no statistically significant differences in any of the fine root parameters measured; however, the Montesanti stand had twice the amount of fine roots (gram per square metre) compared to the Puzzella stand, even though the tree stocking density was only 50% higher. This higher root mass was also shown in the fine root surface area and in the fine root length. The specific root length (centimetre per gram) of 539 for Puzzella and 686 for Montesanti is indicative of the thinner fine roots. The presence of *Phytophthora* infection was confirmed in the *Rhododendron* leaf studies. Using the Montesanti soil, on average, 30% of the leaves became infected compared to 0.5% using the Puzzella soil. A total of 23 and 29 ECM fungal species were determined on a total of 7,530 and 14,663 root tips at the Puzzella and Montesanti stands, respectively (Table 2). Similarly to the fine root mass, the number of root tips at Montesanti was nearly twice that determined at Puzzella. However, there was no significant difference (t test, p= 0.076) in mean number of root tips per square meter between the two stands types (Table 2). A list of all ECM species determined is shown in Table 2. As previously stated, 23 and 29 ECM fungal species were determined at the Puzzella and Montesanti stands, respectively (Table 2). Using the programme ESTIMATES, the predicted number of species at the sites was calculated. The estimated total species richness for Puzzella and Montesanti stands were

Table 1 The identities of fungal taxa symbiotic with the roots of *Castanea sativa* in a stand with healthy trees at Puzzella (P) and in a stand with *Phytophthora* infected trees at Montesanti (M) achieved through morphological characterisation and subsequent BLAST matching of obtained ITS-sequences

Closest matching BLAST id.	Accession number [a]	Similarity (%)	Bit score	Base pairs	ECM consensus taxon	Site
Boletus porosporus Imler	UDB000475	99	1,251	647	*Boletus porosporus*	M
Boletus subtomentous (L.:Fr.) Quél.	UDB000501	98	827	515	*Boletus subtomentous*	P and M
Boletus appendiculatus (Schaeff.: Fr.) Fr.	UDB000652	93	813	611	*Boletaceae* sp. 3	M
Byssocorticium atrovirens (Fr.) Bondartsev and Singer	UDB000075	99	1,013	519	*Byssocorticium atrovirens*	M
Cenococcum geophilum Fr.	UDB002302	98	803	526	*Cenococcum geophilum*	P and M
Cortinarius sp.	EU057110.1	95	1,083	678	*Cortinarius* sp. 1	P
Cortinariaceae sp.	EF619674.1	96	981	612	*Cortinariaceae* sp. 2	P
Cortinarius sp.	EU057117.1	93	1,033	678	*Cortinariaceae* sp. 3	M
Helvellaceae sp.	AJ879682.1	82	560	646	*Helvellaceae* sp.	M
Hydnum rufescens (Pers.) Poir.	AJ547880.1	99	880	485	*Hydnum rufescens*	P
Lactarius chrysorheus Fr.	UDB000864	99	1,118	712	*Lactarius chrysorheus*	M
Lactarius serifluus (DC.: Fr.) Fr.	UDB000868	99	1,380	825	*Lactarius serifluus*	P
Oidiodendron citrinum G.L. Barron[b]	AF307762.1	100	736	412	*Oidiodendron citrinum*	M
Oidiodendron maius G.L. Barron[b]	AB089655	99	781	298	*Oidiodendron maius*	P and M
Paxillus involutus (Batsch) Fr.	UDB001205	100	1,063	812	*Paxillus involutus*	M
Pezizaceae sp.	AY299221.1	91	826	641	*Pezizaceae* sp. 1	P
Russula delica Fr.	AF418605	100	1,211	593	*Russula delica*	P and M
Russula emetica (Schaeff.) Pers	AY228360.1	98	1,227	707	*Russula emetica*	M
Russula lepida Fr.	DQ422013.1	98	926	535	*Russula lepida*	P and M
Russula nigricans (Bull.) Fr.	UDB000337	99	1,289	673	*Russula nigricans*	M
Russula risigallina (Batsch) Sacc.	UDB002505	99	1,259	775	*Russula risigallina*	P and M
Russula rosea Pers.	AY061715.1	99	723	394	*Russula rosea*	M
Russula vesca Fr.	UDB000340	98	377	206	*Russula vesca*	P
Russula azurea (Bres.)	AY061660.1	95	767	473	*Russulaceae* sp. 9	P and M
Sebacina sp.	AF465191.1	91	808	592	*Sebacinaceae* sp.	P and M
Sistotrema muscicola (Pers.) S. Lundell	UDB002254	90	500	497	*Sistotremataceae* sp.	M
Tomentella bryophila (Pers.) M.J. Larsen	UDB000035	99	1,267	661	*Tomentella bryophila*	M
Tomentella sp.	DQ150126.1	96	797	451	*Tomentella* sp. 2	P and M
Tomentella sp.	AJ893298.1	98	652	326	*Tomentella* sp. 3	P
Tomentella sp.	AM159590.1	100	1,164	657	*Tomentella* sp. 4	P and M
Tomentella sp.	AM161535.1	99	928	516	*Tomentella* sp. 5	M
Tomentella sp.	AJ534913.1	98	1,234	703	*Tomentella* sp. 6	M
Tuber borchii Kauffman	DQ402505.1	97	795	460	*Tuber borchii*	P
Tuber sp.	DQ990872.1	99	985	555	*Tuber* sp. 2	M
Uncultured ECM	DQ054563.1	99	1,485	645	Uncultured ECM 1	P
Uncultured ECM	EF434054.1	98	1,173	658	Uncultured ECM 2	P
					Unidentified sp. 1	P
					Unidentified sp. 2	P and M
					Unidentified sp. 3	M
					Unidentified sp. 4	M
					Unidentified sp. 5	P

[a] Accession numbers starting with an "U" represent sequences that were BLAST matched in the UNITE database, the other accession numbers represent sequences in the NCBI database

[b] Oidiodendron species are generally associated with ericoid mycorrhizal plants however the potential of these root endophytes to associate with ectomycorrhizal host plants has previously been demonstrated (see Bergero et al. 2000)

Table 2 Root characteristics, the ECM community and soil parameters from the two investigated *Castanea sativa* stands

Characteristics	Puzzella (healthy)	Montesanti (diseased)
Soil parameters (0–15 cm depth) pH	4.9 (0.2)	4.5 (0.2)
Organic matter contents (%)	1.6 (0.3)	2.9 (0.7)
Moisture contents (%)	19.1 (0.9)	21.6 (1.8)
Leaves infected with *Phytophthora* (%)	0.5 (0.3)	30.7 (1.6)
Castanea sativa fine roots		
Length (cm m^{-2})	60,150 (17,428)	136,432 (36,778)
Surface (cm^2 m^{-2})	14,972 (3,840)	32,552 (8,714)
Dry weight (g m^{-2})	111.6 (31.1)	198.8 (54.5)
ECM community		
Total ECM root tips sampled	7,518	14,663
ECM specific root tip density (tips cm^2)[a]	10.0 (1.4)	10.5 (1.6)
Phylotype richness	23	29
Mean rarified richness[b]		28.8 (0.4)
Mean species per core	5.3 (0.6)	7.0 (1.0)
Estimated richness[b]	47.0 (20.2)	38.0 (6.8)

Values shown are means and in parentheses are standard errors

[a] ECM specific root tip density expressed as tips per square centimetre fine root surface area

[b] Taking the number of ECM root tips sampled at Puzzella (7518) as the standardised sample size

[c] Estimations made with the programme ESTIMATES (Colwell 2006) illustrating Chao-2 estimator values, and analytical standard deviation values for both stands.

47 and 38 species, respectively (Table 2). Differences between the actual and estimated richness can be attributed to the accumulation of new species in each soil core with no levelling off as more samples were analysed. At both stands, the majority of species were rare, found in one or two soil cores only (Table 3).

Frequency and abundance patterns of identified ECM taxa

A total of 11 fungal species were shared between the two stand types. Three of them (*Cenococcum geophilum*, *Oidiodendron maius* and *Russula lepida*) were considered to be relatively frequent fungal species both at Puzzella and Montesanti, whereas the others were infrequent–rare (Table 2). *C. geophilum* was found in nine or ten out of ten cores (Table 2) at Puzzella and Montesanti, respectively, and had more than two tips per square centimetre root surface area (Table 2 and Fig. 3). *O. maius* was found in five or seven out of ten cores at Puzzella and Montesanti, respectively, but formed only 0.21 or 0.53 tips per centimetre root surface area, whereas *R. lepida* was only found in two and three cores but formed one to 1.6 tips per centimetre root surface area. At both stands, the fungi *C. geophilum* and *O. maius* were the only two species found in 50% or more of the sampled soil cores.

Using the values of relative ECM root tip abundance and relative core frequencies, importance values for the species were calculated. The ten most important species for each site are shown in Fig. 1. Both stands differed in their composition of dominant fungal species (Fig. 1a, b), although they shared the ascomycete *C. geophilum* as the outspoken system dominant. However, with 40.8%, the relative abundance of *C. geophilum* was much higher at Montesanti compared to 27.2% at Puzzella. As a result the remaining dominant fungal species in the diseased chestnut stand exhibited lower root tip numbers per core than species in the healthy chestnut stand at Puzzella (Fig. 1a, b). At the Puzzella stand, the second most abundant species was a *Cortinarius* species, which was not found at Montesanti. This is seen as a general pattern if all the species are compared. At Puzzella, the most important species are two *Tomentella* species (two and four), a *Sebacina* species and a *Cortinarius* species (one), which are not present at the Montesanti site (Fig. 4). Similarly, there is a switch in species from *Russula vesca*, *R. lepida* and *Russula azurea* at Puzzella to *Russula nigricans*, *R. lepida* and *Russula delica* at Montesanti. Both *R. vesca* and *R. azurea* were found at the Puzzella site only (Fig. 3).

Community importance of identified ECM families

The ranking of importance values based on fungal family and other inclusive groupings followed a similar pattern for both stand types (Fig. 2). Identified root tips collected in the healthy chestnut stand (Puzzella) were distributed in nine taxonomic categories (eight families+*C. geophilum*), three of which had low importance value rankings (lower than 0.1). Five fungal species found on the Puzzella roots remained unidentified (representing 3.8% of the total root tips). Root tips collected from the *Phytophthora*-infected roots sampled at Montesanti were distributed among 13 taxonomic categories (12 families+*C. geophilum*). At this site, only three fungal species remained unidentified; however, they represented no less than 18.5% of the total root tips and could potentially change the rankings at Montesanti. The fact that the largest contribution to this group of unidentified species at Montesanti was made by one fungal species (Unidentified sp. 3), which was morphologically identified to be a *Cortinarius* species, confirms this.

ECM root morphology

Measurements of the thickness of unramified ECM root tip initials showed that the average diameter at Puzzella was slightly higher measuring 0.328 mm compared to

Table 3 Fungal taxa detected in a *Castanea sativa* stand with healthy trees at Puzzella and in a *Castanea sativa* stand with *Phytophthora* infected trees at Montesanti

Puzzella			Montesanti		
Fungal species	Core frequency	ECM specific tip density (tips cm^{-2} fine root surface area)	Fungal species	Core frequency	ECM specific tip density (tips cm^{-2} fine root surface area)
Cenococcum geophilum	9	2.42	*Cenococcum geophilum*	10	2.83
Oidiodendron maius	5	0.21	*Oidiodendron maius*	7	0.53
Cortinariaceae sp. 2	4	0.26	*Russula delica*	4	0.23
Sebacinaceae sp.	4	0.71	*Russula nigricans*	4	0.42
Tomentella sp. 2	4	0.74	*Unidentified* sp. 3	4	2.10
Cortinarius sp. 1	3	0.59	*Boletus subtomentous*	3	0.05
Russulaceae sp. 9	3	1.26	*Cortinariaceae* sp. 3	3	0.06
Russula vesca	3	0.82	*Oidiodendron citrinum*	3	0.05
Russula lepida	2	1.06	*Russula lepida*	3	1.61
Tomentella sp. 4	2	0.31	Unidentified sp. 4	3	0.07
Unidentified sp. 1	2	0.24	*Helvellaceae* sp.	2	0.18
Boletus subtomentous	1	0.02	*Paxillus involutus*	2	0.29
Lactarius serifluus	1	0.14	*Sebacinaceae* sp.	2	0.49
Pezizaceae sp. 1	1	0.14	*Tomentella* sp. 2	2	0.05
Russula delica	1	0.17	*Tomentella* sp. 6	2	0.15
Russula risigallina	1	0.04	*Tuber* sp.	2	0.28
Sistotremataceae sp.	1	0.13	Unidentified sp. 2	2	0.13
Tomentella sp. 3	1	0.53	*Boletaceae* sp. 3	1	0.04
Tuber borchii	1	0.15	*Boletus porosporus*	1	0.15
Uncultured ECM 1	1	0.01	*Byssocorticium atrovirens*	1	0.06
Uncultured ECM 2	1	0.02	*Hydnum rufescens*	1	0.27
Unidentified sp. 2	1	0.04	*Lactarius chrysorheus*	1	0.12
Unidentified sp. 5	1	0.03	*Russulaceae* sp. 9	1	0.002
			Russula emetica	1	0.05
			Russula risigallina	1	0.08
			Russula rosea	1	0.12
			Tomentella bryophila	1	0.04
			Tomentella sp. 4	1	0.04
			Tomentella sp. 5	1	0.003

Shown is the frequency of occurrence in ten sample cores and the number of root tips per square centimetre fine root surface area

0.299 mm at Montesanti. When the ECM root tip measurements for all ECM fungal species were grouped into three diameter classes it was observed that proportionally less ECM root tips had small diameters (>0.1–<0.25 mm) at Puzzella compared to Montesanti (2.5% vs. 24.0%, respectively). At Montesanti, a small majority (54.9%) of the ECM root tips had thick (>0.3–<0.45 mm) tip diameters. However, the difference between the latter diameter class and the other two classes was less pronounced at Montesanti than at Puzzella, where 76.8% of the ECM root tips had tip diameters between 0.3 and 0.45 mm (Fig. 4). At Montesanti, the mean values of specific ECM root tip density did not differ significantly between the three hyphal diameter classes (ANOVA, P=0.06) with 2.8±1.4 tips per square centimetre for diameter class >0.1–<0.25 mm, 1.9±0.5 tips per square centimetre for diameter class >0.25–<0.30 mm and 5.7±1.4 tips per square centimetre for diameter class >0.30–<0.45 mm (data not shown). In a similar analysis with hyphal diameter data from Puzzella, a significant difference was observed for the mean values of specific ECM root tip between the smallest and the biggest diameter class (Kruskal–Wallis P< 0.05) with 0.2±0.2 tips per square centimetre for diameter class >0.1–<0.25 mm, 3.1±1.1 tips per square centimetre for diameter class >0.25–<0.30 mm and 6.7±1.3 tips per square centimetre for diameter class >0.30–<0.45 mm (data not shown).

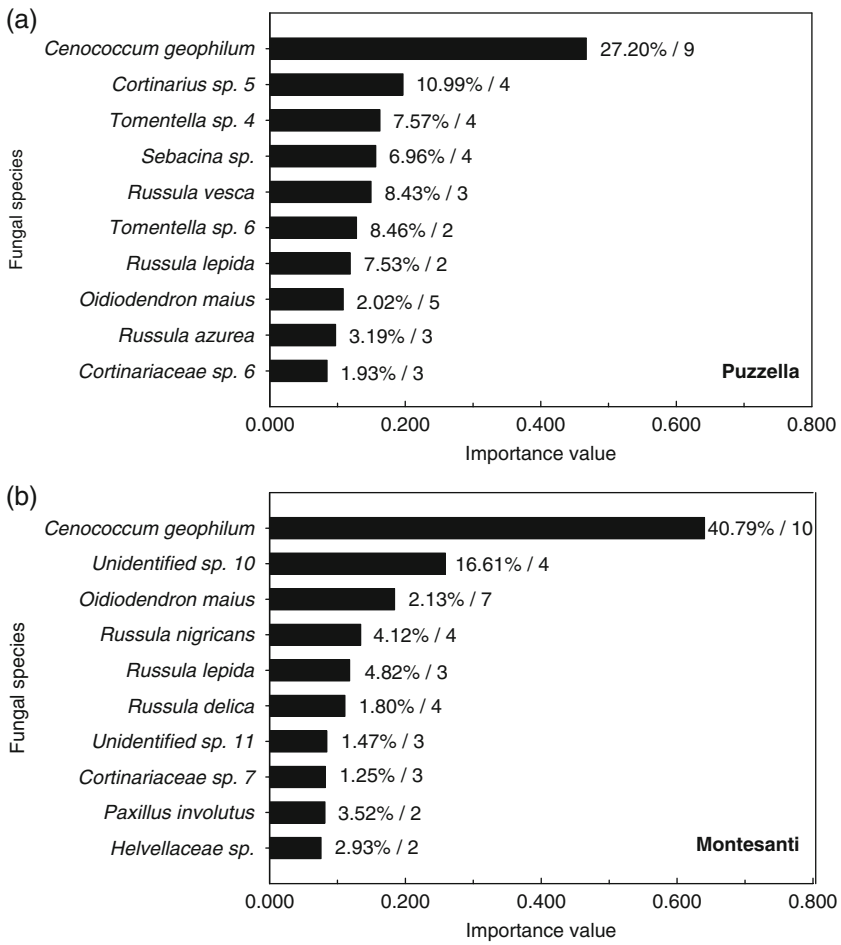

Fig. 1 **a**, **b** Ranked species distributions according to importance values based on the sum of relative ECM root tip abundance and relative core frequencies for the top 10 fungi observed on the roots in ten soil cores sampled at **a** Puzzella a healthy *Castanea sativa* stand and **b** Montesanti a *Castanea sativa* stand with *Phytophthora* infected trees. Numbers at the end of each bar correspond to the percent of total root tips and core frequency for each species

To try to gain further insight into the frequency of ECM species with differing hyphal morphologies, the ECM species were categorised into density classes i.e. number of emanating hyphae per root tip, of none, sparse and dense (Fig. 5). A pattern similar to that observed for the ECM tip diameter classes emerged. At both sites, the number of root tips with dense hyphae was greater compared to those without or with few emanating hyphae. However, the difference between the class representing root tips with many emanating hyphae and the other two classes was more pronounced at Puzzella. At this stand, 15.0% of the root tips had no emanating hypae, 10.9% had few emanating hypae and 74.1% had many emanating hypae (Fig. 5). The mean values of specific ECM root tip density differed significantly between the former two and the latter hyphal density class (Kruskal–Wallis $P<0.05$) with 2.1±0.9, 1.4±1.0 and 6.6±1.3 tips per square centimetre, respectively (data not shown). At Montesanti, 26.8% of the root tips were without emanating hypae, 19.7% had few emanating hypae and 53.5% had abundant emanating hypae (Fig. 5). The mean values of specific ECM root tip density did not differ significantly between the three former hyphal density classes (ANOVA, $P=0.11$) with 2.9±1.3, 2.9±1.5 and 4.8±0.7 per square centimetre, respectively (data not shown).

Discussion

Although no statistically significant differences in the ECM fungal community composition between Puzzella, a healthy *C. sativa* stand, and Montesanti, a *C. sativa* stand with *Phytophthora*-infected trees, were observed, the results clearly show a variation in the species richness and the relative importance of species between the stands. Numerous factors underlay the structure and diversity of mycorrhizal communities (Kernaghan 2005). Natural abiotic conditions, for example, are well known to influence ECM fungal community composition (Erland and Taylor 2002). To ensure the selection of comparable sites for the present study, important features like pH, organic matter and moisture content were therefore considered (see Table 1). Knowing that ECM diversity is also linked to the maturity of stands, it should be noted that a difference in the age of the trees at both stands existed (approximately 80 years and up to 200 years for Montesanti and Puzzella,

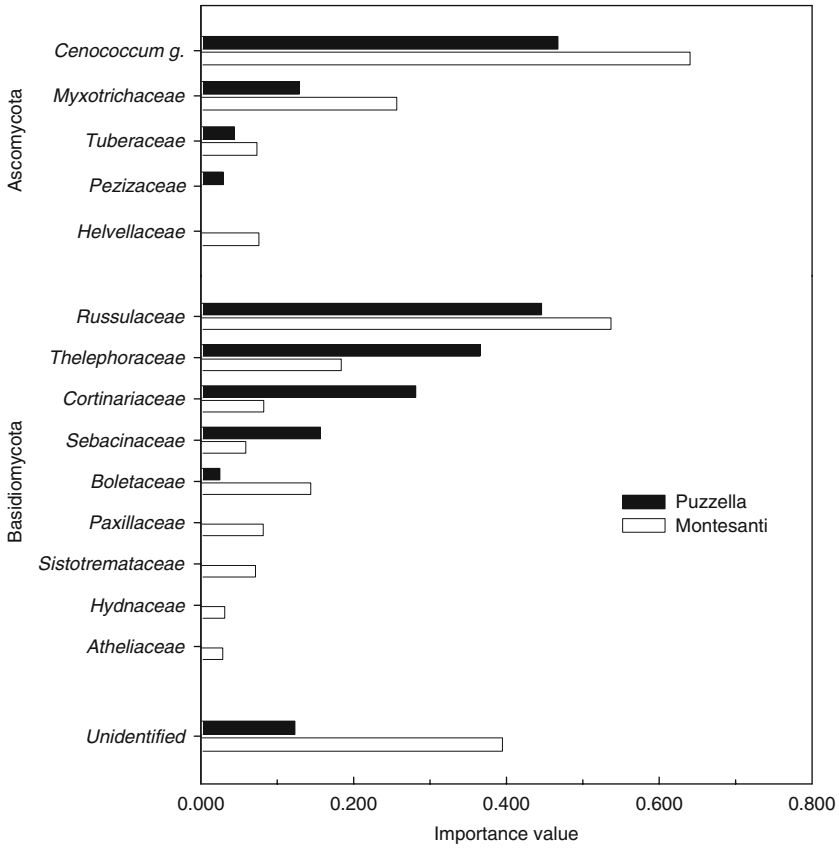

Fig. 2 Comparison of importance values according to familial grouping, fungus type and unidentified fungal species in a *Phytophthora*-infected sweet chestnut stand (Montesanti) and a healthy sweet chestnut stand (Puzzella)

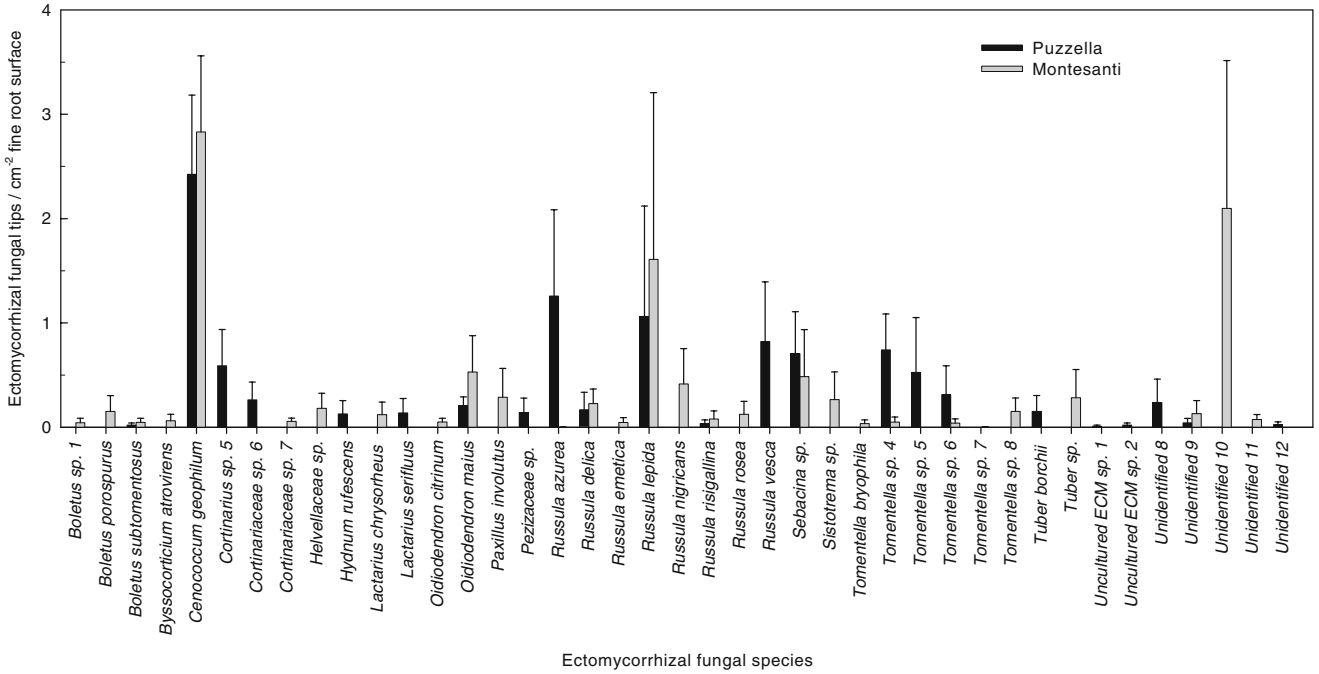

Fig. 3 Mean specific tip density for each ectomycorrhizal fungal species±SE from Puzzella a healthy *Castanea sativa* stand and Montesanti a *Castanea sativa* stand with *Phytophthora* infected trees

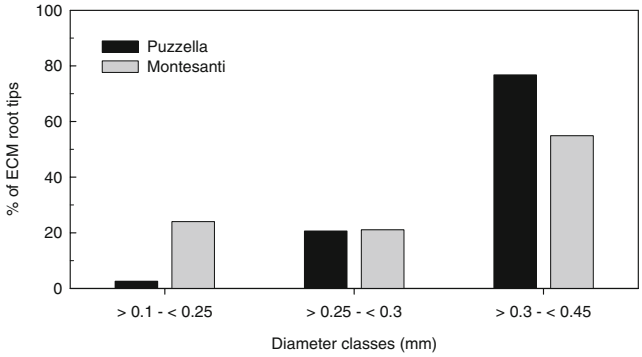

Fig. 4 The number of ECM root tips divided into three root diameter classes of root tip initials expressed as a percentage of the total number of ECM tips sampled at the Puzzella and Montesanti stands

respectively). Fungal species composition tends to change as stands mature (Last et al. 1987), and older forests have a propensity to support a greater number of fungal species than younger forests (Visser 1995; Rao et al. 1997; Smith et al. 2002). However, such observations have normally been a comparison of young and mature stands. In this work, both of the studied stands can be classified as mature stands. The observation that the higher number of measured and predicted ECM fungal species was determined at the younger Montesanti stand, 29 and 47, respectively, compared to 23 and 38 at the older Puzzella stand (Table 1) is striking. It would appear that the healthy trees at Puzzella are more selective than the diseased trees at Montesanti, which are more dependent on ECM response diversity (Elmqvist et al. 2003). However, there were almost twice as many ECM root tips at Montesanti than Puzzella, suggesting that the complexity of the ECM community structure may be related to the number of potential colonisation sites. The reason for the higher root mass and number of root tips at the diseased Montisanti site is not known but may reflect a greater belowground allocation in diseased root systems. Within the limitations of only comparing two sites, albeit with similar environmental characteristics, the differences between the ECM communities could be a response to disease at Montesanti. However, it must be stressed that the roots analysed from the Montesanti stand are the surviving fine roots on damaged trees. Given that the development of disease symptoms, referred to as gradual decline, sometimes lasts for up to 10 years, until the death of the tree (Crawford 1995), the original ECM fungal community at Montesanti could have undergone substantial compositional changes before this study as an indirect result of the environmental stress applied by *Phytophthora* on the trees since the pathogen first infected them.

Typical effects of ink disease on chestnut trees include dieback of branches, defoliation and the reduced photosynthetic performance of remaining leaves (Crawford 1995; Gomes-Laranjo et al. 2004). As the maintenance of symbionts requires a considerable proportion of photosynthates to be allocated belowground (Finlay and Söderström 1992; Smith and Read 1997), a loss of photosynthetic leaf area would more or less directly imply a reduced capacity to maintain root-associated symbionts. Several studies on the impact of artificial defoliation on the capacity of tree seedlings to maintain ECM symbionts showed that the loss of photosynthesising foliar biomass resulted in decreased carbon allocation to the ECM symbiosis (Saikkonen et al. 1999; Kuikka et al. 2003). Artificial defoliation was found to negatively affect ECM symbionts by reducing the production of fungal biomass in the fine roots (Markkola et al. 2004; Stark and Kytöviita 2005) and, in some cases, ECM colonisation of fine root tips (Gehring and Whitham 1991; Gehring et al. 1997; Rossow et al. 1997). The composition of the ECM was also shown to change when the belowground allocation of C was increased under elevated atmospheric CO_2 (Godbold and Berntson 1997; Godbold et al. 1997). Godbold and Berntson (1997) suggested that under conditions of increased atmospheric CO_2 concentration birch saplings could support a more costly mycorrhization. To explain the significant change in the composition of the ECM assemblage toward morphotypes with a higher incidence of emanating hyphae and rhizomorphs, Godbold and Berntson (1997) reasoned that a surplus in C can be invested by the host in associations with more extramatrical mycelium, a morphological feature that may improve nutrient and water acquisition.

When the ECM roots from both investigated sites in the present study are compared morphologically, it becomes apparent that the chestnut trees at the healthy Puzzella stand supported fewer ECM root tips that are very thin (Fig. 5). Fine roots in symbiosis with the ten most important species of the ECM assemblage at Puzzella (Fig. 2a) did not produce ECM root tips thinner than 0.25 mm, whereas association with *Cortinarius* sp. 1, Cortinariaceae sp. 2, *R. lepida*, *Sebacina* sp. or *Tomentella* sp. 4 resulted in ECM

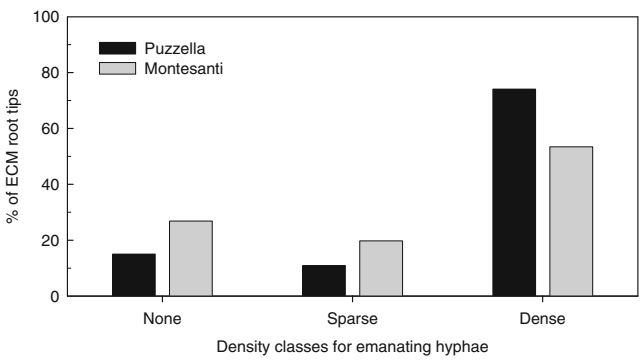

Fig. 5 The number of ECM root tips divided into three hyphal density classes expressed as a percentage of the total number of ECM tips sampled at the Puzzella and Montesanti stands

root tips thicker than 0.30 mm. Conversely, in the ink disease impacted chestnut stand at Montesanti, there appears to be a change in the ECM assemblage towards species with less thick hyphal mantles, since three species (*R. nigricans*, Unidentified sp. 3 and Cortinariaceae sp. 3) from the ten most important fungi in the ECM assemblage at Montesanti (see Fig. 1b) produced ECM root tips thinner than 0.25 mm. A similar shift in the ECM fungal assemblage of Scots pine towards fungal morphotypes possessing low fungal biomass was reported after artificial defoliation (Saikkonen et al. 1999; Saravesi et al. 2008).

From research by Marx and Davey (1969a, b) and Marx (1970), it is known that the fungus mantles of ECM present formidable physical barriers to penetration by *Phytophthora cinnamomi*. Thin ECM mantles will logically contain fewer layers of interwoven hyphae. This could provide more voids, which would diminish the preclusive effect of the ECM mantle to exposure of root tissue to direct contact with the rhizosphere. Thus, species like *R. nigricans*, Unidentified sp. 3 and Cortinariaceae sp. 3 may provide a weaker mechanical barrier. In view of the relative importance of these fungi on the roots of sweet chestnut at the Montesanti stand (Fig. 1b), they might considerably decrease the overall protective ability of the ECM assemblage. Since *R. nigricans*, Unidentified sp. 3 and Cortinariaceae sp. 3 were absent at the healthy Puzzella stand, it is difficult to establish if these fungal species naturally form thin ECM mantles on sweet chestnut roots or if this morphological trait could, e.g., be down to the reduced availability of photosynthates from their hosts. However, comparison of other morphological features for fungal species, which were found on the roots at both sites, seems to undermine the latter theory. For example, the length and the density of hyphae emanating from the ECM mantles of *R. delica* and *O. maius* did not differ between sites. However, this could be a result of a change in allocation of C between root tissue and fungal symbionts in response to disease, with a consequence of high fine root biomass but changes in ECM community structure associated with a reduced availability of photosynthates.

Nevertheless, there were overall more ECM root tips at Puzzella that had dense extramatrical hyphae (Fig. 5). Only the bottom two species of the top ten important fungal symbionts at Puzzella (*Russula azurea* and Cortinariaceae sp. 2) were without emanating hyphae, whereas six of the top eight most important species (*C. geophilum*, *Cortinarius* sp. 1, *O. maius*, *R. vesca*, *Sebacina* sp. and *Tomentella* sp. 4) had dense extramatrical mycelium. At Montesanti, the second most important species (Unidentified sp. 3) had no emanating hyphae, whereas five of the top eight most important species had dense extramatrical mycelium, namely *C. geophilum*, Cortinariaceae sp. 3, *O. maius*, *R. delica* and *R. nigricans*.

Contrary to observed shifts in species composition and related morphological differences between the ECM fungal assemblages at Puzzella and Montesanti, the differences in fine root morphology (length, surface area and the number of root tips) do not seem to fit the general pattern of *Phytophthora* infection and its impact on root vitality (e.g. Wang et al. 2003; Jung et al. 2003; Fleischmann et al. 2004). Although the root samples from the Montesanti stand contained a substantially larger amount of dead fine root (not quantified), the length and surface area of live fine root and the relative number of ECM root tips per square centimetre fine root surface area were also all higher at the *Phytophthora* diseased site (Table 1). Whereas the superior length and surface area of the fine root sampled at Montesanti can possibly be explained by the greater tree stocking density at this stand (150 trees per hectare compared to 100 trees per hectare at Puzzella), this does not apply for the ECM root tip density. Despite the fact that ECM fungal community at Montesanti has clearly not been able to prevent the widespread development of chestnut ink disease, the unaffected ECM root tip density among other findings of this study indicate that the infected trees that were still alive continued investing in a viable ECM fungal community.

As the total diversity estimates imply, nearly 32% and 27% of species may have remained undetected at Puzzella and Montesanti, respectively. This indicates that the number of soil cores sampled and analysed for this study, ten from each site, was too limited. Nonetheless, it is likely that many of the system dominants were identified, and these species bare most of the functional relevance (Dahlberg 2001), with its potential antagonistic significance. Although many ECM fungal taxa fulfil broadly similar ecological functions, it is well established that considerable differences in the physiological function of different ECM fungal species exists (Godbold 2005). It is likely that many ECM fungi will possess defence mechanisms against soil pathogens, but some species will offer better overall protection than others. For example, all the described ECM fungi in the present study developed a protective barrier around the fine roots of their symbiotic host, but not all these hyphal mantles were so robust (Fig. 5).

Extensive screening of fungi has also illustrated that their antibiotic activity varies and is nonexistent in some species (Wilkins and Harris 1944). Unfortunately, only a few of the species that were identified to be associated with sweet chestnut in the present study have been tested for antibiotic mechanisms. Those that have been screened (e.g. *Boletus subtomentous*, *C. geophilum*, *Lactarius chrysorheus*, *P. involutus* and *Russula emetica*) have not necessarily been examined for antagonism against soil-borne *Phythophthora* (Wilkins and Harris 1944). Even if *Phytophthora* was the studied pathogen (e.g. Marx and Davey 1969b), the

investigated host species has rarely been *C. sativa* (Branzanti et al. 1999). Seeing that the protective effects of ECM fungi against root pathogens is dependent upon a combination of the fungal species or isolate, the host species and the pathogen (Chakravarty et al. 1991), the outcome of such screening studies is not easily inferred on the findings in the present study.

The in-depth view that this study offers of the composition of the ECM fungal communities associated with *Phytophthora*-infected and healthy *C. sativa* trees therefore provides a unique foundation for further research into the mycorrhizal ecology of the identified species (e.g. antibiotic production) and their mycorrhizal association (e.g. mantle structure), which is needed to elucidate the true mechanisms behind their antagonistic potential.

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