Mycorrhiza (2005) 15: 235–245
DOI 10.1007/s00572-004-0313-6

ORIGINAL PAPER

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Year-round monitoring of diversity and potential metabolic activity of the ectomycorrhizal community in a beech (*Fagus silvatica*) forest subjected to two thinning regimes

Received: 10 September 2003 / Accepted: 3 May 2004 / Published online: 25 June 2004

Abstract This work was aimed at understanding how the functional diversity of ectomycorrhizas (ECM) is driven by environmental factors and how it adapts to the structure of the forest stand. Superficial fine roots were sampled 21 times during an entire year in two adjacent plots (no thinning and strong thinning) of a mature beech (*Fagus silvatica*) forest. Individual ectomycorrhizal root tips were morphologically characterised and the symbiotic fungi were molecularly identified. ECM were also tested for dehydrogenase and acid phosphatase activities, and soil moisture and temperature were recorded. The results provide a description of ECM community dynamics over a whole year in the two stands. The main conclusions are threefold: (1) the species structure of the ECM community and metabolic activity of each morphotype change depending on the season, temperature and soil moisture, and a number of morphotypes are more abundant and active in winter than in summer, (2) the silviculture treatment (strong thinning) modifies the ectomycorrhizal community structure, and (3) the overall function of the ECM community results from the individual time pattern and specialisation of each morphotype.

Keywords Ectomycorrhiza · Diversity · Activity · Thinning · *Fagus silvatica*

Introduction

The uptake of water and solutes by woody species in forests of the mediterranean, temperate and boreal zones largely depends on the mycorrhizal status of the roots. The fine roots of forest trees occupy the top 10 cm of the soil, where nutrient cycling is the most intense, and are dominated by ectomycorrhizal symbiosis (Bruns 1995; Prévost and Pargney 1995; Bakker et al. 2000). The symbiotic fungi develop an extensive hyphal network, which provides the trees with water and nutrients (Garbaye and Guehl 1997; Smith and Read 1997).

In these forest ecosystems, the richness and diversity of the communities of ectomycorrhizas (ECM) strongly contrasts with the low number of woody species. Scores of fungal species are commonly associated with a single tree, and the heterogeneity between trees results in several hundreds of species at the stand scale (Dahlberg 2001; Horton and Bruns 2001; Jonsson et al. 2001). This diversity also exists in terms of morphology: the emanating mycelium can be hydrophilic or hydrophobic, more or less branched, loose or aggregated into strands, etc. (Voiry 1981; Agerer 1987–1998).

The factors that maintain such a diversity, and which drive the dynamics of the ECM communities, remain incompletely known. Abiotic factors such as fire, soil pH or nitrogen status have been identified as influencing their structure (Dighton and Skeffington 1987; Agerer et al. 1998; Goodman and Trofymow 1998; Peter et al. 2001; Erland and Taylor 2002; Jany et al. 2002). Some studies have also revealed that species of ECM fungi differ in their ability to exploit soil nutrients; this diversity might explain their distribution among different ecological niches (Bruns 1995; Dickie et al. 2002; Erland and Taylor 2002). Studies on the functioning of the symbiotic association under controlled conditions suggest specialisation and complementarity of the different ECM types for functions beneficial to the trees: water uptake, mobilisation and assimilation of nutrients, growth regulators, and protection against pathogenic organisms (Smith and Read 1997; Baxter and Dighton 2001; Dahlberg 2001). Climatic factors have also been shown to influence ECM diversity. Soil moisture and temperature have long been known to affect the structure of ECM communities (Worley and Hacskaylo 1959; Fogel 1980; Erland and Finlay 1992; Shi et al. 2002; Jany et al. 2003). Anthropic factors also affect microbial diversity in the rhizosphere, and therefore ECM communities. Some environmental disturbances caused by human activities (atmospheric CO_2 increase, local ozone pollution, heavy

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metals in the soil, etc.) have direct or indirect effects on biodiversity (Baxter et al. 1999; Erland and Taylor 2002). Similarly, forestry practice (clear-cutting, fire, nitrogen fertilisation, liming, etc.) impacts on the ECM diversity of forest ecosystems (Blaise and Garbaye 1983; Goodman and Trofymow 1998; Kranabetter and Wylie 1998; Baar et al. 1999; Bakker et al. 2000; Byrd et al. 2000; Le Tacon et al. 2001; Peter et al. 2001; Lilleskov et al. 2002).

Here, we studied the links between the diversity of an ECM community and the viability and functionality of beech fine roots twice a month throughout all seasons during an entire year. This approach also aimed at determining to what extent the thinning regime can affect this aspect of microbial diversity and therefore the resilience of the forest stand facing environmental stresses.

Materials and methods

Site characteristics

Investigations were performed in the Souilly experimental forest in north-eastern France (Oswald 1986; Le Goff and Ottorini 1993), which is pure, 80-year-old beech (*Fagus silvatica* Lin.). The loamy soil (30–50 cm deep) with a mull-type humus (pH 5.5–6.5) has been formed on clay derived from compact Jurassic limestone (Oswald 1986). It has no holorganic layer: the A1 horizon, containing a dense mat of superficial fine roots, is immediately under the fresh leaf litter. The whole experimental area was fenced against game damage. Sampling was performed in two 0.4 ha adjacent experimental plots, which had been subjected to highly contrasting silviculture treatments during the 38 years preceding our observations, with a 6-year period: control with no thinning (dead trees only have been removed) vs strong thinning. This had resulted in 705 trees per hectare versus 170, respectively, as shown by the last inventory in 2002. The forest floor is covered with ivy (*Hedera helix*) in the control, while the vegetation is more diverse in the thinned stand: bramble (*Rubus fruticosus*) dominant, with *Asperula odorata* and *Paris quadrifolia*.

Root sampling pattern and sample treatment

Four plots (10 m ×10 m) were selected in each stand (control or thinning), in which root sampling points (more than 1 m from trees or stumps) were pre-identified and tagged. Each plot was equipped with three micropsychrometer probes PST-55–15-SF (Wescor, Logan, Utah) set 5–10 cm deep in the A1 horizon. On 21 occasions throughout a whole year (every 2nd or 3rd week from October 2001 to October 2002, see Fig. 1), temperature and water potential were measured, and one sample of the A1 horizon (20×20 cm and 10 cm deep) was collected in each one of the eight plots. The eight samples were placed in individual isotherm boxes in order to keep them at sampling temperature, immediately transported to a nearby laboratory, and soaked in water. The roots were gently washed and live root tips were examined under a stereomicroscope at 40× magnification. Morphological typing of ECM tips was realised according to Agerer (1987–1998, 1995) and Voiry (1981). Types of ECM branching, colour and texture of mycorrhizal mantle, types of cystidia, colour and abundance of external hyphae, rhizomorphs or strands were described. Two or three tips of the two to five most abundant morphotypes in each sample for each date were frozen in liquid nitrogen and kept at −20°C, awaiting later molecular identification. The root-free soil was air-dried and sieved (2 mm) for the determination of pH (H_2O), total C, total N and available P (according to the method of Olsen and Sommers, 1982) at the INRA soil analysis laboratory (Laboratoire d'analyses des sols, Arras, France).

For each treatment, 84 samples were described over 12 months (21 sampling dates and 4 samples per date per treatment). The ECM morphotype composition was expressed in terms of relative frequency and relative abundance. Relative frequency was defined as the absolute frequency of individual species divided by the sum of absolute frequencies of all species (Gardes and Bruns 1996). The absolute frequency was the number of samples in which a species occurred divided by the total number of samples (Gardes and Bruns 1996; Horton and Bruns 2001). The relative abundance was defined in the same way as for

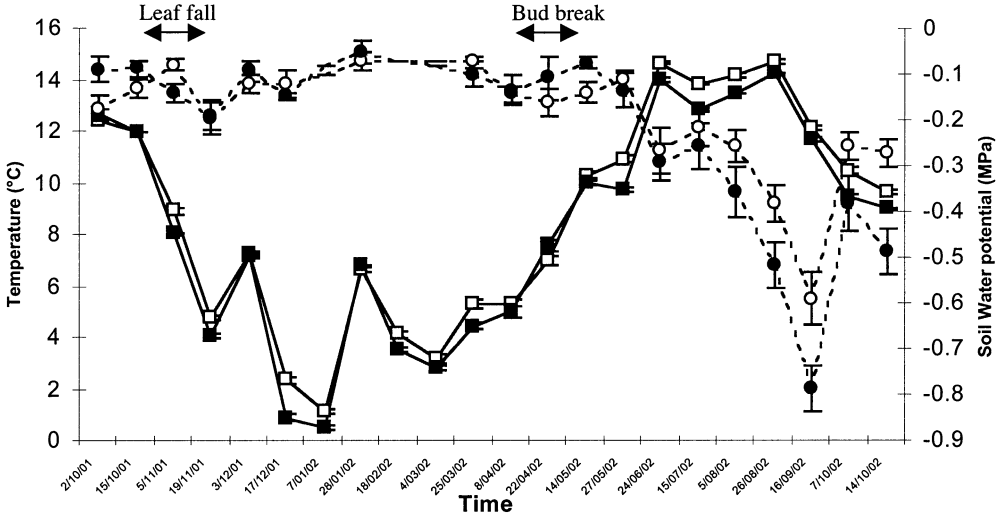

Fig. 1 Soil water potential (*circles*) and soil temperature (*squares*) in the two beech stands during the whole study: *open symbols* thinning (170 trees/ha), *filled symbols* control (705 trees/ha). Each stand was equipped with 12 micropsychrometer probes. *Bars* Standard errors

the relative frequency, but only the two to five dominant morphotypes in each sample (depending on the sample diversity) were considered. Because of the high spatial heterogeneity of ECM distribution, and because destructive soil sampling was randomly performed within the eight sampling plots at each date, the dominant morphotypes may differ among samples. These dominant morphotypes were subjected to the test of mycorrhiza potential metabolic activity (MTS test).

MTS test

The most abundant morphotypes (between two and five types in each sample for each date) were submitted to the dehydrogenase activity test; 24 single unbranched mycorrhizal tips were placed in 24 wells of a 96-well microtitration plate containing 100 µl pH 7.4 phosphate buffer per well (Sigma, St Quentin Fallavier, France). CellTiter 96 AQueous One solution (20 µl/well; Cell Proliferation Assay, Promega, Madison, Wis.) was added to each well and the plate was incubated at 25°C for 4 h. A number of preliminary experiments were run in order to optimise incubation parameters such as temperature, time or concentrations (data not shown). The CellTiter 96 AQueous One reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron-coupling reagent (phenazine ethosulfate; PES). In this test, the MTS tetrazolium compound is bioreduced in formazan by NADPH and NADH produced by dehydrogenase enzymes in metabolically active cells (Dunigan et al. 1995; Zolnai et al. 1998). The quantity of formazan produced, as measured by the absorbance at 490 nm (A_{490}), is linearly proportional to the metabolic activity of the tissues tested. The optical density at 490 nm was measured with a Microplate Reader, Model 550 (Bio-Rad, Hercules, Calif.). Preliminary experiments with ectomycorrhizas submitted to induced desiccation or temperature stress were performed and used to validate the test (data not shown). Control wells containing the reagents but no mycorrhizas were included, and results were expressed as corrected A_{490}/unit area. The projected surface area of the mycorrhizal tips was measured by scanning the 96-well plate followed by image analysis using the Mac/Win Rhizo software (Regent Instruments, Quebec City, Canada). It had been found previously (data not shown) that the projected area of the mycorrhizal root tips is linearly and significantly correlated with their dry weight.

Determination of acid phosphatase activity

The most abundant morphotypes recorded on 26 August, 16 September and 14 October 2002, were submitted to a phosphatase activity test: 24 single unbranched mycorrhizal tips per morphotype were placed in 24 wells of a 96-well microtitration plate containing 100 ml acetate buffer (pH 5) and 50 ml para-nitro-phenyl phosphate (pNPP) solution (30 mM), which is a substrate for acid phosphatases. After incubating at 25°C for 30 min, 50ml 1 M NaOH were added in each well in order to stop the development of the yellow colour due to the paranitrophenol (pNP) resulting from the pNPP lysis, and the A_{415} was measured (adapted from Eleanor and Lewis 1973; Tibett et al. 1998).

DNA analysis

Total DNA was extracted from one to five ectomycorrhizal root tips (frozen and kept in liquid nitrogen) of each morphotype using the DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. The internal transcribed spacer (ITS) region of the fungal nuclear rDNA was specifically amplified using primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Invitrogen, Cergy Pontoise, France) (Gardes and Bruns 1993). A typical PCR amplification reaction consisted of the following components (for 20 µl PCR mix): 2 µl template DNA, 2 µl 1× incubation buffer (Q.BIOgene, containing 1.5 mM $MgCl_2$), 0.4 µl dNTPs mix (10 mM), 0.4 µl primers ITS1F and ITS4 (10 µM), 0.1 µl *Taq* DNA polymerase (5 U/µl, Q.BIOgene) and 14.7 µl sterile distilled water. The PCR amplifications were performed using a Perkin Elmer GeneAmp 9600 thermocycler (Perkin Elmer Instruments, Norwalk, Conn.) under the following parameters: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. After electrophoresis on 1% regular agarose gels in 1% TBE (Tris buffer-EDTA), PCR products were stained with ethidium bromide and visualised under UV light to check the success of amplification. After purification on a 96-well filtration system (MultiScreen-PCR plates, Millipore, Mass.), the amplified fragments were sequenced using the BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City, Calif.) and an ABI genotyper 310 automated sequencer (Applied Biosystems). Sequences were aligned using ITS sequences available in NCBI/GenBank (http://www.ncbi.nlm.nih.gov/) and in our own laboratory databases (http://mycor.nancy.inra.fr/fr/index.html). Moreover, to complete the latter databases, the fruit bodies sampled in the experimental site were identified and the ITS regions of the nuclear rDNA were specifically amplified and sequenced.

Statistical analysis

The analysis of date and treatment effects on the number of morphotypes in root samples was performed by Poisson regression using GENMOD (SAS Institute 1998). The Log function was used as a link in the model. The model validity was checked with the deviance/degree of freedom ratio and with the graph of standardised deviance residuals versus the linear predictor.

Statview software (Alsyd, France) was used for variance analysis of soil temperature, soil water potential and potential metabolic activity values. Potential metabolic activity

data were Log-transformed to normalise the distribution of the residuals and to stabilise the variance. The normality of the data distribution was checked by plotting residuals versus predicted values. Statview was also used for regression analysis.

Results

Soil parameters

Soil analyses did not reveal any difference concerning pH, phosphorus content and C/N ratio between the two thinning treatments. Nevertheless, nitrogen, carbon and organic matter contents were significantly higher (ANOVA) in the control plots than in the thinned plots (Table 1).

Figure 1 shows temperature and water potential variations in the A1 soil horizon. Soil temperatures were often significantly higher in the thinned stand than in the control one, mostly when temperatures were extreme, as in winter or summer. Throughout winter (from leaf fall to bud break), soil water potential remained close to 0 MPa (Fig. 1). In both treatments, soil water potential decreased during summer. The thinning effect (vs control) on soil water potential was very significant (ANOVA) during this period of relative drought, when the soil water potential in the control remained lower than −0.3 MPa and reached −0.8 MPa in the control (from August 15 to October 15). Nevertheless, this dry period was not very severe in contrast to usual summer droughts in north-eastern France (−3 MPa recorded by Breda et al. 1995).

Effect of thinning on ECM community structure and diversity

Sixty-one ECM morphotypes were found and characterised in the beech stands over the 1-year-long survey (Table 2, Fig. 2). The richness of the samples (total number of morphotypes) varied between 6 and 18 depending on the sampling date and the thinning treatment. Sample richness was significantly and positively correlated with the soil water potential in the control stand, but not in the thinned treatment (Fig. 3). Moreover, the global statistical analysis (Poisson regression) performed on the 34 most frequent morphotypes (Fig. 2) revealed a significantly (P=0.048) higher richness in the thinned treatment (about one more species, on average).

The 34 most frequent morphotypes are described in Table 2 (resulting from a combination of morphotyping and ITS rDNA sequencing). Of these, 21 have been successfully identified at the genus or species level after sequencing the PCR-amplified ITS rDNA and matching the sequences either with those of our own sporocarp collection or with those readily available in the NCBI GenBank DNA database. Figure 2 presents the relative frequency and relative abundance of the different types (species within ECM fungal community). The relative frequencies of two species, *Clavulina cristata* and *Cenococcum geophilum*, were highest in both stands. Moreover, the diagrams reveal three groups of species: *C. cristata*, *C. geophilum*, *Hebeloma* sp.1, *Russula* sp.1, *Lactarius subdulcis* and *Tomentella* sp.1 were the most frequent types in the thinned stand, while the seven following ones were more frequent and abundant in the control. The third group was composed of the less frequent morphotypes (more than 40 including "other species", data not shown).

Table 1 Soil properties (A1 horizon, 0–10 cm) in the two forest treatments. Mean values of 16 samples in each treatment. The phosphorus content was determined according to Olsen and Sommers (1982)

	Control (±SE)	Thinning (±SE)
Total carbon (g/kg)[a]	38.59 (±3.89)	21.59 (±1.12)
Total nitrogen (g/kg)[a]	2.82 (±0.23)	1.67 (±0.07)
Carbon/Nitrogen (C/N)	13.58 (±0.63)	12.91 (±0.28)
Phosphorus	0.0083 (±0.0007)	0.0067 (±0.0006)
pH (H_2O)[a]	5.85 (±0.17)	5.25 (±0.06)

[a]Significant difference between the two thinning treatments (P<0.01)

Temporal dynamics of ECM community structure

Figure 4 shows that the relative abundance of the dominant ECM on beech roots changed markedly from winter to summer. *C. geophilum* was poorly represented in winter (7%) in the thinned stand but its relative abundance increased to 19% in summer. In contrast, in the same treatment, *C. cristata* represented 22% of the most abundant species during winter vs only 7% in summer. The morphotypes *Russula* sp.1 and *Laccaria amethystina* were present only during winter in both stands, and a Thelephoraceae, *Tomentella* sp.5 (*Fagirhiza tubulosa*; Agerer 1987–1998), was present (7% of most abundant species) only in the thinned stand during summer (Fig. 4). Statistical analysis (Poisson regression) showed a very significant (P=0.001) date effect on fungal species richness.

Temporal dynamics of the metabolic activities of different ECM morphotypes

The most abundant morphotypes (i.e. from two to five types in each sample for each date) were submitted to the MTS test. Two groups can be defined: on the one hand, *C. cristata*, *L. amethystina* and *Russula* sp. 1 were more abundant and metabolically active in winter than in summer, i.e. during the period when soil temperature was lower than 5°C (Figs. 4, 5); on the other, *C. geophilum*, *Hebeloma* sp., *L. subdulcis* and two *Cortinarius* species were more abundant and potentially active in summer than in winter, i.e. during the period when soil temperature was above 12°C (Figs. 4, 5). The level of potential metabolic activity was not systematically correlated with the abundance of

Table 2 Ectomycorrhizal (ECM) fungi colonizing *Fagus silvatica* roots from the control and thinned stands. Identification was based on morphotyping (Agerer 1987–1998) and sequencing of the internal transcribed spacer (ITS) rDNA followed by BLASTN search in the NCBI GenBank DNA database. The accession numbers are those of our samples (sporocarps or root tips) submitted to GenBank

Number	Taxon	GenBank number	ECM tip morphology
1	*Clavulina cristata*	AY292292	Monopodial mycorrhiza, white, smooth and velvet mantle surface
2	*Cenococcum geophilum*	AY299214	Shiny black mantle
3	*Hebeloma* sp. 1	AY299215	Thin and dirty white mantle with cottony mycelium enveloping particles of soil
4	*Russula* sp. 1	Not sequenced	*R. illota* (Agerer 1987–1998), monopodial extomycorrhiza, white-grey mantle with enclosed air and mineral soil
5	*Lactarius subdulcis*	AY299216	Irregular pyramidal, smooth and orange mantle surface
6	*Tomentella* sp. 1	AY299217	*Fagirhiza spinulosa* (Agerer 1987–1998)
7	*Tomentella* sp. 2	AY299218	Monopodial, bronze and smooth mantle surface
8	Unknown-1 (Agaricale)	AY299219	Monopodial mycorrhiza, grey-white and smooth mantle surface
9	*Cortinarius* sp. 1	AY299220	Pink-white mantle (air enclosed), pink rhizomorphs
10	Unknown-2 (Pezizaceae)	AY299221	Dichotomous ramifications with a white mantle
11	*Tomentella* sp. 3	AY299222	Monopodial ramifications emanating hyphae, brown and smooth mantle
12	*Tomentella* sp. 4	AY299223	Black-brown mantle surface
13	*Inocybe* sp.	AY299224	Ramified tip, light beige mantle, emanating thin cottony hyphae (including air)
14	*Laccaria amethystina*	AY299225	Velvety surface and purple tip
15	*Lactarius* sp. 1	Not sequenced	*L. acris* (Agerer 1987–1998), yellow-brown rough mantle surface, densely branched, covered with soil particles
16	*Lactarius blennius*	AY299226	Smooth mantle, honey patches, veined surface
17	*Cortinarius* sp. 2	AY299227	White mantle (air enclosed), red-brown rhizomorphs
18	*Tuber* sp. 1	AY299228	Brown mantle surface with cystidia on the ectomycorrhizal tips
19	*Tomentella* sp. 5	AY299229	*Fagirhiza tubulosa* (Agerer 1987–1998)
20	*Tomentella* sp. 6	AY299230	*Fagirhiza setifera* (Agerer 1987–1998)
21	Scleroderma-type	Not sequenced	Monopodial-pyramidal with some rhizomorphs and white mantle including air (Agerer 1987–1998)
22	*Tomentella* sp. 7	Not sequenced	Monopodial-pyramidal ramifications, black-brown mantle, very grainy
23	Unknown-3 (Pezizaceae)	AY299231	Brown and smooth mantle, golden hyphae
24	*Tomentella* sp. 8	Not sequenced	White-brown, smooth mantle surface
25	Unknown-4	Not sequenced	Pyramidal, cream velvet mantle
26	*Russula* sp. 2	Not sequenced	*Russula mairei* (Agerer 1987–1998), monopodial, white-orange mantle and short spiny surface
27	*Lactarius* sp. 2	AY299232	Pyramidal, white-yellow and smooth mantle
28	*Tomentella* sp. 9	AY299233	Black mantle with long dark brown spines
29	*Tomentella* sp. 10	Not sequenced	Golden brown and smooth mantle
30	*Byssocorticium atrovirens*	Not sequenced	Monopodial ectomycorrhiza with steel-blue and cottony envelope of emanating hyphae (Agerer 1987–1998)
31	*Tomentella* sp. 11	Not sequenced	Smooth and verdigris mantle
32	Inocybe-type	Not sequenced	Yellow mantle, ramified tip emanating cottony hyphae enveloping air and particles of soil (Agerer 1987–1998)
33	*Cortinarius* sp.3	Not sequenced	White mantle (air enclosed), purple rhizomorphs
34	*Russula* sp. 3	Not sequenced	*Russula ochroleuca*: light mantle with yellow patches (Agerer 1987–1998)
35	Others morphotypes		Unknown or only identified on morphological characteristics (27 morphotypes apparently different)

the morphotypes (data not shown). For some ECM species, such as *Clavulina cristata*, potential activities were significantly higher in the control stand than in the thinned stand (Fig. 5). The mean potential activity of the most abundant ECM morphotypes pooled together (they may differ from date to date) was significantly much higher and more variable in the control stand than in the thinned stand throughout the whole duration of the study (Fig. 6).

In addition to the MTS test, at the three last sampling dates (from 26 August onwards) the metabolic activity of

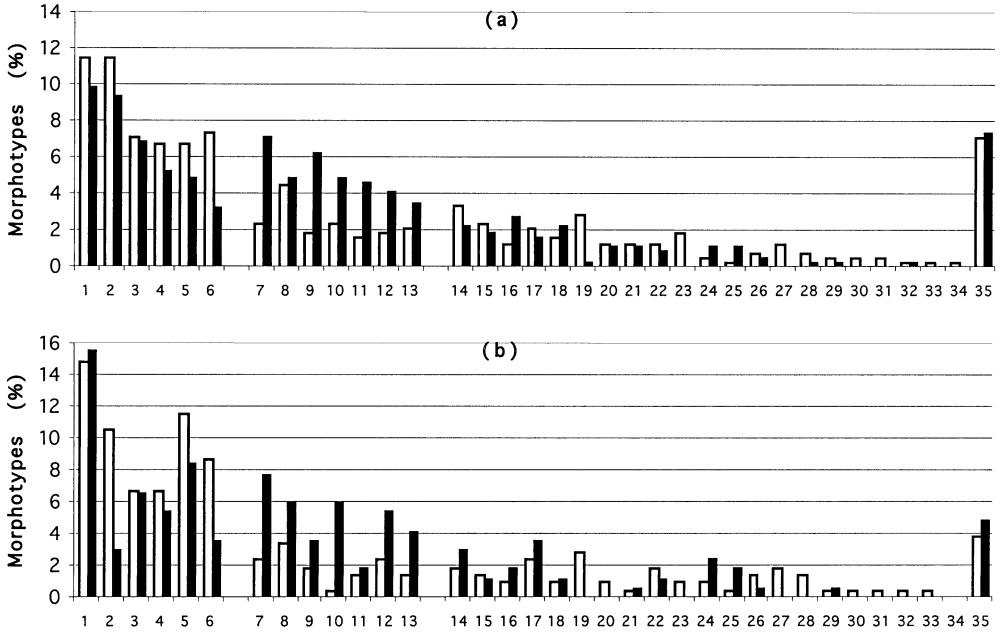

Fig. 2 Structure of the ectomycorrhizal (ECM) community expressed as species relative frequency (**a**) or relative abundance (**b**) in the two beech stands: *white* thinning, *black* control. *1 Clavulina cristata*, *2 Cenococcum geophilum*, *3 Hebeloma* sp.1, *4 Russula* sp.1, *5 Lactarius subdulcis*, *6 Tomentella* sp.1, *7 Tomentella* sp.2, *8* unknown-1, *9 Cortinarius* sp.1, *10* unknown-2, *11 Tomentella* sp.3, *12 Tomentella* sp.4, *13 Inocybe* sp., *14 Laccaria amethystina*, *15 Lactarius* sp.1, *16 Lactarius blennius*, *17 Cortinarius* sp.2, *18 Tuber* sp.1, *19 Tomentella* sp.5, *20 Tomentella* sp.6, *21 Scleroderma*-type, *22 Tomentella* sp.7, *23* unknown-3, *24 Tomentella* sp.8, *25* unknown-4, *26 Russula* sp.2, *27 Lactarius* sp.2, *28 Tomentella* sp.9, *29 Tomentella* sp.10, *30 Byssocorticium atrovirens*, *31 Tomentella* sp.11, *32 Inocybe*-type, *33 Cortinarius* sp.3, *34 Russula* sp.3, *35* other morphotypes

ECM tips was assessed using another biochemical marker: acid phosphatase activity (pNPP test). Figure 7 shows the distribution of these two activities for two morphotypes (*C. geophilum* and *L. subdulcis*) on 16 September. The distribution of the values is very variable depending on the date, morphotypes, and test. At this sampling date, the distribution of non-transformed values was close to normal only for phosphatase activity of *L. subdulcis*, but asymmetric (in favour of low values) in the other cases. However, the distribution of the log-transformed values was close to normal whatever the test or the fungus. The population of *L. subdulcis* ECM had a very high phosphatase activity (94±3.3) compared with the population of *C. geophilum* ECM (10.45±0.81). The mean metabolic activity (DO/cm^2 in the MTS test) was 8.53±0.86 for the same population of *L. subdulcis* ECM and 7.67±1.1 for the population of *C. geophilum* ECM (not significantly different).

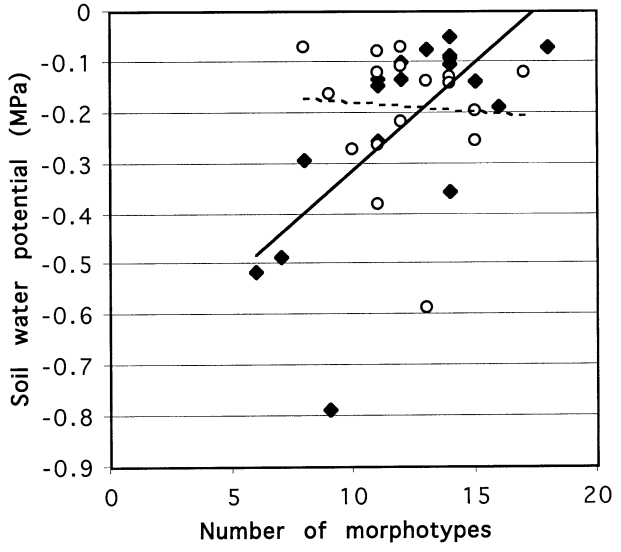

Fig. 3 Correlation between ECM species richness (number of morphotypes) and soil water potential for all sampling dates in the control beech stand (*black lozenges*; *solid line*; R^2=0.457; P<0.01) and in the thinned beech stand (*white circles*; *dotted line*; R^2=0.004; non significant)

Discussion

In 0,8 ha of the beech stand on rich soil studied here, more than 60 ECM morphotypes were found by sampling the top soil horizon twice a month during a whole year. This ECM richness is similar to those reported in other forest ecosystems (Horton and Bruns 1998; Jonsson et al. 2001; Peter et al. 2001; Lilleskov et al. 2002), but the evenness of the ECM community was higher than in most similar studies. This might be due to the fact that we collected observations for a longer period of time, including winter. However, most morphotypes were very infrequent and occurred a few times only. Interestingly, the thinning treatment resulted in significantly higher ECM diversity, and species richness significantly decreased in relation to soil drought in the control stand (unthinned treatment) only. This correlates with the fact that soil water potential decreased faster in this treatment. This suggests that the few ECM

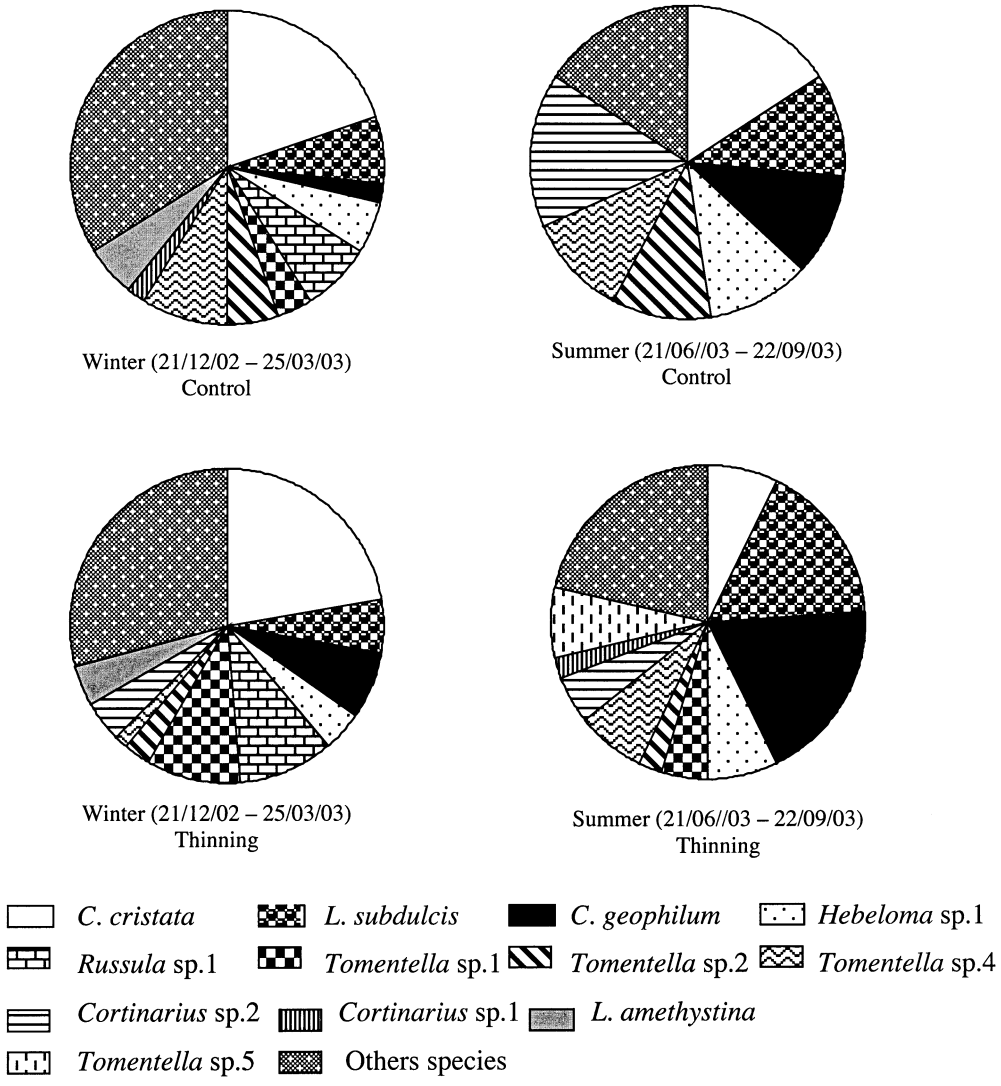

Fig. 4 Seasonal variation in the relative abundance of ECM types in the two beech stands (control and thinned). Mean value of four soil samples per stand for five consecutive sampling data per season is shown

morphotypes remaining during this period (*Cenococcum geophilum*, *Cortinarius* sp.2, *Clavulina cristata*, *Hebeloma* sp. and several *Tomentella* sp.) are more drought resistant than those formed by other fungi, and/or that their formation is favoured by low water potentials.

Among the relatively frequent morphotypes, 17 have been molecularly identified at the genus level only and 4 at the species level. Two species were particularly frequent: *Clavulina cristata* and *Cenococcum geophilum*. The latter is known to be ubiquitous (Pigott 1982; Jany et al. 2002). Concerning *Clavulina cristata*, present results from ITS sequencing of ectomycorrhizas and fruiting bodies demonstrate the symbiotic status of this fungus and confirm the recent finding of Tedersoo et al. (2003). The ECM status of this fungus had long been suggested (Bruns et al. 1998; Peter et al. 2001; Dickie et al. 2002). Other frequent species were Russulaceae such as *Russula* spp. and *Lactarius subdulcis*, the latter known as being specific to beech (Prévost and Pargney 1995), and many Thelephoraceae. The latter group is very abundant in temperate and boreal forest (Peter et al. 2001).

In order to monitor the viability of the ectomycorrhizas, a colorimetric test was used, based on the reduction of the MTS tetrazolium salt into soluble formazan. The results represent potential enzyme capacities because they were obtained at 25°C (optimised incubating temperature) and in a buffer rather than at the temperature and water potential that existed locally in the soil when the morphotypes were sampled. These are standardised laboratory conditions that do not pretend to mimic the pedoclimate (soil temperature in the A1 horizon in this type of forest almost never exceeds 20°C). The ecological meaning of these values is therefore the ability of the tested mycorrhizal root tips to rapidly resume metabolic and functional activities whenever environmental conditions improve. It must also be stressed that the activity recorded by this method results not only from the symbiotic organ but also from the associated micro-organisms (bacteria and free-living micro-fungi). This is why the reaction time must be kept short in order to avoid over-estimating the contribution of bacteria because of their proliferation. The fact that this test can be applied to single ECM tips makes it possible to study the distribution of potential activity values within a morphotype popula-

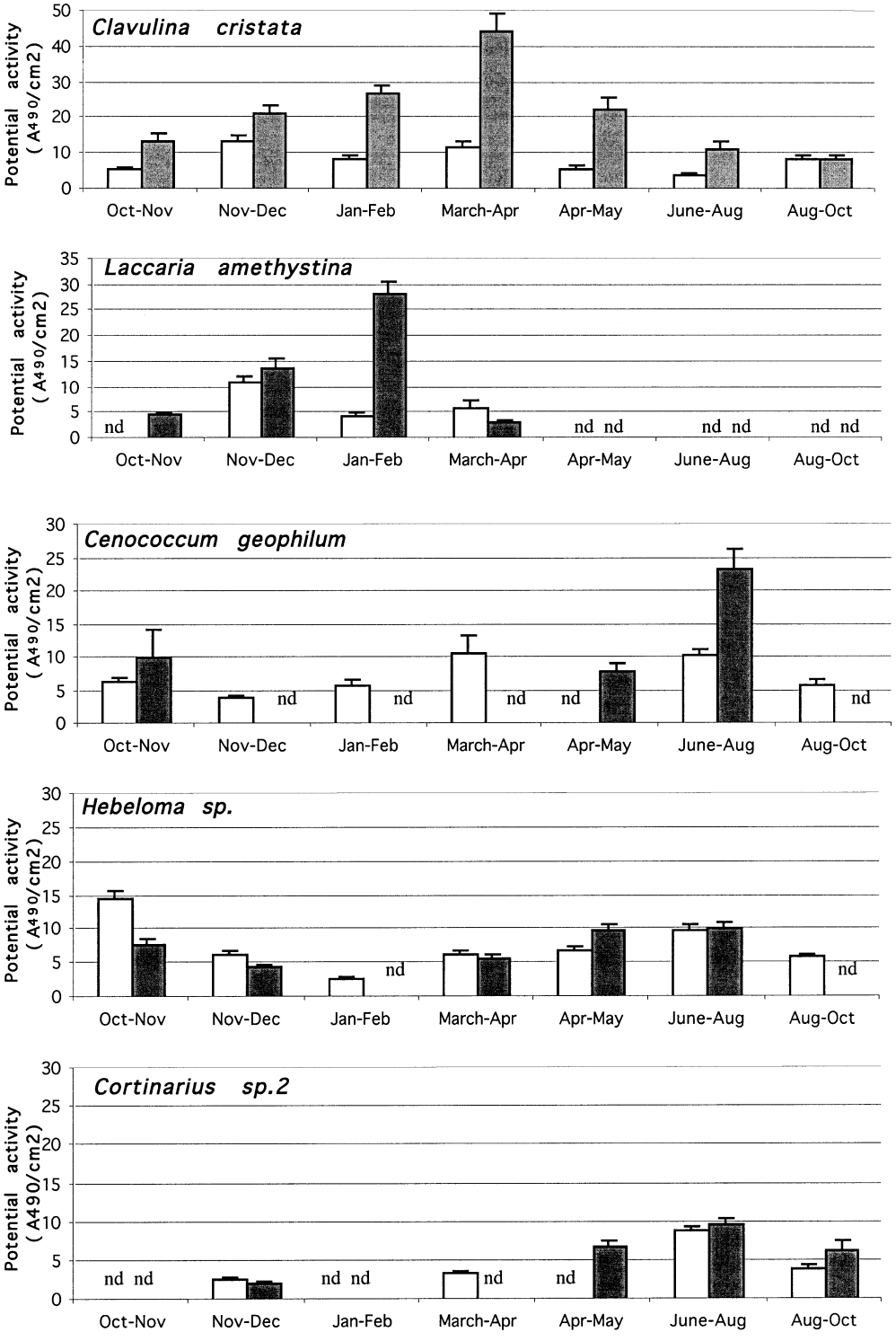

Fig. 5 Potential metabolic activity (MTS test, results in $A_{490\ nm}$ cm^{-2}) of five morphotypes showing contrasted seasonal patterns depending on thinning treatments (*grey bars* unthinned control, *white bars* strong thinning). Each value is the mean (± standard error) of individual ECM tip measurements ($24<n<168$) during the period considered (*nd* no data; number of tips too low)

tion, leading to future developments in functional population dynamics and community structure in order to study the role of ECM symbioses on mature trees in situ rather than on seedlings in controlled conditions as usually practiced.

One of the most original and significant results of this study is the evidence for a dynamic and potentially active ECM community even in winter. This is supported both by significant changes in the population size of particular ECM morphotypes, and by the results of the MTS metabolic test during this period. Some authors have detected enzymatic activities in soil at low temperature (Dormaar et al. 1984; McClaugherty and Linkins 1990), particulary dehydrogenase activities (Ivarson and Sowden 1970; Ross 1970; Dormaar et al. 1984). However, to our knowledge, few studies have addressed in situ tree root or ectomycorrhiza activity during winter in a cold temperate climate. For instance, ECM formed here by three fungal species (*C. cristata*, *L. amethystina* and *Russula* sp.1) were signif-

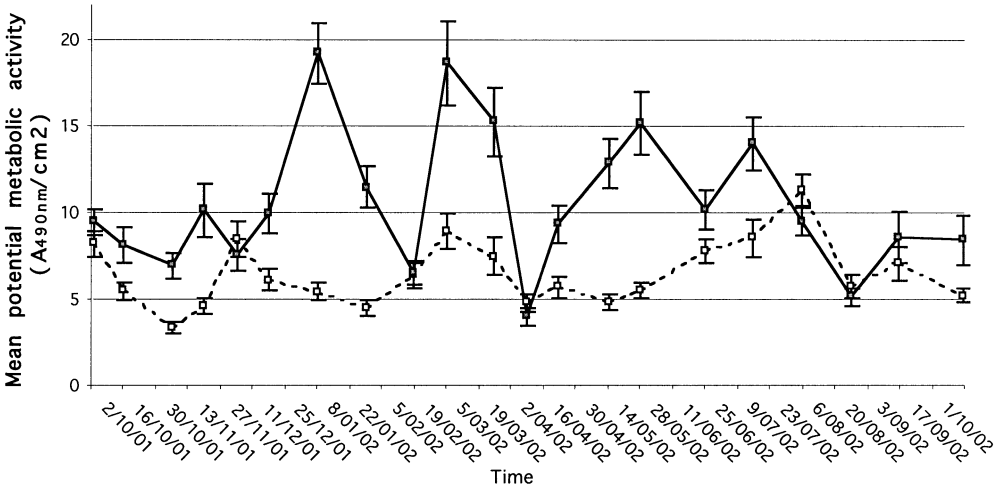

Fig. 6 Mean potential activity of the most abundant ECM morphotypes pooled together (MTS test, results in $A_{490\ nm}$ cm^{-2}) of the ECM community during the whole study. Each plot is the mean (± standard errors; $48<n<322$) of potential metabolic activities of the most abundant morphotypes on the corresponding sampling dates. Treatment effect is highly significant (ANOVA, the data were Log-transformed to normalise the distribution of the residuals and to stabilise the variance; $P=0.0095$). *Solid line* Control, *dotted line* thinning

icantly more abundant and more active in winter than in summer. A consequence of these observations is that the usual way of sampling ECM communities during the tree growth period for diversity studies might dramatically underestimate some functionally-important morphotypes. Another consequence is that the question of the role of these winter ECM morphotypes remains open and deserves further investigation because it challenges the concept of root "vegetative rest" in cold-temperate deciduous forests. It can be hypothesised, for instance, that the winter dehydrogenase activity of *C. cristata*, *L. amethystina* and *Russula*. sp.1 revealed by the MTS test corresponded to the synthesis and storage of fungal reserves and protective sugars such as mannitol or trehalose (Koide et al. 2000), the synthesis of which involves specific dehydrogenases (Martin et al. 1987; Ramstedt et al. 1987; Hampp and Schaeffer 1995). Alternatively, it might reveal a saprotrophic activity of these fungi during winter, in order to acquire carbon directly from the soil organic matter or fresh debris when the host is leafless and photosynthetically inactive.

Another interesting result is the very marked and significant difference in ECM morphotype frequency between the thinned and control stands. When compared to the unthinned control, the six most frequent ECM morphotypes, *Clavulina cristata* or *Cenococcum geophilum*, were more abundant in the stand subjected to strong thinning. In contrast, another group of seven morphotypes (including several *Tomentella* sp.) was less frequent and abundant in the thinned treatment. Thinning also reduced the mean value, and above all the time variations, of potential metabolic activity of the most abundant morphotypes. This might be linked to the smaller variations in soil temperature and water potential recorded in this treatment, re-

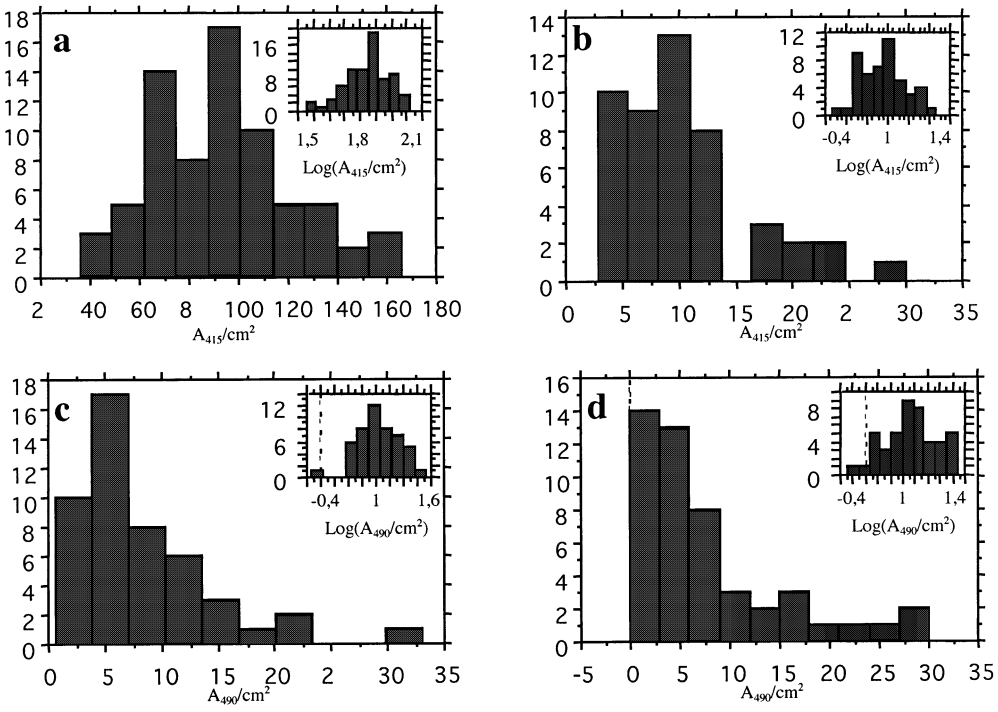

Fig. 7 Distributions of *Lactarius subdulcis* and *Cenococcum geophilum* ECM ($48<n<72$) into classes of dehydrogenases activities (MTS test, results in $A_{490\ nm}$ cm^{-2}) and acid-phosphatase activity (pNPP test, results in $A_{415\ nm}$ cm^{-2}) on 16 September. **a** *L. subdulcis* (phosphatase activity), **b** *C. geophilum* (phosphatase activity), **c** *L. subdulcis* (MTS test), **d** *C. geophilum* (MTS test). *Insets* Distributions of Log-transformed values

sulting in less strain on the ECM community. Indeed, Aussenac and co-workers. (Aussenac and Granier 1988, Aussenac et al. 1995) showed that thinning increased the soil water reserve available for the trees (Breda et al. 1995), and soil moisture is known to affect ECM fungal communities (Erland and Taylor 2002; Shi et al. 2002). Other indirect environmental effects of the strong thinning, such as reduced soil organic matter content and ground vegetation changes, are also likely to have modified the ECM community structure.

The ECM formed by *Cenococcum geophilum* were second in frequency after the *Clavulina cristata* type and its distribution showed a clear seasonal pattern with a peak of abundance and activity in summer, at the beginning of the soil drying period, and more particularly in the control treatment where the soil was drier than in the thinned treatment. This is in accordance with what was already known of *C. geophilum*, i.e. that its ECM remain metabolically active and even colonise new roots and pre-existing mycorrhizas at low soil water potential, when other morphotypes stop functioning and begin to decline (Pigott 1982; Jany et al. 2003).

This study was meant to be an exploratory work and did not pretend to have inference beyond this particular case study. We sampled roots in four plots in each of the two stands (thinned and unthinned) but these treatments were not replicated as another set of two stands. In the same way, the dramatic temporal dynamics of the ECM community we observed during a single year cannot be generalised. Therefore, the results of this case study are a snapshot in time and space, and inference cannot be made to seasonality or to the effect of thinning in other areas.

In conclusion, this work clearly illustrates the spatial and temporal diversity of an ECM community, not only in terms of species diversity, but also in terms of behaviour: seasonality, response to environmental factors and metabolic activities. For instance *Clavulina cristata* and *Cenococcum geophilum*, the two dominant species, displayed very contrasted seasonal patterns: *C. cristata* formed ECM mostly in winter and was more metabolically active during this season, while the *C. geophilum* population built up and expressed maximal activity in summer, especially during the dry period. This diversity can also concern functions, as shown by *Lactarius subdulcis* and *C. geophilum*: in spite of the similar potential metabolic (MTS) activity, the former shows a much higher acid phosphatase activity, indicating a clear difference in the potential for these two ECM types to mobilise phosphorus from soil or litter organic matter. Nevertheless, the results suggest that the concepts of functional complementarity (with time as well as across space) and functional groups may be applicable to ECM communities. This hypothesis requires further testing in field studies by analysing the functional activity of single ECM tips through, for example, transcriptome analysis (Martin 2001) or by isotope determinations (Högberg et al. 1999; Hobbie et al. 2001a,b), or for other functions of root activity and nutrient uptake, such as protease or laccase activity, organic acids and proton release, metal complexing, mineral weathering, etc.

Acknowledgements This research was supported by the GIP ECOFOR ("Biodiversité et Gestion Forestière" programme). We would like to thank Drs. N. Le Goff and J.M. Ottorini (INRA Nancy, France) and the Verdun-Fresne division of the Office National des Forêts (ONF), for the authorisation to use the site of Souilly for this study. We also thank Drs. Francis Martin and Martina Peter (INRA Nancy) for critical reading of the manuscript and discussions, Dr. Benoît Marçais for his decisive help in statistical analysis, Christine Delaruelle (INRA Nancy) for her technical assistance in DNA sequencing and Jean-Pierre Maurice (AMYPHAR, Nancy, France) for the identification of fungal fruiting bodies.

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