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Does forest liming impact the enzymatic profiles of ectomycorrhizal communities through specialized fungal symbionts?

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Abstract Liming (Ca–Mg soil amendment) is a forestry practice used to correct soil acidification and restore health and productivity in declining stands. Liming is known to modify tree mineral nutrition beyond the sole Ca and Mg. We hypothesized that liming also modifies the very functioning of the tree absorbing system (that is the ectomycorrhizal fine roots) in a way that facilitates the mobilization of mineral nutrients, particularly those entrapped in soil organic matter. This hypothesis has been tested here in beech and Norway spruce stands in North-Eastern France. In autumn, we compared the ectomycorrhizal community structure and the enzymatic profiles of ectomycorrhizal root tips in limed and untreated plots by measuring the activities of eight enzymes related to the degradation of soil organic matter. The results show that the ectomycorrhizal community responds to the Ca–Mg amendment and to the resulting soil modifications by modified enzyme activity profiles and ability to mobilize nutrients from soil organic matter. The effects of liming on the belowground functioning of the tree stands result essentially from specialized ECM fungal species such as *Clavulina cristata* (with strong glucuronidase activity), *Lactarius subdulcis* (with strong laccase activity) or *Xerocomus pruinatus* (with strong leucine aminopeptidase activity).

Keywords Ca–Mg soil amendment · Ectomycorrhizal community · Secreted enzymatic activities · Nutrient mobilization · Functional specialization

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Introduction

Liming is a common practice in European forests to improve calcium and magnesium supply and to correct cation deficits. It has been intensely used in the 1980s to counteract forest decline following soil acidification (Ulrich et al. 1979; Hüttl 1989).

In temperate and boreal forests, tree growth and nutrition are highly dependent on ectomycorrhizas (ECMs; Smith and Read 2008). The fungal partners (Ascomycete or Basidiomycete) provide the tree with nutrients derived from both mineral and organic sources. These elements can be taken up by the fungal symbionts from soil minerals through the secretion of low-molecular-weight organic acids (e.g. oxalic acid: Rineau et al. 2008) or fungal siderophores (e.g., hydroxamates) which contribute to mineral weathering (Landeweert et al. 2001; Rineau et al. 2008). Ectomycorrhizal fungi are also able to secrete enzymes to forage for nutrients by degrading organic matter. Methods have been developed to monitor the secretion of these enzymes by individual excised ECM root tips in microplates using fluorescent substrates (Pritsch et al. 2004; Courty et al. 2005; Mosca et al. 2007).

Liming modifies soil organic matter composition (Kreutzer 1995; Rosenberg et al. 2003) and raises soil pH (Kreutzer 1995). These soil parameters have been proven to influence ECM diversity (Erland and Taylor 2002). Indeed, liming increased twofold the ECM root abundance in the humus layers of a Norway spruce stand in southern Germany (Nowotny et al. 1998) and changed the ECM community structure (Qian et al. 1998). In contrast, Blaise and Garbaye (1983) found a reduced ECM abundance in beech plots amended with NPKCaMg.

In a more recent work Rineau and Garbaye (2009) found that liming applied 16 years earlier still resulted in

significant changes of the ECM community composition and structure, in both beech and Norway spruce stands in an acidified soil. These changes were accompanied by profound humus modifications and by the recovery of the previously declining trees. We therefore hypothesized that in addition to its direct effect on tree mineral nutrition through massive Ca and Mg input, liming had modified the very functioning of the tree absorbing system (that is the ECM fine roots) in a way that facilitates the access to major nutrients such as N, P or Ca that are for a large part entrapped in soil organic matter.

As a preliminary attempt to test for this hypothesis, we used the same experimental site and compared, in limed and untreated plots, the ability of the ECM communities to mobilize nutrients from soil organic matter using the micro-plate enzymatic assays developed by Courty et al. (2005).

Materials and methods

Study site

The experimental site of Humont (48°00′00″N, 6°29′28″E, Altitude: 570 m, Vosges forest, North-Eastern France) consists of moderately declining stands of 35-year-old Norway spruce (*Picea abies*) and 60-year-old beech (*Fagus sylvatica*). The allocrisol (typic dystrochrept, USDA Soil Taxonomy System, http://soils.usda.gov/technical/classification/taxonomy/) is formed on sandstone. The liming treatment was carried out by helicopter in 1991 with 757 kg/ha of CaO and 380 kg/ha of MgO. Sixteen years after the treatment, liming had restored tree health, mineral nutrition and vegetation diversity, had shifted humus type from moder to oligomul or from dysmoder to oligomull, in the beech and spruce stands, respectively. It had also strongly enhanced colonization by earthworms as shown by the abundance of mounds. The present soil chemical properties in the treated and untreated plots are recorded in Table 1.

Sampling and sample processing

We chose, separately for spruce and beech, two pairs of 1,000 m^2 plots (12.5 m×8 m), each pair with one plot in the limed area and the other plot outside (untreated area). The plot couples were set in areas homogeneous in terms of topography, stand age, density, and sylviculture (data not shown). They contain ten and 13 trees for beech and spruce, respectively.

Twenty soil cores (4 cm diameter, 18 cm deep, 225 cm3, 2×2.5 m apart from each other, not closer than 0.5 m to the nearest tree) were collected in each plot, before leaf fall on October 9, 2007, in a 5 m×4 m grid. All soil cores were stored at 4 C and processed within 4 days after sampling. Soil cores were washed separately on a 0.5 mm screen and the roots were cut into 1 cm long pieces and observed using a stereomicroscope. Ectomycorrhizal types were first identified morphologically using the descriptions by Agerer (1987–1998). ECM root tips of each morphotype were counted in order to determine their relative abundance: root pieces were randomly picked and the ECMs counted until reaching 100 tips (Garbaye 1990). For each morphotype, seven tips per plot were frozen at −80 C. Their pooled DNA was used to identify the fungal symbionts by sequencing the ITS region (Gardes and Bruns 1993).

The structure of the ECM communities in the Humont experimental site has been extensively described and discussed in Rineau and Garbaye (2009). The particular results concerning the distribution in the four plots of the 23 ECM types encountered during the present study are shown in Table 2.

Table 1 Chemical properties of the soil in the four plots (0–18 cm deep) at the time of sampling, 15 years after liming

Soil variable	Spruce, untreated	Spruce, limed	Beech, untreated	Beech, limed
Total N (g kg^{-1})	4.89	4.83	4.28	3.07
C/N	21.1	19.9	15.8	17.5
Total C (g kg^{-1})	103.0	95.9	67.6	53.7
pH	4.24	4.27	4.02	4.53
H^+ (exchangeable, cmol kg^{-1})	1.44	1.00	0.84	0.80
Al^{3+} (exchangeable, cmol kg^{-1})	8.10	6.74	8.20	5.22
Ca^{2+} (exchangeable, cmol kg^{-1})	0.80	0.93	0.20	1.57
Fe^{3+} (exchangeable, cmol kg^{-1})	0.14	0.10	0.11	0.02
Mg^{2+} (exchangeable, cmol kg^{-1})	0.44	0.44	0.22	0.47
Mn^{2+} (exchangeable, cmol kg^{-1})	0.18	0.24	0.14	0.43
K^+ (exchangeable, cmol kg^{-1})	0.41	0.39	0.35	0.29
Na^+ (exchangeable, cmol kg^{-1})	0.05	0.04	0.03	0.03
P (Duchaufour and Bonneau 1959, g kg^{-1})	0.037	0.041	0.050	0.043

Table 2 Relative abundance (per cent of the total number of vital ECM root tips in 20 soil cores) of the 23 ECM types in the four expérimental plots

Ectomycorrhizal type (associated fungal species)	Beech, untreated	Beech, limed	Spruce, untreated	Spruce, limed
Amanita rubescens	1 %	6%	0%	0%
Cenococcum geophilum	34 %	13%	51%	12%
Clavulina cristata	8 %	19%	0%	53%
Cortinarius sp.	7 %	0%	1%	2%
Dermocybe sp.	1 %	0%	0%	0%
Hygrophorus olivaceoalbus	0 %	0%	1%	0%
Laccaria amethystina	12 %	18%	0%	0%
Lactarius subdulcis	3 %	12%	0%	0%
Lactarius tabidus	0 %	0%	10%	3%
Paxillus sp.	2%	0%	3%	3%
Russula cyanoxantha	0%	1%	0%	0%
Russula nigricans	3%	0%	0%	0%
Russula ochroleuca	2%	0%	7%	2%
Russula sp.	0%	0%	1%	0%
Sebacina epigeia	1%	2%	0%	0%
Tomentella sp.	7%	12%	0%	0%
Tomentella sp.2	2%	0%	0%	0%
Tomentella sublilacina	8%	14%	4%	15%
UECM sp. 1	0%	0%	6%	8%
UECM sp. 2	0%	0%	1%	0%
UECM sp. 3	0%	0%	4%	0%
UECM sp. 4	0%	0%	1%	0%
Xerocomus pruinatus	8%	4%	11%	3%

Enzymatic assays

In each plot, the 20 soil cores have been randomly pooled into four composite samples (i.e. pseudo replicates) obtained by mixing together the content of five cores. Enzymatic activities were measured on all ECM species presenting more than seven healthy root tips after pooling, in order to have enough ECM material (seven tips) for the enzymatic assays. This constraint excluded only two species very poorly represented: *Hygrophorus olivaceoalbus* and unknown species UECM sp. 2 (Table 2). Whenever possible, those seven ECM root tips were excised from different clusters or from different root parts to encompass the individual functional variability of the ECMs (Cairney 1999). We measured the activity of eight secreted enzymes involved in soil organic matter degradation and nutrient mobilization: cellobiohydrolase and β-glucosidase (involved in cellulose degradation), xylosidase and glucuronidase (hemicelluloses), laccase (lignin), *N*-acetyl-glucosaminidase (chitin), leucine aminopeptidase (proteins) and acid phosphatase (organic phosphorus; Leake and Read 1990; Cullen and Kersten 2004; Courty et al. 2005). Enzyme assays were performed using the protocol defined by Courty et al. (2005). The enzymatic substrates, except laccase, were C-labeled with the fluorimetric probes 7-amino-4-methylcoumarin for leucine aminopeptidase assay and 4-methylumbelliferone for the others. Fluorescence measurements were carried out at 360 nm excitation and 465 nm emission with a fluorescence spectrophotometer microplate reader (Victor3, Wallac Perkin Elmer Life Sciences, Villebon-sur-Yvette, France). Spectrophotometric measurements for laccase activity were done at 415 nm with the plate reader Hercules 550 (Bio-Rad, CA, USA), and using the dye 2-2′-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS).

When enzymatic assays were finished, ECM tips were transferred into a fresh clear microplate containing 100 µl of water per well, and the projection surface area of each ECM tip was determined using the automated image analysis software WINRHIZO 2003b (Regent instruments, Inc., Canada; Buée et al. 2005). All measured activities were normalized per square centimeter of surface area of individual ECM tips and expressed in µmol cm^{-2} min^{-1}.

Such enzymatic assays actually measure the total secreted potential activity of the ECM considered as a mixed organ composed of plant and fungal tissues and associated bacteria. All these components potentially contribute to the measured enzymatic activities. Here, we did not try to separate the activities due to each partner,

because the mobilization of nutrients by ECMs and its benefit to the tree results from all these activities together (Courty et al. 2005).

Data processing

A given ECM type was characterized by: (1) the mean value per each one of the four pseudoreplicate in each plot (i.e., for five pooled soil cores) for each one of the eight enzymatic activities (e, in µmol cm^{-2} min^{-1}), (2) the projection surface area of all of its root tips (s, in cm^2), and (3) its relative abundance in each soil core (a, in %). We calculated E, the enzymatic activity of a given ECM type in each soil core ($E=s\times e$, in µmol/l of soil). In each plot, we then calculated the total enzymatic activity as the sum of potential activities of the 20 soil cores: $\Sigma(E\times a)$.

The contribution of an ECM type to this aggregated trait, that is the part of the total enzymatic activity due to each ECM type in a given plot, has been calculated as the total enzymatic activity of a given type in a plot divided by the sum of the activities of this enzyme for all the ECM types in the plot.

Statistics

The normality of the potential enzymatic activity per pseudoreplicate was assessed using a Shapiro–Wilk's test. As data were not normally distributed and in order to avoid hazardous transformations, we used the non-parametric test U of Mann and Whitney, which requires no assumption of normality, to compare the mean total enzymatic activities of the four pseudoreplicates in limed and untreated plots. Statistical analyses were done using the R software (http://www.r-project.org; Ihaka and Gentleman 1996).

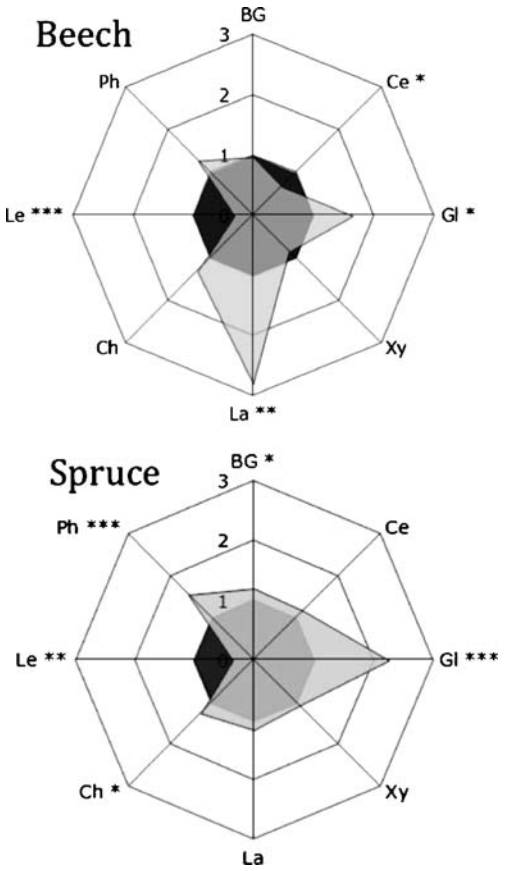

Fig. 1 Comparison between the mean enzymatic activities of the ECM community in beech (*top*) and spruce (*bottom*) limed and untreated plots. Eight enzymatic activities have been measured on seven ECM tips of each ECM type following the procedure of Courty et al. (2005): *BG* ß-glucosidase, *Ce* cellobiohydrolase, *Gl* glucuronidase, *Xy* xylosidase, *La* laccase, *Ch* chitinase, *Le* leucine aminopeptidase, *Ph* acid phosphatase. The polar graphs represent the ratios of the total enzymatic activity of a community of 100 ECMs in the limed plot versus that in the control plot in µmol min^{-1} (see detail of the calculation in "Materials and methods" section). Significant differences (Mann–Whitney U test) are shown by asterisks: *$p<0.05$, **$p<0.01$, ***$p<0.001$

Results

Results showed different patterns for spruce and beech (Fig. 1).

Concerning the spruce ECM communities, an enzymatic activity involved in plant cell wall degradation by removing lateral chains of hemicellulose (glucuronidase) was significantly twofold higher in the limed plot. Acid phosphatase activity was also significantly higher in the limed plots (×1.5) whereas leucine aminopeptidase activity was lower (×0.5).

Regarding the beech ECM communities, liming significantly reduced the potential activity of leucine aminopeptidase (×0.3), and that of cellobiohydrolase (×0.7), but the most dramatic effect was the enhancement of laccase activity (×2.8).

We then calculated the contribution of each ECM type to the total potential enzymatic activities of the community (Fig. 2). The major contributing ECM types were clearly different in untreated and limed plots. *Cenococcum geophilum* was, overall, the main contributor to total enzyme activities in the untreated plots, irrespective of the tree host. Nevertheless, its contribution was close to its relative abundance (more than 30%, except for chitinase and particularly leucine aminopeptidase which was almost totally due to *Xerocomus pruinatus*), meaning that its potential enzyme activities were close to the mean value of the community.

In the limed spruce plot, all enzyme activities (except laccase and leucine aminopeptidase which were due for more than 50% to *Tomentella sublilacina* and to *X.*

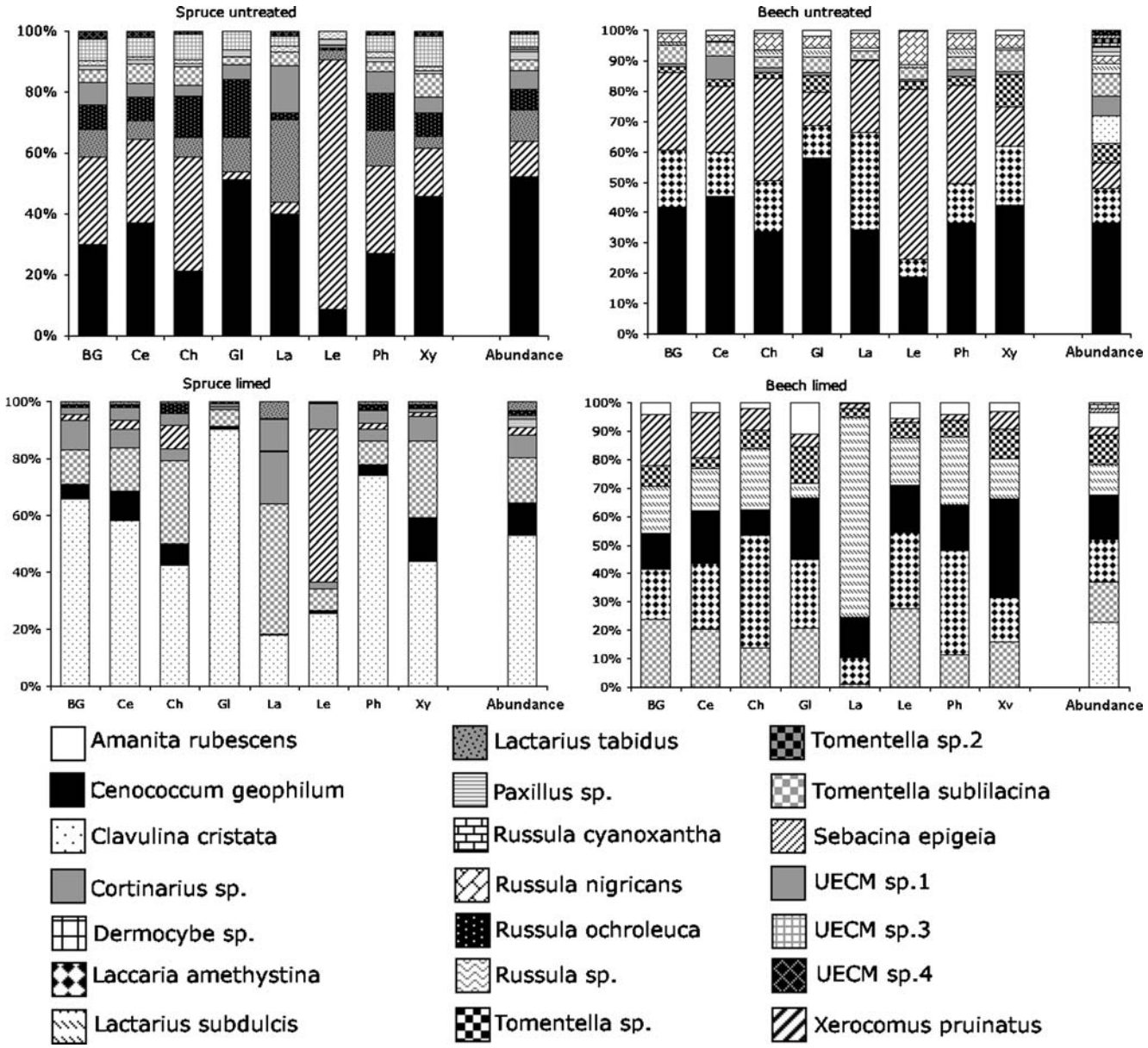

Fig. 2 Relative abundance (%) and mean contribution (%) of the different ECM types to the eight potential enzyme activities in the ECM community. Contribution of morphotype m (Cm) is calculated as $Cm=(a_m \times e_m)/\sum$, where e_m is the mean enzymatic activity of the morphotype m (μmol tip^{-1} min^{-1}), a_m its relative abundance (%), and $\sum$, the enzymatic activity of a community of 100 ECMs in the plot (μmol min^{-1}). Replicates: n=20 (abundances), and n=4 (contributions). Enzyme abbreviations: *BG* ß-glucosidase, *Ce* cellobiohydrolase, *Gl* glucuronidase, *Xy* xylosidase, *La* laccase, *Ch* chitinase, *Le* leucine aminopeptidase, *Ph* acid phosphatase

pruinatus, respectively) were predominantly due to the most abundant ECM type, *Clavulina cristata*. This ECM type was almost the only contributor of glucuronidase activity (90%), which was strongly enhanced in the limed plot at the community scale (Fig. 1).

Concerning the limed beech plot, the abundance and the activities of most of the enzymes tested were more or less equally distributed among the six co-dominant species *Lactarius subdulcis, C. geophilum, Laccaria amethystina, C. cristata, Tomentella* sp., and *Tomentella* sp. 2. However, *L. subdulcis* contributed to more than 70% of the laccase activity, which increased in the limed plot at the scale of the community (Fig. 1), whereas laccase activity in the untreated beech plot was as low as its relative abundance.

The ECM type *X. pruinatus* usually presented low relative abundances but high contributions to enzymatic activities, particularly for leucine aminopeptidase (53% and 82% in the untreated plots). In the same way, the ECM morphotype *T. sublilacina* was a high contributor of laccase activity in the spruce limed plot (46%).

Discussion

The ECM types encountered here can be sorted into two different functional types according to their enzymatic profiles in autumn, at the end of the growing season. *C. geophilum* and the majority of the ECM types found in this study can secrete all of the eight enzymes but always in low quantities. In contrast, more specialized ECM types, such as *L. subdulcis*, *C. cristata*, or *X. pruinatus*, secrete particularly high quantities of one enzyme (laccase, glucuronidase and leucine aminopeptidase, respectively). However, these autumn results should not be generalized to other periods of the year without caution: Courty et al. (2008) have found that the ECM community structure in a deciduous oak forest changed significantly across seasons.

The change of ECM community composition due to liming (Rineau and Garbaye 2009) was accompanied by an obvious change of ECM community functioning, estimated as the potential secretion of extracellular enzymes able to degrade organic matter.

In the case of spruce, we found a twofold-higher potential secretion of glucuronidase at the scale of the total ECM community. This was, above all, due to the high abundance of *C. cristata*, which contributed to most of the glucuronidase activity of the community. Among the four enzymes involved in cellulose and hemicellulose degradation, glucuronidase was the only one secreted by a specialist ECM type. Glucuronidase, as xylosidase, is a glucuroxylan-degrading enzyme, which cuts the linkage between the terminal xylose at the non-reducing chain end and a 4-*O*-methylglucuronic residue (Cullen and Kersten 2004). No glucuronidase activity was measured in another basidiomycete, *Heterobasidion annosum* (Maijala et al. 1995), and no glucuronidase genes have been found in the genomes of *Coprinopsis cinerea* (a saprotrophic species) or *Laccaria bicolor* (an ECM species), whereas these genes are widespread among bacteria and Ascomycetes. However, we cannot rule out a bias due to the fact that Basidiomycete fungi have not been studied as extensively as Ascomycetes and bacteria in this respect. Because ECMs are known to be associated with bacteria in the ectomycorrhizosphere (Frey-Klett et al. 2005), it is thus possible that the strong potential glucuronidase activity observed in ECMs of the basidiomycete *C. cristata* is due to bacteria specifically associated with this fungus. This would be consistent with the general aspect of *C. cristata* ECMs, always as clusters embedded in clay or silt and probably containing many bacterial colonies. The occurrence of saprotrophic ascomycetes colonizing the surface of ECMs of *C. cristata* and which might contribute to the glucuronidase activity is less probable, because the surface of the ECMs were homogeneous regarding the hyphal mantle structure. Moreover, we did not observe multiple PCR bands after DNA amplification of this ECM morphotype.

In the case of beech, liming also significantly reduced leucine aminopeptidase activity (×0.5). But the most conspicuous effect was the enhancement of laccase activity. *L. subdulcis* was the main potential contributor to laccase activity in the limed beech plots, and was the most active laccase producer among all the ECM fungi tested. Liming not only increased the relative abundance of *L. subdulcis* ECMs but also stimulated their potential laccase activity. Laccase is an oxidative, unspecific polyphenol-degrading enzyme (Cullen and Kersten 2004), produced by numerous fungi, particularly white-rot ones (Reid 1995). Several laccase genes have been found in the genome of *L. bicolor*, an ECM fungus (Martin et al. 2008, Courty et al. 2009). Nevertheless, some extracellular laccases are also involved in basidiome development and interactions with pathogens (Burke and Cairney 2002). Some laccase isoenzymes can be constitutive and others regulated (Cullen and Kersten 2004). The increased potential laccase activity of *L. subdulcis* ECMs in the beech limed plot can thus be interpreted as the enhancement of lignin polyphenol degradation ability by the tested ECM community. We can hypothesize that it is due to an increased abundance of polyphenols in the limed plots, what is consistent with the higher leaf biomass produced and with the particularly high polyphenol content in beech litter. The frequent presence of large clusters of *L. subdulcis* ECMs under piles of dead wood, in packs of poorly fragmented leaves and in dead wood pieces in our sampling site (extensive qualitative observations in addition of soil core sampling, results not shown) was also consistent with this behavior of a laccase-secreting white-rot fungus. *Lactarius quietus*, an oak-specific ECM, which is, as *L. subdulcis*, abundant, highly clusterized, and smooth (contact-exploration type, according to Agerer 2001), also showed close contact to dead leaves and white-rot behavior (Courty et al. 2007). It suggests that contrary to what Colpaert and Van Laere (1996) showed, who demonstrated *in vitro* that *Thelephora terrestris* and *Suillus bovinus* did not have polyphenol oxydase activity, some ECM root tips are capable to degrade lignin and, as white-rot fungi, can have an important role in the degradation of phenolic compounds.

We also observed a stimulating effect of liming on the potential acid phosphatase activity of the whole ECM community in spruce plots. Condron et al. (1992) showed that liming reduces the availability of soil organic P. Moreover, phosphatases catalyses the hydrolysis of organic P into inorganic and available P (Burns 1978), and phosphatase enzymes are inducible under conditions of low inorganic P concentration (Antibus et al. 1992; Tibett et al. 1998). It is thus likely that the increased potential acid phosphatase activity of the whole ECM community was caused by the reduced inorganic P availability in the soil of the limed plots. Huber et al. (2006) showed that liming significantly decreased P needle contents. Reduction of P

nutrition has also been recorded in liming experiments in very similar and nearby forests (Renaud et al., pers. com). Because tree phosphorus nutrition is highly dependent on the ECM symbiosis, this reduction of P tree nutrition is consistent with the increased ECM phosphatase activity and decreased P availability in the limed plots.

Finally, liming reduced the total leucine aminopeptidase activity of the ECM communities, even if this effect was not significant in beech. It was a consequence of the reduced abundance of the ECM type *X. pruinatus*, particularly active for this enzyme at the time of sampling. According to the Vepsäläinen et al. (2001), leucine aminopeptidase activity is a good marker of the overall protease activity of a soil because it is very strongly correlated with a range of other protein-degrading enzymes. Abuzinadah and Read (1986) have demonstrated, using mycelial cultures in vitro, that some ECM fungi ('protein fungi') are able to use proteins as the sole nitrogen source; but no *Xerocomus* was among the species tested.

In conclusion, these preliminary results are consistent with our initial hypothesis that the ectomycorrhizal community responds to the Ca–Mg amendment and to the resulting soil modifications by profound changes of activities of some secreted enzymes involved in the mobilization of nutrients from soil organic matter. They also suggest that the effects of liming on the belowground functioning of the spruce and beech stands studied here result essentially from the presence/absence of specialized ECM fungal species such as *C. cristata* (glucuronidase), *L. subdulcis* (laccase), or *X. pruinatus* (leucine aminopeptidase). In order to comfort these preliminary clues and to test fully for the hypothesis, a wider investigation is underway, using more extensive sampling in a network of forest soil amendment experiments under various environmental conditions.

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