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Thèse

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En Biologie Végétale et Forestière
Par

François Rineau

Etude des conséquences du chaulage sur la structure et le fonctionnement des communautés d'ectomycorrhizes des forêts des Vosges

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INTRODUCTION GENERALE



Carpophores de *Laccaria laccata* poussant sur des turricules de vers de terre dans la parcelle de hêtre chaulé à Humont. (photo : F. Rineau)

Le dépérissement forestier des années 1980

Au début des années 1980, les forêts d'épicéas d'europe centrale furent confrontées à des symptômes de jaunissement et de défoliation. Ceux-ci ont été immédiatement attribués aux pluies acides et largement médiatisés, et furent un sujet de vif débat scientifique pendant une dizaine d'années, jusqu'à ce que le chaulage soit reconnu comme une pratique forestière efficace pour restaurer la santé des arbres.

Les premiers symptômes de dépérissement apparurent dans les forêts de Tchéquie endommagées par les pluies acides à cause des grandes quantités de SO_2 produites par la combustion des lignines (Bonneau, 2007). Ces symptômes gagnèrent progressivement la Bavière, les Ardennes (Nys, 1989), la forêt noire, et, finalement, les Vosges, et furent attribués à la production de SO_2 dans ces territoires fortement industrialisés. En 1989, 37% des 190 000 Ha de conifères de la région étaient touchés (Richter & Nageleisen, 2007). Cependant, le suivi de la pollution de l'air dans les Vosges révéla que les quantités de SO_2 étaient au moins deux fois plus faibles que le seuil de toxicité (Bonneau, 2007). La véritable cause du dépérissement forestier n'était donc pas encore élucidée.

On proposa alors une hypothèse alternative: ce dépérisement forestier était la conséquence de la sécheresse de 1976. Cependant, des défoliations et des jaunissements d'aiguilles étaient observés dans des stations où les arbres étaient plantés de manière très dense et sur un sol peu épais (sols de montagne). Mais le décalage de plusieurs années entre la sécheresse et la phénologie de l'arbre empêchait d'établir des relations claires entre ces deux phénomènes (Lévy & Becker, 1987).

C'est à ce moment qu'une troisième cause possible à ce déclin forestier fut identifiée. Des analyses foliaires sur des épicéas dépérissants en forêt noire révélèrent de sévères carences en magnésium (Zöttl & Mies, 1983). Ces carences provoquent le jaunissment de l'extrêmité des aiguilles, ou encore des taches jaunissantes entre les nervures chez le hêtre, avec une

transition brutale du jaune au vert, suivie, dans les cas plus graves, d'une coloration brune ou d'un déssèchement complet des aiguilles (Bonneau, 1995). De plus, ces carences foliaires furent inversement corrélées avec les quantités de calcium et de magnésium échangeables dans le sol; de plus les symptômes de dépérissement étaient plus fréquents sur des roches-mères gréseuses ou granitiques pauvres en cations (Landmann & Nageleisen, 2001 ; Nechwatal & Oswald, 2003). L'acidité naturelle des précipitations dans les Vosges (pH entre 4.3 and 5.4) a donc conduit au drainage des cations divalents liés aux colloïdes du sol et à leur remplacement par l'aluminium, dont certaines formes sont toxiques pour les plantes. Une grande partie de ces forêts vosgiennes perdaient ainsi 2% du magnésium et 0,5% du calcium par an (Dupouey et al., 1998). Un suivi temporel des analyses de sol ont elles aussi démontré une acidification progressive des sols (Göttlein, 1992; Hallbäcken and Tamm, 1986).

Le dépérissement forestier fut alors attribué à une combinaison de ces trois facteurs. En effet, la plupart du Mg dans les jeunes aiguilles provient de la minéralisation de l'humus à la surface du sol. Une sécheresse stoppe cette minéralisation et le Mg nécéssaire au développement des nouvelles aiguilles est prélevé dans les aiguilles des années précédentes. De plus, dans les sols acides, les horizons minéraux ont de très faibles capacités de rétention du Mg provenant de l'humus. Par conséquent, on déduisit que la sécheresse de 1976 avait provoqué de sévères carences dans les jeunes aiguilles et que la nutrition minérale des arbres s'est dégradé à cause de la perte progressive de Mg des horizons superficiels à cause des pluies acides et de l'acidification des sols (Bonneau, 2007).

Le chaulage: une méthode de restauration des forêts dépérissantes

Les amendements calco-magnésiens sont une pratique forestière utilisée pour compenser le manque de cations dans les sols par une application directe de Ca et Mg sur le sol forestier.

L'amendement peut être amené sous forme de CaO ou bien en mélange avec $CaCO_3$, $Ca(OH)_2$, CaO, $CaSO_4$ ou $MgSO_4$, en fonction des propriétés chimiques du sol à amender (Bonneau, 1995). L'amendement peut être apporté à la main, par soufflerie ou par hélicoptère (Bonneau, 1995), et a été utilisé en routine en Allemagne dans le milieu des années 80 à des doses de 3 à 4 t/ha (Worken & Brumme, 1997). Jusqu'alors le chaulage a été assez peu utilisé en France, et limité à des essais en forêt, en particulier dans les Ardennes, les Vosges (Renaud et al., 2000) et en Normandie. Dans tous les cas, le chaulage a durablement restauré la nutrition minérale des arbres. Les effets du chaulage sur les sols forestiers sont maintenant bien connus et peuvent être classés en cinq catégories:

**effets sur la biologie du sol:* Ils sont principalement concentrés dans les horizons superficiels, où le chaulage a le plus d'influence sur l'acidité du sol. On remarque une respiration plus forte dans l'humus. Le chaulage augmente l'abondance des lombrics et la biomasse bactérienne, en particulier la microflore nitrifiante (Anderson, 1998), au détriment des champignons (Kreutzer, 1995). La communauté de nématodes passe par conséquent d'une communauté de mycophages à une aure plutôt bactériophage (Arpin, 2007). Malgré tout, on peut observer d'avantage de champignons parasitant les racines. On observe aussi une diminution de la vitalité des mycorrhizes, et une redistribution de la profondeur du système racinaire dans les forêts chaulées vers une légère diminution de la densité des racines fines en surface et augmentation de la densité entre 5 et 15cm. On observe de plus un ralentissement du turnover des racines dans les placeaux chaulés.

**effets sur la composition chimique du sol:* le chaulage augmente le pH des solutions de sol dans les horizons superficiels et induit la déprotonation des groupements –COOH des acides humiques (Kreutzer, 1995). Cela conduit à une plus grande capacité d'échange cationique et donc à la rétention de Ca et Mg par les colloïdes du sol. Le remplacement d'Al^{3+}, cation acide et toxique, par Ca^{2+} et Mg^{2+} dans le complexe d'échange et son élimination des horizons

superficiels par lessivage diminue l'acidité échangeable, la concentration de Al^{3+} échangeable et par conséquent la toxicité aluminique (Ingerslev, 1997). Le chaulage diminue aussi les concentrations de Mn, Cd et Zn dans la solution de sol en provoquant leur préciptation sous forme d'oxydes, et augmente les concentrations totales de Fe et Cu qui forment des complexes stables avec la matière organique. Ce changement dans la composition chimique du sol peut s'accompagner par un lessivage des nitrates dans des peuplements n'ayant pas la capacité de se revitaliser, donc d'immobiliser les nitrates dans la biomasse. Dans les zones chaulées, les taux de minéralisation augmentent avec l'activité biologique (Huber et al., 2006).

**structure du sol*: le chaulage, en stimulant l'activité des lombrics, en particulier les espèces anéciques qui incorporent la matière organique jusqu'à une profondeur de 20cm (Judas et al., 1997), améliore l'aération des horizons supérieurs du sol. Cela a pour effet d'améliorer la structure du sol et la capacité de rétention d'eau (Schack-Kirchner & Hildebrand, 1998).

**humus*: le chaulage provoque la transformation des mor ou moder en humus de type mull (Kreutzer, 1995; Renaud et al., 2000).

**nutrition des arbres*: les analyses foliaires, qui révèlent la qualité de la nutrition minérale des arbres, montrent une meilleure nutrition en Ca (Huber et al., 2006) ou en Ca et Mg (Belkacem et al., 1992; Renaud et al., 2000)

**végétation*: le chaulage réduit le taux de germination des espèces acidophiles mais stimule celle des espèces ubiquistes (Olsson & Kelner, 2002). La flore passe d'une communauté acidophile à une autre plus nitrophile dans les zones chaulées (Hallbäcken & Zhang, 1998). En revanche, lorsque les doses d'amendements sont plus faibles, les espèces herbacées acidophiles sont conservées. Un effet identique du traitement est observé chez les mousses, de manière dose-dépendante (Dulière et al., 2000). Le chaulage est à nouveau un sujet d'actualité

en foresterie pour améliorer la production de biomasse, grâce à son impact modéré sur l'écosystème et à son coût relativement faible (Bonneau, 1995).

La symbiose ectomycorrhizienne

La majorité des racines fines de la plupart des essences forestières d'intérêt économique des forêts boréales et tempérées (pin, sapin, chêne, hêtre, épicéa) sont en association symbiotique avec des champignons appartenant aux Asco- et aux Basidiomycètes. Le partenaire fongique forme avec la racine un organe mixte appelé ectomycorrhize (ECM), caractérisé par un manteau de mycélium qui recouvre l'extrêmité des racines fines (Smith & Read, 1997). La symbiose permet à l'arbre d'exploiter un volume de sol plus important grâce au mycélium extramatriciel qui transfère alors des nutriments et de l'eau aux racines (Garbaye et Guehl, 1997). Un même arbre peut être en association avec une grande diversité d'espèces d'ECMs, dont chacune est plus ou moins spécialisée et complémentaire pour la récupération et la translocation des nutriments. La diversité fonctionelle de la communauté d'ectomycorrhizes est donc un paramètre très important pour l'adaptation de la population d'arbres à des ressources limitées ou bien à des perturbations de l'écosystème.

Les ECMs assimilent les composés carbonés, azotés et phophatés, ainsi que les nutriments disponibles dans le sol, tels Ca, Mg, K, et les transfèrent à la racine via leur mycélium (Smith & Read, 1997). On considère que les ECMs sont impliquées dans l'assimilation des nutriments par l'arbre car elles occupent la majorité de l'extrémité des racines fines, qui constitue l'appareil absorbant des arbres. Par conséquent tous les nutriments transitent, de manière active ou passive, à travers le manteau des mycorhizes. La disponibilité de ces nutriments est parfois limitée dans les sols forestiers, et ils peuvent être d'origine à la fois organique ou minérale.

L’assimilation des nutriments organiques par les ECMs

Dans les sols forestiers, la plus abondante source de nutriments est la litière, qui est composée de cellulose, d’hémicellulose et de lignine, et qui comprend une grande diversité de composés nécéssaires à la nutrition de l’arbre. La dégradation de ces macromolécules est réalisée en majorité par les champignons, grâce à la sécrétion de puissantes enzymes hydrolytiques pariétales (Kirk & Cullen, 1998; Cullen & Kersten, 2005), et, dans une moindre mesure, par les bactéries (Gramss et al., 1999). Les champignons saprotrophes ne sont pas les seuls organismes impliqués dans le cycle des nutriments dans les sols forestiers: les ECMs y jouent aussi un rôle important et présentent beaucoup de similitudes avec les champignons saprotrophes dans leur capacité à assimiler des nutriments (Leake & Boddy, 2002). En effet, les ECMs produisent elles aussi des enzymes pariétales qui récupèrent les nutriments à partir de la matière organique (Dighton, 1983), mais en plus faibles quantités que les champignons saprotrophes. Des plus faibles activités de la cellulase et de la cellobiohydrolase ainsi qu’une activité phosphomonestérase identique ont été détectées dans des cultures pures d’ECMs en comparaison avec des cultures pures de champignons dégradeurs de litière (Colpaert & Van Laere, 1996). De la même manière, les ECMs produisent de faibles mais significatives quantités de phénol-oxydases en comparaison avec les champignons saprotrophes (Bending & Read, 1997). Des activités polyphénol-oxydases on été aussi détectées chez les ECMs (Burke & Cairney, 2002). De plus, la comparaison de séquences d’ADN entre ECMs et champignons de la pourriture blanche a révélé que les gènes codant pour les enzymes ligninolytiques (lignine et manganèse peroxydases, laccase multicopper oxidases) étaient répandus chez un large panel d’espèces d’ECMs (Chen et al., 2001 ; Luis et al., 2004). Les ECMs sont aussi impliquées dans la dégradation des cuticules, des parois cellulaires, des phénols et des tanins (Caldwell et al., 1991; Bending & Read, 1997; Günther et al., 1998). Ces résultats suggèrent

que les ECMs possédent de nombreux caractères de champignons décomposeurs (Read & Perez-Moreno, 2003).

Parmi les enzymes produites par les ECMs, les endoglucanases, les cellobiohydrolases et les ß-glucosidases convertissent la cellulose en molécules de glucose. Les endoglucanases clivent les liaisons internes dans les chaînes de cellulose. Les cellobiohydrolases complètent cettte dégradation en hydrolysant progressivement les nouvelles chaînes ainsi formées par leurs extrêmités et forment ainsi des molécules de cellobiose, formées de deux glucoses liées en ß 1-4. Enfin, les ß-glucosidases découpent la cellobiose en deux molécules de glucose (Kirk & Cullen, 1998). La dégradation des hémicelluloses suit un processus plus complexe et implique huit familles d'enzymes différentes en fonction de la molécule formant le maillon de la chaîne d'hémicellulose. En ce qui concerne la dégradation des lignines, l'hydrolyse de ce composé nécessite de puissantes enzymes oxydatives à cause des nombreuses liaisons C-C et éther qui la composent. Les trois principaux groupes d'enzymes de dégradation de la matière organique sont les lignines peroxydases, les manganèse peroxydases et les laccases (Kirk & Cullen, 1998). Des gènes codant pour ces trois familles d'enzymes et proches de celles de champignons de la pourriture blanche comme *Phanerochaete chrysosporium* (pour la lignine peroxydase) et *Pleurotus ostreatus* (pour la manganèse peroxydase) on été retrouvées dans le génome du champignon ectomycorrhizien *Laccaria bicolor*, ce qui suggère que ce champignon est potentiellement capable de dégrader la lignine. Des activités de polyphénol oxydases ont aussi été détectées dans des cultures pures d'ECMs tels que *Suillus bovinus*, *Pisolithus tinctorius*, et *Paxillus involutus* (Burke & Cairney, 2002), ainsi que des activités de manganèse peroxydase chez *Tylospora fibrillosa* (Chambers et al., 1999).

La faible disponibilité de l'azote est une caractéristique des écosystèmes forestiers dominés par les arbres à symbiose ectomycorrhizienne (Smith & Read, 1997). De plus, la concentration en formes solubles de l'azote dans les sols (ammonium et nitrate) est très faible.

Plus de 95% de l'azote dans les sols est en effet sous forme organique dans les forêts tempérées (Chalot & Brun, 1998). La symbiose mycorrhizienne est donc cruciale pour la nutrition azotée des arbres dans ces écosystèmes, car les ECMs peuvent récupérer de l'azote à partir des deux principales sources d'azote organique de l'humus forestier: la chitine et les protéines. Des activités protéases ont été mesurées pour beaucoup d'espèces mycorrhiziennes: *Suillus variegatus, S. bovinus, Piloderma croceum, Pisolithus tinctorius, Cenococcum geophilum, Amanita rubescens, Lactarius subdulcis, Hebeloma crustuliniforme, Paxillus in volutus,* and *Thelephora terrestris* (Smith & Read, 1997). Il existe beaucoup de types de protéases: par exemple, celles sécrétées par le champigon ectomycorrhizien *Cenococcum geophilum* appartiennent à la famille des sérines protéases. De la même manière, les chitinases permettent de solubiliser l'azote inclus dans la chitine (chaîne de N-acétyl glucosamine, donc contenant un atome d'azote par monomère) (Lindahl & Finlay, 2006). Des activités N-acétyl-glucosaminidases ont été mesurées sur des apex d'ECMs excisés (Pritsch et al., 2004).

Des tests enzymatiques utilisant des substrats fluorescents ou colorés ont été développés pour mesurer les activités enzymatiques potentielles d'apex mycorrhiziens excisés (Courty et al., 2005) ou sur des systèmes racinaires *in situ* (Dong et al., 2007).

L'assimilation des nutriments minéraux par les ECMs (contribution à une revue en préparation pour Soil Biology and Biochemistry)

Besides nitrogen, a major tree-growth limiting element (Aerts, 2002), forest trees also need inorganic nutrients of mineral origin such as PO_4^{3-}, Ca^{2+}, Mg^{2+}, Fe^{3+}, K^{+}. Ectomycorrhizal fungi contribute significantly to the uptake of these ions (Smith & Read, 1997), most of which

Table 1: List of mycorrhizal fungi described for their ability to solubilize minerals

Ectomycorrhizal species	Type of experiment	Fungal origin	Mineral source	Mechanism of mineral dissolution involved	References
Boletus edulis	Mycelium	Culture	-	Siderophores (hydroxamates)	Szaniszlo et al., 1981
Cenococcum geophilum	Mycelium	Culture	Vermiculite, Phlogopite	Siderophores (ferricrocin) Proton, Organic acids (oxalate)	Yuan et al., 2004 Haselwandter & Winkelmann, 2002
Cortinarius glaucopus	Mycelium	Culture	Quartz, potassium feldspar, apatite, TCP, marble	Organic acids (oxalate)	Rosling et al., 2005
Gautieria monticola	Hyphal mats	Forest soil	-	Organic acids (oxalate)	Griffiths et al., 1994
Hebeloma crustuliniforme	Seedlings	Forest soil (E horizon)	-	Organic acids (oxalate, citrate, propionate) Siderophores (ferricrocin)	van Hees et al., 2006b, 2006c
Hebeloma longicaudum	Seedlings	Culture	-	Organic acids (oxalate)	van Sch□ll et al., 2006b
Hysterangium crassum	Hyphal mats	Forest soil	-	Organic acids (oxalate)	Cromack et al., 1979
Hysterangium setchelli	Hyphal mats	Forest soil	-	Organic acids (oxalate)	Griffiths et al., 1994
Laccaria bicolor	Mycelium	Culture	-	Organic acids (oxalate)	Cumming et al., 2001
Lactarius subdulcis	Ectomycorrhiza	Forest soil	-	Organic acids (oxalate)	Rineau et al., in press
Paxillus involutus	Mycelium	Culture	Orthophosphate	Proton, Organic acids (oxalate)	Arvieu et al., 2003
			-	Organic acids (oxalate)	Lapeyrie et al., 1987 van Sch□ll et al., 2006b
			Phlogopite, Quartz, potassium feldspar, apatite, TCP, marble	Organic acids (oxalate)	Paris et al., 1996Ź; Rosling et al., 2005
		Forest soil (E horizon)	-	Organic acids (oxalate, citrate, formate, malonate)	van Hees et al., 2003, 2005
		Culture	Biotite, Microcline	Organic acids (oxalate, citrate)	Wallander & Wickman, 1999
Piloderma byssinum	Mycelium	Culture	Quartz, potassium feldspar, apatite, TCP, marble	Organic acids (oxalate)	Rosling et al., 2005
Piloderma croceum	Mycelium	Culture	Apatite (TCP)	Organic acids (oxalate)	Mahmood et al., 2001
	Seedlings	Culture	-	Organic acids (oxalate)*	van Sch□ll et al., 2006b

derive from the dissolution of soil mineral particles: apatite contains phosphorus, feldspars, micas and hornblendes provide Ca, Mg and K, while Fe^{3+} is found in mica and in secondary minerals such as iron oxides (Landeweert et al., 2001). The ability of the ECM fungi to dissolve minerals was reported for identified and unidentified mycorrhizal isolates (Table 1; Paris et al., 1996; Mahmood et al., 2001). Pure cultures of some of these fungal species appeared capable of dissolving tri-calcic phosphorus or biotite. Evidence of the fungal impact on the dissolution of minerals was also described for seedlings infected by ECM fungi. The transformation of chlorite and mica into clay minerals, as well as the dissolution of apatite, was reported for seedlings infected with *Piloderma croceum* and *Suillus variegatus*, respectively (Arocena et al., 2004; Wallander, 2000a). In the same way, an experiment with tree seedlings showed that the ECM fungus *Paxillus involutus* significantly contributed to the dissolution of muscovite (source of K) and increased root potassium contents (van Schöll et al., 2006a). Interestingly, ectomycorrhizal fungi associated with seedlings have also been shown to recover phosphorus from apatite (Wallander et al., 2000a) and potassium from biotite and microcline (Wallander & Wickmann, 1999).

The relevance of these mechanisms at the ecosystem scale has been stressed by Blum et al. (2002) who showed that a large part of the calcium used by the trees was mobilized from apatite by ECM fungi. Another manifestation of fungal mineral dissolution could be the formation of smooth tunnels of constant diameter in feldspar and hornblende grains (Hoffland et al., 2002). These tunnels are often colonized by fungal hyphae. Because feldspar tunnel density is positively correlated with ECM density, Hoffland et al. (2003) emitted the hypothesis that ectomycorrhizal hyphae were involved in mineral dissolution. Nevertheless, the contribution of tunneling to weathering is probably low because it occurs mainly in very old soils and for a very low fraction of total soil minerals (Smits et al., 2005).

Table 1: List of mycorrhizal fungi described for their ability to solubilize minerals (part 2)

Ectomycorrhizal species	Type of experiment	Fungal origin	Mineral source	Mechanism of mineral dissolution involved	References
Piloderma fallax	Mycelium	Culture	Quartz, potassium feldspar, apatite, TCP, marble	Organic acids (oxalate)	Rosling et al., 2005
Piloderma spp.	Mycelium	Culture	Apatite (TCP)	Organic acids (oxalate)*	Mahmood et al., 2001
Pisolithus spp.	Mycelium	Culture	Vermiculite Phlogopite	Proton, Organic acids (oxalate)	Yuan et al., 2004
Pisolithus tinctorius	Mycelium	Culture	-	Organic acids (Oxalate, tartrate, formate, glycolate)	Cumming et al., 2001
		Culture	Apatite (TCP)	Mineral dissolution	Jayakumar & Tan, 2005
		Culture	Phlogopite	Organic acids (oxalate)	Paris et al., 1996
	Seedlings	Culture	-	Organic acids (citrate)	Tahara et al., 2005
Rhizopogon luteolus	Mycelium	Culture	-	Organic acids (oxalic acid, citric acid, succinic acid), Siderophores (catecholates, hydroxamates)	Machuca et al., 2007
Rhizopogon roseolus	Mycelium	Culture	Orthophosphate	Proton, Organic acids (oxalic acid)	Arvieu et al., 2003
	Seedlings	Forest soil (A horizon)	Soil + Hydroxyapatite or Calcium carbonate	Proton, Organic acids (oxalate)	Casarin et al., 2003
	Seedlings	Mediterranean chromic cambisol	Soil +/- CaCO3 and P	Proton, Organic acids (oxalate)	Casarin et al., 2004
Scleroderma verrucosum	Mycelium	Culture	-	Organic acids (oxalate, citrate, succinate), Siderophores (catecholates, hydroxamates)	Machuca et al., 2007
Suillus bovinus	Mycelium	Culture	-	Organic acids (oxalate, acetate)	Ping Sun et al., 1999
Suillus collinitus	Mycelium	Culture	Orthophosphate	Proton, Organic acids (oxalate)	Arvieu et al., 2003
	Seedlings	Culture	-	Proton	Rigou et al., 1995
Suillus granulatus	Seedlings	Forest soil (E horizon)	-	Organic acids (oxalate, citrate, formate)	van Hees et al., 2005
	Mesh bags	Culture	Apatite	Organic acids (oxalate)*	Wallander et al., 2003

However, these elements stored in mineral particles are not readily available for microorganisms. ECM fungi, as many bacteria and plants, need to release them through complexolysis and acidolysis. Complexolysis is a variant of hydrolysis where negatively charged molecules attach to mineral cations through electrostatic and covalent forces (Haas et al., 2006). This reaction leads to the solubilization of nutrients from the mineral surface. Acidolysis (i.e the destructuration of the crystallin structure of the mineral via the action of protons) also releases mineral nutrients in the soil solution. Organic acids of low molecular weight (LMWOAs) are considered as the main agents of mineral dissolution, because of their dual acidifying and complexing properties (Ochs et al., 1994; Barker et al., 1998). A significant part of the LMWOAs in soils are components of the root exudates *sensu lato* (Jones, 1998), suggesting an important role of the fungal symbionts in the case of ectomycorrhizal trees. Oxalate is one of the most widespread and abundant organic acids in forest soils (Jones et al., 2003). The secretion of oxalate has been reported for various ECM species (Table 1), such as *Paxillus involutus*, *Piloderma spp.*, and *Suillus spp*., contrary to *Hebeloma cylindrosporum* and *Amanita muscaria* that do not produce this acid (Casarin et al., 2003; Rosling et al., 2004). Because they do not produce oxalate crystals in presence of Ca, the ECM fungi *Tylospora fibrillosa, Cenococcum geophilum* and *Thelephora terrestris* are also suspected not to produce oxalate. However, there is a high diversity of ECM fungi for which oxalate production was observed in pure culture, in symbiotic association with seedlings or as hyphal mats or ectomycorrhizal root tips root tips in forest soils (Table 1). Oxalate production is also associated with increased dissolution of common soil minerals such as apatite (Wallander, 2000a; Wallander et al., 2003), biotite (Wallander, 2000b), phlogopite (Paris et al., 1996) and microcline (Wallander & Wickmann, 1999). Even if many publications deal with this subject, it is hard to compare the quantities of oxalate secreted by ECMs because of the different experimental conditions used. Most experiments revealed

Table 1: List of mycorrhizal fungi described for their ability to solubilize minerals (part 3)

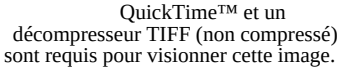
Ectomycorrhizal species	Type of experiment	Fungal origin	Mineral source	Mechanism of mineral dissolution involved	References
Suillus luteus	Mycelium	Culture	-	Organic acids (oxalate, citrate, succinate, malonate), Siderophores (catecholates, hydroxamates)	Machuca et al., 2007
Suillus variegatus	Seedlings	Culture	Biotite, Apatite	Organic acids (oxalate, citrate)	Wallander & Wickman, 1999Ź; Wallander, 2000
Thelephora terrestris	Mesh bags	Forest soil	Apatite	Organic acids (oxalate)*	Wallander et al., 2003
Tylospora fibrillosa	Mycelium	Culture	Apatite (TCP)	Organic acids (oxalate)*	Mahmood et al., 2001
Xerocomus sp.	Ectomycorrhiza	Forest soil	-	Organic acids (oxalate)	Rineau et al., 2008
Unidentified	Forest soil	Forest soil	Apatite	Mineral dissolution	Blum et al., 2002
	Forest soil	Forest soil	-	Siderophores (Hydroxamates)	Ess¸n et al., 2006 Holmstrom et al., 2004
	Forest soil (Podzol)	Forest soil	Biotite, Hornblende	Tunnelling	Hoffland et al., 2002
	Soil along a slope	Forest soil	Feldspar	Tunnelling	Hoffland et al., 2003
	Mesh bags	Forest soil	Apatite, Biotite	Mineral dissolution	Wallander & Hagerberg, 2004
	Mesh bags	Forest soil	Apatite, Biotite	Organic acids (oxalate)*	Wallander et al., 2002
	Mesh bags	Forest soil	Microcline, Biotite	Mineral dissolution	Wallander et al., 2006
	Seedlings	Forest soil	Biotite limed soil	Organic acids (oxalate)	Wallander, 2000b

*presence of oxalate crystals

oxalate concentrations ranging from 10 to 100 μM. But many of these measurements were integrated over the whole root system of young seedlings and for periods over 24h, and thus highly underestimated the real concentrations in the vicinity of the root tips or mycelium patches, where short-lifespan molecules as oxalate are mainly released (Jones, 1998; Wallander, 2006). However, if oxalate is the most abundant organic anion detected, it is not the only one secreted by ECM fungi. A wide range LMWOAs such as citrate, formate, malate, malonate or succinate are produced by many ECMs such as *Paxillus involutus, Suillus spp., Pisolithus tinctorius* and *Scleroderma verrucosum*, and can also act as mineral complexing agents (Wallander & Wickman, 1999; van Hees et al., 2003; van Hees et al., 2005; Tahara et al., 2005; Machuca et al., 2007 ; see also Figure 2).

Fungi have developed, as well as many bacteria, a specialized type of complexation by siderophores for the uptake of ferric iron (Fe^{3+}) which is essential for various cell processes like respiration, DNA synthesis, metabolism, and is also an essential cofactor for catalases, cytochromes and peroxidases (Neilands, 1995). Most of the soil ferric iron is complexed in mineral particles. Fungi, and in particular ECM ones, produce siderophores belonging only to the hydroxamate family (Szaniszlo et al., 1981; Watteau & Berthelin, 1994; Machuca et al., 2007). Evidence of siderophore production by ECM fungi has been reported for mycelia of *Suillus granulatus, Hebeloma crustuliniforme, Pisolithus tinctorius, Amanita muscaria, Boletus edulis, Suillus brevipes, S. punctipes, S. tomentosus, S. luteus, Cenococcum geophilum* and *Rhizopogon luteolus*, and for ectomycorrhizal root tips of *Xerocomus sp.* and *Lactarius subdulcis* (Szaniszlo & Powell, 1981; Haselwandter & Winkelmann, 2002; van Hees et al., 2006c; Machuca et al., 2007; Rineau et al., 2008, see chapter 3, table 2). Siderophores have very high affinity constants for iron but are produced in very small amounts by ECMs: ferricrocin, an hydroxamate siderophore, was produced in very low concentrations by *Cenococcum geophilum* and *Hebeloma crustuliniforme* (Haselwandter &

Figure 1. Some of the main molecules secreted by ECM fungi which dissolve minerals. ECM species which were reported to secrete them are written in italic below each molecule.



Winkelmann, 2002; van Hees et al., 2006c). Finally, nanomolar concentrations of two siderophores, ferrichrome and ferricrocin, were assumed to be produced by unidentified ECM species in the upper layers of a forest soils (Hölmström et al., 2004; Essén et al., 2006).

Organic anions can also complex ferric iron, but probably play a less important role than siderophores in iron uptake. To date, a number of approximately 30 species of ectomycorrhizal fungi have been tested for their ability to dissolve minerals. Sixteen ECM species were found to have a significant organic acid production, belonging to the genera *Paxillus, Suillus, Hebeloma, Piloderma, Pisolithus, Lactarius,* and *Cortinarius*. In contrast no organic acid production was observed for some ECM species belonging to the *Tylospora, Cenococcum , Thelephora , Hebeloma and Amanita genera*. There is no report of any ECM species that does not significantly complex iron. Nevertheless, all these data have been obtained with fungi in pure cultures or in pot experiments, and probably differ from what really happens in forest soils. Measurements in mesh bags with minerals-tests revealed significant mineral dissolution rates due to ECM fungi in a boreal podzol (Wallander et al., 2002, 2003, 2006; Wallander & Hagerberg, 2004). Other field studies revealed significant organic acid or siderophore production by hyphal mats of ECMs (Cromack et al., 1979; Griffiths, 1994) or freshly excised ectomycorrhizal root tips (Rineau et al., 2008, chapter 3; Blum et al., 2002; Hölmström et al., 2004; Essén et al., 2006).

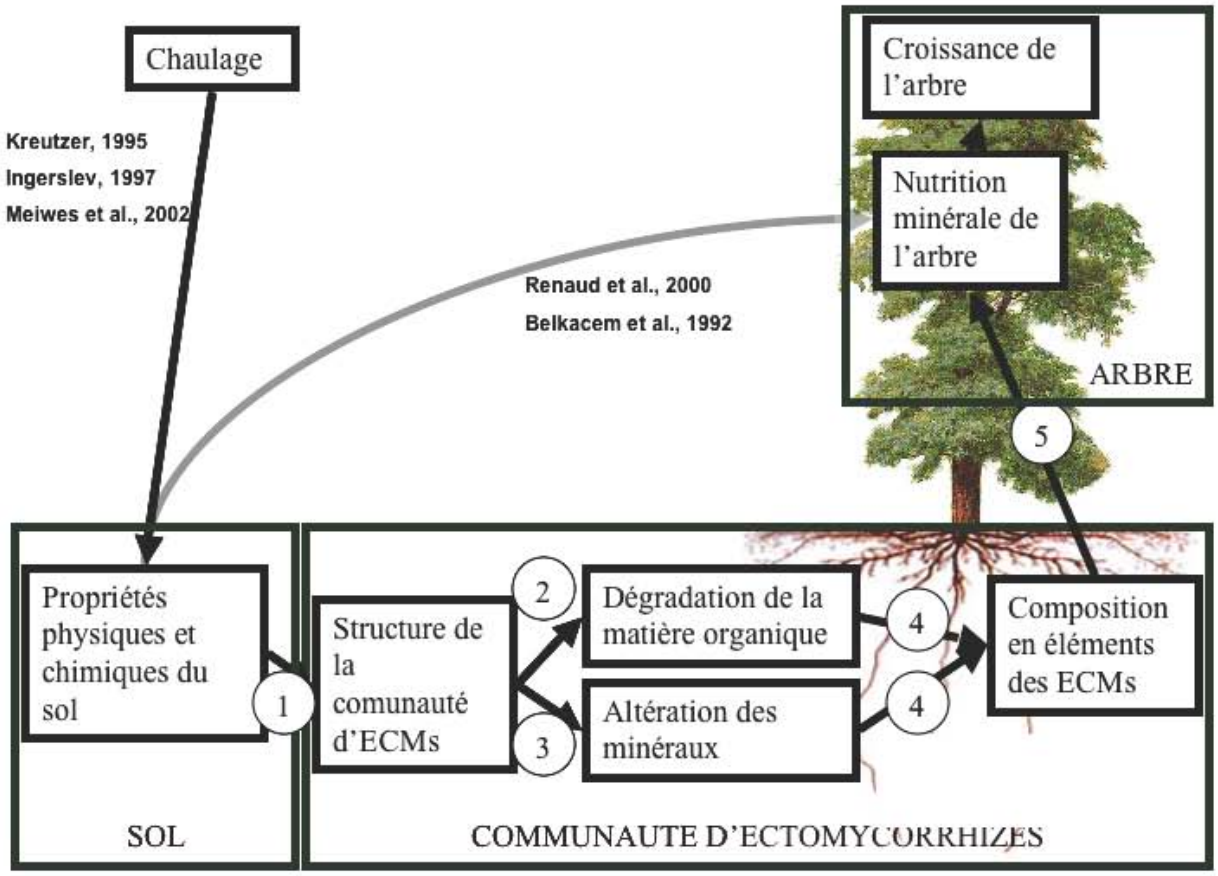


Figure 2: Schéma des effets du chaulage sur les caractéristiques chimiques du sol, les communuatés d'ECMs et la nutrition des arbres. Les numéros entourés d'un cercle correspondent aux différents chapitres de la thèse.

Objectifs de la thèse

Il est maintentant bien établi que le chaulage en forêt restaure la nutrition des arbres en Ca et Mg sur sol acide. De plus, le chaulage modifie profondément les propriétés physiques et chimiques du sol, la matière organique, la composition en minéraux du sol, la flore et la faune. Sachant que les propriétés chimiques du sol, en particulier le pH, influencent considérablement l'écologie des ECMs (van der Heijden & Sanders, 1998), nous nous sommes d'abord intéressés à l'influence de cette augmentation du pH et de la disponibilité de certains nutriments sur la structure de la commuauté d'ECMs, du point de vue à la fois des carpophores et des apex mycorrhiziens (chapitre 1). Le chaulage modifie aussi la composition de la matière organique, et les ECMs sont capables d'accéder aux nutriments enfermés dans la matière organique via la sécrétion d'enzymes hydrolytiques. Par conséquent, nous nous sommes intéressés à savoir si la capacité des ECMs à dégrader la matière organique a pu être modifiée par le chaulage, et si une modification de la strucuture de la communauté d'ECMs pouvait être accompagnée d'un changement dans son fonctionnement (chapitre 2). Les ECMs jouent aussi un rôle important dans l'assimilation de nutriments inorganiques par les arbres, via la dissolution des minéraux. En considérant que le chaulage augmente la disponibilité de certains nutriments comme Ca et Mg, nous nous sommes posés la question des effets de cette pratique forestière sur la dégradation des minéraux en mesurant la capacité des ECMs de hêtre à sécréter de l'oxalate (agent majeur de l'altération des minéraux par les champignons) et à chélater du fer (microélément dont la récupération implique la sécrétion de molécules spécialisées, appelées sidérophores) (chapitre 3). De plus, la composition en éléments des ECMs peut être le reflet de l'assimilation effective des nutriments par les ECMs. Nous avons donc vérifié si cette modification de l'altération potentielle des minéraux était suivie par un changement significatif dans la composition en éléments dans le manteau des ECMs de l'espèce caractérisée par son très haut potentiel de sécrétion d'oxalate (chapitre 4). Pour finir,

nous avons tenté de mettre en perspective les résultats obtenus sur les communautés d'ectomycorrhizes avec d'autres résultats sur le sol et la nutrition des arbres, afin de vérifier l'hypothèse selon laquelle le chaulage, en plus de l'apport direct de Ca et du Mg, améliorerait indirectement la nutrition de l'arbre en modifiant la commnauté d'ECMs.



Fruiting bodies of *Russula brunneoviolacea* growing in the beech limed forest stand in Humont. (photo : F. Rineau)

The forest tree decline in the 1980's

At the beginning of the 1980's, many forests of central europe were confronted with symptoms of needle yellowing and defoliation. Immediately attributed to acid rain and thus to industrial pollution, this forest decline was widely covered by the media, and has been a subject of sharp debate for the scientific community for a decade, until liming was proved to be an efficient way to restore tree health.

First symptoms appeared in czechoslovakian spruce forests damaged by acid rain because of the high quantities of SO_2 produced by lignite combustion (Bonneau, 2007). These symptoms gradually reached Bavaria, Ardennes (Nys, 1989) and then Schwartzwald and the Vosges, and were attributed to SO_2 production in these industrial territories. In 1989, 37% of the 190 000 ha of softwood were affected by this forest disease (Richter & Nageleisen, 2007). Nevertheless, monitoring of air pollution in the Vosges revealed that the quantities of SO_2 were at least 2 times lower than the toxicity threshold (Bonneau, 2007). The cause of tree decline was thus not yet identified.

An alternative hypothesis was that defoliation was the consequence of 1976 drought. Indeed, spruce defoliation and needle yellowing were observed in dense tree stands growing on superficial substrates (i.e mountain soils). But the delay of several years between drought and tree phenology impeded prevented from establishing clear relationships between the two phenomenons (Lévy & Becker, 1987).

A third possible cause of this forest decline was identified at the same time. Foliar analyses of yellowing spruces in Schwartzwald revealed strong deficiencies in magnesium (Zöttl & Mies, 1983). Tree magnesium deficiency causes yellowing of the needle tip or yellowish spots between veins, with brutal transition to the green colour, followed by brownish colour or by desiccation of the needle (Bonneau, 1995). These foliar nutrient imbalances were also correlated with soil exchangeable calcium and magnesium. In the same way, deperishment

was more frequent in nutrient-poor sandstone and granite bedrocks (Landmann & Nageleisen, 2001; Nechwatal & Oswald, 2003). Moreover, the natural rain acidity in the Vosges forests (pH between 4.3 and 5.4) leads to the drainage of divalent cations linked to soil colloids and their replacement by aluminium, certain forms of which are toxic for plants. Many of these deperishing forest stands had cation losses of 2% of Mg and 0.5% of Ca per year (Dupouey et al., 1998). Analyses of time series also demonstrated a progressive soil acidification (Göttlein, 1992; Hallbäcken and Tamm, 1986).

The cause of forest decline was thus attributable to a combination of these three factors. Actually, most of the Mg in young needles comes from surface humus mineralization. A drought stops this mineralization and the required Mg is transferred from 1-year-old needles to the younger ones. Moreover, in acidic soils, mineral layers have very low retention abilities of Mg derived from humus. Thus, the 1976 drought caused strong Mg deficiencies in the young needles and mineral nutrition was declined in the following years because of the progressive loss of Mg from the soil due to acid rain and soil acidification (Bonneau, 2007).

Forest liming as a method for restoring the health of declining forest stands

Calci-Magnesian amendments are used as a forest management practice to compensate for cation depletion by spreading Ca and Mg directly over forest soils. Calcium and magnesium can be applied as pure compounds or mixes between $CaCO_3$, $Ca(OH)_2$, CaO, $CaSO_4$ or $MgSO_4$, depending on the chemical properties of the forest soil (Bonneau, 1995). Lime can be applied by hand, by towed blowers or by helicopter (Bonneau, 1995) and has routinely been used in Germany in the mid 1980's at the doses of 3 to 4 t/ha (Worken & Brumme, 1997). To date, such liming has been relatively rare in France and limited to forest experiments, especially in the Ardennes, Vosges (Renaud et al., 2000) and Normandie. In all cases, lime

application restored tree mineral nutrition on a long term basis. The effects of calcareous amendments on forest soils are now well documented and can be classified into 6 categories:

*_impact on soil biology_: Biological modifications are concentrated into the upper soil layers, where liming has more influence on soil acidity. Increased respiration is observed in humus. Earthworm abundance, bacterial biomass and microbial NH_4 and NO_2 oxidizers are stimulated by liming (Anderson, 1998), at the detriment of fungi (Kreutzer, 1995). Nevertheless, liming can cause an increase of root rot, decrease mycorrhizal vitality, and shifting the structure of root system, towards a slight reduction of fine root density in the topsoil layer and an increase of the root density in the 5-15cm. The root turnover is also reduced after liming. The nematode community tends to shift from a mycophagous to a bacteriophagous one (Arpin, 2007).

*_impact on soil chemistry_: Liming increases the pH of soil solutions in the upper layers and induces the deprotonation of -COOH groups of organic humic compounds (Kreutzer, 1995). It leads to higher cation exchange capacity and thus Ca and Mg retention in soils colloids. The replacement of the detrimental acidic cation Al^{3+} by Ca^{2+} and Mg^{2+} in the ion exchange complex and its elimination from the upper layers by leaching reduces exchangeable acidity, concentration of exchangeable Al^{3+} and thus Al toxicity (Ingerslev, 1997). Liming reduces the soils solution concentration of Mn, Cd and Zn due to their precipitation as oxides, and increased the total soil concentration of Fe and Cu, which form stable complexes with organic matter. The change in soil chemistry could be accompanied by nitrate leaching in populations which are not able of revitalization. The mineralization rates increase with biological activity (Huber et al., 2006).

*_soil structure_: Liming, by stimulating biological activity, and especially earthworms, which incorporate organic matter to a depth of 20cm (Judas et al., 1997), increases top soil aeration

and thus improves soil structure and soil water retention capacity (Schack-Kirchner & Hildebrand, 1998).

**humus*: calcareous amendments transform mor or moder into mull-type humus (Kreutzer, 1995; Renaud et al., 2000).

**tree nutrition*: The nutritional status of limed trees, as shown by the concentrations of elements in leaves or needles, indicates improved Ca (Huber et al., 2006) or Ca-Mg nutrition (Belkacem et al., 1992; Renaud et al., 2000).

**vegetation*: Liming reduces the germination rate of acidophilic species but stimulated that of widespread ones (Olsson & Kelner, 2002). The ground flora, including the moss community, shifts from acidophilic to more nitrophilic in limed areas (Hallbäcken & Zhang, 1998), and this effect is dose-dependant (Dulière et al., 2000). However, when liming is provided at lower doses, the acidophilic species are conserved.

Liming is now once again a topical subject for forest management as a tool to improve the production of wood biomass, because of its mild impact on the diversity of forest ecosystems, and its relatively low cost (around 9% of the cost of the total sylviculture costs: Bonneau, 1995).

The ectomycorrhizal fungi

The fine roots of most of the tree of economical interest in the temperate and boreal forests (pine, fir, oak, beech, spruce) are symbiotic with a large group of Asco- and Basidiomycete fungi. The fungal partner forms with the root a mixed organ called ectomycorrhiza (ECM), characterized by a mycelial mantle which covers the root tip, and more diffused emanating mycelium (Smith & Read, 1997). The symbiotic association allows to explore a higher soil volume via the extraradical mycelium, and transfers water and nutrients to the root (Garbaye

and Guehl, 1997). The same tree can be associated with many ECM species, and all of those are more or less specialized and complementary for nutrient uptake and translocation. The functional diversity of the ECM community is thus a critical parameter for the ability of the tree population to forage for limiting soil resources and to adapt to environmental disturbances.

Ectomycorrhizal fungi assimilate C, N and P compounds, and nutrients available in soil as Ca, Mg, K and translocate them to the roots through the extensive vegetative mycelium (Smith & Read, 1997). ECMs are obviously involved in nutrient uptake by the tree because they cover the majority of fine roots where the tree absorbs nutrients, and thus all nutrients have to transit through fungal mantle, via active or passive mechanisms. These nutrients are sometimes sparingly available in temperate forest soils, and can be from organic (i.e included in organic matter) or inorganic (i.e contained in mineral particles) origin.

Organic nutrient uptake by ECMs

In forest soils, the most abundant source of nutrients is plant litter, composed of cellulose, hemicellulose and lignin, which encloses a high diversity of compounds containing a lot of elements required for plant nutrition. The degradation of these macromolecules is mostly achieved by fungi, through the secretion of powerful cell-wall bound hydrolytic enzymes (Kirk & Cullen, 1998; Cullen & Kersten, 2005), and, to a lesser extent, bacteria (Gramss et al., 1999). Saprotrophic fungi are not the sole organisms involved in nutrient cycling in forest soils: ECM fungi also play a major role and present high similarities with saprotrophs in their active foraging for nutrients (Leake & Boddy, 2002). Indeed, ECMs are capable of producing cell-wall bound enzymes to scavenge nutrients from organic matter (Dighton, 1983), even if in lower quantities than saprotrophic fungi. In comparison with leaf saprotrophic basidiomycetes, lower cellulase, cellobiohydrolase and similar phosphomonoesterase

activities were produced by ECM fungi in pure cultures (Colpaert & Van Laere, 1996). In the same way, ECMs produced low but significant activities of phenol-oxidases compared with saprotrophic fungi (Bending & Read, 1997). Moreover, there is obvious evidence of polyphenol-oxidases (PPO) secretion by ECMs (Burke & Cairney, 2002). Moreover, comparing DNA sequences between ECMs and white-rot fungi revealed that the genes coding for ligninolytic enzymes (lignin and manganese peroxidases, laccase multicopper oxidases) were widespread in a large taxonomic range of ECM fungi (Chen et al., 2001 ; Luis et al., 2004). The ECM fungi are also involved in cuticle, plant cell wall, phenol and tannin degradation (Caldwell et al., 1991; Bending & Read, 1997; Günther et al., 1998). These results suggest that ECM fungi express many 'decomposing abilities' (Read & Perez-Moreno, 2003).

Among enzymes produced by ECM fungi, endoglucanases, cellobiohydrolases, and ß-glucosidases can convert cellulose into glucose. Endoglucanases hydrolyses internal bonds in cellulose chains. Cellobiohydrolase continues the hydrolysis progressively at both ends of existing chains and those created by endoglucanases, and forms cellobiose, a molecule composed of two glucoses linked in ß 1-4. ß-glucosidases cleaves the cellobiose into two glucoses (Kirk & Cullen, 1998). The degradation of hemicellulose is more complex and involves eight different enzyme families depending on the molecule forming the unit of the hemicellulose polymer. Concerning lignin degradation, the lignin-degrading systems needs powerful oxidative enzymes because of C-C links and ether bonds. The three main groups of lignin degrading enzymes are lignin peroxidases, manganese peroxidases and laccases (Kirk & Cullen, 1998). Genes coding for these three enzyme families and close to those of the white-rot fungi *Phanerochaete chrysosporium* (lignin peroxidase) and *Pleurotus ostreatus* (manganese peroxidase) are present in the genome of the ECM fungus *Laccaria bicolor*, suggesting the potential ability of this fungus to degrade lignin. Polyphenol oxidase activities

have been detected in pure cultures of the ECM fungi *Suillus bovinus*, *Pisolithus tinctorius*, and *Paxillus involutus* (Burke & Cairney, 2002) and manganese peroxidase in *Tylospora fibrillosa* (Chambers et al., 1999).

The low availability of N is a characteristic of forest ecosystems dominated by ECM trees (Smith & Read, 1997). Moreover, the concentration of the soluble forms of N in soils (ammonium and nitrate) is very low; more than 95% of the soil N is in organic form in temperate forest ecosystems (Chalot & Brun, 1998). The ECM symbiosis is thus of critical importance for tree N nutrition because ECMs can have access to N from the two main organic sources abundant in forest humus: chitin and proteins. Protease activities have been reported for some ECM species: *Suillus variegatus, S. bovinus, Piloderma croceum, Pisolithus tinctorius, Cenococcum geophilum, Amanita rubescens, Lactarius subdulcis, Hebeloma crustuliniforme, Paxillus involutus,* and *Thelephora terrestris* (Smith & Read, 1997). Proteases includes a variety of enzymes: those secreted by the ECM fungus *Cenococcum geophilum* belong to the serine protease family. In the same way, chitnolytic enzymes render available organic N incorporated into fungal or insect chitin (chain of N-acetylglucosamine, containing one atom of N per monomer) (Lindahl & Finlay, 2006), and N-acetyl-glucosaminidase activities have already been reported for excised ECM root tips (Pritsch et al., 2004).

Enzyme assays using fluorescent or colorimetric substrates have been developed in order to measure enzyme activities of excised ECMs (Courty et al., 2005) or *in situ* in root systems (Dong et al., 2007).

Table 1: List of mycorrhizal fungi described for their ability to solubilize minerals

Ectomycorrhizal species	Type of experiment	Fungal origin	Mineral source	Mechanism of mineral dissolution involved	References
Boletus edulis	Mycelium	Culture	-	Siderophores (hydroxamates)	Szaniszlo et al., 1981
Cenococcum geophilum	Mycelium	Culture	Vermiculite, Phlogopite	Siderophores (ferricrocin) Proton, Organic acids (oxalate)	Yuan et al., 2004 Haselwandter & Winkelmann, 2002
Cortinarius glaucopus	Mycelium	Culture	Quartz, potassium feldspar, apatite, TCP, marble	Organic acids (oxalate)	Rosling et al., 2005
Gautieria monticola	Hyphal mats	Forest soil	-	Organic acids (oxalate)	Griffiths et al., 1994
Hebeloma crustuliniforme	Seedlings	Forest soil (E horizon)	-	Organic acids (oxalate, citrate, propionate) Siderophores (ferricrocin)	van Hees et al., 2006b, 2006c
Hebeloma longicaudum	Seedlings	Culture	-	Organic acids (oxalate)	van Schöll et al., 2006b
Hysterangium crassum	Hyphal mats	Forest soil	-	Organic acids (oxalate)	Cromack et al., 1979
Hysterangium setchelli	Hyphal mats	Forest soil	-	Organic acids (oxalate)	Griffiths et al., 1994
Laccaria bicolor	Mycelium	Culture	-	Organic acids (oxalate)	Cumming et al., 2001
Lactarius subdulcis	Ectomycorrhiza	Forest soil	-	Organic acids (oxalate)	Rineau et al., in press
Paxillus involutus	Mycelium	Culture	Orthophosphate	Proton, Organic acids (oxalate)	Arvieu et al., 2003
			-	Organic acids (oxalate)	Lapeyrie et al., 1987 van Schöll et al., 2006b
			Phlogopite, Quartz, potassium feldspar, apatite, TCP, marble	Organic acids (oxalate)	Paris et al., 1996Ź; Rosling et al., 2005
		Forest soil (E horizon)	-	Organic acids (oxalate, citrate, formate, malonate)	van Hees et al., 2003, 2005
		Culture	Biotite, Microcline	Organic acids (oxalate, citrate)	Wallander & Wickman, 1999
Piloderma byssinum	Mycelium	Culture	Quartz, potassium feldspar, apatite, TCP, marble	Organic acids (oxalate)	Rosling et al., 2005
Piloderma croceum	Mycelium	Culture	Apatite (TCP)	Organic acids (oxalate)	Mahmood et al., 2001
	Seedlings	Culture	-	Organic acids (oxalate)*	van Schöll et al., 2006b

Inorganic nutrient uptake by ECMs (contribution to a multi-author review in preparation for Soil Biology and Biochemistry)

Besides nitrogen, a major tree-growth limiting element (Aerts, 2002), forest trees also need inorganic nutrients of mineral origin such as $PO4^{3-}$, Ca^{2+}, Mg^{2+}, Fe^{3+}, K^{+}. Ectomycorrhizal fungi contribute significantly to the uptake of these ions (Smith & Read, 1997), most of which derive from the dissolution of soil mineral particles: apatite contains phosphorus, feldspars, micas and hornblendes provide Ca, Mg and K, while Fe^{3+} is found in mica and in secondary minerals such as iron oxides (Landeweert et al., 2001). The ability of the ECM fungi to dissolve minerals was reported for identified and unidentified mycorrhizal isolates (Table 1; Paris et al., 1996; Mahmood et al., 2001). Pure cultures of some of these fungal species appeared capable of dissolving tri-calcic phosphorus or biotite. Evidence of the fungal impact on the dissolution of minerals was also described for seedlings infected by ECM fungi. The transformation of chlorite and mica into clay minerals, as well as the dissolution of apatite, was reported for seedlings infected with *Piloderma croceum* and *Suillus variegatus*, respectively (Arocena et al., 2004; Wallander, 2000a). In the same way, an experiment with tree seedlings showed that the ECM fungus *Paxillus involutus* significantly contributed to the dissolution of muscovite (source of K) and increased root potassium contents (van Schöll et al., 2006a). Interestingly, ectomycorrhizal fungi associated with seedlings have also been shown to recover phosphorus from apatite (Wallander et al., 2000a) and potassium from biotite and microcline (Wallander & Wickmann, 1999).

The relevance of these mechanisms at the ecosystem scale has been stressed by Blum et al. (2002) who showed that a large part of the calcium used by the trees was mobilized from apatite by ECM fungi. Another manifestation of fungal mineral dissolution could be the formation of smooth tunnels of constant diameter in feldspar and hornblende grains (Hoffland

Table 1: List of mycorrhizal fungi described for their ability to solubilize minerals (part 2)

Ectomycorrhizal species	Type of experiment	Fungal origin	Mineral source	Mechanism of mineral dissolution involved	References
Piloderma fallax	Mycelium	Culture	Quartz, potassium feldspar, apatite, TCP, marble	Organic acids (oxalate)	Rosling et al., 2005
Piloderma spp.	Mycelium	Culture	Apatite (TCP)	Organic acids (oxalate)*	Mahmood et al., 2001
Pisolithus spp.	Mycelium	Culture	Vermiculite Phlogopite	Proton, Organic acids (oxalate)	Yuan et al., 2004
Pisolithus tinctorius	Mycelium	Culture	-	Organic acids (Oxalate, tartrate, formate, glycolate)	Cumming et al., 2001
		Culture	Apatite (TCP)	Mineral dissolution	Jayakumar & Tan, 2005
		Culture	Phlogopite	Organic acids (oxalate)	Paris et al., 1996
	Seedlings	Culture	-	Organic acids (citrate)	Tahara et al., 2005
Rhizopogon luteolus	Mycelium	Culture	-	Organic acids (oxalic acid, citric acid, succinic acid), Siderophores (catecholates, hydroxamates)	Machuca et al., 2007
Rhizopogon roseolus	Mycelium	Culture	Orthophosphate	Proton, Organic acids (oxalic acid)	Arvieu et al., 2003
	Seedlings	Forest soil (A horizon)	Soil + Hydroxyapatite or Calcium carbonate	Proton, Organic acids (oxalate)	Casarin et al., 2003
	Seedlings	Mediterranean chromic cambisol	Soil +/- CaCO3 and P	Proton, Organic acids (oxalate)	Casarin et al., 2004
Scleroderma verrucosum	Mycelium	Culture	-	Organic acids (oxalate, citrate, succinate), Siderophores (catecholates, hydroxamates)	Machuca et al., 2007
Suillus bovinus	Mycelium	Culture	-	Organic acids (oxalate, acetate)	Ping Sun et al., 1999
Suillus collinitus	Mycelium	Culture	Orthophosphate	Proton, Organic acids (oxalate)	Arvieu et al., 2003
	Seedlings	Culture	-	Proton	Rigou et al., 1995
Suillus granulatus	Seedlings	Forest soil (E horizon)	-	Organic acids (oxalate, citrate, formate)	van Hees et al., 2005
	Mesh bags	Culture	Apatite	Organic acids (oxalate)*	Wallander et al., 2003

et al., 2002). These tunnels are often colonized by fungal hyphae. Because feldspar tunnel density is positively correlated with ECM density, Hoffland et al. (2003) emitted the hypothesis that ectomycorrhizal hyphae were involved in mineral dissolution. Nevertheless, the contribution of tunneling to weathering is probably low because it occurs mainly in very old soils and for a very low fraction of total soil minerals (Smits et al., 2005).

However, elements stored in mineral particles are not readily available for microorganisms. ECM fungi, as many bacteria and plants, need to release them through complexolysis and acidolysis. Complexolysis is a variant of hydrolysis where negatively charged molecules attach to mineral cations through electrostatic and covalent forces (Haas et al., 2006). This reaction leads to the solubilization of nutrients from the mineral surface. Acidolysis (i.e the destructuration of the crystallin structure of the mineral via the action of protons) also releases mineral nutrients in the soil solution. Organic acids of low molecular weight (LMWOAs) are considered as the main agents of mineral dissolution, because of their dual acidifying and complexing properties (Ochs et al., 1994; Barker et al., 1998). A significant part of the LMWOAs in soils are components of the root exudates *sensu lato* (Jones, 1998), suggesting an important role of the fungal symbionts in the case of ectomycorrhizal trees. Oxalate is one of the most widespread and abundant organic acids in forest soils (Jones et al., 2003). The secretion of oxalate has been reported for various ECM species (Table 1), such as *Paxillus involutus*, *Piloderma spp*., and *Suillus spp*., in contrast to *Hebeloma cylindrosporum* and *Amanita muscaria* which do not produce this acid (Casarin et al., 2003; Rosling et al., 2004). Because they do not produce oxalate crystals in presence of Ca, the ECM fungi *Tylospora fibrillosa, Cenococcum geophilum* and *Thelephora terrestris* are also suspected not to produce oxalate. However, there is a high diversity of ECM fungi for which oxalate production was observed in pure culture, in symbiotic association with seedlings or as hyphal mats or ectomycorrhizal root tips root tips in forest soils (Table 1).

Table 1: List of mycorrhizal fungi described for their ability to solubilize minerals (part 3)

Ectomycorrhizal species	Type of experiment	Fungal origin	Mineral source	Mechanism of mineral dissolution involved	References
Suillus luteus	Mycelium	Culture	-	Organic acids (oxalate, citrate, succinate, malonate), Siderophores (catecholates, hydroxamates)	Machuca et al., 2007
Suillus variegatus	Seedlings	Culture	Biotite, Apatite	Organic acids (oxalate, citrate)	Wallander & Wickman, 1999Ź; Wallander, 2000
Thelephora terrestris	Mesh bags	Forest soil	Apatite	Organic acids (oxalate)*	Wallander et al., 2003
Tylospora fibrillosa	Mycelium	Culture	Apatite (TCP)	Organic acids (oxalate)*	Mahmood et al., 2001
Xerocomus sp.	Ectomycorrhiza	Forest soil	-	Organic acids (oxalate)	Rineau et al., 2008
Unidentified	Forest soil	Forest soil	Apatite	Mineral dissolution	Blum et al., 2002
	Forest soil	Forest soil	-	Siderophores (Hydroxamates)	Ess¸n et al., 2006 Holmstrom et al., 2004
	Forest soil (Podzol)	Forest soil	Biotite, Hornblende	Tunnelling	Hoffland et al., 2002
	Soil along a slope	Forest soil	Feldspar	Tunnelling	Hoffland et al., 2003
	Mesh bags	Forest soil	Apatite, Biotite	Mineral dissolution	Wallander & Hagerberg, 2004
	Mesh bags	Forest soil	Apatite, Biotite	Organic acids (oxalate)*	Wallander et al., 2002
	Mesh bags	Forest soil	Microcline, Biotite	Mineral dissolution	Wallander et al., 2006
	Seedlings	Forest soil	Biotite limed soil	Organic acids (oxalate)	Wallander, 2000b

*presence of oxalate crystals

Oxalate production is also associated with increased dissolution of common soil minerals such as apatite (Wallander, 2000a; Wallander et al., 2003), biotite (Wallander, 2000b), phlogopite (Paris et al., 1996) and microcline (Wallander & Wickmann, 1999). Even if many publications deal with this subject, it is hard to compare the quantities of oxalate secreted by ECMs because of the different experimental conditions used. Most experiments detected oxalate concentrations ranging from 10 to 100 μM, values in accordance with the oxalate concentrations measured in soil solutions (Jones et al., 2003). But many of these measurements were integrated over the whole root system of young seedlings and for periods over 24h, and thus highly underestimating the real concentrations in the vicinity of the root tips or mycelium patches, where short-lifespan molecules as oxalate are mainly released (Jones, 1998; Wallander, 2006). However, if oxalate is the most abundant organic anion detected, it is not the only one secreted by ECM fungi. A wide range LMWOAs such as citrate, formate, malate, malonate or succinate are produced by many ECM species such as *Paxillus involutus, Suillus spp.*, *Pisolithus tinctorius* and *Scleroderma verrucosum*, and can also act as mineral complexing agents (Wallander & Wickman, 1999; van Hees et al., 2003; van Hees et al., 2005; Tahara et al., 2005; Machuca et al., 2007 ; see also Figure 2).

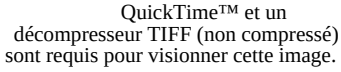
Fungi have developed, as well as many bacteria, a specialized type of complexation by siderophores for the uptake of ferric iron (Fe^{3+}), which is essential for various cell processes like respiration, DNA synthesis, metabolism, and is also an essential cofactor for catalases, cytochromes and peroxidases (Neilands, 1995). Most of the soil ferric iron is complexed in mineral particles. Fungi, and in particular ECM ones, produce siderophores belonging only to the hydroxamate family (Szaniszlo et al., 1981; Watteau & Berthelin, 1994; Machuca et al., 2007). Evidence of siderophore production by ECM fungi has been reported for mycelia of *Suillus granulatus, Hebeloma crustuliniforme, Pisolithus tinctorius, Amanita muscaria, Boletus edulis, Suillus brevipes, S. punctipes, S. tomentosus, S. luteus, Cenococcum*

geophilum and *Rhizopogon luteolus*, and for ectomycorrhizal root tips of *Xerocomus sp.* and *Lactarius subdulcis* (Szaniszlo & Powell, 1981; Haselwandter & Winkelmann, 2002; van Hees et al., 2006c; Machuca et al., 2007; Rineau et al., 2008, see chapter 3, table 2).

Siderophores have very high affinity constants for iron but are produced in very small amounts by ECMs: ferricrocin, an hydroxamate siderophore, was produced in very low concentrations by *Cenococcum geophilum* and *Hebeloma crustuliniforme* (Haselwandter & Winkelmann, 2002; van Hees et al., 2006c). Finally, nanomolar concentrations of two siderophores, ferrichrome and ferricrocin, were assumed to be produced by unidentified ECM species in the upper layers of a forest soils (Hölmström et al., 2004; Essén et al., 2006).

Organic anions can also complex ferric iron, but probably play a less important role than siderophores in iron uptake. To date, a number of approximately 30 species of ECM fungi have been tested for their ability to dissolve minerals. Sixteen ECM species, belonging to the genera Paxillus, Suillus, Hebeloma, Piloderma, Pisolithus, Lactarius, and Cortinarius, were found to have a significant organic acid production. In contrast no organic acid production was observed for some ECM species belonging to the Tylospora, Cenococcum , Thelephora , Hebeloma and Amanita genera. There is no report of any ECM species that does not significantly complex iron. Nevertheless, all these data have been obtained with fungi in pure cultures or in pot experiments, and probably differ from what really happens in forest soils. Measurements in mesh bags with minerals-tests revealed significant mineral dissolution rates due to ECM fungi in a boreal podzol (Wallander et al., 2002, 2003, 2006; Wallander & Hagerberg, 2004). Other *in situ* studies revealed significant organic acid or siderophore production by hyphal mats (Cromack et al., 1979; Griffiths, 1994) or ectomycorrhizal root tips (Rineau et al., 2008, chapter 3; Blum et al., 2002; Hölmström et al., 2004; Essén et al., 2006).

Figure 1. The molecules secreted by ECM fungi which dissolve minerals. ECM species which were reported to secrete them are written in italic below each molecule.



Aims of the thesis

It is now well established that forest liming restores tree Ca and Mg nutrition in acidic soils. Moreover, liming profondly changes soil physical and chemical properties, as well as organic matter and soil mineral composition, and significantly modifies flora and soil fauna communities. As soil chemical properties, especially pH, considerably influence ECM ecology (van der Heijden & Sanders, 1998), we first wondered how increases in pH and nutrient availability can affect the ECM community structure. This question was addressed in chapter 1, using ECM root tips and fruiting bodies. Liming also modifies organic matter composition, and ECMs are able to access nutrients scavenged from organic matter via the secretion of hydrolytic enzymes. Thus, we wanted to know if the ECM ability to degrade organic material was modified by liming, and if a change in the ECM community structure was followed by a change in the functioning of the community. This question was addressed in chapter 2. The ECMs also play an important role in the uptake of inorganic nutrients by trees, through activities of mineral weathering. As liming increases the availability of many nutrients such as Ca and Mg, we addressed the question of the effects of this forest practice on mineral degradation by measuring the ability of beech ECMs to secrete oxalate (a major molecule of mineral weathering) and to chelate iron (a microelement, whose uptake involves the production of specialized molecules, called siderophores) in chapter 3. Moreover, the elemental composition of ECMs can reveal the effective nutrient assimilation by ECMs. In chapter 4, we investigated whether the change of nutrient mobilization after liming was followed by a significant change in elemental composition of one beech ECM morphotype characterized by its very high potential oxalate secretion, measured with microanalysis techniques. Finally, in chapter 5, we put all these results together in order to test the hypothesis that forest liming indirectly improves tree mineral nutrition by modifying the ECM community, in addition to directly providing extra Ca and Mg.

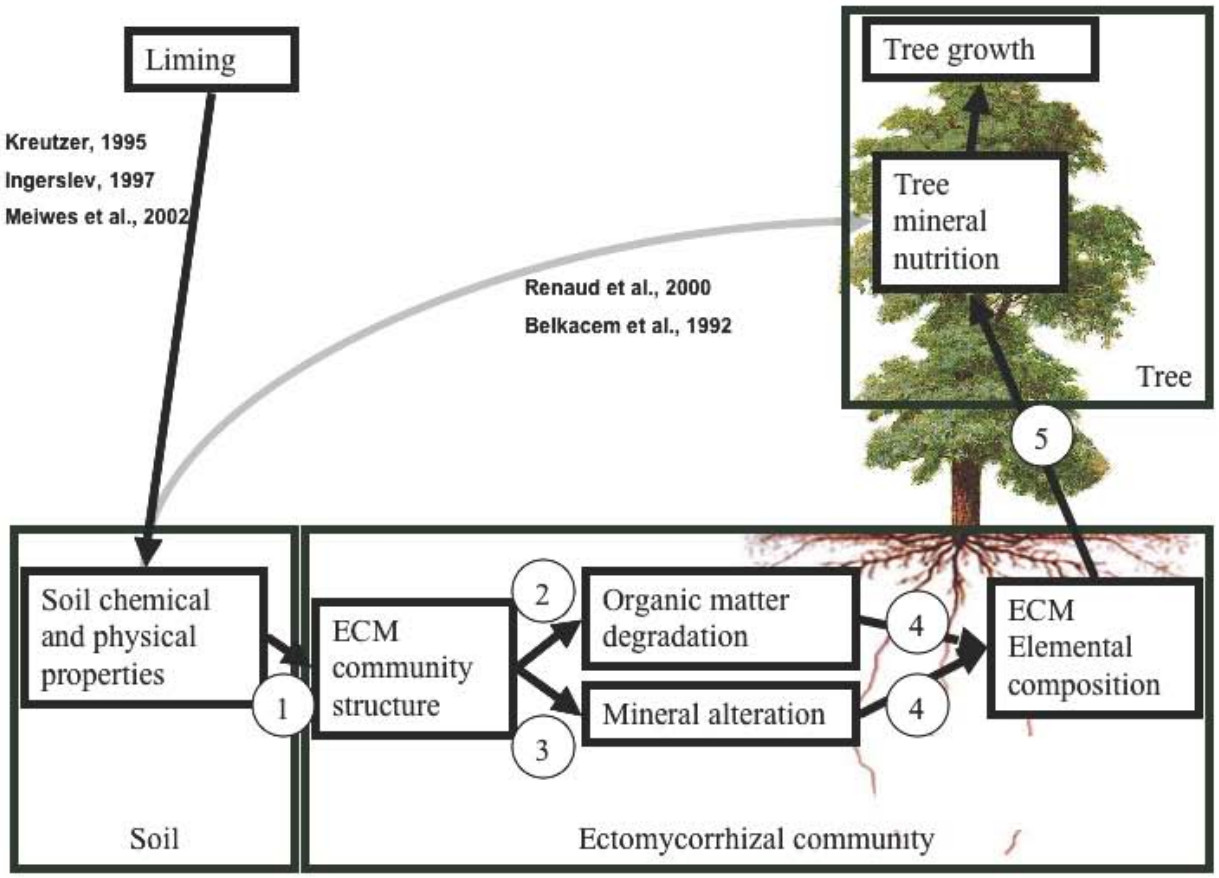


Figure 2: Diagram of the effects of liming on soil chemical composition, ECM communities, and tree nutrition. Encircled numbers refers to the chapters of the thesis.

References

Aerts, R., 2002. The role of various types of mycorrhizal fungi in nutrient cycling and plant competition. In Mycorrhizal ecology, Ecological studies, vol. 157, M.G.A. van der Heijden, I. Sanders (Eds.), Springer-Verlag Berlin Heidelberg, 470p.

Anderson, T. H., 1998. The influence of acid irrigation and liming on the soil microbial biomass in a Norway spruce (Picea abies [L.] K.) stand. Plant and Soil, 199 : 117-122.

Arocena JM, Gottlein A, Raidl S, 2004. Spatial changes of soil solution and mineral composition in the rhizosphere of Norway-spruce seedlings colonized by Piloderma croceum. Journal of Plant Nutrition and Soil Science, 4, 479-486.

Arpin, P., 2007. Influence d'un amendement calcique sur les peuplements de nématodes dans les massifs forestiers du PNR Normandie-Maine. Séminaire Sols forestiers acides: Amendements calciques et fonctionnement de l'écosystème, Carrouges, France.

Arvieu JC, Leprince F, Plassard C, 2003. Release of oxalate and protons by ectomycorrhizal fungi in response to P- deficiency and calcium carbonate in nutrient solution. Annals of forest science, 60, 815-821.

Barker, W.W., Welch, S.A., Banfield, J.F., 1997 Biogeochemical weathering of silicate minerals. In Geomicrobiology: Interactions between Microbes and Minerals. pp 391-428. Mineralogical Soc America, Washington.

Belkacem, S., Nys., C., Gelhaye, D., 1992. Effets d'une fertilisation et d'un amendement sur l'immobilisation d'éléments dans la biomasse d'un peuplement adulte d'épicéa commun (*Picea abies* L Karst). Annals of Forest Science 49, 235-252.

Bending, G. D., Read, D., 1997. Lignin and soluble phenolic degradation by ectomycorrhizal and ericoid mycorrhizal fungi. Mycological Research, 101: 1348-1354.

Blum JD, Klaue A, Nezat CA, Driscoll CT, Johnson CE, Siccama TG, Eagar C, Fahey TJ, Linkens GE, 2002. Mycorrhizal dissolution of apatite as an important calcium source in base-poor forest ecosystems. Nature, 417, 729-731.

Bonneau, M., 1995. Fertilisation des forêts dans les milieux tempérés. ENGREF, Nancy, 368p.

Bonneau, M., 2007. Le dépérissement des forêts des années 80 : Roche mère, pluies acides et climat. In « Forêt et pollution », Ed. Andrée Corvol., GHFF, Paris.

Borken, W., Brumme, R.,1997. Liming practice in temperate forest ecosystems and the effects on CO_2, N_2O and CH_4 fluxes. Soil use and management, 13: 251-257.

Lévy, G., Becker, M., 1987. Silver fir dieback in the Vosges, France: fundamental role of water deficits. Annales des Sciences Forestieres, 44 : 403-416.

Burke, R. M., Cairney, J. W. G., 2002. Laccases and other polyphenol oxidases in ecto- and ericoid mycorrhizal fungi. Mycorrhiza, 12: 105-116.

Caldwell, B. A., Castellano, M. A., Griffiths, R. P., 1991. Fatty acid esterase production by ectomycorrhizal fungi. Mycologia, 83 : 233-236.

Casarin V, Plassard C, Souche G, Arvieu JC, 2003. Quantification of oxalate ions and protons released by ectomycorrhizal fungi in rhizosphere soil. Agronomie, 23, 461-469.

Casarin V, Plassard C, Hinsinger P, Arvieu JC, 2004. Quantification of ectomycorrhizal fungal effects on the bioavailability and mobilization of soil P in the rhizosphere of Pinus pinaster. New Phytologist, 163, 177-185.

Chalot, M., Brun, A., 1998. Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas. FEMS Microbiology reviews 22: 21-44.

Chambers, S. M., Burke, R. M., Brooks, P. R., Cairney, J. W. G., 1999. Molecular and biochemical evidence for manganese-dependent peroxidase activity in Tylospora fibrillosa. Mycological research, 103 : 1098-1102.

Chen, D. M., Taylor, A. F. S., Burke, R. M., Cairney, J. W. G, 2001. Identification of genes for lignin peroxidases and manganese peroxidases in ectomycorrhizal fungi. New Phytologist, 152: 151-158.

Colpaert, J. V., Van Laere, A., 1996. A comparison of the extracellular enzyme activities of two ectomycorrhizal and a leaf-saprotrophic basidiomycete colonizing beech leaf litter. New Phytologist, 133: 133-141.

Courty, P.E., Pritsch, K., Schloter, M., Hartmann, A., Garbaye, J. 2005. Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. New Phytologist 167, 309-319.

Cromack K, Sollins P, Graustein WC, Speidel K, Todd AW, Spycher G, Li CY, Todd RL, 1979. Calcium oxalate accumulation and soil weathering in mats of the hypogeous fungus Hysterangium crassum. Soil Biology and Biochemistry, 11, 463-468.

Cullen D, Kersten PJ, 2004. Enzymology and molecular biology of lignin degradation. *The Mycota III : biochemistry and molecular biology*. Berlin Heidelberg : Springer-Verlag.

Dighton, J., 1983. Phosphatase production by mycorrhizal fungi. Plant and Soil, 71: 455-462.

Dong, S., Brooks, D., Jones, M. D., Grayston, S. J., 2007. A method for linking *in situ* activities of hydrolytic enzymes to associated organisms in forest soils. Soil Biology and Biochemistry, 30: 1-9.

Dupouey, J. L., Thimonier, A., Lefevre, Y., le Tacon, F., Bonneau, M., Dambrine, E., Poszwa, A., Landmann, G., 1998. Desaturation and nitrogen enrichment of forest soils in north-eastern France in the last few decades. Revue Forestiere Francaise 50, 5, 391-402.

Dulière, J.F., De Bruyn, R., Malaisse, F., 2000. Changes in the moss layer after liming in a Norway spruce (Picea abies [L.] Karst.) stand of Eastern Belgium. Forest Ecology and Management 136, 97-105.

Essen SA, Bylund D, Holmstrom SJM, Moberg M, Lundstrom US, 2006. Quantification of hydroxamate siderophores in soil solutions of podzolic soil profiles in Sweden. Biometals, 19, 269-282.

Garbaye, J., Guehl, J. M., 1997. The role of ectomycorrhizas in water use by forest trees. Revue forestière Française, 49: 110-120.

Göttlein, A., 1992. Changes of soil-pH in the forest district of Rothenbuch since 1982. Forstwissenschaftliches centralblatt, 111 : 169-178.

Gramss, G., Ziegenhagen, D., Sorge, S., 1999. Microbial ecology, 37: 140-151.

Griffiths RP, Baham JE, Caldwell BA, 1994. Soil solution chemistry of ectomycorrhizal mats in forest soil. Soil Biology and Biochemistry, 26, 331-337.

Günther, H., Perner, B., Gramss, G., 1998. Activities of phenol oxidizing enzymes of ectomycorrhizal fungi in axenic culture and in symbiosis with Scots pine (Pinus sylvestris L.). Journal of basic microbiology, 38 : 197-206.

Haas, J. R., Bailey, E. H., Purvis, O. W., 2006. Bioaccumulation of metals by lichens: Uptake of aqueous uranium by Peltigera membranacea as a function of time and pH. American mineralogist, 83 : 1494-1502.

Hallbäcken, L., Tamm, C. O., 1986. Changes in soil acidity from 1927 ti 1982-1984 in a forest area of south-west Sweden. Scandinavian Journal of Forest Research. 1 : 219-232.

Hallbäcken, L., Zhang, L. Q., 1998. Effects of experimental acidification, nitrogen addition and liming on ground vegetation in a mature stand of Norway spruce (Picea abies [L.])Karst.) in SE Sweden. Forest Ecology and Management 108, 201-213.

Haselwandter K, Winkelmann G, 2002. Ferricrocin-an ectomycorrhizal siderophore of Cenococcum geophilum. BioMetals, 15, 73-77.

Hoffland E, Giesler R, Jongmans T, van Breemen N, 2002. Increasing feldspar tunneling by fungi across a north Sweden podzol chronosequence. Ecosystems, 1, 11-22.

Hoffland E, Giesler R, Jongmans AG, Van Breemen N, 2003. Feldspar tunneling by fungi along natural productivity gradients. Ecosystems, 6, 739-746.

Holmstrom SJM, Lundstrom US, Finlay RD, van Hees PAW, 2004. Siderophores in forest soil solution. Biogeochemistry, 71, 247-258.

Huber C, Weiss W, Gottlein A, 2006. Tree nutrition of Norway spruce as modified by liming and experimental acidification at the Höglwald site, Germany, from 1982 to 2004. *Plant and Soil* **63**:861-869.

Ingerslev, M., 1997. Effects of liming and fertilization on growth, soil chemistry and soil water chemistry in a norway spruce plantation on a nutrient-poor soil in Denmark. Forest Ecology and Management, 92: 55-66.

Jayakumar P, Tan TK, 2005. Phosphorus solubilization by ectomycorrhizal Pisolithus tinctorius in pure culture and in association with Acacia mangium. Symbiosis, 39, 125-130.

Jones, D. L., 1998. Organic acids in the rhizosphere – a critical review. Plant and Soil 205, 1, 25-44.

Jones DL, Dennis PG, Owen AG, van Hees PAW, 2003. Organic acid behavior in soils-Misconceptions and knowledge gaps. Plant and Soil, 248. 31-41.

Judas, M., Schauermann, J., Meiwes, K. J., 1997. The inoculation of Lumbricus terrestris L. in an acidic spruce forest after liming and its influence on soil properties. Soil Biology and Biochemistry, 23: 677-679.

Kirk, T. K., Cullen, D., 1998. Enzymology and molecular genetics of wood degradation by white-rot fungi. In Environmentally friendly technologies for the pulp and paper industry, Eds. R. A. Young & M. Akhtar, John Wiley & Sons Inc, pp 273-307.

Kreutzer K, 1995. Effects of forest liming on soil processes. *Plant and soil* **168**:447-470

Landeweert R, Hoffland E, Finlay RD, Kuyper T, van Breemen N, 2001. Linking plants to rocks. ectomycorrhizal fungi mobilize nutrients from minerals. Trends in Ecology & Evolution 16, 248-254.

Lapeyrie F, Chilvers GA, Bhem CA, 1987. Oxalic acid synthesis by the mycorrhizal fungus Paxillus involutus (Batsch.Ex Fr.) Fr. New Phytologist, 106, 139-146.

Landmann G., Nageleisen L.M. (2001) - *La santé de la sapinière française, notamment vosgienne : bilan rétrospectif et analyse prospective.* In "le sapin : enjeux anciens, enjeux actuels", sous la direction d'Andrée Corvol, Groupe d'histoire des forêts françaises, Parc Naturel Régional des Ballons des Vosges, l'Harmattan, pp. 89-101.

Leake, J. R., Boddy, D. L., 2002. Interactions between ectomycorrhizal ad saprotrophic fungi. Mycorrhizal ecology, 157: 345-372.

Lindahl, B. D., Finlay, R. D., 2006. Activities of chitinolytic enzymes during primary and secondary colonization of wood by basidiomycetous fungi. New Phytologist, 169 : 389-397.

Luis, P., Walther, G., Kellner, H., Martin, F., Buscot, F., 2004. Diversity of laccase genes from basidiomycetes in a forest soil. Soil Biology and Biochemistry, 36: 1025-1036.

Machuca A, Pereira G, Aguiar A, Milagres AMF, 2007. Metal-chelating compounds produced by ectomycorrhizal fungi collected from pine plantations. Letters in Applied Microbiology, 44, 7-12.

Mahmood S, Finlay RD, Erland S, Wallander H, 2001. Solubilisation and colonisation of wood ash by ectomycorrhizal fungi isolated from a wood ash fertilised spruce forest. FEMS Microbiology Ecology, 35, 151-161.

Nechwatal, J., Oswald, W. F., 2003. Evidence for the involvment of biotic factors in the yellowing disease of Norway spruce (Picea abies L. Karst) at higher elevations of the Bavarian forest. Plant and Soil, 254: 403-414.

Neilands JB, 1995. Siderophores. Structure and fonction of microbial iron transport compounds. Jour. Biol. Chem, 270. 26723-26726.

Nys, C., 1989. Fertilizer application, forest decline and yield of Norway spruce (Picea abies) in the French Ardennes. Revue forestière française, 41 : 336-347.

Ochs M., Cosovic B., Stumm W., 1994. Coordinative and hydro-phobic interaction of humic substances with hydrophilic Al2O3 and hydrophobic mercury surfaces. Geochimica et Cosmochimica Acta, 58, 639–650.

Olsson BA, Kellner O, 2002. Effects of soil acidification and liming on ground flora establishment after clear-felling of Norway spruce in Sweden. *Forest Ecology and Management* **158**:127-139.

Paris F, Botton B, Lapeyrie F, 1996. In vitro weathering of phlogopite by ectomycorrhizal fungi .2. Effect of K+ and Mg2+ deficiency and N sources on accumulation of oxalate and H+. Plant and soil, 179, 141-150.

Ping Sun Y, Unestam T, Lucas SD, Johanson KJ, Kenne L, Finlay R, 1999. Exudation-reabsorption in a mycorrhizal fungus, the dynamic interface for interaction with soil and soil microorganisms. Mycorrhiza, 9, 137-144.

Pritsch K, Raidl S, Marksteiner E, Blaschke H, Agerer R, Schloter M, Hartmann A, 2004. A rapid and highly sensitive method for measuring enzyme activities in single mycorrhizal tips using 4-methylumbelliferone-labelled fluorogenic substrates in a microplate system. *Journal of Microbiological Methods* **58**:233-241.

Read, D. J., Perez-Moreno, J., 2003. Mycorrhizas and nutrient cycling in ecosystems-a journey towards relevance? New Phytologist, 157: 475-492.

Renaud, J.P., Picard, J.F., Richter, C., Nys, C. 2000. Restauration de sols forestiers acides par un amendement calco-magnesien. Cas du Massif Vosgien et de l'Ardenne. Rapport DERF–ONF–INRA, 39pp.

Richter, C., Nageleisen, L. M., 2007. Dépérissement forestier et amnedements : exemple du massif vosgien. Séminaire Sols forestiers acides: Amendements calciques et fonctionnement de l'écosystème, Carrouges, France.

Rigou L, Mignard E, Plassard C, Arvieu JC, Remy JC, 1995. Influence of ectomycorrhizal infection on the rhizosphere pH around roots of maritime pine (Pinus pinaster Soland in Ait.). New Phtologis, 130, 141-147.

Rineau, F., Courty, P. E., Uroz S., Buée, M., Garbaye, J., 2008. Simple microplate assays to measure iron mobilization and oxalate secretion by ectomycorrhizal tree roots. Soil Biology and Biochemistry, 40: 2460-2463.

Rosling A, Lindahl BJ, Taylor FS, Finlay R, 2004. Mycelial growth and substrate acidification of ectomycorrhizal fungi in response to different minerals. FEMS Microbiology Ecology, 47, 31-37.

Rosling, A., Finlay, R. D., 2005. Responses of ectopmycorrhizal fungi to mineral substrates. Geochimica et cosmochimica acta, 69 : 232-232.

Schack-Kirchner, H., Hildebrand, E. E., 1998.Changes in soil structure and aeration due to liming and acid irrigation. Plant and Soil, 199: 167-176.

Smith SE, Read DJ, 1997. *Mycorrhizal symbiosis*. Ed 2, Academic press, London.

Smits, M. M., Hoffland, E., Jongmans, A. G., van Breemen, N., 2005. Contribution of mineral tunneling to total feldspar weathering. Geoderma, 125: 59-69.

Szaniszlo PJ, Powell PE, Reid CPP, Cline GR, 1981. Production of hydroxamate siderophore iron chelators by ectomycorrhizal fungi. Mycologia, 73, 1158-1174.

Tahara K, Norisada M, Tange T, Yagi H, Kojima K, 2005. Ectomycorrhizal association enhances Al tolerance by inducing citrate secretion in Pinus densiflora. Soil science and plant nutrition, 51, 397-403.

van der Heijden, M. G. A., Sanders, I. R., 1998. Mycorrhizal Ecology. Ecological studies, vol. 157, M.G.A. van der Heijden, I. Sanders (Eds.), Springer-Verlag Berlin Heidelberg, 470p.

van Hees PAW, Godbold DL, Jentschke G, Jones DL, 2003. Impact of ectomycorrhizas on the concentration and biodegradation of simple organic acids in a forest soil. European journal of soil science, 54, 697-706

van Hees PAW, Jones DL, Jentschke G, Gobold DL, 2005. Organic acid concentrations in soil solution: effects of young coniferous trees and ectomycorrhizal fungi. Soil Biology and Biochemistry, 37, 771-776.

van Hees PAW, Rosling A, Finlay RD, 2006b. The impact of trees, ectomycorrhiza and potassium availability on simple organic compounds and dissolved organic carbon in soil. Soil Biology and Biochemistry, 38, 1912-1923.

van Hees PAW, Rosling A, Essen S, Godbold DL, Jones DL, Finlay RD, 2006c. Oxalate and ferricrocin exudation by the extramatrical mycelium of an ectomycorrhizal fungus in symbiosis with Pinus sylvestris. New Phytologist, 169, 367-377.

van Schöll L, Smits MM, Hoffland E, 2006a. Ectomycorrhizal weathering of the soil minerals muscovite and hornblende. New Phytologist, 171, 805-814.

van Schöll L, Hoffland E, van Breemen N, 2006b. Organic anion exudation by ectomycorrhizal fungi and Pinus sylvestris in response to nutrient deficiencies. New Phytologist, 170, 153-163.

Wallander H, 2000a. Uptake of P from apatite by Pinus sylvestris seedlings colonised by different ectomycorrhizal fungi. plant and soil, 218, 249-256.

Wallander H, 2000b. Use of strontium isotopes and foliar K content to estimate weathering of biotite induced by pine seedlings colonised by ectomycorrhizal fungi from two different soils. Plant and Soil, 222, 215-229.

Wallander H, Mahmood S, Hagerberg D, Johansson L, 2003. Elemental composition of ectomycorrhizal mycelia identified by PCR-RFLP analysis and grown in contact with apatite or wood ash in forest soil. FEMS Microbiology Ecology, 44, 57-65.

Wallander H, Hagerberg D, 2004. Do ectomycorrhizal fungi have a significant role in weathering of minerals in forest soil? Symbiosis, 37, 249-257.

Wallander H, Hagerberg D, Aberg G, 2006. Uptake of Sr-87 from microcline and biotite by ectomycorrhizal fungi in a Norway spruce forest. Soil Biology and Biochemistry, 38, 2487-2490.

Wallander H, Wickman T, 1999. Biotite and microcline as potassium sources in ectomycorrhizal and non-mycorrhizal Pinus sylvestris seedlings. Mycorrhiza, 9, 25-32.

Yuan L, Huang JG, Li Xl, Christie P, 2004. Biological mobilization of potassium from clay minerals by ectomycorrhizal fungi and eucalypt seedling roots. Plant and soil, 262, 351-361.

Zöttl, H. W., Mies, E., 1983. Nähelementversorgung und schastoffbelastung von fichtenökosystemen in Südschwarzwald unter immissionseinfluss. Mitteilungen der Duetschen bodenkundliche gesellschaft, 38: 429-433.

CHAPTER 1. CONSEQUENCES OF LIMING ON ECM COMMUNITY STRUCTURE

***Article 2** " Forest liming durably impact the communities of ectomycorrhizas and fungal epigeous fruiting bodies". Accepted in Annals of Forest Science.

***Article 3** " Effects of liming on ectomycorrhizal community structure in relation to soil horizons and tree hosts". Accepted in Fungal ecology.



Sporocarps of *Russula ionochlora* found in the spruce limed stand in Humont. (photo : J-P Maurice & F. Rineau)

Article 2 " Forest liming durably impact the communities of ectomycorrhizas and fungal epigeous fruiting bodies". Accepted in Annals of Forest Science.

Abstract:

- Liming is a forestry practice used to counteract forest decline in acidic soils. It consists of direct Ca and Mg input to forest soil, which restores tree mineral nutrition, but also modifies microbial communities in soil. The aim of this study was to assess the effects of liming on both belowground (ectomycorrhizal root tips) and aboveground (epigeous sporocarps) fungal communities.
- Results showed that the modification of soil chemical properties (pH, and Ca-Mg contents versus total free Al and Fe concentrations) was a stronger factor of ECM community structuring than tree host. The species appearing in limed plots were ubiquist or known as good competitors and replaced acidophilic and stress species.
- At the sporocarp level, tree host was a stronger factor of community structuring than soil chemical properties associated with liming. On the whole, there was a shift in the community composition from a typical acidophilic forest fungal community of medium altitude in the untreated plots to a less typical one, with the reduced dominance of acidophilic fungi while many late-stage forest species appeared.
- We finally suggest a marker species (*Russula ochroleuca*) to assess both above and belowground effects of liming on ectomycorrhizal communities.

Key words: **Mycorrhiza / Ecosystem management**

Résumé:

- Le chaulage est une pratique forestière utilisée pour restaurer la nutrition minérale des arbres apparaissant sur sol acide. Il consiste en un apport direct de Ca et Mg au sol forestier, ce qui restaure la nutrition minérale de l'arbre, mais aussi modifie les communautés microbiennes du sol. Cette étude évalue les effets du chaulage sur les communautés fongiques hypogées (apex ectomycorrhiziens: "ECM") et épigées (carpophores).
- Les résultats montrent que la modification des des propriétés chimiques du sol (pH et concentrations en Ca-Mg échangeables versus concentrations en Al et Fe échangeables) est un facteur de structuration de la communauté d'ECMs plus fort que l'arbre hôte. Les espèces qui sont apparues dans les placeaux chaulés sont ubiquistes ou compétitrices et ont remplacé des espèces acidophiles ou connues pour être associées à des conditions de stress.
- Concernant les carpophores, l'arbre hôte est un facteur de structuration de la communauté plus fort que les propriétés chimiques du sol associées au chaulage. Dans l'ensemble, on a observé une modification de la communauté fongique, passant d'une communauté typique de forêt acide de moyenne altitude dans les placeaux témoins vers une autre moins spécifique, caractérisée par une moindre dominance d'espèces acidophiles et l'apparition de nombreuses espèces de forêt mature.
- Nous suggérons enfin une espèce marqueur (*Russula ochroleuca*) qui permet d'évaluer facilement les effets du chaulage sur les communautés de champignons mycorrhiziens, aussi bien du point de vue des apex mycorrhiziens que des carpophores.

Mots-clés: **Mycorhize / Management des écosystèmes**

Introduction

Symptoms of forest decline due to soil acidification have been reported in many forests of central Europe for the past 20 years (Ulrich, 1983). Spruce needle yellowing and beech defoliation were consequences of cation starvation due to free calcium and magnesium losses in acidic soils with low weathering rates. Liming (i.e direct input of Ca and Mg in forest soils) proves to be efficient to restore tree mineral nutrition, and induces drastic and long-term changes in soil structure and chemical properties (Kreutzer, 1995). Calcareous amendments reduce the acidity of upper soil layers, increase cation exchange capacity and soil base saturation, especially in humus layers (Kreutzer, 1995 ; Renaud et al., 2000 ; Frank & Stuanes, 2003), and stimulates soil fauna (particularly earthworms) (Persson, 1988). Liming could also shift the humus type from mor to mull (Kreutzer, 1995).

Liming is now once again a topical subject for forest management as a tool to improve the production of wood biomass in nutrient-poor forest ecosystems. As this forest practice will be applied to large forest areas, ecological consequences of calcareous amendments are thus of critical importance. Renaud et al. (2000) showed increased plant species richness due to the apparition of meso-acidophilic to neutrophilic or nitrophilic species in limed spruce and fir forests. However, the abundance decrease of acidophilic species followed by appearance of widespread neutro- and nitrophilic ones could lead to a shift of patrimonial value of the ecosystem.

Because fungi are strongly influenced by soil organic matter and soil properties, the effect of liming on ectomycorrhizal (ECM) communities has already been surveyed. Liming enhanced sporocarp production of *Hygrophorus pustulatus* (Agerer et al., 1998) in spruce stands, and of *Lycoperdon gemmatum* in beech ones (Garbaye et al., 1979). Contrary to that, sporocarps of the acidophilic (but widespread) fungus *Russula ochroleuca* decreased in abundance in limed spruce stands (Agerer et al., 1998). In terms of ectomycorhizal root tips, very diverse effects

of liming were recorded, probably because of site-dependant response to amendment. A promoting effect of liming on ECM root tip abundance was reported in oak plots (Bakker et al., 2000) and in the humic layers of a Norway spruce stand (Nowotny et al., 1998), but the opposite effect was observed in a mixed oak and beech forest (Blaise & Garbaye, 1983). Haug & Feger (1990) showed no significant effect of liming on relative frequency of mycorrhizal and non-mycorrhizal roots. When focusing on the relative abundances of certain ECM species, liming increased the abundance of ECMs of *Piceirhiza nigra* and *Tuber puberulum* in spruce forests (Qian et al, 1998). Investigation on the effects of liming on the exploration type of ECMs (according to Agerer, 2001) showed the reduced abundance of ECMs with abundant external mycelium (medium- and long-distance exploration types) in limed plots (Blaise & Garbaye, 1983). Nevertheless, most of the works performed to date, assessed effects of liming on a short time scale (less than 10 years) and without parallel survey of ECM root tips and the fruiting bodies (FB) of symbiotic and saprophytic fungal species.

Here, we addressed the hypothesis that, as for plants, liming shifts the ECM community structure from acidophilic to a more widespread and neutrophilic type. The aim of this paper is thus to evaluate the mid-term (i.e. 15 years) impact of liming on the structure of both belowground (mycorrhizal root tips) and aboveground (epigeous sporocarps) fungal communities and to relate it with soil chemical properties.

Material & Methods

Study site

The experimental site of Humont (48°00’00’’N, 6°29’28’’E, Elevation: 570 m, Vosges mountains, North-Eastern France) consists in moderately declining stands of 35-year old Norway spruce (*Picea abies*) and 60-year-old beech (*Fagus sylvatica*). The liming treatment

was carried out in part of these stands by helicopter in 1991 with 757 kg/ha of CaO and 380 kg/ha of MgO, as grounded dolomitic rock, which is a relatively low dose compared to most calcareous amendments in eastern europe. The allocrisol (typic dystrochrept, USDA, 1999) is formed on sandstone. Four blocks were defined, two under beech (B) and two under spruce (S); in each block a couple of plots (limed: L, and untreated: U) were defined, resulting in 8 plots of about 20m x 20m each. Fifteen years after the treatment, liming had resulted in a shift of the humus type from moder to oligomull, restored tree health, mineral nutrition and vegetation diversity and strongly increased earthworm colonisation (Renaud et al, 2000).

Soil analyses

The topsoil layers of five soil cores (8cm in diameter, 20cm deep) were taken in each plot in October 2006. The five cores were then pooled per plot, air-dried at ambient temperature during one week and sieved at 2mm. We then measured, for each composite sample, the total contents of N, organic matter, pH, and concentrations of exchangeable Al^{3+}, Mg^{2+}, Fe^{3+}, Ca^{2+}, Mn^{2+}, K^{+} and Na^{+}. Soil phosphorus contents were estimated using the Duchaufour method, consisting in a double acid and basic extraction, which solubilized both organic and mineral P components (Duchaufour & Bonneau, 1959).

Estimation of the ECM root tip community structure

Soil core sampling was carried out on October 2006 (before leaf fall, date 1), May 2007 (after bud break, date 2) and October 2007 (before leaf fall, date 3). At each date, ECM sampling was done in one of the two blocks for beech and spruce, in order to measure ECM community structure at least once for each treatment. Three soil cores (8 cm diameter, 15cm deep, 750cm^3) were randomly collected in each sampled plot for date 1. Twenty soil cores (4 cm diameter, 18cm deep, 225 cm^3) were randomly collected in each sampled plot for date 2.

Twenty soil cores of the same size were collected in each sampled plot for date 3 in a 5 * 4 grid (distance between two soil cores: 2.5 m). As the aim of this study was to estimate the effects of liming on ECM communities, the effect of season (spring vs autumn) on community structure was not taken into account here, and the spring 2007 sampling was considered as an independent time replication. Considered that the ECM community structure is variable in a very small space scale (Dahlberg, 2001), three soil cores spaced by a few meters and thoroughly investigated took into account as much as variability in the ECM community than 20 smaller soil cores. The community structure obtained with the three sampling schemes was thus comparable.

Ectomycorrhizal morphotypes were then identified according to Agerer (1987-98). For each collected morphotype, seven tips were frozen at -80°C and used, when possible, for extending the morphotype identification by DNA sequencing of the ITS region (Gardes & Bruns, 1993). Sequences were aligned using ITS sequences available in the NCBI (http://www.ncbi.nlm.nih.gov/) and UNITE (http://unite.ut.ee/) databases. The morphological description and the results of DNA identification of all morphotypes are given in the supplementary table 1.

Ectomycorrhizal tips of each species were exhaustively counted in soil cores at date 1. For the date 2 samples, roots were washed and homogeneously spread in a large Petri dish (20 cm diameter) containing tap water. Then ECM tips of each species were exhaustively counted in a 1/8 sector of the dish. For the sample date 3, roots were washed, homogeneously spread in the same type of Petri dish, and cut in small pieces (1cm long). Root pieces were then randomly picked and ECMs counted until we reached 100 tips (Garbaye, 1990). For each date, values of abundance were then transformed into relative abundances by dividing the total number of ECM root tips belonging to a given species by the total number of root tips found in the plot. We then calculated the mean relative abundance for each species between

the three sampling dates. We then compared the mean relative abundances of all species during the whole sampling campaign.

Counting of sporocarps

Only conspicuous, epigeous sporocarps were counted; hypogeous and hidden resupinate ones were ignored. At each date, fungal sporocarps were identified and counted in at least one block under spruce or beech, in order to measure sporocarp community structure at least one time for each block. Fruiting bodies were absent in May 2007, so the sampling date 2 was postponed to the beginning of July 2007 for sporocarp counting. In addition, we assessed the presence / absence of saprophytic species in each block. Areas from $1600m^2$ to $3200m^2$, equally distributed between treatments, were surveyed by the same two people, for up to 1 hour per block, at each sampling date.

Statistics

The Shannon diversity index (H') was calculated as: $H' = -\sum ((Ni / N) * \log2 (Ni / N))$, and the Simpson's equitability index was calculated as: $D = \sum Ni (Ni - 1) / (N (N-1))$, where Ni was the abundance of the species i and N the total number of individuals. Diversity indices (Shannon index, Simpson index) were then compared between limed and untreated plots for each tree host using a student t test.

Fungal communities structures in each treatment were compared using canonical correspondence analysis (CCA) based on soil analyses with relative abundances of the species (ECM root tips and sporocarps of ECM fungi) and presence / absence data (saprophytic species). Statistical calculations and representations were done using the R software (http://www.r-project.org) (Ihaka & Gentleman, 1996).

	ECM root tips								Sporocarps							
Tree host	Beech				Spruce				Beech				Spruce			
Treatment	Limed		Untreated		Limed		Untreated		Limed		Untreated		Limed		Untreated	
Replicate	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Amanita battarrae (Amba)									6.45%							
Amanita citrina (Amci)											2.67%				1.05%	
Amanita crocea (Amcr)									3.23%							
Amanita eliae (Amel)									16.12%							
Amanita rubescens (Amru)	5.58%	0.36%	0.08%	1.49%							1.33%			3.60%		
Amanita sp. (Amsp)					0.35%			0.49%								
Amanita spissa (Amspi)										11.11%			0.46%			
Amanitopsis submembranacea (Amsub)									3.23%		1.33%			0.72%		
Boletus edulis (Boel)											1.33%					
Cantharella sp. (Casp)			0.04%													
Cantharellus cibarius (Caci)																0.64%
Cenococcum geophilum (Cege)	13.23%	26.03%	71.73%	34.07%	15.39%	45.75%	50.99%	69.59%								
Clavulina cristata (Clcr)	18.68%	17.97%	5.60%	8.43%	54.56%	3.28%	1.38%		3.23%				91.30%	0.72%	0.26%	
Cortinarius acutus (Coac)													0.46%		0.79%	
Cortinarius anomalus (Coan)		0.63%	0.80%				0.01%	0.24%								
Cortinarius argentatus (Coar)											1.33%					
Cortinarius delibutus (Code)											9.33%	11.11%				
Cortinarius evernius (Coer)														0.72%		
Cortinarius lebretonii (Coer)												11.11%	0.46%		1.31%	
Cortinarius sp. (Cosp)			0.14%	7.11%	0.66%		0.25%									
Dermocybe sp. (Desp)		0.24%	8.21%	1.21%												
Elaphomyces muricatus (Elmu)														2.16%		
Gomphidius glutinosus (Gogl)																2.55%
Hygrophorus olivaceoalbus (Hyol)			3.52%		0.02%	14.91%	3.18%						1.37%	15.11%	32.29%	15.92%
Hygrophorus pustulatus (Hypu)														5.76%		11.46%
Inocybe asterospora (Inas)												11.11%				
Inocybe lanuginosa (Inla)																0.64%
Inocybe napipes (Inna)									3.23%			11.11%	0.46%	0.72%	0.26%	
Inocybe petiginosa (Inpe)									3.23%							
Laccaria amethystina (Laam)	18.06%			12.02%					6.45%		1.33%	11.11%		6.47%	0.26%	
Laccaria laccata (Lala)									9.68%	55.56%	1.33%	11.11%				
Lactarius camphoratus (Laca)											2.67%				1.31%	14.01%
Lactarius helvus (Lahe)														2.88%		
Lactarius sp. (Lasp)			0.96%													
Lactarius sp.2 (Lasp2)			1.19%													
Lactarius sp.3 (Lasp3)					1.99%											
Lactarius sp.6 (Lasp6)		9.67%														
Lactarius subdulcis (Lasu)	11.60%	21.05%	1.45%	3.20%					3.23%		2.67%	11.11%				
Lactarius tabidus (Lata)					0.87%	4.68%	2.89%	1.12%			2.67%		0.46%	23.74%	2.10%	

Table 1 (Part 1)

Results

Description of the ECM fungal communities

Throughout the whole duration of the study, 40 morphotypes of ECM root tips were observed, and sporocarps of 52 species of ECM fungi and of 70 species of saprophytic fungi were found.

Concerning the ECM community, the most abundant morphotype was *Cenococcum geophilum*, always more abundant in the untreated plots (34 to 69% vs 13 to 46% in the limed one, as seen in Table 1). In the same way, *Russula ochroleuca* ECMs were almost absent in the limed plots (0.5% abundance in one spruce limed plot) but relatively abundant and present in all the untreated ones (1 to 11%). The limed plots were dominated by *Clavulina cristata* ECMs (except for SL-B one), under beech as well as under spruce, except in one replicate of the spruce limed plots. *Lactarius subdulcis* and *Tomentella sublilacina* were also found as codominant species in the beech limed plots. Ectomycorrhizae of *Xerocomus pruinatus* were found at low abundances but in all plots, whatever in limed or untreated ones. The ECM fungus *Laccaria amethystina* was not frequent but locally abundant in some beech plots.

Concerning sporocarps, *Russula ochroleuca*, *Laccaria amethystina* and *Inocybe napipes* were found in all the treatments, and *Russula ochroleuca* was the only species found in all the blocks (Table 1). The fungal species *Amanita citrina* was the only species to be present only in untreated plots, whatever the tree host, and absent in limed ones. In the spruce untreated plot, the sporocarp community was dominated by *Russula ochroleuca* (51% of relative abundance, in mean between the two plots) and to a lesser extent *Hygrophorus olivaceoalbus* (24%). In spruce limed plots, the community was dominated by *Clavulina cristata* (46% in mean, but especially dominant in one replicate with 91% abundance), *Russula ochroleuca* (19%), and *Lactarius tabidus* (12%), but with large differencies between the two plots.

	ECM root tips								Sporocarps							
Tree host	Beech				Spruce				Beech				Spruce			
Treatment	Limed		Untreated		Limed		Untreated		Limed		Untreated		Limed		Untreated	
Replicate	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Paxillus sp. (Pasp)				1.87%	0.83%		3.85%	0.39%								
Piceirhiza sp. (Pisp)					2.33%			1.20%								
Russula aeruginosa (Ruae)																2.55%
Russula brunneoviolacea (Rubr)									3.23%		1.33%					
Russula cyanoxantha (Rucy)	1.32%								25.79%		1.33%				0.52%	
Russula cyanoxantha var. pelteraui (Rucyp)											1.33%					
Russula densifolia (Rude)															0.26%	
Russula fageticola (Rufa)											33.34%					
Russula heterophylla (Ruhe)									3.23%							
Russula ionochlora (Ruio)															0.79%	
Russula lilacea (Ruli)									3.23%							
Russula nigricans (Runi)				2.81%								11.11%				
Russula ochroleuca (Ruoc)			2.32%	1.60%	0.54%		13.00%	4.81%	3.23%	22.22%	28.01%	11.11%	1.83%	35.25%	55.40%	46.50%
Russula parazurea (Rupa)										11.11%					0.26%	
Russula puellaris (Rupu)														0.72%		
Russula risigalina (Ruri)									3.23%							
Russula sp. 2 (Rusp2)							0.21%									
Russula turci (Rutu)													0.46%			
Russula vesca (Ruve)													0.91%			0.64%
Russula violeipes (Ruvi)															0.26%	
Sebacina epigaea (Seep)	1.50%			0.88%												
Tomentella sp. (Tosp)	11.72%			7.00%												
Tomentella sp. 2 (Tosp2)				1.98%				5.54%								
Tomentella sublilacina (Tosu)	14.48%	15.56%	0.05%	7.94%	4.70%		1.00%									
Tricholoma saponaceum (Trsa)											1.33%					
Tricholoma sp. (Trsp)						14.26%										
Tylopilus felleus (Tyfe)															0.26%	
UECM sp.1 (UE1)					6.99%	6.35%	12.79%	7.11%								
UECM sp.10 (UE10)																
UECM sp.11 (UE11)						5.27%										
UECM sp.12 (UE12)						0.27%										
UECM sp.13 (UE13)						0.81%										
UECM sp.14 (UE14)						0.16%										
UECM sp.15 (UE15)		1.81%														
UECM sp.2 (UE2)					2.71%		0.85%	6.84%								
UECM sp.5 (UE5)			0.15%													
UECM sp.6 (UE6)							0.90%									
UECM sp.7 (UE 7)			2.73%													
UECM sp.8 (UE8)							1.12%									
UECM sp.9 (UE9)							0.28%									
Xerocomus badius (Xeba)													1.83%	1.44%	2.10%	5.10%
Xerocomus chrysenteron (Xech)											5.33%					
Xerocomus ferrugineus (Xefe)															0.26%	
Xerocomus pruinatus (Xepr)	3.82%	6.67%	1.01%	8.38%	8.07%	4.25%	7.29%	2.67%							0.26%	
Diversity (Shannon index)	1.82	2.08	1.19	2.20	1.58	1.70	1.68	1.19	1.15	2.44	2.06	2.20	0.45	1.88	1.18	1.61
Equitability (Simpson index)	0.82	0.86	0.47	0.84	0.66	0.74	0.70	0.50	0.62	0.88	0.79	0.89	0.16	0.79	0.58	0.72

Table 1 (Part 2)

Table 1. Relative abundance of ECM fungi in each of the 8 plots. S: spruce, B: Beech, U: untreated, L: limed, A: replicate A, B: replicate B. The intensity of the grey shading increases with higher values. The abbreviation name used for each species in figures 1 and 2 is given in brackets in the first column.

Amanita rubescens and many other rare species (<3% relative abundance : *Amanitopsis submembranacea, Cortinarius evernius, Elaphomyces muricatus, Lactarius helvus, Russula puellaris, Russula turci*) appeared in these limed spruce plots, while the abundance of the fungus *Xerocomus* was slightly repressed (from 2 to 5% in untreated plots vs 1 to 2% in limed ones).

Consequences of liming under beech were less obvious because total abundances were not so high. In the untreated plots, the community was dominated by *Russula ochroleuca* (20% in mean between the two plots), *Russula fageticola* (17%) and *Cortinarius delibutus* (10%). In beech limed plots, the two latter species were absent and the community was dominated by *Laccaria laccata* (32%), *Russula ochroleuca* (13%) and *Russula cyanoxantha* (13%). We observed the apparition of *Clavulina cristata* and many *Amanita* and *Russula* species (*Amanita spissa, Amanita battarae, Amanita eliae, Russula heterophylla, Russula lilacea, Russula parazurea*); moreover, the relative abundance of other *Amanita* spp. (*Amanitopsis submembranacea*) and *Russula* spp. (*Russula brunneoviolacea, Russula cyanoxantha*) was promoted by liming. Oppositely, the abundance of *Lactarius subdulcis* and *Inocybe napipes* was higher in the untreated plots, while many *Cortinarius spp.* (*Cortinarius delibutus, Cortinarius lebretonii, Cortinarius argentatus*) were absent in the limed plots.

There was no significant effect of liming on ECM or FB diversity (Shannon index) or equitability (Simpson index) (Table 1).

Clavulina cristata was a dominant species of both below- (ECM root tips) and aboveground (sporocarps) communities, showing its high fructification rate, contrary to *Lactarius subdulcis*, dominant in terms of ECM root tips but without any fructification in the limed beech plots.

Code	SU-A	SU-B	SL-A	SL-B	BU-A	BU-B	BL-A	BL-B
N	4.89	3.80	4.83	4.56	3.86	4.28	4.17	3.07
C/N	21.10	20.80	19.90	21.00	16.40	15.80	18.70	17.50
C	103.00	79.10	95.90	96.00	63.40	67.60	78.10	53.70
pH	4.24	4.01	4.27	4.38	4.16	4.02	4.60	4.53
H+	1.44	1.16	1.00	0.76	0.76	0.84	0.76	0.80
Al3+	8.10	8.91	6.74	8.39	6.95	8.20	4.86	5.22
Ca2+	0.80	0.37	0.93	0.82	0.15	0.20	3.57	1.57
Fe3+	0.14	0.22	0.10	0.07	0.09	0.11	0.02	0.02
Mg2+	0.44	0.25	0.44	0.39	0.24	0.22	0.96	0.47
Mn2+	0.18	0.10	0.24	0.05	0.06	0.14	0.44	0.43
K+	0.41	0.29	0.39	0.27	0.30	0.35	0.41	0.29
Na+	0.05	0.04	0.04	0.04	0.04	0.03	0.05	0.03
P2O5	0.17	0.17	0.19	0.22	0.27	0.23	0.19	0.20

Table 2. Chemical properties of the topsoil in the 8 sampling plots. Concentrations in C, N and available P are given in $g.kg^{-1}$. Concentrations in exchangeable H^{+}, Al^{3+}, Ca^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, K^{+} and Na^{+} are given in $cmol+.kg^{-1}$. The available P has been measured using the method of Duchaufour and Bonneau (1959)

Canonical analysis-ECM root tips

Results of a CCA based on relative abundances of ECM root tips showed a strong opposition between limed and untreated plots (Figure 1). The first canonical component (33% of the total variance, x axis) strongly opposed exchangeable Mn^{2+}, Ca^{2+} and Mg^{2+}contents, and pH, to Al^{3+}, N, C and P_2O_5 contents (Figure 1, Correlations). Predictions of canonical coordinates also showed the differentiation between limed and untreated plots following the first canonical component for beech and ii) a differentiation between a limed plot (SL-A) and the other spruce ones (Figure 1, ECM root tips). The correspondence analysis (CA) based on ECM root tip relative abundances revealed the opposition between species found in SL-A and both limed beech plots (*Russula cyanoxantha, Tomentella sublilacina, Amanita rubescens, Clavulina cristata, Lactarius subdulcis*) against those found in SL-B and all untreated plots (*Cantharellus sp., UECM spp. 1 to 9, Russula ochroleuca, Cenococcum geophilum*) (Figure 1, ECM root tips). The second canonical component (17% of the total variance, y axis) was not strongly correlated with one environmental variable: it was to some extent correlated with available soil Na^+, K^+, H^+ and Fe^{3+} concentrations. The second canonical component also predicted an opposition between limed (especially SL-B) and untreated plots; the CA also showed a distinct group of species close to the SL-B plot (*Hygrophorus olivaceoalbus, Tricholoma sp., UECM spp. 11 to 14, Lactarius tabidus*).

Canonical analysis-Sporocarps of ECM fungi

Results of a CCA based on relative abundances of ECM sporocarps clearly isolated SL-A and BL-A plots. The first canonical component (24% of the total variance, x axis) was positively correlated to concentrations in soil available K and negatively correlated with soil P concentration (Figure 2, Correlations). Predictions of first canonical coordinate isolated

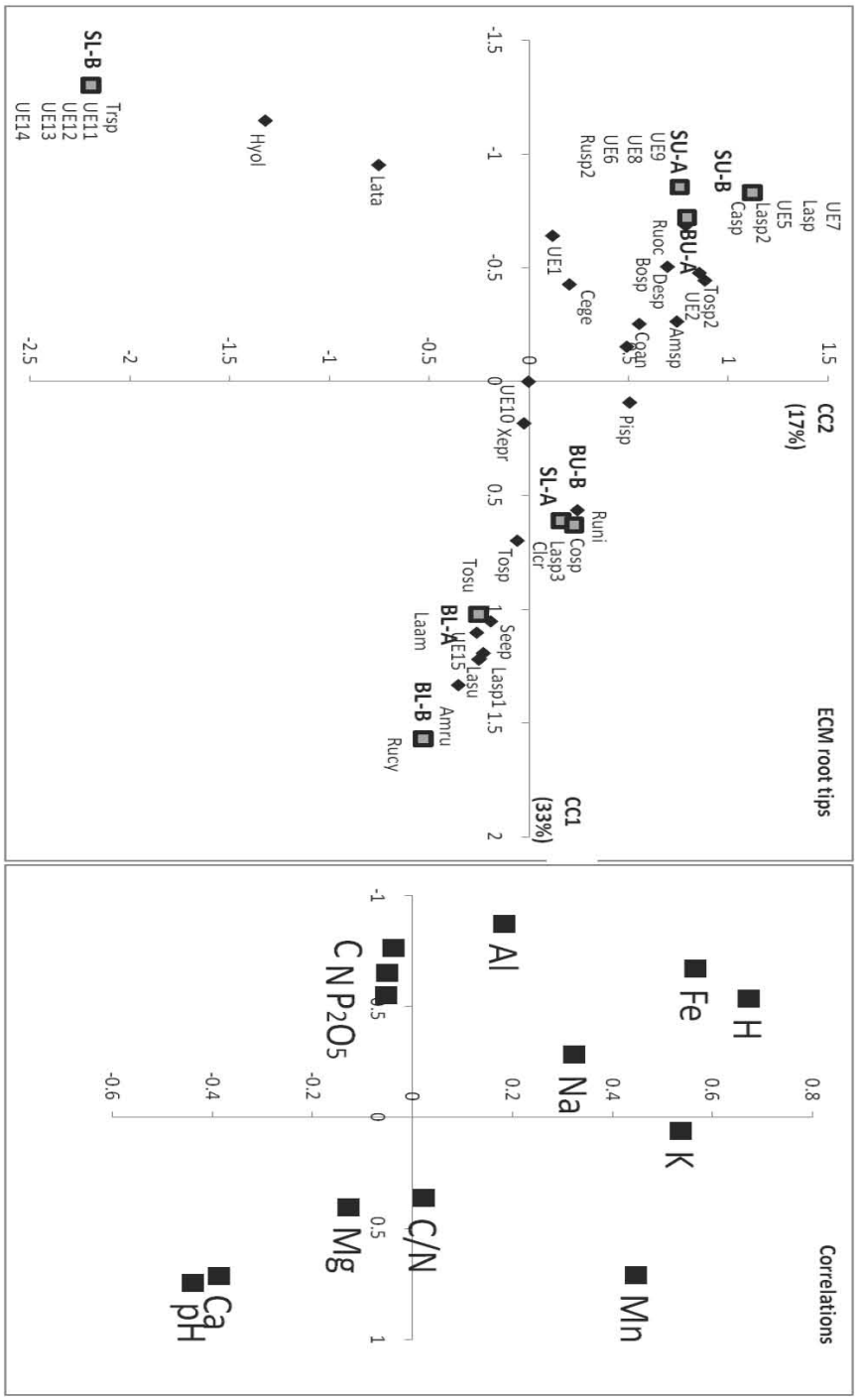


Figure 1. Canonical analysis based on the relative abundance of ECM root tips in each plot. Correlations: correlation circle between soil nutrient contents. ECM root tips: projection of the species and the 8 investigated plots on the plan of the two first canonical components. The first component (CC1) explained 33% of the total variance, and the second one (CC2) 17% (total: 50%). S: spruce, B: Beech, U: untreated, L: limed, A: replicate A, B: replicate B. The name of each species is abbreviated according to Table 1.

clearly the SL-A plot from the other ones (Figure 2, ECM sporocarps). The correspondence analysis (CA) based on sporocarp abundances also isolated species found in SL-A plot (particularly *Clavulina cristata,* and to a lesser extent *Russula vesca* and *Cortinarius acutus*). The second canonical component (20% of the total variance, y axis) strongly opposed pH, exchangeable Mn, Ca and Mg concentrations, to Na, Al, H, C and N contents (Figure 2, Correlations). Even if BU-B was close to BL-A, predictions with the second canonical coordinate gradually isolated the beech limed plots from the untreated ones (Figure 2, ECM sporocarps). The CA also distinguished species found only in the limed beech plots (*Amanita battarae, Amanita crocea, Amanita eliae, Amanitopsis submembranacea, Inocybe petiginosa, Russula cyanoxantha, Russula heterophylla, Russula lilacea, Russula risigalina*) (Figure 2, species). The species and plot ordination appeared as a horseshoe, showing that the sampled plots were probably distributed along an environmental gradient (Ramette, 2007).

Canonical analysis-Sporocarps of saprophytic fungi

The first canonical component (22% of the total variance, x axis) isolated the spruce plots from beech ones, and was correlated with pH, Ca^{2+} and Mn^{2+}, and negatively correlated with Al^{3+} (Figure 3, Correlation). The second canonical component (19% of the total variance, y axis) was correlated with K and negatively correlated with soil P contents. Nevertheless, prediction of canonical components showed no distinction between limed and untreated plots. There was a clear block effect for spruce.

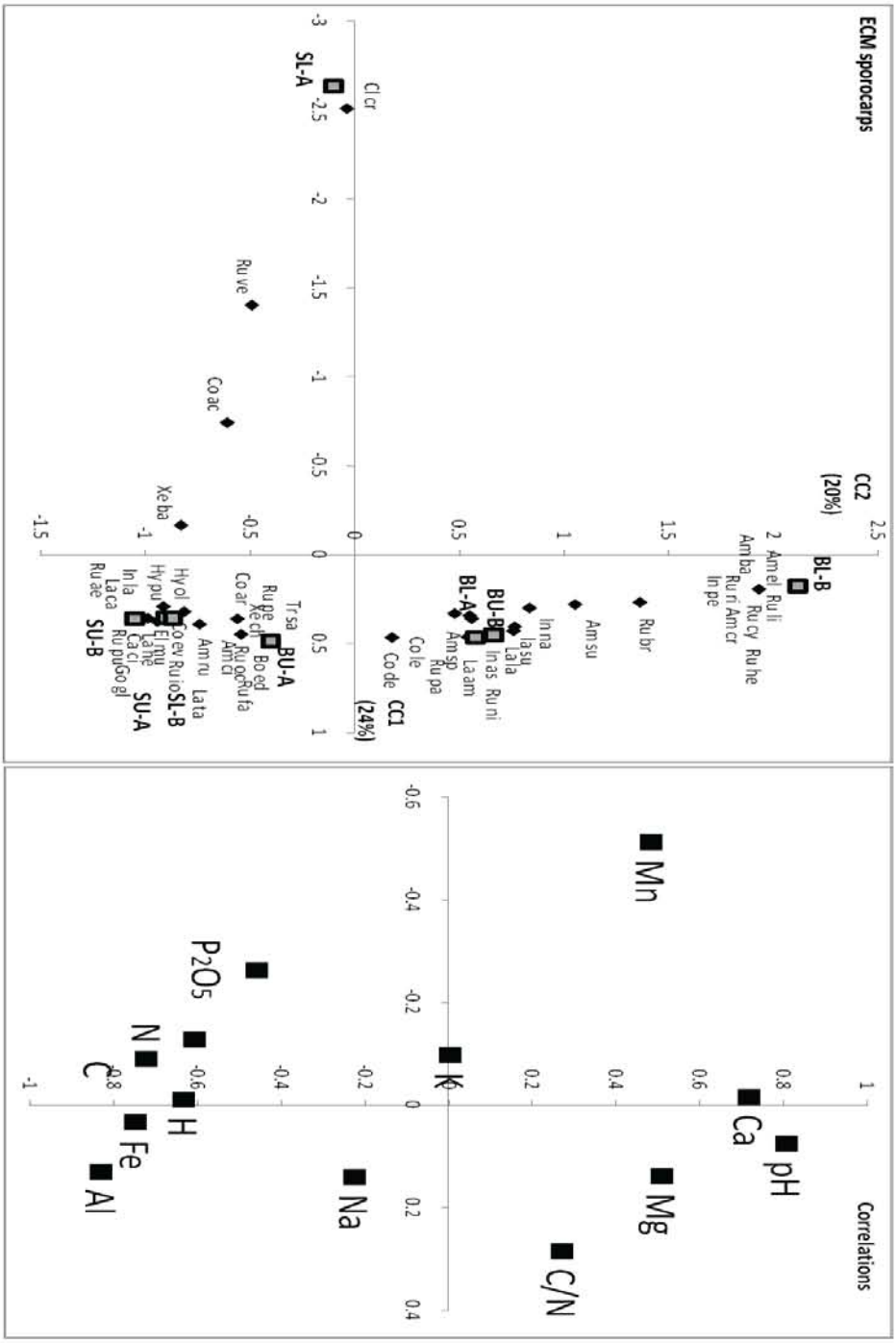


Figure 2. Canonical analysis based on the relative abundance of sporocarps of ECM species in each plot. Correlations: correlation circle between soil nutrient contents. ECM root tips: projection of the species and the 8 investigated plots on the plan of the two first canonical components. The first component (CC1) explained 24% of the total variance, and the second one (CC2) 20% (total: 44%). S: spruce, B: Beech, U: untreated, L: limed, A: replicate A, B: replicate B. The name of each species is abbreviated according to Table 1.

Discussion

There was an inversion in the environmental variables associated with the two main canonical components between ECMs and fruiting bodies. Soil parameters relevant with the effect of liming (increased Ca^{2+}, Mg^{2+}, pH, and decreased Al^{3+}, total N and total C) were correlated with the first canonical component for ECMs and with the second one for fruiting bodies. In contrast, increased exchangeable K^{+} content and decreased in available P content were both correlated with the first canonical component for fruiting bodies and with the second one for ECMs, meaning that liming was a stronger factor of community structuring than tree host for ECMs, but not for sporocarps.

Concerning ECMs, there were many common species between spruce and beech plots. Among them, two ECM species were particularly repressed by liming: the most abundant ECM species in the overall community (*Cenococcum geophilum*), and another frequent ECM species (*Russula ochroleuca*). *Cenococcum geophilum* has often been reported as the dominant species of the ECM community (Tedersoo et al, 2003; Courty et al, 2005; Dickie & Reich, 2005; Baier et al, 2006). Generally a strong dominance of *Cenococcum geophilum* ECMs is reported in stressed forest stands, which is consistent with the status of moderate decline of the forest studied here. Acidophilic morphotypes as *Russula ochroleuca* were also repressed in limed plots. A similar decrease of abundance of this ECM in a liming experiment has already been reported (Qian et al., 1998). In contrast, liming also promoted relative abundance of the two ECM morphotypes *Clavulina cristata* and *Tomentella sublilacina* in both spruce and beech plots. These two species have often been found in surveys of ECM root tips and are considered as ubiquist (Taylor & Bruns, 1999; Köljalg et al., 2000; Frey et al., 2004; Buée et al., 2005; Tedersoo et al., 2006). Moreover, *Tomentella sublilacina* is an excellent competitor in mature forests (Taylor & Bruns, 1999). Liming also promoted relative abundance of the beech-specific and widespread ECM fungus *Lactarius subdulcis* (Courty et

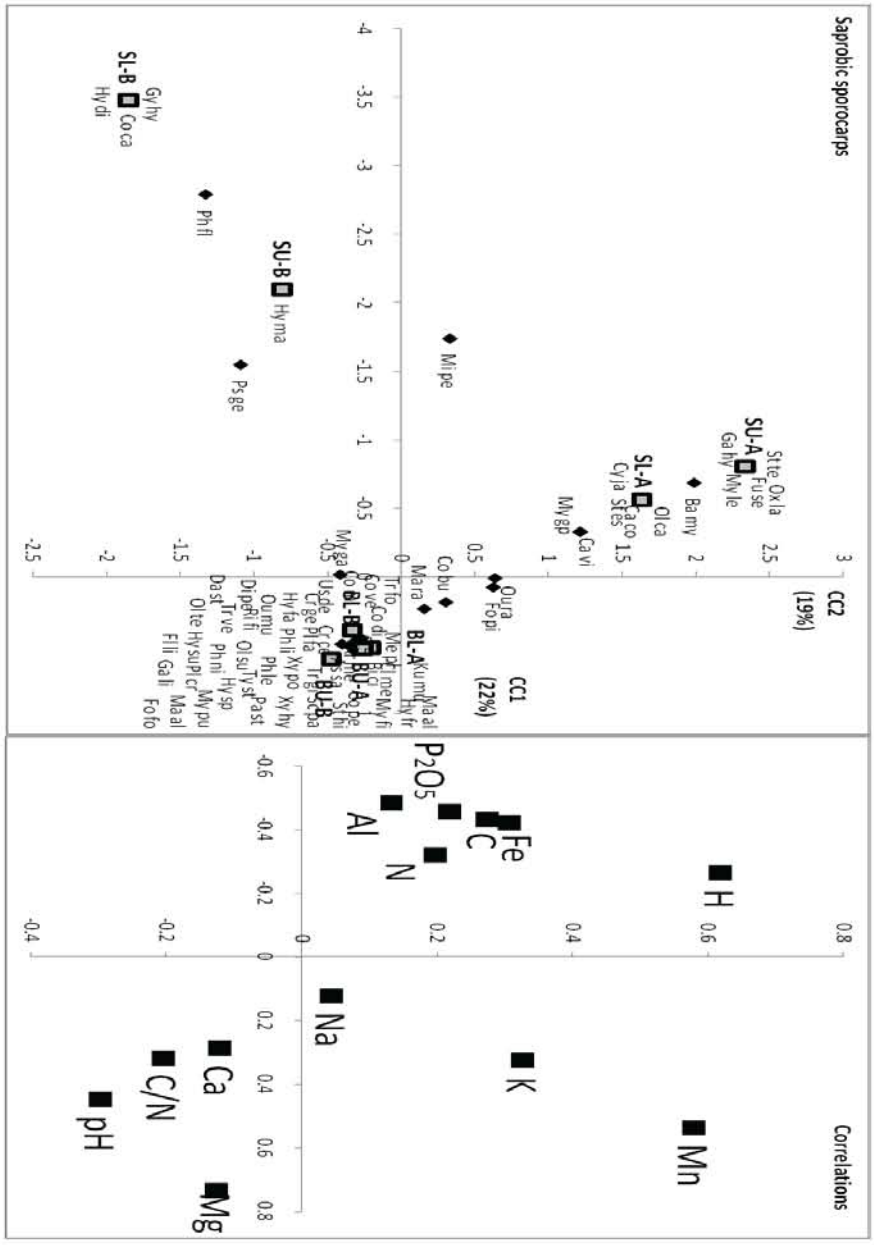


Figure 3. Canonical analysis based on the presence / absence of sporocarps of saprophytic species in each plot. Correlations: correlation circle between soil nutrient contents. ECM root tips: projection of the species and the 8 investigated plots on the plan of the two first canonical components. The first component (CC1) explained 22% of the total variance, and the second one (CC2) 19% (total: 41%). S: spruce, B: Beech, U: untreated, L: limed, A: replicate A, B: replicate B. The name of each species is abbreviated with the first two letters of its genus name, followed by a space and the first two letters of its species name (see also supplementary table 2).

al., 2005). The main consequences of liming on ECM communities have therefore been the shift from *Cenococcum geophilum* and acidophilic species (i.e and *Russula ochroleuca*) to a community dominated by ubiquist and highly competitive species. It is probable that the presence of these ubiquist species was due to the emergence of more nutrient-rich ecological niches in the limed areas, characterized by increased pH and contents of exchangeable Ca and Mg, as observed on our sample site.

Community structuring of ECM fruiting bodies appeared more distinct between spruce and beech. Opposition between the SL-A plot and all the other ones was a prominent factor of community structuring. This plot was characterized by the very high abundance of *Clavulina cristata* and by a slight reduction of the abundance of *Xerocomus badius*, a mesoacidophilic fungus (Köttke et al., 1998). A possible "genet effect" (presence in one plot only because of a limited genet size) for *Clavulina cristata* is unlikely, because its sporocarps covered almost all the limed plot, and were nearly absent from the untreated one.

The second factor of sporocarp community structuring gradually opposed beech limed and untreated plots; the limed plot coordinates were correlated with parameters characteristic of limed soils (exchangeable Ca and Mg, pH). Moreover, we observed that the 3 acidophilic species *Amanita citrina, Cortinarius delibutus* and *Russula fageticola* (in order of decreasing acidophilic optimum) were absent in the limed plots. The two latter ones were codominant species of the beech untreated plots, but none of the 3 species were found as ECM root tips. The relative abundance of *Russula ochroleuca* sporocarps also clearly decreased in the limed spruce plots, as observed by Agerer et al. (1998). As in the spruce limed plots, the abundance of an ubiquist fungus (*Laccaria laccata*) was also promoted by liming in beech. Nevertheless, acidophilic species as *Russula brunneoviolacea* also occurred in beech limed plots, showing that underlying soil acidity remained even after liming.

Species name	Code in figure 3	Beech				Spruce			
		Limed		Untreated		Limed		Untreated	
		A	B	A	B	A	B	A	B
Armillaria mellea	Ar me								X
Ascocoryne sarcoides	As sa								X
Baeospora myosura	Ba my	X		X					
Bisporella citrina	Bi ci						X		
Calocera cornea	Ca co			X					
Calocera viscosa	Ca vi	X		X					X
Clitocybe metachroa	Cl me					X			
Collybia butyracea	Co bu			X			X	X	
Collybia distorta	Co di							X	
Collybia peronata	Co pe					X			
Coltrichia perennis	Col pe					X			
Cordyceps capitata	Co ca				X				
Coriolus versicolor	Co ve							X	
Coprinus micaceus	Co mi							X	
Creopus gelatinosus	Cr ge							X	
Crepidotus cesatii	Cr ce						X		
Cystoderma jasonis	Cy ja			X					
Dacrymyces stillatus	Da st								X
Ditiola pezizaeformis	Di pe								X
Flammulaster limulatoides	Fl li					X			
Fomes fomentarius	Fo fo					X	X	X	
Fomitopsis pinicola	Fo pi	X				X	X		
Fuligo septica	Fu se	X							
Galerina hypnorum	Ga hy	X							
Ganoderma lipsiense	Ga li								X
Gymnopilus hybridus	Gy hy				X				
Hypholoma dispersum	Hy di				X				
Hypholoma fasciculare	Hy fa						X	X	X
Hypholoma marginatum	Hy ma		X						
Hypholoma sublateritium	Hy su						X		X
Hypoxylon fragiforme	Hy fr								X
Hypoxylon sp	Hy sp							X	X
Kuehneromyces mutabilis	Ku mu					X	X	X	X
Marasmiellus ramealis	Ma ra			X		X		X	X
Marasmius alliaceus	Ma al					X			X
Megacollybia platyphylla	Me pl					X	X	X	X
Microomphale perforans	Mi pe	X	X	X	X				
Mycena filopes	My fi					X	X	X	X
Mycena galericulata	My ga		X			X	X	X	X
Mycena galopus	My gp	X		X					X
Mycena leptophylla	My le	X							
Mycena pura	My pu						X		
Oligoporus caesius	Ol ca			X					
Oligoporus subcaesius	Ol su							X	X
Oligoporus tephroleucus	Ol tp					X			
Oudemansiella mucida	Ou mu								X
Oudemansiella radicata	Ou ra	X		X		X	X		X
Oxyporus laetomarginatus	Ox la	X							
Panellus stipticus	Pa st							X	X
Pholiota flammans	Ph fl		X		X				
Pholiota lenta	Ph le							X	
Pholiota limonella	Ph li								X
Physosporus nitreus	Ph ni								X
Plicatura crispa	Pl cr					X			
Plicaturopsis faginea	Pl fa								X
Pseudohydnum gelatinosum	Ps ge				X				X
Rickenella fibula	Ri fi								X
Schizopora paradoxa	Sc pa								X
Stereum hirsutum	St hi					X	X	X	X
Strobilurus esculentus	St es			X					
Strobilurus tenacellus	St te	X							
Trametes gibbosa	Tr gi								X
Trametes versicolor	Tr ve					X		X	X
Tremella foliacea	Tr fo						X		
Tyromyces stipticus	Ty st						X		
Ustulina deusta	Us de								X
Xylaria hypoxylon	Xy hy						X	X	X
Xylaria polymorpha	Xy po						X	X	X

Table 3. Distribution and code name (for the figure 3) of the 70 species of saprophytic fungi found in the study.

Some places in the overall limed area showed recolonization by *Sphagnum* species, showing a probable local soil reacidification, often localized in hollows. It was the case in the SL-B plot, whose fungal community was very close to untreated plots. Moreover, we found very acidophilic species as *Lactarius helvus*, in this plot. This means that liming effect on fungal communities is not uniform, probably because of the heterogeneous distribution of liming material in the soil, or by soil moisture and drainage in some places. The uneven distribution of some fungal species among sampling plots (such as *Clavulina cristata*) may also be due to spatial soil heterogeneity in nutrient distribution, which is expected to significantly vary at a 5m scale in forest soils (Gallardo, 2003). The SL-A plot, showing a strong dominance of *Clavulina cristata* in both ECM and sporocarp communities, presented high concentrations of exchangeable Mn: this might reflect preference of this ECM fungus to Mn-rich soils. It is also likely that, knowing that ECM fungi vary a lot in niche preference size (Toljander et al, 2006), earthworm bioturbation after liming decreases the micro-scale soil heterogeneity, and thus destroys potential niches for fungi of less ecological amplitude. The bacterial communities, which are also affected by liming (Bäckman et al., 2003), could also indirectly elicit or repress some fungal species and thus influence fungal community structure.

The relative abundance of *Russula ochroleuca* sporocarps was affected by liming, as well as its fruiting behaviour. The sporocarps were widespread all over the 4 untreated plots, whereas they were always restricted to the base of trees in the limed ones, where most of the acidity occurs through stem flow. This suggests *Russula ochroleuca* as a good marker species of above and belowground effects of liming on ECM communities, using its ECM root tip relative abundance in several limed and untreated soils cores, and the position of its fruiting bodies in whole untreated and limed plots. This could be a quick and useful tool for foresters, because ECM root tips of *Russula ochroleuca* are frequent and easy to identify (pale yellow surface covered with bright yellow dots), as well as its sporocarps. Moreover, the spatial

repartition of this species shows that local acidity can occur even in the limed plots and thus the soil spatial heterogeneity observed here can highly influence fungal community structure.

A similar liming-induced reduction of the abundance of acidophilic species at the profit of ubiquist, neutrophilic or competitive ones was observed on forest ground vegetation (Hallbäcken & Zhang, 1998) and mosses (Dulière et al., 2000). In the same way, high-dose liming decreased germination of acidophilic plant species (such as *Vaccinium myrtillus*) but increased germination of widespread ones (*Primula veris, Senecio sylvaticus*) (Olsson & Kellner, 2002). Nevertheless, liming did not have any significant effect on sporocarp and ECM community diversity (Shannon index) and equitability (Simpson index), even while the ECM diversity (Shannon index) had a tendency to decrease in limed plots. These results strongly tend to highlight the trivialization of all the communities after liming, due to the establishment of more nutrient-rich, and thus less selective, ecological niches.

Finally, among the environmental factors studied here, tree host was the most important for controlling the presence/absence of sporocarps of saprophytic fungal species. This has been mentioned in the literature for most of the lignin degrading basidiomycete species (Osono, 2007). Nevertheless, one non-specific species (*Clitocybe nebularis*) was present only in limed plots; other *Clitocybe spp.* and *Collybia spp.*, known as unspecific to a tree host (Osono, 2007), were not affected by liming. The presence of many saprophytic fungi could be affected by interactions with other soil microbes (Osono, 2007), C/N ratio (Lindahl et al., 2007), litter thickness (Yamashita & Hijii, 2006) or earthworm presence (Osono, 2007), and even if all these factors were more or less influenced by liming, we did not observe a specific pattern of saprophytic community structure in the limed plots. We did not observe a significant reduction of fungal diversity (Shannon index and Simpson index) in plots where N concentration increased.

Some other soil factors can affect sporocarp production were not measured here, and could interfere with liming for structuring fungal communities. For example, soil humidity is a major factor of fungal fructification (Kawakami et al., 2004), as well as soil temperature (Li, 1979), or CO_2 concentration (Dahlberg & Van Etten, 1982). The age of the root system sampled can also be of critical importance for evaluating the ECM community structure (Gibson & Deacon, 1988). Finally, fungal species differ in their distribution: some can occur in a very patchy way, whereas other ones can be solitary. This is true as well as for sporocarps and for ECM root tips. The sampling strategy used here may overestimate the abundance of these patchy species compared to solitary ones. All these concerns, plus the lifespan of a mycorrhiza (one week to one month) compared to a sporocarp one (one day to one week), makes the sporocarp sampling more sensitive to climate than ECM one. This can be a reason why the ECM communities studied here appears more sensitive to liming than sporocarp ones.

In conclusion, liming influenced very differently the structure of the communities of ECM root tips and sporocarps of symbiotic and saprophytic fungi. This forest practice had no obvious effect on saprophytic communities, but reduced the abundance of some acidophilic and meso acidophilic fruiting bodies of symbiotic fungi. This is compensated by the increased abundance of ubiquist species (*Clavulina cristata* and in a lesser extent *Laccaria laccata*). Finally, the same effect of liming (replacement of acidophilic and stress species by ubiquist and highly competitive ones) was observed in ECM root tip communities, and it was the main factor of community structuring, whatever the tree host. The appearance of ubiquist fungi was correlated with the development of new ecological niches, characterized by higher pH and exchangeable Ca^{2+}, Mg^{2+} and Mn^{2+} concentrations. Nevertheless, the re-acidification of limed plots was observed in some depressed areas. These results show that, as for plants, liming leads to the trivialization of symbiotic fungal communities, even 15 years after the treatment.

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References

Agerer R., 1987-1998. Colour atlas of ectomycorhizae. Einhorn-Verlag Eduard Dietenberger, Munich.

Agerer R., 2001. Exploration types of ectomycorrhizae: a proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. Mycorrhiza 11: 107-114.

Agerer R., Taylor A.F.S., Treu R., 1998. Effects of acid irrigation and liming on the production of fruit bodies by ectomycorrhizal fungi. Plant Soil 199: 83-89.

Bäckman J.S.K., Hermansson A., Tebbe C.C., Lindgren P.E., 2003. Liming induces growth of a diverse flora of ammonia-oxidising bacteria in acid spruce forest soil as determined by SSCP and DGGE. Soil Biol. Biochem. 35: 1337-1347.

Baier R., Ingenhaag J., Blaschke H., Gottlein A., Agerer R., 2006. Vertical distribution of an ectomycorrhizal community in upper soil horizons of a young Norway spruce (Picea abies [L.] Karst.) stand of the Bavarian Limestone Alps. Mycorrhiza 16: 197-206.

Bakker M.R., Garbaye J., Nys C., 2000. Effect of liming on the ectomycorrhizal status of oak. For. Ecol. Manag.126 : 121-131.

Blaise T., Garbaye J., 1983. Effets de la fertilisation minerale sur les ectomycorhizes d'une hetraie. Oecolog. Plantar. 18: 165-169.

Buée M., Vairelles D., Garbaye J., 2005. Year-round monitoring of diversity and potential metabolic activity of the ectomycorrhizal community in a beech (*Fagus silvatica*) forest subjected to two thinning regimes. Mycorrhiza 15: 235-245.

Courty P.E., Pritsch K., Schloter M., Hartmann A., Garbaye J. 2005. Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. New Phytol. 167: 309-319.

Dahlberg A., 2001. Community ecology of ectomycorrhizal fungi: an advancing interdisciplinary field. New Phytol. 150: 555-562.

Dahlberg K.R., van Etten J.L., 1982. Physiology and biochemistry of fungal sporulation. Ann. Rev. Phytopathol. 20: 281-301.

Dickie I.A., Reich P.B., 2005. Ectomycorrhizal fungal communities at forest edges. J. Ecol. 93: 244-245.

Duchaufour Ph., Bonneau M.,1959. Une méthode nouvelle de dosage du phosphore assimilable dans les sols forestiers. Bul. AFES 4: 193-198.

Dulière J.F., De Bruyn R., Malaisse F., 2000. Changes in the moss layer after liming in a Norway spruce (Picea abies [L.] Karst.) stand of Eastern Belgium. For. Ecol. Manag. 136: 97-105.

Frank J., Stuanes A. O., 2003. Short-term effects of liming and vitality fertilization on forest soil and nutrient leaching in a scots pine ecosystem in Norway. For. Ecol. Manag. 176: 371-386.

Frey S.D., Knorr M., Parrent J.L., Simpson R.T., 2004. Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. For. Ecol. Manag. 196: 159-171.

Gallardo A., 2003. Spatial variability of soil properties in a floodplain forest in northwest spain. Ecosystems 6: 564-576.

Garbaye J., 1990. Pourquoi et comment observer l'état mycorhizien des plants forestiers. Revue Forestière Française XLII:35-47

Garbaye J., Kabre A., Le Tacon F., Mousain D., Piou D., 1979. Fertilisation minérale et fructification des champignons supérieurs en hêtraie. Ann. For. Sci. 36: 151-164.

Gardes M., Bruns T.D., 1993. ITS primers which enhanced specificity fot basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. 2: 113-118

Gibson F., Deacon J.W., 1988. Experimental study of establishment of ectomycorrhizas in different regions of birch root systems. T. Brit. Mycol. Soc. 91: 239-251.

Hallbäcken L., Zhang L. Q., 1998. Effects of experimental acidification, nitrogen addition and liming on ground vegetation in a mature stand of Norway spruce (Picea abies [L.])Karst.) in SE Sweden. For. Ecol. Manag. 108: 201-213.

Haug I., Feger K.H., 1990. Effects of fertilization with MgSO4 and (NH4)2SO4 on soil solution chemistry, mycorrhiza and nutrient content of fine roots in a Norway spruce stand. Water Air Soil Poll. 54: 453-467.

Ihaka R., Gentleman R., 1996. R: A Language for Data Analysis and Graphics. J. Comput. Graph. Stat. 5: 299-314

Kawakami A., Matsumoto N., Naito S., 2004. Enviroonmental factors influencing sporocarp formation in *Typhula ishikariensis*. J. Gen. Plant. Pathol. 70: 1-6.

Köljalg U., Dahlberg A., Taylor A.F.S., Larsson E., Hallenberg N., Stenlid J., Larsson K.H., Fransson P.M., Karen O., Jonsson L., 2000. Diversity and abundance of resupinate thelephoroid fungi as ectomycorrhizal symbionts in Swedish boreal forests. Mol. Ecol. 9: 1985-1996.

Kottke, I., Qian, X.M ;, Pritsch, K., Haug, I., Oberwinkler, F., 1998. *Xerocomus badius-Picea abies*, an ectomycorrhiza of high activity and element storage capacity in acidic soil. Mycorrhiza 7: 267-275.

Kreutzer K., 1995. Effects of forest liming on soil processes. Plant Soil 168:447-470

Li C.Y., 1979. Light and temperature induced sporocarp formation of *Phellinus weirii*. Can. J. For. Res. 9: 535-538.

Lindahl B., Ihrmark K., Boberg J., Trumbore S.E., Högberg P., Stenlid J., Finlay R.D., 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytol 173: 611-620.

Nowotny I., Dähne J., Klingelhöfer D., Rothe G.M., 1998. Effect of artificial soil acidification and liming on growth and nutrient status of mycorrhizal roots of Norway spruce (Picea abies [L.])Karst.). Plant Soil 199: 29-40.

Olsson B.A., Kellner O., 2002. Effects of soil acidification and liming on ground flora establishment after clear-felling of Norway spruce in Sweden. For. Ecol. Manag. 158: 127-139.

Osono T., 2007. Ecology of ligninolytic fungi associated with leaf litter decomposition. Ecol. Res. 22: 955-974.

Persson T., 1988. Effects of liming on soil fauna in forests. A literature review. Swed. Environ. Prot. Board Report. Rep. 3418.

Qian X.M., Köttke I., Oberwinkler F., 1998. Influence of liming and acidification on the activity of the mycorrhizal communities in a Picea abies (L.) Karst. Stand. Plant Soil 199: 99-109.

Ramette A., 2007. Multivariate analyses in microbial ecology. FEMS Microbiol. Ecol. 62: 142-160

Renaud J.P., Picard J.F., Richter C., Nys C., 2000. Restauration de sols forestiers acides par un amendement calco-magnesien. Cas du Massif Vosgien et de l'Ardenne. Rapport DERF–ONF–INRA, 39pp.

Taylor D.L., Bruns T.D, 1999. Community structure of ectomycorrhizal fungi in a Pinus muricata forest: minimal overlap between the mature forest and resistant propagule communities. Mol. Ecol. 8: 1837-1850.

Tedersoo L., Köljalg U., Hallenberg N., Larsson K.H., 2003. Fie scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. New Phytol. 159: 153-165.

Tedersoo L., Suva T., Larsson E., Köljalg U., 2006. Diversity and community structure of ectomycorrhizal fungi in a wooded meadow. Mycol. Res. 110: 734-748.

Toljander J.F., Eberhardt U., Toljander Y.K., Paul L.R., Taylor A.F.S., 2006. Species composition of an ectomycorrhizal fungal community along a local nutrient gradient in a boreal forest. New Phytologist, 170: 873–884.

Ulrich B., 1983. Effects of acid deposition. Acid deposition, pp.31-41.

Article 3 " Effects of liming on ectomycorrhizal community structure in relation to soil horizons and tree hosts" (accepted in Fungal ecology).

Abstract: Liming is a forestry practice used to correct tree cation deficiency induced by soil acidity. Ectomycorrhizal (ECM) community structure and functioning is closely linked to soil nutrient availability, which is strongly affected by liming. The aim of this study was to assess the impact of liming on ECM community structure depending on soil horizon and tree host. Acidophilic species occurring in untreated plots such as *Russula ochroleuca* were absent from limed ones and replaced by more generalist morphtoypes. The abundance of ECM root tips in the untreated plots was higher in topsoil layers, whereas most of the ECM root tips in the limed plots were in the organomineral layer, whatever the tree host. Liming was the first factor of community structuration before tree host.

Key words: Calcareous amendments, Symbiotic fungi, Community structure.

Introduction

Forest decline on acidic soils has been reported in the East of France and in central Europe for the past 30 years (Ulrich et al., 1979; Erland & Taylor, 2002). Symptoms of spruce needle yellowing or beech decline were due to soil cation starvation, especially in calcium and magnesium, which are leached in drainage water because of increased acidic deposition (Hüttl, 1989). Compensatory liming (i.e the direct input of calcium and magnesium oxides in forest soil by hand or by helicopter) proved to be efficient in restoring tree mineral nutrition (Ulrich et al, 1979; Renaud, J. P., et al., pers. com). Liming increases pH, Ca and Mg supplies, soil base saturation, and shifts humus type from moder to oligomull (Kreutzer, 1995). Liming is a common practice in European forests to improve calcium and magnesium supply and to correct soil acidification, and has been intensely used in the 1980s to counteract forest decline following acid rain. It is now once again a topical subject for forest management as a tool to improve the production of wood biomass, because of its supposed mild impact on the functioning of forest ecosystems (Hüttl, 1989).

In temperate and boreal forests, tree growth and nutrition is highly dependant on mutualistic associations with fungi : more than 95% of fine tree roots are in symbiotic association with mycelium, forming mixed organs called ectomycorrhizae (ECM) (Smith & Read, 1997). The fungal partners (Ascomycete or Basidiomycete) provide the tree with nutrients, such as nitrogen, phosphorus, calcium or magnesium. These nutrients are derived from both mineral and organic sources.

Liming reduces humus accumulation, and, as a consequence, C and N contents in soil (Kreutzer, 1995; Rosenberg et al., 2003). Liming also increases soil pH and reduces soil Al^{3+} concentration (Kreutzer, 1995; Renaud J. P., et al., pers. com). These soil parameters have been proved to influence ECM diversity (Erland & Taylor, 2002). Indeed, in Norway spruce experiments, liming increased two-fold ECM root abundance in the humus layers (Nowotny

et al., 1998) and disturbed the ECM community structure (Qian et al., 1998), by decreasing the abundance of *Russula ochroleuca* and *Tylospora* sp. root tips and increasing those of *Piceirhiza nigra* and *Tuber puberulum*. In other experiments, liming caused a replacement of the ECM species, and had no effect on ECM diversity at high doses (8.75 ton.Ha-1), whereas diversity decreased at lower doses (3.25 and 4.28 ton.Ha-1) (Taylor & Finlay, 2003). In contrast, Blaise & Garbaye (1983) found a reduced ECM abundance in limed beech plots. It also appears that liming has a gradually decreasing influence on pH with soil depth (Kreutzer, 1995). The aim of this study was thus to assess the consequences of liming on the structure of ECM communities.

Material & 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 liming treatment was carried out by helicopter in 1991 with 757 kg/ha of $CaCO_3$ and 380 kg/ha of $CaMg(CO_3)_2$, which was a relatively low dose compared to what is often applied in central Europe (Hüttl, 1989; Kreutzer, 1995).

The allocrisol (typic dystrochrept, USDA, 1999) is formed on sandstone. Fifteen years after the treatment, liming had restored tree health, mineral nutrition and vegetation diversity, had shifted humus type from moder to oligomull and had strongly enhanced earthworm colonisation (Renaud et al., com. pers). Liming also increased soil pH and divalent cations (Ca^{2+} and Mn^{2+}) concentration, whereas Al^{3+} concentration had decreased (Table 1). The

concentration of exchangeable Mg2+ was higher in the beech limed plot than in untreated one, whereas it remained unchanged in the spruce limed plot (Table 1).

Sampling and sample processing

The limed area is a mixed forest stand composed of spruce (*Picea abies*), beech (*Fagus sylvatica*) and fir (*Abies alba*) monospecific patches. We chose, separately for spruce and beech, two pairs of plots of about 400 m^2: one plot was in the limed area, the other outside (untreated area), forming 4 plots: spruce untreated, spruce limed, beech untreated and beech limed. Plot couples were set in areas homogeneous in terms of topography, soil water flow, tree age and sylviculture.

In order to measure the effect of liming depending on the soil horizons, we collected three soil cores (8 cm diameter, 15 cm deep, 750 cm3) in each plot, before leaf fall on October 9, 2006. The spatial arrangement of these soil cores was randomly arranged. Then, soil cores were sliced into 3 subsamples corresponding to the fragmented litter, humic and organomineral layers.

Each subsample was sieved separately on a 0.5 mm screen and then gently washed with tap water, and observed under stereomicroscope. Ectomycorrhizal morphotypes were first identified morphologically using Agerer's (1987-98) descriptions. For each collected morphotype, seven tips were frozen at -80°C and used for confirming the morphotype identification by DNA sequencing of the ITS region (Gardes & Bruns, 1993). All soil cores were stored at 4° C.

In order to measure the effect of liming on the ECM community structure depending on soil depth, ECM tips of each species were exhaustively counted in each subsample of the 3 cores in each plot.

Molecular identification

The total genomic DNA of 7 pooled ECM tips stored at -80°C for each morphotype was extracted with the Dneasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France) following the manufacturer's instructions. Morphotypes were not molecularly identified for each subsample and plot, because of cases of double bands and unsuccessful amplifications (Table 2). Nevertheless, even if some morphotyping errors might have occurred, closely looking morphotypes most of the time proved to belong to the same fungal taxon according to DNA sequencing. The Internal Transcribed Spacer (ITS) region of the fungal nuclear rDNA was specifically amplified using the primer couples ITS1F / ITS4 (Invitrogen, Cergy Pontoise, France), or ITS1F / ITS2 (producing shorter DNA amplicons) for recalcitrant sequences. The PCR reactions were performed in a Gene Amp 9600 thermocycler (Perkin Elmer instruments, Norwalk, Conn.) in a mix composed of 2.5 µl of each primer, 20µl of Eppendorf mastermix (Dutscher, Brumath, France) and 25 µl of milliQ water. The PCR reactions were done using 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 1 min, with a final extension at 72° C for 10 min. Amplification success was checked in a 1% agarose electrophoresis gel in 1% TBE (Tris-Buffer-EDTA) stained with ethidium bromide and visualized under UV light. Purifications were done by adding 50µl of PEG solution (26.2% Poly Ethylene Glycol 8000, 6.6 mM $MgCl_2$, 0.6 M NaO-Acetate) to the amplified DNA solution, then incubating at ambient temperature for 5 min and centrifuging at 13000 rpm (15000G) for 15min. The pellet was then rinsed with 70% ethanol and centrifuged twice at 13000 rpm (15000G) for 5 min. The pellet containing purified DNA was re-eluted in 20µl of ultrapure sterile water. The amplified fragments were sequenced using the Quick start mix (Kit DTCS, Beckman Coulter, USA) in a CEQ8000 sequencer (Beckman Coulter, USA). Sequences were aligned using ITS sequences available in the NCBI

Table 1. Chemical composition of the topsoil of the 4 sampling plots. Concentrations in C, N and P_2O_5 are given in $g.kg^{-1}$. The P_2O_5 was extracted following the Duchaufour method. Concentrations in H^+, Al^{3+}, Ca^{2+}, Fe^{3+}, Mg^{2+} and K^+ are given in $cmol+.kg^{-1}$ and corresponds to the exchangeable fraction of these cations.

Tree host	Treatment	Code	N	C/N	C	pH	H^+	Al^{3+}	Ca^{2+}	Fe^{3+}	Mg^{2+}	Mn^{2+}	K^+	Na^+	P_2O_5
Spruce	Untreated	SU-A	4,89	21,10	103,00	4,24	1,44	8,10	0,80	0,14	0,44	0,18	0,41	0,05	0,17
	Limed	SL-A	4,83	19,90	95,90	4,27	1,00	6,74	0,93	0,10	0,44	0,24	0,39	0,04	0,19
Beech	Untreated	BU-B	4,28	15,80	67,60	4,02	0,84	8,20	0,20	0,11	0,22	0,14	0,35	0,03	0,23
	Limed	BL-B	3,07	17,50	53,70	4,53	0,80	5,22	1,57	0,02	0,47	0,43	0,29	0,03	0,20

Table 2. Details on the molecular identification of the 17 ectomycorhizal types encountered throughout the study. When molecular identification was successful, Blast results are given in the table (sequence length and Genebank accession number). Amplification results for each morphotype in the four plots were reported (S: Spruce, B: Beech, U: Untreated, L: Limed, +: successful amplification, -: unsuccessful amplification, //: double-band amplification).

Fungal taxon	accession number	Identity	Length	SU	SL	BU	BL
Clavulina sp.	DQ974710.1	90%	375/418		+		
Hygrophorus olivaceoalbus	AF430252.1	98%	212/215	+		-	
Lactarius subdulcis	AJ889966.1	99%	612/613			-	+
Russula ochroleuca	AM087261.1	99%	595/598	+		+	
Tomentella sublilacina	DQ822833.1	99%	630/635		-		+
Xerocomus pruinatus	AJ889933.1	98%	536/542	+	-	-	-
Cortinarius sp.	AJ889941.2	87%	413/417			+	-
Boletale sp.	AY237179.1	88%	352/396	+			
Cantharella sp.	AM161510.1	99%	612/614			+	
Cenococcum geophilum				-	-	-	-
Unidentified ECM1				-	-		
Dermocybe sp.						-	
Piceirhiza sp.					-		
Unidentified ECM2				-	-		
Lactarius sp.1						//	
Lactarius sp.2						//	//
Lactarius sp.3					//		

(http://www.ncbi.nlm.nih.gov/) and UNITE (http://unite.ut.ee/) databases. This protocol has been slightly adapted from Buée et al (2005).

Statistics

Diversity indices (species richness, Shannon's diversity and Simpson's equitability) were calculated per soil core and the mean values were compared in limed and untreated plots. The species richness index is the number of species present in the treatment. The Shannon diversity index (H') was calculated as: $H' = -\sum ((Ni / N) * \log2 (Ni / N))$, and the Simpson's equitability index was calculated as: $D = \sum Ni (Ni - 1) / (N (N-1))$, where Ni was the abundance of the species i and N the total number of individuals.

The correspondence analysis was performed on presence/absence data of ECM morphotypes in each layer of each treatment. As the results showed a "horsehoe effect", we performed a detrended analysis on the same dataset. Analysis of variance was performed on the abundance of each ECM morphotype and on diversity indices, depending on soil layer and tree host, after a log(1-x) transformation. Statistical analyses were done using the R software (http://www.r-project.org) (Ihaka & Gentleman, 1996).

Results

Effects of liming on ECM species composition in the 3 soil layers of each treatment

The effects of liming on ECM community structure were measured using a detrended correspondence analysis based on presence/absence of each ECM morphotype in the 3 soil layers of all the 12 soil cores (i.e 36 samples in total). The first axis (40.1% of the total

Table 3. Analysis of variance of the effects of treatment, soil layer and their interaction on the abundance of each ECM morphotype and on diversity indices. Diversity was calculated as Species Richness (SR), Shannon diversity index (Shan), and Simpson equitability index (Simp) in each soil core. The effects of treatment and soil layer were also tested on total abundance of ECMs (N) in each sample. Significant effects of treatment and soil layer are reported with stars. ***: p<0.001, **: p<0.01, *: p<0.05. Cl=*Clavulina sp*, Cg=*Cenococcum geophilum*, Co=*Cortinarius sp.*, Dsp=*Dermocybe sp.*, Ho=*Hygrophorus olivaceoalbus*, Ls=*Lactarius subdulcis*, Lsp1=*Lactarius sp.1*, Lsp2=*Lactarius sp.2*, Lsp3=*Lactarius sp.3*, Psp=*Piceirhiza sp.*, Ro = *Russula ochroleuca*, Bsp=*Boletale sp.*, Tsp=*Tomentella sp.*, UE1=*Unidentified ECM 1*, UE2=*Unidentified ECM 2*, Xp=*Xerocomus pruinatus*.

	Ro	Ho	Cl	Bsp	Cg	Lsp1	Psp	N	UE1	Ls	Other species	SR	Shan	Simp
Treatment	***	***	***	**	**	*	*	*						
Layer		***							*	*				
Interaction		***										*	*	

variance, Figure 1) separated 7 of the 9 beech limed samples (BLOL2, BLOH1, BLOH2, BLOH3, BLOM1, BLOM2, BLOM3). These samples belong to humic as well as organomineral layers and to one litter layer only of the 3 soil cores from beech limed plot, and were associated with two ECM morphotypes : *Lactarius subdulcis* and *Tomentella sublilacina*. Together, the two first DCA axes clearly separated all the limed spruce samples from the untreated samples (the second axis representing 30.2% of the total variance, Figure 1). The cluster of limed spruce samples was associated with the ECM morphotypes *Lactarius sp. 3* and *Piceirhiza sp.*. The morphotype *Clavulina sp.* was associated with limed samples from both beech and spruce. Concerning the spruce untreated samples, axis 2 tends to separate the organomineral soil layers from the others. The samples from litter and humic layers had low values on axis 2 (from -0.83 to -0.46), whereas those from organomineral layers presented higher values on the same axis (-0.65, -0.02 and 1.08).

Effect of liming on the ECM species abundance depending on soil depth and tree host

Ectomycorrhizal root tips were exhaustively counted in each soil layer (fragmented litter layer, humic layer and organomineral layer) of 3 soil cores in each plot. A total number of 9 out of 17 the morphotypes were identified successfully, whereas 5 out of the 17 morphotypes were not because of unsuccessful amplifications and 3 out of 17 because of presence of double bands (Table 2).

The raw abundance data per plot (Figure 2) showed that the total abundance of mycorrhizal root tips in the two untreated plots was decreasing with depth, whereas we observed the opposite in the two limed plots, whatever the tree host. This was due to a significant reduction of the total abundance of ECMs in the OL and OH layers in the limed plots (Table 3) whereas total number of ECM root tips remained unchanged in the OM layer of the limed plots. Liming caused a minor reduction of the thickness of litter and humic layers (from 40 to 30

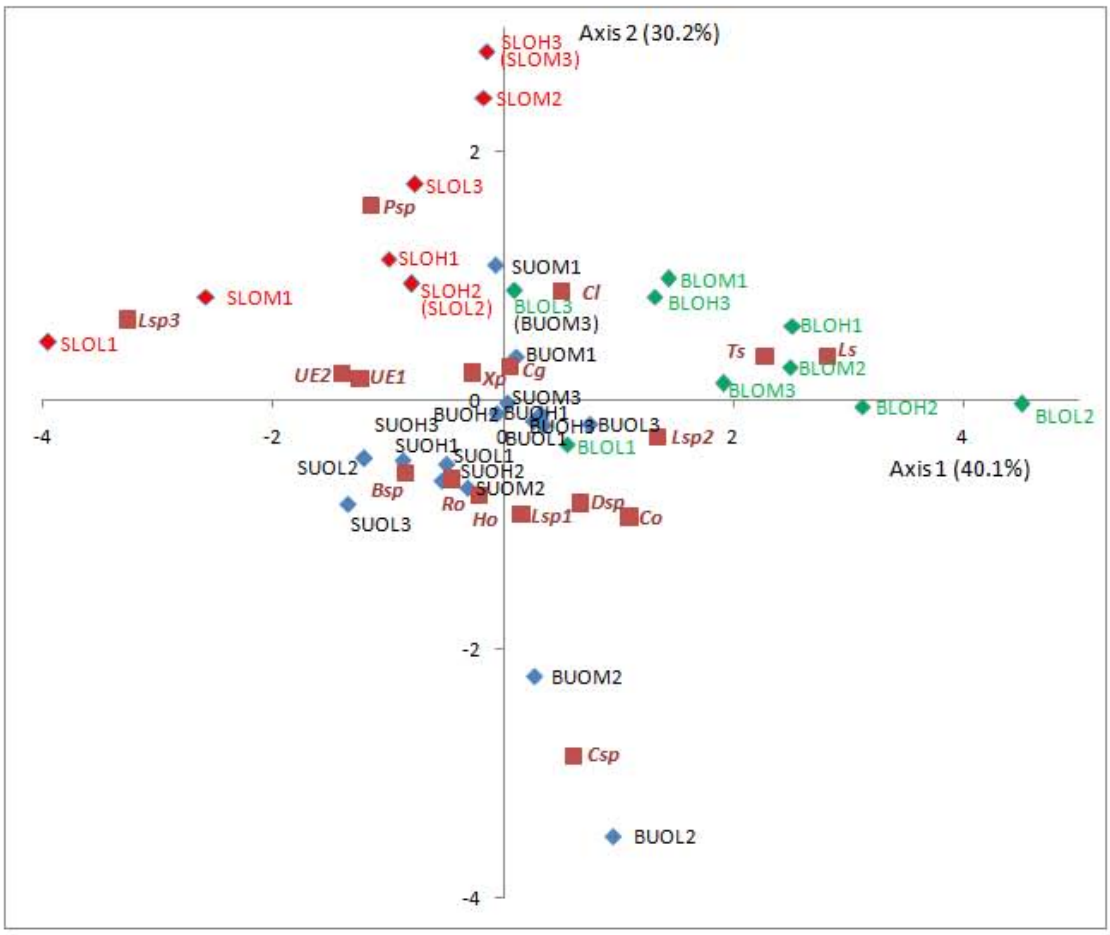


Figure 1. Detrended correspondence analysis on the presence / absence of each ECM morphotype on each soil core and soil layer. Cl=*Clavulina sp.*, Cg=*Cenococcum geophilum*, Co=*Cortinarius sp.*, Dsp=*Dermocybe sp.*, Ho=*Hygrophorus olivaceoalbus*, Ls=*Lactarius subdulcis*, Lsp1=*Lactarius sp.1*, Lsp2=*Lactarius sp.2*, Lsp3=*Lactarius sp.3*, Csp=*Cantharella sp.*, Psp=*Piceirhiza sp.*, Ro = *Russula ochroleuca*, Bsp=*Boletale sp.*, Ts=*Tomentella sublilacina.*, UE1=*Unidentified ECM 1*, UE2=*Unidentified ECM 2*, Xp=*Xerocomus pruinatus*. S=Spruce, B=Beech, U=Untreated, L=Limed, OL=Litter layer, OH=Humic layer, OM=Organomineral layer. The ECM morphotypes are represented as garnet squares. Samples coming from spruce limed plots are represented as red diamonds and samples coming from beech limed plots as green diamonds. Superimposed points are labelled in brackets.

mm under spruce and from 40 to 31.6 mm under beech, Figure 2). The variance analysis of the effects of liming and soil depth on ECM abundances showed that the treatment significantly influenced the abundance of *Russula ochroleuca*, *Hygrophorus olivaceoalbus, Boletale sp.*, *Lactarius sp.*1, *Cenococcum geophilum*, *Clavulina sp.* and *Piceirhiza sp.* (Table 3). The acidophilic morphotypes *Russula ochroleuca* and *Hygrophorus olivaceoalbus* completely disappeared in the limed plots, such as the morphotypes *Boletale sp.*, and *Lactarius sp.1*. The most dominant type in the two untreated plots was *Cenococcum geophilum*, (42% and 72% of total abundance in spruce and beech plot, respectively), which was repressed in the two limed ones (15% and 25% of total abundance in spruce and beech plot, respectively). Acidophilic morphotypes were replaced by new morphotypes: *Clavulina sp.* (for both spruce and beech), *Lactarius subdulcis* (for beech only), both more abundant with depth, and *Piceirhiza sp.*(for spruce only).

The soil layer significantly influenced the abundance of *Hygrophorus olivaceoalbus* (with an interaction with the effect of treatment), *Unidentified ECM sp.1* and *Lactarius subdulcis* (Table 3).The ECM morphotypes *Hygrophorus olivaceoalbus* and *Unidentified ECM 1* were more abundant in the litter layers, as well as for spruce and beech, contrary to those of *Lactarius subdulcis*, which were more abundant in the organomineral layers.

There was no significant effect of treatment or soil layer on species richness, diversity and equitability indices (Table 3).

Discussion

One of the most striking effects of liming was the inversion of the soil occupation profile of the ECM community. We observed very similar effects of liming on the vertical structure of the ECM community under the two tree hosts. The total abundance of the ECM community in

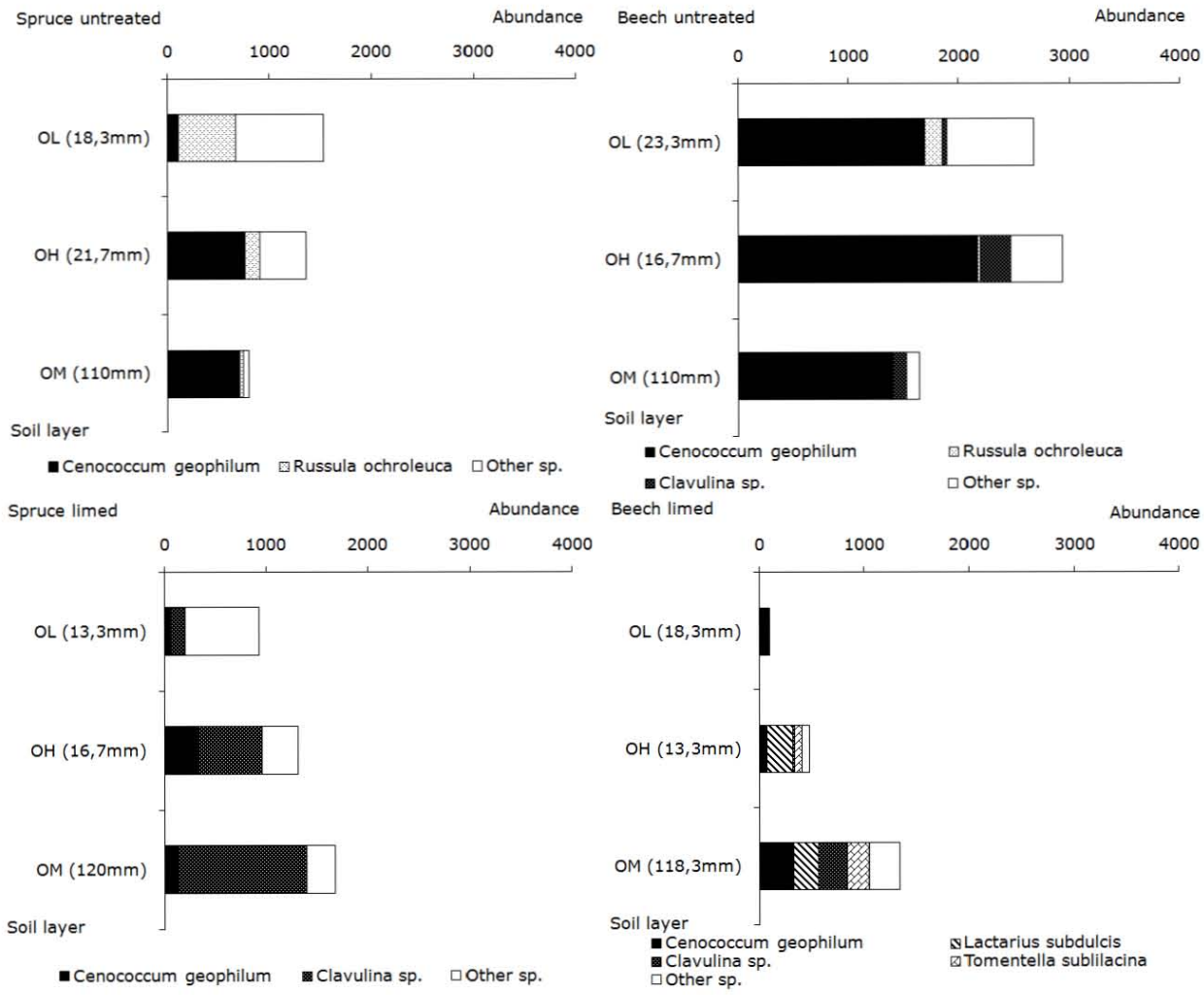


Figure 2. Abundance of ECM types depending on soil layer. The three soil cores collected in each plot were sliced in three subsamples corresponding to the soil layers (OL: fragmented litter, OH: humic layer and OM: organomineral layer). The abundance of each ECM types was measured in each subsample by exhaustive counting and pooled per plot for each layer. Each ECM type is represented by a different symbol and the depth of each soil layer is reported in brackets.

the two untreated plots was decreasing from surface to deeper horizons, whereas it was increasing with depth in the two limed plots. These two different patterns were observed because of a strong reduction of the abundances of ECM tips in the litter and humic layers in samples coming from limed plots, when the abundances in the organomineral layers remained roughly the same. This shift was due to i) the disappearance of some acidophilic species (*Russula ochroleuca*, *Hygrophorus olivaceoalbus*, Courtecuisse & Duhem, 1994) and the strong decrease of *Cenococcum geophilum* in the limed plots, and to ii) the new occurrence of widespread and generalist species in terms of preferences in soil acidity (*Clavulina sp.* for spruce, *Lactarius subdulcis* for beech). The higher tree resistance to drought observed in the limed plots (C. Nys, pers. com.) could be a consequence of the presence of fine roots and ECMs taking refuge in deeper horizons where water potential remains higher.

Interestingly, the first factor of ECM community structuration was liming, before tree host, as shown by multivariate analysis. Moreover, the effect of liming on the ECM community composition was more important in organomineral layers for spruce and in both humic and organomineral layers for beech. It was unexpected because generally the most important factor of structuration of ECM community is tree host.

Surprisingly, the effect of liming on *Lactarius subdulcis* abundance was not significant, as well as the effects of soil layer on the abundance of *Clavulina sp.*, even if raw data showed quite higher abundances of these ECMs on limed beech plot and on organomineral layers, respectively. This could be due to the high variability of the abundance of these ECM morphotypes among soil samples; nevertheless, the effects of liming and soil layer on *Lactarius subdulcis* were revealed by the multivariate analysis, showing that these factors influenced its presence, even without significant effect on their abundance. Diversity indices were not significantly affected by liming, probably because dominant ECM morphotypes in

Supplementary table. Morphological description of the 17 ectomycorhizal types encountered throughout the study.

Fungal taxon	ECM tip morphology and Agerer's exploration type (2001)
Clavulina cristata	Monopodial pyramidal mycorrhiza, forming abundant and dense mycorhizal systems, without rhizomorphs, with bent ends, whitish or pale yellowish mantle, cottony and densely covered with soil particles; short-distance exploration type
Hygrophorus olivaceoalbus	Unramified mycorhiza, solitary, without rhizomorphs, white, straight, slightly constricted in the base; contact exploration type
Lactarius subdulcis	Monopodial-pyramidal mycorhiza, with low density and large branches, abundant and dense, without rhizomorphs, with orange smooth surface, secreting white latex; contact exploration type
Russula ochroleuca	Monopodial-pyramidal mycorhiza, with variable aspects under spruce or beech, but always without rhizomorphs, with smooth surface and yellowish mantle dots; contact exploration type
Tomentella sublilacina	Monopodial-pyramidal mycorhiza, with low-density and short to long branches, abundant, without rhizomorphs, brown and glistening mantle, with narrow black and bended emanating hyphae, loosely wooly; short-distance exploration type
Xerocomus pruinatus	Monopodial-pyramidal mycorrhiza, with long branches, present in few numbers, with infrequent long and white rhizomorphs, hydrophobic brownish to pale yellow and silvery mantle, with smooth surface; medium-distance exploration type
Cortinarius anomalus	Irregularly pinnate mycorrhiza, with dense hyphal mats, tapening ends, with frequent fan-like white rhizomorphs, pale brownish loosely cottony and hydrophobic surface; medium-distance exploration-type
Paxillus sp.	Monopodial-pyramidal mycorhiza, with high density and short to long sized branches, solitary, with short white rhizomorphs, sinuous ends, stringy hydrophobic mantle, with cortical cells visible at the ends, brown and covered with white or silvery mycelium; medium-distance exploration type
Cenococcum geophilum	Unramified mycorrhiza, solitary, without rhizomorphs, with inflated ends, black, grainy and glistening surface, with frequent black and bended emanating hyphae, forming black sclerotia; short-distance exploration type
Unidentified ECM1	Monopodial-pinnate mycorhiza, with short to long branches, abundant and dense, without rhizomorphs, cortical cells visible, brown and cottony mantle, white and sometimes constricted at the tip, with very abundant white emanating hyphae; short-distance exploration type
Dermocybe sp.	Monopodial-pyramidal mycorrhiza, with low-density and short side branches, with tapening and sinuous ends, solitary but often densely arranged, brownish with bright yellow places, without rhizomorphs; contact exploration type
Fagirhiza sp.	Unramified mycorrhiza, solitary, without rhizomorphs, black, grainy and glistening surface, with short white and straight emanating hyphae; short-distance exploration type
Unidentified ECM2	Monopodial-pyramidal mycorrhiza, with long branches, present in few numbers, without rhizomorphs, hydrophobic pale pink and silvery mantle, with loosely cottony surface; contact exploration type
Lactarius sp.1	Monopodial-pyramidal mycorrhiza, with low density and medium size branches, solitary, with very few small and white emanating hyphae, with smooth pale orange to pale brownish-orange surface; short-distance exploration type
Lactarius sp.2	Monopodial-pyramidal mycorrhiza, with low density and high size branches, solitary, with very few small and white emanating hyphae, with smooth orange surface; short-distance exploration type
Lactarius sp.3	Monopodial-pyramidal mycorrhiza, with smooth pale orange surface; contact exploration type

untreated plots (*Cenococcum geophilum* and *Russula ochroleuca*) were replaced by other dominant ones (*Clavulina sp., Lactarius subdulcis*).

The calcareous amendment strongly affected the ECM community composition. The untreated plots were dominated by *Cenococcum geophilum* ECMs in both beech and spruce stands. *Cenococcum geophilum* is known to be resistant to drought because of its melanized and thick cell walls (Pigott, 1982; di Pietro et al., 2007), widespread, and often reported as the dominant species of the community (Tedersoo et al, 2003; Courty et al, 2005; Dickie & Reich, 2005; Baier et al, 2006). Generally a strong dominance of *Cenococcum geophilum* ECMs is reported in stressed forest stands, which is consistent with the status of moderate decline of the forest studied here. In the spruce limed plots, the main abundant ECM species was *Clavulina sp.*, which was also very common as fruiting bodies. *Clavulina sp.* is a cantharelloid fungus which forms short-distance exploration type ECMs (i.e with extraradical mycelium but without rhizomorphs, increasing the volume of soil exploited by the fungus in a short distance from the root; Agerer, 2001), and is widespread in deciduous as well as in coniferous forests (Agerer, 2001; Dentinger & Mc Laughlin, 2006; Kjoller, 2006). The most common ECM species found in limed beech plot was *Lactarius subdulcis*, which forms a contact ECM type (Agerer, 2001), is specific to beech (Prévost and Pargney, 1995; Buée et al, 2005), and is widespread among temperate beech forests.

Under spruce and beech, acidophilic ECM types (*Russula ochroleuca, Hygrophorus olivaceoalbus*) were repressed in the limed plots, where generalist species (*Clavulina sp., Lactarius subdulcis*) were present and strongly dominant. A similar liming-induced reduction of the abundance of acidophilic species at the profit of generalist, neutrophilic or competitive ones was observed on forest ground vegetation (Hallbäcken & Zhang, 1998) and mosses (Dulière et al., 2000). In the same way, liming decreased the germination of acidophilic plant species (such as *Vaccinium myrtillus*) but increased that of widespread and more generalist

ones (*Primula veris, Senecio sylvaticus*) (Olsson & Kellner, 2002). Our results concerning ECM communities showed the same trends as plant and mosses ones, i.e a shift towards less specific and acidophilic ones.

Acknowledgements

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References

Agerer R, 1987-1998. *Colour atlas of ectomycorhizae*. Einhorn-Verlag Eduard Dietenberger, Munich.

Agerer R, 2001. Exploration types of ectomycorhizae. *Mycorrhiza* **11**:107-114.

Baier R, Ingenhaag J, Blaschke H, Gottlein A, Agerer R, 2006. Vertical distribution of an ectomycorrhizal community in upper soil horizons of a young Norway spruce (*Picea abies* [L.] Karst.) stand of the Bavarian Limestone Alps. *Mycorrhiza* **16**:197-206.

Blaise T, Garbaye J, 1983. Effets de la fertilisation minerale sur les ectomycorhizes d'une hetraie. *Oecologia Plantarum* **18**:165-169.

Buée M, Vairelles D, Garbaye J, 2005. Year-round monitoring of diversity and potential metabolic activity of the ectomycorrhizal community in a beech (*Fagus silvatica*) forest subjected to two thinning regimes. *Mycorrhiza* **15**:235-245.

Courtecuisse R, Duhem B, 1994. Les champignons de France. Eclectis, Paris.

Courty PE, Pritsch K, Schloter M, Hartmann A, Garbaye J, 2005. Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. *New Phytologist* **167**:309-319.

Dentinger BTM, Mc Laughlin DJ, 2006. Reconstructing the *Clavariaceae* using nuclear large subunit rDNA sequences and a new genus segregated from *Clavaria*. *Mycologia* **98**:746-762.

di Pietro M, Churin JL, Garbaye J, 2007. Differential ability of ectomycorrhizas to survive drying. *Mycorrhiza* **17**:547-550.

Dickie IA, Reich PB, 2005. Ectomycorrhizal fungal communities at forest edges. *Journal of Ecology* **93**:244-245.

Dulière JF, De Bruyn R, Malaisse F, 2000. Changes in the moss layer after liming in a Norway spruce (Picea abies [L.] Karst.) stand of Eastern Belgium. *Forest Ecology and Management* **136**:97-105.

Erland S, Taylor AFS, 2002. Diversity of ecto-mycorrhizal fungal communities in relation to the abiotic environment. in: Ecological studies, Vol. 157, van der Heijden MGA, Sanders IR (Eds.) *Mycorrhizal ecology*. Springer Berlin Heidelberg New York.

Gardes M, Bruns TD, 1993. ITS primers which enhanced specificity fot basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**:113-118.

Hallbäcken L, Zhang, LQ, 1998. Effects of experimental acidification, nitrogen addition and liming on ground vegetation in a mature stand of Norway spruce (Picea abies [L.])Karst.) in SE Sweden. *Forest Ecology and Management* **108**:201-213.

Hüttl RF, 1989. Liming and fertilization as mitigation tools in declining forests ecosystems. *Water Air Soil Pollut.* **44**:93-118.

Ihaka R, Gentleman R, 1996. R: A Language for Data Analysis and Graphics. *Journal of Computational and Graphical Statistics* **5**:299-314.

Kjoller R, 2006. Disproportionate abundance between ectomycorrhizal root tips and their associated mycelia. *FEMS Microbiology ecology* **58**:214-224.

Kreutzer K, 1995. Effects of forest liming on soil processes. *Plant and soil* **168**:447-470

Nowotny I, Dähne J, Klingelhöfer D, Rothe GM, 1998. Effect of artificial soil acidification and liming on growth and nutrient status of mycorrhizal roots of Norway spruce (Picea abies [L.])Karst.). *Plant and Soil* **199**:29-40.

Olsson BA, Kellner O, 2002. Effects of soil acidification and liming on ground flora establishment after clear-felling of Norway spruce in Sweden. *Forest Ecology and Management* **158**:127-139.

Pigott CD, 1982. Survival of mycorrhiza formed by Cenococcum geophilum FR. in dry soils. *New Phytologist* **92**:513-517.

Prevost A, Pargney JC, 1995. Comparison of natural ectomycorrhizas between beech (*Fagus sylvatica*) and 2 fungi (*Lactarius blennius var viridis* and *Lactarius subdulcis*) .1. Morphological and cytological characteristics. *Annals of Forest Science* **52**:131-146.

Qian XM, Kottke I, Oberwinkler F, 1998. Influence of liming and acidification on the activity of the mycorrhizal communities in a Picea abies (L.) Karst. Stand. Plant and Soil 199:99-109

Rosenberg W, Nierop KGJ, Knicker H, de JAger PA, Kreutzer K, Weiss T, 2003. Liming effects on the chemical composition of the organic surface layer of a mature Norway spruce stand (Picea abies [L.] Karst.). *Soil Biology and Biochemistry* **35**:155-165.

Smith SE, Read DJ, 1997. *Mycorrhizal symbiosis*. Ed 2, Academic press, London.

Taylor AFS, Finlay RD, 2003. Effects of liming and ash application on below ground ectomycorrhizal community structure in two Norway spruce forests. *Water, Air and Soil Pollution* **3**: 63-76.

Tedersoo L, Koljalg U, Hallenberg N, Larsson KH, 2003. Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. *New Phytologist* **159**:153-165.

Ulrich B, Mayezr R, Khana PK, 1979. Die deposition von Luftverunreinigungen und ihre auswirkungen in walsokosystemim solling. *Schriften Forstl. Fa. Univ. Göttingen* **58** :1-291.

Conclusion du chapitre I et introduction au chapitre II

Nous avons montré dans le chapitre I que le chaulage modifiait les caractéristiques chimiques du sol, ansi que la structure de la communauté des apex mycorrhiziens, et, dans une moindre mesure, celle de leurs fructifications.

La plupart des apex racinaires mycorrhizés sont cantonnés aux horizons supérieurs du sol, tout comme les effets du chaulage sur la composition chimique du sol et sur la faune. D'une part, le chaulage modifie profondément la composition de la matière organique (apport accru de litière et de nutriments dûs à à une biomasse foliaire plus forte, accélération de l'activité des bactéries, toxicité aluminique plsu faible…). D'autre part, les ECMs peuvent produire des enzymes hydrolytiques pariétales qui récupèrent les nutriments à partir de la matière organique. Il est par conséquent probable que le chaulage, en modifiant la quantité de nutriments dans les horizons supérieurs du sol, influence la structure verticale de la communauté d'ECMs, ainsi que la nutrition de ces ECMs via l'assimilation d'éléments d'origine organique grâce à des enzymes hydrolytiques.

Nous avons donc mesuré, dans le chapitre II, les effets du chaulage sur i) la structure verticale de la communauté d'ECMs et ii) les activités potentielles de dégradation de la matière ganique par les ECMs.

Conclusion of chapter I and introduction to chapter II

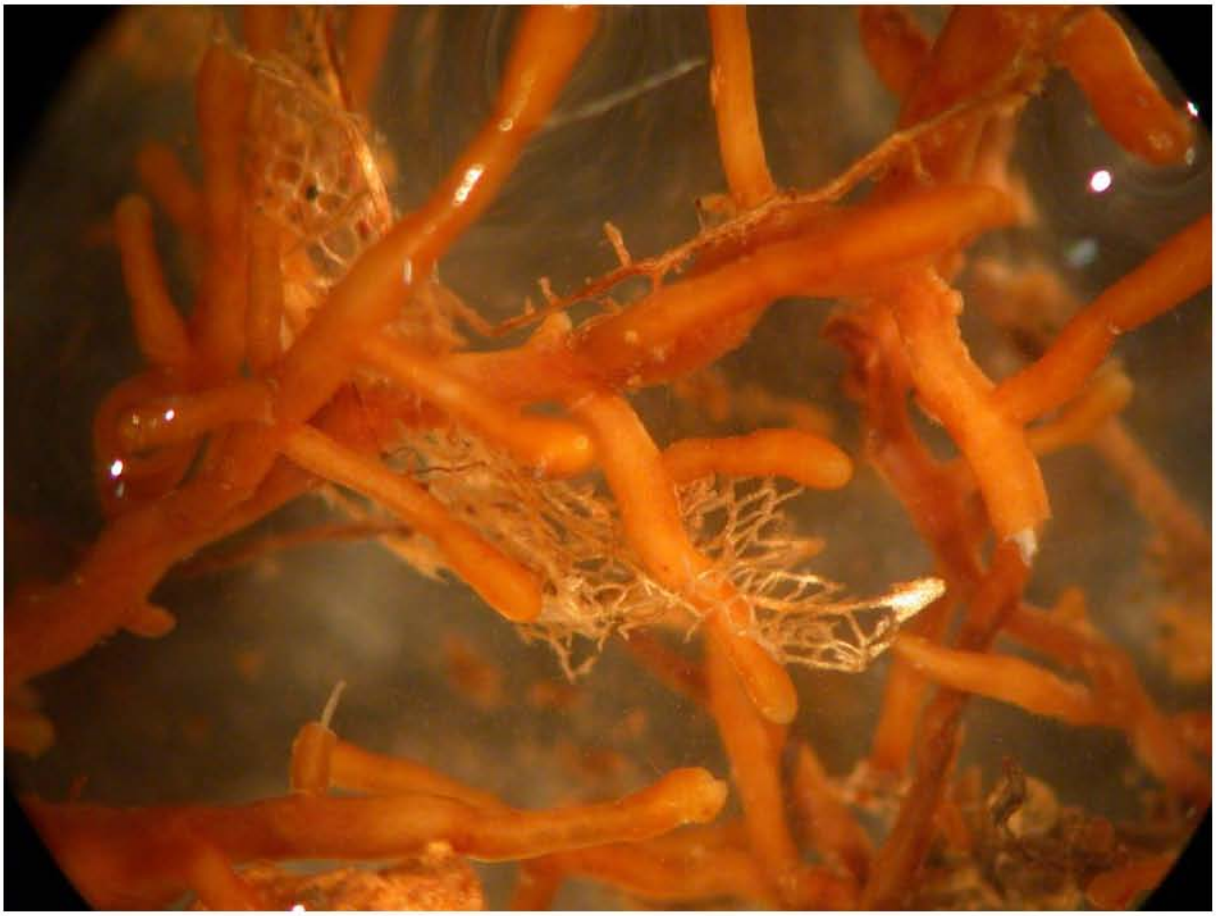
In chapter I, we have shown that liming modified the soil chemical composition, as well as the structure of the community of mycorrhizal root tips, and, to a lesser extent, of epigeous symbiotic sporocarps.

Most of the ECM root tips are confined in the upper soil layers, as are the effects of liming on soil chemical composition and fauna. In one hand, liming considerably changes the composition of organic matter (increased organic matter and nutrient input due to a higher leaf volume, acceleration of bacterial activity, decreased Al toxicity…). In the other hand, ECMs are capable of producing cell-wall bound enzymes to scavenge nutrients from organic matter. It is thus probable that liming, by modifying the organic nutrient quantity in the upper soil layers, would have an influence on the vertical structure of the ECM community and also change the nutrition of ECMs via uptake of nutrients from organic matter through the secretion of hydrolytic enzymes.

Therefore, in chapter II, we assessed the effects of liming on i) the vertical structure of the ECM community and ii) the potential enzymatic activities of organic matter degradation by ECMs.

CHAPTER II. CONSEQUENCES OF LIMING ON POTENTIAL ACTIVITIES OF ORGANIC MATTER DEGRADATION BY ECMS

***Article 4**. " Does forest liming impact the enzymatic profiles of ectomycorrhizal communities through specialized fungal symbionts? " (Mycorrhiza, in press)



Macrophotography of a cluster of *Lactarius subdulcis* ECMs penetrating beech litter in the beech limed plot of Humont forest. (photo : F. Rineau)

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 mobilisation 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).

Key words:

Ca-Mg soil amendment, Ectomycorrhizal community, Secreted enzymatic activities, Nutrient mobilisation, functional specialisation

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 & 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 & Taylor, 2002). Indeed, liming increased two-fold 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 & Garbaye (1983) found a reduced ECM abundance in beech plots amended with NPKCaMg.

In a more recent work Rineau and Garbaye (2009) we found that liming applied 16 years earlier still resulted in significative 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 microplate enzymatic assays developed by Courty *et al.* (2005).

Material & 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 colonisation 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 1000 m^2 plots (12.5 m x 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 10 and 13 trees for beech and spruce, respectively.

Twenty soil cores (4 cm diameter, 18 cm deep, 225 cm3, 2 x 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 x 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 Agerer's (1987-98) descriptions. 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.

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

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 & Bonneau 1959, g kg^{-1})	0,037	0.041	0.050	0.043

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, 1990a; 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-labelled 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 measuments for laccase activity were done at 415nm 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 cm^2 of surface area of individual ECM tips and expressed in $\mu mol.cm^{-2}.min^{-1}$.

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

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%

not try to separate the activities due to each partner, because the mobilisation of nutrients by ECMs and its benefit to the tree results from all these activities together (Courty *et al.*, 2005).s

Data processing

A given ECM type was characterized by: i) the mean value per each one of the four pseudo-replicate in each plot (i.e for 5 pooled soil cores) for each one of the 8 enzymatic activities (e, in $\mu mol.cm^{-2}.min^{-1}$), ii) the projection surface area of all of its root tips (s, in cm^2), and iii) 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*e, in μmol per liter of soil). In each plot we then calculated the total enzymatic activity as the sum of potential activities of the 20 soil cores: Σ(E*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 & 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 & Gentleman, 1996).

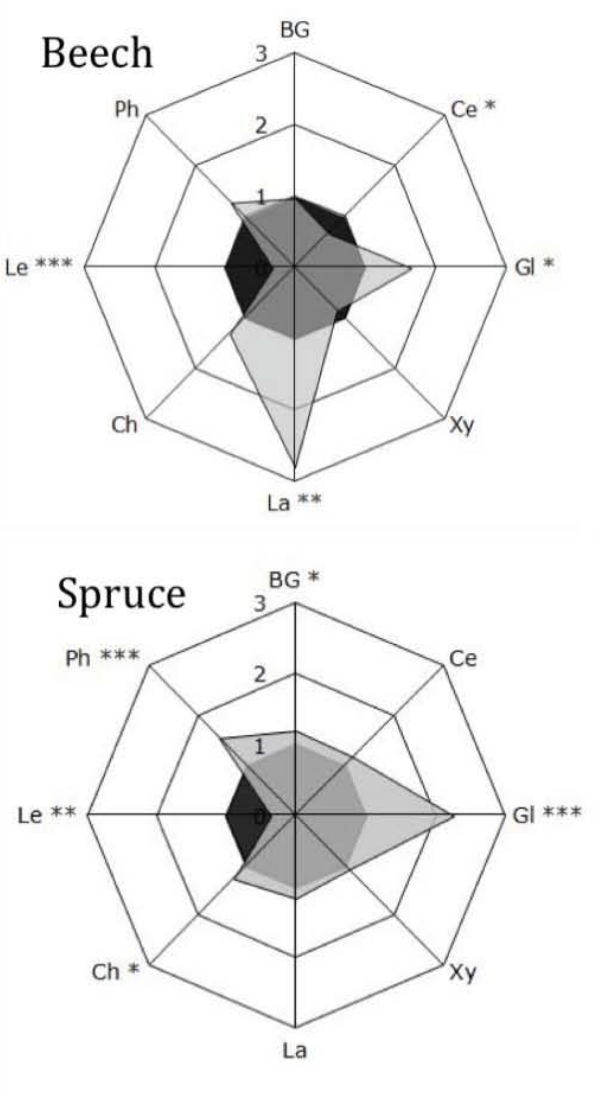


Figure 1 — Comparison between the mean enzymatic activities of the ECM community in beech (top) and spruce (bottom) limed and untreated plots. Eight enzymatic activities have been measured on seven ECM tips of each ECM type following the procedure of Courty *et al.* (2005) : BG, ß-Glucosidase ; Ce, Cellobiohydrolase; 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). Significant differences (Mann & Whitney's 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 two-fold higher in the limed plot. Acid phosphatase activitiy was also significantly higher in the limed plots (x 1,5) whereas leucine aminopeptidase activity was lower (x 0.5).

Regarding the beech ECM communities, liming significantly reduced the potential activity of leucine aminopeptidase (x 0.3), and that of cellobiohydrolase (x 0.7), but the most dramatic effect was the enhancement of laccase activity (x 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 (excepted laccase and leucine aminopeptidase which were due for more than 50 % to *Tomentella sublilacina* and to *Xerocomus 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, Cenococcum geophilum, Laccaria amethystina, Clavulina cristata, Tomentella* sp.

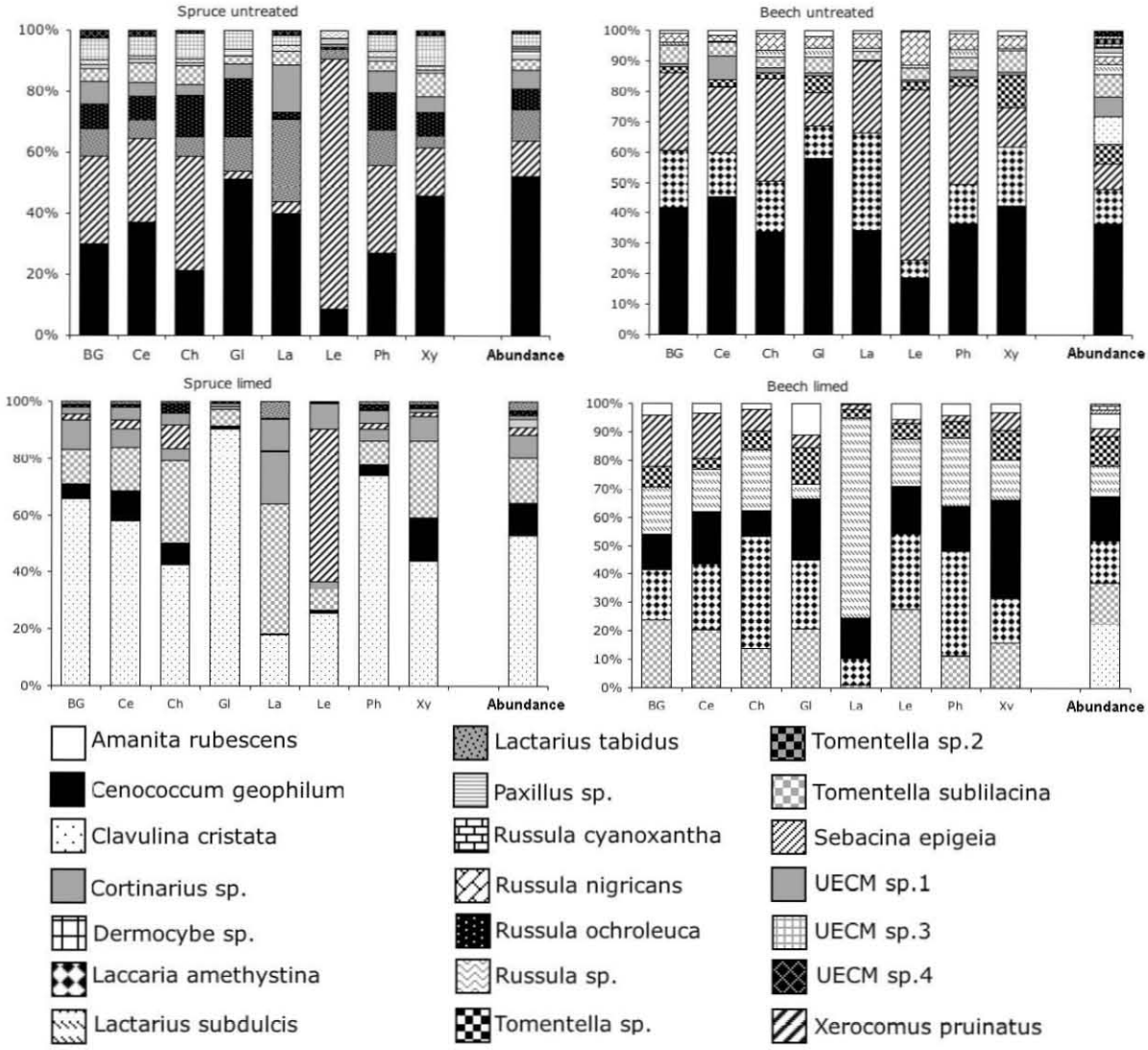


Figure 2 — Relative abundance (%) and mean contribution (%) of the different ECM types to the eight potential enzyme activities in the ECM community. Contribution of morphotype m (Cm) is calculated as $Cm = (a_m * e_m) / \sum$, where e_m is the mean enzymatic activity of the morphotype m ($\mu mol.tip^{-1}.min^{-1}$), a_m its relative abundance (%), and $\sum$, the enzymatic activity of a community of 100 ECMs in the plot ($\mu mol.min^{-1}$). 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.

and *Tomentella* sp. 2. However, *Lactarius 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 *Xerocomus 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 *Tomentella 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. *Cenococcum 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 *Lactarius subdulcis, Clavulina cristata* or *Xerocomus 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 stsructure in a deciduous oak forest changed significantly across seasons.

The change of ECM community composition due to liming (Rineau et 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 two-fold higher potential secretion of glucuronidase at the scale of the total ECM community. This was above all due to the high abundance of *Clavulina 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, 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 be studied as extensively as Ascomycetes and bacteria in this respect. Because ECMs are known to be associated with bacteria in the ectomycorhizosphere (Frey-Klett et al, 2005), it is thus possible that the strong potential glucuronidase activity observed in ECMs of the basidiomycete *Clavulina cristata* is due to bacteria specifically associated with this fungus. This would be consistent with the general aspect of *Clavulina cristata* ECMs, always as clusters embedded in clay or silt and probably containing many bacterial colonies. The occurence of saprotrophic ascomycetes colonizing the surface of ECMs of *Clavulina 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 (x 0.5)

But the most conspicuous effect was the enhancement of laccase activity. *Lactarius 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 *Lactarius subdulcis* ECMs but also stimulated their potential laccase activity. Laccase is an oxidative, unspecific polyphenol-degrading enzyme (Cullen & Kersten,

2004), produced by numerous fungi, particularly white rot ones (Reid, 1995). Several laccase genes have been found in the genome of *Laccaria 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 & Cairney, 2002). Some laccase isoenzymes can be constitutive and others regulated (Cullen & Kersten, 2004). The increased potential laccase activity of *Lactarius 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 *Lactarius 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 behaviour of a laccase-secreting white-rot fungus. *Lactarius quietus*, an oak-specific ECM, which is, as *Lactarius subdulcis*, abundant, highly clusterized, and smooth (contact-exploration type, according to Agerer, 2001), also showed close contact to dead leaves and white-rot behaviour (Courty et al, 2007). It suggests that, contrary to what Colpaert & 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 hydrolsis 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., 1991; 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 dependant 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 *Xerocomus 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 mobilisation 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 specialised ECM fungal species such as *Clavulina cristata* (glucuronidase), *Lactarius subdulcis* (laccase) or *Xerocomus 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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References

Abuzinadah RA, Read DJ, (1986) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. I. Utilisation of peptides and proteins by ectomycorrhizal fungi. New Phytol 103, 481-493.

Agerer R, (1987-1998) Colour atlas of ectomycorrhizae. Einhorn-Verlag Eduard Dietenberger, Munich.

Agerer R (2001. Exploration types of ectomycorhizae. Mycorrhiza 11:107-114.

Antibus RK, Sinsabaugh RL, Linkins AE (1992) Phosphatase activities and phosphorus uptake from inositol phosphate by ectomycorrhizal fungi. Can J Botany 70:794-801.

Baier R, Ingenhaag J, Blaschke H, Göttlein A, Agerer R (2006) Vertical distribution of an ectomycorrhizal community in upper soil horizons of a young Norway spruce (Picea abies [L.] Karst.) stand of the Bavarian Limestone Alps. Mycorrhiza 16:197-206.

Blaise T, Garbaye J (1983) Effets de la fertilisation minérale sur les ectomycorhizes d'une hêtraie. Oecol Plantarum 18:165-169.

Burke RM, Cairney JWG (2002) Laccases and other polyphenol oxidases in ecto- and ericoid mycorrhizal fungi. Mycorrhiza 12:105-116.

Burns RG (1978) Enzyme activity in soil: some theoretical and practical considerations. Soil Enzymes. Ed R G Burns, Academic Press, New York.

Cairney JWG (1999) Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. Mycorrhiza 9:125-135.

Colpaert JV, van Laere A (1996) A comparison of the extracellular enzyme activities of two ectomycorrhizal and a leaf-saprotrophic basidiomycete colonizing beech leaf litter. New Phytol 134:133-141.

Condron LM, Tiessen H, Trasar-Cepeda C, Moir JO, Stewart JWB (1992) Effects of liming on organic matter decomposition and phosphorus extractability in an acid humic Ranker soil from northwest Spain. Biol and Fer of Soils 15:279-284.

Courtecuisse R, Duhem B (1994) Les champignons de France. Eclectis, Paris.

Courty PE, Bréda N, Garbaye J, 2007. Relation between oak tree phenology and the secretion of organic matter degrading enzymes by Lactarius quietus ectomycorrhizas before and during bud break. Soil Biol Biochem 39, 1655-1663.

Courty PE, Pritsch K, Schloter M, Hartmann A, Garbaye J (2005) Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. New Phytol 167:309-319.

Courty PE, Franc A, Pierrat JC, Garbaye J, 2008. Temporal changes in the ectomycorrhizal community in two soil horizons in a temperate oak forest. Appl Environ Microbiol 5792-5801.

Courty PE, Hoegger PJ, Kilaru S, Kohler A, Buée M, Garbaye J, Martin F, Kües U (2009) Phylogenetic analysis, genomic organization, and expression analysis of multi-copper oxidases in the ectomycorrhizal basidiomycete Laccaria bicolor. New Phytol, in press

(doi : 10.1111/j.1469-8137.2009.02774.x).

Cullen D, Kersten PJ (2004) Enzymology and molecular biology of lignin degradation. The Mycota III : biochemistry and molecular biology. Berlin Heidelberg : Springer-Verlag.

Dentinger BTM, Mc Laughlin DJ (2006) Reconstructing the Clavariaceae using nuclear large subunit rDNA sequences and a new genus segregated from Clavaria. Mycologia 98:746-762.

di Pietro M, Churin JL, Garbaye J (2007) Differential ability of ectomycorrhizas to survive drying. Mycorrhiza 17:547-550.

Dickie IA, Reich PB (2005) Ectomycorrhizal fungal communities at forest edges. J Ecol 93:244-245.

Duchaufour Ph, Bonneau M (1959) Une méthode nouvelle de dosage du phosphore assimilable dans les sols forestiers. Bulletin de l'Association Française pour l'Etude du Sol 4 (193-198.

Dulière JF, De Bruyn R, Malaisse F (2000) Changes in the moss layer after liming in a Norway spruce (Picea abies [L.] Karst.) stand of Eastern Belgium. Forest Ecol Manag 136:97-105.

Erland S, Taylor AFS (2002) Diversity of ecto-mycorrhizal fungal communities in relation to the abiotic environment. in: Ecological studies, Vol. 157, van der Heijden MGA, Sanders IR (Eds.) Mycorrhizal ecology. Springer Berlin Heidelberg New York.

Frey-Klett P, Chavatte M, Clausse ML, Courrier S, Le Roux C, Raaijmakers J, Martinotti MG, Pierrat JC, Garbaye J (2005) Ectomycorrhizal symbiosis affects functional diversity of rhizosphere fluorescent pseudomonads. New Phytol 165:317-328.

Garbaye J (1990) Pourquoi et comment observer l'état mycorhizien des plants forestiers. Revue Forestière Française XLII:35-47.

Gardes M, Bruns TD (1993) ITS primers which enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol 2:113-118.

Hallbäcken L, Zhang, LQ (1998) Effects of experimental acidification, nitrogen addition and liming on ground vegetation in a mature stand of Norway spruce (Picea abies [L.])Karst.) in SE Sweden. Forest Ecol Manag 108:201-213.

Huber C, Weiss W, Gottlein A (2006) Tree nutrition of Norway spruce as modified by liming and experimental acidification at the Höglwald site, Germany, from 1982 to 2004. Plant Soil 63:861-869.

Hüttl RF (1989) Liming and fertilization as mitigating tools in declining forests ecosystems. Water Air Soil Poll 44:93-118.

Ihaka R, Gentleman R (1996) R: A Language for Data Analysis and Graphics. J Comput Graph Stat 5:299-314.

Kjoller R (2006) Disproportionate abundance between ectomycorrhizal root tips and their associated mycelia. FEMS Microbiol Ecol 58:214-224.

Kreutzer K (1995) Effects of forest liming on soil processes. Plant Soil 168:447-470

Landeweert R, Hoffland E, Finlay R, Kuyper T, van Breemen N (2001) Linking plants to rocks. Ectomycorrhizal fungi mobilizes nutrients from minerals. Trends Ecol Evol 16:248-254.

Leake JR, Read DJ (1990) Proteinase activity in mycorrhizal fungi II. The effects of mineral and organic nitrogen sources on induction of extracellular proteinase in Hymenoscyphus ericae (Read) Korf & Kernan. New Phytol 116:123-128.

Nowotny I, Dähne J, Klingelhöfer D, Rothe GM (1998) Effect of artificial soil acidification and liming on growth and nutrient status of mycorrhizal roots of Norway spruce (Picea abies [L.])Karst.) Plant Soil 199:29-40.

Maijala R, Raudaskoski M, Viikari L (1995) Hemicellulolytic enzymes in p-strains and s-strains of Heterobasidion annosum. Microbiol-UK 141:743-750.

Martin F, Aerts A, Ahren D, Brun A, Danchin EGJ, Duchaussoy F, Gibon J, Kohler A, Lindquist E, Pereda V, Salamov A, Shapiro HJ, Wuyts J, Blaudez D, Buee M, Brokstein P, Canbäck B, Cohen D, Courty PE, Coutinho PM, Delaruelle C, Detter JC, Deveau A, DiFazio S, Duplessis S, Fraissinet-Tachet L, Lucic E, Frey-Klett P, Fourrey C, Feussner I, Gay G, Grimwood J, Hoegger PJ, Jain P, Kilaru S, Labbe J, Lin YC, Legue V, Le Tacon F, Marmeisse R, Melayah D, Montanini B, Muratet M, Nehls U, Niculita-Hirzel N, Oudot-Le MP, Peter M, Quesneville H, Rajashekar B, Reich M, Rouhier N, Schmutz J, Yin T, Chalot M, Henrissat B, Kues U, Lucas S, Van de Peer Y, Podila G, Polle A, Pukkila PJ, Richardson PM, Rouze P, Sanders IR, Stajich JE, Tunlid A, Tuskan G, Grigoriev (2008) Symbiosis insights from the genome of the mycorrhizal Basidiomycete Laccaria bicolor. Nature 452:88-92.

Mosca E, Montecchio L, Scattolin L, Garbaye J (2007) Enzymatic activities of three ectomycorrhizal types of Quercus robur L. in relation to tree decline and thinning. Soil Biol Biochem 39:2897-2904.

Olsson BA, Kellner O (2002) Effects of soil acidification and liming on ground flora establishment after clear-felling of Norway spruce in Sweden. Forest Ecol Manag 158:127-139.

Pigott CD (1982) Survival of mycorrhiza formed by Cenococcum geophilum FR. in dry soils. New Phytol 92:513-517.

Prevost A, Pargney JC (1995) Comparison of natural ectomycorrhizas between beech (Fagus sylvatica) and 2 fungi (Lactarius blennius var viridis and Lactarius subdulcis) .1. Morphological and cytological characteristics. Ann For Sci 52:131-146.

Pritsch K, Raidl S, Marksteiner E, Blaschke H, Agerer R, Schloter M, Hartmann A (2004) A rapid and highly sensitive method for measuring enzyme activities in single mycorrhizal tips using 4-methylumbelliferone-labelled fluorogenic substrates in a microplate system. J Microbiol Meth 58:233-241.

Qian XM, Kottke I, Oberwinkler F (1998) Influence of liming and acidification on the activity of the mycorrhizal communities in a Picea abies (L.) Karst. Stand. Plant Soil 199:99-109

Reid ID (1995. Biodegradation of lignin. Can J Botany 73:1011-1018.

Rineau F, Courty PE, Uroz S, Buée M, Garbaye J (2008) Simple microplate assays to measure iron mobilization and oxalate secretion by ectomycorrhizal tree roots. Soil Biol Biochem 40, 2460-2463.

Rineau F, Garbaye J (2009) Liming modifies ECM community structure : effects of soil horizon and tree host. Fungal Ecology (in press).

Rosenberg W, Nierop KGJ, Knicker H, de Jager PA, Kreutzer K, Weiss T (2003) Liming effects on the chemical composition of the organic surface layer of a mature Norway spruce stand (Picea abies [L.] Karst.) Soil Biol Biochem 35:155-165.

Smith SE, Read DJ (2008) Mycorrhizal symbiosis. Ed 3, Academic press, London.

Tedersoo L, Köljalg U, Hallenberg N, Larsson KH (2003) Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. New Phytol 159:153-165.

Tibett M, Sanders FE, Cairney JWG (1998) The effect of temperature and inorganic phosphorus supply on growth and acid phosphatase production in arctic and temperate strains of ectomycorrhizal Hebeloma spp. in axenic culture. Mycol Res 102:129-133.

Ulrich B, Mayer R, Khanna PK, 1979. Die Deposition von Luftverunreinigungen und ihre Auswirkungen in Waldökosystemem im Solling. Schriften Forstl Fa Univ Göttingen **58** :1-291.

Vepsäläinen M, Kukkonen S, Vestberg M, Sirviö, Niemi RM, 2001. Application of soil enzyme activity test kit in a field experiment. Soil Biol Biochem 33, 1665-1672.

Conclusion du chapitre II et introduction au chapitre III

Nous avons donc observé que le chaulage modifiait les activités potentielles de dégradation de la matière organique par la communauté d'ECMs. Ceci est cohérent avec une modification de la nutrition du partenaire fongique, mais aussi pour la nutrition de l'arbre en N et P. De plus, la dégradation de certains composés de la matière organique par les ECMs peut modifier la dégradation de l'humus et de la litière, ce qui contribuerait au changement du type d'humus, et par conséquent la disponobilité et la mobilisation de certains nutriments d'origine minérale.

Cependant, la nutrition des arbres forestiers nécessite qussi des nutriments d'origine inorganique, comme les ions PO_4^{3-}, Ca^{2+}, Mg^{2+}, Fe^{3+}, K^{+}. Les champignons ectomycorrhiziens contribuent significativement à la récupération de ces nutriments, en accélérant la dissolution des minéraux de par la sécrétion d'oxalate, un agent majeur de l'altération minérale par les champignons, et via la sécrétion de molécules de faible poids moléculaire, pour la récupération du Fe^{3+}. Sachant que le chaulage modifie fortement les caractéristiques chimiques du sol (augmentation du Ca^{2+} et Mg^{2+}échangeable, diminution du pH et de la concentration en Al^{3+}…), il est probable que le chaulage influence aussi la récupération des nutriments par les ECMs. Cependant, la plupart des mesures de sécrétion d'oxalate par les ECMs ont été réalisées en culture pure ou des expériences en pot, et l'étude de communautés d'ECMs nécessite l'utilisation de tests à haut débit.

Le chapitre III est donc consacré au développement de tests en microplaques permettant de mesurer le potentiel d'altération des minéraux par les communautés d'ECMs via la sécrétion d'oxalate et la chélation du fer. Ces tests sont ensuite appliqués à Humont pour mesurer l'effet du chaulage sur l'activité potentielle d'altération des minéraux par les communautés d'ECMs du hêtre.

Conclusion of chapter II and introduction to chapter III

Therefore, liming modifies the potential activities of organic matter degradation by the ECM community. It is consistent with a modification of the nutrition of the fungal partner, but also for tree N and P nutrition. Moreover, the degradation of some components of organic matter by ECMs can modify the humus and litter degradation, which probably contributes to humus change, and therefore modify the availability and mobilization of many mineral nutrients.

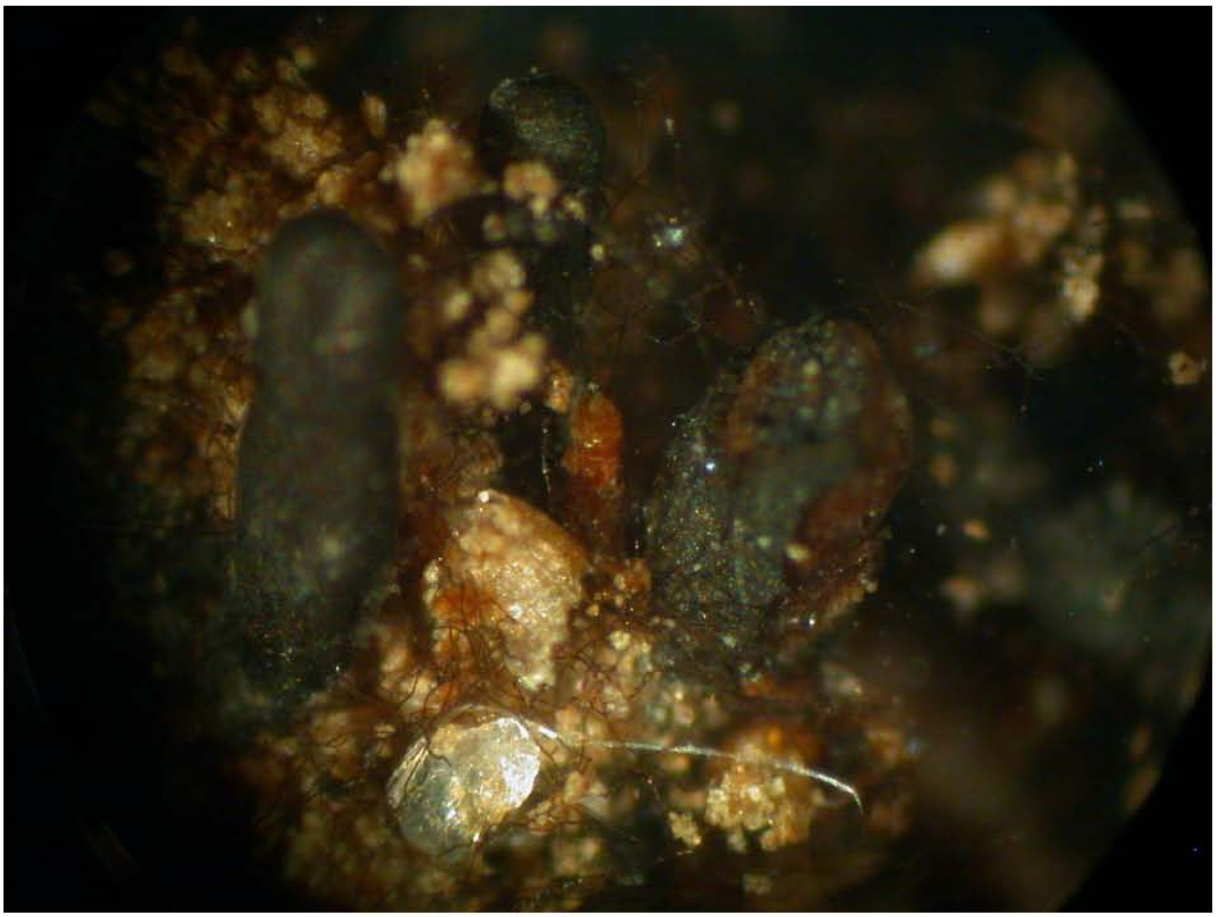
Nevertheless, forest trees also need inorganic nutrients of mineral origin such as PO_4^{3-}, Ca^{2+}, Mg^{2+}, Fe^{3+}, K^{+}. Ectomycorrhizal fungi contribute significantly to the uptake of these ions, by enhancing mineral dissolution, through the secretion of oxalate, a major agent of fungal mineral weathering, and via the secretion of low molecular weight chelating compounds for iron uptake. Considering that liming strongly modifies soil chemical composition (increase in exchangeable Ca^{2+} and Mg^{2+}, decrease of pH and Al^{3+}…), it is probable that liming have also an influence on nutrient uptake by ECMs. Nevertheless, most of the measurements of oxalate secretion by ECMs has been done with pure cultures or in pot experiments, and high-throuhput techniques for estimation of mineral weathering by ECM communities are lacking.

Thus, in chapter III, we developed microplate assays to measure the potential mineral dissolution by ECMs through the secretion of oxalate and iron complexation. These microplate tests were then used to measure the consequences of liming on potential oxalate secretion and iron complexation by beech ECM communities.

CHAPTER III. CONSEQUENCES OF LIMING ON POTENTIAL ACTIVITIES OF MINERAL WEATHERING BY ECM COMMUNITIES

***Article 5** " Simple microplate assays to measure iron mobilization and oxalate secretion by ectomycorrhizal tree roots" (accepted in Soil Biology and Biochemistry)

***Article 6** " Effects of liming on potential mineral weathering activities of beech ectomycorrhizal root tips" (submitted to Soil Biology and Biochemistry)



Macrophotography showing the emanating hyphae of *Cenococcum geophilum* ECMs in contact with soil mineral particles, in the spruce untreated plot, in May 2006. (photo : F. Rineau)

Article 5: Simple microplate assays to measure iron mobilization and oxalate secretion by ectomycorrhizal tree roots (accepted in Soil Biology and Biochemistry)

Abstract

The ability of ectomycorrhizal (ECM) fungi to mobilize soil inorganic nutrients has been well documented for a diversity of species. However, most of these studies were performed with tree seedlings or fungal cultures, making them hardly comparable. We propose here three microplate assays to compare iron chelation, free iron uptake and oxalate production by ECMs freshly sampled in mature forest ecosystems. These assays proved to be sensitive enough to detect significant differences between two common ECM types. *Lactarius subdulcis* was less efficient than *Xerocomus sp.* for accessing to free or complexed iron, but produced 100 times more oxalate. These preliminary results open the way to studying the contribution of ECM communities to nutrient cycling in forest ecosystems.

Key words: Ectomycorrhiza; Iron mobilization; Siderophores; Oxalate

Introduction

The fine roots of the trees in temperate forests form, with specialized fungi, symbiotic organs called ectomycorrhizas (ECMs) (Smith and Read, 1997), which contributes to the mineral nutrition of the trees by solubilizing soil minerals (Gadd, 2007) through the exudation of complexing compounds and low molecular weight organic acids (LMWOAs) (Landeweert et al., 2001).

Among the micronutrients derived from soil minerals, ferric iron (Fe^{3+}) is essential for various cellular processes as respiration, DNA synthesis and metabolism (Neilands, 1995). It is abundant in soils but mostly as insoluble forms, placing organisms into “famine in a land of

plenty" (Dancis et al., 1990). In order to have access to the soil iron reservoir, ECMs produce small molecules (M_r<1500) with high affinity for ferric iron, called siderophores (Landeweert et al., 2001; Howard, 2004; Kraemer, 2004). Siderophores secretion is repressed by high ferric iron concentration and induced by iron starvation (Neilands, 1995). LMWOAs are important mineral weathering agents, because of their dual acidifying and chelating ability (Landeweert et al., 2001). Oxalic acid, and to a lesser extent citric acid, are the most abundant LMWOAs produced by ECM fungi (Lapeyrie, 1987; van Hees et al., 2006) and can solubilize minerals by protonating or chelating cations (Landeweert et al., 2001).

In the present context of growing interest for deciphering the functional diversity of ECM communities in mature forest stands (Buée *et al.* 2005, Mosca *et al.* 2007), simple and high-throughput microplate assays are needed to quantify the ability of different ECM types to mobilize iron, as already done for enzymatic activities (Courty et al., 2005) or the resistance to desiccation (di Pietro et al., 2007).

The secretion of siderophores and other iron-chelating agents is commonly assessed in pure fungal or bacterial cultures and *in vitro* assays (Fekete et al., 1989; Frey-Klett et al., 2005; Machuca et al., 2007) using the chrome azurol sulfonate (CAS) reagent method of Schwyn & Neilands (1987). Oxalate secretion by ECM fungi has already been quantified *in vitro* or in pot experiments by high performance liquid chromatography (Wallander et al., 2000; Arvieu et al., 2003). However, these two methods have never been applied to ECMs sampled from forest soils.

The aim of the present work was therefore to develop sensitive microplate colorimetric tests to measure the ability of freshly-excised ECM tips to chelate complexed iron, to mobilize free iron, and to produce oxalate. The assays were tested on ECMs formed by *Xerocomus sp* and *Lactarius subdulcis*, sampled in two different sites in a mixed forest in North-Eastern France. About ten mycorrhizal short roots from each one of these two ECM

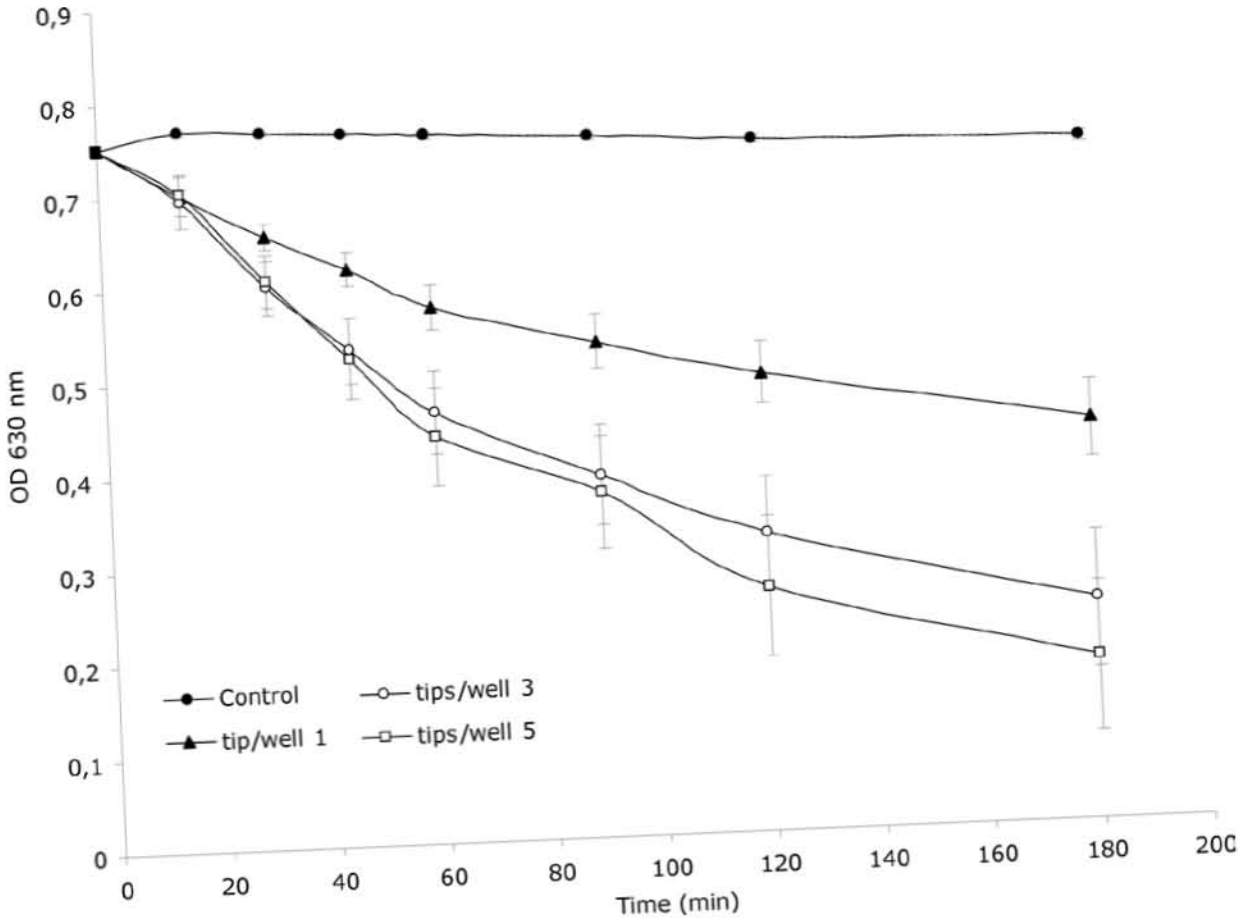


Figure 1 — Time course of iron mobilization from the CAS-Fe complex (measured as absorbance at 630 nm) by one, three or five *Xerocomus* sp ECMs per well in the microplate assay. Bars represent standard errors (n = 8). The absorbance at 630 nm of the blue CAS-Fe complex is a function of the concentration of iron chelating agents in the solution (Schwyn & Neilands, 1987).

morphotypes were molecularly identified by sequencing the ITS region of the fungal nuclear rDNA (Buée *et al.* 2005). One single amplicon was observed for each ECM type, showing that the morphological identification was reliable.

Microplate test n°1: Determination of complexed iron mobilized by ECMs using the CAS

Ability of ECM root tips to mobilize complexed iron was assessed by adapting the test of Schwyn and Neilands (1987) to the microplate system. In this assay, ferric iron is weakly complexed with CAS. The CAS-Fe complex, which absorbs at 630 nm (A_{630}), is broken down by stronger biological complexing agents such as organic acids or siderophores. We first optimized the CAS assay by measuring, in a 96-well microplate, the variations of A_{630} with one, three or five *Xerocomus sp* ECM tips per well, along a three-hour time course (Figure 1). The decrease of A_{630} was almost linear from 0 to 60 min. The slopes of the curves diminished afterwards whatever the number of ECM tips per well. All along the three hours, the reduction of A_{630} was significantly higher with three to five ECM tips per well than with one tip only, but does not differ significantly between three and five tips. In order to avoid the proliferation of bacteria during the assay, the incubation time must be kept as short as possible: three tips per well and 60 min incubation time were chosen as the best compromise between a significant and robust response, a short incubation time and a reasonable number of ECMs of the desired type to be picked from each root sample.

Figure 2 shows the application to this method to ECMs of *Xerocomus sp.* and *Lactarius subdulcis* sampled in the field. The measurements were in all cases significantly higher than the base line, showing the high sensitivity of the test. Moreover, *Xerocomus sp.* chelated significantly more iron than *Lactarius subdulcis*, showing that the test was sensitive enough to discriminate two ECM types (Fig. 2, white bars). The two ECM were able to

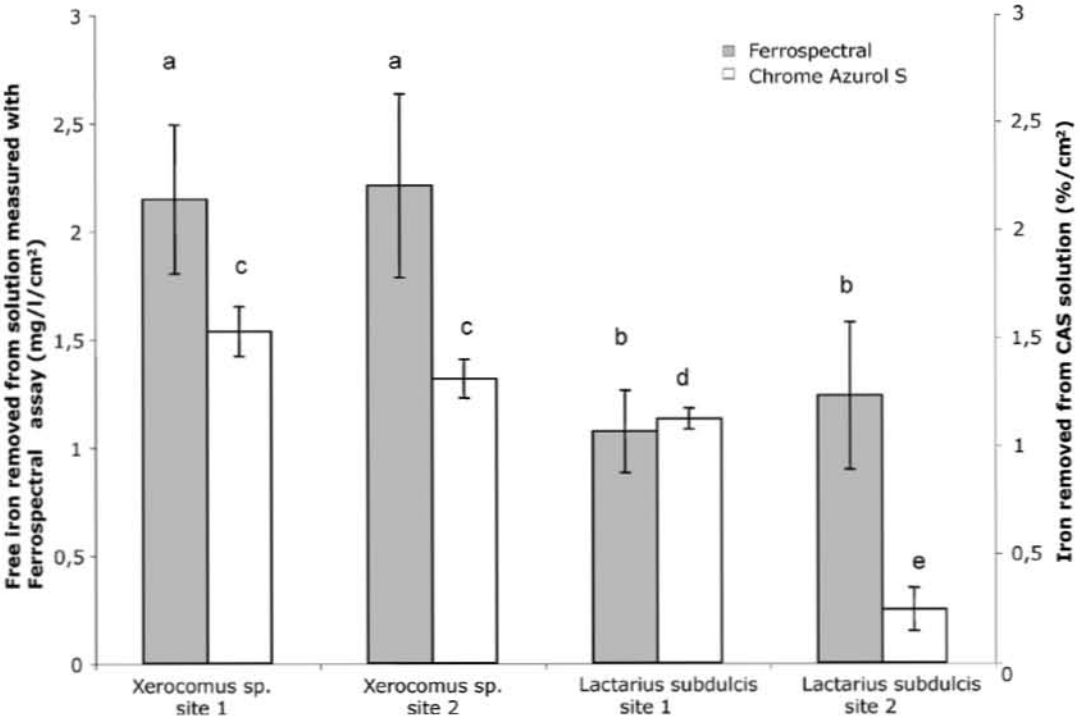


Figure 2 — Effect of *Xerocomus* sp and *L. subdulcis* ECMs on trapping free iron from the solution (left scale and grey bars, using the Ferrospectral® assay with three ECM tips per well, expressed in mg of Fe trapped.l^{-1}.cm^{-2}.h^{-1}) and on mobilizing iron from the CAS-Fe complex (right scale and white bars, using the CAS assay with three ECM tips per well, expressed as relative absorbance of the blue CAS-Fe complex, in%.cm^{-2}.h^{-1}).

mobilize iron from the CAS-Fe complex inducing a decrease of the absorbance from 1 to 2%. Iron chelation was significantly different in the two sample sites for *Lactarius subdulcis*, probably as a consequence of different soil properties. (Figure 2 Legend, part 2)

For Ferrospectral® assay, three ECM tips per well were incubated in 180 µl of $FeCl_3$, 2.7 mg l^{-1} (which corresponds to 0.9 mg l^{-1} of Fe) at 20°C for 1 hour. The apices were then removed from the plate with tweezers and 20 µl of Ferrospectral® were added to each well. The amount of total iron (Fe^{2+} plus Fe^{3+}) concentration in each well was immediately determined by measuring the absorbance at 595 nm with a Bio-Rad model 550 microplate reader (Uroz et al., 2007).

For the CAS assay, three ECM tips per well were incubated in 100 µl of CAS solution (60 µM HDTMA, 15 µM $FeCl_3$, $6H_2O$, 75 µM Chrome azurol sulfonate, 0.25 M piperazine, pH 6.5) at 20°C for 1 hour, then removed from the microplate with tweezers.

ECM tips were scanned directly in a new microplate filled with water using the automated image analysis software WINRHIZO 2003b (Regent instruments. Inc., Canada) which measures the surface area of each ECMs (Buée *et al*, 2005). All values were corrected by surface area of ECM tips (in cm^2) in order to facilitate comparisons between ECM types.

Small bars represent standard errors (n = 8). The effects of ECM type and sampling site on quantity of free iron trapped and iron chelation were assessed using the non-parametric Kruskall & Wallis rank sum test because data were not normal (Shapiro-Wilk's normality test). Values which do not significantly differ are annotated with the same letter.

Microplate test n°2: Determination of free iron trapped by ECMs using the Ferrospectral® reagent

The uptake of free iron (i.e. iron removed by absorption and/or adsorption by ECMs from a solution containing Fe^{3+} ions) was assessed using ferrospectral® (Merck), a highly iron-reactive agent used for measuring iron concentration in solution. This probe has been already used *in vitro* to assess the quantity of iron released from minerals by some bacterial isolates (Uroz et al., 2007). Contrary to the previous assay with CAS, the ferrospectral® assay was used here to measure the amount of iron trapped directly from the solution.

Calibration curves were built to determine the relationship between absorbance and iron concentration (Uroz et al., 2007). The difference with the blank, expressed in Fe concentration ($mg.l^{-1}$), is a measure of the ability of the ECM type studied to adsorb and/or absorb soluble iron. Three ECM root tips of *Xerocomus sp* and *Lactarius subdulcis* were used for the ferrospectral® test as for the CAS assay (Figure 2).

The ferrospectral® assay was reproducible, as shown by the standard errors between replicates (n=8) which were around 10 % of the mean. The two ECM types were able to trap between 1 to 2% of the free iron present in the solution (as shown in Figure 2, grey bars). Moreover, this test was sensitive enough to discriminate significantly *Xerocomus sp.* and *Lactarius subdulcis* ECMs in their ability to access to free iron. We did not observe any significant spatial variability between the two sites for the two ECM types.

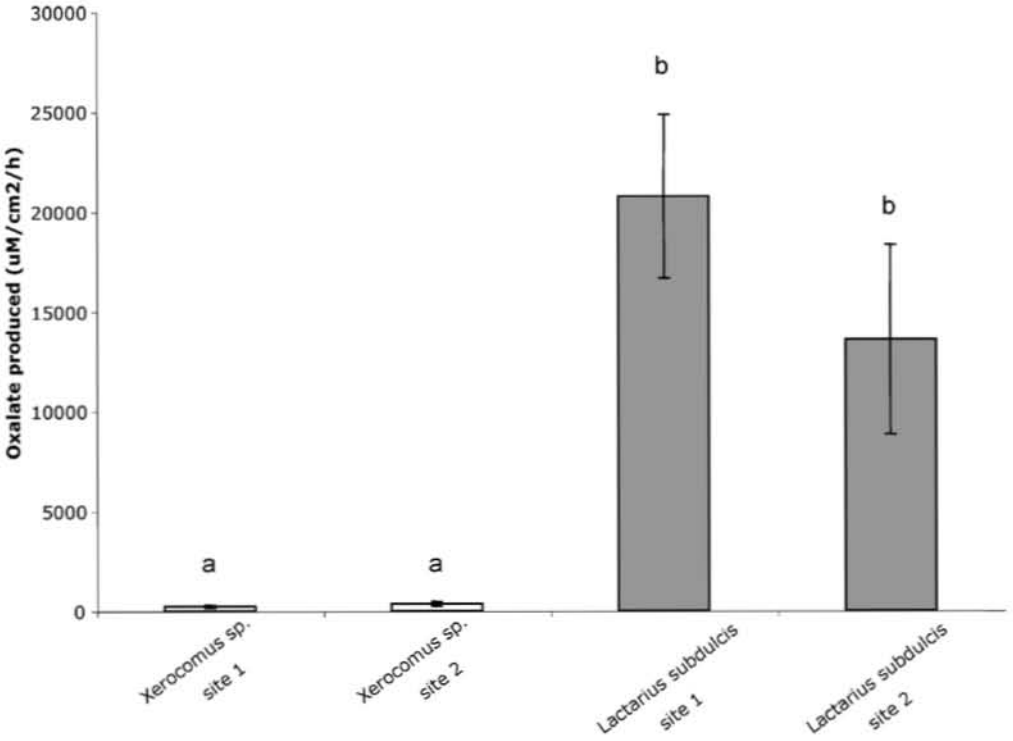


Figure 3 — Oxalate production (measured using the Urinalysis® diagnostics kit and expressed as µM of oxalate produced.$cm^{-2}.h^{-1}$) by *Xerocomus sp* and *Lactarius subdulcis* ECMs. Small bars represent standard errors (*n* = 8). The titration curve showed a linear relationship between A_{590} and the oxalate concentration in the incubation solution from 0 to 600 µM (Y = 0.0382 + 0.0026X, R^2=0.99, data not shown). We adapted the manufacturer's protocol to our root samples as follows. One ECM tip was incubated in 100 µl of MilliQ water for 1hour. Fifty µl of the incubation solution were transferred into a new microplate containing 50 µl of the dilution solution (EDTA 10mM, Buffer pH 7.6 +/- 0.1), 20 µl of oxalate reagent A (DMAB 3.2 mM, MBTH 0.22 mM, Buffer pH 3.1 ± 0.1) and 4 µl of oxalate reagent B (oxalate oxidase from barley: 3000 u l^{-1}, peroxidase from horseradish: 100 000 u l^{-1}). The reading of the absorbance at 590 nm took place immediately because the color is unstable. As for the two previous assays, all values were corrected by surface area of ECM tips (in cm^2) in order to facilitate comparisons between ECM types. Small bars represent standard errors (*n* = 8). The effects of ECM type and sampling site on quantity of free iron trapped and iron chelation were assessed using the non-parametric Kruskall & Wallis rank sum test because data were not normal (Shapiro-Wilk's normality test). Values which do not significantly differ are annotated with the same letter.

Microplate test n°3: Determination of oxalate secretion by ECMs

The ability of ECM tips to secrete oxalate was assessed using the urinalysis® diagnosis kit (Trinity Biotech France SARL, Champigny sur Marne). The test is based on oxalate oxidation by oxalate oxidase into carbon dioxide and hydrogen peroxide, which is transformed, in the presence of peroxidase, into a blue indamine dye which has an absorbance maximum at 590 nm. Then, the intensity of the blue color which develops during the reaction is proportional to the oxalate concentration in the sample. As for ferrospectral ® and CAS assays, the test was sensitive enough to discriminate significantly the two ECM types tested (Figure 3). Moreover, we showed that *Lactarius subdulcis* ECMs were able to produce 100 times more oxalate than those of *Xerocomus sp.* (0.2 to 0.3 $mM.cm^{-2}.h^{-1}$ for *Xerocomus sp* and 13 to 20 $mM.cm^{-2}.h^{-1}$ for *L. subdulcis*).

Comparisons between LMWOAs and siderophores for iron chelation using the CAS assay

We showed that ECMs were able to produce chelating agents able to break the CAS-Fe complex, and to produce high amounts of oxalate. Nevertheless, organic acids as oxalate, citrate or malate, and siderophores, are both capable of leaching iron from mineral surface (Gadd, 2007). In order to compare siderophores and LMWOAs in their ability to chelate iron, we assessed the effects of four iron-mobilizing agents on the CAS-Fe complex: oxalate and citrate (organic acids commonly secreted by fungi: van Hees et al., 2003), catechol (a synthetic siderophore), and deferoxamine (DFAM, a synthetic analog of siderophore found in *Streptomyces*). Figure 4 shows that deferoxamine was the most efficient iron-chelating agent at concentrations as low as 5 mM, closely followed by citrate. Catechol and oxalate did not reach the same efficiency even at concentrations over 40 mM. Nevertheless, DFAM is present

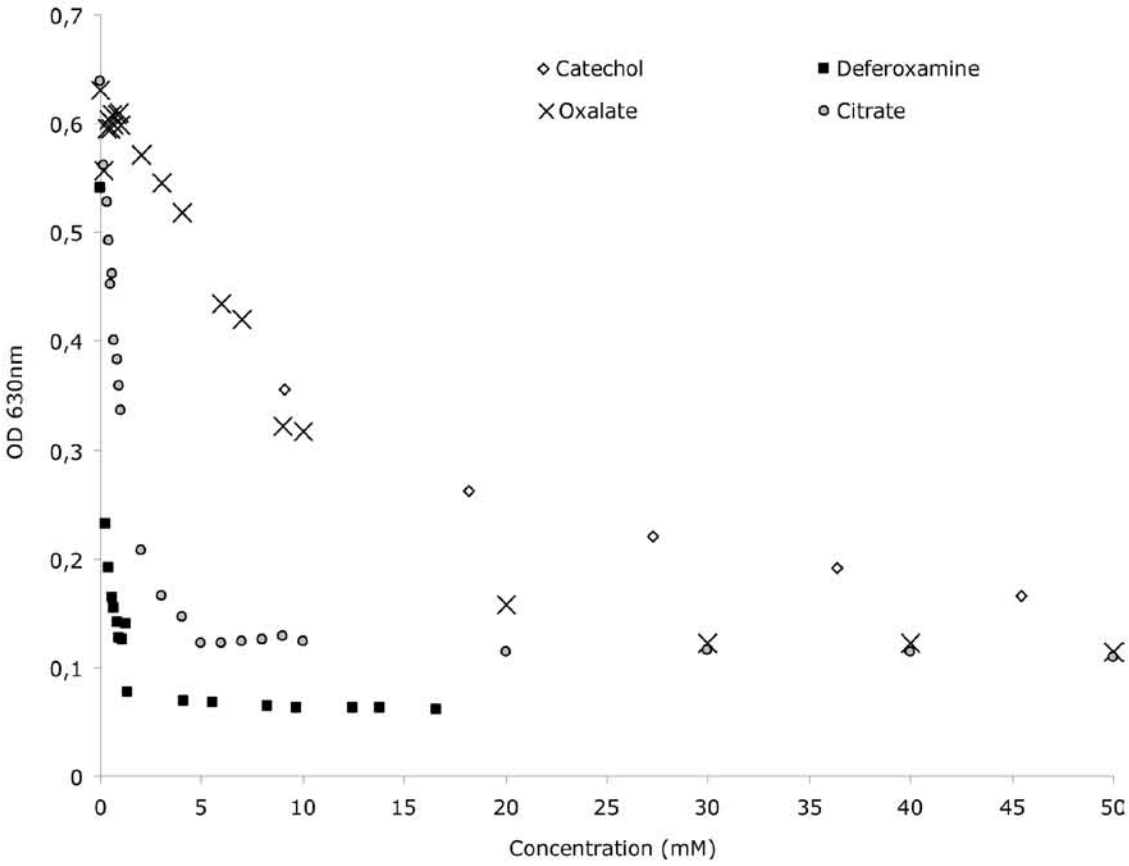


Figure 4 — Concentration effect of four iron chelating molecules on the CAS-Fe complex. The amount of iron released from the CAS-Fe complex after one hour incubation is proportional to the absorbance of the CAS-Fe complex at 630nm. A: the four iron complexing molecules tested. B: response curves (A_{630} of the CAS-Fe complex against concentration) for the four complexing agents.

in soils in 10^{-7} to 10^{-8} M concentrations (Kraemer, 2004), when oxalate can reach concentrations of 50μM (Jones et al., 2003), suggesting that oxalate plays a more important role in iron chelation by ECMs.

Aknowledgements

We thank Dr. C. Nys for providing the experimental site of Humont and for his expertise about liming, and the *Office National des Forêts* for allowing us to sample roots in the experimental plots. This work was made possible by grants coming from INRA and Lorraine Region. We also thank anonymous reviewers for their useful comments.

References

Arvieu, J. C., Leprince, F., Plassard, C., 2003. Release of oxalate and protons by ectomycorrhizal fungi in response to P-deficiency and calcium carbonate in nutrient solution. Annals of Forest Science 60, 815-821.

Buée, M., Vairelles, D., Garbaye, J., 2005. Year-round monitoring of diversity and potential metabolic activity of the ectomycorrhizal community in a beech (F. sylvatica) forest subjected to two thinning regimes. Mycorrhiza 15, 235-245.

Courty, P.E., Pritsch, K., Schloter, M., Hartmann, A., Garbaye, J., 2005. Activity profiling of ectomycorhiza communities in two forests soils using multiple enzymatic tests. New Phytologist 167, 309-319.

Dancis, A., Klausner, R. D., Hinnesbuch, A. G., Barriocanal, J. G., 1990. Genetic evidence that Ferric Reductase is required for iron uptake in *Saccharomyces cerevisiae*. Molecular and Cellular Biology 10, 2294-2301.

di Pietro M., Churin J.L., Garbaye J., (2007). Differential ability of ectomycorrhizas to survive drying. Mycorrhiza 17, 547-550.

Fekete, F. A., Chandhoke, V., Jellison, J., 1989. Iron-binding compounds produced by wood-decaying basidiomycetes. Applied and Environmental Microbiology 55, 2720-2722.

Frey-Klett, P., Chavatte, M., Clausse, M. L., Courrier, S., Le Roux, C., Raaijmakers, J., Martinotti, M. G., Pierrat, J. C., Garbaye, J., 2005. Ectomycorrhizal symbiosis affects functional diversity of rhizosphere fluorescent pseudomonads. New Phytologist 165, 317-328.

Gadd, G. M., 2007. Geomycology: biogeochemical transformatins of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycological Research 111, 3-49.

Howard, D. H., 2004. Iron gathering by zoopathogenic fungi. FEMS Immunology and Medical Microbiology 40, 95-100.

Jones D. L., Dennis P. G., Owen A. G., van Hees P. A. W., 2003. Organic acid behavior in soils-Misconceptions and knowledge gaps. Plant and Soil, 248. 31-41.

Kraemer, S. M., 2004. Iron oxide dissolution and solubility in the presence of siderophores. Aquatic Sciences 66, 3-18.

Landeweert, R., Hoffland, E., Finlay, R. D., Kuyper, T., van Breemen, N., 2001. Linking plants to rocks. Ectomycorrhizal fungi mobilize nutrients from minerals. Trends in Ecology and Evolution 16, 248-254.

Lapeyrie, F., Chilvers, G. A., Bhem, C. A., 1987. Oxalic acid synthesis by the mycorrhizal fungus *Paxillus involutus* (Batsch. Ex Fr.) Fr. New Phytologist 106, 139-146.

Machuca, A., Pereira, G., Aguiar, A., Milagres, A. M. F., 2007. Metal-chelating compounds produced by ectomycorrhizal fungi collected from pine plantations. Letters in Applied Microbiology 44, 7-12.

Mosca E., Montecchio L., Scattolin L., Garbaye J., 2007. Enzymatic activities of three ectomycorrhizal types of Quercus robur L. in relation to tree decline and thinning. Soil biology and Biochemistry 39, 2897-2904

Neilands J. B., 1995. Siderophores. Structure and fonction of microbial iron transport compounds. Jour. Biol. Chem, 270. 26723-26726.

Schwyn, B. and Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry 160, 47–56.

Smith, S. E., Read, D. J., 1997. Mycorrhizal Symbiosis, Ed 2, Academic press, London, 606 pp.

Uroz, S., Calvaruso, C., Turpault, M. P., Pierrat, J. C., Mustin, C., Frey-Klett, P., 2007. Mycorrhizosphere effect on the genotypic and metabolic diversity of the soil bacterial communities involved in mineral weathering in a forest soil. Applied and Environmental Microbiology 73, 3019-3027.

van Hees P. A. W., Godbold D. L., Jentschke G., Jones D. L., 2003. Impact of ectomycorrhizas on the concentration and biodegradation of simple organic acids in a forest soil. European journal of soil science 54, 697-706

van Hees, P. A. W., Rosling, A., Essén, S., Godbold, D. L., Jones, D. L., Finlay, R. D., 2006. Oxalate and ferricrocin exudation by the extramatrical mycelium of an ectomycorrhizal fungus in symbiosis with *Pinus sylvestris*. New Phytologist 169, 367-368.

Wallander, H., 2000. Uptake of P from apatite by *Pinus sylvestris* seedlings colonized by different ectomycorrhizal fungi. Plant and Soil 218, 249-256.

Article 6: Effects of liming on potential mineral weathering activities of beech ectomycorrhizal root tips (submitted to Soil Biology and Biochemistry)

Abstract

Liming is used to counteract forest decline induced by soil acidification. It consists in Ca and Mg input in forest soil and restores tree mineral nutrition, but also modifies the availability of nutrients in soil. Ectomycorrhizal (ECM) fungi are involved in mineral nutrient uptake by trees and can recover them through dissolution of mineral surface. Oxalate and siderophore secretion are considered as the main agents of mineral weathering by ECMs. Here, we studied the effects of liming on the potential oxalate secretion and iron complexation by beech ECM root tip communities. Results show that freshly excised ECM root tips belonging to the species *Lactarius subdulcis* presented the unexpectedly high mean potential oxalate exudation rate of 178 +/- 21 $\mu M.tip^{-1}.h^{-1}$. As this ECM species distribution is very dense, it is likely that in the field oxalate concentrations in the vicinity of its clusters could be very high. This point out that not only extraradical mycelium but also ECM root tips of certain species can contribute significantly to mineral weathering. Liming highly increased the mean potential oxalate production by ECM root tips, and this activity was mainly driven by *Lactarius subdulcis* ECMs. Potential activity of iron mobilization by ECM root tips was significantly lower in limed plots. As the mean oxalate secretion by ECM morphotype was not significantly correlated to its mean iron mobilization, we conclude that iron complexation was due to either other organic acids or to siderophores.

Key words: ectomycorrhiza, beech, liming, oxalate, iron mobilization

Introduction

Since the 1970's, forest tree decline in acidic soils has been reported for many countries of central Europe. Affected areas showed symptoms of beech defoliation, which was attributed to Ca^{2+} and Mg^{2+} deficiencies (Ulrich, 1983). Natural rainfall acidity in these forests lead to the leaching of divalent cations exchanged on soil colloids, resulting in the loss of 2% of Mg and 0.5% of Ca per year (Dupouey et al., 1998). Direct calcium and magnesium amendment (liming) has then been used to durably restore tree mineral nutrition. Liming not only restores tree health, but also causes considerable change in the soil ecosystem: it reduces the acidity of upper soil layers, increases cation exchange capacity and soil base saturation, especially in humus layers (Frank & Stuanes, 2003), and stimulates soil fauna, in particular earthworms (Persson, 1988). Liming also shifts the humus type from mor or moder to mull (Kreutzer, 1995).

Ectomycorrhizae (ECMs) play a predominant role in soil nutrient transfer to the trees (Smith & Read, 1997). The root-associated symbiotic fungi are able to retrieve soil Ca^{2+} and Mg^{2+}, but also Fe^{3+}, Pi, K^{+} and other nutrients complexed in soil minerals, through different mechanisms of weathering such as acidolysis or complexolysis (Landeweert et al., 2001). Acidolysis of mineral grains through the secretion of organic acids (especially oxalate for the majority of fungi: Gadd, 1999) is suspected to play an important role in nutrient uptake by ECMs, because of the dual acidifying and complexing abilities of these acids (Landeweert et al., 2001). ECMs, as many rhizosphere bacteria, are also able to retrieve ferric iron, which is an essential cofactor for catalases, cytochromes or peroxidases (Neilands, 1995), but is scarce as an available, soluble form (10^{-17} M free Fe^{3+} at pH 7, Dancis *et al*, 1990). ECM fungi produce low-molecular weight chelating agents (M_r<1500) with high affinity for ferric iron, called siderophores (Neilands, 1995; Howard, 2004; Kraemer, 2004). The secretion of siderophores is repressed by high ferric iron concentration and induced by iron starvation

(Neilands, 1995), and most fungal species can secrete various types of siderophores (Haas, 2003).

Thus, ECMs clearly contribute to nutrient mobilization from minerals in forest ecosystems, but it is also well known that the ECM communities are very diverse (Smith & Read, 1997) and that their structure is very responsive to environmental disturbance, including soil amendment and liming (Erland & Taylor, 2002; Rineau & Garbaye, 2009). It is therefore likely that many ecosystem functions of ECM communities are modified by the practice of forest liming, indirectly impacting tree nutrition and growth.

Here, we hypothesize that liming modifies the ability of the root tip ECM community to weather minerals and mobilize nutrients. In order to experimentally address this question, we compared the functional structure of the ECM community in the two treatments (limed vs. untreated) of a liming experiment in a declining beech stand. We focused on two activities relevant with mineral weathering: potential oxalate production and potential iron complexation, using recently-developed microplate assays with individual ECM root tips sampled *in situ*.

Materials and methods

Site and forest stand

The experimental site of Humont (48°00’00’’N, 6°29’28’’E, Altitude: 570 m, Vosges forest, in North-Eastern France) is a 60-year-old beech forest (*Fagus sylvatica*). The acidic brown soil (typic dystrochrept) is formed on red sandstone. The liming treatment was carried out in a 500 by 400m area by helicopter in 1991 with 757 kg/ha of $CaCO_3$ and 380 kg/ha of $CaMg(CO_3)_2$. Sixteen years later, pH had shifted from 4.4 to 4.7 and humus had shifted from a dysmoder to a typical oligomull in the amended area, which was also characterized by

abundant earthworms, a significant increase of exchangeable Ca and Mg and a significant drop of exchangeable Al (Data not shown).

Sampling and sample processing

Three replicates, each with a limed and untreated plot (20m * 20m), were defined. Couples of plots within each replicates were chosen as close as possible in terms of soil structure, slope and tree density. Sampling took place in May 2008. Eight trees were randomly chosen in replicates 1 and 3, and seven ones in replicate 2. One $5cm^3$ soil core (1cm diameter, 5cm depth) was taken at 1m of the base of each tree. All soil cores were stored at 4°C and treated within 2 days after sampling. Fine roots were washed out of the soil cores on a 0.5 mm sieve and observed using a stereomicroscope. The fungal species associated with the different ECM morphotypes were identified according to Agerer (1987-98) and further specific molecular identification as described below. Within each soil core, all of the ECM tips were collected with tweezers, separately placed in the 96 wells of microplates filled with 100µl of milliQ water, and stored at 4°C before test processing. The potential oxalate secretion and iron mobilization assays were done using the whole ECM community of all soil cores.

Measurements of potential oxalate secretion and iron mobilization by ECMs

The measurement of potential oxalate secretion and iron mobilization by ECMs was done following the protocols of Rineau et al. (2008). The ECMs were incubated for one hour in 100µl of milliQ water for the oxalate assay (1 tip per well) at room temperature. The test (urinalysis® diagnostics kit, Trinity Biotech France SARL, Champigny sur Marne) is based on oxalate oxidation by oxalate oxidase into carbon dioxide and hydrogen peroxide, which is transformed, in the presence of peroxidase, into a blue indamine dye which has an absorbance maximum at 590 nm. The intensity of the blue color which develops during the reaction is

proportional to the oxalate concentration in the sample. After 1 hour incubation, ECMs were removed from the plate and pooled 3 per well for each morphotype within each soil core for the potential iron mobilization assay. The reason what the ECMs were pooled for potential iron mobilization assay is that CAS is not sensitive enough to allow measurements on 1 ECM root tip within 1 hour. Three tips per well and 60 min incubation time were chosen as the best compromise between a significant and robust response, a short incubation time and a reasonable number of ECMs of the desired type to be picked from each root sample (Rineau et al., 2008). The iron mobilization assay is adapted from the chrome azurol S (CAS) assay (Schwyn & Neilands, 1987), where the complex CAS-Fe^{3+}-HDTMA mimics a soil mineral particle: its absorbance at 630nm decreases when a strong chelator (i.e a siderophore) removes the iron from the dye. The pooled ECMs were incubated for one hour in a solution of CAS at room temperature. The two assays were thus done with the same ECM root tips, allowing measurements of total secreted oxalate and total iron mobilization in the same community. Before running the CAS assay, in order to prevent the chemical deterioration by the CAS solution, 1 tip of each of the 29 morphotypes was collected, frozen at -80°C and used for confirming the morphotype identification by DNA sequencing of the ITS region (Gardes & Bruns, 1993).

Molecular identification

The total genomic DNA of ECM tips was extracted with the Dneasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France) following the manufacturer's instructions. The Internal Transcribed Spacer (ITS) region of the fungal nuclear rDNA was specifically amplified using nested PCR with the primer couples ITS1f / ITS4 and ITS1 / ITS4 (Invitrogen, Cergy Pontoise, France). The PCR reaction was performed in a Gene Amp 9600 thermocycler (Perkin Elmer instruments, Norwalk, Conn.) in a mix composed of 3µl of genomic DNA, 2.5µl of each

primer, 20µl of 5 prime mastermix (Dutscher, Brumath, France) and 25µl of milliQ water. The PCR reactions were done using the following parameters: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 50°C for 30s and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. Amplification success was checked in a 1% agarose electrophoresis gel in 1% TBE (Tris-Borate-EDTA) stained with ethidium bromide and visualized under UV light. Purifications were done with milliQ water using Millipore filtration microplates. The amplified fragments were sequenced using the Quick start mix (Kit DTCS, Beckman Coulter, USA) in a CEQ8000 sequencer (Beckman Coulter, USA). Sequences were aligned using ITS sequences available in the NCBI (http://www.ncbi.nlm.nih.gov/) and UNITE (http://unite.ut.ee/) databases. This protocol has been slightly adapted from Buée et al (2005).

Data treatment and statistics

The potential secretion of oxalate by ECM root tips was expressed in $\mu M.tip.h^{-1}$. Concerning the CAS assay, the absorbance of the CAS solution was proportional to the quantity of ferric iron complexed with CAS, i.e inversely proportional to iron complexation by ECM exsudates (Rineau et al., 2008). Iron complexation was thus expressed in % of CAS discoloration per tip and per hour (CAS $units.tip.h^{-1}$).

The normality of oxalate secretion and iron mobilization activities were assessed using the Shapiro-Wilk's test. Data were not normal and no significant normalization was possible because of many inactive ECMs (null values). Therefore, we used the non-parametric Wilcoxon test to measure the effect of the treatment on the mean potential activities of oxalate secretion and iron mobilization by the ECM community (comparison between two mean values), and the Kruskall-Wallis test to assess the effect of treatment (limed vs untreated) on

Table 1. Abundance and mean potential oxalate production and iron complexing of the 25 morphotypes found in the study. The abundance is given as the total number of root tips of one morphotype in all soil cores for each of the two treatments (untreated vs limed). The mean potential activity of oxalate production is given as the mean activity of all ECM tips of one morphotype per treatment. The mean potential activity of iron complexing is given as the mean activity of all ECM tips of one morphotype per treatment. When molecular identification was successful, Blast results are given in the table (Accession number, score and e-value). Species are sorted by decreasing abundance. The species name of morphotypes found is this study which were highly similar to another one sequenced previously were given in brackets.

Morphotype	Abundance in limed plots	Abundance in untreated plots	Total abundance	Accession number	Fungal species name	Score	e-value	Mean CAS activity in the limed plots (in CAS units.tip-1.h-1)	Mean CAS activity in the untreated plots (in CAS units.tip-1.h-1)	Mean oxalate secretion in the limed plots (in µM.tip.h-1)	Mean oxalate secretion in the untreated plots (in µM.tip.h-1)
Cenococcum geophilum	10	239	249					0.00%	0.28%	0	4
(Tomentella sublilacina)	70	50	120					0.45%	0.61%	0	5
Lactarius subdulcis	115	5	120	AJ889965.1	*Lactarius subdulcis*	1425	0	1.18%	3.43%	177	14
(Clavulina cristata)	30	4	34					1.47%	0.00%	5	0
Russula ochroleuca	8	25	33	EU350580.1	*Russula ochroleuca*	759	0	1.01%	0.74%	8	3
Unidentified sp.2	18	12	30					0.00%	1.26%	23	0
Unidentified sp.1	2	19	21					-	1.17%	65	2
Russula puellaris	7	11	18	UDB000010	*Russula puellaris*	452	e-128	1.37%	0.96%	18	3
(Cortinarius sp.)	0	16	16					-	1.79%	-	0
Unidentified sp.7	15	0	15					0.00%	-	0	-
(Laccaria amethystina)	0	13	13					-	4.88%	-	14
Unidentified sp.4	11	0	11	UDB000547	*Tricholoma sciodes*	315	e-86	2.69%	-	0	-
Xerocomus pruinatus	1	10	11	UDB000018	*Xerocomus pruinatus*	1067	0	-	3.55%	0	4
Amanita rubescens	0	9	9	UDB000038	*Amanita rubescens*	880	0	-	4.29%	-	0
Unidentified sp.8	0	9	9					-	12.19%	-	27
Unidentified sp.5	4	2	6					0.00%	0.00%	0	0
Unidentified sp.3	5	0	5					3.28%	-	1	-
Unidentified sp.6	3	1	4					-	-	0	0
Lactarius rostratus	3	0	3	UDB000852	*Lactarius rostratus*	1029	0	-	-	0	-
Unidentified sp.12	0	3	3					-	-	-	0
Scleroderma bovista	2	0	2	UDB002179	*Scleroderma bovista*	492	e-140	-	-	0	-
Unidentified sp.9	0	2	2					-	-	-	0
Unidentified sp.10	0	2	2					-	-	-	0
Unidentified sp.11	0	2	2					-	-	-	0
Thelephora sp.	1	0	1	UDB000211	*Thelephora sp.*	208	e-54	-	-	0	-

mean oxalate secretion and iron mobilization activities by each ECM species (non-parametric analysis of variance).

The correlation between mean oxalate secretion and mean iron mobilization by each ECM morphotype was assessed using the Spearman correlation test. Statistical analyses were done using the R software (http://www.r-project.org) (Ihaka & Gentleman, 1996).

Because these potential activities were measured for all the ECM root tips in all soil cores, we calculated total (sum for all tips, i.e whole community) oxalate secretion and iron mobilization in each treatment.

Results

Description of the ECM community

A total number of 739 ECM root tips, belonging to 25 ECM morphotypes and contained in 46 soil cores, were studied (Table 1). Four of the morphotypes found in this study gave no amplification. They were impossible to be distinguished from the morphotypes *Clavulina cristata., Cortinarius sp., Laccaria amethystina* and *Tomentella sublilacina,* which were easy to determine, repeatedly found close to the same plots, and successfully molecularly identified in surrounding plots for other studies. We therefore assigned to these four morphotypes their species names in brackets, in order to distinguish them from those successfully sequenced in this study. The 3 species : *Cenococcum geophilum, Tomentella sublilacina* and *Lactarius subdulcis* represented 66% of the total abundance of ECM root tips and were thus considered as codominant. The second most abundant species after *Lactarius subdulcis* was *Clavulina cristata* (4 times less abundant:120 vs 34 tips, respectively). Thus, we present here the results

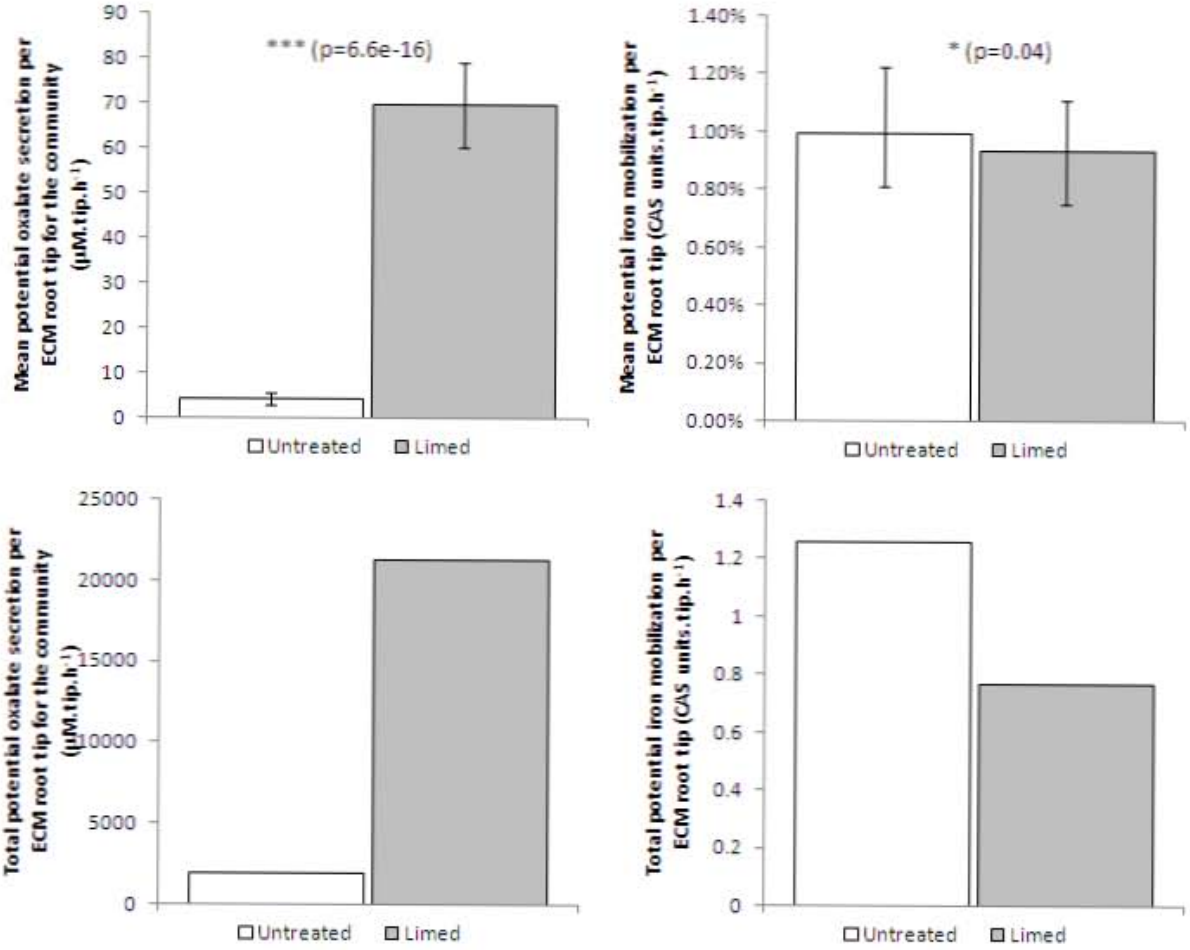


Figure 1. Mean oxalate secretion (in $\mu M.tip^{-1}.h^{-1}$), mean potential iron mobilization (in CAS $units.tip^{-1}.h^{-1}$), total potential oxalate secretion (in $\mu M.\ h^{-1}$) and total potential iron mobilization (in CAS $units.\ h^{-1}$) of the sampled ECM communities in the limed and untreated plots. Bars represent standard error. The total potential activities of the community were calculated as the sum of the activities of all the tips in the limed plots and in the untreated ones, respectively. Significant differences in mean oxalate secretion and iron mobilization activities were assessed using the non parametric test of Wilcoxon. The value of the probablility of the test is given above the bars.

separately for the 3 codominant species mentioned above and we have pooled the results concerning the others. In terms of occupation of the root system, *Cenococcum geophilum* ECMs were isolated but homogeneously distributed all over the beech fine roots, whereas *Lactarius subdulcis* ECMs formed dense clusters of 5 to several dozens of root tips, making the distribution of this ECM species very heterogeneous among soil cores.

Potential oxalate secretion and iron complexing by the whole ECM communities

No calcium oxalate crystals were observed at the surface of ECM root tips. There was a significant positive effect of liming on the mean potential oxalate secretion by ECMs (69.7 +/- 9.3 $\mu M.tip.h^{-1}$, n=305, in limed plots; 4.3 +/- 1.4 $\mu M.tip.h^{-1}$, n=434, in untreated ones, +/- standard error, $p=6.6e^{-16}$) (Figure 1). The mean potential oxalate secretion was very low in the untreated plots. In opposite, liming significantly reduced the mean potential iron chelation by ECMs (0.93 +/- 0.23 CAS $units.tip.h^{-1}$, n=82 in limed plots; 0.99% +/- 0.18 CAS $units.tip.h^{-1}$, n=127, in untreated ones, +/- standard error, p=0.04). When all the individual ECM root tip potential activities were added per treatment, total oxalate secretion in limed plots was 11 times higher than in untreated ones (21256 $\mu M.h^{-1}$ in limed plots for 305 ECM root tips; 1875 $\mu M.h^{-1}$ in untreated ones for 434 ECM root tips). The total iron complexing ability was slightly lower (1.6 times) in limed. The mean activities of iron complexing and oxalate secretion by individual ECM species were not significantly correlated ($p>0.05$).

Effects of liming on potential oxalate production by ECM root tips

Lactarius subdulcis was by far the most active morphotype for potential oxalate secretion (178 +/- 21 $\mu M.tip^{-1}.h^{-1}$ in mean for all his root tips, Figure 2). However, the activity of this morphotype was significantly lower in the untreated plots (14 +/- 8 $\mu M.tip^{-1}.h^{-1}$ in mean). The

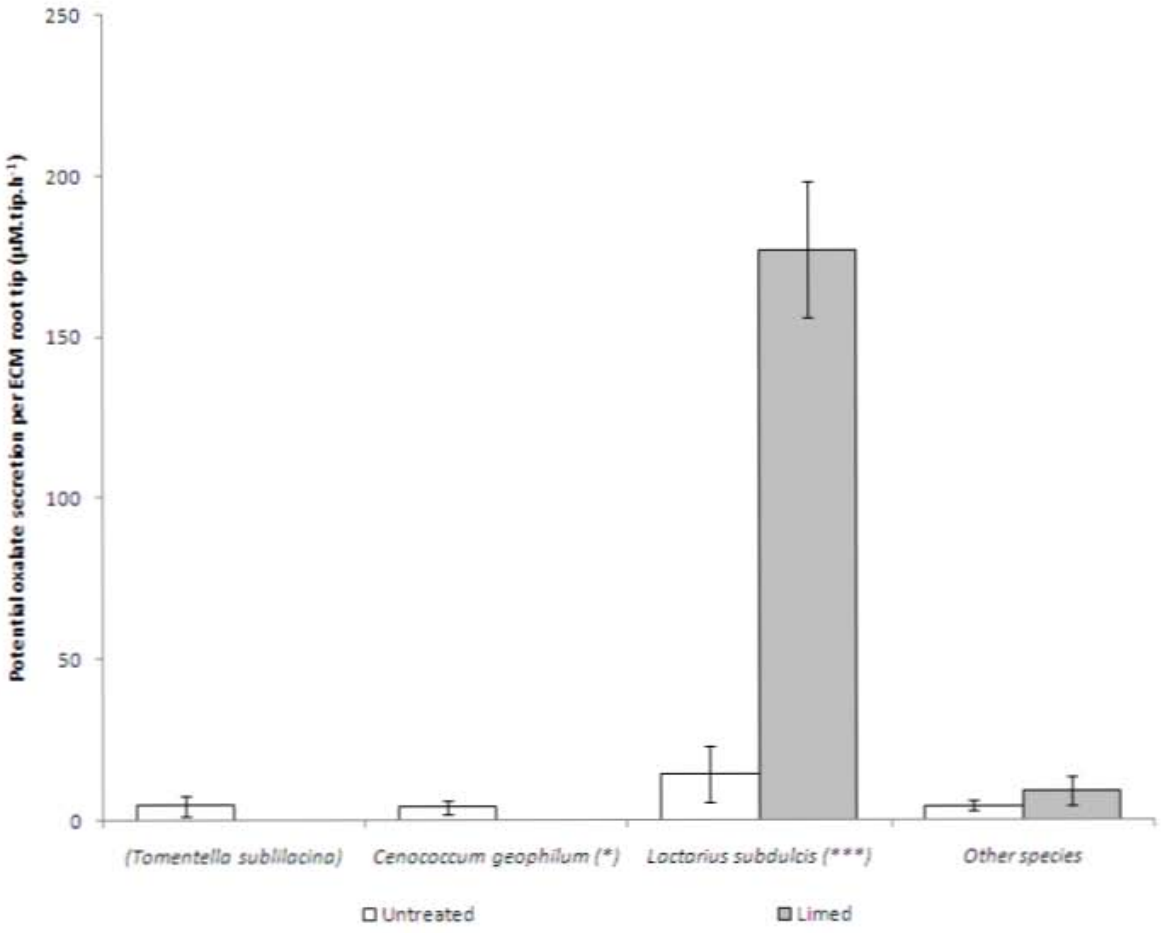


Figure 2. Mean potential oxalate secretion (in $\mu M.tip^{-1}.h^{-1}$) of the ECM morphotypes in the limed and untreated plots. Results are presented separately for the 3 co-dominant morphotypes, and other morphotypes have been pooled into the "Other species" category. The species name of morphotype found in this study which was highly similar to another one sequenced previously were given in brackets. Bars represent standard error. The effect of liming on the mean activity of each of the 4 categories (*Tomentella sublilacina*, *Cenococcum geophilum*, *Lactarius subdulcis*, Other species) has been assessed using the non-parametric Kruskall & Wallis test (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

potential oxalate secretion by *Cenococcum geophilum* and *Tomentella sublilacina* (4 +/- 2 $\mu M.tip^{-1}.h^{-1}$ and 5 +/- 3 $\mu M.tip^{-1}.h^{-1}$, respectively) was low in untreated plots and almost null in limed plots. There was no significant effect of liming on potential oxalate secretion by other ECM species.

Effects of liming on potential iron mobilization by ECM root tips

Most of the ECM species were able to chelate significant amounts of iron (Table 1). The most active ECM morphotype was *Lactarius subdulcis* (3.43 +/- 0.16 CAS $units.tip^{-1}.h^{-1}$ in mean, Figure 3). The two other most abundant species, *Tomentella sublilacina* and *Cenococcum geophilum* had lower activities (between 0.28 +/- 0.11 CAS $units.tip^{-1}.h^{-1}$ and 0.61 +/- 0.43 CAS $units.tip^{-1}.h^{-1}$). However, and contrary to potential oxalate secretion activities, the less abundant species (bar "other species" in Figure 3) had quite high mean potential activities of iron complexation (2.46 +/- 0.70 CAS $units.tip^{-1}.h^{-1}$) but these activities were highly variable. The effects of liming on potential iron chelation showed the same trend for all the ECM species, i.e the reduced potential activity in the limed plots. However, this effect was not significant for *Tomentella sublilacina* ECMs.

All the activities of oxalate secretion and iron complexation were quite variable, mainly because of the high occurence of null values. For example, for *Cenococcum geophilum*, 219 out of 239 tips did not produce oxalate and 69 groups of ECMs (pooled per 3) of 75 had no activity of iron complexation; for *Lactarius subdulcis*, the proportions of inactive ECM tips were only 24/120 and 24/37, respectively. When considering only non-null values, the variability of the data was considerably lower.

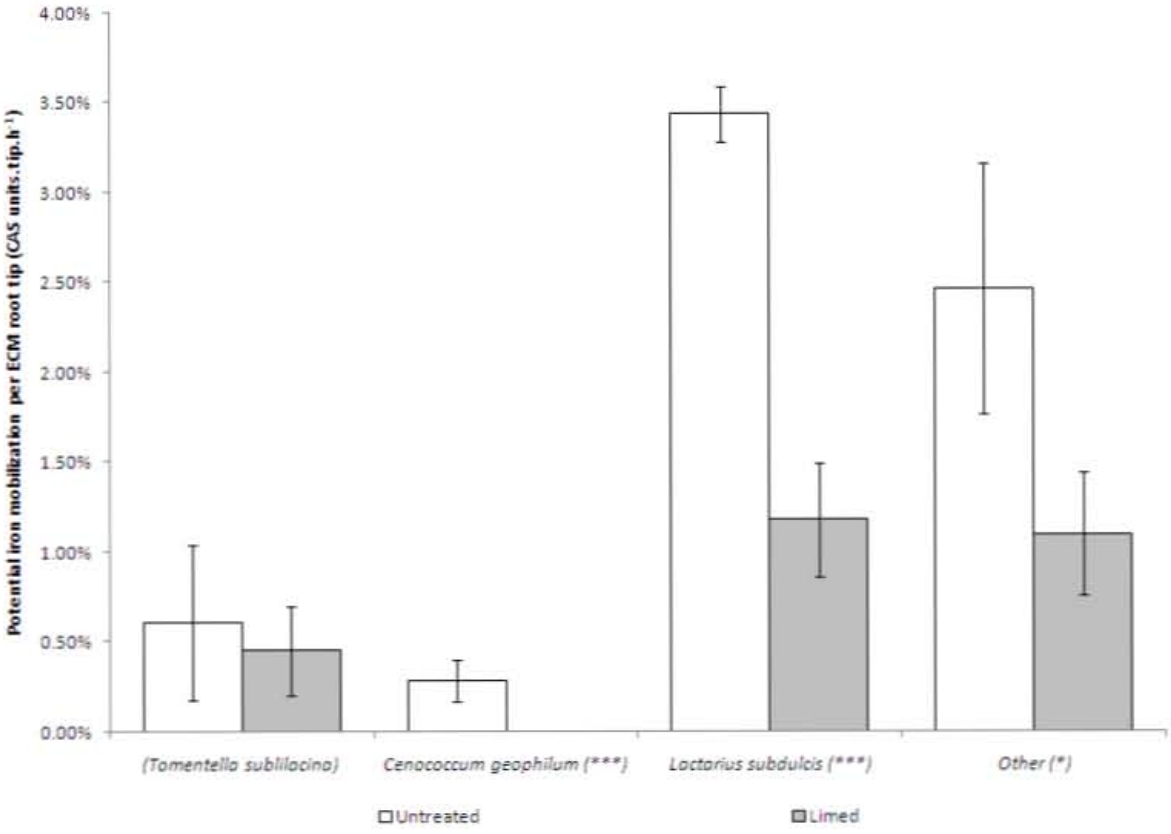


Figure 3. Mean potential iron mobilization (in CAS units.tip^{-1}.h^{-1}) of the ECM morphotypes in the limed and untreated plots. Results are presented separately for the 3 codominant morphotypes, and other morphotypes have been pooled into the "Other species" category. The species name of morphotype found in this study which was highly similar to another one sequenced previously were given in brackets. Bars represent standard error. The effect of liming on the mean activity of each of the 4 categories (*Tomentella sublilacina*, *Cenococcum geophilum*, *Lactarius subdulcis*, Other species) has been assessed using the non-parametric Kruskall & Wallis test (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

Discussion

The most striking result of this study was the unexpected high potential secretion of oxalate by individual ECM root tips. The observed oxalate concentrations in our assays were around 180µM per hour in mean for a *Lactarius subdulcis* ECM root tip in starving conditions (100µl of sterile water). The mean residence time of organic acids in the soil is very short: 3 to 69h, according to van Hees et al. (2005). Here, a higher residence time due to stabilization of oxalate in calcium oxalate crystals is unlikely because we did not observe any of these crystals throughout the study. Thus, if we consider the very small average migration distance of LMWOAs (from 0.01 to 14mm; van Hees et al., 2005), the potential exudation rates were measured here are likely to produce huge oxalate concentration in the vicinity of the ECM root tips. Moreover, the ECMs of *Lactarius subdulcis* belong to the contact exploration type (Agerer, 2001), i.e with a smooth mantle and a few emanating hyphae, meaning that they mobilize nutrients in their close vicinity. Regarding the distribution of this ECM species in large and dense patches of 5 to several dozens of root tips, it is very likely that oxalate concentration within these patches is very high. This emphasizes the fact that even if mycorrhizal mycelium is supposed to be the main component of fungal mineral weathering, the ECM root tips of certain species such as *Lactarius subdulcis* can have locally a high impact on this process too. Moreover, the total potential activity of oxalate secretion by the ECM root tip community in the limed treatment was very high (21mM.h^{-1}). It shows that the cumulated activity of about 300 ECM root tips in a small soil volume (23 soil cores of 5cm^3, i.e 115cm^3) can be of ecological importance for potential mineral weathering.

Liming significantly enhanced the mean and the total potential oxalate productions by ECM root tips. This result was unexpected, because we supposed that oxalate production was

favored by the scarcity of inorganic nutrients (Landeweert et al., 2001), which was corrected in the limed plots for at least Ca^{2+} and Mg^{2+} (data not shown, see Rineau et al, 2009). Thus, the direct input of available Ca and Mg should have been followed by a reduced potential oxalate secretion. However, this increased potential activity of the overall community was due to one ECM type only, *Lactarius subdulcis*, which was both the most abundant and the most oxalate active in the limed plots. Besides the pH effect which may have affected this ECM fungus by decreasing the acidic stress in the limed plots and thus enhancing its metabolic activity and its abundance, this increased oxalate production could be explained by two different and probably linked mechanisms: i) The stimulation of organic acid secretion by $CaCO_3$, due to the incorporation of HCO_3^- for oxalate biosynthesis. A similarly increased oxalate secretion by the ECM fungus *Rhizopogon roseolus* was already observed in a culture medium implemented with $CaCO_3$ (Casarin et al., 2003; Arvieu et al., 2003). ii) The physiological response to increased nitrogen mineralization, as reported in liming experiments on acidic forest soils (Kreutzer, 1995; Bäckman, 2003). Oxalate secretion by mycorrhizal seedlings has been found to be correlated with the rate of microbial mineralization (van Hees et al., 2003). In the same way, the mycelium of *Paxillus involutus* grown in a medium with NO_3^- and Ca^{2+} produced large quantities of oxalic acid (Lapeyrie et al., 1987).

Because liming increases exchangeable iron concentration (Kreutzer, 1995) and because a high iron concentration generally represses siderophore secretion (Raaska & Mattila, 1995), we expected a significantly lower potential iron mobilization in limed plots. Nevertheless, there was a spectacular drop of available ferric iron (from 0.11 to 0.02 $cmol.kg^{-1}$) in the limed plots (data not shown). Contrary to what was expected, liming decreased both ferric iron availability and potential iron mobilization by ECM root tips. This could be explained by the

fact that ferric iron was in excess even in limed plots, which is possible because the forest is based on red sandstone, naturally rich in Fe^{3+}.

Most of the ECM morphotypes encountered (15 out of 18) were able to breakdown more than the threshold (2%) of the CAS-Fe complex. This is in accordance with the literature which predicts that most of the ECM fungi induce CAS discoloration (Szaniszlo et al., 1981). Iron complexing in soils is not due to siderophores only: organic acids, as oxalate or citrate, can also complex iron (Winkelmann, 2002; Jones *et al*, 2003). Nevertheless, there was no correspondance between high potential oxalate secretion and high iron complexing by ECMs: either these ECMs produces other organic acids, such as citrate, which is a stronger iron complexing agent than oxalate (Rineau et al., 2008), or iron complexing is due to siderophores. Moreover, siderophores bind Fe^{3+} in soils much more effectively than organic acids do (Kalinowski et al, 2000), even if citrate and siderophores are suspected to act synergetically for mineral dissolution (Reichard et al., 2007). The ECMs species tested here presented diverse responses to liming, from induction or stimulation to decrease or repression of iron complexing. As siderophore secretion is down-regulated by high free Fe^{3+} concentrations (around 10µM: Neilands, 1984), it is probable that each ECM morphotype produces different siderophores, each of them characterized by different optimum iron concentrations. Experimental evidence of differential siderophore secretion by ECM pure cultures in response to iron addition has been shown by Machuca et al., 2007: different siderophore concentrations in the medium were obtained with *Suillus luteus, Scleroderma verrucosum* and *Rhizopogon luteolus*, but also with two strains of *Rhizopogon luteolus* when 35µM of iron was added.

Oxalate secretion and iron mobilization by ECMs were characterized by a high variability within species, due to the high proportion of null values. Therefore, our results points out the

high intraspecific variability in the ability of ECMs to weather minerals. This variability cannot be due to a lack of precision of the method, because the oxalate concentrations in each well are accurately measured and the variability due to the method is very low. An explanation for this variability could thus be the spatial micro-heterogeneity of mineral resources within soil, leading to the activation of ECMs in the close vicinity of minerals only. This is in accordance with Jones et al. (2003), who strongly suggested that the heterogeneity of organic acids concentration in soils has been underestimated.

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References

Agerer R., 1987-98. Colour atlas of ectomycorhizae. Einhorn-Verlag Eduard Dietenberger, Munich.

Arvieu J.C., Leprince F., Plassard C., 2003. Release of oxalate and protons by ectomycorrhizal fungi in response to P-deficiency and calcium carbonate in nutrient solution. *Annals of Forest Science* **60**: 815-821.

Bäckman J.S.K., Hermansson A., Tebbe C.C., Lindgren P.E., 2003. Liming induces growth of a diverse flora of ammonia-oxidising bacteria in acid spruce forest soil as determined by SSCP and DGGE. *Soil Biology and Biochemistry* **35**: 1337-1347.

Casarin V., Plassard C., Souche G.,Arvieu J.C., 2003. Quantification of oxalate ions and protons released by ectomycorrhizal fungi in rhizosphere soil. *Agronomie* **23**: 461-469.

Dancis A., Klausner R.D., Hinnesbuch A.G., Barriocanal J.G., 1990. Genetic evidence that Ferric Reductase is required for iron uptake in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **10**: 2294-2301.

Dupouey J.L., Thimonier A., Lefevre Y., le Tacon F., Bonneau M., Dambrine E., Poszwa A., Landmann G., 1998. Desaturation and nitrogen enrichment of forest soils in north-eastern France in the last few decades. *Revue Forestiere Francaise* **50**: 391-402.

Erland S., Taylor A.F.S., 2002. Diversity of ectomycorrhizal fungal communities in relation to the abiotic environment. Mycorrhizal Ecology, Ecological studies vol. 157, Eds. M. G. A. van der Heijden & I. Sanders, Springer-Verlag Berlin Heidelberg, pp. 163-200.

Frank J., Stuanes A.O., 2003. Short-term effects of liming and vitality fertilization on forest soil and nutrient leaching in a scots pine ecosystem in Norway. *Forest ecology and management* **176**: 371-386.

Gadd G., 1999. Fungal production of citric and oxalic acid: importance in metal speciation, physiology and biogeochemical processes. *Advances in Microbial Physiology* **41**: 47-92.

Gardes M., Bruns T.D., 1993. ITS primers which enhanced specificity fot basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113-118.

Haas H., 2003. Molecular genetics of fungal siderophore biosynthesis and uptake. the role of siderophores in iron uptake and storage. *Applied Microbiology and Biotechnology* **62**: 316-330.

Howard D.H., 2004. Iron gathering by zoopathogenic fungi. *FEMS Immunology and Medical Microbiology* **40**: 95-100.

Jones D.L., 1998. Organic acids in the rhizosphere – a critical review. *Plant and Soil* **205**: 25-44.

Jones D.L., Dennis P.G., Owen A.G., van Hees P.A.W., 2003. Organic acid behavior in soils-Misconceptions and knowledge gaps. *Plant and Soil* **248**: 31-41.

Kalinowski B.E., Liermann L.J., Givens S., Brantley L.S., 2000. Rates of bacteria-promoted solubilization of Fe from minerals. a review of problems and approaches. *Chemical Geology* **169**: 357–370.

Kraemer S.M., 2004. Iron oxide dissolution and solubility in the presence of siderophores. *Aquatic Sciences* **66**: 3-18.

Kreutzer K., 1995. Effects of forest liming on soil processes. *Plant and Soil* **168**: 447-470.

Landeweert R., Hoffland E., Finlay R.D., Kuyper T., van Breemen N., 2001. Linking plants to rocks. Ectomycorrhizal fungi mobilize nutrients from minerals. *Trends in Ecology and Evolution* **16**: 248-254.

Lapeyrie F. Chilvers G.A. Bhem C.A., 1987. Oxalic acid synthesis by the mycorrhizal fungus *Paxillus involutus* Batsch. Ex Fr.. Fr. *New Phytologist* **106**: 139-146.

Machuca A., Pereira G., Aguiar A., Milagres A.M.F., 2007. Metal-chelating compounds produced by ectomycorrhizal fungi collected from pine plantations. *Letters in Applied Microbiology* **44**: 7-12.

Neilands J.B., 1984. Siderophores of bacteria and fungi. Microbiological sciences **1**: 9-14.

Neilands J.B., 1995. Siderophores. Structure and fonction of microbial iron transport compounds. *Jour. Biol. Chem* **270**: 26723-26726.

Paris F., Botton B., Lapeyrie F., 1996. In vitro weathering of phlogopite by ectomycorrhizal fungi .2. Effect of K+ and Mg2+ deficiency and N sources on accumulation of oxalate and H+. *Plant and soil* **179**: 141-150.

Persson T., 1988. Effects of liming on soil fauna in forests. A literature review. Swed. Environ. Prot. Board Report. Rep. 3418.

Raaska L., Mattila-Sandholm T., 1995. Effects of iron level on the antagonistic action of siderophores from non-pathogenic *Staphylococcus* sp. *Journal of Industrial Microbiology* **15**: 480-485.

Rineau F., Courty P.E., Uroz S., Buée M., Garbaye J., 2008. Simple microplate assays to measure iron mobilization and oxalate secretion by ectomycorrhizal tree roots. *Soil Biology and Biochemistry* **40**: 2460-2463.

Rineau F., Garbaye J., 2009. Effects of liming on ectomycorrhizal community structure in relation to soil horizons and tree hosts. Fungal Ecology, in press.

Schwyn B., Neilands J.B., 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**: 47–56.

Smith S.E., Read D.J., 1997. Mycorrhizal symbiosis, Ed 2, Academic press, London, 606 pp.

Szaniszlo P.J., Powell P.E., Reid C.P.P., Cline G.R., 1981. Production of hydroxamate siderophore iron chelators by ectomycorrhizal fungi. *Mycologia* **73**: 1158-1174.

Ulrich B. 1983. Effects of acid deposition. Acid deposition 31-41.

van Hees P.A.W., Godbold D.L., Jentschke G., Jones D.L., 2003. Impact of ectomycorrhizas on the concentration and biodegradation of simple organic acids in a forest soil. *European journal of soil science* **54**: 697-706

van Hees P.A.W., Jones D.L., Jentschke G., Gobold D.L., 2005a. Organic acid concentrations in soil solution: effects of young coniferous trees and ectomycorrhizal fungi. *Soil Biology and Biochemistry* **37**: 771-776.

van Hees P.A.W., Jones D.L., Nyberg L., Holmström S.J.M., Godbold D., Lundström U.S., 2005b. Modelling low molecular weight organic acid dynamics in forest soils. *Soil Biology and Biochemistry* **37**: 517-531.

van Hees P.A.W., Rosling A., Essèn S., Godbold D.L., Jones D.L., Finlay R.D., 2006. Oxalate and ferricrocin exudation by the extramatrical mycelium of an ectomycorrhizal fungus in symbiosis with Pinus sylvestris. *New Phytologist* **169**: 367-377.

Wallander H., 2000. Uptake of P from apatite by Pinus sylvestris seedlings colonised by different ectomycorrhizal fungi. *Plant and Soil* **218**: 249-256.

Winkelmann G., 2002.. Microbial siderophore-mediated transport. *Biochemical Society Transactions* **30**: 691-696.

Conclusion du chapitre III et introduction du chapitre IV

Dans le chapitre III, nous avons démontré que le chaulage modifiait le potentiel d'altération des minéraux par les communautés d'ECMs, et que le morphotype *Lactarius subdulcis* était responsable de l'essentiel de potentiel d'altération.

Cependant, cela ne nous renseigne pas sur la mobilisation effective de de ces nutriments par les ECMs. Un moyen d'approcher la mesure des différences de cette mobilisation entre les placeaux chaulés et non chaulés est d'évaluer les concentrations des divers éléments composant le manteau des apex mycorrhiziens, ce qui est permis par des techniques de micronanalyse semi-quantitative.

Nous avons donc mesuré, dans le chapitre IV, les effets du chaulage sur la concentration en éléments dans le manteau des apex mycorrhizés par *Lactarius subdulcis*.

Conclusion of chapter III and introduction to chapter IV

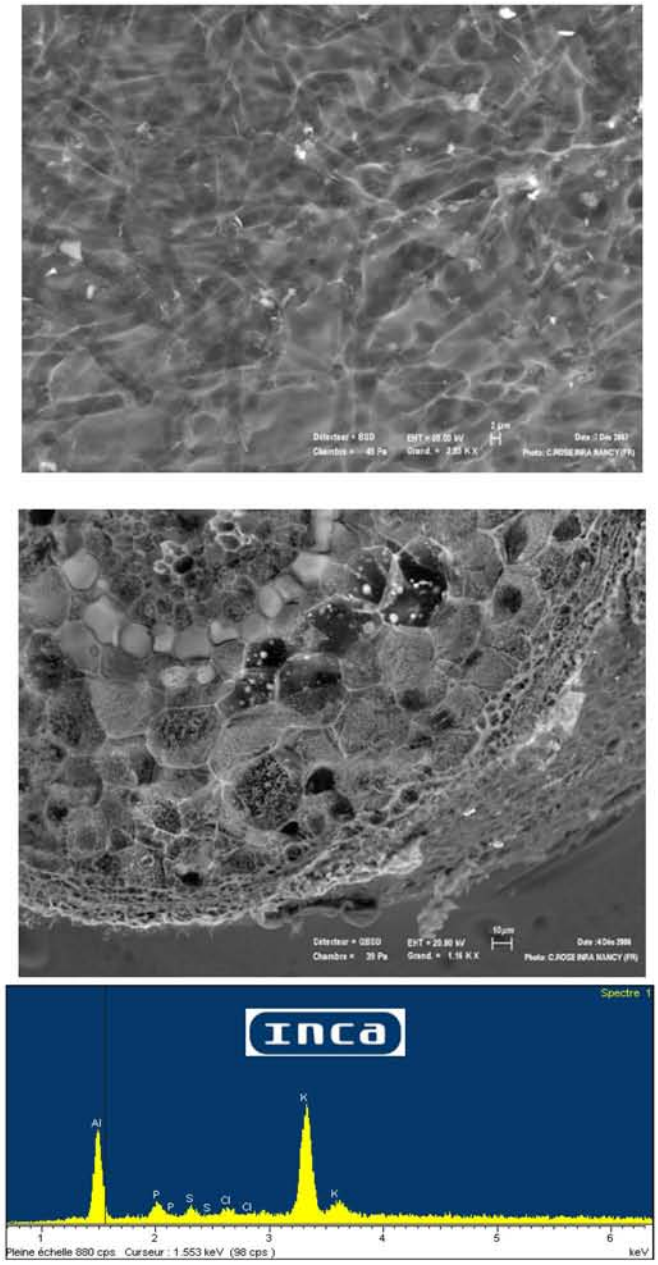
Chapter III showed that liming significantly modifies the potential mineral alteration by ECM communities, especially through the large oxalate secretion by the ECM morphotype *Lactarius subdulcis*.

However, the potential alteration of minerals does not inform on the effective uptake of these nutrients by the ECMs. A way to measure differences between the mobilization of nutrients by ECMs in limed and untreated plots would be to measure the concentrations of elements in the ECM fungal mantle, which is allowed by semi-quantitative microanalysis techniques.

Therefore, in chapter IV, we assessed the effects of liming on the concentration of elements in the fungal mantle of *Lactarius subdulcis* ECMs.

CHAPTER IV. CONSEQUENCES OF LIMING ON ELEMENTAL COMPOSITION OF ECTOMYCORRHIZAE

*__Article 7__ " Mineral concentrations of *Lactarius subdulcis*-beech ectomycorrhizae measured with EDS and WDS are enhanced by liming " (in preparation for Mycological Research)



Pictures of *Lactarius subdulcis* ECM mantle (top, x 2580), transvere section (middle, x 1160), and dispersive analysis spectrum of an ECM mantle (bottom). (Photo: C. Rose)

Abstract

Liming is a forest practice used to counteract forest decline induced by soil acidification. It consists of direct Ca and Mg inputs to forest soil, which restores tree mineral nutrition, but also modifies the availability of mineral nutrients in soil. Ectomycorrhizal (ECM) fungi are involved in mineral nutrient uptake by trees and can recover these minerals through alteration of mineral surfaces. In this study we determined how liming influences mineral element contents (i.e nutrient storage) in the ECMs of *Lactarius subdulcis*, the dominant symbiont in a beech stand, using energy- (EDS) and wavelength-dispersive spectroscopy (WDS). Results showed that ECMs contained significantly higher quantities of Ca, Mg, Mn, K, Si, Al and Fe in limed plots. Moreover, the P contents decreased significantly from the tip to the base of the ectomycorrhizas. These results are discussed with regard to mycorrhizal physiology and tree nutrition.

Key words: *Lactarius subdulcis*, EDS, WDS, mineral nutrition

Introduction

Many forests of central Europe have been declining since the 1970's because of Ca and Mg deficiencies. Soil and rainfall acidity lead to the leaching of up to 2% of Mg and 0.5% of Ca per year (Dupouey et al., 1998). Liming consists of direct $CaCO_3$ and $CaMg(CO_3)_2$ application to forest soil in order to correct acidity and restore Ca and Mg concentrations (Renaud et al., 2000; Frank & Stuanes, 2003). Indeed, the nutritional status of limed trees, as shown by element concentrations in leaves or needles of limed trees, indicates an improvement of Ca (Huber et al., 2004) or both Ca and Mg nutrition (Belkacem et al., 1992; Renaud et al., 2000). Nutrients are absorbed by the tree through fine roots. In temperate and boreal forests, more than 95% of these fine roots are in symbiotic association with fungi, forming a mixed organ called an ectomycorrhiza (ECM) (Smith & Read, 1997). The fungal

symbionts provide the trees with water and nutrients mobilized from organic matter and minerals. ECMs are able to recover Ca, Mg, or P but also Mn or Fe through direct alteration of the mineral surface (Gadd, 2007). Oxalate is suspected to be a key mediator of this mineral alteration and is secreted by many ECMs (Landeweert et al., 2001).

In the Humont forest, the ECM morphotype *Lactarius subdulcis* appeared as dominant in limed plots and beech fine root tips infected by this ECM produced significant quantities of oxalate (Rineau et al., 2008). As mycorrhizal oxalate secretion has been demonstrated to improve the uptake of mineral elements by the tree host *in vitro* (Yuan et al., 2004), this ECM is suspected to play a significant role in inorganic nutrient uptake through mineral alteration. Consequently, it would be of great interest to know if this higher potential mobilization is followed by increased nutrient storage in the fungal mantle. Inductively coupled argon plasma spectrometry (ICP) can be used to measure quantity of elements stored within the fungal mantle, but requires a lot of fungal material (Hodson & Wilkins, 1991; Kottke et al., 1998). In contrast, EDS (Energy-Dispersive-Spectroscopy) and WDS (Wavelength-Dispersive-Spectroscopy) provides methods of elemental analyses at the microscopic level (Hess et al., 1995) and thus allows accurate measurements on a single ECM root tip (Bucking et al., 1998 ; Köttke et al., 1998 ; Rumberger et al., 2004 ; Rumberger et al., 2005).

The aim of this work was thus to determine how liming influences the mineral element contents (i.e nutrient storage) in ECMs of *Lactarius subdulcis*, an abundant and active secretor of oxalate in beech stands. We also measured the different scales of variation of nutrient contents of ECMs : between sampling plot and through a longitudinal transect along mycorrhizal tips. Measurements of these elemental contents were achieved using EDS for all elements and WDS for Ca, Mg and Mn, which were chosen because of their implication in the response of trees to liming and their relatively low concentrations in ECMs.

Materials and methods

Site and forest stand

The experimental site of Humont (48°00'00''N, 6°29'28''E, Altitude: 570 m, Vosges forest, in the North-Eastern France) is composed of 60-year-old beech (*Fagus sylvatica*). The acidic brown soil (typic dystrochrept) is formed on sandstone. The liming treatment was carried out in a 500x400m forest stand by helicopter in 1991 with 757 kg/ha of CaCO3 and 380 kg/ha of CaMg(CO3)2. Sixteen years later, humus had shifted from a dysmoder to a typical oligomull in spruce amended plots in the same forest stand, with abundant earthworms, together with a significant increase of exchangeable Ca and Mg and a significant drop of exchangeable Al (Renaud et al., 2000).

Sampling and sample processing

Sampling took place in January 2008. Five plots of 50x50cm were defined in the limed area and five others in the untreated one. A minimum distance of 5m separated each plot from others. Clusters of *Lactarius subdulcis* ECMs were collected from each plot and immediately placed at 4°C in MilliQ water, still embedded in their surrounding soil, in order to prevent them from osmotic loss. In the lab, ECM tips were excised with forceps and carefully and quickly washed with MilliQ water under a stereomicroscope. The *Lactarius subdulcis* ECMs were identified using Agerer's (1987-2002) descriptions. Ten ECMs were randomly collected into clusters coming from each plot. A total number of 100 ectomycorrhizal root tips of *Lactarius subdulcis* were collected. The ECMs were then freeze dried under vacuum in the measurement chamber to allow microscopical observations.

Measurements of mineral concentrations by Energy-Dispersive- Spectroscopy (EDS) and Wavelength-Dispersive-Spectroscopy (WDS)

An atom excited by an electron beam emits X-ray with characteristic wavelength and energy. The EDS system detects all the energies of the X-rays emitted by all the elements in a sample at the same time, but with a limited sensitivity (Hess et al., 1995). The WDS system allows measurements at one wavelength (i.e one element) at each time, and needs high beam currents to obtain enough count rates. It is thus more accurate then EDS but also more time-consuming (Hess et al., 1995). Thus, we focused the WDS analysis on 3 elements relevant to liming: Ca, Mg and Mn. In effect, the concentration of these three elements in top soil layers was especially enhanced by liming (Nys, pers.com). The EDS technique does not allow accurate measurements of elements of atomic number lower than 11 (Na), because they produce X-rays too low in energy (less than 1KeV) (Hess et al., 1995).

The X-ray microanalytical studies were carried out under standardized conditions in a Leo 1450 VP (Zeiss) LEO-Scanning electron microscope fitted with the Oxford INCA ATW (for EDS) and the Oxford INCA wave spectrometer (for WDS) systems. The spectra were collected between 0 and 20keV with a Si (Li) X-ray detector with a $10mm^2$ polymeric window. We set the Peltier stage temperature at -30°c and the chamber pressure at 50 Pa for EDS and 10^{-4} Pa for WDS. Samples were coated with C to give electrical conductivity. We set the working distance at 15mm and the spectra acquisition time at 80s. We took five measurements on each ECM, consisting of 60µm square windows regularly spaced by 100µm along the longitudinal axis of the mycorrhiza, from the tip to the basis. After every tenth ECM, we realized quantitative optimization of the standard current using an external standard of pure Co.

Even after careful washing, some soil particles can remain at the ECM surface and influence the results. We analysed the soil embedded around some ECMs of *Lactarius subdulcis* and

found mainly Si. Silicon (Si) has a large efficient peak section, which causes high energy peaks even if present in small quantities. The Si pollution due to soil particles was thus revealed by noticeably higher Si signal than in clean ECMs. These discrepancies in Si concentrations were obtained above 14500ppm. Therefore, we chose to remove from analyses all the measurements where Si concentrations were higher than 14500 ppm.

Data treatment and statistics

The elemental composition of ECMs was expressed in % of weight concentration (part per million (ppm) of dried material weight). The normality of data was assessed using a Shapiro-Wilk's test. Because the data were not normal even after transformation, we used the non-parametric Kruskall-Wallis test to assess the effect of treatment (2: limed vs untreated), plot (10: 5 in untreated area, 5 in limed area), and position within the root tip (5, regularly spaced of 100µm step away from the apex) on elemental concentrations in the mantle of *Lactarius subdulcis* ECMs. Statistical analyses were done using the R software (http://www.r-project.org) (Ihaka & Gentleman, 1996).

Results

We measured the elemental contents of a total number of 47 and 46 ECMs in limed and untreated plots, respectively.

Effect of liming on the elemental contents in the mantle of the Lactarius subdulcis ECMs

We performed 218 and 213 measurements of ECM mantle elemental contents in untreated and limed plots, respectively. Thirty three other values were removed because of their high Si contents (see Material and Methods, 2.3). Liming significantly increased the elemental

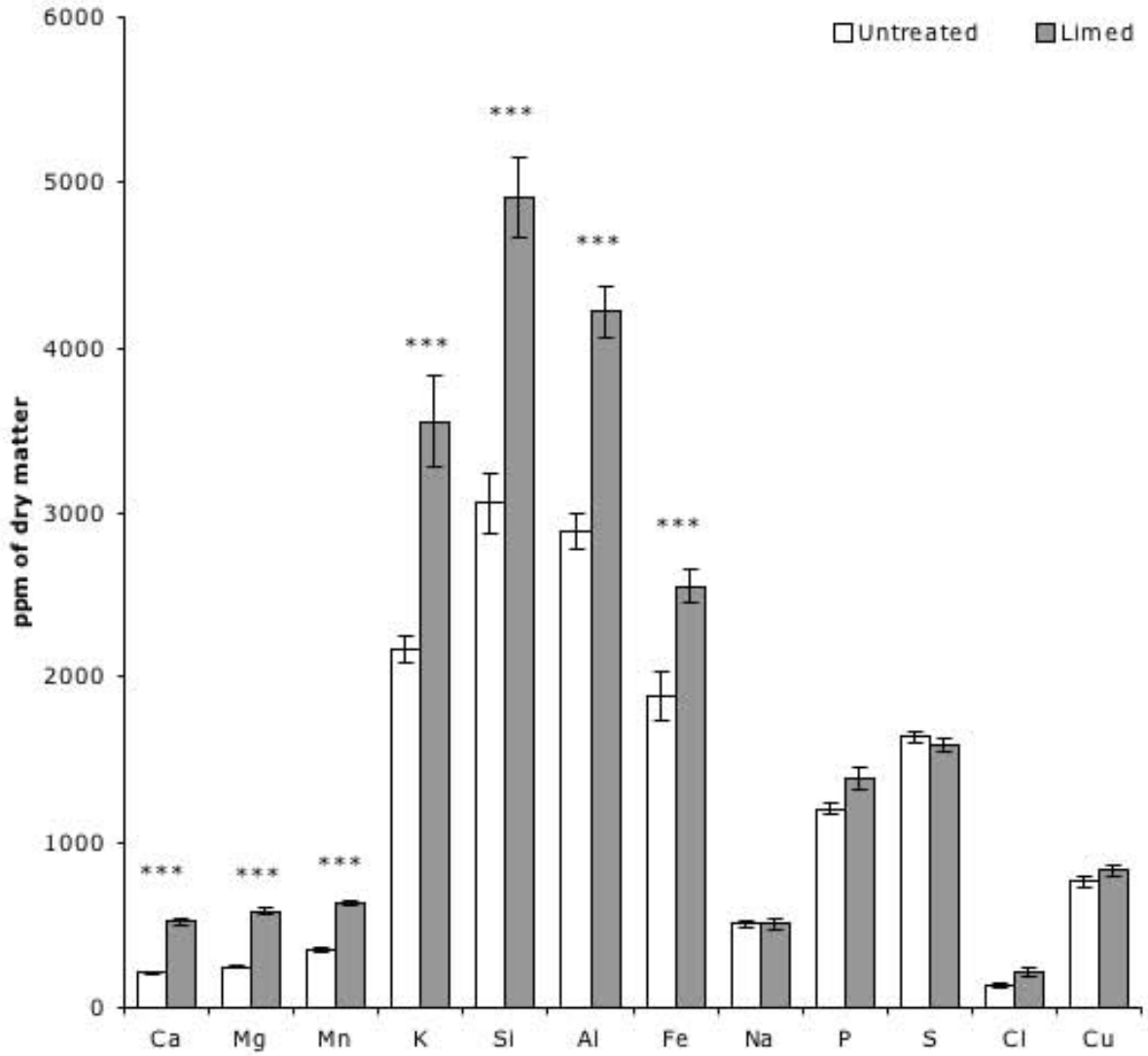


Figure 1: Amounts (in ppm of dry matter) of elements found in the 15µm outer hyphal sheath of *Lactarius subdulcis* ECMs coming from untreated (white bars) and limed (grey bars) plots. The amounts of Ca, Mg and Mn were measured with WDS analyser. Bars= standard error. n=218 and n=213 in untreated and limed plots, respectively. Variance analysis of effect of liming on each elemental content was assessed using a non-parametric Shapiro-Wilk's test. Significance codes: ***= $p<0.001$; **= $p<0.01$; *= $p<0.05$

contents of Ca, Mg, Mn, K, Si, Al, and Fe, and had no effect on Na, P, S, Cl and Cu (Figure 1). The stronger response to liming was seen with the elements measured with WDS: Ca (x 2.44), Mg (x 2.36), and Mn (x1.81) (Figure 1). The concentration of some other elements, measured with EDS (K, Si, Al, Fe), were 1.36 to 1.64 times enhanced in ECMs from the limed plots.

Mean elemental composition (excepting C, O and N) of an ECM of *Lactarius subdulcis* in the studied sampling site was thus high levels of Si (3974ppm) and Al (3549ppm), a lot of K (2851ppm), Fe (2213ppm), S (1612ppm) and P (1292ppm), some Cu (797ppm), and small quantities of Na (506ppm), Mn (491ppm), Mg (411ppm), Ca (364ppm) and Cl (175ppm).

Effect of sampling plot on the elemental contents in the mantle of the Lactarius subdulcis ECMs

We did between 35 and 50 measurements in each of the 10 sampling plots (between 8 and 10 ECM root tips in each plot). There was a significant plot effect on the concentration of all the elemental contents of ECMs (Figure 2). The ECMs coming from plot n°8 were particularly rich in K and P. There was also a large outlier for iron in plot n°1. The Ca/Mg and Si/Al ratios were close to 1 in each plot.

Effect of distance from apex on Lactarius subdulcis elemental contents in the mantle

For each one of the 5 positions on the ECM mantle, we performed 82 to 92 measurements. Only P was significantly affected by the distance from the apex (Figure 3). Phosphorus had a maximal concentration of 1440ppm at the apex, and this concentration gradually decreased to 1155ppm at 300µm away from the apex (Figure 3). The distribution of S followed exactly the same pattern, but with no significant differences between positions. In the same way, Si, Al, Na and K showed a similar spatial distribution along the ECMs, with maximal concentrations

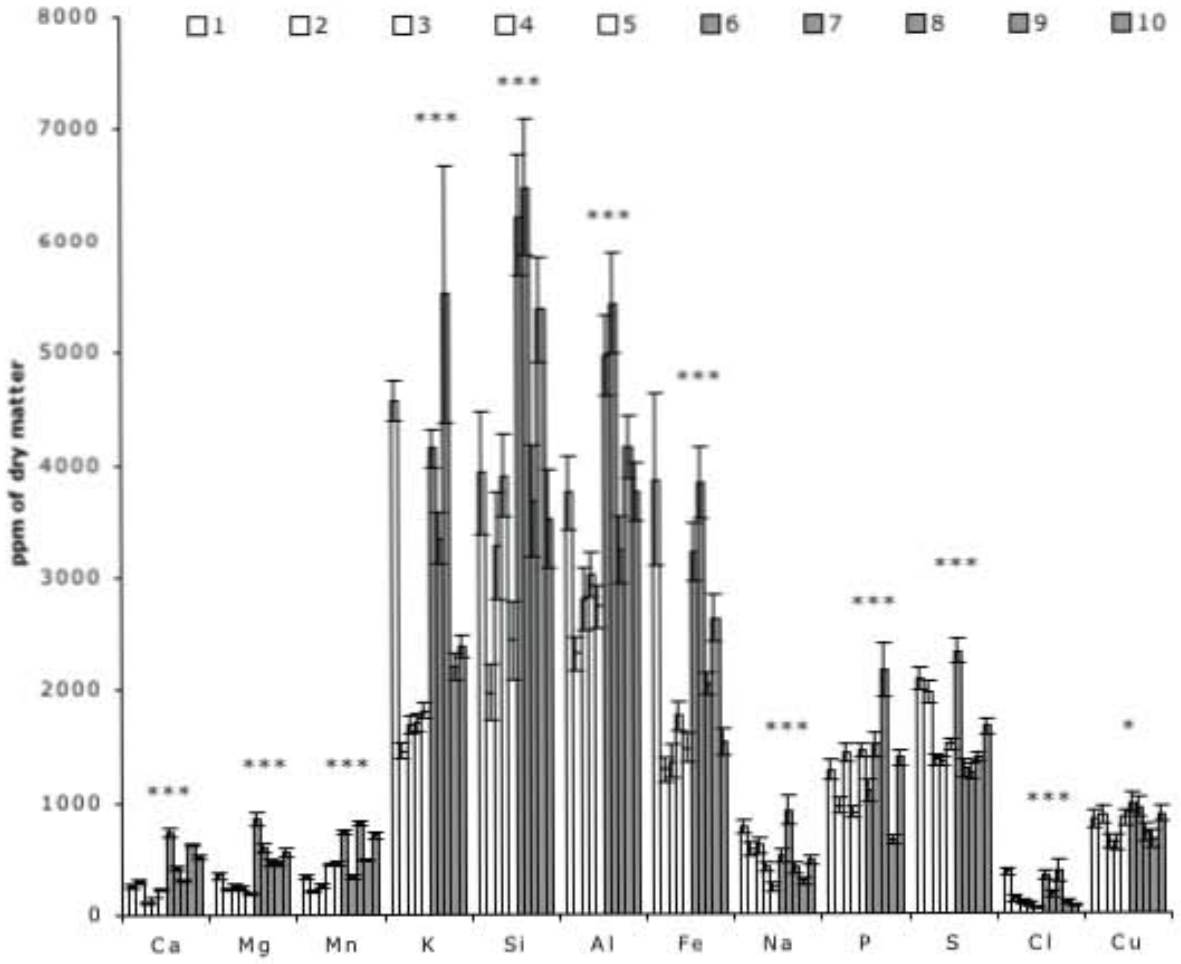


Figure 2: Amounts (in ppm of dry matter) of elements found in the 15µm outer hyphal sheath of *Lactarius subdulcis* ECMs on the 10 sampled plots. The amounts of Ca, Mg and Mn were measured with WDS analyser. The plots 1 to 5 (white bars) were untreated, the plots 6 to 10 (grey bars) were limed. Bars= standard error. n=37 for plot 1, n=44 for plot 2, n=40 for plot 3, n=47 for plot 4, n=50 for plot 5, n=41 for plot 6, n=35 for plot 7, n=48 for plot 8, n=40 for plot 9, n=49 for plot 10. Variance analysis of effect of liming on each elemental content was assessed using a non-parametric Shapiro-Wilk's test. Significance codes: ***= $p<0.001$; **= $p<0.01$; *= $p<0.05$

at the apex and at 400µm away, and minimal ones at 200µm, whatever the size of the ECM. Calcium and Cu were in constant proportions all along the 400µm of the ECM. Magnesium concentrations were slightly lower close to the apex (from 383ppm at the apex to 434ppm at 300µm).

Discussion

The calcium concentrations measured here are not due to oxalate crystals formation often reported in ECM mycelium (Lapeyrie et al., 1987; Wallander et al., 2002) because we did not observe any of these crystals.

The nutrient amounts of K, Mg, Mn and Na in the ECMs of *Lactarius subdulcis* analysed here were of the same size scale as those of ECM roots of beech in a podzolic soil in sweden analysed by ICP (Tyler et al., 2004). Previous studies showed that nutrient amounts of Al, Ca, Mg, S, in beech ECMs analysed by ICP were correlated with EDS analyses (Rumberger et al., 2005). Nevertheless, our ECMs were 80 and 40 times more concentrated in Cu and Al, and 50 times less concentrated in Ca. This could be due to a difference in soil chemical compositions between a boreal podzol and a mountainous dystrochrept. Moreover, ICP measures the element concentrations in the whole root tip, including plant tissues, whereas EDS reflects only the element concentrations in the fungal sheath. The electron beam can penetrate samples down to a depth of 10-15µm. The elemental concentrations presented here are thus relevant to the composition of most of the hyphal sheath thickness of *Lactarius subdulcis* ECMs (between 15 and 25µm depending on the diameter of the ECM in our sample site).

Liming significantly enhanced the amounts of Ca, Mg, Mn, K, Si, Al and Fe in *Lactarius subdulcis* ECMs. The ECM root tips of these species has been shown to secrete significant quantities of oxalate in the same stands (Rineau et al., 2008). Oxalate secretion is suspected to

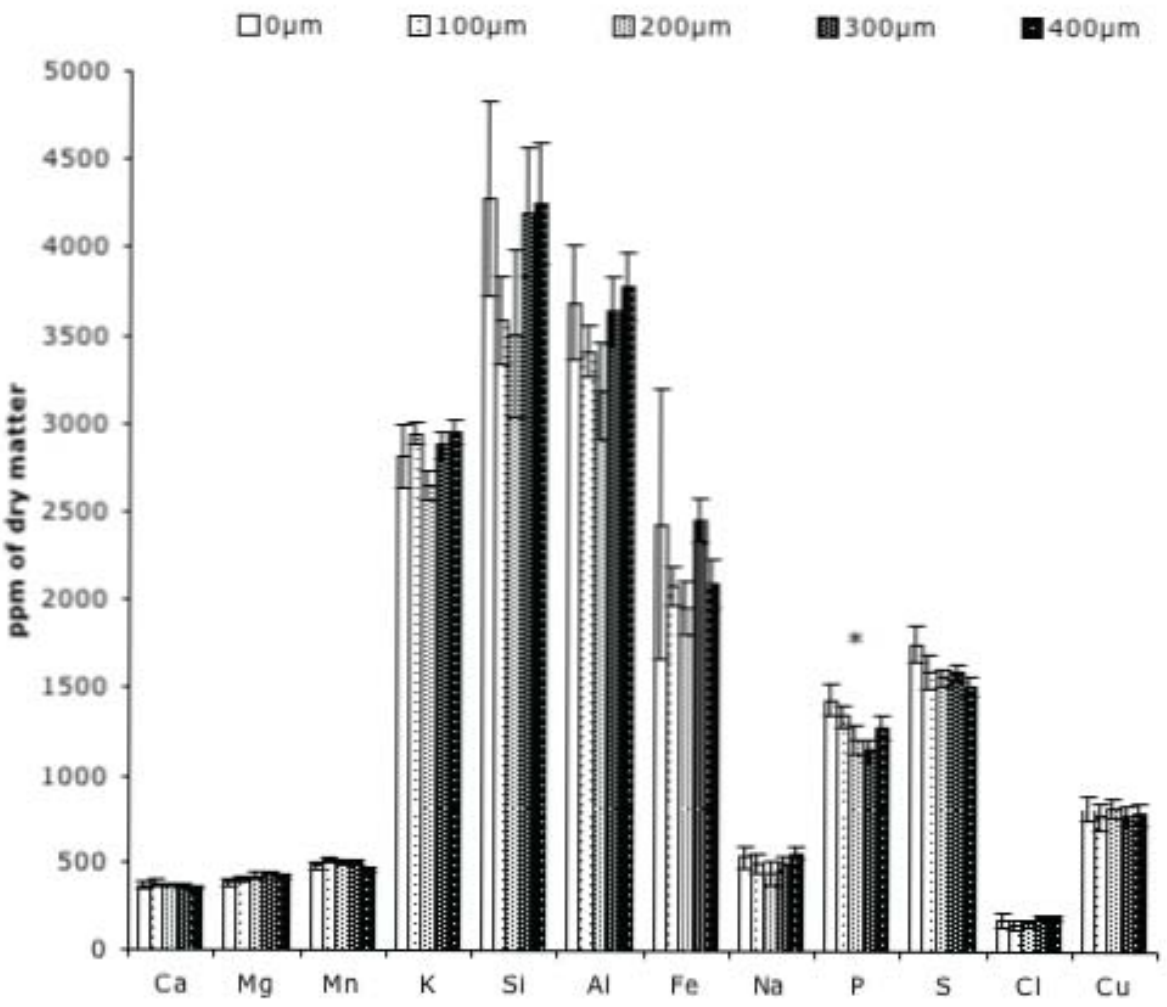


Figure 3: Amounts (in ppm of dry matter) of elements found in the 15µm outer hyphal sheath of *Lactarius subdulcis* ECMs in windows regularly spaced by 100µm along the longitudinal axis of the mycorrhiza, from the tip (0µm) to the direction of the basis (till 400µm). The amounts of Ca, Mg and Mn were measured with WDS analyser. The plots 1 to 5 (wide bars) were untreated, the plots 6 to 10 (grey bars) were limed. Bars= standard error. n=92 for measurement at the tip (0µm), n=88 for measurement at 100µm, n=82 for measurement at 200µm, n=86 for measurement at 300µm, n=83 for measurement at 400µm. Variance analysis of effect of liming on each elemental content was assessed using a non-parametric Shapiro-Wilk's test. Significance codes: ***= $p<0.001$; **= $p<0.01$; *= $p<0.05$

play a crucial role in ECM-mediated mineral weathering (Landeweert et al., 2001). The hypothesis that the higher amounts of elements in the fungal mantle of ECMs resulted from increased oxalate production by *Lactarius subdulcis* ECMs in the limed plots is thus likely.

We observed a significant increase of Ca and Mg concentrations in *Lactarius subdulcis* ECMs in limed plots. A similar increase of Ca and Mg contents in spruce mycorrhizal root tips was observed in a limed spruce forest (Nowotny et al., 1996). In the same way, liming generally enhances exchangeable Ca and Mg in soil, as well as Ca and Mg contents in leaves (Kreutzer, 1995). Here, the same results were observed for spruce needles in the same Humont forest (Renaud et al., 2000). Because the presence of a fungal mantle represses the rapid (4mn) and passive portion of Ca and Mg diffusion into roots (Bucking et al., 2002), ECMs are suspected to be involved in active uptake of these elements by trees. *Lactarius subdulcis* ECMs could thus play a significant role in the improvement of beech mineral nutrition by increasing Ca and Mg uptake in limed plots, resulting in enhanced Ca and Mg contents in fungal sheath.

We also observed a significant increase of Al concentration in *Lactarius subdulcis* ECMs in limed plots. Moreover, Al was the most abundant element stored in ECM mantle with Si. The presence of this toxic element in such high concentrations can be the consequence of aluminium detoxification by ECMs, already observed in *Piceirhiza gelatinosa*-spruce ECMs (Köttke et al., 1998). This Al accumulation was localized to the fungal sheath and could be inferred from a high metal adsorption capacity of *Lactarius subdulcis* ECMs. An Al chelation by secreted oxalic acid at the mantle surface is also likely, and could be a major factor of Al tolerance for the tree host (Jentschke & Godbold, 2000). It may also be due to the presence of Al-rich pigments, as suspected in *Paxillus involutus* ECMs (Hodson and Wilkins, 1991). Moreover, Al seems to affect Ca and Mg uptake by mycorrhizal seedlings (Jentschke & Godbold, 2000), which can explain the low Ca and Mg concentrations in untreated plots, where Al contents are higher.

Silicon was the most abundant element found in *Lactarius subdulcis* ECMs, and its concentration was enhanced by liming. Such high Si concentrations in living fungal material were unexpected. High Al and Si concentrations were also found in *Paxillus involutus*-spruce ECMs by Hodson & Wilkins (1991), who hypothesized an Al detoxification by Si, as observed for sorghum grown in water culture (Galvez et al., 1987). The high Si amounts observed here could counterbalance the Al toxicity, present in equivalent quantities in the *Lactarius subdulcis* ECMs. This Si can be from mineral origin, but can also be indirectly recovered from the enzymatic degradation of beech litter, as beech is known to accumulate high quantities of Si in leaves (Gérard et al., 2008).

We also found large amounts of K in the *Lactarius subdulcis* ECMs, which was the third most abundant element found after Si and Al. It is well known that ECMs plays a crucial and active role in K uptake by trees (Yuan et al., 2004). A similar increased K content of spruce ECMs was also observed in a limed forest stand in (Nowotny et al., 1996).

Phosphorus was found to be more abundant at the tip of the ECMs. The presence of polyphosphate granules was unlikely, because P content was not correlated with any other element concentration. The higher P contents observed in the tip of ECMs, were probably due to increased biological activity at the root tip, where fungal tissues are the youngest, and contains more ATP and nucleic acids. Moreover, Cairney & Alexander (1992) found that older *Tylospora fibrillosa* ECMs had lower P absorption rates than young ones, suggesting that older ECM tissues would contain less P.

Finally, we observed that nutrient amounts of *Lactarius subdulcis* ECMs were not uniform within the untreated or the limed areas. The abundance of all elements found in *Lactarius subdulcis* ECMs (Si, Al, K, Fe, S, P, Cu, Na, Mn, Mg, Ca and Cl) were significantly different between plots at 5m distances. This spatial variability could result from i) different nutrient needs between tree hosts in each plot. Indeed, the beech foliar concentrations in K, Ca, Mn

and Mg were found significantly different between trees in Northeastern France, and varied from 8 to 26% (Duquesnay et al., 2000). ii) differential ability of several genets of *Lactarius subdulcis* for nutrient uptake, assuming that the genet size of this ECM is lower than 5m, which was already shown for some Russuloid species of genet size between 1.1 and 9.3 m^2 (Redecker et al., 2001). iii) a direct dependance in nutrient sources, assuming that these sources significantly differs at a 5m scale, which is in accordance with spatial variability threshold of nutrients in the top 10 cm of a forest, between 5 and 10m (Gallardo, 2003). iv)or a combination between these three factors.

Acknowledgements

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References

Agerer, R., 1987-2002. Colour atlas of ectomycorhizae. Einhorn-Verlag Eduard Dietenberger, Munich.

Belkacem, S., Nys., C., Gelhaye, D., 1992. Effets d'une fertilisation et d'un amendement sur l'immobilisation d'éléments dans la biomasse d'un peuplement adulte d'épicéa commun (*Picea abies* L Karst). Annals of Forest Science 49, 235-252.

Bücking, H., Beckmann, S., Heyser, W., Kottke, I., 1998. Elemental contents in vacuolar granules of ectomycorrhizal fungi measured by EELS and EDXS. A comparison of different methods and preparation techniques. Micron 29, 53-61.

Bücking, H., Kuhn, A.J., Schröder, W.H., Heyser, W., 2002. The fungal sheath of ectomycorrhizal pine roots: an apoplastic barrier for the entry of calcium, magnesium, and potassium into the root cortex? Journal of experimental botany 53, 1659-1669.

Cairney, J.W.G., Alexander, I.J., 1992. A study of aging of spruce [Picea-sitchensis (bong) carr] ectomycorrhizas .3. Phosphate absorption and transfer in aging picea-sitchensis Tylospora-fibrillosa (burt) donk ectomycorrhizas. New phytologist 122, 159-164.

Cullen, D., Kersten, P.J., 2004. Enzymology and molecular biology of lignin degradation. The Mycota III : biochemistry and molecular biology. Berlin Heidelberg, Springer-Verlag, 2004, 249-273

Duquesnay, A., Dupouey, J.L., Clement, A., Ulrich, E., Le Tacon, F., 2000. Spatial and temporal variability of foliar mineral concentration in beech (Fagus sylvatica) stands in northeaster France. Tree Physiology 20, 13-22.

Gallardo, A., 2003. Spatial variability of soil properties in a floodplain forest in northwest spain. Ecosystems 6, 564-576.

Galvez, L., Clark, R.B., Gourley, L.M., Maranville, J.W., 1987. Silicon interactions with manganese and aluminium toxicity in sorghum. Journal of Plant Nutrition 10, 1139-1147.

Hodson, M.J., Wilkins D.A., 1991. Localizaion of aluminium in the roots of Norway spruce [Picea abies (L.)Karst.] inoculated with *Paxillus involutus* Fr. New Phytologist 118, 273-278.

Huber, C., Kreutzer, K., Röhle, H., Rothe, A., 2004. Response of artificial acid irrigation, liming and N-fertilization on elemental concentrations in needles, litter fluxes, volume increment, and crown transparency of a N saturated Norway spruce stand. Forest Ecology and Management 200, 3-21.

Ihaka, R., Gentleman, R., 1996. R: A Language for Data Analysis and Graphics. Journal of Computational and Graphical Statistics 5, 299-314.

Jentschke, G., Goldbold, D. L., 2000. Metal toxicity and ectomycorrhizas. Physiologia Plantarum, 109 : 107-116.

Kottke, I., Qian, X.M ;, Pritsch, K., Haug, I., Oberwinkler, F., 1998. *Xerocomus badius-Picea abies*, an ectomycorrhiza of high activity and element storage capacity in acidic soil. Mycorrhiza 7, 267-275.

Kreutzer, K., 1995. Effects of forest liming on soil processes. Plant and Soil 168-169, 447-470.

Landeweert, R., Hoffland, E., Finlay, R. D., Kuyper, T., van Breemen, N., 2001. Linking plants to rocks. Ectomycorrhizal fungi mobilize nutrients from minerals. Trends in Ecology and Evolution 16, 248-254.

Lapeyrie, F., Chilvers, G. A., Bhem, C. A., 1987. Oxalic acid synthesis by the mycorrhizal fungus *Paxillus involutus* (Batsch. Ex Fr.) Fr. New Phytologist 106, 139-146.

Nowotny, I., Dähne, J., Klingelhöfer, D., Rothe, G.M., 1996. Effects of artificial soil acidification and liming on growth and nutrient status of mycorrhizal roots of Norway spruce (Picea abies (L.)Karst.). Plant and soil 199, 29-40.

Redecker, D., Szaro, T.M., Bowman, R.J., Bruns, T.D., 2001. Small genets of Lactarius xanthogalactus, Russula cremoricolor and Amanita franchetii in late-stage ectomycorrhizal successions. Molecular Ecology 10, 1025-1034.

Renaud, J. P., Picard, J. F., Richter, C., Nys, C., 2000. Restauration de sols forestiers acides par un amendement calco-magnésien : cas du Massif Vosgien et des Ardennes. Rapport DERF–ONF–INRA, 47 p.

Rineau, F., Courty, P. E., Uroz S., Buée, M., Garbaye, J., 2008. Simple microplate assays to measure iron mobilization and oxalate secretion by ectomycorrhizal tree roots. Soil Biology and Biochemistry, 40: 2460-2463.

Rumberger, M.D., Münzenberger, B., Bens, O., Ehrig, F., Lentzsch, P., Hüttl, R.F., 2004. Changes in diversity and storage function of ectomycorrhiza and soil organoprofile dynamics after introduction of beech into Scots pine forests. Plant and Soil 264, 111-216.

Rumberger, M.D., Lentzch, P., Münzenberger, B., Hüttl, R.F., 2005. Nutrient amounts of ectomycorrhizae analysed by EDX using ESEM and ICP. Mycorrhiza 15, 307-312.

Hess, W.M., Hess, C.Z., Standing, M.D., 1995. X-Ray microanalysis. Molecular Methods in Plant Pathology. Lewis Publishers, Boca Raton, New York, Tokyo, pp3-9.

Smith, S. E., Read, D. J., 1997. Mycorrhizal symbiosis, Ed 2, Academic press, London, 606 pp.

Tyler, G., 2004. Ionic charge, radius, and potential control root/soil concentration ratios of fifty cationic elements in the organic horizon of a beech (Fagus sylvatica) forest podzol. Science of the total environment 329, 231-239.

Wallander, H., Johansson, L., Pallon, J., 2002. PIXE analysis to estimate the elemental composition of ectomycorrhizal rhizomorphs grown in contact with different minerals in forest soil. FEMS microbiology ecology 39, 147-156.

Yuan, L., Huang, J., Li, X., Christie, P., 2004. Biological mobilization of potassium from clay minerals by ectomycorrhizal fungi and eucalypt seedling roots. Plant and Soil 262, 351-361.

CHAPITRE V. SYNTHESE GENERALE ET PERSPECTIVES



Photographies des placeaux chaulés et témoins à Humont, prises en Juillet 2006. En haut à gauche : hêtre témoins. En haut à droite : hêtres chaulés, à dix mètres du placeau de hêtres témoins. En bas à gauche : aspect du houppier des hêtres dans le placeau témoin, montrant une défoliation de certains arbres. En bas à droite : aspect du houppier des hêtres dans le placeau chaulé.

1. Conclusions générales à propos de l'étude effectuée à Humont

Résumé des résultats

Les objectifs de cette étude étaient de déterminer les effets du chaulage sur la structure et le fonctionnement des communautés d'ectomycorrhizes (ECMs) sous hêtre et épicéa. Les résultats ont montré que les amendements calci-magnésiens :

i) diminuaient l'abondance des carpophores d'espèces acidophiles, quel que soit l'arbre hôte (chapitre 1)

ii) rédusaient l'abondance des apex mycorhizés par des morphotypes acidophiles, et augmentaient celle des morphotypes ubiquistes (*Clavulina cristata* pour l'épicéa, *Lactarius subdulcis* pour le hêtre) ; de plus, ces morphotypes sont confinés dans l'horizon organo-minéral (chapitre 2)

iii) augmentaient la sécrétion potentielle d'enzymes impliquées dans la dégradation de la matière organique (glucuronidase pour l'épicéa, laccase pour le hêtre) à l'échelle de la communauté d'ECMs. Les espèces ubiquistes apparaissant dans les placeaux chaulés sont spécialisés dans la sécrétion de ces deux enzymes et contribuent à la grande majorité de leur sécrétion (chapitre 2)

iv) augmentaient la sécrétion d'oxalate par les communautés d'ECMs. *Lactarius subdulcis*, de loin le morphotype le plus actif pour la sécrétion d'oxalate, est donc suspecté de jouer un rôle important dans l'altération des minéraux par la communauté d'ECMs de hêtre (chapitre 3).

v) accroissaient les quantités de Ca, Mg, Mn, K, Al, Si et Fe dans le manteau des ECMs de *Lactarius subdulcis* (chapitre 4)

Le chaulage modifie donc la structure et le fonctionnement des communautés d'ECMs, en réduisant l'abondance d'ECMs acidophiles et peu actifs en terme de dégradation de la matière

organique, et en induisant l'apparition d'autres morphotypes ubiquistes et hautement spécialisés. De plus, le potentiel d'altération des minéraux par les communautés d'ECMs sous hêtre augmente, et conduit à une augmentation des concentrations de nutriments dans le manteau des ECMs de *Lactarius subdulcis*, le morphotype le plus actif pour la sécrétion d'oxalate.

Cependant, ces résultats ne nous informent aucunement sur le rôle potentiel des ECMs dans l'amélioration de la nutrition des arbres après chaulage. Nous avons donc décidé de comparer qualitativement les effets du chaulage sur la composition chimique du sol, le fonctionnement de la communauté d'ECMs (activités potentielles de dégradation de la matière organique et de l'altération des minéraux), et sur les analyses foliaires, qui sont le témoin de la qualité de la nutrition minérale de l'arbre. Ces comparaisons ont été faites uniquement pour le hêtre car nous n'avons effectué aucune mesure de potentiel d'altération des minéraux ni de contenu en éléments des ECMs sous épicéa.

Deux placeaux d'environ 20m x 20m ont été choisis dans les parcelles chaulée et témoin. Toutes les mesures ont été effectuées dans les mêmes placeaux, que ce soit pour le sol, les communautés d'ECMs, ou pour les feuilles.

Les analyses de sol ont été réalisées par le Laboratoire des Cycles Biogéochimiques (INRA Nancy). Les horizons A de cinq carottes de sol de 8cm de diamètre ont été prélevées dans les placeaux chaulés et témoins en Octobre 2006. Les analyses de sols ont été effectuées selon le protocole décrit dans le chapitre 1 (partie "Soil analyses"), et les analyses de communautés d'ECMs selon le protocole décrit dans le chapitre 2 (partie "Material and Methods"). Nous nous sommes aussi intéressés aux activités enzymatiques moyennes des ECMs appartenant au morphotype *Lactarius subdulcis* à cause de son rôle clé dans le potentiel d'altération des minéraux et les activités enzymatiques, de son abondance dans le placeau chaulé et de sa présence à la fois dans le placeau chaulé et le placeau témoin.

Tableau 1. Effets du chaulage sur la composition chimique des sols, les activités enzymatiques moyennes par carotte de la communauté d'ECMs, les activités enzymatiques moyennes de *Lactarius subdulcis*, les activités potentielles d'altération des minéraux de la communauté d'ECMs, les activités potentielles d'altération des minéraux par *Lactarius subdulcis*, les concentrations moyennes en éléments dans le manteau des ECMs de *Lactarius subdulcis*, et sur les contenus en éléments des feuilles dans les placeaux de hêtre à Humont. + : augmente dans le placeau chaulé ; - : diminue dans le placeau chaulé ; 0 : pas de différence entre placeau chaulé et témoin.

Elément concerné	Paramètre mesuré	Technique	Effet chaulage
Carbone	C du sol	Analyses de sol	-
	BGlucosidase activité totale (communauté d'ECMs)	Tests en microplaque	-
	Cellobiohydrolase activité totale (communauté d'ECMs)	Tests en microplaque	-
	Glucuronidase activité totale (communauté d'ECMs)	Tests en microplaque	0
	Xylosidase activité totale (communauté d'ECMs)	Tests en microplaque	-
	Laccase activité totale (communauté d'ECMs)	Tests en microplaque	+
	BGlucosidase activité moyenne (Lactarius subdulcis)	Tests en microplaque	+
	Cellobiohydrolase activité moyenne (Lactarius subdulcis)	Tests en microplaque	+
	Glucuronidase activité moyenne (Lactarius subdulcis)	Tests en microplaque	-
	Xylosidase activité moyenne (Lactarius subdulcis)	Tests en microplaque	-
	Laccase activité moyenne (Lactarius subdulcis)	Tests en microplaque	+
	C foliaire	Analyses élémentales	0
Azote	N du sol	Analyses de sol	-
	Chitinase activité totale (communauté d'ECMs)	Tests en microplaque	0
	Leucine aminopeptidse activité totale (communauté d'ECMs)	Tests en microplaque	-
	Chitinase activité moyenne (Lactarius subdulcis)	Tests en microplaque	+
	Leucine aminopeptidase activité moyenne (Lactarius subdulcis)	Tests en microplaque	+
	N foliaire	Analyses élémentales	0
Phosphore	P organique du sol	Analyses de sol	-
	Phosphatase acide activité totale (communauté d'ECMs)	Tests en microplaque	+
	Phosphatase acide activité moyenne (Lactarius subdulcis)	Tests en microplaque	+
	Contenu du manteau en P (Lactarius subdulcis)	EDS	+
	P foliaire	ICP	-
Fer	Fe du sol	Analyses de sol	-
	Chélation du fer activité moyenne (communauté d'ECMs)	Tests en microplaque	-
	Chélation du fer activité totale (communauté d'ECMs)	Tests en microplaque	+
	Chélation du fer activité moyenne (Lactarius subdulcis)	Tests en microplaque	-
	Chélation du fer activité totale (Lactarius subdulcis)	Tests en microplaque	+
	Contenu du manteau en Fe (Lactarius subdulcis)	EDS	+
	Fe foliaire	ICP	-
Calcium et magnésium	Ca du sol	Analyses de sol	+
	Mg du sol	Analyses de sol	+
	Sécrétion d'oxalate moyenne (communauté d'ECMs)	Tests en microplaque	+
	Sécrétion d'oxalate totale (communauté d'ECMs)	Tests en microplaque	+
	Sécrétion d'oxalate moyenne (Lactarius subdulcis)	Tests en microplaque	+
	Sécrétion d'oxalate totale (Lactarius subdulcis)	Tests en microplaque	+
	Contenu du manteau en Ca (Lactarius subdulcis)	WDS	+
	Contenu du manteau en Mg (Lactarius subdulcis)	WDS	+
	Ca foliaire	ICP	+
	Mg foliaire	ICP	+
Autres	Al du sol	Analyses de sol	-
	Mn du sol	Analyses de sol	+
	K du sol	Analyses de sol	-
	Na du sol	Analyses de sol	-
	Contenu du manteau en Al (Lactarius subdulcis)	EDS	+
	Contenu du manteau en Mn (Lactarius subdulcis)	WDS	+
	Contenu du manteau en K (Lactarius subdulcis)	EDS	+
	Contenu du manteau en Na (Lactarius subdulcis)	EDS	0
	Al foliaire	ICP	-
	Mn foliaire	ICP	-
	K foliaire	ICP	-
	Na foliaire	ICP	0

Les analyses foliaires ont été réalisées par l'UMR 1137 (Ecologie et écophysiologie forestières) et le Laboratoire de biogéochimie des écosystèmes forestiers (INRA Nancy). Les feuilles ont été collectées sur dix arbres choisis au hasard dans le placeau chaulé et sur vingt arbres dans le placeau témoin (dix sains plus dix dépérissants) en Juillet 2007. Les feuilles ont été séchées à 65°C, broyées, homogénéisées et analysées séparément. Les concentrations totales en C et N ont été mesurées avec un analyseur élémentaire NCS2500 (CE instrument Thermo Quest, Italie); les autres éléments (C, S, P, K, Ca, Mg, Mn, Na, Al et Fe) ont été minéralisés dans l'acide perchlorique et mesurés par ICP.

Résultats et discussion

Les effets du chaulage sur l'écosystème forestier sont détaillés dans le tableau 1 et résumés dans la figure 1. Les concentrations moyennes en éléments dans le sol et les feuilles sont données dans le tableau 2.

*Cycle du carbone

Les contenus en C des horizons A du sol sont plus faibles dans les placeaux chaulés (Tableau 1). L'activité laccase potentielle de l'ensemble de la commnuauté augmente dans le placeau chaulé, alors que toutes les autre activités impliquées dans la mobilisation du C d'origine organique (activités de dégradation des celluloses et hémicelluloses : ß glucosidase, cellobiohydrolase, glucuronidase, xylosidase) diminuent ou restent stables. Il n'y a aucun effet du traitement sur les contenus en C des feuilles. Une diminution des contenus en C de l'horizon A a déjà été remarquée dans les placeaux chaulés de dispositifs similaires (Höglwald) sous épicéa (Kreutzer, 1995), et ont été attribuées à une accélération du turnover de la matière organique et au transfert du C organique dissous aux horizons minéraux, en particulier via l'action des lombrics. De plus, dans le placeau chaulé du dispositif du

Höglwald, l'horizon formé par les lombrics (Olu) était significativement enrichi en composés aromatiques (Kreutzer, 1995), qui sont des substrats des laccases. Un phénomène identique est probable dans le dispositif de Humont, où l'activité des lombrics est forte, et peut être responsable de la forte activité laccase observée dans le placeau chaulé. En revanche, l'augmentation de la biomasse foliaire dans le placeau chaulé conduit à un apport de litière fraîche plus élévé et donc à des apports plus importants de cellulose et d'hémicellulose dans la matière organique. Or toutes les activités de dégradation de ces substrats par les ECMs ont régressé dans le placeau chaulé. Ceci peut alors s'expliquer par une augmentation de l'activité des champignons de la pourriture brune, qui réaliserainet l'essentiel de la dégradation des celluloses et des hémicelluloses dans le placeau chaulé.

D'autre part, la forte activité laccase observée dans le placeau chaulé accélère probablement la dégradation de la matière organique, ce qui contribue au changement d'humus, et permet de libérer de nombreux nutriments minéraux inclus dans la matière organique.

*Azote

Le chaulage a diminué les concentrations en N de l'horizon A (Tableau 1). Cet effet du chaulage a déjà été noté par Kreutzer (1995) dans un dispositif semblable. Cela a été accompagné par une diminution de l'acivité Leucine aminopeptidase de la communauté d'ECMs par carotte, mais est resté sans effet sur l'activité chitinase totale. L'activité moyenne de leucine aminopeptidase et de chitinase des ECMs de *Lactarius subdulcis* a augmenté dans le placeau chaulé. Enfin, les contenus en azote dans les feuilles n'ont pas été modifiés de mannière flagrante par le chaulage. Il apparaît donc que la forte diminution d'activité leucine aminopeptidase observée dans le placeau chaulé pour l'ensemble de la communauté d'ECMs n'a pas eu de réel effet sur les contenus en N des feuilles, ce qui n'est guère surprenant au vu de la relativement faible quantité d'azote stockée dans les protéines du sol par rapport à la

celle stockée dans la chitine (la chitine est le principal composant du mycélium des champignons et de la cuticule des nombreux arthropodes qui vivent dans le sol). La diminution de la quantité d'azote dans le sol est probablement une conséquence de son lessivage de l'horizon A aux horizons plus profonds (Huber et al., 2006), où il est minéralisé. Il est intéresant de noter que la diminution de l'azote total du sol n'a induit aucun changement dans l'activité chitinase de la communauté d'ECMs. Cela peut signifier que la quantité de chitine ne change pas dans le sol chaulé et que seul l'azote minéral (NO_3^- et NH_4^+) est affecté par le traitement, et contribue d'avantage à la nutrition de l'arbre. On peut aussi émettre l'hypothèse que le chaulage, en provoquant une plus forte minéralisation de l'azote, augmente les concentrations de NH_4^+ et NO_3^- dans les sols, et que ces éléments, facilement disponibles pour l'arbre, seraient responsables des plus faibles activités d'assimilation de l'azote d'origine organique.

*Phosphore

Les concentrations de l'horizon A en P disponible diminuent après chaulage, alors que l'activité phosphatase potentielle de la communauté d'ECMs augmente, ainsi que l'activité phosphatase moyenne des ECMs de *Lactarius subdulcis*, et des contenus en P du manteau de ces mêmes ECMs. Comme pour l'azote et le carbone, il est probable que la diminution de la concentration en P résulte de l'accélération du turnover de la matière organique, avec une assimilation plus importante du P par les microorganismes dans le placeau chaulé. Une relation inverse entre la disponibilité du P inorganique et l'activité phosphatase des ECMs a déjà été signalée (Antibus et al., 1992). La diminution de la quantité de P échangeable dans le sol a probablement stimulé l'activité phosphatase des ECMs, en particulier pour *Lactarius subdulcis*, ce qui a conduit à des contenus en P plus élevés dans le manteau de ces mêmes ECMs. Cependant, cette augmentation de l'assimilation potentielle du P par les ECMs a été

suivie d'une diminution des contenus en P des feuilles, et la nutrition en P des arbres, déjà sous le niveau optimal dans le placeau témoin, a atteint le seuil critique dans le placeau chaulé (Tableau 2). Cela signifie que l'activité phosphatase n'est peut-être pas corrélée avec une augmentation réelle de l'assimilation du P par la communauté d'ECMs dans son ensemble, ou bien que le P dans le manteau des ECMs de *Lactarius subdulcis* bénéficie seulement au partenaire fongique.

*Fer

Comme observé dans le dispositif du Höglwald (Kreutzer, 1995), le chaulage a diminué la concentration en Fe échangeable dans les horizons supérieurs du sol. La capacité de chélation de la communauté d'ECMs a augmenté dans le placeau chaulé, ainsi que celle de *Lactarius subdulcis*, et les contenus en Fe du manteau des ECMs appartenant à ce morphotype. La chélation de Fe par des sidérophores est réprimée par des fortes concentrations en fer ferrique et sont induites par des carences en fer (Neilands, 1995). Cela est en accord avec le plus grand potentiel de chélation du fer de la commnuauté d'ECMs observé dans le placeau chaulé. Cependant, comme pour le P, une augmentation de l'assimilation du Fe par les ECMs n'a pas résulté dans une diminution des contenus folaiires en Fe. Bonneau (1995) a fixé le seuil de carence en fer dans les feuilles de hêtre à 40mg/kg et l'optimum à 90mg/kg dans les forêts de l'Est de la France. Il apparaît que la plupart des hêtres à Humont, que ce soit dans les placeaux traités ou les placaux témoins, ont une nutrition en fer supérieure à l'optimum (140 et 170mg/kg , respectivement, Tableau 2). Par conséquent, le chaulage modifie probablement l'équilibre de l'échange de fer entre l'ECM et l'arbre et peut ainsi conduire à la récupération de fer suppléméntaire pour le métabolisme du champignon.

*Calcium et magnésium

Le chaulage a augmenté les quantités de Ca et Mg échangeables dans l'horizon A du sol. L'augmentation de Ca échangeable a été particulièrement forte en comparaison à celle de Mg, sans doute à cause des quantités différentes apportées par le chaulage (757 kg/ha de CaCO3 et 380 kg/ha de CaCaMg(CO3)2). Cette augmentation de la quantité de cations échangeables a été suivie par une plus forte sécrétion d'oxalate par les ECMs, dûe principalement à *Lactarius subdulcis*, une augmentation des concentrations en Ca et Mg dans le manteau des mycorrhizes de *Lactarius subdulcis*, ainsi que par une augmentation de la quantité de Ca et Mg dans les feuilles. Cela suggère fortement que l'apport de Ca et Mg a été suivi par la stimulation de la sécrétion d'oxalate par *Lactarius subdulcis*, de loin le plus important sécréteur d'oxalate de la communauté, et a amélioré la dissolution des minéraux en place et/ou facilité la complexation des formes dissoutes de Ca et Mg par ces ECMs. Cela a été suivi d'une meilleure nutrition en Ca et Mg des arbres. La plupart du système absorbant des arbres (racines fines) est en association avec des ECMs (Smith & Read, 1997). De plus, la sécrétion d'oxalate par les ECMs est positivement corrélée avec la nutrition minérale de semis *in vitro*. Il est donc probable que les ECMs, en particulier celles de *Lactarius subdulcis*, soient directement impliquées dans l'amélioration à long terme de la nutrition du hêtre en Ca et Mg.

*Aluminium

La concentration en Al échangeable diminue dans l'horizon A du placeau chaulé, probablement à cause de son remplacement par Ca et Mg dans le complexe absorbant. Cependant, les concentrations d'Al trouvées dans le manteau de *Lactarius subdulcis* sont plus fortes dans ce même placeau, alors que les concentrations d'Al dans les feuilles diminue. La présence de cet élément toxique en relativement fortes concentrations dans les ECMs de *Lactarius subdulcis* (aussi bien dans le placeau témoin que dans le placeau chaulé, malgré

l'effet du traitement) peuvent contribuer à la tolérance à l'Al observée dans les sols acides, comme il a été démontré pour des semis de *Pinus taeda* associé à *Pisolithus tinctorius* (Moyer-Henry et al., 2005). L'augmentation concomitante des concentrations en Al et Si dans le manteau des ECMs de *Lactarius subdulcis* peut être aussi un moyen d'inhibition de la toxicité aluminique par le Si, comme il a déjà été observé pour des racines de maïs ou de sorgho (Galvex et al., 1987; Kidd et al., 2001). De fortes concentrations en Si et Al ont aussi été trouvées dans des mycorhizes de *Paxillus involutus* sous épicéa, par Hodson & Wilkins (1991), qui ont alors émis l'hypothèse de détoxification de l'Al par Si. Il est intéressant de noter que cette séquestration de l'Al par les ECMs de *Lactarius subdulcis* a été augmentée dans le placeau chaulé, agissant en synergie positive avec le traitement.

*Potassium

Le chaulage a réduit la concentration en K dans l'horizon A, ainsi que les contenus en K des feuilles, qui restent cependant dans une gamme de valeurs optimale (7 et 9 mg/kg, tableau 2). Cependant, les concentrations en K dans le manteau des ECMs de *Lactarius subdulcis* ont augmenté. Les modifications de la concentration en K sont difficiles à interpréter car l'assimilation du K par les cellules fongiques passe par de nombreuses protéines de transport ou d'échange, de par son rôle crucial dans la régulation du potentiel osmotique de la cellule (Garraway & Evans, 1984). Cette augmentation de la concentration en K dans le manteau des ECMs peut conduire à un plus fort potentiel osmotique du manteau fongique et ainsi être interprété comme une adaptation à une plus forte absorption d'eau par les ECMs, à cause d'une demande en eau plus forte des arbres chaulés (biomasse foliaire plus forte).

*Manganèse

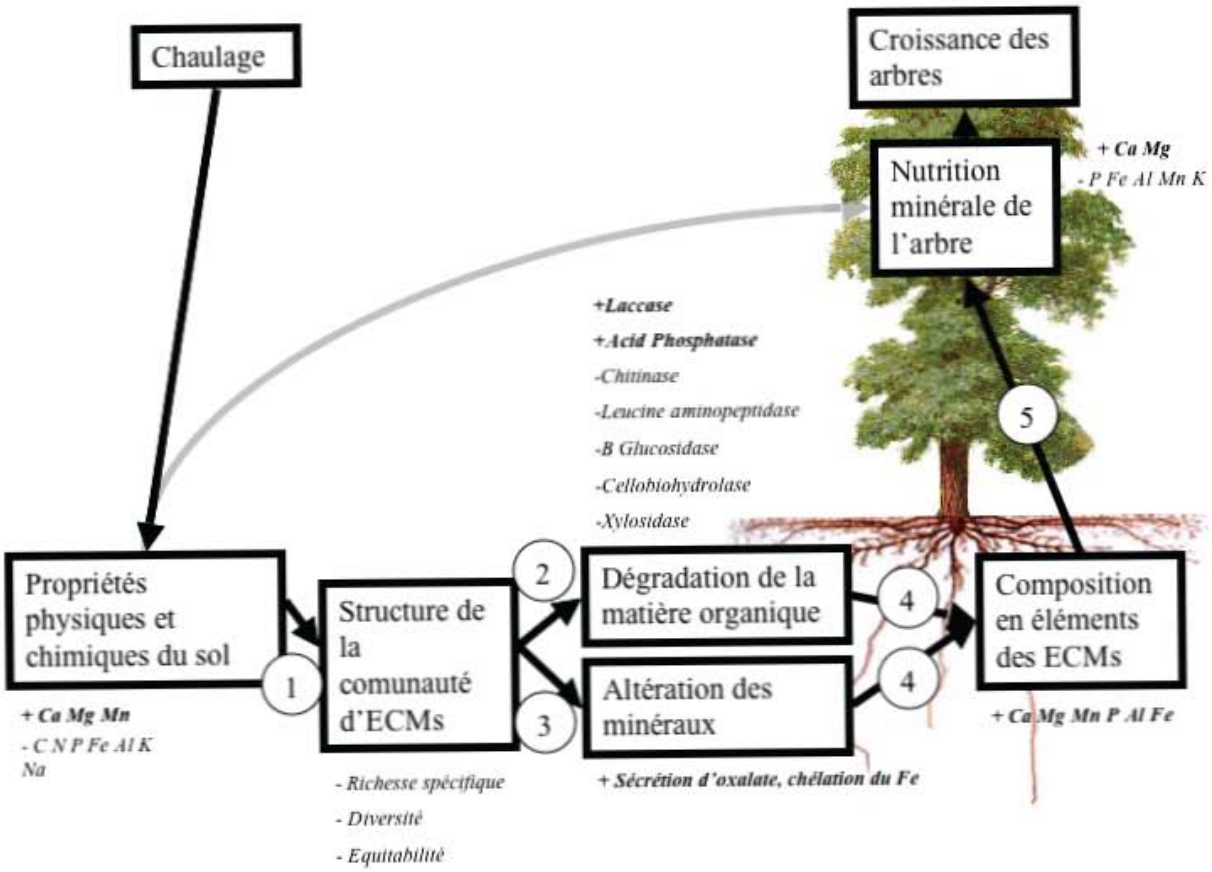


Figure 1. Représentation des effets du chaulage sur la composition chimique du sol, les commnautés d'ECMs et la nutrition de l'arbre. Les numéros entourés correspondent aux chapitres de la thèse. Les résultats obtenus au cours de cette étude sont notés en italique sous les titres correspondants.

Les quantités de Mn dans l'horizon A du placeau chaulé ont augmenté, ainsi que les concentrations en Mn dans le manteau des ECMs de *Lactarius subdulcis*, mais les teneurs en Mn ont diminué dans les feuilles de hêtre, et sont restées assez élevées (tableau 2). Curieusement, la nutrition en Mn des champignons impliquerait des mécanismes d'absorption identiques à ceux du Mg (Garraway & Evans, 1984). Il semblerait donc que les ECMs de *Lactarius subdulcis* sont capables d'augmenter leur absorption de Mn dans le placeau chaulé (par exemple via la sécrétion d'oxalate), ce qui conduit à une plus grande concentration en Mn dans le manteau de la mycorrhize, mais que celle-ci ne la transfère pas, ou peu, à l'arbre. Il est donc probable que, comme pour l'Al, le Mn est accumulé dans le manteau des ECMs lorsqu'il est en l'excès, à cause de sa toxicité potentielle.

Ces résultats montrent que le chaulage modifie non seulement la structure et le fonctionnement de la communauté d'ECMs, mais aussi que les ECMs jouent un rôle dans l'amélioration de la nutrition de l'arbre après chaulage, en fournissant de plus fortes quantités de Ca et Mg via une plus forte dissolution et complexation des minéraux. A la lumière de ces résultats, nous pouvons alors annoter le schéma du résumé de cette étude (figure 1). Ces résultats démontrent aussi que le remplacement d'un seul morphotype comme *Lactarius subdulcis* peut modifier considérablement le bénéfice potentiel de la symbiose pour l'arbre.

2. Quel est le degré de variabilité inter-sites des effets du chaulage sur les communautés d'ECMs ?

Les effets du chaulage étudiés à Humont sont spécifiques à une forêt de hêtres de 60 ans, en moyenne montagne, basée sur du grès rouge. Cependant, on trouve des sols forestiers acides dans beaucoup d'autres types d'environnements : le chaulage est ainsi susceptible d'être effectué dans des conditions de sol et de sylviculture très différentes. Afin d'estimer la variabilité des effets du chaulage sur les communautés d'ECMs, nous avons mesuré les effets du chaulage sur la structure de la communauté d'ECMs et sur ses activités enzymatiques potentielles dans une forêt de hêtre plus jeune et basée sur un sol plus riche, dans une autre région du Nord-Est de la France. Ce travail a été réalisé en collaboration avec Hélène Genet (Laboratoire d'Ecophysiologie forestière, équipe de Phytoécologie, INRA Nancy).

Caractéristiques du site d'échantillonage

Le site expérimental des Potées (49°85'25''N, 4°46'80''E, altitude: 380m, forêt des Ardennes, Nord-Est de la France) est consituté de hêtres (*Fagus sylvatica*) de 30 ans plantés en ligne. Contrairement à Humont, les hêtres ne sont pas dépérissants. Le chaulage (Ca et Mg) a été apporté à la main en 1996, soit 5 ans après Humont. Le sol est de type luvisol et est formé sur une roche-mère faite de schistes horizontaux. Contrairement à Humont, le chaulage n'a pas eu d'effet sur le type d'humus, qui est resté de type oligomull dans le placeau chaulé, mais a tout de même amélioré la nutrition des arbres (Tableau 2). La colonisation du sol par les lombrics est faible, que ce soit dans le placeau chaulé ou dans le placeau témoin.

Le sol témoin des Potées est caractérisé par des concentrations plus élevées qu'à Humont en limons, Ca et Mn, tandis que les concentrations en C, N, Al, K, Fe, en argiles, en sables, et la capacité d'échange cationique (CEC) sont plus faibles qu'à Humont (Tableau 2). La nutrition

Tableau 1. Diversité moyenne des morphotpyes d'ECMs par carotte de sol dans les deux placeaux (chaulé et témoin) du site des Potées en Juillet 2007. La diversité a été calculée en terme de richesse spécifique (RS), indice de diversité de Shannon, et indice d'équitabilité de Simpson pour chaque carotte de sol. Les barres d'erreur représentent l'erreur type. Les différences significatives entre indices de diversité pour chaque placeau sont évaluées à l'aide d'un test t de Student. Les différences significatives sont notées par des astérisques. ***: p<0.001; **, p<0.01; *p<0.05.

	Richesse spˇcifique	Shannon	Simpson
H□tre tˇmoin	4,25 (0,25)	1,11 (0,08)	0,59 (0,03)
H□tre chaulˇ	2,45 (0,39) ***	0,49 (0,12) ***	0,29 (0,08) ***

Tableau 2. Analyses de sols et analyses foliaires à Humont et aux Potées. En ce qui concerne les analyses de sol, les concentrations en C, N, P, H, CEC, argiles, limons et sables sont exprimées en $g.kg^{-1}$. Les concentrations en Al, Ca, Mg, K, Mn, Na et Fe échangeables sont exprimées en $cmol+.kg^{-1}$. Pour les analyses foliaires, toutes le sconcentrations sont exprimées en $g.kg^{-1}$. Les valeurs inférieures aux concentrations optimales selon Bonneau (1995) sont notées en gras.

Type d'analyse	Sol				Feuilles				Concentration optimale	Seuil critique	Seuil de carence
Site	Les potˇes		Humont		Les Potˇes		Humont				
Traitement	Tˇmoin	Chaulˇ	Tˇmoin	Chaulˇ	Tˇmoin	Chaulˇ	Tˇmoin	Chaulˇ			
C	54,80	88,05	67,60	53,70	506,17	492,35	499,08	481,71	-	-	-
N	3,38	5,55	4,28	3,07	24,51	25,00	22,27	20,95	20-25	15	NA
Al	4,83	2,67	8,20	5,22	0,10	0,11	0,15	0,11	NA	NA	NA
Ca	0,56	4,76	0,20	1,57	5,30	7,71	**1,58**	7,04	5-8	NA	NA
Mg	0,21	0,74	0,22	0,47	**0,91**	**1,33**	**0,59**	**1,68**	2	NA	1
K	0,22	0,39	0,35	0,29	**6,13**	8,49	9,67	**7,49**	8-13	NA	NA
Mn	0,37	0,84	0,14	0,43	3,24	2,20	1,69	0,98	0,25	NA	NA
Na	0,03	0,04	0,03	0,03	0,20	0,16	0,11	0,11	NA	NA	NA
P	0,27	0,26	0,23	0,20	**1,29**	**1,42**	**1,41**	**1,30**	1,5-1,8	1,3	NA
Fe	0,04	0,05	0,11	0,02	0,16	0,20	0,17	0,14	0,09	NA	0,04
Argiles <2µm	200,00	238,00	351,00	338,00							
Limons fins 2-20µm	340,00	350,50	243,00	213,00							
Limons grossiers 20-50µm	341,00	344,50	126,00	103,00							
Sables fins 50-200µm	55,00	43,50	183,00	225,00							
Sables grossiers 200-2000µm	64,00	23,50	97,00	121,00							
pH	3,90	4,35	4,02	4,53							
pH KCl	3,44	3,84	3,62	3,80							
C/N	16,20	16,00	15,80	17,50							
H	1,08	0,96	0,84	0,80							
CEC	6,09	9,82	9,42	6,96							

des hêtres est en moyenne meilleure qu'à Humont en ce qui concerne Ca, Mg, Mn et Na, en revanche les feuilles contiennent moins d'Al et de K. Dans le placeau chaulé, le sol des Potées est plus pauvre en argile, sable et aluminium; toutes les concentrations en nutriments échangeables sont plus fortes qu'à Humont (Tableau 2). De la même manière, tous les contenus en nutriments des feuilles sont égales ou supérieures dans le placeau chaulé des Potées par rapport à Humont, en particulier pour Fe et Mn, et Mg excepté (Tableau 2). Les caractéristiques du sol et la nutrition des arbres aux Potées sont par conséquent celles d'un site moins pauvre, en terme de cations, et où la nutrition des hêtres est meilleure, par rapport à Humont. Les effets du chaulage sont moins spectaculaires qu'à Humont, mais résultent en une meilleure nutrition minérale de l'arbre.

Nous avons choisi deux placeaux de 40m x 30m, un dans la zone chaulée, un autre dans la zone témoin. Nous avons collecté 11 (dans le placeau chaulé) et 12 (dans le placeau témoin) carottes de sol par placeau (8cm de diamètre, 15 cm de profondeur, 750cm^3), chacune à 1m du pied d'un arbre différent, en Juillet 2007. Les carottes de sol ont alors été tamisées séparément à 0,5mm et délicatement lavées avec l'eau du robinet, puis observées à la binoculaire. Les morphotypes ectomycorrhiziens ont été identifiés selon les descriptions d'Agerer (1987-98). Toutes les carottes de sol ont été stockées à 4°C et traitées dans les 4 jours suivant l'échantillonnage.

Les mesures d'abondance et d'activités enzymatiques des ECMs ont été effectuées comme décrit dans le chapitre II Material & Methods, *Estimation of ECM abundance*, *Enzymatic assays* et *Data treatment.*

Résultats et discussion

Comme pour Humont, le chaulage a significativement modifié la structure de la communauté d'ECMs, mais cela a été plus intense dans la forêt des Potées : nous avons observé une

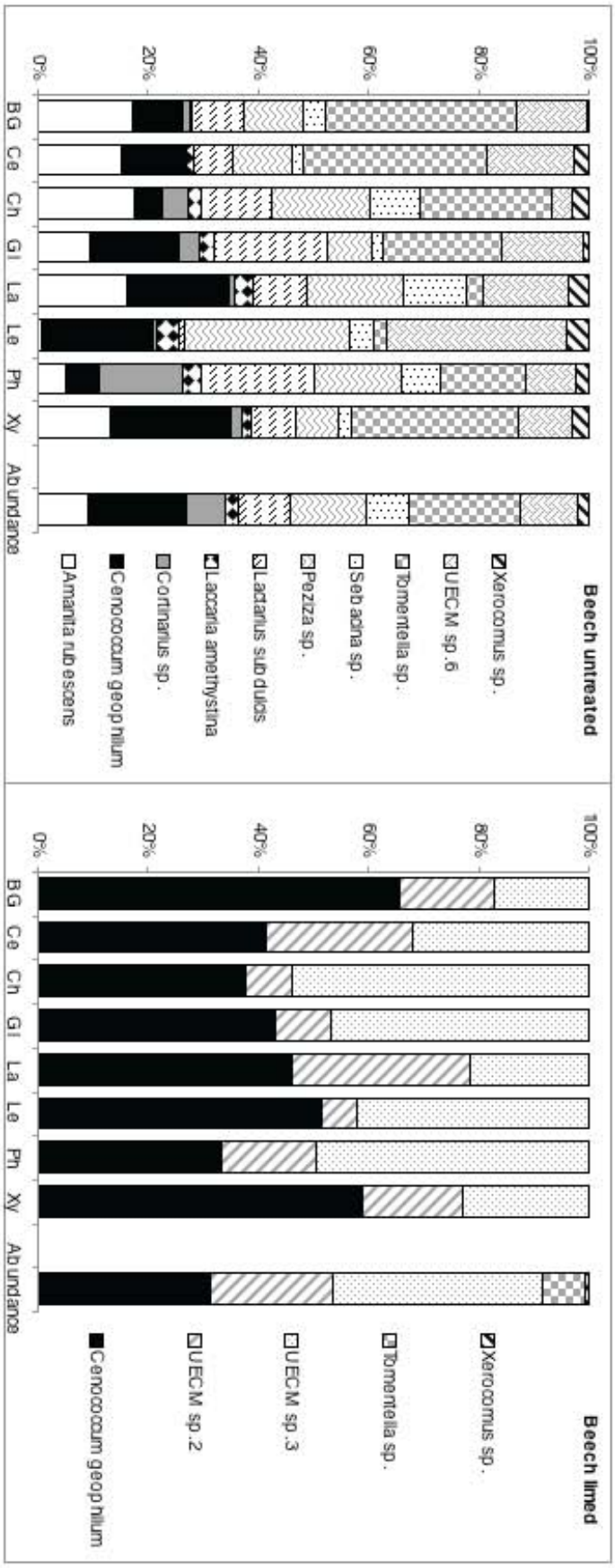


Figure 2. Abondance relative (%) et contribution moyenne à chaque activité enzymatique potentielle de la communauté d'ECMs (%) de chaque morphotype. Les abondances ont été mesurées sur 10 carottes de sol par placeau. Les racines ont été lavées, réparties de manière homogène dans une boîte de pétri, et coupées en petits morceaux d'environ 1cm de long. Les segments de racines ainsi obtenus sont pris au hasard et on compte les ECMs jusqu'à atteindre un effectif de 100 (Garbaye, 1990). La barre « Abondance » représente l'abondance relative de chaque morphotype dans les 11 carottes de sol regroupées. La contribution représente la part de l'activité enzymatique totale dûe à un morphotype. La contribution du morphotype m (Cm) est calculée comme suit :

$Cm = (a_m * e_m) / \sum$

Où e_m est l'activité enzymatique moyenne du mùorphotype m ($\mu mol.apex^{-1}.min^{-1}$), a_m son abondance relative (%), et $\sum$, l'activité enzymatique d'une communauté de 100 ECMs dans le placeau ($\mu mol.min^{-1}$). BG: ß-Glucosidase, Ce: Cellobiohydrolase, Gl: Glucuronidase, Xy: Xylosidase, La: Laccase, Ch: Chitinase, Le: Leucine aminopeptidase, Ph: Acid Phosphatase. Beech untreated: hêtre témoin; Beech limed : hêtre chaulé.

réduction très significative de tous les indices de diversité dans le placeau chaulé (Tableau 1). Contrairement à Humont, *Cenococcum geophilum* était plus abondant dans le placeau chaulé (35% d'abondance relative, Figure 2, barre « Abundance »), que dans le placeau témoin (16% d'abondance relative) ; les abondances dans le placeau témoin étaient bien équilibrées entre les espèces (17% pour *Tomentella sp*., 12% pour *Peziza sp*., 9% pour *Lactarius subdulcis*, 8% pour *Amanita rubescens*…), alors que le placeau chaulé était dominé par *Cenococcum geophilum*. De plus, contrairement au placeau témoin, dans la plupart des carottes de sol provenant du placeau chaulé, nous n'avons pas atteint le nombre total de 100 ECMs. Le morphotype *Lactarius subdulcis* était peu abondant (9% d'abondance relative dans le placeau témoin) et était totalement absent du placeau chaulé. Tout se passe donc comme si la symbiose ectomycorrhizienne, à la fois en terme d'abondance et de diversité, avait été réprimée dans le placeau chaulé, et que les arbres se libéraient de leurs ECMs à cause de la grande quantité de nutriments disponibles dans le sol.

En ce qui concerne le fonctionnement de la communauté d'ECMs, toutes les activités enzymatiques par carotte de sol sont plus faibles dans le placeau chaulé (Figure 3), ce qui est une conséquence directe de la plus faible abondance des ECMs dans ces placeaux (de 87,7 ECMs par carotte dans le placeau témoin contre 21,9 dans le placeau chaulé). Contrairement à Humont, *Xerocomus sp.* A très peu contribué à toutes les activités enzymatiques, et *Lactarius subdulcis* a très peu contribué à l'activité laccase. Tous les morphotypes présentent des contributions équivalentes pour les huit activités enzymatiques : on ne distingue pas d'espèces spécialistes (Figure 2).

Ces résultats prouvent que les effets du chaulage sur la structure des communautés d'ECMs dépendent fortement des caractéristiques du site. Des apports de Ca et Mg sur des sols de nature différente et sur des arbres d'âge différent peut créer des niches écologiques très distinctes, et par conséquent favoriser la colonisation d'autres espèces d'ECMs. Les

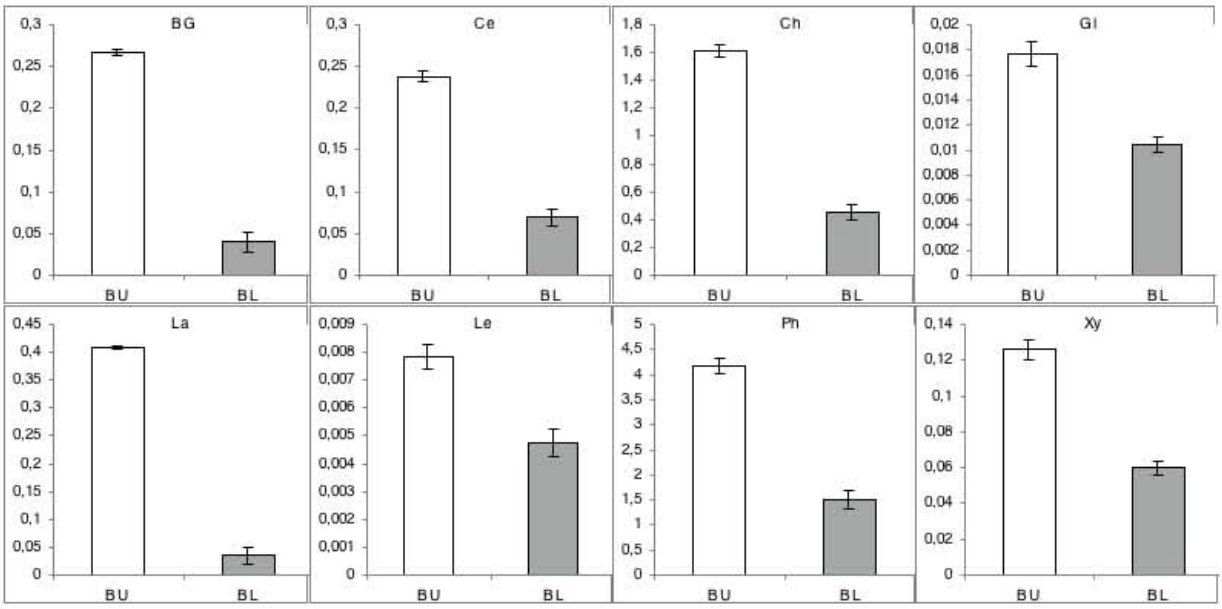


Figure 3. Activités enzymatiques totales de la communauté d'ECMs par carotte de sol (valeur moyenne pour 12 carottes dans le placeau témoin et pour 11 carottes dans le placeau chaulé) dans chaque placeau de la forêt des Potées en Juillet 2007. Huit activités enzymatiques potentielles ont été mesurées (BG: ß-Glucosidase, Ce: Cellobiohydrolase, Gl: Glucuronidase, Xy: Xylosidase, La: Laccase, Ch: Chitinase, Le: Leucine aminopeptidase, Ph: Acid Phosphatase). L'activité enzymatqiue totale a été mesurée comme suit:

E=a*e

Où a est l'abondance relative d'un morphotype donné dans une carotte de sol, e l'activité enzymatqiue moyenne de sept ECMs de chaque morphotype, selon le protocole de Courty et al. (2005) (en $\mu mol.cm^{-2}.h^{-1}$), et E l'activité enzymatique potentielle d'un morphotype donné dans une carotte de sol (en $\mu mol.\ h^{-1}$). Dans chaque placeau on calcule alors l'activité enzymatique totale (Σ) par carotte de sol en additionnant les valeurs de E de tous les mophotypes présents à l'intérieur de cette carotte. Placeau chaulé (BL): barres grises (n=11), placeau témoin (BU): barres blanches (n=12). Les barres d'erreur représentent l'erreur type.

divergences entre le site de Humont et celui des Potées, en particulier en ce qui concerne la texture du sol et le Ca et Al échangeables ainsi que le P disponible, et le ratio Al/cations basiques, peuvent expliquer les différences dans la composition de la communauté d'ECMs. De plus, l'influence des lombrics est beaucoup plus faible aux Potées qu'à Humont, et par conséquent on n'observe pas de formation d'un horizon Olu enrichi en composés phénoliques. Cela peut expliquer la plus faible activité laccase et la très faible contribution de *Lactarius subdulcis* à l'activité laccase et peut-être l'absence de *Lactarius subdulcis* du placeau chaulé.

Donc, tout se passe comme si le chaulage, lorsqu'il est appliqué à un sol forstier relativement riche, conduit à une amélioration notable de l'accessibilité des nutriments, rendant la symbiose ectomycorrhizienne moins utile pour l'arbre, qui diminue alors son allocation d'énergie à la fabrication et à la maintenance de racines fines et à la symbiose ecomycorrhizienne. Cependant, dans les sols pauvres, comme à Humont, où la carence en cations est sévère, le chaulage induit la stimulation de la symbiose ectomycorrhizienne en favorisant des morphotypes spécialistes pour la dégradation de la matière organique et très efficaces pour l'altération potentielle des minéraux, aidant l'arbre à restaurer sa nutrition minérale.

CHAPTER V. GENERAL SYNTHESIS AND PERPECTIVES



Pictures of untreated and limed beech stands in Humont, taken in july 2006. Top left : untreated beeches. Top right : Limed beeches, 10 meters away from the untreated stand. Bottom left : aspect of the beech canopy in the untreated stand, showing some defoliation in the top of some trees. Bottom right : aspect of the beech canopy in the limed stand.

1. General conclusion from the work in Humont

Review of the results

The aims of this study were to determine the effects of liming on the structure and the functioning of beech and spruce ectomycorrhizal (ECM) communities. Results showed that calci-magnesian amendments:

i) reduced the sporocarp abundance of acidophilic species, whatever the tree host (chapter 1)

ii) decreased the abundance of ECM tips of morphotypes formed by acidophilic fungal species and promoted ubiquist ones (*Clavulina cristata* for spruce, *Lactarius subdulcis* for beech) ; these ECM morphotypes were especially confined in the organomineral layer (chapter 2)

iii) increased the potential secretion of enzymes involved in organic matter degradation (glucuronidase for spruce, laccase for beech) at the scale of the ECM community. The ubiquist newcomer ECM morphotypes were specialized in the secretion of these two enzymes and were nearly the sole contributors to these enzymatic activities (chapter 2).

iv) increased oxalate secretion by beech ECM communities. The *Lactarius subdulcis* ECM was by far the most active morphotype for this oxalate secretion, and was thus suspected to play an important role in mineral weathering by the beech ECM community (chapter 3).

v) increased the amounts of Ca, Mg, Mn, K, Al, Si and Fe contained in the mantle of *Lactarius subdulcis* ECMs (chapter 4).

Thus, liming significantly modifies the ECM community structure and functioning. It reduced the abundance of ECM morphotypes acidophilic and poorly active in terms of organic matter

degradation, and induced the appearance of ubiquist and highly specialized ECM morphotypes. Moreover, the potential mineral dissolution by beech ECM community was higher, and lead to a higher mineral nutrient content in the mantle of *Lactarius subdulcis*, the most active ECM morphotype in the beech stands.

However, these results did not tell anything about a potential role of ECMs in the improvement of tree nutrition after liming. We thus decided to compare qualitatively the effects of liming on soil chemical composition, ECM community functioning (potential enzymatic and mineral alteration activities), and foliar analyses, which reflects the tree mineral nutrition. These data were computed for beech only because no data on mineral weathering and ECM mantle contents were recorded for spruce ECMs.

Two plots of approximately 20m x 20m were chosen in limed and untreated (control) beech stands. All the measurements were done in the same plots, as well as for soil, ECM community and leaves.

Soil analyses were performed by the Laboratoire des Cycles Biogéochimiques (INRA Nancy). The topsoil layer (A) of five soil cores (8cm diameter) was taken in the limed and in the untreated plot in October 2006. Soil analyses were performed as described for October 2007 in the first chapter, in the part “Soil analyses”, and ECM community analyses as described in the second chapter, in the part “Material and methods”. We also focused on the mean enzymatic activities of *Lactarius subdulcis* because of its key role in potential mineral weathering, enzymatic activities, its abundance in limed plot and its presence in both limed and untreated plot. Foliar analyses were performed by the UMR 1137 (Ecologie et écophysiologie forestières) and Laboratoire de biogéochimie des écosystèmes forestiers (INRA Nancy). Leaves were collected in ten randomly choosed trees in the limed plot and in

Table 1. Recapitulation of qualitative effects of liming on soil chemical composition, mean potential enzymatic activities of ECM community per soil core, *Lactarius subdulcis* mean enzymatic activities, potential mineral weathering abilities of ECM community, *Lactarius subdulcis* mean mineral weathering abilities, mean mineral contents of the mantle of *Lactarius subdulcis* ECMs and foliar analyses in the beech plots in Humont. + : increased in limed plot ; - : decreased in limed plot ; 0 : no difference in limed and untreated plot.

Nutrient concerned	Measurement	Technique	Response to liming
Carbon	Soil C	Soil analyses	-
	Total BGlucosidase activity (ECM community)	Microplate assays	-
	Total Cellobiohydrolase activity (ECM community)	Microplate assays	-
	Total Glucuronidase activity (ECM community)	Microplate assays	0
	Total Xylosidase activity (ECM community)	Microplate assays	-
	Total Laccase activity (ECM community)	Microplate assays	+
	Mean BGlucosidase activity (Lactarius subdulcis)	Microplate assays	+
	Mean Cellobiohydrolase activity (Lactarius subdulcis)	Microplate assays	+
	Mean Glucuronidase activity (Lactarius subdulcis)	Microplate assays	-
	Mean Xylosidase activity (Lactarius subdulcis)	Microplate assays	-
	Mean Laccase activity (Lactarius subdulcis)	Microplate assays	+
	Foliar C	Elemental analyses	0
Nitrogen	Soil N	Soil analyses	-
	Total chitinase activity (ECM community)	Microplate assays	0
	Total Leucine aminopeptidase activity (ECM community)	Microplate assays	-
	Mean chitinase activity (Lactarius subdulcis)	Microplate assays	+
	Mean Leucine aminopeptidase activity (Lactarius subdulcis)	Microplate assays	+
	Foliar N	Elemental analyses	0
Phosphorus	Soil organic P	Soil analyses	-
	Total Acid Phosphatase activity (ECM community)	Microplate assays	+
	Mean Acid Phosphatase activity (Lactarius subdulcis)	Microplate assays	+
	Mantle P content (Lactarius subdulcis)	EDS	+
	Foliar P	ICP	-
Iron	Soil Fe	Soil analyses	-
	Mean Iron chelation activity (ECM community)	Microplate assays	-
	Total Iron chelation activity (ECM community)	Microplate assays	+
	Total Iron chelation activity (Lactarius subdulcis)	Microplate assays	-
	Mean Iron chelation activity (Lactarius subdulcis)	Microplate assays	+
	Mantle Fe content (Lactarius subdulcis)	EDS	+
	Foliar Fe	ICP	-
Ca, Mg	Soil Ca	Soil analyses	+
	Soil Mg	Soil analyses	+
	Mean Oxalate secretion (ECM community)	Microplate assays	+
	Total Oxalate secretion (ECM community)	Microplate assays	+
	Total Oxalate secretion (Lactarius subdulcis)	Microplate assays	+
	Mean Oxalate secretion (Lactarius subdulcis)	Microplate assays	+
	Mantle Ca content (Lactarius subdulcis)	WDS	+
	Mantle Mg content (Lactarius subdulcis)	WDS	+
	Foliar Ca	ICP	+
	Foliar Mg	ICP	+
Other nutrients	Soil Al	Soil analyses	-
	Soil Mn	Soil analyses	+
	Soil K	Soil analyses	-
	Soil Na	Soil analyses	-
	Mantle Al content (Lactarius subdulcis)	EDS	+
	Mantle Mn content (Lactarius subdulcis)	WDS	+
	Mantle K content (Lactarius subdulcis)	EDS	+
	Mantle Na content (Lactarius subdulcis)	EDS	0
	Foliar Al	ICP	-

twenty trees (ten healthy plus ten declining) in the untreated one in July 2007. Foliar tissues were dried at 65°C, grounded, homogenized, and analyzed separately. Total C&N contents were measured using the elemental analyser NCS2500 (CE instrument Thermo Quest, Italie); other elements (C, S, P, K, Ca, Mg, Mn, Na, Al and Fe) were mineralized in perchloric acid and measured by ICP.

Results and discussion

The effects of liming on forest ecosystem are summarized in figure 1. Mean element concentration in soil and beech leaves is given in table 2.

*Carbon cycling

Soil C contents decreased in the A layer of the limed plot (Table 1). We observed an increased total laccase activity of the community in the limed plot, whereas all other potential activities involved in the mobilization of organic C (activities of cellulose and hemicellulose degradation: ß glucosidase, cellobiohydrolase, glucuronidase, xylosidase) decreased or were unchanged. There was no effect of liming on the total C content in beech leaves. A similar decrease of the total soil C in the A horizon was already observed in a similar experiment (Höglwald) with spruce (Kreutzer, 1995) and was attributed to the accelerated organic matter turnover and to the transfer of dissolved organic carbon in mineral layers in limed plot, especially through earthworm bioturbation. Interestingly, in the limed plot of the Höglwald experiment, the soil layer formed by earthworm bioturbation (Olu) was significantly enriched in aromatic compounds (Kreutzer, 1995), that are substrates of laccases. A similar enrichment is likely to have also occured in our sampling site, where earthworm activity was high, and could be responsible of the high laccase activity observed in the limed plot. The increase in

foliar biomass due to liming leads to higher leaf fall and thus increased cellulose and hemicellulose inputs in organic matter. Nevertheless, the potential ability of ECMs to degrade celluloses and hemicelluloses decreased in the limed plots. It could be explained by an enhanced activity of brown-rot fungi. The increased laccase activity observed in the limed plot can induce faster humus and litter degradation, contributing to humus change, and improving the availability and mobilization of many mineral nutrients.

*Nitrogen nutrition

Liming decreased soil N contents in the A layer (Table 1). This consequence of liming was already reported by Kreutzer (1995) in a similar liming experiment. It was accompanied by a decrease of the total leucine aminopeptidase activity, but was without any effect on the total chitinase activity per soil core of the ECM community. The *Lactarius subdulcis* ECM population increased its mean activities of chitinase and leucine aminopeptidase in the limed plot. Finally, foliar N contents were not clearly affected by liming. It appears that the sharp decrease in leucine aminopeptidase activity of the ECM community observed in the limed plot did not have any effect on foliar N contents, what is not surprising considering the potentially relatively low amounts of proteins in soil compared to chitin ones (chitin is the main component of fungal mycelium and dead arthropods). The total soil N decrease is probably a consequence of N leaching from A horizon to deeper soil layers (Huber et al., 2006), where N is mineralized (Kreutzer, 1995). It is interesting to note that the decrease of total soil N did not induce any change in the total chitinase activity of the ECM community. It could mean that chitin supply in limed soil does not change and that mineral N only is affected by the treatment, and could be relevant with the similar total N contents in leaves. We could also hypothesize that liming, by inducing higher N mineralization, increased the

amounts of NH_4^+ and NO_3^- in soil. This higher availability in forms of easily available N may be responsible of the lower activities of organic N uptake.

*Phosporus nutrition

The contents in available phosphorus decrease in the A horizon after liming, whereas the total potential acid phosphatase activity of the community increased, as well as the mean phosphatase activity of *Lactarius subdulcis*. It was followed by the increased P content in the mantle of *Lactarius subdulcis* root tips. As for C and N, it is probable that the decreased P concentration results from accelerated organic matter turnover, with an increased uptake of inorganic P by microorganisms in the limed plot. An inverse relationship between inorganic P availability and acid phosphatase activity of ECM fungi has already been reported (Antibus et al. 1992). The lower concentration of available soil P probably stimulates the acid phosphatase activity of ECMs, especially for *Lactarius subdulcis*, leading to higher P contents in its ECM mantle. Nevertheless, this increase in potential P uptake was followed by a decrease in leaf P contents, and P nutrition, already under the optimal range, reached the critical threshold in the limed plot (Table 2). It means that phosphatase activity may not be correlated with effective increase in P uptake by the whole community, or that P is stored in ECM mantle of *Lactarius subdulcis* at the benefit of the sole fungus partner.

*Iron nutrition

As for the Höglwald experiment (Kreutzer, 1995), liming has reduced exchangeable Fe concentration in the upper soil layers. The iron chelating ability of the whole ECM community increased in the limed plot, as the iron complexation ability of *Lactarius subdulcis*, and iron contents in its ECM mantle. Iron chelation through siderophore secretion is repressed by high ferric iron concentration and induced by iron starvation (Neilands, 1995):

it is consistent with the higher iron chelating potential observed in limed plot. Nevertheless, as for P, an increase in Fe uptake by ECMs resulted in a decrease of foliar Fe contents. Bonneau (1995) fixed the threshold of iron deficiency in leaves at 40mg/kg and optimum at 90mg/kg for beech in eastern French forests. It appeared that most of the beech trees in Humont, as well as in limed and untreated plots, had an iron nutrition higher than the optimum (140 and 170mg/kg , respectively, Table 2). Thus, liming probably modifies the balance of Fe exchange between the ECM and the tree and could lead to the recover of supplementary Fe for fungal metabolism.

*Calcium and magnesium nutrition

As expected, liming increased the amounts of exchangeable Ca and Mg in soil A layer. Exchangeable Ca increase was especially high compared to Mg, because of different amounts of Ca and Mg material supplied (757 kg/ha of CaCO3 and 380 kg/ha of CaCaMg(CO3)2). This increase of exchangeable cations was followed by higher oxalate secretion by ECMs, mostly due to *Lactarius subdulcis*, an increase in Ca and Mg in ECM mantle of *Lactarius subdulcis*, and finally by an increase in Ca and Mg content in beech leaves. This strongly suggests that the Ca and Mg input was followed by the stimulation of oxalate secretion of *Lactarius subdulcis*, by far the most important oxalate secretor of the community, and thus improved mineral dissolution and the complexation of dissolved forms of Ca and Mg by these ECMs, resulting in a better Ca and Mg nutrition by the trees, as shown by the higher Ca and Mg contents in ECMs and in leaves. Most of the tree absorbing system (fine roots) is associated with ECM fungi (Smith & Read, 1997). Moreover, the oxalate secretion by ECMs is positively correlated with seedling mineral nutrition *in vitro*. It is therefore probable that ECMs, and especially *Lactarius subdulcis* ones, are directly involved in the long-term improvement of beech Ca and Mg nutrition.

*Aluminium

The concentration of exchangeable Al was lower in the topsoil of the limed plot, probably because of its replacement by Ca and Mg in the absorbing complex. Nevertheless, amounts of Al in *Lactarius subdulcis* ECMs increased after liming, whereas Al foliar contents decreased. The presence of this toxic element in relatively high concentrations in mycorrhizae of *Lactarius subdulcis* (as well as in limed and untreated plots, despite the effect of the treatment) can contributes to Al tolerance in acidic soils, as found in loblolly pine associated with *Pisolithus tinctorius* (Moyer-Henry et al., 2005). The concomitant increase in Al and Si contents in ECM mantle of *Lactarius subdulcis* could be a mean of silicon-induced amelioration of Al toxicity, as observed for maize roots or sorghum grown in water culture (Galvex et al., 1987; Kidd et al., 2001). High Al and Si concentrations were also found in *Paxillus involutus*-spruce ECMs by Hodson & Wilkins (1991), who emitted the hypothesis of Al detoxification by Si. It is interesting to note that this aluminium sequestration by ECMs was increased in the limed plot, acting iteratively with the treamtent.

*Potassium

Liming reduced the concentration of K in the soil A layer, as well as foliar K contents, which however remained in the optimal range (7 and 9 mg/kg, table 2). Nevertheless, K concentrations in the mantle of ECMs of *Lactarius subdulcis* increased. Modifications in K concentrations were difficult to intrepret because K uptake by fungal cells is mediated by many carrier proteins and exchange proteins, because of its crucial role for regulation of osmotic cellular potential (Garraway & Evans, 1984). This increase in K concentration in ECM fungal sheath could result in an increased osmotic potential of fungal mantle and could be interpreted as an adaptation to a higher water uptake by ECMs, because of higher water needs of the limed trees (increased leaf biomass).

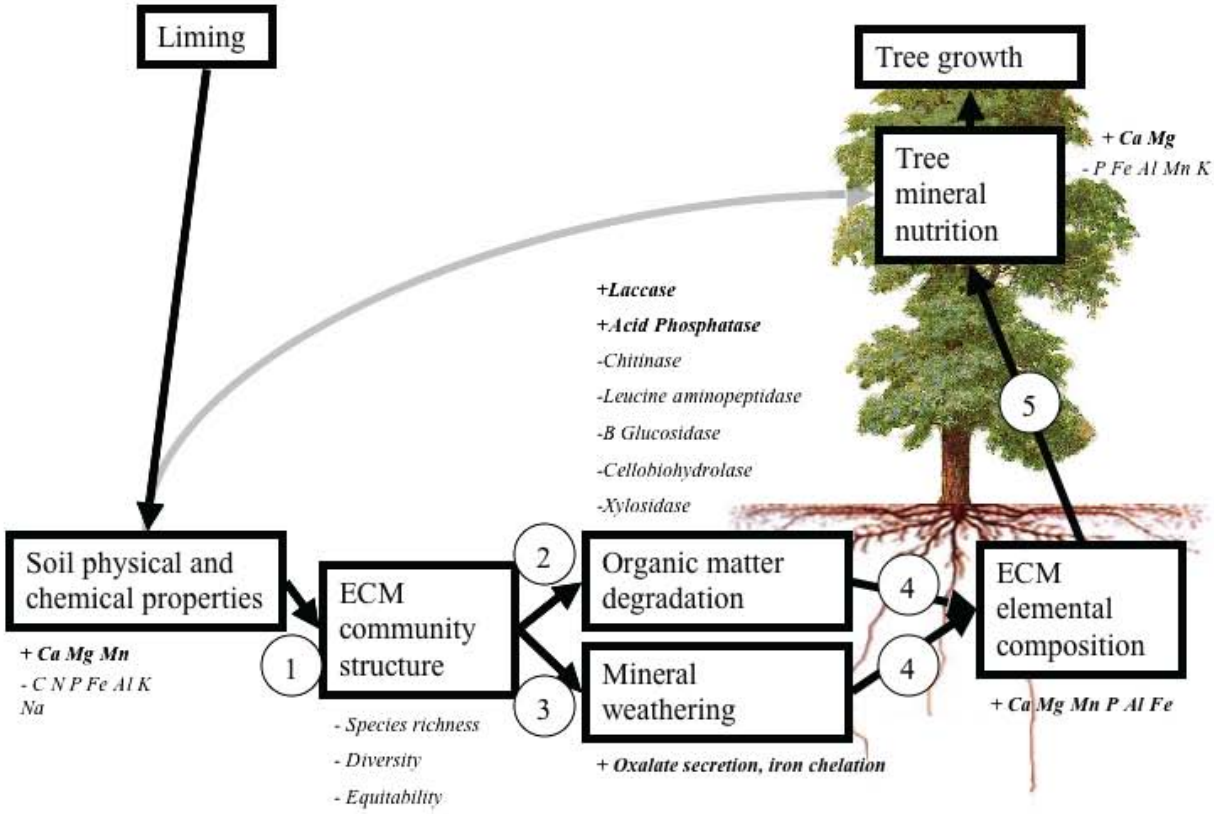


Figure 1. Representation of the effects of liming on soil chemical composition, ECM communities, and tree nutrition. Circled numbers refer to the chapter of the thesis. Results obtained in this study are in italic besides the corresponding boxes.

*Manganese

The amounts of Mn increased in the upper layers of the limed plot, as well as in the mantle of *Lactarius subdulcis* ECMs, but decreased in beech leaves in the limed plot, and presented high foliar concentrations (Table 2). Interestingly, Mn nutrition in fungi involves the same uptake mechanisms than Mg one (Garraway & Evans, 1984). It seems that *Lactarius subdulcis* ECMs were able to increase their Mn uptake in the limed plot (through for example the secretion of oxalate), resulting in higher Mn contents in the ECM mantle, but did not transfer it to the tree. It is thus probable that, as for Al, Mn is accumulated in the sheath of ECMs when it reaches excessive concentrations in leaves, because of its potential toxicity.

These results shows that liming not only modifies the ECM community structure and functioning, but also that ECMs play a role in the improvement of tree nutrition after liming, by providing higher quantities of Ca and Mg via enhanced mineral dissolution and complexation. It allowed us to refine the diagram which summarizes the structure of this work (Figure 1). These results also stress the fact that the replacement of a sole ECM morphotypes (i.e *Lactarius subdulcis*) considerably modify the potential benefit of the symbiosis for the tree.

2. What is the degree of site-specificity in the effect of liming on ECM communities?

The effects of liming studied in Humont are specific to a 60 year-old beech forest stand, in low mountains, based on red sandstone. However, because acidic forest soils also exist in many other environments, liming is susceptible to take place in very different soil and sylviculture conditions. In order to assess the variability of liming effects on ECM communities, we measured effects of liming on ECM community structure and potential enzymatic activities in a younger planted beech forest based on a richer soil in another region of northeastern France. This work has been done in collaboration with Hélène Genet (Laboratoire d'Ecophysiologie forestière, équipe de Phytoécologie, INRA Nancy).

Site and tree stand characteristics

The experimental site of Les Potées (49°85'25''N, 4°46'80''E, elevation: 380m, Ardennes forest, North-Eastern France) consists in stands of regularly planted 30-year old beech (*Fagus sylvatica*). In contrast to Humont, beech were not declining in Les Potées. The liming treatment (Ca and Mg) was carried out by hand in 1996, 5 years after Humont. The luvisol is formed on roughly horizontal schist. In contrast with Humont, liming did not have any effect on the humus type, which remained an oligomull in the limed plot, but improved tree mineral nutrition (Table 2). Earthworm colonization was low in both untreated and limed plots.

The untreated soil in Les Potées was characterized by higher contents in silts, Ca and Mn, whereas C, N, Al, K, Fe, clay, sand, and CEC were lower than in Humont (Table 2). Beech nutrition was in the average better in Ca, Mg, Mn and Na than in Humont and a little lower in Al and K. In the limed plot, the soil in Les Potées was poorer than Humont in clay, sand, and Al; all the concentrations of exchangeable nutriments were higher than in Humont (Table 2). In the same way, all the nutrient contents in beech leaves were equal or higher in the limed

Table 1. Mean ectomycorrhizal diversity per soil core in the two (untreated and limed) beech plots in Potées forest on July 2007. Diversity was calculated as Species richness (SR), Shannon diversity index, and Simpson equitability index in each soil core. Standard deviation is given in brackets. Significant differences in diversity indices between plots were assessed using a Student's t test. Significant differences are reported with asterisks. ***: $p<0.001$; **, $p<0.01$; *$p<0.05$.

	Species richness	Shannon	Simpson
Beech untreated	4,25 (0,25)	1,11 (0,08)	0,59 (0,03)
Beech limed	2,45 (0,39) ***	0,49 (0,12) ***	0,29 (0,08) ***

Table 2. Soil and foliar analyses in Humont and Les Potées. Concerning soil analyses, concentrations of C, N, P, H, CEC, clays, silts and sands are given in $g.kg^{-1}$. Concentrations in exchangeable Al, Ca, Mg, K, Mn, Na and Fe are given in $cmol+.kg^{-1}$. Concerning foliar analyses, concentrations are given in $g.kg^{-1}$. Values below the optimum given by Bonneau (1995) are given in bold.

Type of analysis	Soil				Leaves				Optimal concentration	Critical threshold	Starvation threshold
Site	Les potˇes		Humont		Les Potˇes		Humont				
Treatment	Untreated	Limed	Untreated	Limed	Untreated	Limed	Untreated	Limed			
C	54,80	88,05	67,60	53,70	506,17	492,35	499,08	481,71	-	-	-
N	3,38	5,55	4,28	3,07	24,51	25,00	22,27	20,95	20-25	15	NA
Al	4,83	2,67	8,20	5,22	0,10	0,11	0,15	0,11	NA	NA	NA
Ca	0,56	4,76	0,20	1,57	5,30	7,71	**1,58**	7,04	5-8	NA	NA
Mg	0,21	0,74	0,22	0,47	**0,91**	**1,33**	**0,59**	**1,68**	2	NA	1
K	0,22	0,39	0,35	0,29	**6,13**	8,49	9,67	**7,49**	8-13	NA	NA
Mn	0,37	0,84	0,14	0,43	3,24	2,20	1,69	0,98	0,25	NA	NA
Na	0,03	0,04	0,03	0,03	0,20	0,16	0,11	0,11	NA	NA	NA
P	0,27	0,26	0,23	0,20	**1,29**	**1,42**	**1,41**	**1,30**	1,5-1,8	1,3	NA
Fe	0,04	0,05	0,11	0,02	0,16	0,20	0,17	0,14	0,09	NA	0,04
Clay <2µm	200,00	238,00	351,00	338,00							
Fine silt 2-20µm	340,00	350,50	243,00	213,00							
Coarse silt 20-50µm	341,00	344,50	126,00	103,00							
Fine sand 50-200µm	55,00	43,50	183,00	225,00							
Coarse sand 200-2000µm	64,00	23,50	97,00	121,00							
pH	3,90	4,35	4,02	4,53							
pH KCl	3,44	3,84	3,62	3,80							
C/N	16,20	16,00	15,80	17,50							
H	1,08	0,96	0,84	0,80							
CEC	6,09	9,82	9,42	6,96							

plot of Les Potées, especially for Fe and Mn, and except for Mg (Table 2). The soil characteristics and beech nutrition in Les Potées were therefore those of a more fertile site, in terms of cations, and of a healthy beech stand in terms of mineral nutrition, compared to Humont. The effects of liming were less spectacular than in Humont, but resulted in a similar beech mineral nutrition.

We chose two plots of 40x30m: one in the limed area, the other in the untreated one.

We collected 10 soil cores per plot (8 cm diameter, 15cm deep, 750cm^3), each one at 1 meter from the foot of one tree, on July 2007. Soil cores were sieved separately at 0.5 mm and then gently washed with tap water, and observed under stereomicroscope. Ectomycorrhizal morphotypes were identified using Agerer's (1987-98) descriptions. All soil cores were stored at 4°C and treated within 4 days after sampling.

The measurements of ECM abundances and enzymatic activities were performed as described in chapter II, Material & Methods, *Estimation of ECM abundance*, *Enzymatic assays* and *Data treatment.*

Results and discussion

As in Humont, liming significantly modified the ECM community structure, but it was more intense in Les Potées forest: there was a sharp significant reduction of all the diversity indices in the limed plot (Table 1). Contrary to Humont, *Cenococcum geophilum* was more abundant in the limed plot (35% abundance, Figure 2, bar « Abundance ») than in the untreated one (16% abundance); the abundances in the untreated plot were equitable between species (17% for *Tomentella sp.*, 12% for *Peziza sp.*, 9% for *Lactarius subdulcis*, 8% for *Amanita rubescens*…), whereas the limed plot was dominated by *Cenococcum geophilum*. Moreover, contrary to the untreated plot, in most of the soil cores from limed plot we did not reach the

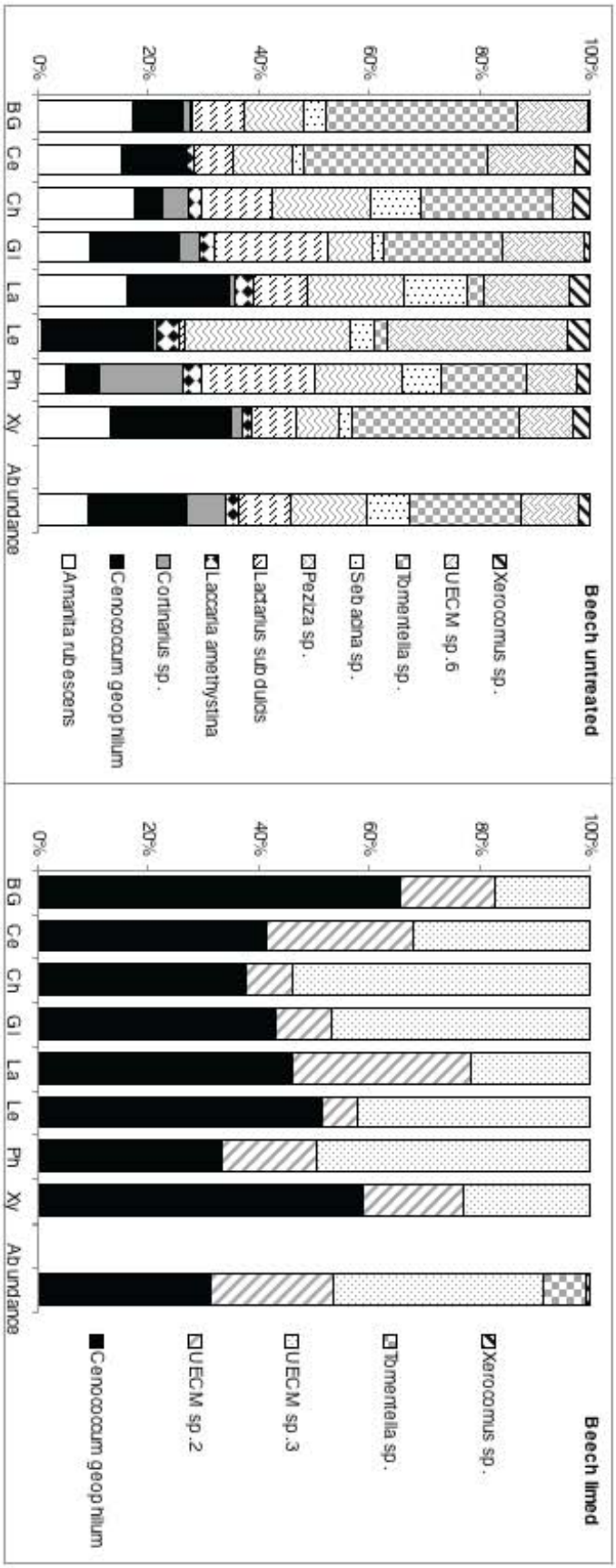


Figure 2. Relative abundance (%) and mean contribution to each potential enzyme activity in the ECM community (%) of each morphotype. Abundances were measured on 10 soil cores per plot. Roots were washed, homogeneously spread in a Petri dish, and cut in small pieces (1cm long). Root pieces were then randomly picked and ECMs counted until we reached 100 tips (Garbaye, 1990). The bar "Abundance" represents the relative abundance of each morphotype in the pooled 10 soil cores. Contribution represents the part of the total enzymatic activity due to each morphotype. Contribution of the morphotype m (Cm) is calculated as:

$Cm = (a_m * e_m) / \sum$

Where e_m was the mean enzymatic activity of the morphotype m ($\mu mol.tip^{-1}.min^{-1}$), a_m its relative abundance (%), and $\sum$, the enzymatic activity of a community of 100 ECMs in the plot ($\mu mol.min^{-1}$). n=20 (abundances), and n=4 (contributions). BG: ß-Glucosidase, Ce: Cellobiohydrolase, Gl: Glucuronidase, Xy: Xylosidase, La: Laccase, Ch: Chitinase, Le: Leucine aminopeptidase, Ph: Acid Phosphatase.

number of 100 ECMs (data not shown). Interestingly, *Lactarius subdulcis* was present in small numbers (9% abundance in the untreated plot) but was totally absent from limed plot. Everything happens as if the ECM symbiosis was repressed, in terms of abundance and diversity, in the limed plot, and if the trees got rid of their fungal partners because of easily available nutrient supply.

Considering the functioning of the ECM community, all the total enzymatic activities per soil core were repressed in the limed plot (Figure 3). It was a direct consequence of the decrease of the mean abundance of ECM tips observed in the limed plot (from 87,7 per core in the untreated plot to 21,9 in the limed one). Contrary to Humont, *Xerocomus sp.* displayed very low contributions for all the activities tested, and *Lactarius subdulcis* contributed very poorly to laccase activity. However, all the morphotypes tested presented roughly similar contributions for the eight enzymatic activities: it appeared that there was no specialist (Fig 2).

These results show that the effects of liming on the ECM community structure are highly dependant on the site characteristics. Calcium and Mg inputs in soils of different nature can create very different soil ecological niches, and thus favour or not the colonization by different ECM species. The differences between the sites of Les Potées and Humont, especially concerning soil texture and exchangeable Ca, Al and available P, and Al/basic cations ratio, could explain the difference in ECM community composition. Moreover, earthworm colonization is considerably lower in Potées forest, and consequently there is no formation of a Olu horizon enriched in phenolic compounds : it could explain the low overall laccase activity, the very low contribution of *Lactarius subdulcis* to laccase activity and maybe the absence of *Lactarius subdulcis* in limed plot.

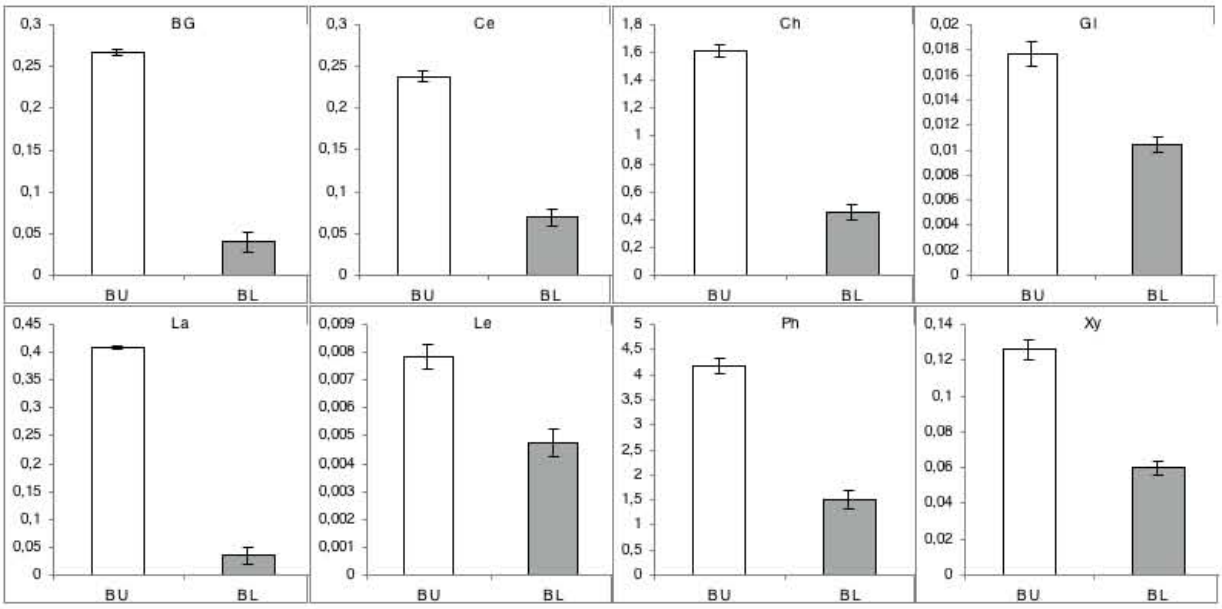


Figure 3. Total enzymatic activities of the ECM community per soil core (mean value for 12 cores in the untreated plot and 11 cores in the limed plot) in each plot in Potées forest on July 2007. Eight potential enzymatic activities were measured (BG: ß-Glucosidase, Ce: Cellobiohydrolase, Gl: Glucuronidase, Xy: Xylosidase, La: Laccase, Ch: Chitinase, Le: Leucine aminopeptidase, Ph: Acid Phosphatase). Total enzymatic activities were calculated using the following formula:

$E=a*e$

where a was the relative abundance of a given ECM type in one soil core, e was the mean enzymatic activity on seven ECM tips of each ECM morphotype, following the protocol of Courty et al (2005) (in $\mu mol.cm^{-2}.h^{-1}$) and E was the potential enzymatic activity of a given ECM type in a soil core (in $\mu mol.\ h^{-1}$). In each plot we then calculate the total potential enzymatic activity (Σ) in the soil core by adding the E values of all ECM type present within this soil core.

Limed: grey bars (n=11), Untreated: white bars (n=12). Bars represents standard error.

Thus, everything happens as if liming, when applied in a relatively rich forest acidic soil, lead to a substantial improvement of nutrient access, making ECM symbiosis useless for the tree and decreasing its allocation to fine root establishment and maintenance and to ECM symbiosis. Nevertheless, in poor soils, as in Humont, where cation starvation is severe, liming induces the development of ECM symbiosis with specialists for organic matter degradation, and highly efficient species for mineral nutrient uptake, which assists the tree in restoring its mineral nutrition.

References

Antibus RK, Sinsabaugh RL, Linkins AE (1992) Phosphatase activities and posphorus uptake from inositol phosphate by ectomycorrhizal fungi. Canadian journal of Botany 70:794-801

Bonneau M (1995) Fertilisation des forêts dans les pays tempérés. ENGREF Nancy, 368p.

Duchaufour Ph., Bonneau M.,1959. Une méthode nouvelle de dosage du phosphore assimilable dans les sols forestiers. Bul. AFES , 4, 193-198.

Galvex, L.; Clark, R. B.; Gourley, L. M.; Maranville, J. W., 1987. Silicon interactions with manganese and aluminium toxicity in sorghum. Journal of Plant Nutrition, 10 : 1139-1147.

Garraway M. O., Evans R. C., 1984. Fungal Nutrition & Physiology. A Wiley Interscience publication. 400p.

Hodson, M.J., Wilkins D.A., 1991. Localizaion of aluminium in the roots of Norway spruce [Picea abies (L.)Karst.] inoculated with *Paxillus involutus* Fr. New Phytologist 118, 273-278.

Huber C, Weiss W, Gottlein A (2006) Tree nutrition of Norway spruce as modified by liming and experimental acidification at the Höglwald site, Germany, from 1982 to 2004. Plant and Soil 63:861-869

Kidd PS, Llugany M, Poschenrieder C, Gunse B, Barcelo J., 2001. The role of root exudates in aluminium resistance and silicon-induced amelioration of aluminium toxicity in three varieties of maize (Zea mays L.). Journal of Experimental Botany, 52: 1339-1352.

Kottke, I., Qian, X.M ;, Pritsch, K., Haug, I., Oberwinkler, F., 1998. *Xerocomus badius-Picea abies*, an ectomycorrhiza of high activity and element storage capacity in acidic soil. Mycorrhiza 7, 267-275.

Kreutzer, K., 1995. Effects of forest liming on soil processes. Plant and Soil 168-169, 447-470.

Moyer-Henry K., Silva i., Macfall J., Johannes E., allen n;, Goldfarb B., Rufty T., 2005. Accumulation and localization of aluminium in root tips of loblolly pine seedlings and the associated ectomycorrhiza *Pisolithus tinctorius*. Plant, cell and Environment 28, 111-120.

Neilands J. B., 1995. Siderophores. Structure and fonction of microbial iron transport compounds. Jour. Biol. Chem, 270. 26723-26726.

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Ed 2, Academic press, London :606p

APPENDIX



Sporocarps of *Russula violeipes* in the spruce limed stand of the Humont forest. (Photo : F. Rineau)

Appendix : Map of the plots investigated for survey of ECM communities in the thesis

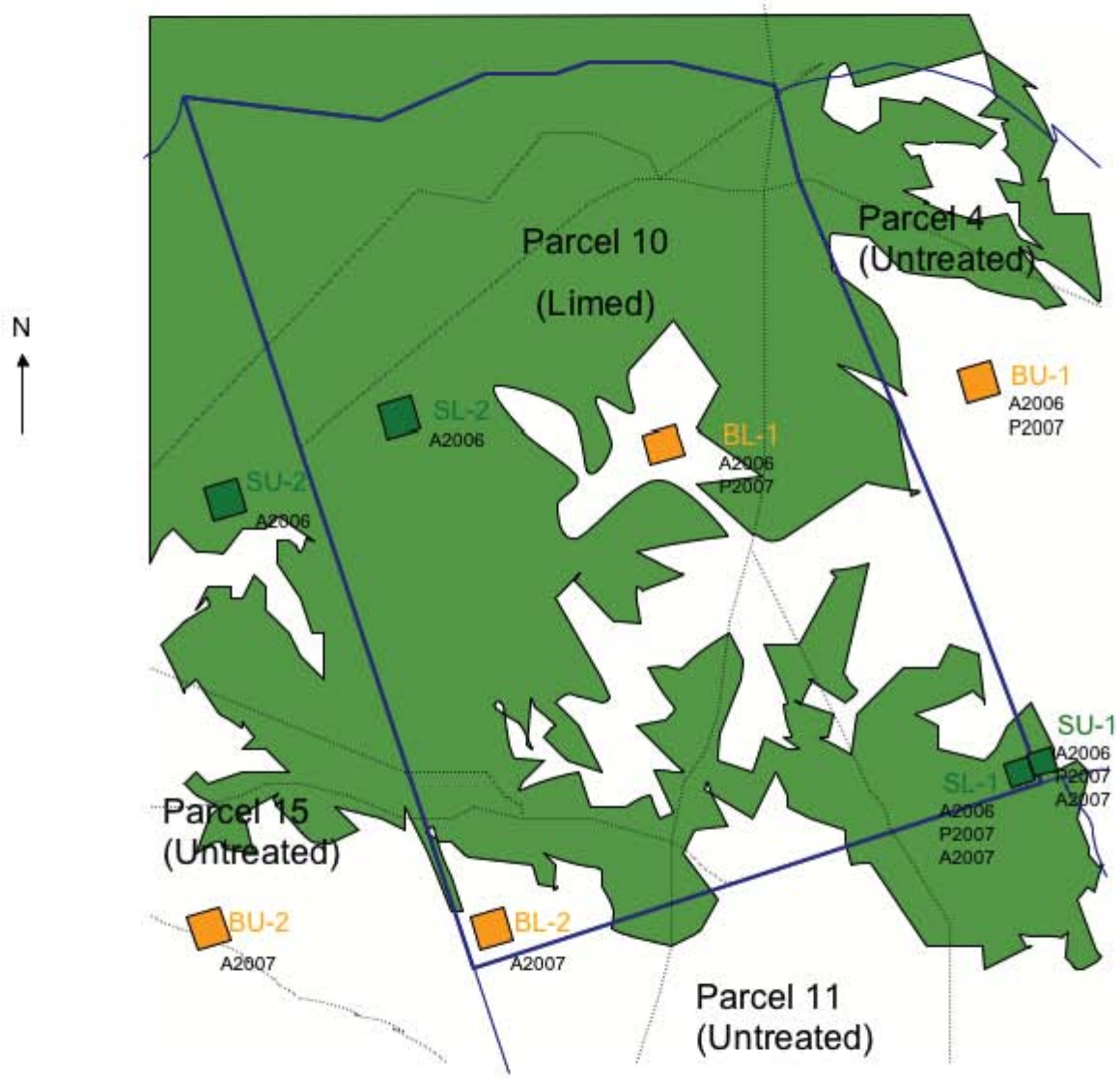


Figure 1: Overview of the sampling site in Humont. Parcel boundaries are represented in blue. Parcel 10 was limed, the others were untreated. Spruce stands are green and beech stands are white. The spruce plots are represented with green squares, the beech ones with orange ones. The date when ECM were sampled from the plot was given below. The arrow indicates the North. Scale : 1cm=50m.

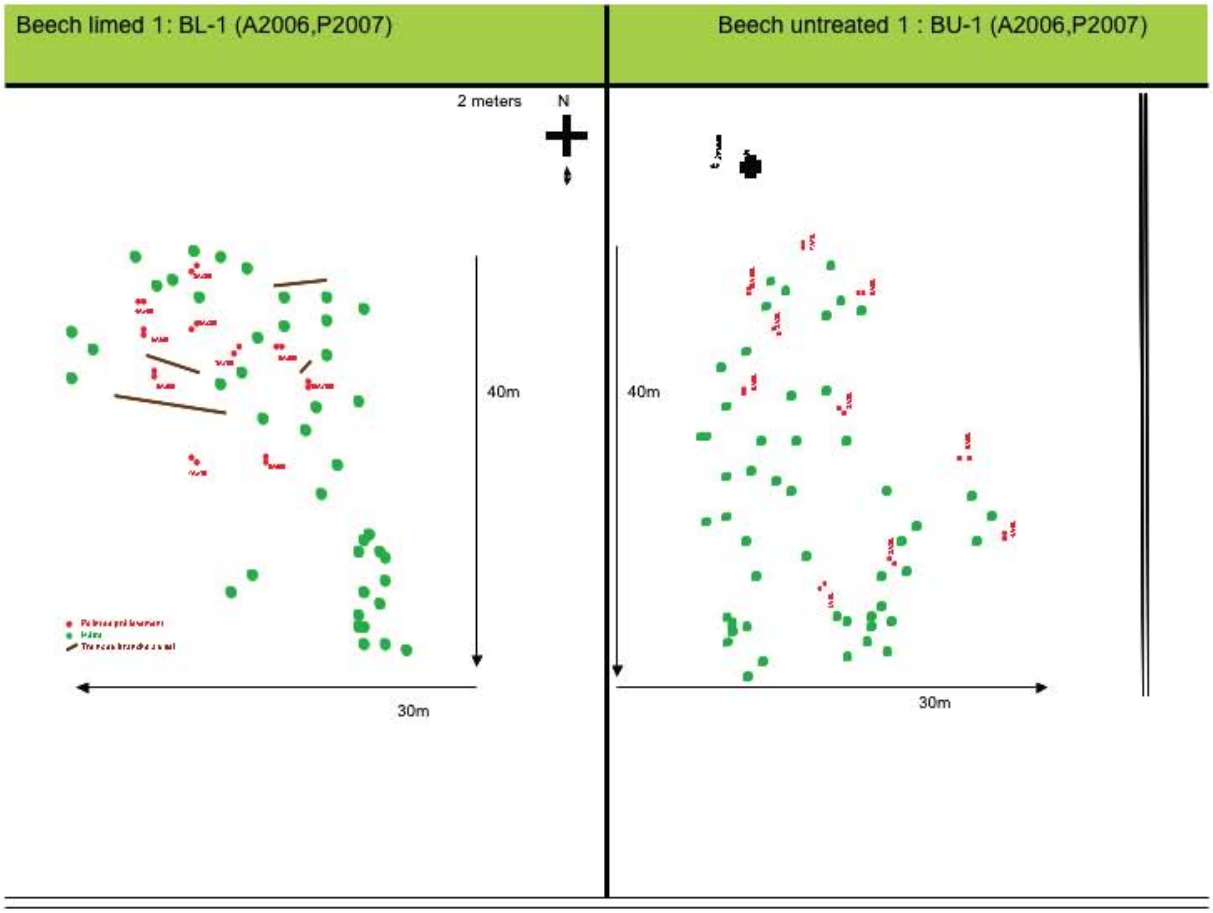


Figure 2: Overview of the beech plots BL-1 and BU-1. Soil cores are represented with red dots, beech trees with green dots and fallen stems or large branches with brown bars.

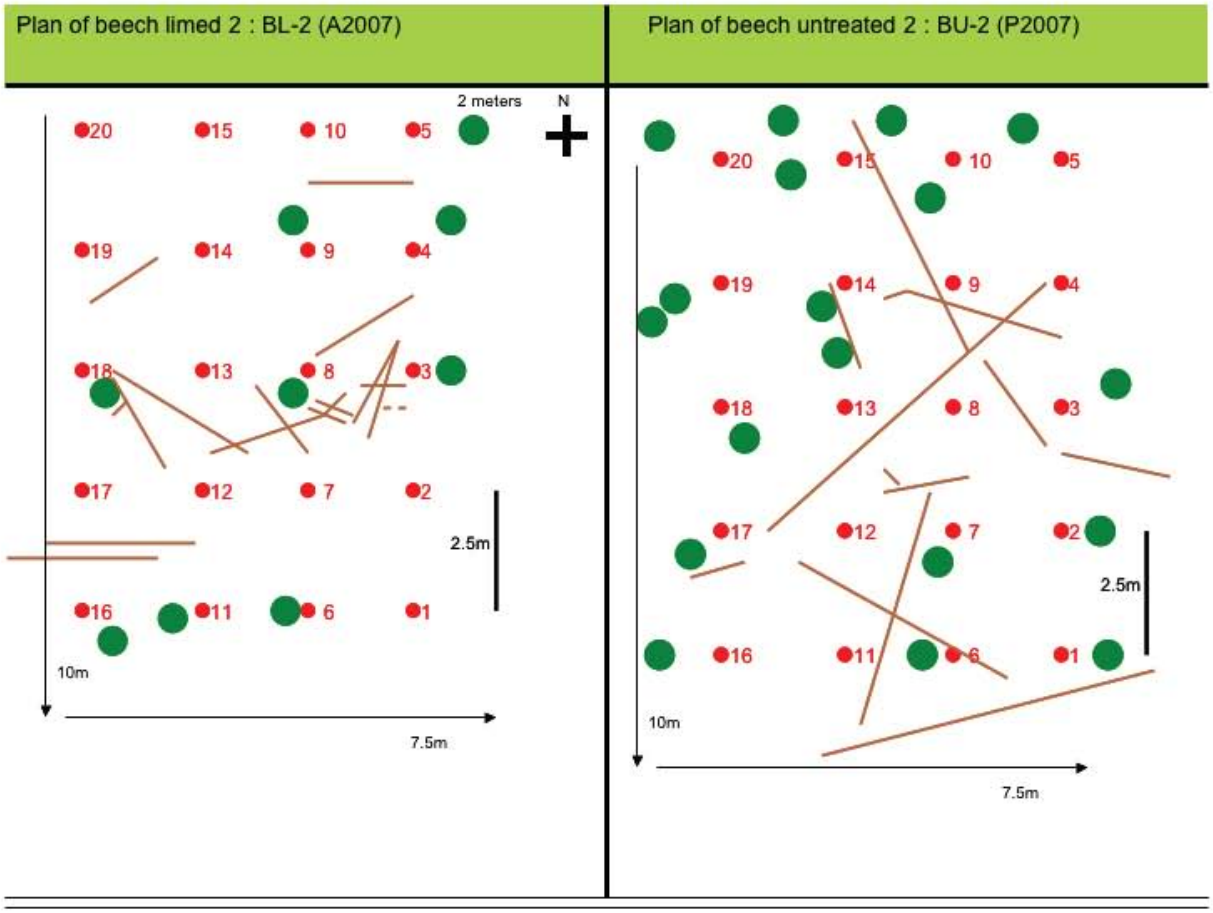


Figure X : Overview of the beech plots BL-2 and BU-2. Soil cores are represented with red dots, beech trees with green dots and fallen stems or large branches with brown bars.

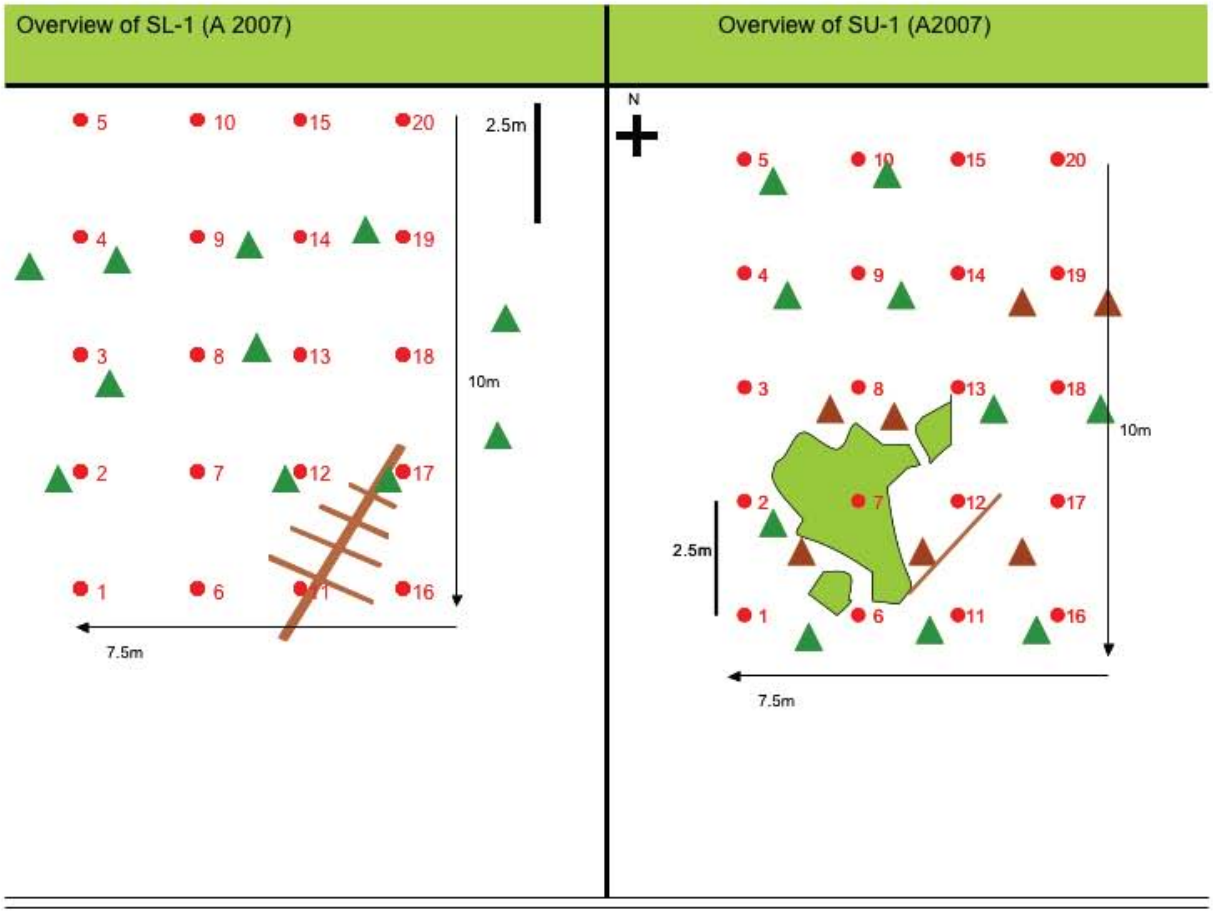


Figure 3: Overview of the spruce plots SL-1 and SU-1. Soil cores are represented with red dots, spruce trees with green triangles, dead spruce trees with brown triangles, areas covered with mosses with light green, and fallen stems or large branches with brown bars.

Abstract

Liming is a forestry practice consisting in Ca and Mg amendment used to correct tree cation deficiency induced by soil acidity. Ectomycorrhizae (ECM) provides the tree with nutrients derived both from mineral and organic sources. The ECM community structure and functioning is closely linked to soil nutrient availability, which is considerably modified by liming. The aims of this study were to assess, in declining beech and spruce stands, the impact of liming on ECM community structure and functioning, and to determine if ECMs take part in the improvement of tree mineral nutrition. Results showed that liming was the second factor of sporocarp community structuration after tree host. Liming also decreased the abundance of ECM tips of acidophilic morphotypes and induced the dominance of more ubiquist ones (*Clavulina cristata* for spruce, *Lactarius subdulcis* for beech). These morphotypes were confined in the organomineral layer and specialized in the potential secretion of glucuronidase and laccase, respectively. For beech, liming significantly increased the potential oxalate secretion by ECMs, which highly depends on the activity of *Lactarius subdulcis*. This morphotype is thus suspected to play an important role in mineral weathering, all the more that it accumulated more nutrients in its mantle in the limed plots. Qualitative comparison of liming effects on soil properties, ECM community functioning and beech nutrition showed that ECMs, especially a single morphotype as *Lactarius subdulcis*, can play an important part in the improvement of tree health, especially concerning Ca and Mg uptake. Liming effects appeared to be site-specific depending on the soil fertility.

Résumé

Le chaulage est en un apport de Ca et Mg utilisé pour corriger les carences en cations des arbres dûes à l'acidité du sol. Les ectomycorrhizes (ECMs) apportent à l'arbre des nutriments d'origine organique et inorganique. Le fonctionnement de la communauté d'ECMs dépend de la disponibilité des éléments dans le sol, qui est modifiée par le chaulage. Les objectifs de cette étude sont donc de mesurer l'impact du chaulage sur la structure et le fonctionnement des communautés d'ECMs de hêtre et d'épicéa dépérissants, et de déterminer leur rôle dans l'amélioration de la nutrition minérale de l'arbre. Les résultats montrent que le chaulage est le deuxième facteur de structuration de la communauté des carpophores après l'arbre hôte. Le chaulage réduit l'abondance de mycorrhizes de morphotypes acidophiles au profit d'autres plus ubiquistes (*Clavulina cristata* sous épicéa et *Lactarius subdulcis* sous hêtre), confinées dans l'horizon organominéral et spécialisées dans la sécrétion potentielle de glucuronidase et de laccase, respectivement. Le chaulage augmente la sécrétion potentielle d'oxalate des ECMs sous hêtre, qui dépend en grande partie de l'activité des mycorrhizes de *Lactarius subdulcis*. Ce morphotype joue sans doute un rôle important dans l'altération des minéraux, d'autant plus que l'on observe une plus grande quantité d'éléments minéraux stockée dans ses ECMs. La comparaison qualitative des propriétés du sol, du fonctionnement des communautés d'ECMs, et de la nutrition des hêtres suggère que les ECMs, en particulier *Lactarius subdulcis*, jouent un rôle dans l'amélioration de la santé des arbres via l'assimilation de Ca et Mg, mais ces effets varient selon le type de sol.