Responses of Ectomycorrhizal Fungi to Mineral Substrates

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Abstract

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Boreal forest soils are complex, heterogeneous growth substrates where organic and mineral components provide nutrient resources for soil organisms and plants. Mineral nutrients are cycled between living and dead organic components of the forest soil and weathering of soil minerals provides an important input of new resources, compensating for losses from the ecosystem. Predicting soil responses to changing climate and management practices is important to determine their effect on forest production. Models for this purpose are largely based on the concept of the soil solution as the interface controlling soil processes such as weathering and nutrient uptake by plants, whereas soil microbiology recognises microbial processes as the driving force in soil nutrient cycling.

In boreal forests most tree root tips are colonised by ectomycorrhizal fungi. The mycelia of these symbiotic fungi mediate nutrient uptake by their tree hosts. These fungi are abundant in the organic layer of forest soils and ectomycorrhizal research has therefore largely focused on nutrient uptake from this horizon. Minerals in the soil may, however, also serve as nutrient resources for ectomycorrhizal fungi. Through combined chemical and physical processes fungi can affect nutrient availability by weathering minerals. This thesis describes a field experiment investigating the distribution of different ectomycorrhizal fungi in organic and mineral forest soil horizons, *in vitro* studies of fungal acidification of artificial substrates with different mineral element composition, microcosm studies of growth and carbon allocation in intact ectomycorrhizal systems colonising heterogeneous mineral substrates and a preliminary investigation of changes in surface micro-topography of minerals colonised by ectomycorrhizal hyphae. Half of the fungal species identified in the forest soil occurred exclusively in the mineral horizons. Mycelial growth, carbon allocation and substrate acidification by fungi colonising different mineral substrates *in vitro* and in microcosms appeared to be influenced by mineral element composition. Interpretation of possible modification of mineral surface micro-topography is more difficult but together the results obtained suggest that ectomycorrhizal fungi may contribute to the development of microenvironments on colonised mineral surfaces, where increased weathering can take place. Processes regulating nutrient availability in such microenvironments are different from those estimated from the bulk soil solution.

Keywords: autoradiography, calcite marble, *Hebeloma crustuliniforme*, *Piloderma fallax*, *Pinus sylvestris*, podzol, potassium feldspar, scanning electron microscopy, quartz

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Appendix

Papers I - IV

The thesis is based on the following papers, which will be referred to using bold Roman numerals.

I. Rosling A, Landeweert R, Lindahl BD, Larsson K-H, Kuyper TW, Taylor AFS & Finlay RD (2003) Vertical distribution of ectomycorrhizal fungal taxa in a podzol soil profile. New Phytologist 159, 775 - 783.

II. Rosling A, Lindahl BD & Finlay RD (0000) Carbon allocation to ectomycorrhizal roots and mycelium colonising different mineral substrates. Submitted to New Phytologist.

III. Rosling A, Lindahl BD, Taylor AFS & Finlay RD (2003) Mycelial growth and substrate acidification of ectomycorrhizal fungi in response to different minerals. FEMS Microbiology Ecology (In Press)

IV. Rosling A, Daniel G, Unestam T & Finlay RD (0000) Alteration of micro-topography of calcite marble surfaces as a result of ectomycorrhizal hyphal growth. Submitted to Canadian Journal of Microbiology.

I. List of figures, tables and boxes

II. Aim of the thesis

This thesis examines responses of ectomycorrhizal fungi to mineral substrates. The central hypothesis is that different ectomycorrhizal fungi are able to regulate their growth and activity in response to different mineral substrates, creating micro-environments of intense weathering on colonised mineral surfaces. Field and laboratory experiments have been conducted at different levels: from the field level distribution of ectomycorrhizal fungi in a podzol soil profile to the micrometer scale of individual fungal hyphae growing on mineral surfaces. The aims of the experiments were:

To examine systematically changes in ectomycorrhizal community composition on root tips in different horizons of a boreal forest podzol soil profile (**I**).

To investigate patterns of root and mycelial proliferation and carbon allocation in intact ectomycorrhizal systems colonising heterogeneous mineral substrates in microcosms (**II**).

To study species dependent responses of ectomycorrhizal fungi to different minerals in their growth substrates, by measuring mycelial growth and substrate acidification (**III**).

To study the effect of growing hyphae on mineral surface micro-topography (**IV**).

III. Abbreviations

bp – base pairs

CPM – counts per minute

ITS – internal transcribed spacer

PCR – polymerase chain reaction

rDNA – ribosomal DNA

RFLP – restriction fragment length polymorphism

SEM – scanning electron microscopy

SEM-EDS – element diffraction spectrometry

SEM-SE – secondary electrons

TCP – *tri*-calcium phosphate

T–RFLP – terminal restriction fragment length polymorphism

IV. Glossary of useful terms

Adsorption – Adhesion of substances to surfaces.
Anion –Ion with net negative charge.
Ascomycota - Largest group of fungi (*ca.* 45% of all known species). Spores are formed inside asci. Include yeast, many moulds, morels and truffles.
Basidiomycota – Second largest group of fungi (*ca.* 35% of all known species). Spores formed on basidia. Include species forming fruit-bodies like mushrooms, puffballs and toadstools.
Biominerals – Crystals precipitated as a result of complex formation between organic anions and metals ions, *e.g.* calcium oxalate crystals on fungal hyphae.
Cation – Ion with net positive charge.
Crystal – A particular pattern or arrangement of atoms that is continuously repeated in a three dimensional structure.
Etch-pits – Depressions in a mineral surface as a result of weathering.
Hypha – Tubular filament that is the structural growth unit of filamentous fungi.
Lithosphere – The non-living, non-organic part of the environment, such as rocks and the mineral fraction of soil in the Earth's crust.
Metal – Good conductors of heat and energy and can form cations.
Micro-topography – Surface topography at a microscopic.
Mineral – A naturally occurring homogeneous solid with a definite chemical composition and an ordered atomic arrangement. (Box 1)
Mineral nutrient – Elements that originates from minerals which organisms need as nutrients, *i.e.* P, Mg, K.
Morphotyping – Identification method for the fungal partner of ectomycorrhizal root tips. Based on examination of morphological and chemical characteristics.
Mycelium – Network of hyphae, the characteristic vegetative phase of many fungi.
Mycorrhiza – The symbiotic association between a fungi and a plant.
Mycorrhizosphere - The soil influenced by mycorrhizal roots and mycelia.
Nutrients – Substances that organisms need as a food source (*i.e.* N, P, Mg, K).
Parental material – The primary mineral composition of soil, from which the present minerals of the soil have derived during soil formation processes.
Pedogenesis – Soil formation.
Primary mineral – Rock-forming minerals in igneous or metamorphic rock. Formed at elevated temperature and pressure.
Re-crystallisation – Synthesis of new crystals.
Rhizomorphs – Differentiated hyphae aggregates, formed among basidiomycetes.
Rhizosphere – The soil influenced by roots.
Saprotroph – Organism that feeds on dead organic matter.
Secondary mineral – Mineral weathering products, such as clay.
Siderophore – Organic compound that form complexes with iron, released by plants, fungi and bacteria.
Sorption – Retention of material at surface by adsorption or absorption.
Substrate – Structural and nutritional matrix in which roots and mycelia grow.
Tectonic events – Movements of tectonic plates shaping the earths crust.
Translocation – Energy dependent process of moving nutrients from the site of absorption to other parts of fungal mycelium.

1. **Sammanfattning**

I begynnelsen bestod jorden av sten, vatten och en atmosfär utan syre. De mikrobiologiska livsformerna, som funnits på jorden i minst 3.5 miljarder år, skapade förutsättningarna för växt och djurrikets utveckling (Brock *et al.*, 1994). Vittring av sten och fotosyntesens omvandling av koldioxid till syre och organisktmaterial har skapat syrehaltig atmosfär och näringsrika jordar (Ehrlich, 1998; Sterflinger, 2000). Alla levande organismer behöver näringsämnen från mineraler för sin tillväxt och aktivitet. I de flesta ekosystem cirkuleras dessa mineralnäringsämnen mellan levande och döda biologiska komponenter i jorden (Barbour *et al.*, 1987). Vittring av sten tillför kontinuerligt mineralnäringsämnen till de levande organismerna och bevara därmed markens bördighet (White & Brantley, 1995).

Svampar är ett eget rike bland mikroorganismerna. Till skillnad från bakterierna har svamparna cellkärna och de bildar ofta flercelliga organismer. Genom att bilda svamptrådar (hyfer) som grenar sig och bildar nätverk (mycel) kan svamparna kolonisera t ex jord och ved. Svamparna delas in i funktionella grupper beroende på vilken kolkälla de huvudsakligen utnyttjar. Patogena svampar tar up kol genom att infektera levande växter och djur. Saprotrofiska svampar tar upp kol genom att bryta ned dött organiskt material. Mykorrhiza svampar får sitt kol genom att leva i symbios med rötterna hos levande växter (Fig. 2) (Jennings & Lysek, 1996). Ektomykorrhizasvampar lever i symbios med framför allt barrträd. Nästan alla fina rötter i skogsmarken är koloniserade av ektomykorrhizasvampar (Taylor, 2002) och deras artrikedom är hundratals gånger högre än hos de träd de lever i symbios med (Dahlberg *et al.*, 2000). Från ektomykorrhizarötterna växer svampens mycel ut i jorden. Mycelet tar upp näringsämnen och vatten som transporteras till trädet i utbyte mot att svampen får socker från trädets fotosyntes. Sockret använder svampen både som energi källa och substrat för att bygga upp nytt mycel och för att utsöndra organiska syror och enzymer som ökar mycelets näringsupptag från marken.

Flera olika sorters svampar kan vittra sten (Sterflinger, 2000) genom en kombination av fysiska och kemiska mikroprocesser (Banfield *et al.*, 1999). Sedan länge har det föreslagits att ektomykorrhizasvampar kan öka trädens upptag av mineralnäringsämnen genom att vittra sten (Cromack *et al.*, 1979; Jongmans *et al.*, 1997; Landeweert *et al.*, 2001). Den aktiva vittringens kvantitativa betydelse för skogens näringsupptag är dock omtvistad (Sverdrup *et al.*, 2002).

Målsättningen med denna avhandling är att öka förståelsen för ektomykorrhizasvampars aktiva vittring av sten i skogsmark. Hypotesen är att mycel kan skapa lokala mikromiljöer för intensiv vittring på mineralytor som koloniserats av mycel. Avhandlingen studerar ektomykorrhizasvamparnas roll i vittring från flera utgångspunkter och omfattar både en fältstudie och laborativa experimentella.

Många skogsjordar i Sverige är podzoliserade, vilket innebär att distinkta horisonter med olika mineralogiska och kemiska egenskaper har utvecklats (Fig. 1). I en sån profil varierar artsammansättningen av ektomykorrhizasvampar

mellan jordhorisonterna. Hälften av 22 dokumenterade arter förekom endast på rötter i mineraljorden och bland dessa återfinns tre nya arter (**Artikel I**). För att på ett riktigt sätt analysera artsammansättningen i marken är det, till skillnad från rådande praxis, nödvändigt att vid provtagning inkludera mineraljorden. I ett laboratorieexperiment växte vissa ektomykorrhizasvampar mer i mineraljord jämfört med standard substratet torv (**Artikel II**). Det mesta av det kol som svamparna fick från sitt värdträd, transporterades genom rötter och mycel till mineraljorden. Svampen växte även mer och transporterade mer kol till fläckar av kalifältspat-sand, jämfört med fläckar av kvarts-sand (**Artikel II**). Myceltillväxt orsakar lokal försurning av substratet, genom bland annat utsöndring av syror, vilket är en förutsättning för att svampen ska kunna orsaka vittring. Olika arter uppvisar olika mönster av försurning i förhållande till myceldensiteten när agarsubstratet är berikat med olika mineral-pulver (**Artikel III**). Efter att mycelet från en ektomykorrhizasvamp hade fått kolonisera en slipad marmoryta i fyra månader studerades ytan i elektronmikroskop. Hyferna som lyftes bort lämnade spår i ytan. Jämfört med resten av ytan var spåren jämnare och tycktes vara nedsänkta i ytan (**Artikel IV**). Dessa resultat tyder på att närvaron av hyfer har haft en direkt effekt på mineral strukturen i marmor ytan.

Avhandlingens slutsats är att mineralers sammansättning och struktur påverkar tillväxt och aktivitet hos vissa ektomykorrhizasvampar. Mycel som koloniserar mineraler kan kraftigt försura miljön i zonen mellan hyfer och mineralytor. Därigenom skapas en intensiv vittringsmiljö som i sin tur påverkar sammansättningen och strukturen hos mineralerna. Det kvarstår dock att kvantifiera ektomykorrhizasvamparnas vittringskapacitet och i vilka ekosystem som den är avgörande för skogsträdens näringsupptag.

2. Introduction

2.1. General introduction: Responses of fungi to the abiotic environment

Interactions between living and non-living material have determined Earth's development throughout evolutionary and geological history (Ehrlich, 1998; Sterflinger, 2000). Recent research suggests that life may even have emerged in close association with mineral surfaces. Natural pores in feldspars, with diameters of 0.4 - 0.6 μm, could have served as rudimentary cellular structure enabling the formation of the first self-replicating biomolecules by preventing dilution and providing protection from hydrolysis and UV radiation (Parsons *et al.*, 1998). Fossil evidence of microbial life exists from about 3.5 billion years ago. Microbial abundance and diversity appears to have increased dramatically approximately 1 billion years later when the development of oxygenic photosynthesis resulted in oxygen being accumulated in the atmosphere (Brock *et al.*, 1994). Vascular plants evolved relatively recently, approximately 400 million years ago, and colonised terrestrial ecosystems by associating with mycorrhizal fungi (Blackwell, 2000).

The harsh terrestrial conditions on the early Earth have been altered by biological activity into the buffered terrestrial soil systems we have today. Photosynthesis resulted in deposition of atmospheric carbon into the lithosphere, mainly through the formation of sedimentary limestone, and followed later by organic matter accumulation in soil, and the formation of peat, coal and petroleum (Richards, 1987). The development of land living plants increased transfer of carbon compounds from the air to the soil, dramatically increasing weathering of silicate minerals (Drever, 1994). Today, nutrients are accumulated in biological material in soils of many terrestrial ecosystems and maintained in organic form by being cycled between living and non-living organic components of the ecosystem (Barbour *et al.*, 1987). Mineral weathering, however, remains important to provide new inputs of mineral nutrients and maintain soil fertility in natural systems (White & Brantley, 1995).

Soil chemistry and calculations of weathering budgets are largely based on the concept of soil solution as the interface controlling soil processes such as weathering and nutrient uptake by plants (Sverdrup & Warfvinge, 1995). Soil microbiology on the other hand recognizes microbial processes as the driving force in soil nutrient cycling (Richards, 1987). Fungi are known as biogeochemical agents (Sterflinger, 2000) influencing weathering through physical and chemical processes (Banfield *et al.*, 1999). Direct weathering and nutrient uptake by ectomycorrhizal fungi colonising mineral particles has been suggested as a possible pathway for element uptake by forest trees (Landeweert *et al.*, 2001). However, the quantitative importance of fungal weathering in forest nutrition remains controversial (Sverdrup *et al.*, 2002).

This thesis takes an interdisciplinary approach to obtain a comprehensive understanding of the possible mineral weathering activities of ectomycorrhizal fungi in the boreal forest ecosystem. Field and laboratory experiments have been conducted to examine the conceptual idée that mycelial growth and activity of different ectomycorrhizal fungi respond to different mineral substrates, thereby creating micro-environments of intense weathering at colonised mineral surfaces. To support this idée the introduction provides an overview of factors, such as mineral structure, weathering conditions and mycelial activity in soil, influencing ectomycorrhizal fungi responses to different mineral substrates.

2.2. Weathering of primary minerals

Most rocks are built up of primary minerals that were formed at high temperature and pressure. Granites and gneisses make up the majority of Swedish bedrock (Magnusson *et al.*, 1963) and contain primary minerals such as quartz, feldspar, mica and apatite. Tectonic events and pressure from the inland ice cover, together with thermal cycles in rocks have resulted in the development of fissures. When water fills the fissures freezing and thawing cycles result in mechanical disintegration of the rock into smaller particles (Schulze, 1989). Primary minerals weather because they are not stable under the climatic conditions prevailing at the surface of Earth today (Banfield *et al.*, 1999). As primary minerals break down during weathering, cations and anions are released and weathering residuals recombine to form secondary minerals, such as clays and oxides, which are more stable under current environmental conditions. In particular, aluminium, iron and manganese form oxide, hydroxide or oxyhydroxide minerals that are stable in the soil environments (Schulze, 1989).

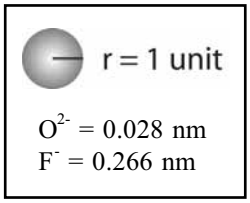
Weathering proceeds through simultaneous dissolution, transport and precipitation processes occurring when minerals are in contact with a solution, most commonly water. Both temperature and pH of the solution as well as the mineral particle size strongly influence the weathering rate (White & Brantley, 1995). The formation of secondary minerals is largely controlled by the chemical composition and structure of the primary mineral (Hochella & Banfield, 1995). When colonising mineral particles, bacteria and fungi may increase moisture retention at the surface, induce local acidification and take up elements, there by have a direct influence on the mineral surface chemistry. Microorganisms are thus important components in mineral weathering both in soil and in the above ground environments (Barker *et al.*, 1997).

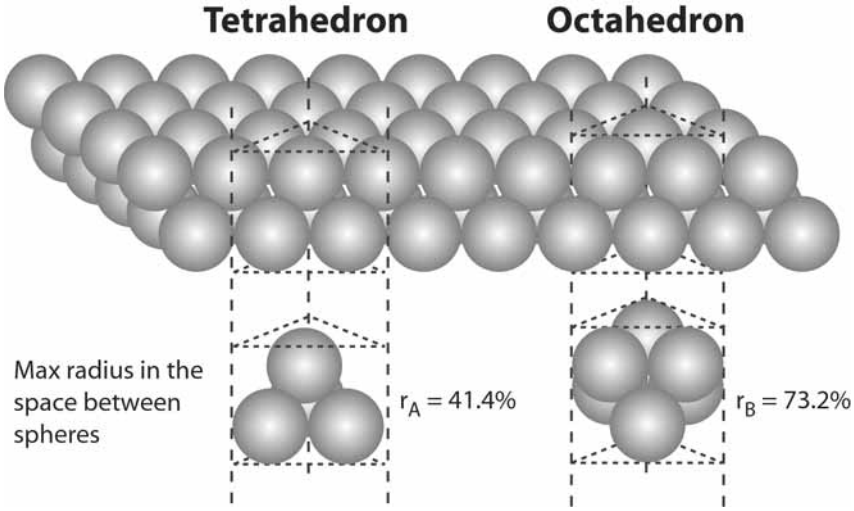
2.2.1. Structure and surface reactivity of primary minerals

Minerals are naturally occurring solids with a defined chemical composition and an ordered atomic arrangement. Both the chemical composition and the crystal structure are used to define a mineral (Schulze, 1989). Box 1 presents an overview of silicate mineral structure. Oxygen constitutes close to 90% of the volume in the Earth's crust and the closest packing of O^{2-} ions is the basis for most mineral structures. Spheres can either be packed as tetrahedron (4 spheres) or octahedron (6 spheres) structures with a central space in the middle of the

Box 1 – Closest packing of O^{2-} ions is the basis for most mineral structures, (Schulze, 1989).

Spheres build up minerals, predominantly O^{2-} but F^- also occur in phyllosilicate structures. When packing spheres as close together as possible empty spaces are created between them. Four spheres form a tetrahedron, with a space A inside, while six spheres form an octahedron with a space B inside.

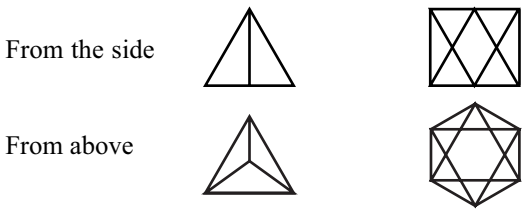




The ions that can fit in the A and B space

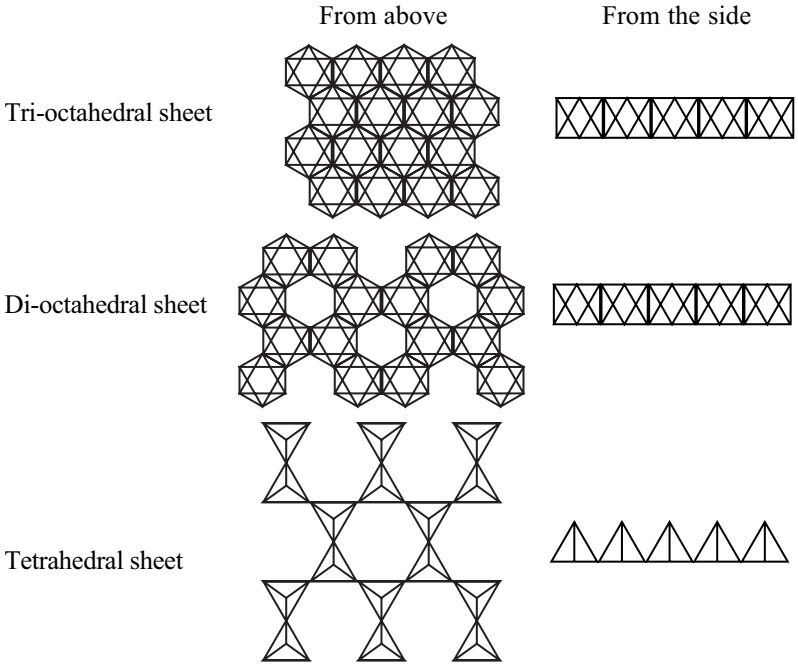
r = % of the O^{2-} r		
Si^{4+}	27.8	Si^{4+}
Al^{3+}	36.4	Al^{3+}
	45.7	Fe^{3+}
	47.1	Mg^{2+}
	48.6	Ti^{4+}
	52.9	Fe^{2+}
	57.1	Mn^{2+}
	69.3	Na^{+}
	70.7	Ca^{2+}

Polyhedral models used to visualise mineral structures

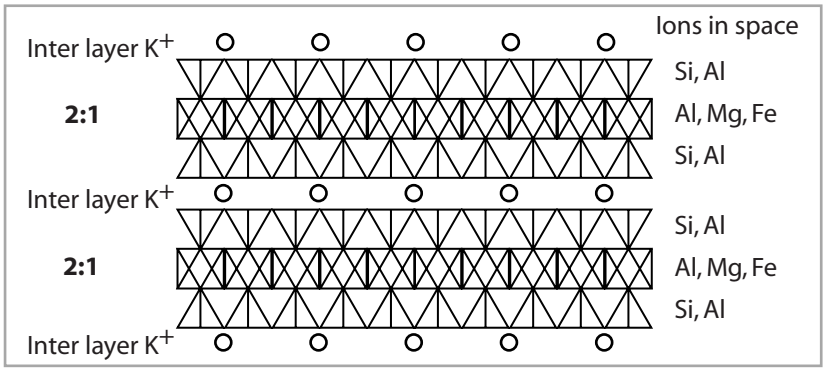


Box 1 continued

Minerals are built of tetrahedral and octahedral units. They will have different charge depending on what ions occupy their internal space. The charge determines the packing of units into sheets, with different degrees of sharing ions from the init structure. Tetrahedral or octahedral sheets are the structural backbone of phyllosilicates.



Phyllosilicates are minerals that are built up by different combinations of tetrahedral or octahedral sheets. Sheets are organised into layers of 1:1 with one tetrahedral sheet and one octahedral sheet or 2:1 with one tetrahedral on each side of an octahedral sheet. The mineral is built up of several layers and depending on the net charge of the layers the inter-space between them is balanced by ions or water. Mica is an example of a 2:1 phyllosilicate.



structure. To neutralising the charge cations fill these spaces and their size and charge determines whether they can occupy the smaller tetrahedron or the larger octahedron space. Almost all tetrahedron spaces are occupied by Si^{4+} cations, and silica is the second most common element in the earth's crust, contributing just over 2% of its volume. Aluminium cations can occupy both octahedral and tetrahedral spaces whereas other cations are located only in the octahedral voids (Krauskopf & Bird, 1995). Remaining charge in tetrahedral and octahedral structures is balanced by sharing oxygen ions, thereby creating charge-balanced networks or layers, which constitute the structural backbone of silicate minerals (Banfield & Hamers, 1997). Layered minerals, *i.e.* micas, are weather primarily through exchange of interlayer ions and are thus easier to weathering compared to network minerals that are structurally more stable (Schulze, 1989).

Processes at the mineral – solution interface determine weathering, the surface size and activity of mineral particles are therefore important factors determining weathering rates. Chemical and enzymatic reactions taking place on mineral surfaces are strongly affected by the element composition, charge and micro-topography of the mineral surface. The relationship between reactivity and shape alters the equilibrium and activation energy of chemical reactions in ways not accounted for by solution chemistry alone. The reactive surface area is the proportion of the total surface area that is involved in weathering or other processes (Hochella & Banfield, 1995). By assuming that particles are symmetrical spheres the surface area is estimated from the particle size. This ignores the structural and chemical heterogeneity of mineral surfaces and may explain observed non-linear relationships between weathering rates and estimated surface area (Brantley & Chen, 1995). For the purpose of estimates of weathering rates in the laboratory, reactive surface areas can be approximated by average surface area as determined by standard laboratory techniques of nitrogen adsorption (Lasaga, 1995). Mineral particles do not only have external surfaces, but internal pores with diameters of 2 - 50 nm also contribute to the total surface area of many silicate minerals (Brantley & Mellott, 2000). Pores in primary minerals may result from conditions during mineral formation. Crystallisation under wet conditions has been suggested to result in pore formation through fluid inclusions (Walker *et al.*, 1995). Feldspar crystals where the structure is disturbed by pores are more reactive in a weathering environment than non-disturbed crystals (Hochella & Banfield, 1995). Mineral particles may contain different kinds of mineral crystals that are packed together and the crystal boundaries are points of weakness in the particle. Weathering processes change the morphology of the mineral surface by grain edge rounding, widening existing pores and formation of etch-pits. This results in increased surface area but a large proportion of the new surface is unreactive, such as etch-pit walls (Walker *et al.*, 1995; Gautier *et al.*, 2001).

As a result of weathering, primary mineral particles in soil are coated in secondary minerals and the element composition of the surfaces may be significantly different from that of the bulk mineral. Further more, mineral surface charges are balanced by organic matter adsorption (Ullman et al., 1996). Laboratory studies of mineral weathering rates commonly analyse newly ground mineral particles. The dissolution rates of fresh mineral surfaces are much higher

than those of previously exposed surfaces. Fresh surfaces are rare in soil and this may partially explain the commonly observed discrepancy between laboratory and field observations of mineral dissolution rates (Hochella & Banfield, 1995).

2.2.2. Factors determining mineral weathering

The kinetics of element dissolution from a mineral to the solution depends on the specific structure and composition of the mineral (Casey & Ludwig, 1995). As a result of element dissolution the ionic concentrations in the solution will eventually reach a level of saturation. At that point precipitation of the elements into secondary minerals will remove ions from the solution. In reality these processes are simultaneously occurring and which of the processes that dominate under certain circumstances depends on the saturation state at the mineral – solution interface (White & Brantley, 1995). Looser crystal packing as a result of increased calcium and aluminium content dramatically increased the rate of feldspar dissolution compared to feldspar with lower content of the same ions (Ullman *et al.*, 1996).

Biological activities influence all steps of weathering, for example moisture retention at mineral surfaces, solution acidity and ion equilibrium, and ion complex formation through the release of organic polymers (Marschner, 1998). Laboratory estimates of both biotic and abiotic weathering reactions generally result in higher predicted rates than those detected in field studies. This large discrepancy is a combined result of laboratory experimental design and problems of bulk estimates in field observations. Laboratory systems largely fail to reproduce field conditions with respect to the circulation at the mineral – solution interface, the direct effect of microbes adhering to mineral surfaces, the complexity of interacting microbial communities and, possibly most importantly, the supply of unrealistically high concentrations of carbon and nutrients in laboratory experiments (Barker *et al.*, 1997). Bulk measurements of field soil solution composition fail to estimate local concentrations in the acidic extracellular mucilage at the microbe – mineral interface (Barker & Banfield, 1996). In this section, biogeochemical processes will be further discussed in relation to weathering.

The pH dependence of mineral dissolution varies for different minerals. Under acidic conditions the impact of pH on mineral dissolution results from the activity of hydrogen ions adsorbed to the mineral surface (Lasaga, 1995). It is not only the rate but also the mechanism of mineral dissolution that are altered with decreasing pH, as determined by element ratios in solution and residual material (Welch & Ullman, 1993). Element uptake via proton pumps results in decreased pH around metabolically active roots (Stryer, 1995). Similarly, low pH around hyphal tips has been demonstrated and suggested to be the result of the high activity in the growing tip (Jackson & Heath, 1993). Respiration by plant roots and soil microorganisms produces carbon dioxide, which dissolves in water and results in the production of carbonic acid. This also decreases the pH of the solution and thus contributes to weathering through proton attack (Chang, 1994). In addition to pH, the ion concentration balance at the mineral – solution interface significantly affects weathering rates. Preferential uptake of ions by roots and hyphae create

concentration gradients by depleting some and concentrating other ions (Marschner, 1998).

Weathering as a result of organic acid exudation by fungi and bacteria, was recognized early in studies of biogeochemical processes (*e.g.* Duff *et al.*, 1963; Henderson & Duff, 1963). In solution, low molecular weight organic acids dissociate to release protons and provide metal complex-forming organic anions (Gadd, 1999). Aluminium silicates are dissolved by a combination of proton and ligand attack, primarily at the aluminium sites on the mineral surface. This destabilizes the mineral structure and silica is released to the solution. In acidic solutions organic anions, such as oxalate, succinate and citrate, increase mineral dissolution more than anions of inorganic acids (Ullman *et al.*, 1996).

The ability to produce and exude low molecular weight organic acids is widespread in the fungal kingdom (Gadd, 1999). Oxalic acid is suggested to be the main organic acid exuded by mycorrhizal fungi (Lapeyrie *et al.*, 1991). Accumulation of calcium oxalate crystal on ectomycorrhizal hyphae in the field, indicate that oxalic acid is an important agent in biological weathering resulting increased phosphorus availability to plants (Graustein & Cromack, 1977). Field estimates of organic acid concentrations in the soil solution are however, generally too low to cause weathering of minerals, such as feldspar (Drever & Stillings, 1997). This is likely to be a result of current methods used to measure organic acids concentrations in field samples, which systematically underestimates the real concentrations by not taking into account possible large spatial variations and microbial control of the production and respiration of organic acids (Jones *et al.*, 2003).

Siderophores are organic polymers released by plants, fungi and bacteria in response to iron deficiency. Strong complexes are formed with Fe^{3+} and these are then taken up through specific transporters in the plasma membrane in some plants, fungi and bacteria (Shenker *et al.*, 1995; Marschner, 1998). High etching rates of amorphous and crystalline silicates were observed when these were colonised by the fungi *Penicillium notàtum* and *Aspergillus amstellodami*. The intense etching was suggested to be a result of the presence of siderophores in the cell walls of the fungi (Callot *et al.*, 1987). Strong complex formation with elements in the mineral structure, such as binding of siderophores to iron, reduces the stability of the mineral structure and thereby enhancing weathering (Ehrlich, 1998).

Weathering does not take place unless the mineral surface is in contact with a solution. This prerequisite may be fulfilled through the improved moisture retention in extracellular mucilage produced by fungi and bacteria (Hirsch *et al.*, 1995; Barker *et al.*, 1998). Many fungal hyphae are commonly extensively coated in rich extracellular mucilage enabling adhesion of the fungi to surfaces as well as to other hyphae (Jones, 1994). The mucilage consists of organic polymers, such as carbohydrates, proteins and lipids exuded by the fungi, but the composition varies between different fungi (Jones, 1994; Cooper *et al.*, 2000). Less variation is found in mucilage of bacteria and algae, where polysaccharides are the major component (Jones, 1994). Fungal mucilage has mainly been studied in the context of spore germination and hyphal adhesion of plant pathogens and wood decay fungi *e.g.*

(Chaubal *et al.*, 1991; Abu *et al.*, 1999). The biogeochemical importance of extracellular mucilage has however, been studied in bacteria, predominantly in aquatic biofilm systems (Little & Wagner, 1997). While strong attachment of some polymers to a mineral surface may inhibit dissolution, other polymers form complexes with components of the mineral surface, resulting in reduced stability and thus increased dissolution of the mineral (Ullman *et al.*, 1996). Polysaccharides can change the weathering rate of minerals by a factor of three, either enhancing or suppressing the process (Banfield *et al.*, 1999). The water-holding capacity of extracellular mucilage may be one of the major weathering effects resulting from microbial attachment to mineral surfaces (Barker *et al.*, 1998). Formation of biomineral, such as calcium oxalate, is commonly observed in fungal mucilage and has been suggested to be a method by which fungi regulate external calcium concentrations (Connolly *et al.*, 1999).

2.2.3. Soil formation, focusing on podzol soils

Quaternary deposits, such as clays and silt deposits, formed by sedimentation of particles in seas and lakes generally give rise to very fertile soils. These are mainly used for agriculture production. Boreal forest ecosystems are commonly restricted to poorer soils formed on more coarsely grained glacial deposits, such as tills. These soils are further discussed in this thesis. Soils are comprised of mineral grains, organic matter, water and air and are formed through accumulation of organic matter and by weathering of rock through exposure to climate and living organisms (Barbour *et al.*, 1987). The mineral composition of a soil depends on the parental material and the degree of weathering (McBride, 1989).

Podzol soils characteristically develop under boreal forests (Fig. 1). Acid foliage and slow decomposition rates in these ecosystems lead to the development of a surface layer of organic matter, where partial decomposition results in formation of high molecular weight organic acids such as fulvic acids, which percolate with rain-water through the soil. The underlying, upper mineral soil is weathered as soluble complexes are formed between the organic acids and ions of iron and aluminium, creating the eluvial E horizon. The organic matter-metal complexes have low charge and can percolate further through the profile. Metal ions continue to adhere to the complexes and these eventually become charged and precipitate below the E horizon, creating a characteristic rust coloured, illuvial B horizon overlying the C horizon parent material. Few burrowing animals thrive in these soils and mixing is thus limited, leading to the conservation of visible horizons in the soil profile (references within: Lundström *et al.*, 2000; van Breemen *et al.*, 2000b). This chemical model of podzolisation has been challenged by the biodegradation theory emphasising metal complex formation by low molecular weight organic acids that percolate through the profile until they are degraded by microorganisms (Lundström *et al.*, 2000a; Lundström *et al.*, 2000b).

Soil formation proceeds as elements are vertically translocated in the soil profile, downward through percolation and upwards through capillary rise (Barbour *et al.*, 1987). The involvement of fungi in this process has been suggested. Observed high concentrations of dissolved aluminium and iron in the organic horizon of podzol soils were explained as a result of translocation of elements

through the ectomycorrhizal mycelia from deeper mineral horizons to plant roots in the organic horizon. As a result of selective uptake by plant roots, elements that were not taken up, such as Al and Fe, would accumulate in the organic horizon (Lundström *et al.*, 2000b). In a base-poor forest ecosystem, ectomycorrhizal trees were suggested to take up apatite-derived calcium through mycelial translocation from apatite sources in the parental material (Blum *et al.*, 2002). There is however, no reason to consider ectomycorrhizal fungi as the only soil fungi involved in this process (Connolly *et al.*, 1999, and references there in). The potential for interaction between fungal hyphae and mineral surfaces is enormous. Both in the eluvial and the illuvial soil of a forest podzol total lengths of active fungal hyphae have been estimated to be 28 and 10 m g-1 soil, respectively (Söderström, 1979). In these soil horizons the surface area of mineral particles available for interaction with fungal hyphae is also high and these interactions are likely to have a significant influence on weathering and nutrient uptake.

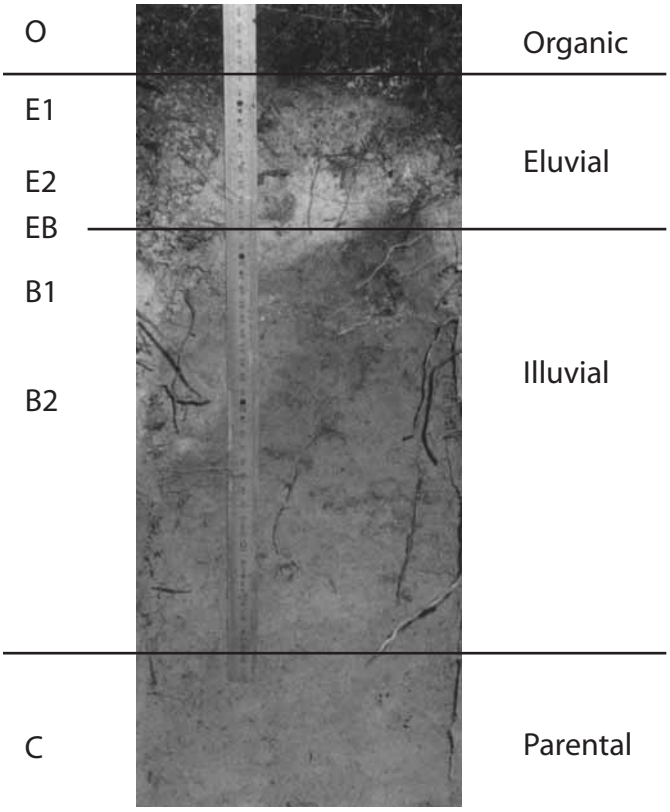


Fig. 1.
Podzol soil profiles are stratified into distinct horizons. Soil horizon abbreviations as used in **Paper I**, are given to the left in the figure. An organic horizon (O) has accumulated on top of the mineral soil. The upper mineral soil is bleached (Eluvial) and subdivided into E1 where organic matter is readily visible and E2 with less organic material. Below that, iron and aluminium have accumulated and an illuvial horizon has developed, strongly illuvial in B1 and less in B2. When the border between eluvial and illuvial soil was not distinct an additional horizon EB was defined. The parental material (C) is found at the bottom of the soil profile. Photo: Renske Landeweert.

2.3. Fungi as biogeochemical agent

Biogeochemical cycling is the exchange of mineral nutrient elements between the non-living and the living components of the ecosystem. Uptake of mineral nutrient elements is essential for the growth of all living organisms and their availability in soil is greatly increased by plant and microbial activities affecting geochemical processes (Richards, 1987). Biological weathering is not exclusively a directed action to obtain mineral nutrient elements; even CO_2 produced during respiration may dissolve in water and induce mineral dissolution through the formation of carbonic acid (Chang, 1994). Biogeochemical weathering has wide implications for both basic researches in areas such as pedogenesis and applied areas such as mobility of environmental contaminants, storage of atomic waste and stability of construction works (Barker *et al.*, 1997). At the beginning of the last century, it was first suggested that microbes were involved in the "decay" of stones *e.g.* (Paine *et al.*, 1933). Because of their ubiquitous occurrence in all habitats, bacteria have been the focus of most studies on the importance of microorganisms in geological processes (Ehrlich, 1998). A significant body of literature on fungi as geological agents is however also available, comprehensively reviewed by Sterflinger (2000).

The capacity of lichens to physically weather rock surfaces is the most striking of biological weathering processes and is relatively well studied (Barker *et al.*, 1997). Lichens growing on sandstone can actively weather their substrate, both at the colonised surface and inside the mineral through penetration. Moisture and temperature are important variables controlling biological activity in rock substrates (Wessels & Wessels, 1995). In the cold and dry environment of the Ross Desert in Antarctica, lichens living inside rock induce weathering by producing oxalic acid, thereby affecting nutrient availability as well as the structural stability and moisture retention of the habitat (Johnston & Vestal, 1993). Fungal hyphae of crust forming lichens can penetrate 10 mm down into the rock surface by exploiting mineral grain boundaries and micro-fissures in the rock (Barker & Banfield, 1996). The structure and element composition of the biotic and abiotic components in intact lichen – rock aggregates was examined in cross-sections using different electron microscopy techniques (Ascaso *et al.*, 1998). Disintegration of the stone structure as a result of the microbial colonisation was demonstrated and calcium was found to migrate from the mineral to accumulate in cell wall structures. The weathering capacity of lichens inhabiting calcareous rock can largely be assigned to the activity of the fungal partner of the association (Ascaso *et al.*, 1998). Structure and distribution of secondary minerals formed during biological weathering by rock inhabiting lichens, has been found to be less determined by the primary mineral structure, compared to secondary minerals formed during weathering which does not involve a biological component (Banfield *et al.*, 1999). These results demonstrate that weathering conditions in biologically mediated micro-environments may be dramatically different from those determining weathering under abiotic conditions.

2.3.1. A short introduction to fungi

Fungi are eukaryotic organisms and form a kingdom separate from plants and animals. The familiar "mushrooms" are fruitbodies of species within the major phyla ascomycota and basidiomycota. Together these comprise about 80% of the all described fungal species and are either single celled organisms growing as yeast, or multi-cellular organisms growing as filamentous hyphae (Berbee & Taylor, 1999). When using the term fungi, this thesis refers to fungal species within the ascomycota and basidiomycota. Trophic groups of fungi are based on what their major carbon source is. Pathogenic fungi – infecting living plants and animals, saprotrophic fungi – degrading dead organic material and mycorrhizal fungi – forming symbiotic associations with living plant roots (Jennings & Lysek, 1996). Metabolic activities of many fungi contribute to the process of soil formation and maintenance of soil fertility. Examples of such activities are degradation of plant debris and transport of nutrients and water in the soil system. Fungi by no means act alone in these processes, but exist in close association with other soil organisms such as bacteria, nematodes and other components of the soil fauna (Richards, 1987).

The hyphal growth mode of filamentous fungi is well adapted to explore and exploit nutrient sources in the highly heterogeneous soil environment (Robson, 1999). Hyphae exhibit turgor-driven polarised growth, with expansion restricted to the tip. As the hyphae expand into un-colonised substrates branches are produced in order to ensure efficient colonisation of the substrate. Behind the growing tip the hyphal wall is rigid and resistant to internal turgor pressure. As resources in the mycelial centre are exhausted, growth is restricted to the mycelial front and the mycelium may become differentiated (Robson, 1999). Hyphae can be hydrophobic and the degree of hydrophobicity can vary within a single mycelium depending on the stage of differentiation. The hyphal tip commonly being the most hydrophilic, enabling uptake of inorganic and organic nutrients from the soil (Unestam, 1991). The hyphal tip is both a site of intense growth, as well as a site of nutrient acquisition from the substrate, and thus consumes a large proportion of the mycelial resources (Unestam & Sun, 1995).

In many basidiomycetes, hyphal aggregates, called rhizomorphs, are formed behind the mycelial front. In these, elonged vessels may develop, surrounded by closely packed hydrophobic hyphae. Rhizomorphs are units of spread, survival and long-distance translocation of nutrients and water in the mycelia (Jennings & Lysek, 1996). By applying concurrent explorative and exploitative growth strategies the mycelia of rhizomorph-forming basidiomycetes grow and differentiate in response to the spatial distribution of nutrient and moisture resources within the growth substrate (Ritz & Crawford, 1990). A fungal mycelium acts as a single, interconnected functional unit, translocating resources within the network of hyphae and rhizomorphs. The ability of mycelia to connect different mineral and carbon sources enables translocation of heterogeneously distributed nutrients and moisture through the mycelia. This makes resource utilisation more effective and increases stress tolerance in filamentous fungi compared to single celled organisms (Hirsch *et al.*, 1995).

2.3.2. Minerals as fungal habitats

Terrestrial rock surfaces are either exposed to the atmosphere, *i.e.* sub-aerial, or covered by soil, *i.e.* subterranean. Both below and above ground, mineral surfaces provide support, structure and protection for bacteria and fungi. Microorganisms condition their habitats, and the structure and mineralogy of mineral surfaces to which they attach are altered by both physical force and chemical processes (Gorbushina *et al.*, 1993; Barker & Banfield, 1996; Barker *et al.*, 1998).

A large part of the literature on fungal activity in structural minerals derives from studies of sub-aerial bio-deterioration of stone houses and constructions (Dornieden *et al.*, 2000; Sterflinger, 2000). To survive under the harsh environmental conditions existing on bare rock surfaces, organisms need effective protection against radiation and desiccation. This is achieved through the production of protective surface layers, composed of different extracellular products, such as compact layers of mucilage, extracellular metabolic products, pigments and biominerals, *i.e.* calcium oxalate crystals. Many free-living and symbiotic ascomycetes, such as lichens, are highly stress tolerant in this respect and subsequently dominate these environments (Sterflinger, 2000; Gorbushina *et al.*, 2003).

Fungi are also abundant in the less hostile subterranean system, where their biogeochemical activity may be similar to that in sub-aerial systems. Extensive mycelia may connect different substrates and mineral substrates are possible niches for soil living fungi as long as carbon can be obtained from elsewhere. Compared to bacteria and algae the mycelial growth mode of fungi is an advantage when acting as biological weathering agents. Biological weathering by a number of mould fungi has been demonstrated and constitute the first steps in the weathering of basaltic rock in cold environments (Etienne & Dupont, 2002). Vertical translocation of elements from mineral soil to organic top layers by saprotrophic (Connolly *et al.*, 1999) and mycorrhizal (van Breemen *et al.*, 2000b) mycelia has been suggested to play a major role in forest nutrient cycling and soil formation. The weathering activity of ectomycorrhizal fungi will be further discussed below in Section 2.4.5.

2.3.3. Regulation of organic acid production in fungi

Wood preservation using toxic metals to prevent fungal decomposition and the interest in possible bioremediation of metal-contaminated soils has stimulated much research in the field of metal tolerance in plants and fungi. In soil systems, increased exudation of organic acids, by plants, fungi and bacteria, has been demonstrated in response to high concentrations of toxic metals, such as aluminium (Gadd, 1993; Ma *et al.*, 1997; Hamel *et al.*, 1999). Phosphorus deficiency in plants is associated with changes in carbon metabolism, and, among other reactions, the production and exudation of organic acids are increased (Ryan *et al.*, 2001). Phosphorus deficiency has been suggested to induce similar responses in fungi. Dissolution of insoluble phosphates has been demonstrated in association with release of oxalic acid by ectomycorrhizal fungal mycelia (Lapeyrie *et al.*, 1991). In experiments by Paris *et al.* (1996) magnesium and potassium deficiency significantly increased oxalic acid exudation in the external mycelium

of *Paxillus involutus* (Batsch.: Fr) Fr. and *Pisolithus tinctorius* (Pers.) Coker & Couch compared to non-deficient conditions. Oxalic acid production in *P. tinctorius* was increased regardless of the nitrogen source supplied, whereas *P. involutus* increased oxalic acid production when nitrogen was supplied as NH^{4+} but not when the N source was NO^{3-} (Paris *et al.*, 1996). Wallander & Wickman (1999) examined the production of organic acids in response to potassium deficiency using pine seedlings that were non-mycorrhizal or colonised by either *Suillus variegatus* (Sw.: Fr.) O. Kuntze or *P. involutus*. Plants receiving potassium either from biotite or from microcline were compared to controls receiving no potassium. The production of malic, oxalic and citric acids was only significantly greater than in the non-mycorrhizal controls when potassium was supplied as biotite, and only in systems with *S. variegatus* (Wallander & Wickman, 1999). Element deficiency does not always induce increased organic acid in fungi and deficiency responses may also be difficult to separate from general stress responses.

Fungal biomass has a high capacity for sorption of metals. The capacity varies between species and strains and is a result of physico – chemical properties of the cell wall constituents. Mycelial pigmentation, for example melanins in the cell wall, strongly increases biosorption compared to non-pigmented mycelia (Fomina & Gadd, 2003). Heavy metal tolerance of plants is often increased when they are colonised by ectomycorrhizal fungi. It has been suggested that this is a result of the high metal retention capacity of the fungal mycelium and fungal production of complex forming organic compounds (Marschner, 1998). In pot experiments, the growth and nutrient uptake of pine seedlings was examined when the systems were exposed to different levels of nickel and cadmium. Seedlings colonised by *Laccaria bicolor* (Maire) Orton grew significantly better in all metal treatments compared to non-mycorrhizal seedlings (Ahonen-Jonnarth & Finlay, 2001). In a number of experiments on responses to elevated aluminium concentrations, oxalic acid production was commonly increased in pine seedlings colonised by ectomycorrhizal fungi such as *Suillus bovinus* (L.: Fr.) Roussel, *Rhizopogon roseolus* (Corda) Th. M. Fr. and *P. involutus*, compared to non-mycorrhizal control seedlings (Ahonen-Jonnarth *et al.*, 2000).

Calcium gradients are involved in maintaining hyphal polarity and controlling apex growth (Robson, 1999). To maintain hyphal polarity, internal calcium concentrations are highly regulated by pumping calcium out of the cytoplasm, either into vacuoles or through the cell membrane into the external environment. High concentrations of calcium may induce stress in fungi and can be alleviated by precipitation of calcium oxalate outside the cells (Jackson & Heath, 1993). In **Paper III**, the restricted mycelial growth and high substrate acidification, observed for *Cortinarius glaucopus* (Sch.: Fr.) Fr. grown on plates enriched with *tri*-calcium phosphate (TCP) could be the result of calcium stress.

The production and exudation of oxalic acid may fulfil a range of different functions in the physiology and ecology in different groups of fungi. For instance the form and availability of carbon and nitrogen sources influence the production of oxalic acid (Dutton & Evans, 1996). With more oxalate generally being produced when nitrogen is supplied as nitrate, compared to ammonium (Gharieb *et*

al., 1998; Gadd, 1999). This pattern, however, varies for different fungi (Casarin *et al.*, 2003). Increased production has been found in response to excess carbon compared to other elements in the fungal growth substrate (Gadd, 1999) and suggested to be a result of incomplete oxidation of sugars (Richards, 1987). *Rhizoctonia solani* Kühn was used to analyse fungal carbon requirements for producing and exuding organic acid, indicated by dissolution of TCP in the media. Unless the glucose concentration was 2% w/v in a source available to the fungus, *R. solani* did not dissolve TCP in sink part of the mycelium (Jacobs *et al.*, 2002). Oxalic acid production is involved in the degradation of plant-derived organic matter by saprotrophic fungi *e.g.* (Connolly & Jellison, 1995; Palfreyman *et al.*, 1996; Ruijter *et al.*, 1999) and in the plant colonising activity of pathogenic fungi (Dutton & Evans, 1996; Clausen *et al.*, 2000). Through ion complex formation, exuded oxalic acid results in the formation of biominerals on the surface of many fungal hyphae. It has been suggested that this may protect hyphae from dehydration, as well as providing a physical barrier against grazing micro fauna (Arocena *et al.* 2001, and references there in). Although organic acids, such as oxalic acid, play an important role in biogeochemical weathering, the many underlying mechanisms for its production in fungi are not fully characterised.

2.4. Ectomycorrhizal fungi in the boreal forest ecosystem

Most of the terrestrial Northern hemisphere is covered by the boreal forest biome in which the above ground plant species diversity is relatively low and dominated by coniferous trees (Barbour *et al.*, 1987). In Swedish boreal forest the overstorey consists mainly of Norway spruce (*Picea abies* [L.] Karst.) and Scots pine (*Pinus sylvestris* L.) (Söderström, 1971). Establishment, health, survival and decomposition of forest trees are largely dependent on the activity of fungi, which constitute a large component of the active biomass in boreal forest ecosystem.

2.4.1. Ectomycorrhizal symbiosis

Mycorrhiza is the mutualistic, symbiotic association between soil fungi and plant roots (Smith & Read, 1997). Ectomycorrhizal association is the predominant form of mycorrhiza in boreal forest trees. Other kinds of mycorrhiza, such as ericoid- and endomycorrhiza, exist in the boreal forest ecosystem but will not be considered further in this thesis. Upon ectomycorrhizal colonisation a fungal sheath called the mantle covers the short roots of the host tree (Fig. 2a). To maximise the contact between the plant and the fungi, hyphae colonise the intercellular space between cortical root cells, forming the Hartig net (Fig. 2b). From the mantle, hyphae extend out into the surrounding substrate (Fig 2a). The mycorrhizal short root is the functional unit of the symbiosis where exchange of nutrients, carbon and water between the symbiotic partners take place (Smith & Read, 1997). Saprotrophic and mycorrhizal fungi are not separate groups from an evolutionary perspective, indicating that the ability of fungi to form symbiotic associations with plants is a life strategy that has appeared from ancestral saprotrophic life strategies several times during evolutionary history (Hibbet *et al.*, 1997). The mycorrhizal strategy to derive carbon from its living host releases them

from competition with other soil fungi for deriving carbon from sources of dead organic material.

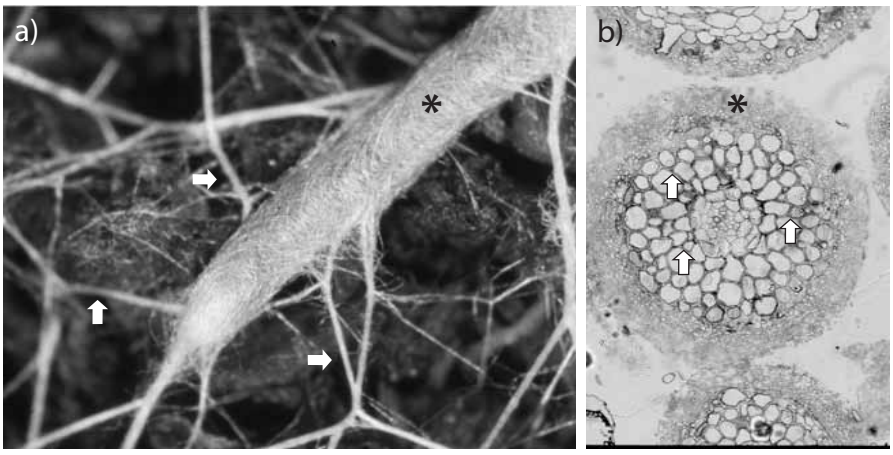


Fig. 2.
Colonisation of root tips by ectomycorrhizal fungi results in the formation of the ectomycorrhizal short root. The ectomycorrhizal short root is the functional unit of the ectomycorrhizal symbiosis where nutrients, carbon and water are exchanged between the symbiotic partners. a) The ectomycorrhizal short root is covered by hyphae, forming the mantle (*). Extramatrical hyphae extend out from the mantle into the surrounding substrate (arrows). b) A cross section of an ectomycorrhizal root tip. The mantle (*) surrounds the root and fungal hyphae colonise the space between the epidermal and cortical root cells (arrows). Photo: Andy F.S. Taylor

The identification of ectomycorrhizal fungal species on short roots is a difficult task. The accuracy of identification has been greatly improved through the development of molecular techniques and the use of DNA sequence databases (Horton & Bruns, 2001). High throughput surveys, however, remain largely dependent on initial grouping of roots based on morphological characteristics, *i.e.* morphotyping. Identification by morphotyping depends largely on personal experience and skill and rough morphotyping and vaguely defined fungal groups often prevent meaningful comparisons between different studies. Using a combination of morphotypic and molecular identification techniques is, at present, the most reliable approach with which to study ectomycorrhizal community composition. More recent studies approaching the issue of the species distribution in mycorrhizal mycelia, rather than roots, within different soil compartments have used molecular methods to identify fungal DNA in soil (Landeweert *et al.*, 2003). The exponentially growing size of public DNA sequence databases increases the chances of identifying unknown samples. New problems however arise as taxonomically misidentified sequences accumulate in these databases. Improved taxonomic identification and critical analysis of obtained sequence homologies are necessary when using public databases for the purpose of species identification (see Vilgalys, 2003).

2.4.2. Ectomycorrhizal fungal communities in soil

In contrast to host plants, the fungal diversity is high in boreal forests. In Sweden, more than one thousand ectomycorrhizal fungal species are known (Dahlberg *et*

al., 2000). In soil, the majority of fine tree root, commonly more than 95%, are colonised by ectomycorrhizal fungi (Taylor, 2002). Individual mycelia of ectomycorrhizal fungi may become large over time with genets of *S. bovinus* extending at least 17.5 m in forest stands older than 70 years (Dahlberg & Stenlid, 1994) and to a depth of at least 20 cm (Zhou & Hogetsu, 2002). Studies of fungal distribution in soil thus have to take into account factors of spatial and temporal variation, as well as the size of individuals when designing sampling strategies (Taylor, 2002). In boreal forest soils, the highest fine root density is found in the organic and upper mineral soil horizons (Persson, 1980; Sylvia & Jarstfer, 1997; Makkonen & Helmisaari, 1998). Tree roots are, however, found at greater depths (Jackson *et al.*, 1996). Most studies of ectomycorrhizal fungal communities have restricted sampling to the upper, organic part of the soil profile (Horton & Bruns, 2001) thereby ignoring the ectomycorrhizal community in deeper mineral soils.

Only a few studies have examined the vertical distribution of ectomycorrhizal fungi in soils, comparing the community composition in different soil horizons. Earlier studies have used morphologically defined ectomycorrhizal taxa in organic and mineral soil either directly in soil samples (Egli, 1981; Goodman & Trofymow, 1998; Fransson *et al.*, 2000) or on bait seedlings in organic and mineral substrates (Danielsson & Visser, 1989; Heinonsalo *et al.*, 2001). Their results suggest that there may be large differences in species composition between the organic layer and the mineral soil. A resent study used combined morphotyping and sequencing of the internal transcribed spacer in the ribosomal DNA (ITS rDNA) to examine the fine scale distribution of ectomycorrhizal fungi in different components of the forest floor, including coarse woody debris and E horizon mineral soil (Tedersoo *et al.*, 2003). The study demonstrated that the ectomycorrhizal community at the site was highly variable at a 5 cm scale and ascomycetes, including Helotiales sp., dominated the community in mineral soil. Multiple factors are involved in determining the ectomycorrhizal community composition in soil and the functional implications of the high diversity and variation remain largely unknown (Dahlberg, 2001). Micro-scale analysis of the spatial distribution of ectomycorrhizal species in combination with analysis of micro-spatial soil characteristics may, in the future, reveal functional variation in the ectomycorrhizal community.

Whereas the mycorrhizal root tip is the functional unit of the mycorrhizal association, the hyphal tips remain the functional units of fungal interaction with the soil through exudation and uptake. Mycelial distribution is thus interesting when examining possible functional connections between ectomycorrhizal fungal species and local conditions in the soil. Including both mycorrhizal and saprotrophic fungi, hyphal density is higher in the organic soil (16 500 m g^{-1}) compared to eluvial (650 m g^{-1}) and illuvial (390 m g^{-1}) horizons of podzol soils. The proportion of active hyphal length relative the total hyphal length, may however be higher (4.3%) in eluvial soil compared to organic (2.4%) and illuvial (2.6%) soil (Söderström, 1979). The difficulty of quantifying the mycorrhizal and saprotrophic components separately has been a major problem in the interpretation of field data. The development of molecular identification techniques has however made identification of species possible from mycelial colonising complex substrates. Identification of ectomycorrhizal fungi through terminal restriction

fragment length polymorphism (T–RFLP) analysis of DNA extracted from soil mycelium, has been used to demonstrate differences in ectomycorrhizal species composition between different components of the forest floor (the litter, fermentation and humus layers) and the B horizon of the mineral soil in a North American *Pinus resinosa* Ait. stand (Dickie *et al.*, 2002). In a podzol profile studied in this thesis, the ectomycorrhizal community composition was shown to change depending on the soil horizon, both with regard to fungi colonising ectomycorrhizal root tips (**Paper I**) and fungi in extramatrical mycelium (Landeweert *et al.*, 2003). However, when using T–RFLP for three-dimensional mapping of the distribution of ectomycorrhizal root tips in a Japanese *Larix kaempferi* (Lindl.) Carrière stand (Zhou & Hogetsu, 2002), no clear vertical distribution patterns were found.

2.4.3. Carbon allocation and nutrient translocation in mycelia of ectomycorrhizal fungi

Carbon resources in the forest ecosystem originate from the photosynthetic activity of plants. The synthesised carbon compounds are allocated to growth, respiration and exudation in the plant (Marschner, 1998). A substantial proportion, 20 - 25%, of the photosynthates allocated to tree roots is required for the growth and maintenance of the mycorrhizal fungi (Smith & Read, 1997). Current photosynthate is allocated mainly to sites of active growth (Erland *et al.*, 1990). Together, roots, mycorrhizal fungi and the associated microbial community respire a large part of the carbon derived from photosynthesis in trees, accounting for approximately half of the soil respiration in a boreal pine forest in the north of Sweden (Högberg *et al.*, 2001).

Mycelial proliferation and the formation of mycelial patches in ectomycorrhizal systems can be induced by a heterogeneous substrate, as demonstrated by introduction of leaf litter in the peat substrate of a Larix seedling colonised by *Boletinus cavipes* (Klotzch ex Fr.) Kalchbr. (Read, 1991). Newly formed mycelial patches of *S. bovinus*, extending from a colonised *P. sylvestris* seedling, were strong sinks of host-derived carbon (Bending & Read, 1995a). Within 48 h, close to 60% of the host derived carbon allocated to mycorrhizal mycelia of *P. involutus* colonising *P. sylvestris* seedlings was allocated to mycelial patches proliferating in discrete sources of organic matter (Leake *et al.*, 2001).

As a result of intense mycelial colonisation of patches of organic matter, both *S. bovinus* and *Thelephora terrestris* Ehrh.: Fr. decreased the relative content of nitrogen and potassium in the patches. *S. bovinus* also decreased the phosphorus content of organic patches. Reduction in nitrogen and potassium content was most pronounced in patches colonised by *S. bovinus*, which also exhibited more intense mycelial proliferation compared to *T. terrestris* (Bending & Read, 1995b). In *Betula pendula* Roth seedlings colonised by *P. involutus*, mycelial exploitation of beech, birch and pine litter for 90 days, led to a significant decrease in the phosphorus content of all litter types and to enhanced growth of the seedling compared to non litter controls (Perez-Moreno & Read, 2000).

Fungi transport external nutrients across the plasma membrane using facilitated diffusion, active transport or ion channels (Robson, 1999). Phosphorus is

concentrated in vacuoles by simultaneous balancing of charge differences with cations such as magnesium (Mg^{2+}) and particularly potassium (K^{+}) (Bücking & Heyser, 1999). Phosphorus-rich vacuoles can be transported via a motile tubular vacuole system (Ashford & Allaway, 2002) that could theoretically enable the long-distance transport seen in extensive mycelial systems (Finlay & Read, 1986). Long-distance translocation of carbon compounds towards growing hyphal tips in fungal mycelia has largely been assigned to pressure-driven bulk flow in the hyphae, from sites of nutrient and water uptake to areas of high nutrient demand (Jennings & Lysek, 1996). Mycelial carbon translocation as a prerequisite for substrate acidification were observed in laboratory systems by growing *R. solani* from the centre of a model system of rings of discrete agar droplets in a plastic dish. Jacobs *et al.* (2002) were able to demonstrate that translocation of glucose from one ring of droplets enabled dissolution of TCP in both interior and exterior rings of droplets.

2.4.4. Biotic interactions of hyphae

Bacterial communities in soil are influenced by a number of factors, such as moisture, nutrients and carbon availability. Release of photosynthetically derived carbon and input of organic matter by roots and mycorrhizal hyphae alters the local soil environment to allow microbial proliferation. The soil – root interface, the rhizosphere (*e.g.* Marschner, 1995), and the soil – hyphal interface, the mycorrhizosphere (Rambelli, 1973), commonly differ from the bulk soil with higher carbon availability, lower pH, changed redox potential and increased concentrations of low molecular weight organic compounds (Marschner, 1998). Microbial activity may further be stimulated by stabilised moisture conditions in the mycorrhizosphere compared to the bulk soil. Under drought conditions, nocturnal water transfer from deep host roots to external mycorrhizal mycelia has been demonstrated (Querejeta *et al.*, 2003) and drops are commonly observed on aerial hydrophobic hyphae of ectomycorrhizal fungi (Unestam & Sun, 1995). In studies of mycorrhizal fungi inoculated with different bacteria, the mycorrhizosphere environment has been reported to have both synergistic and antagonistic effects on microbial activity (Leyval & Berthelin, 1989).

Combined biotic and abiotic factors are involved in the regulation of bacterial activity, as demonstrated by Olsson & Wallander (1998). Growing bait seedlings of *P. sylvestris* in either boreal forest humus or illuvial mineral soil Heinonsalo *et al.* (2001) demonstrated that the numbers of bacteria increased from the bulk soil through intermediate values in the rhizosphere soil to reach the highest numbers in the mycorrhizosphere soil. This was true for both soil types, with average numbers of bacteria being higher in the organic soil compared to the mineral soil. In experimental systems ectomycorrhizal seedlings of *Pinus contorta* Douglas ex Loudon were grown in a mixture of sandy soil and pure quartz sand, all ectomycorrhizal fungi tested were demonstrated to decrease the bacterial activity, measured as thymidine incorporation, by 20 - 50% compared to non-mycorrhizal controls (Olsson *et al.*, 1996). When the substrate consisted of a mixture of peat and sand amended with the mineral microcline bacterial activity was reduced as a result of mycelial colonisation of the substrate, for both *P. involutus* and *S. variegatus* colonising pine seedlings compared to non-mycorrhizal controls.

When instead amending the growth substrate with either biotite or apatite both bacterial number and activity was increased in the presence of *S. bovinus* (Olsson & Wallander, 1998). These somewhat contradicting results demonstrate that there is no general correlation between mycelial colonisation by ectomycorrhizal fungi and bacterial activity in a substrate. Bacterial activity is probably influenced by combined biotic and abiotic factors.

In soil, mycelia of both mycorrhizal and saprotrophic fungi interact and compete for substrate. In microcosms where the mycelial vigour of *S. bovinus* is decreased as a result of the presence of the wood decomposing fungus *Phanerochaete velutina* (DC.: Fr.) P. Karts., less carbon was allocated to the mycelium and allocation was slower than in non-disturbed mycelia (Leake *et al.*, 2001). The carbon pools available to the different mycelia largely determine the outcome of competitive interactions. As one mycelium achieves competitive superiority over another, it may utilize the other mycelium as a source of nutrients (Lindahl *et al.*, 2001).

2.4.5. The role of ectomycorrhizal fungi in weathering

There are several reasons for considering ectomycorrhizal involvement in mineral weathering processes in forest soils. Ectomycorrhizal mycelia are the major organs for nutrient uptake to forest trees in the boreal ecosystem. The mycelial colonisation of the soil substrate increases the absorptive surface area of the root system, but through enzymatic activity the fungi also have qualitative effects on nutrient availability (Smith & Read, 1997). Högberg *et al.* (2001) demonstrated a close coupling between current photosynthesis allocated to soil, the production of ectomycorrhizal fruit-bodies and soil respiration. A considerable fraction of the carbon fixed by photosynthesis in the boreal forests is allocated to ectomycorrhizal mycelia (Finlay & Söderström, 1992; Leake *et al.*, 2001) and is ultimately deposited in the soil. In forest soils, some ectomycorrhizal fungi, such as *Hysterangium crassum* [Tul. & Tul.] Fischer, *Hysterangium setchellii* (Fischer) and *Gautieria monticola* (Harkness), are known to form dense mycelial mats, strongly affecting the nutrient availability and weathering rate of the colonised mineral (Graustein & Cromack, 1977; Cromack *et al.*, 1979; Entry *et al.*, 1991; Griffiths *et al.*, 1994). On the basis of calcium – strontium ratios in soil water, minerals in the soil and different mycorrhizal and non-mycorrhizal trees, Blum *et al.* (2002) concluded that direct calcium uptake by ectomycorrhizal fungi weathering apatite in the parental material, could compensate calcium loss in base-poor ecosystems. Data on element ratios should, however, be interpreted with care, because of high variation of calcium – strontium ratios in different plant tissues and limited understanding of the cycling of these elements in plants (Watmough & Dillon, 2003).

In the soil, accumulation of calcium oxalate crystals has been documented in mycorrhizal mats of *H. crassum* (Graustein & Cromack, 1977; Cromack *et al.*, 1979) and in the mycorrhizosphere of *Piloderma* sp. (Arocena *et al.*, 2001). Oxalic acid is suggested to be the organic acid most abundantly released by ectomycorrhizal mycelia (Lapeyrie *et al.*, 1991). In mycorrhizal mat soils, Griffiths *et al.* (1994) found a significant positive correlation between oxalate and

PO_4^{3-} concentrations, suggesting that fungal exudation of organic acids enhanced weathering and solubility of PO_4^{3-} in the soil. They concluded that the fungi released oxalic acid in excess of that which was precipitated by Ca^{2+} and that this caused intensive local weathering that increased the availability of PO_4^{3-} and SO_4^{2-}. In field and laboratory studies, weathering effects have been detected on clay minerals as a result of colonisation by mycorrhizal fungi (Cromack *et al.*, 1979; Paris *et al.*, 1995).

Dissolution of different insoluble phosphorus sources has been demonstrated in pure cultures of ectomycorrhizal fungi (Lapeyrie *et al.*, 1991; Chang & Li, 1998; Mahmood *et al.*, 2001). The phosphorus rich mineral apatite has been demonstrated to increase growth of phosphorus deficient pine seedlings, and a larger effect was seen for seedlings growing in association with symbiotic mycorrhizal fungi compared to non-mycorrhizal controls (Wallander, 2000). The introduction of mesh bags with apatite amended sand stimulated production of ectomycorrhizal root tips in a forest soils with poor phosphorus status (Hagerberg *et al.*, 2003). In pot experiments both mycorrhizal and non-mycorrhizal pine seedlings could mobilise potassium from biotite. Seedlings colonised by the ectomycorrhizal fungus *S. bovinus* were the most efficient, and a significant positive correlation was observed between foliar potassium and the amount of fungal mycelium, as estimated by the ergosterol content of the substrate (Wallander & Wickman, 1999). Organic acid production in fungi depends on the amount of carbon available to the fungus (Jacobs *et al.*, 2002). If nutrient deficiency in the plant increases the carbon allocated to the root system, this carbon may be allocated to mycorrhizal fungi and result in increased exudation of organic compound by the hyphae. Under field conditions, however, poor potassium and phosphorus status of the trees did not result in result in increased production of ectomycorrhizal mycelia (Hagerberg *et al.*, 2003).

Tubular pores, 3 - 10 µm wide with parallel walls and rounded ends, have been observed in thin sections of weatherable minerals in field samples from various European podzol soils (Jongmans *et al.*, 1997). Because the tunnels were sometimes occupied by fungal hyphae, it was suggested that they could be formed though the direct weathering activity of ectomycorrhizal hyphae (van Breemen *et al.*, 2000a). Examining mineral grains in a podzol chronosequence of 190 - 7800 years, tunnels were found to occur only in the upper 2 cm of the E horizon mineral soil and only in soils of 2700 years or older. At 2700 years most biotite has been dissolved in the upper mineral soil and feldspar tunneling was suggested to be a result of limited availability of calcium and potassium (Hoffland *et al.*, 2002). The pores are suggested to constitute localised sites of intense weathering and by penetrating mineral particles fungi and associated bacteria would avoid competition for nutrients in the soil solution (van Breemen *et al.*, 2000a). Fungal involvement in the formation of tunnels remains uncertain and has been heavily criticised by soil chemists (Sverdrup *et al.*, 2002). Whether formed by fungi or not, the contribution of these tunnels to the total weathering of feldspar has been estimated to be less than 1% (Smits *et al.*, 2003) and may be negligible with respect to total forest nutrient budgets. However this does not mean that the total biologically induced weathering of minerals is insignificant in relation to forest nutrient budgets. The phenomenon is also of great interesting for understanding

physical interactions of fungal hyphae with mineral surfaces and micro-environmental conditions at the fungal mineral interface. Although it has so far not been possible to identify the fungal species growing inside tubular pores, in a preliminary study presented in this thesis ectomycorrhizal species have been identified from hypha growing inside cracks and fissures of rocks in boreal forests (Appendix A).

Exudation in fungal mycelia is concentrated at the growing hyphal tips (Robson, 1999), where excess water and metabolic by-products are deposited outside the hyphae (Unestam & Sun, 1995). In the field, a large part of the exuded metabolites is probably consumed by rich microbial communities commonly found in association with mycorrhizal mycelia in the soil (Perotto & Bonfante, 1997). Micro-environments with high carbon and low nutrient availability, known to stimulate the production of organic acids by many soil living bacteria (Ullman *et al.*, 1996), could commonly occur in the close vicinity of mycorrhizal hyphae. Bacterial attachment to minerals is commonly mediated by increased polymer production, which creates local micro-environments on the surface where weathering agents may be concentrated due to the restricted exchange of solution (Ullman *et al.*, 1996). In studies of rock-inhabiting lichens, mineral grains are found to be detached from the rock surface and incorporated into the lichen structure. These minerals are coated on all sides by extracellular mucilage produced by the community of bacteria, algae and fungi. In such micro-environment, mucilage composition regulates the different steps of mineral weathering (Barker & Banfield, 1996). Bacteria are commonly observed in close association with mycorrhizal hyphae growing on mineral surfaces (**Paper IV**) and in future studies, there may be reason to further examine the impact of fungal associated bacteria in weathering processes in forest soil.

From earlier research, as presented in this introduction it can be concluded that microbial colonisation of mineral surfaces may induce intense weathering. Ectomycorrhizal fungi have several characteristics suggesting their involvement in biogeochemical processes. The work presented in this thesis addresses two topics of major significance for understanding weathering by ectomycorrhizal fungi. The species dependence of ectomycorrhizal fungal responses to different mineral substrates was examined in the field (**Paper I**) and in laboratory (**Paper III**). Patterns of ectomycorrhizal fungal mycelial colonisation of solid mineral substrates were examined by mycelial carbon allocation (**Paper II**) and with respect to mineral surface micro-topography (**Paper IV**). The results of the current studies provide a further step in understanding the mineral weathering capacity of ectomycorrhizal fungi.

3. Results and discussion

3.1. Identification of ectomycorrhizal roots and mycelia in mineral substrates

The first step in examining the role of ectomycorrhizal fungi in weathering of minerals is to identify which fungal species colonise mineral substrates in the field. Minerals are important components of the forest soil, forming the bedrock and the major part of the mineral soil underlying the upper organic horizons (Section 2.2.3.) as well as stones and boulders embedded in the soil or at the soil surface. Identification of ectomycorrhizal fungi in two of these mineral habitats, mineral soil and fissures in boulders, is presented here. **Paper I** examines the vertical distribution of ectomycorrhizal fungi colonising root tips in different soil horizons in a continuous 52 cm deep podzol profile. In a pilot study, the species identity of fungal hyphae and rhizomorphs colonising fissures in boulders was compared to that of hyphae colonising ectomycorrhizal root tips in the adjacent moss cover. Boulders were sampled from the upper part of the forest soil and were covered only by a thin moss layer. A full description of the materials and methods of this pilot study is presented in **Appendix A**.

3.1.1. Vertical distribution of ectomycorrhizal fungi in a podzol profile

Fine root density is highest in the organic and upper mineral soil horizons of boreal forest soils (Persson, 1980; Sylvia & Jarstfer, 1997; Makkonen & Helmisaari, 1998). Usually all of these root tips are colonised by ectomycorrhizal fungi (Taylor, 2002). This apparent association between mycorrhizal distribution and organic matter has resulted in that much ectomycorrhizal research has focused on mycelial responses to organic substrates (*e.g.* Read, 1991), but mycelial responses to mineral substrates may be just as important. In the podzol soil profile examined in **Paper I**, two thirds of all root tips were found in the mineral soil (Fig. 3). The degree of ectomycorrhizal colonisation in different soil horizons varied between 60% and 98%, and was not systematically related to the depth in the profile. In this podzol profile, close to 70% of all ectomycorrhizal root tips were found in the mineral soil. The results of **Paper I** highlight the importance of mineral soil as a growth substrate for ectomycorrhizal fungi in the field.

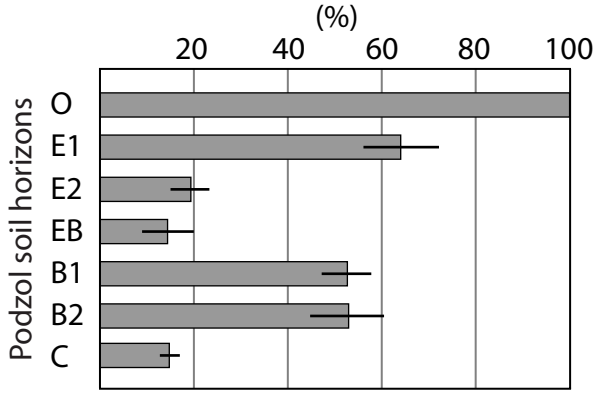


Fig. 3.
Average total number of root tips in each soil horizon (O, E1, E2, EB, B1, B2 and C) expressed as the percentage of total number of root tips in the organic horizon. Error bars represent standard error of the mean (n = 3). (**Paper I**)

Apart from the number of ectomycorrhizal root tips in mineral soil, the species composition of ectomycorrhizal communities has been demonstrated to vary in organic and mineral soils (Egli, 1981; Danielsson & Visser, 1989; Goodman & Trofymow, 1998; Fransson *et al.*, 2000; Heinonsalo *et al.*, 2001; Tedersoo *et al.*, 2003). Identifying the fungal colonisers of root tips (**Paper I**) and species occurring as extramatrical mycelia (Landeweert *et al.*, 2003) in the different horizons of a typically stratified podzol soil (Fig. 1), enabled comparison of ectomycorrhizal community composition in different mineral substrates as well as an organic substrate. The results of these studies demonstrate that in this podzol, the ectomycorrhizal fungal communities colonising both root tips and forming extramatrical mycelia differ depending on the soil horizon. Half of the 22 taxa colonising root tips were found exclusively in the mineral soil (Table 1) (**Paper I**). Four taxa, *Tylospora* spp., *Cortinarius* spp., *Piloderma reticulatum* and *Piloderma* sp. JS15686, were found to colonise root tips throughout the profile. Two taxa, *Inocybe* sp. and *Piloderma byssinum*, were found only in the organic horizon. Five more taxa were identified from root tips in the organic horizon, *Tomentellopsis submollis*, *Piloderma fallax*, *Hygrophorus olivaceoalbus*, *Russula decolorans* and *Dermocybe* spp.; these also colonised the upper mineral soil, including the E1, E2 and EB horizons. *Lactarius utilis* and three hitherto un-described *Piloderma* sp. (1, 2 & 3) were found on root tips in the central part of the profile, *i.e.* from the E2 horizon down to the B2. The new *Piloderma* species were assigned to this genus based on the position of the sequences obtained from root tip material within a sequence homology tree of sequences from *Piloderma* sporocarps (K.-H. Larsson, unpublished). *Suillus luteus* was found to colonise root tips throughout the mineral soil, from the E2 horizon and down to the C horizon. Two taxa that were only found in the B2 horizon and could not be identified by comparing the obtained sequences with those in public sequence databases. *Wilcoxina* sp., *Russula adusta* and *Tricholoma portentosum* were only identified on root tips in the parental C horizon. From the results in **Paper I** it can be concluded that fungal taxa colonising ectomycorrhizal root tips in mineral soil are different from those colonising root tips in the organic soil. Many of the ectomycorrhizal taxa predominantly colonising mineral soil may still be un-described, as indicated by the finding of three new *Piloderma* species and two other unidentified taxa in **Paper I**. Apart from the main colonisers of ectomycorrhizal root tips, secondary colonisers were also commonly detected through PCR amplification of multiple bands from root tip DNA samples. These results are discussed further in Box 2.

In parallel with **Paper I**, Landeweert *et al.* (2003) examined the ectomycorrhizal community composition as mycelia colonising the different soil horizons of the podzol profile. Nearly all of the ectomycorrhizal species for which sequences were obtained from root-free soil samples were also recorded on root tips. There were large differences in the species abundance detected in the two studies. This could partially be explained by the fact that the roots and mycelia were extracted from different but adjacent soil samples. Four of the taxa identified on root tips, *P. fallax*, *R. decolorans*, *H. olivaceoalbus* and *Piloderma* sp. 2, (**Paper I**) had the same profile distribution pattern when identified in extracts of root-free soil (Landeweert *et al.*, 2003).

Table 1.
Vertical distribution of ectomycorrhizal taxa in a podzol profile. The presence of the taxa in any of the horizons (O, E1, E2, EB, B1, B2 and C) is indicated by X.
Modified from **Paper I**.

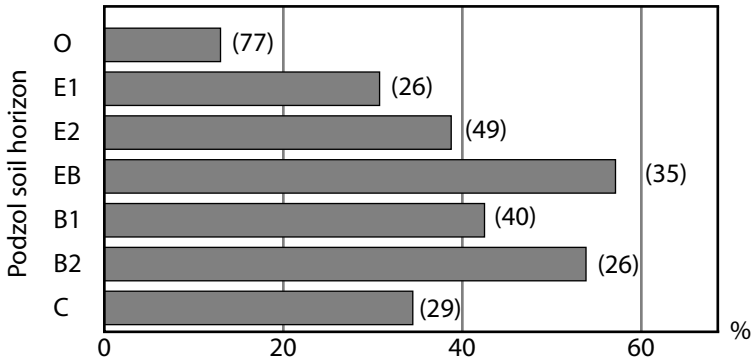
Ectomycorrhizal taxa	**O**	**E1**	**E2**	**EB**	**B1**	**B2**	**C**
Tylospora spp.	X	X	X	X		X	X
Cortinarius spp.	X		X	X	X	X	X
Piloderma reticulatum	X	X	X	X		X	X
Piloderma sp. JS15686	X	X			X	X	
Inocybe	X						
Piloderma byssinum	X						
Tomentellopsis submollis	X	X					
Piloderma fallax	X	X	X				
Hygrophorus olivaceoalbus	X	X	X				
Russula decolorans	X	X	X	X			
Dermocybe spp.	X	X	X	X			
Tomentelloid			X				
Lactarius utilis			X	X	X		
Piloderma sp. 2			X	X	X	X	
Piloderma sp. 3				X	X	X	
Piloderma sp. 1				X	X	X	
Suillus luteus			X	X	X	X	X
unID#15						X	
unID#12						X	
Wilcoxina							X
Russula adusta							X
Tricholoma portentosum							X

The observed vertical distribution of ectomycorrhizal fungi (**Paper I**), suggests that the proliferation of certain species may be influenced by the conditions in the different soil horizons. Ectomycorrhizal community composition is highly variable in space and time and the factors controlling fungal distribution in soil are not well understood (Dahlberg, 2001). The community composition of host trees is a major determinant of the composition of the ectomycorrhizal community (Molina *et al.*, 1992). The generally greater rooting depth of pine compared to spruce (Mikola *et al.*, 1966) could partially explain the variation in community composition with depth. Pine specific *Suillus* species are, for instance, almost entirely restricted to the mineral soil (**Paper I**). This may explain why previous studies, sampling only the upper organic horizons, have found a very low level of root colonisation by these species in relation to their fruit body production (Dahlberg *et al.*, 1997). **Paper I** clearly demonstrates that the mineral soil is an important habitat for certain ectomycorrhizal fungi in the field, and their activity in this substrate could be important for biogeochemical processes.

Box 2 - Endophytes in ectomycorrhizal root tips

PCR amplification with the universal primers ITS1 and ITS4 produces multiple PCR products in 38% of the root tips subjected to molecular identification. Most were double bands and these were separated and identified by sequencing (**Paper I**). Sequences from the double colonisers commonly matched sequences from the monophyletic group Helotiales spp. The order Helotiales has a broad geographic distribution and covers a broad ecological spectrum of pathogens, endophytes, ecto- and ericoid mycorrhizal ascomycetes (Vrålstad *et al*., 2002) including sterile endophytic fungi, such as dark septate endophytes (Jumpponen, 2001). Identical ITS sequences within the Helotiales spp. have been amplified from different kinds of mycorrhiza on different host plants (Vrålstad *et al*., 2002).

The occurrence of double banding was not entirely random with respect to the taxa or soil horizon in which they occurred. The frequency of double-banded PCR products was highest, above 50%, in samples from the EB and the B2 horizons and lowest, with 13%, among root tips sampled in the organic horizon, see figure below.



The percentage of double-banded PCR products out of the total number of PCR amplifications (in parenthesis) from DNA extracts of root tip sampled in the different podzol soil horizons (O, E1, E2, EB, B1 B2 & C).

The documented double colonisers did not affect the morphology of the root tip where they occurred, either because secondary colonisation had just started or because the second fungi existed as rather inactive endophytes within the ectomycorrhizal root tips. The ability of a single fungal species to form both ericoid and ectomycorrhizal associations has been suggested to function as an inoculum base from deeper ectomycorrhizal root tips for ericaceous colonisers (Bergero *et al.*, 2000). Mycelia and ectomycorrhizal root tips surviving in the deeper mineral soil could serve as a post disturbance inoculum base (Grogan *et al.*, 2000). Existing as endophytes inside roots, including ectomycorrhizal roots could be an opportunistic strategy of the documented double colonisers, enabling survival until conditions change and new growth strategies can be employed to expand and reproduce.

3.1.2. Fungal hyphae colonising fissures within a boulder

In a pilot study, species identification by PCR–RFLP and sequencing was used to match hyphal fragments from within fissures in a moss-covered boulder with fungi colonising root tips in the moss-cover (**Appendix A**). RFLP patterns were successfully obtained from 50 out of 96 sampled ectomycorrhizal root tips and from 29 out of 70 sampled hyphal fragments. Comparison of RFLP patterns of samples from root tips with those from hyphal fragments (Fig. 4) demonstrated that the fungal species in these two fractions were largely different. There was, however, one cluster where root tips and hyphal samples grouped together. Sequencing samples from this group gave one root tip sequence of 326 base pairs (bp) and one hyphal sequence of 442 bp. The two sequences were identical over 306 bp and gave a 97% match to a *Tomentella* sp. sequence in the GenBank database at NCBI using the BLAST program (Altschul *et al.*, 1997). Three other samples of hyphal fragments were also sequenced and compared to sequences in GenBank. For one there was no good match, the second matched *Russula vinosa* with 98% sequence homology over 272 bp and the third matched a *Hymenoscyphus* sp. with 97% sequence homology over 243 bp. Because of the small sample size and the short sequences these results must be regarded as preliminary. Nevertheless, the study demonstrates that hyphae of ectomycorrhizal fungi may colonise fine fissures within boulders, which roots cannot penetrate. Colonised fissures represent potential environments for ectomycorrhizal weathering.

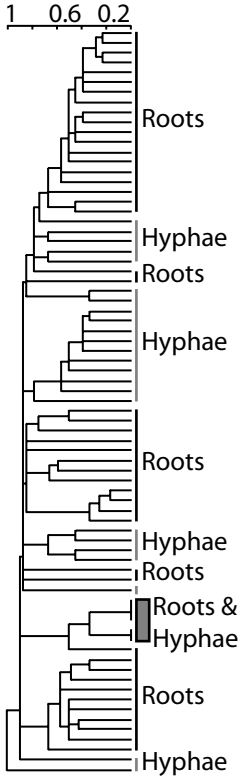


Fig. 4.
PCR–RFLP homology between fungal species samples as hyphae within fissures in boulders and from ectomycorrhizal root tips in the moss layer covering the boulders (**Appendix A**). Branches are grouped and named as either Roots or Hyphae, depending on the origin of the samples. Most groups are separate for root and hyphae respectively. One group however, encompassed both roots and hyphal samples, as highlighted by a black and grey box.

3.2 Mycelial growth and carbon allocation in mineral substrates

To determine the potential of ectomycorrhizal fungi as biogeochemical actors, their mycelial activity in different mineral substrates needs to be studied. Ectomycorrhizal fungi that colonise root tips and produce extramatrical mycelia in mineral soils will allocate carbon to this habitat. Allocated carbon is used to build up mycelial biomass and to mediate mycelial activities, such as nutrient uptake mediated through exudation of organic compounds that condition the environment surrounding the hyphae (Section 2.4.3.). Intact ectomycorrhizal microcosm systems, containing both host plant and symbiotic fungi, were used to examine patterns of carbon allocation to different mineral substrates (**Paper II**). By supplying $^{14}CO_2$ to the shoot, the distribution of newly assimilated carbon could be monitored non-destructively using electronic autoradiography, and destructively determined through sample combustion and measurements of radioactivity in a scintillation counter. The ectomycorrhizal fungi *Hebeloma crustuliniforme* (Bull.) Quél. (isolate code - UP184) and *Piloderma fallax* (Liberts) Stalpers (UP121) were used in these experiments (**Paper II**). The *H. crustuliniforme* was isolated from an ectomycorrhizal root tip in a mycelial mat colonising the underside of a moss sheet lifted from a boulder in a mixed pine forest on rocky granite soils in Lunsen, Uppsala, Sweden. *P. fallax* is commonly observed in the organic – mineral interface, the particular isolate used in this thesis was isolated in Flakaliden, Sweden.

3.2.1. Carbon allocation of roots and mycelia to complex mineral substrate

Pine seedlings colonised by either *H. crustuliniforme* or *P. fallax* were grown in vertically divided flat bed microcosms, with *Sphagnum* peat and E1 horizon material as contrasting organic and mineral substrates (**Paper II**). Both fungi grow well in pure peat systems (Fig. 5a & b). When mycorrhizal plants were introduced at the interface between the two substrates root and mycelial growth was more intense in the mineral substrate compared to the organic substrate, (Fig. 5c & d). The systems were radioactively labelled by one week of incubation in a $^{14}CO_2$-enriched atmosphere, followed by electronic autoradiography scanning (Fig. 5e & f). The long continuous labelling resulted in approximately 80% of the accumulated ^{14}C being detected in the below ground compartment for both ectomycorrhizal fungi. In systems with *H. crustuliniforme*, approximately 60% of the total ^{14}C in the systems was incorporated into the extramatrical mycelia, as measured in the root free substrate. The corresponding figure for *P. fallax* was 50%. This activity recovered from mycelia, is high compared with earlier studies using pulse labelling, where typically 2 - 12% of the activity is detected in the mycelium (*e.g.* Leake *et al.* 2001). The discrepancy could be explained by higher carbon use efficiency in the mycorrhizal mycelium compared with the plant tissue. During the one week of continuous labelling, it is likely that respiration in the plant tissue resulted in considerable respiratory loss from roots (Högberg *et al.*, 2002). Assuming that the carbon use efficiency of mycelia is higher compared to that of the roots this would result in proportionally more of the label being retained in the fungal mycelium after a long incubation period.

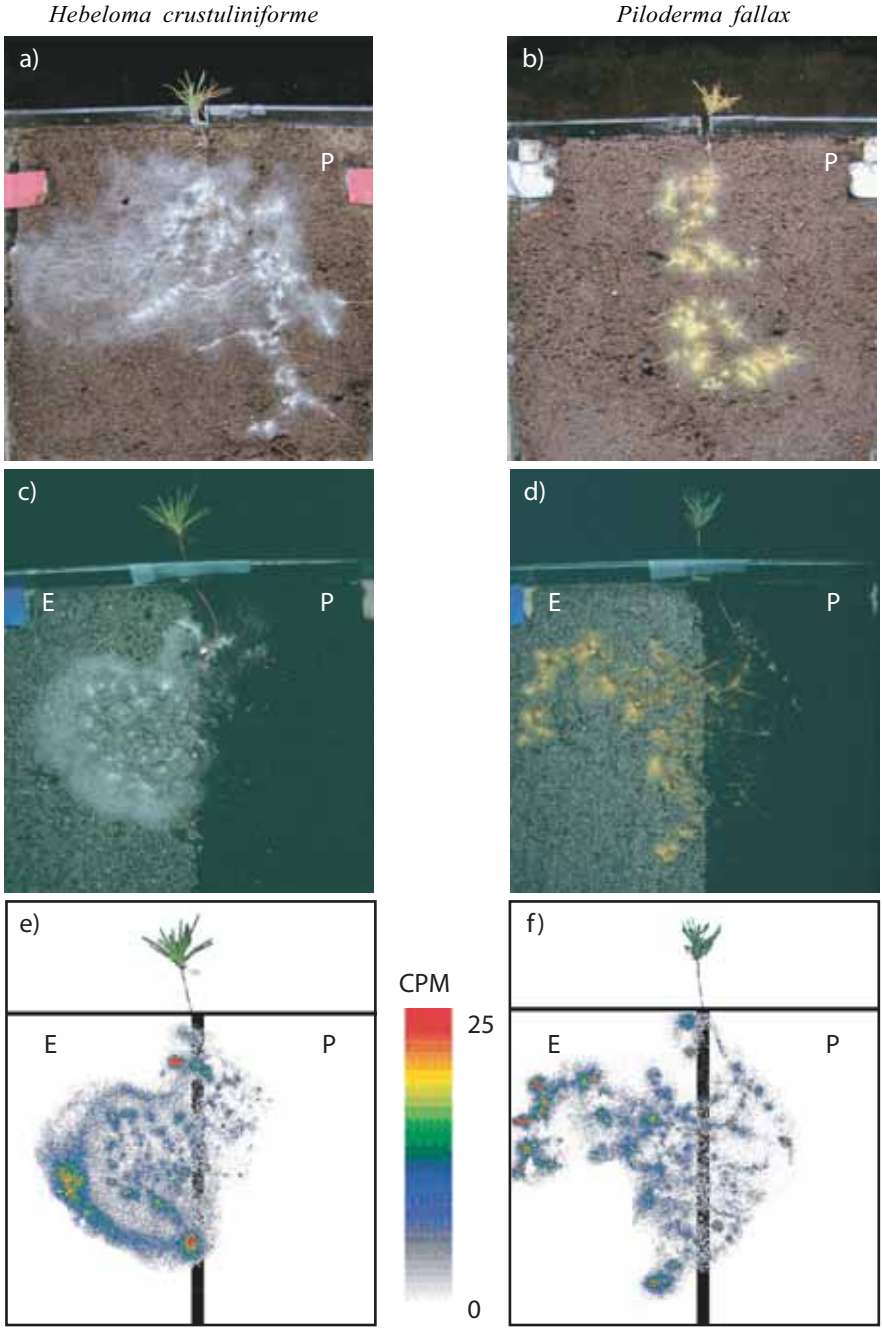


Fig. 5.
a & b) Ectomycorrhizal pine seedlings colonised by either *H. crustuliniforme* or by *P. fallax* grow well in reference microcosms with peat (P). c & d) In vertically divided microcosms with E horizon mineral soil (E) and peat, both fungi grow more vigorously in E compared to P. e & f) Visualisation of the radioactivity distribution in the systems after one week of labelling. The colour bar indicates the detected intensity levels in CPM (counts per minute). (**Paper II**)

Of the ^{14}C in the below ground compartment, 70 – 80% was detected in the E horizon mineral soil (Fig. 6). For both fungi, the total amount of ^{14}C allocated to extramatrical mycelium in E horizon mineral soil was significantly higher than that allocated to extramatrical mycelium in peat (**Paper II**). The experimental design used in this study takes into account the fact that the mycelia is a functionally interconnected unit where the activity in one part of the mycelia affects the activity in other parts (Section 2.3.1.). When challenged with different substrates, as when growing in heterogeneous soil, the mycelium will proliferate in substrate types that exhibit the most favourable conditions. In the current experiment, E horizon mineral soil was a more favourable substrate for both fungi compared to *Sphagnum* peat. The tested substrates are complex and not well characterised and the experiment provides no explanation for the underlying environmental conditions inducing the observed difference in carbon allocation. These results however demonstrate that certain ectomycorrhizal fungi colonising mineral substrates can allocate a substantial amount of carbon to this habitat.

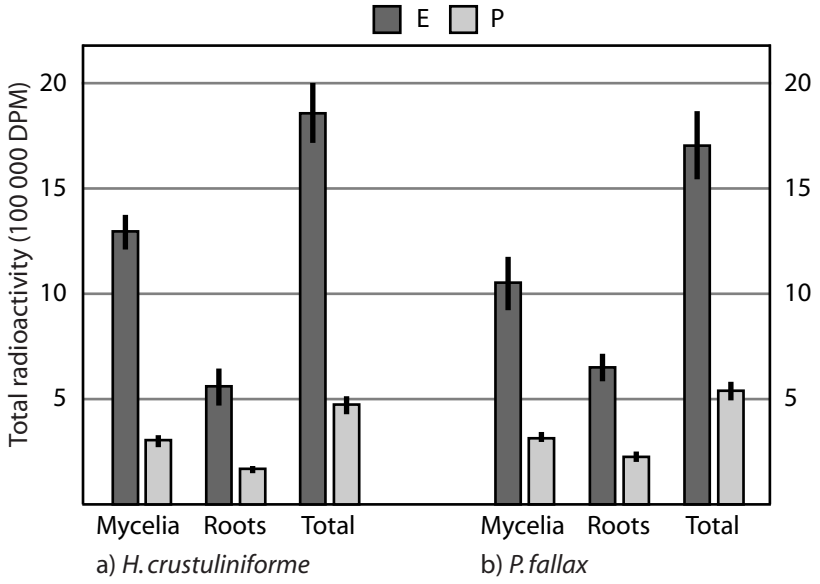


Fig. 6.
Activity measured as disintegrations per minute (DPM) from the destructive harvest of vertically divided microcosms. Mean activity is presented as 100 000 DPM (±SE) in the two contrasting substrates, E horizon mineral soil (E) and peat (P), showing distribution between mycelia and roots as well as the total activity, for a) *H. crustuliniforme* and b) *P. fallax*, n = 5. (**Paper II**)

3.2.2. Carbon allocation of roots and mycelia to pure mineral substrate

Within microcosms containing pine seedlings colonised by *H. crustuliniforme* root and mycelial proliferation in introduced patches of pure potassium feldspar was more intense compared to patches of pure quartz. The seedlings were pulse labelled with $^{14}CO_2$ and incubated for 72 h before autoradiographic scanning and destructive measurements of activity. Significantly more ^{14}C ($p = 0.021$) was allocated to dishes with potassium feldspar compared to dishes with quartz

(Fig. 7). A system, representative of the five replicates is presented in Fig 8. During the 72 h of incubation, carbon allocated to the below ground compartment was concentrated in mycorrhizal roots and closely associated mycelia (Fig. 8b). Potassium feldspar (Huang, 1989) is more readily weathered than quartz (Drees *et al.*, 1989) and is a possible source of mineral nutrient elements, mainly potassium, but also magnesium and sodium. Selective mycelial exploitation of organic patches (Bending & Read, 1995) has been demonstrated to be accompanied by mobilisation and removal of elements from the patches (Lindahl *et al.*, 1999; Perez-Moreno & Read, 2000). Since element depletion of pure mineral sources is a process which is detectable on the geological rather than biological time scale, this was not analysed in the experiment. The causal reason for the observed difference cannot be exclusively concluded from the experiment. In order to examine differences in mycelial colonisation of minerals with different element composition, the pH, particle size and water holding capacity of the two substrates were kept similar. These results suggest that the growth of certain ectomycorrhizal fungi may be regulated in response to element composition of solid minerals, as earlier demonstrated for bacteria (Rogers, 2003). More intense bacterial colonisation was observed on silicate glass surfaces with lower aluminium content (< 5 wt% Al2O3) compared to surfaces with higher aluminium content. In the experiment, pH was controlled to ensure similar surface charges for all minerals (Rogers, 2003). Increased bacterial feldspar weathering has been demonstrated when the feldspar contained inclusions of phosphorus rich apatite, compared to feldspar where no inclusions were present (Rogers *et al.*, 1998).

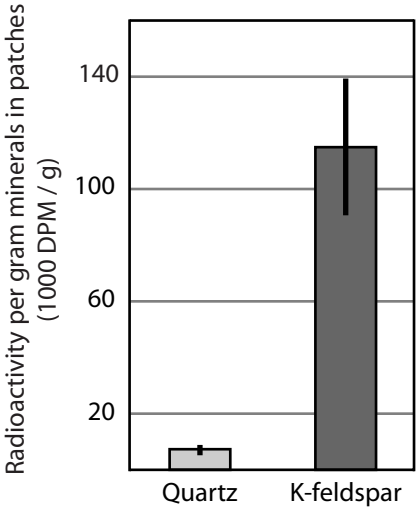


Fig. 7.
Mean radioactivity, measured in thousands of disintegrations per minute (DPM ± SE) per gram mineral, in patches of the two contrasting mineral substrates, quartz and potassium feldspar (K-feldspar) colonised by ectomycorrhizal roots and mycelia of *H. crustuliniforme* colonising $^{14}CO_2$-labelled *Pinus sylvestris* seedlings. (**Paper II**)

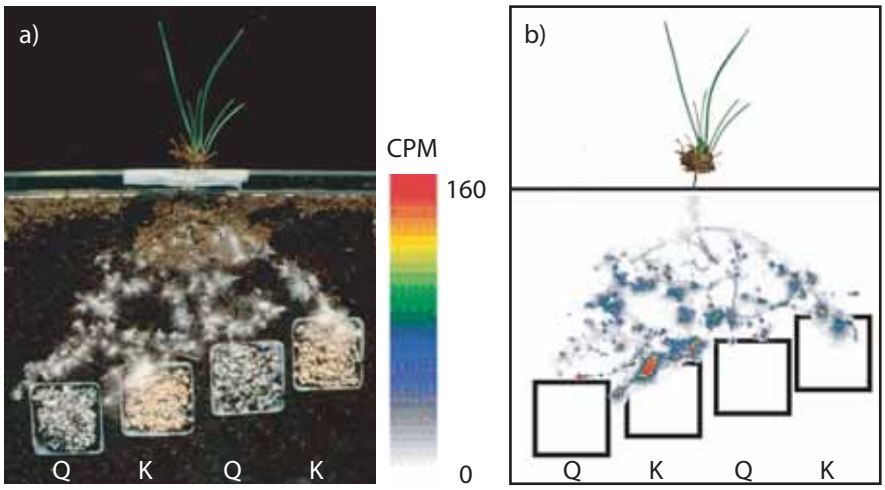


Fig. 8.
Flatbed peat microcosms containing *Pinus sylvestris* seedlings colonised by *H. crustuliniforme* and pure mineral patches of either potassium feldspar (K) or quartz (Q). a) Fifteen weeks after introducing mineral patches at the growing mycelial front, the shoots were pulse labelled with 0.74 Mbq $^{14}CO_2$. The distribution of radioactivity in the systems was detected by non-destructive electronic autoradiography. b) Electronic autoradiographic scan images overlaying schematic views of the mineral patches in the microcosms demonstrate that more of the current carbon assimilates are allocated to root tips and mycelia associated with patches of potassium feldspar compared to patches of quartz. The colour bar indicates the intensity levels in CPM. (**Paper II**)

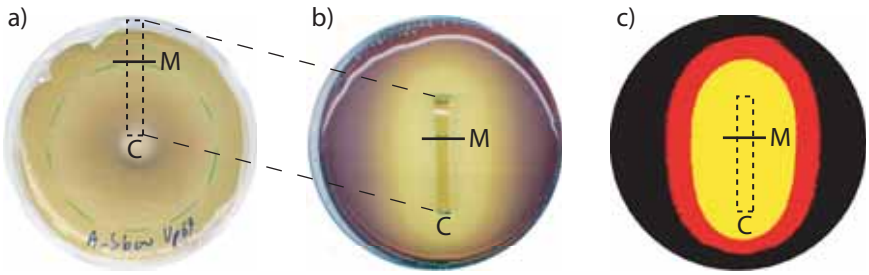


Fig. 9.
Estimation of substrate acidification by growing fungal mycelia, using a three step colorimetric method. a) A fungal mycelium growing out from a central inoculum plug (C). At harvest, the fungal mycelium was removed and a rectangular slice was cut in the underlying agar, from (C) through the mycelial front (M) to the edge of the plate. b) The slice was put on top of a purple pH indicator plate (pH 7). As acidity diffused from the agar slice, pH dropped in a zone around the slice, shifting the colour of the indicator plate to yellow at pH values below 5.2. Plates were scanned after 24 h. c) Image analysis produced schematic images of the zones and enabled quantification of the size of pH shift zone by counting the number of yellow pixels. (**Paper III**)

3.3. Ectomycorrhizal mycelial responses to elements in mineral substrates

As discussed above, ectomycorrhizal mycelia may proliferate in mineral substrates throughout a wide range of habitats in boreal forest soils. To relate ectomycorrhizal colonisation of minerals to its functional significance in weathering processes, variations in mycelial growth and activity in response to different minerals were examined. Responses of fungal hyphae to minerals may be influenced by both the elemental composition of the mineral as well as its surface structure characteristics. To eliminate the effect of surface properties on mycelial responses, the experiments described in **Paper III** were designed to analyse mycelial growth and acidification of the substrate in response to different mineral powders enriching an agar substrate. The acidity at the mineral – solution interface is an important variable in weathering and substrate acidification is commonly increased in the vicinity of roots and hyphae (Section 2.2.2).

Laboratory studies of weathering capacity in fungi have been dominated by the displacement method (*e.g.* Henderson & Duff, 1963). After hyphal growth, the presence or absence of clearing zones in mineral powder enriched agar plates is used to score for presence or absence of weathering capacity in the tested isolates. Clearing zones are the result of mineral dissolution, transport of elements from the zone and re-crystallisation in other parts of the substrate or in the mycelia. Using such systems, the ability of ectomycorrhizal fungi to dissolve limestone and marble (Chang & Li, 1998), as well as insoluble phosphates, (Lapeyrie *et al.*, 1991; Mahmood *et al.*, 2001) has been demonstrated. The displacement method however fails to produce quantitative data and the detection of clearing zones may be obstructed by fungal coloration of the growth substrate. Because substrate acidification is an important factor in biologically induced weathering a novel colorimetric method (Fig. 9, above) was developed to estimate substrate acidification by mycelia in response to enrichment of the agar substrate with different minerals (**Paper III**). One saprotrophic fungus and seven different ectomycorrhizal fungal species were grown on agar enriched with five different minerals. Substrate acidification in the different treatments was compared to an acidity standard produced by known concentrations of oxalic acid. In experimental systems using ectomycorrhizal pine seedlings, the pH of rhizosphere soil has been demonstrated to be strongly correlated with the amount of oxalate in the soil (Casarin *et al.*, 2003). Fungal induced substrate acidity was quantified by comparisons with substrate acidity of known oxalic acid concentrations (Fig. 10).

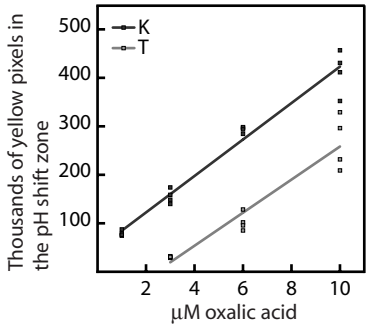


Fig. 10.
Standard curves for 1, 3, 6 and 10 µM oxalic acid applied to plates containing either of four different minerals. Increasing concentrations resulted in increased size of the yellow pH shift zone. Linear regressions for potassium feldspar (K) overlap with apatite and quartz. The high buffering capacity of TCP (T) shifted this curve to the right in the diagram. (**Paper III**)

In certain species, such as *Amanita muscaria* (L.: Fr.) Hook., *H. crustuliniforme*, *Mycena galopus* (Pers.: Fr.) Kumm., *P. fallax*, *P. involutus* and *S. bovinus*, mycelial growth, measured as mycelial density, varied according to the mineral with which the agar was enriched (Fig. 11). Variations in estimated substrate acidification in response to mineral enrichment were highly species dependent and not simply proportional to the fungal biomass in the different species – mineral combinations (Fig. 12). Variation of substrate acidification per unit mycelial density within species indicates that release of protons and carbon dioxide alone cannot account for the measured substrate acidification. Other mechanisms such as production and exudation of organic acids may thus influence the induced substrate acidification.

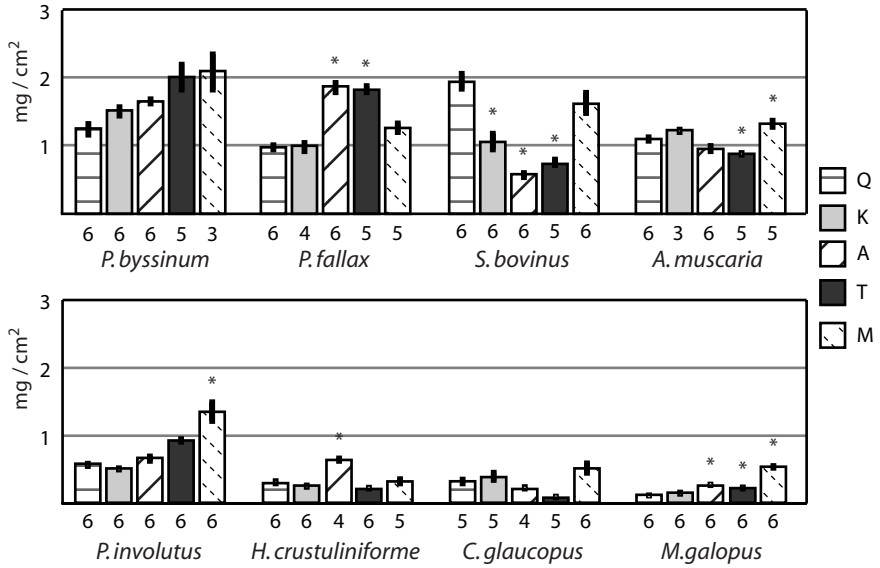


Fig. 11.
Average mycelial density in mg / cm^2 (± SE) for each of the eight fungi grown on media enriched with five different minerals, quartz (Q), potassium feldspar (K), apatite (A), tri-calcium phosphate (T) and marble (M). Mycelial size and dry weight were determined when at least half of the replicates in each treatment had a mycelial diameter of 4.5 cm. Fungal isolates appear from the left according to decreasing average mycelial density. Numbers of replicates (n) are given for each mineral in the histogram. Average mycelial densities for minerals within a fungal species significantly different from that of the quartz control are indicated by * ($p = 0.005$). (**Paper III**)

The processes underlying acidification at the root soil interface have been comprehensive review by Hinsinger *et al.* (2003). Increased substrate acidification by roots is frequently demonstrated in response to environmental stresses, such as nutrient deficiency, particularly iron and phosphorus, and toxicity of metal ions such as aluminium (Hinsinger *et al.*, 2003). Fungi exhibit similar responses to toxic metals, but the degree of response varies between fungal species and even between different isolates of the same species (Gadd, 1993; Ahonen-Jonnarth *et al.*, 2000). Further more, ectomycorrhizal fungal species may exhibit different substrate responses when grown in pure culture compared to when they are living in association with a host plant. Such differences have been demonstrated in

studies of nickel tolerance, where *Scleroderma flavidum* E. & E. increased tolerance of its Birch host but was unable to grow in pure culture at elevated nickel concentrations (Jones & Hutchinson, 1988a, b). Environmental stress due to high calcium concentrations in the substrate may be alleviated by precipitation of calcium oxalate outside the cells (Jackson & Heath, 1993). The sparse growth and high substrate acidification of *C. glaucopus* (Fig. 12) could be a result of toxic levels of Ca^{2+} in the TCP enriched plates, however specific stress responses may be difficult to separate from general ones. Toxic levels may be very different for different fungi and other fungi in the study did not respond in the same was as *C. glaucopus*.

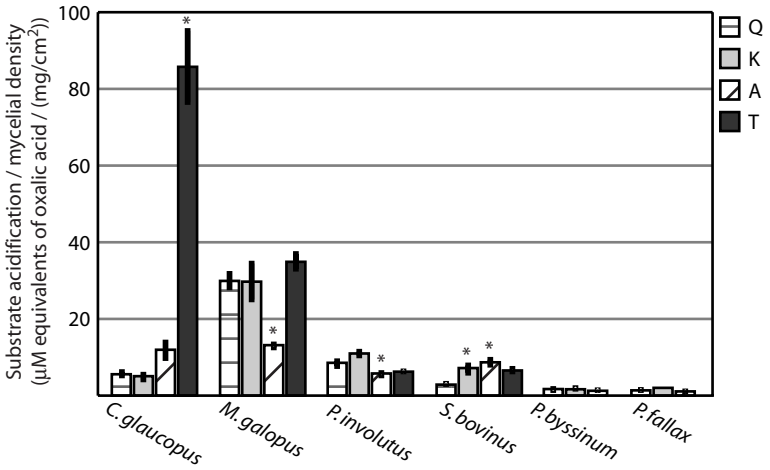


Fig. 12.
Substrate acidification per unit mycelial density after fungal growth on agar enriched with one of four different minerals; quartz (Q), potassium feldspar (K), apatite (A) and *tri*-calcium phosphate (T). Substrate acidification is estimated as µM equivalents of oxalic acid. Substrate acidification was below the detection limit of 3 µM oxalic acid in treatment T for the two *Piloderma* species. Mean substrate acidification per unit mycelial density on minerals within fungal species that differed significantly from that of the quartz control are indicated by * ($p=0.005$). The mean for each treatment is given as equivalents of oxalic acid µM / (mg/cm^2), (± SE). (**Paper III**)

3.4. Colonisation of mineral particles by ectomycorrhizal hyphae

Differing degree of ectomycorrhizal mycelial and root proliferation in spatially heterogeneous microcosms containing different mineral substrates were found in **Paper II**, this demonstrates that certain ectomycorrhizal fungi may regulate their mycelial carbon allocation in relation to the elemental composition of different minerals. Carbon compounds are utilised to build up mycelial biomass or exuded to condition the extracellular environment of the hyphae. Species dependent substrate acidification per unit mycelial density has been demonstrated in response to different mineral enrichment of agar substrates (**Paper III**). Physical interaction of hyphae with mineral surfaces is potentially important to create micro-

environments where mineral weathering can be directly influenced by fungal activity.

3.4.1. Colonisation of mineral surfaces by ectomycorrhizal hyphae

Scanning electron microscopy (SEM) was used to examine hyphal growth on marble surfaces in peat microcosms with pine seedlings colonised by *H. crustuliniforme*. After four months of mycelial colonisation, partial removal of hyphae resulted in visible tracks on the polished marble surface (Fig. 13) (**Paper IV**), suggesting that hyphal interaction may result in alterations of the mineral surface micro-topography. In SEM observations at high magnification, fungal tracks appear to be at a slightly lower depth than the surrounding surface. Because SEM data are open to different interpretations, verification and possibly quantification of these observations by surface topography measurements are desirable ways of complementing results obtained by SEM. Marble consists primarily of $CaCO_3$, an easily dissolved mineral, which does not commonly occur in boreal forest soils. To examine the generality of mineral surface alteration as a result of direct hyphal interaction, other experiments were performed on relevant field minerals such as biotite, potassium feldspar, apatite, calcite, hornblende and labradorite. Preliminary results from these additional studies of surface interaction with hyphae are presented in this section.

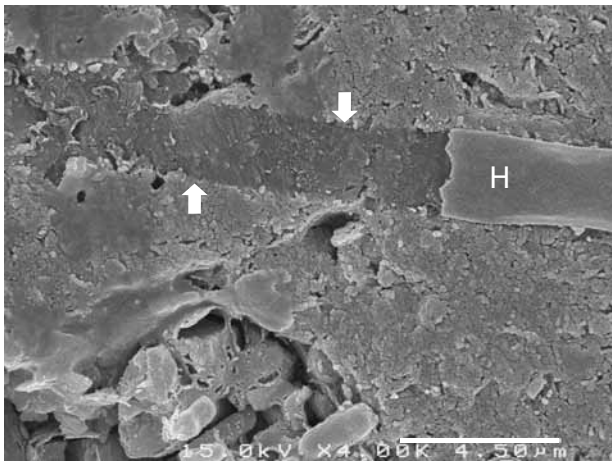


Fig. 13. The micro-topography of tracks remaining after hyphal removal (between arrows) compared to the surrounding surface structure, as visualised by SEM-SE. The appearance of tracks depends largely on the surface structure at the site of hyphal (H) growth. The surface in the track appears smoother than that of the surrounding surface (arrows). Scale bar 4.5 µm. (**Paper IV**)

SEM was used to examine hyphal interactions on potassium feldspar and biotite surfaces in samples prepared by fixation and critical point drying to preserve the three-dimensional structure of the hyphae. A full description of the materials and methods used in this experiment is presented in **Appendix B**. The surface of potassium feldspar was only sparsely colonised by mycelia. In some parts of the surface, the growing mycelia had produced extensive amounts of extracellular mucilage (Fig. 14a). Bacteria were often observed in the close vicinity of mucilage producing hyphae. As in the case of observations presented in **Paper IV**, mucilage cover of the surface was patchy. Hyphal growth appeared to respond to the micro-topography of the mineral surface. Hyphae were commonly observed to follow edges and grow in fissures in the potassium feldspar surface (Fig. 14b). This is

probably because the open flat surface is a rather exposed environment for the fungi to colonise, whereas fissures and other uneven features of the surface provide more surface contact for the growing mycelia.

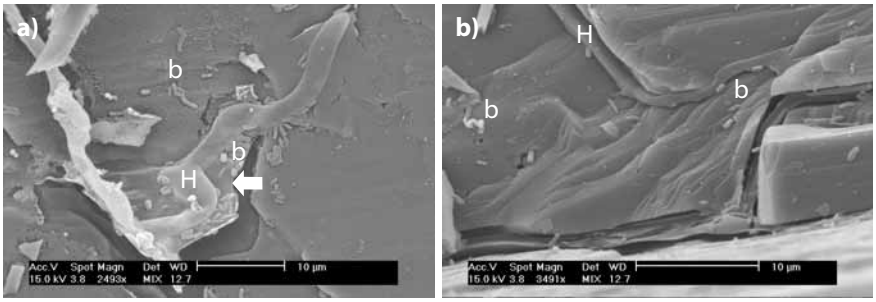


Fig. 14.
Mycelium of *H. crustuliniforme* after colonisation of a potassium feldspar surface for seven months. The sample was prepared by fixation and critical point drying followed by gold coating and analysis by SEM (**Appendix B**). Hyphae (H) and bacteria (b) are visible on the images. Scale bar 10 µm. a) The hyphal surface contact is mediated by a film of extracellular mucilage (arrow) and bacteria are seen in the mucilage. b) Hyphae are commonly observed to grow along fissures in the potassium feldspar surface.

Because of the layered structure of biotite, sample preparation resulted in disintegration of the original organisation of the mycelial interaction with biotite flakes. The mycelial surface colonisation of biotite was thus more difficult to interpret compared to potassium feldspar. Biotite was generally well colonised by mycelia. Hyphal sized marks were sometimes observed on the biotite surface (Fig. 15) but whether these were a result of hyphal growth on the surface could not be concluded from these experiments.

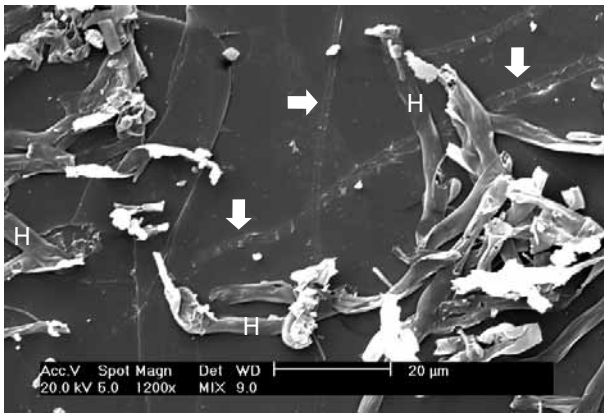


Fig. 15.
SEM-SE image of a biotite surface, after seven months of mycelial colonisation by *S. variegatus*. Sample preparation according to **Appendix B**. disturbed the three-dimensional organisation of the mycelium colonising the biotite minerals. Hyphae (H) and hyphal sized marks (arrows) are visible in the surface. Scale bar 10 µm.

3.4.2. Colonisation of particles in mineral soil by ectomycorrhizal hyphae

In microcosms containing pine seedlings colonised by ectomycorrhizal fungi, a substantial part of the below ground carbon was allocated to roots and mycelia proliferating in E1 mineral soil compared to peat (**Paper II**). To examine hyphal

interactions with particles in the mineral soil, an ectomycorrhizal root tip colonised by *H. crustuliniforme* was sampled from the E horizon mineral soil of a vertically divided microcosm. Cutting of a root approximately one cm behind the tip and lifting it out of the E1 substrate produced a sample with several protruding ectomycorrhizal short roots and extensive extramatrical mycelium with numerous mineral particles connected to it. The sample was prepared for SEM analysis, according to **Appendix B**. Small grains commonly cover hyphae colonising the E1 mineral (Fig. 16a). Findings from other studies of mycorrhizal hyphae (*e.g.* Cromack *et al.*, 1979) led us to assume that these were crystals of calcium oxalate. Mineral particles (M) were trapped within the hyphal network colonising the mineral soil (Fig. 16a). Considering the many steps of sample preparation, mineral particles that remained connected to the mycelium throughout the procedure can be supposed to have been well attached from the start. Where hyphae (H) were observed in direct contact with mineral particles, the surface crystals were commonly absent (Fig. 16b). A similar pattern has been observed previously (Graustein & Cromack, 1977; Cromack *et al.*, 1979; Arocena *et al.*, 2001; **Paper IV**) and could be a result of hyphae translocating dissolved calcium from the site of dissolution to dispose of it in biomineral form in other parts of the mycelium (Connolly *et al.*, 1999).

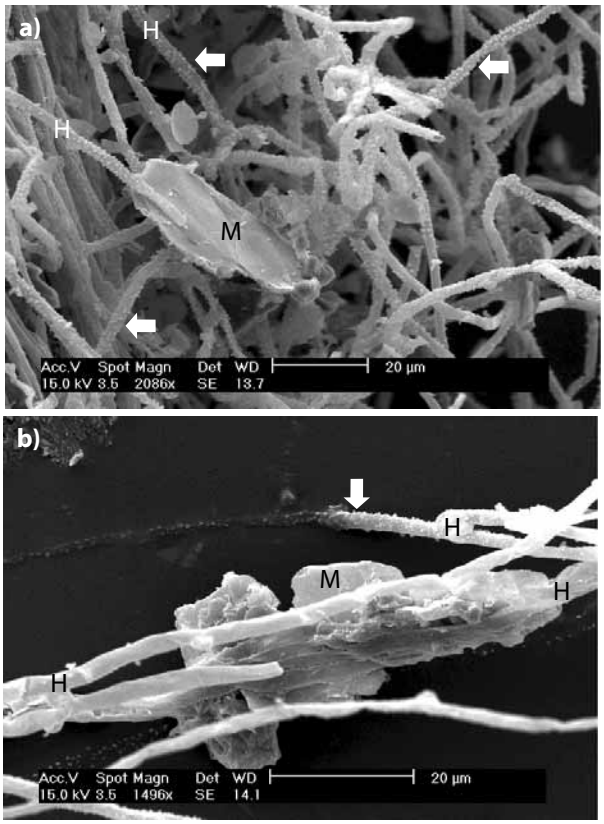


Fig. 16. Ectomycorrhizal pine roots colonised by *H. crustuliniforme*, were sampled from the E1 mineral soil in a microcosm experiment (**Paper II**) and subjected to sample preparation for SEM analysis (**Appendix B)**. Scale bar 20 µm. Hyphae (H) interacting with mineral particles (M). On most hyphae, small grains, probably crystals of calcium oxalate, are visible (arrows) a) Hyphae in the mantle associate with a mineral particle. b) No crystals are seen on extramatrical hyphae in direct contact with mineral particles.

Mineral particles may be integrated into the mycelial mantle of the ectomycorrhizal root (Fig. 17a). Hyphal sized tracks (arrow) in the surface (Fig. 17b) are similar to those observed after hyphal removal from marble surfaces (**Paper IV).** Tracks could be the result of hyphae previously growing on the surface.

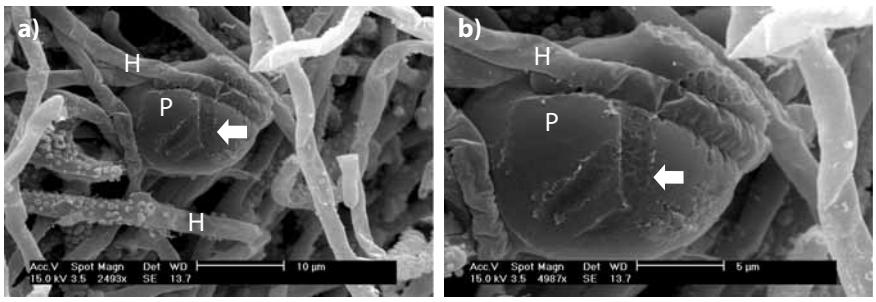


Fig. 17.
An ectomycorrhizal pine root colonised by *H. crustuliniforme,* sampled from E horizon mineral soil in a microcosm (**Paper II**) and prepared for SEM analysis (**Appendix B**). a) Hyphae (H) in the ectomycorrhizal mantle interacting with a particle (P) from the E1 mineral soil. Hyphal sized tracks are visible in the surface of the particle (arrow). Scale bar 10 μm. b) Close up of the particle and tracks. Scale bar 5 μm.

Minerals may be integrated into the mantle and possibly even the outer part of the root (Fig. 18a). Such minerals may be directly affected by root and fungal activity for a longer time. At the arrowhead (Fig. 18a) and in close up in Fig 18b, crystals are precipitated, and these are possibly the result of secondary mineral formation as a result of weathering of the mineral grains.

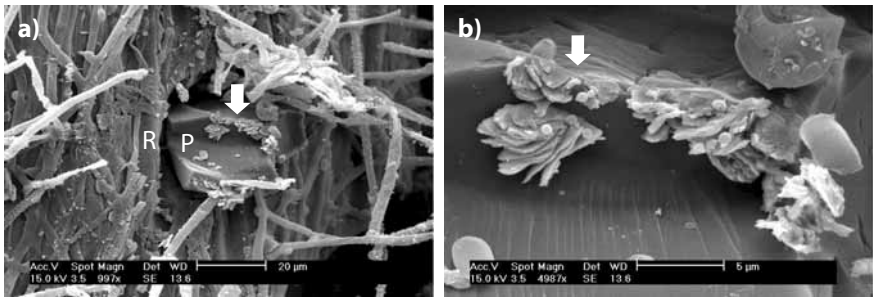


Fig. 18.
Ectomycorrhizal pine roots colonised by *H. crustuliniforme*, sampled from the E1 mineral soil (**Paper II**) and prepared for SEM analysis (**Appendix B)**. a) A mineral particle (P) is integrated in the mycelial mantle and possibly also in the outer layer of the root (R). Precipitation of secondary minerals (arrow) is observed on the particle. Scale bar 20 μm. b) Close up of the mineral particle (P). Scale bar 5 μm.

3.4.3. Element composition of mineral surfaces colonised by hyphae of ectomycorrhizal fungi

In an ongoing study, polished pieces of apatite, calcite, hornblende and labradorite have been used to characterise the mineral surface micro-topography before and after exposure to growing hyphae of *H. crustuliniforme*. The aim of the

experiment was to determine whether mycelial colonisation and possible alteration of the mineral surface is determined by existing weaknesses in the surface structure. A full description of the materials and methods of this ongoing study is presented in **Appendix C**. Comparing the same position on the mineral surface, before and after mycelial growth, will enable examination of how mineral structure affects mycelial colonisation and how mycelial colonisation affects the mineral surface. The material in this study has only been partially analysed and only preliminary results are available. In Fig. 19 the comparison procedure is exemplified, using an apatite surface before (a) and after (b) six months of colonisation by *H. crustuliniforme*. Micro-fissures and irregularities in the polished apatite surface are visible from the start (Fig. 19a). The presence of irregularities facilitates the re-localisation of the same position after mycelial colonisation. The apatite surface appears not to be affected by six months of mycelial colonisation (Fig. 19b). A possible increase in the number of etch-pits (at white arrow in Fig 19a & b) in the surface could be suggested from the comparison. Quantification may however be difficult since the resolutions of the two images are different. This is a result of the image from before mycelial colonisation were scanned directly on the mineral surface, whereas the images after mycelial colonisation are were carbon coated before the scan. After mycelial colonisation surface coating is a prerequisite to obtain contrast in the images.

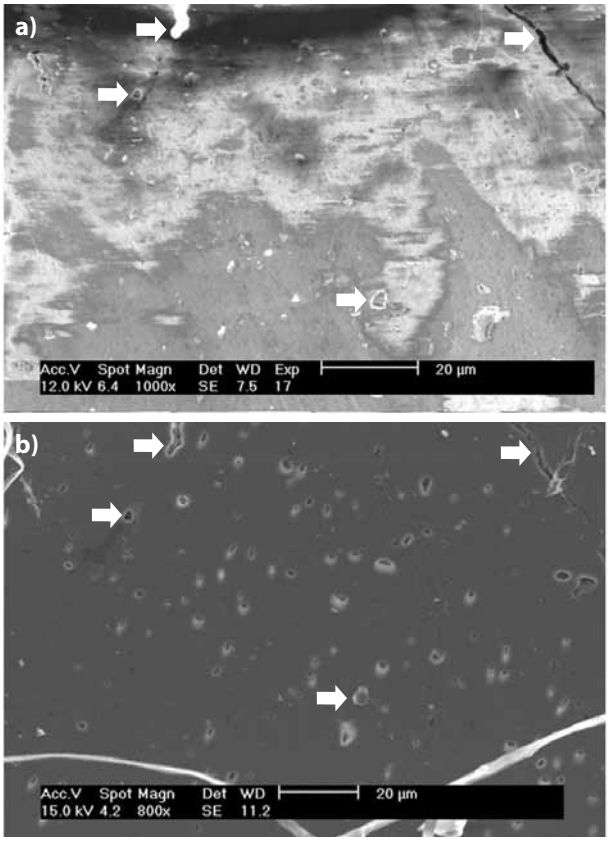


Fig. 19.
The same positions on a polished apatite surface were imaged by SEM-SE both before and after six months exposure to growing mycelia of *H. crustuliniforme* colonising a pine seedling in a peat microcosm. Apparent surface characters (arrows) were used to identify the same positions after mycelial growth. Scale bar 20 µm. a) The appearance of the initial uncoated apatite surface. b) After six months, the mineral sample was cut out of the colonising mycelium, air-dried and carbon coated before SEM imaging was repeated. The general impression is that the number of etch-pits in the apatite surface has increased as a result of mycelia colonisation. (**Appendix C**)

Using SEM–element diffraction spectrometry (EDS), element composition was analysed in the mineral directly under hyphae and in the adjacent mineral (Fig. 20). Mineral dissolution resulting from hyphal growth on the surface, followed by selective transport of ions from the dissolution site could result in differences in the element composition of the mineral below hyphae compared to un-colonised mineral. However no differences in element composition could be observed in this preliminary analysis of hornblende.

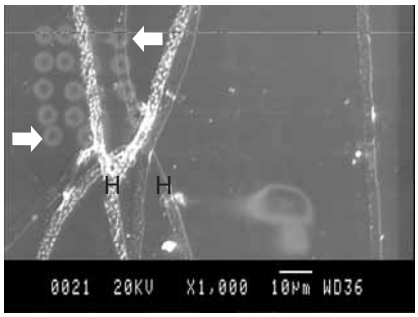


Fig. 20.
SEM–EDS spot analysis was used to determine how mycelial colonisation affected the surface element composition. Electron-excited areas in the surface (arrows) remain after analysis, enabling accurate positioning of the information on element composition. Element composition under hyphae (H) (arrow pointing left) and next to hyphae (arrow pointing right) was examined in air-dried and carbon coated hornblende samples that had been exposed to mycelium of *H. crustuliniforme* growing from a pine seedling in a peat microcosm. (**Appendix C**)

Mineral alteration, if any, may be restricted to the mineral surface, and thus not to be detectable by SEM–EDS analysis since information on element composition is collected from a deeper pear-shaped volume in the mineral. The actual volume depends on the mineral density and the applied voltage and other techniques may have to be applied in order limit the measurements to the uppermost layer of the mineral surface. Apatite surfaces were generally well colonised by mycelia, but at a finer spatial scale surfaces were not evenly colonised and some areas were less well colonised than others. Further studies using SEM–EDS may reveal whether there is a relationship between differences in surface element composition and different degrees of colonisation. Apart from mineral characteristics the degree of mycelial colonisation is largely affected by the moisture and stability of the mycelial growth environment. When harvesting mineral pieces from the peat microcosms, intense colonisation of the peat-covered sides of the mineral pieces was commonly observed. The environment within the peat is more similar to the conditions in soil, compared to the analysed polished mineral surfaces that were exposed to air. After 13 months of contact with peat and colonising mycelia massive colour alterations were observed on the sides of apatite pieces, from the initial brown-red surface colure of apatite into a white coating with red lines. The colure alteration is probably a result of secondary minerals being formed on the apatite surface as a result of weathering. The aim of the experiment to examine the weathering effects induce by ectomycorrhizal mycelial surface colonisation by eliminating other factors influencing weathering, such as substrate moisture, may have prevented a successful experimental design. Future studies of ectomycorrhizal weathering of minerals must take into account the conditions in the soil under witch weathering natural occurs.

4. Conclusions

This thesis demonstrates that:

1. As many as half of the ectomycorrhizal taxa colonising root tips in a boreal forest podzol may be primarily associated with the mineral soil horizons.

2. An accurate description and quantification of ectomycorrhizal communities in boreal podzol soils cannot be achieved unless mineral soil samples are included.

3. The mycelial growth of certain ectomycorrhizal fungi is stimulated by mineral components in the growth substrate.

4. In the heterogeneous soil environment, ectomycorrhizal fungi may selectively allocate carbon *via* rhizomorphs and hyphae to different mineral types.

5. Ectomycorrhizal fungi differ in their tendency to acidify mineral substrates with different element composition.

6. Ectomycorrhizal hyphae can alter the micro-topography of easily weatherable minerals surfaces *e.g.* calcite marble.

Together these findings support the idea that the growth and activity of ectomycorrhizal fungi on minerals in their growth substrate contribute to the development of micro-environments on colonised mineral surfaces, where increased weathering can take place.

5. References

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Appendix A, Identification of fungal species from hyphae and rhizomorphs, collected from fissures in boulders.

Material and Methods

Sampling of stones and corresponding moss, soil and root cover

Samples were collected during April 1998, from mixed pine forests on rocky granite soils in Lunsen in Uppsala, Sweden. Lifting the moss cover on boulders and inspecting the underside of the moss sheet, we examined the fungal colonisation of the rock surface. Samples were taken where extensive proliferation of ectomycorrhizal root tips and extending mycelia was found. Fissures in the selected boulders were split open with a pickaxe. The obtained pieces of rock were approximately 100 cm^3 and 2000 cm^3. The moss sheet was returned into its original position and cut off five cm outside the edge of the released rock fraction. Rock – moss packages were stored in darkness at + 4 ˚C. Roots were extracted from the moss sheet within a week and hyphae were collected from the exposed surface of the opened fissure within one month.

Collection of roots and rhizomorphs

Soaking for 15 min and subsequent rinsing in water removed excess soil from the roots in the moss sheet. Sampling of ectomycorrhizal root tips was carried out with the aim of collecting single root tips representing all species present in the sample, as defined by coarse morphotyping. A total of 96 mycorrhizal root tips was sampled.

The surface of opened fissures were found to be extensively colonised by fungal hyphae and rhizomorphs, by examination in a dissection microscope. Hyphal fragments, commonly rhizomorphs, were manually collected using tweezers, operating at 40x magnification. Small individual fragments were transferred to a _ ml droplet of sterile, double-distilled water in a PCR reaction tube.

DNA extraction and PCR amplification of the rDNA ITS region from root tips

DNA was extracted from individual root tips (Gardes & Bruns, 1993) excluding the initial freeze-thawing step. Following a modification of the protocol described by (Henrion *et al.*, 1994) the ITS region of the rDNA was amplified by PCR (Mullis & Faloona, 1987). The universal primers ITS4 (White *et al.*, 1990) and the fungal specific primer ITS1-F (Gardes & Bruns, 1993) were used. PCR was performed in 20 or 30 µl and the final concentrations of the reaction mix were the following: 0.2 mM of all four nucleotides, 0.3 µM of each primer, 3.1 mM MgCl2 and 0.175 u / µl of DNA polymerase (ExpandTM High Fidelity PCR System, Boehringer Mannheim, GmbH, Germany). Always including negative controls to detect possible contaminations. Optimal DNA template concentrations were established individually for each sample by testing amplification success using dilution series. DNA template was added as 25% of the final reaction

volume. The PCR program started with denaturation at 94 °C for 2 minutes, followed by 25 - 35 cycles of 94 °C for 15 s, 50 °C for 30 s and 72 °C for 60 s, finishing with 72 °C for 7 minutes, performed in a Perkin Elmer Gene Amp PCR System 2400 or a PC-960G Gradient Thermal Cycler.

From rhizomorph samples the ITS region was amplified directly, without prior DNA extraction, by nested PCR. Using the same parameters as described above, the first amplification was performed using ITS1-F and ITS4. A dilution, commonly 1:10 000 or 1:100 000, of the first PCR product was used as a template for the second amplification using the primers ITS5 (White *et al.*, 1990) and ITS4.

The quantity and quality of PCR products were examined by gel electrophoresis on 1,6% agarose gel at 4.7 V/cm for 1.5 hours (Gardes & Bruns, 1993) stained with ethidium bromide and visualised in UV light and documented on black and white Polaroid TM film.

Restriction fragment length polymorphism, RFLP

Restriction fragment analysis was performed on the samples where pure and strong DNA fragment were amplified by PCR. The fragments were cut with the restriction enzymes Hinf I, Mbo I and Taq I (Promega) for 2 hours at 37 °C. Gel electrophoresis was performed and visualised as above except that a 2.3% agarose gel (Metaphor agarose, FMC Bio Products) was used. The RFLP gel photos were scanned with Vista Scan (model S8.) and saved as TIFF format files and subsequently analysed by Taxotron (RestrictoScan® 1994, RestrictoTyper®1996, Adanson600 1994 and Dendrograf® 1994) from The Pasteur Institute, Paris, France. Fragments patterns were compared to the departmental reference library (Kårén *et al.*, 1997). DNA was sequenced according to the procedure of **Paper I**. Sequences were compared to the GenBank sequence database at NCBI using the BLAST program (Altschul *et al.*, 1997) on 2003-10-01.

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Appendix B, Examining hyphal interactions with surfaces of potassium feldspar and biotite.

Material and Methods

Mineral samples in microcosms

Potassium feldspar samples were broken with a hammer to obtain natural crack surfaces and biotite was cut with a pair of scissors, pieces with a diameter of *ca.* 0.5 cm were collected. The minerals were embedded in water agar in plastic cups (d = 1 cm), ensuring that the mineral surface was level with the water agar. These were introduced at the mycelial front in two months old peat microcosm with a pine seedling colonised by *H. crustuliniforme*. Microcosms were set up according to **Paper IV**. Alien mycorrhizal fungi, identified as *S. variegatus* by sequencing according to **Paper I,** spontaneous colonised parts of the root system.

Sample preparation and scanning electron microscopy (SEM)

One piece of potassium feldspar colonised by *H. crustuliniforme* and a number of pieces of biotite colonised by the alien *S. variegatus* were sampled approximately seven months after introduction in the microcosm. The minerals were well covered by mycorrhizal hyphae and had been so since soon after the introduction of the mineral. The dishes were cut out of the microcosm with a razorblade.

Samples were fixed and critical point dried to preserve the three-dimensional structure of the hyphae colonising the minerals. Samples were carefully kept from drying out throughout the process. Samples were fixed in 3% glutaraldehyde and 2% paraformaldehyde in 0.1M Na Cacodylate at pH 7.2, for two days. After pre-fixation samples were washed for 3 x 10 minutes in 0.1M buffer at pH 7.2. Thereafter samples were post-fixed in 2% osmium tetroxide overnight at 4 °C. After washing in distilled water for 2 x 10 minutes, samples were dehydrated stepwise in 20, 40, 60, 80, 90, 99.5% ethanol, each step for 15 minutes. Dehydration was completed by stepwise exchange of ethanol with acetone (2:1, 1:2 and finally only acetone, for 15 minutes each). Critical point drying was performed using a Polaron E3000 device, a carbon dioxide bomb at 35 °C and 1200 lb/in^3 overnight. Finally samples were mounted on stubs using double cellotape and gold coated using a Polaron E5000 sputter device. When the hyphal interaction with the mineral surface had been analysed by SEM the hyphae on potassium feldspar samples were partially removed with an adhesive tape and the minerals were again gold coated and observed. The removed hyphae on the tape were also mounted and gold coated to examine what adhered to the surface of the hyphae. The loose structure of biotite did not allow this approach for the biotite samples. The general surface characteristics of biotite and potassium feldspar were established by examining the parts of the mineral surface that had not been exposed to hyphae. Settings and equipment for scanning electron microscopy are described in **Paper IV**.

Appendix C, Mineral surface micro-topography, of apatite, calcite, hornblende and labradorite, before and after exposure to growing hyphae of *H. crustuliniforme*.

Material and Methods

Mineral samples

Pure minerals of apatite, calcite, hornblende and labradorite were obtained from Professor Ulla Lundström at Mid Sweden University. The minerals were cut into squares, approximately 2 mm high and 2 x 3 mm wide. The pieces were glued to a 1 mm thick glass plate to enable holding of the pieces while the upper surface was polished. Kjell Helge at Minoprep in Hunnebostrand, Sweden, performed cutting and polishing.

To enable comparison of mineral surface micro-topography before and after exposure to growing hyphae, defined locations on the mineral surface was examined by scanning electron microscopy (SEM). SEM was performed in the secondary electron mode, using a Philips ESEM or Hitachi 4200 FE-SEM operated at 12 or 15kV. Magnifications of 800x were used for the examination. Images were also captured at lower magnification to produce a site map of the surface to facilitate localising the same surface area after exposure to fungal hyphae. The mineral surfaces were not coated before SEM analysis and charging of the surface was a common problem disturbing the image quality. Voltage and spot size was modified to minimize surface charge problems and the time exposing each site was kept to a minimum. At least one side of each mineral piece was characterised by this approach. This was, however, rather time consuming and was eventually only done on four out of six pieces of each mineral.

Minerals in microcosms

Six peat microcosm systems with pine seedlings colonised by *H. crustuliniforme* were set up according to the method described in **Paper IV**. One piece of each mineral type was introduced into each system. The location of scanned and non-scanned mineral pieces in the six microcosms is presented in the Table below. When ectomycorrhizal seedlings were well established in the peat microcosms, pieces of marble were introduced at the mycelial front. The pieces were pushed into the peat so that the upper, polished surface was level with the peat surface. After 6 and 13 months respectively, half of the systems were harvested.

At harvest the mycelial coverage of the mineral pieces was documented by taking digital images of the systems and close up images of the individual pieces in a stereo–microscope equipped with a digital camera. The mineral pieces were cut out of the colonising mycelia using a razor blade. The pieces were air-dried before being mounted on a stud and carbon coated.

Table 1.
Microcosm systems were numbered 1 - 6. One piece of each mineral type, apatite, labradorite, calcite and hornblende, was introduced in each system. Whether the introduced mineral piece had been scanned before introduction is indicated by Scan followed by a number. Pieces that had not been scanned are indicated by No scan. The number of months (m) of mineral incubation in the microcosms before the microcosm was harvested is given under Harvest

System	Apatite	Labradorite	Calcite	Hornblende	Harvest (m)
1	No scan	No scan	Scan001	Scan001	13
2	No scan	No scan	Scan101	Scan101	13
3	Scan001	Scan001	Scan201	Scan201	6
4	Scan101	Scan101	Scan301	Scan301	6
5	Scan201	Scan201	No scan	No scan	6
6	Scan301	Scan301	No scan	No scan	13

Post hyphal-colonisation analysis of mineral surfaces

Hyphal interaction with the mineral surfaces and possible colonisation of pre exposure scanned parts of the surface was performed using the same equipment as described above. SEM-element diffraction spectrometry (EDS) point analysis of the element composition in the upper part of the surfaces was performed. SEM-EDS point analysis was performed in collaboration with Hanna Lind at the Department of Inorganic Chemistry at Stockholm University, Sweden.