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Plant-driven weathering of apatite – the role of an ectomycorrhizal fungus

M. M. SMITS,[1,2] S. BONNEVILLE,[3,4] L. G. BENNING,[3] S. A. BANWART[5] AND J. R. LEAKE[1]

[1]*Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield, UK*
[2]*Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium*
[3]*Earth and Biosphere Institute, School of Earth and Environment, University of Leeds, Leeds, UK*
[4]*Biogeochimie – Systeme Terre, Departement des Sciences de la Terre et de l'Environnement, Universite Libre de Bruxelles, Bruxelles, Belgium*
[5]*Kroto Research Institute, North Campus, University of Sheffield, Broad Lane, Sheffield, UK*

ABSTRACT

Ectomycorrhizal (EcM) fungi are increasingly recognized as important agents of mineral weathering and soil development, with far-reaching impacts on biogeochemical cycles. Because EcM fungi live in a symbiotic relationship with trees and in close contact with bacteria and archaea, it is difficult to distinguish between the weathering effects of the fungus, host tree and other micro-organisms. Here, we quantified mineral weathering by the fungus *Paxillus involutus*, growing in symbiosis with *Pinus sylvestris* under sterile conditions. The mycorrhizal trees were grown in specially designed sterile microcosms in which the supply of soluble phosphorus (P) in the bulk media was varied and grains of the calcium phosphate mineral apatite mixed with quartz, or quartz alone, were provided in plastic wells that were only accessed by their fungal partner. Under P limitation, pulse labelling of plants with $^{14}CO_2$ revealed plant-to-fungus allocation of photosynthates, with 17 times more ^{14}C transferred into the apatite wells compared with wells with only quartz. Fungal colonization increased the release of P from apatite by almost a factor of three, from 7.5 (±1.1) × 10^{-10} mol m^{-2} s^{-1} to 2.2 (±0.52) ×10^{-9} mol m^{-2} s^{-1}. On increasing the P supply in the microcosms from no added P, through apatite alone, to both apatite and orthophosphate, the proportion of biomass in roots progressively increased at the expense of the fungus. These three observations, (i) proportionately more plant energy investment in the fungal partner under P limitation, (ii) preferential fungal transport of photosynthate-derived carbon towards patches of apatite grains and (iii) fungal enhancement of weathering rate, reveal the tightly coupled plant–fungal interactions underpinning enhanced EcM weathering of apatite and its utilization as P source.

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Corresponding author: M. M. Smits. Tel.: +32 11 268224; fax: +32 11 26801; e-mail: mark.smits@uhasselt.be

INTRODUCTION

Phosphorus (P) is an essential element in all living cells, where it performs three vital functions in (i) the 'backbone' of the nucleic acids DNA and RNA, (ii) phospholipids, comprising cell membranes and (iii) ATP–ADP, the energy shuttles of cells (Marschner, 1995). In natural ecosystems, the primary source of P is provided by the weathering of minerals, the most important of which is the calcium phosphate apatite (Walker & Syers, 1976; Guidry & MacKenzie, 2000; Peltzer *et al.*, 2010), which accounts for over 95% of all P in the Earth's crust (Jahnke, 1992). It occurs as an accessory mineral in most igneous rocks, and associated soils, but often in small quantities (0.1–1%), both in the form of free grains, and as inclusions in other minerals (Deer *et al.*, 1997). During soil development on stable land surfaces over tens to hundreds of thousands of years, the apatite stocks become progressively depleted by weathering, but in the early stages of soil formation, they play a vital role in providing P to ecosystems (Walker & Syers, 1976; Zehetner *et al.*, 2008; Peltzer *et al.*, 2010). In many soils, P is the limiting nutrient for primary production, either directly or indirectly via limiting nitrogen fixation (Vitousek & Howarth, 1991). Furthermore, apatite weathering on the continental land masses is the primary source for the flux of P into marine ecosystems, ultimately determining marine primary production (Guidry & MacKenzie, 2000; Filippelli, 2008).

In addition to its critical role in controlling biogeochemical cycling of P between the continents and oceans, an increased understanding of the role of apatite weathering in supplying

plants with P has gained particular urgency in the context of the finite supplies of commercially available rock phosphate used to produce fertilizer (Gilbert, 2009). Global demand for P fertilizer is increasing to sustain and enhance the productivity of food crops, biofuels and timber production required by an increasing human population that has been projected to exhaust known stocks of rock phosphate suitable for manufacture of superphosphate fertilizer within 50–100 years (Cordell *et al.*, 2009). More recent evidence suggests that there may be much larger stocks of economically extractable high-grade rock phosphate that would enable fertilizer production rates to be sustained well into next century (Elser & Bennett, 2011). Nonetheless, these reserves are finite, increasingly expensive to purchase, and geographically concentrated in Morocco/Western Sahara with serious implications for global food security and sustainability, as a result of the carbon costs of mining, processing and transport to reach areas of agricultural production (Gilbert, 2009; Elser & Bennett, 2011; Syers *et al.*, 2011).

Against this background, there is urgent need to better understand the mechanisms by which plants in natural ecosystems acquire P from apatite. Forest ecosystems play an increasingly pivotal role in future sustainability, as commercial plantations can make a major contribution to sequestering carbon dioxide from the atmosphere, whilst providing timber and renewable fuel resources. However, many European forests have become progressively more P limited, in response to reactive nitrogen deposition arising from agricultural and industrial emissions of reduced and oxidized nitrogen compounds (Prietzel & Stetter, 2010), so their productivity and capacity to sequester carbon may be increasingly constrained by their rate of mobilization of P from soil minerals. There is therefore an emerging need in forestry to establish the potential to substitute P fertilizers required to maintain agricultural and biofuel production, with less strategically vital mineral sources of P such as the abundant rocks that contain apatite at concentrations too low for economic production of fertilizer. One of the most ancient and important adaptations of plants to increase the uptake of P from soil is the formation of symbiotic mycorrhizal associations between soil fungi and the roots of over 80% of plant species (Smith & Read, 2008; Wang *et al.*, 2010). Of particular interest is the boreal forest – the world's largest vegetation system that provides over 35% of global forest cover (Bonan, 2008) and which is dominated by ectomycorrhizal (EcM) pine, spruce and fir established on young soils that are not depleted in apatite as they have developed on landscapes last glaciated only 10 000 years ago (Read *et al.*, 2004).

Ectomycorrhizal fungi are known to be involved in the release and uptake of rock-derived nutrients, including P from apatite (for reviews see Hoffland *et al.*, 2004; Finlay *et al.*, 2009). These symbionts suppress the formation of root hairs and form sheaths that completely envelop over 95% of the fine root tips (Fransson *et al.*, 2000), with hyphae extending inwards between the cortical cells, from which they obtain sugars, and outwards into surrounding soil, from which they obtain nutrient elements (Smith & Read, 2008). In EcM associations, virtually all plant nutrient, water uptake and carbon release from fine roots occur via mycorrhizal fungal mycelium because the roots themselves have little or no direct contact with soil solution or soil particles (Leake *et al.*, 2008). EcM mycelial systems can extend decimetres away from root tips (Agerer, 2001) and form an absorptive network that may commonly extend to 200–400 m g^{-1} soil (Leake *et al.*, 2004). To support this fungal network, 20–30% of total photosynthate carbon fixed by the host trees is allocated to these fungi (Högberg & Högberg, 2002), which they use to grow, secrete organic acids and ligands/chelators, and actively uptake and transport nutrients. The ability of EcM fungi to connect between plant roots and microsites on mineral surfaces via mycelial networks that pass through bulk soil enables plant photosynthate energy to be directed to, and weathering products exported from, specific mineral surfaces at the scale of individual hyphae (5–15 μm diameter) (Leake *et al.*, 2008; Smits *et al.*, 2008). In a microcosm study, EcM fungi colonized more intensively and allocated more photosynthetically derived carbon into patches with potassium feldspar grains than patches with quartz (Rosling *et al.*, 2004), and trees extract more potassium from muscovite when colonized by the fungus *Paxillus involutus* (van Schöll *et al.*, 2006). In EcM forests established on sand dunes in North Michigan, significant differences in fungal cover on mineral grains occurred in the order plagioclase > microcline > quartz (Smits, 2009).

Mycorrhizal colonization has been shown to increase P uptake from apatite into trees (Wallander *et al.*, 1997; Wallander, 2000), but it remains unclear how much of the increased mineral weathering is because of soil chemistry and other micro-organisms, as the effects of EcM have not been isolated from these potentially confounding factors. On the other hand, whilst pure culture studies of EcM fungi on mineral weathering can directly attribute effects of the fungi (e.g. Paris *et al.*, 1995; Balogh-Brunstad *et al.*, 2008), the growth, morphology and physiology of fungi differ markedly in the absence of a host plant, *in vitro* on agar or in liquid media, compared with the physiology and biological functioning when growing symbiotically in natural environments (Rayner, 1991; Jennings, 1995; Smith & Read, 2008). Furthermore, a fundamental characteristic of this fungal–plant symbiosis is the source–sink relationship driving exchanges of photosynthates and nutrients. Leaving the plant partner out disconnects the fungal mycelium from an important sink for nutrients, and the mass flow pathway of water loss from plant shoots that drives some of the fungal uptake of nutrients. To grow the fungi without a plant necessitates the use of an external energy source (generally glucose) supplied throughout the media, suppressing the formation of multicellular hydrophobic mycelial cords that are characteristic of many EcM fungi in soil and

are used for long-distance reciprocal transport of assimilates from plants and nutrients from soil (Agerer, 2001; Hobbie & Agerer, 2010).

As plant energy powers the EcM metabolism, we have hypothesized that it is the quantity of plant photosynthate energy allocated to the fungus in contact with specific minerals that ultimately determines the weathering activity of the fungal partner (Leake *et al.*, 2008; Brantley *et al.*, 2011). It has recently been suggested that EcM weathering can respond to host nutrient demands, but the evidence remains somewhat equivocal (Rosenstock, 2009). To rigorously test this hypothesis for the first time, we deployed a microcosm design that we have previously developed to study single hypha–mineral interactions (Bonneville *et al.*, 2009, 2011; Saccone *et al.*, in press) with EcM fungi grown in symbiotic association with a host tree under axenic conditions (see Leake *et al.*, 2008). In the present study, we sought to test three hypotheses: (i) that a greater proportion of plant energy would be invested in an EcM fungal partner under plant P limitation, (ii) there will be preferential fungal transport of photosynthate-derived carbon to apatite grains compared with quartz grains and (iii) the enhanced photosynthate energy allocation to EcM fungal hyphae colonizing apatite grains is associated with enhanced mineral weathering rates.

METHODS

Minerals

The fluorapatite [$Ca_{11.5}(PO_4)_6(F_{1.3}Cl_{0.02}\ OH_{3.7})$] originated from Minas Gerais, Brasil (obtained from Krantz Company, Bonn, Germany). The identity was confirmed with X-ray diffraction (XRD; Philips PW1050, Cu K α, Eindhoven, the Netherlands), and composition was determined with X-ray fluorescence (XRF, Axios, Panalytical, Almelo, the Netherlands). The apatite was crushed in an agate mortar, and the 150- to 250-μm fraction (wet sieved using stainless steel sieves) was ultrasonically cleaned in demineralized water. The Brunauer–Emmett–Teller (BET) surface area of the apatite in N_2 was measured using a Gemini V2365 system (Micromeritics Instrument Corp., Norcross, GA, USA) and found to be 0.02 $m^2\ g^{-1}$. In these experiments, the apatite was mixed with processed high-quality (SiO_2: >99.7%) silica sand (Redhill T; WBB Minerals, Sandbach, UK), sieved to the same size fraction (150–250 μm).

Ectomycorrhizal inoculation

Pinus sylvestris seeds were surface sterilized and aseptically germinated (Leake *et al.*, 2001) on 8% water agar, in a controlled environment chamber (18 h day with photon flux density of 250 μm $m^2\ s^{-1}$ and 15 °C, 10 °C night, relative humidity 60–75%). Three weeks after germination, the tree seedlings were transferred to sterile Petri dishes with their roots on 10% MMN agar (see Table S1) pre-inoculated with the EcM fungus *Paxillus involutus*. The shoots emerged via a hole in the side of the lid, which was sealed around the stem with autoclaved, sterile lanolin. After 20 weeks, the plants had formed EcM roots and were ready to be transplanted into experimental microcosms. Control non-mycorrhizal trees were grown in parallel in Petri dishes, but without the fungus.

Microcosm experiments

Microcosms for weathering studies comprised square Petri dishes (10 × 10 cm) that were filled with 100 mL of 1% agar, containing inorganic nutrients either with or without orthophosphate (+P, −P, see Table S1). The agar was covered with a sheet of cellophane, and on the top, an extra 20 mL of the nutrient agar was added to immobilize a monolayer of acid-washed sieved (2.0 < 2.4 mm) perlite grains that were inserted into four polypropylene wells that provided discrete weathering arenas (Fig. 1). The tree seedlings were transplanted with their roots on the perlite and shoots emerging outside through a slot cut in the side of the dishes, sealed around the stem with sterile lanolin, and the lid and base of the dishes held together and sealed with strips of parafilm to maintain axenic conditions.

In the first experiment ('*Weathering experiment*'), all four wells were filled with either 1 g of sterilized (4 h at 180 °C) quartz sand mixed with apatite (5% w/w) ('Ap') or with 1 g sterilized quartz sand only ('Qu'). Each well was moistened with 170 μL autoclaved demineralized water (corresponding to 70% of water holding capacity of the quartz sand). Both 'Ap' and 'Qu' wells were supplied to '+P' and '−P' microcosms, giving four treatments, with five replicate microcosms each. Additionally, apatite and quartz wells were incubated in replicate microcosms of the same design but with a non-mycorrhizal tree.

In the second experiment ('*C-allocation experiment*'), two of the four wells were filled with 10% (w/w) apatite amended quartz sand, and the two other wells with quartz sand only (see Fig. 1). Four replicated microcosms were set up with −P nutrient agar.

All microcosms were covered in aluminium foil to exclude light from the root and mycorrhizosphere compartment, but with their shoots uncovered, and placed for 20 weeks in a controlled environment chamber (18 h day with photon flux density of 250 μm $m^2\ s^{-1}$ and 15 °C, 10 °C night, relative humidity 60–75%). After 10 weeks of growth, sterile water was aseptically added to the agar via a syringe with a fine needle once a week to replenish losses from evapotranspiration from the shoots.

Analyses

On a weekly basis, individual wells were closely inspected and any fungal colonization was recorded. After the planned

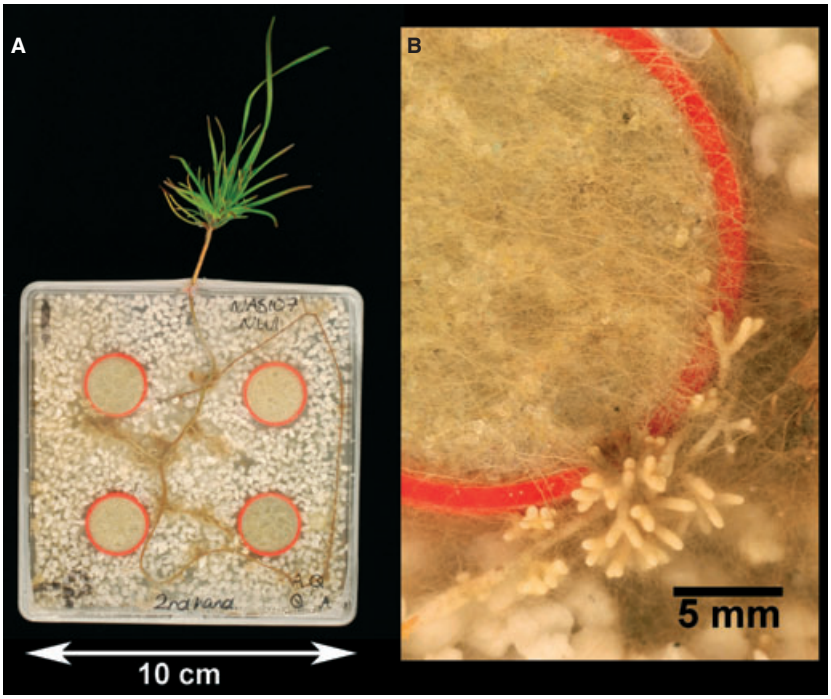

Fig. 1 (A) Sterile microcosm containing *Pinus sylvestris* ectomycorrhizal with *Paxillus involutus*, with plastic wells containing 10% (w/w) apatite in quartz sand (top left and bottom right) and two wells with quartz sand only (top right, bottom left). (B) Detail of an apatite containing well, colonized by *Paxillus involutus*.

20-week growth period, the microcosms were moved to an illuminated fume cupboard (18-h light, photon flux density 250 μmol m^{-2} s^{-1}). In the middle of one of the 18 h day periods, the microcosms were individually sealed into 0.675-L gas-tight, clear Perspex boxes with a layer of moistened tissue paper on the base, into each of which was released 0.74 kBq $^{14}CO_2$ as described by Leake *et al.* (2001). At the end of the photoperiod, the boxes were unsealed by the removal of a bung plugging a 9-mm-diameter hole in the lids.

The microcosms were dissected after 48 h, as previous microcosm work showed that ^{14}C allocation to EcM mycelium reaches a peak 24–48 h after shoots start fixing $^{14}CO_2$ (Leake *et al.*, 2001). First, the wells were removed from the microcosms and transferred into air-tight jars containing an open Eppendorf tube with 0.5 mL 2 M KOH solution to trap accumulated CO_2 from root and fungal respiration, including that dissolved in moisture. After 1 h, during which time $^{14}CO_2$ accumulated in the microcosms in moisture in the wells will be depleted, the traps were renewed. The second set CO_2 traps were incubated with the wells from 1 to 23 h to determine fluxes of $^{14}CO_2$ from fungal respiration after severance of connections to the host plants, after which time the contents of the wells were freeze-dried. Meanwhile, the other components (shoot, roots and perlite together with external mycelium) were also freeze-dried. All freeze-dried samples were weighed and split into subsamples for ^{14}C analysis and for nutrient analysis.

In the *C-allocation experiment*, after ^{14}C labelling of the plants as described for the *weathering experiment*, digital autoradiographs of a microcosm were taken four times between 24 and 48 h later (Packard Instant Imager; Packard Canberra Instruments, Meriden, CN, USA). All the microcosms were then dissected in the same way as in the *weathering experiment*, except EcM root tips were carefully dissected from the remainder of the root systems and analysed separately.

The amount of ^{14}C in the KOH traps was measured using liquid scintillation counting, the Eppendorf tubes being vortexed to homogenize and suspend the absorbed carbonate, with 50-μL aliquots removed and added to 1 mL Ultima gold (PerkinElmer, Buckinghamshire, UK), and counted in a Packard Tricarb 3100TR. The amount of ^{14}C in subsamples of the freeze-dried plant parts, perlite and well contents were determined by sample oxidation (Packard 307 sample oxidizer) and scintillation counting (Packard Tricarb 3100TR) as described by Leake *et al.* (2001).

Subsamples (0.05 g) of shoots and roots were digested in 1 mL concentrated H_2SO_4 and 0.6 mL H_2O_2 at 320 °C. Control wells with 5% w/w apatite, incubated in non-mycorrhizal microcosms, were shaken for 10 min in 5 mL demineralized water to determine the amount of soluble P present. Orthophosphate in digested samples and mineral rinsates was measured by ammonium molybdate and ascorbic acid reagent (John, 1970) by spectrophotometry at 882 nm.

As a proxy measure of fungal biomass in the perlite substrate, the grains were finely ground; the fungal chitin was hydrolysed in 6 M HCl at 80 °C and quantified by spectrophotometry (following Plassard *et al.*, 1982). It was not possible to measure chitin content in the apatite wells because of analytical interference with apatite.

Statistical analysis

Plant and fungal biomass and P-content data of the 'weathering experiment' were analysed using ANOVA with the factors +/− orthophosphate and +/− apatite, followed by Tukey post hoc tests (Zar, 1984), to resolve differences between the means of the four treatments. Biomass and P-content data were natural-log-transformed to increase homogeneity of variance and normality, but are presented as actual means. For the 'C-allocation experiment', ^{14}C accumulation and $^{14}CO_2$ release were compared in the quartz and apatite wells using a two-way ANOVA to test for the main effects of apatite vs. quartz wells, with the replicate microcosms included as a random factor to account for the reduced degrees of freedom in the nested design. All analyses were performed in SPSS 17.0.

RESULTS

Weathering experiment

The fungus developed networks extending from the dichotomously branched, fungal-sheathed root tips, to form multicellular hyphal cords that grew over almost the entire perlite layer, dividing down into individual hyphae (Fig. 1A,B), reflecting the natural growth of EcM fungi in soils (Smith & Read, 2008). Fungal hyphal growth was especially intense in wells containing apatite grains, and the EcM root tips closest to these locations often showed a very high frequency of branching (Fig. 1B). Within the first 5 weeks, more than 30% of the wells were colonized by the fungus, increasing to 95% by week 20 of the experiment (Fig. S1). On average, it took 9.8 (±3.2) weeks from the start of the experiment, before a well became colonized. Neither the presence of apatite in the well nor the absence of P in the nutrient solution affected the time taken for the fungus to first grow into a well (ANOVA, $F(3,9) = 0.67$, $P = 0.59$).

The presence of apatite did not significantly ($P > 0.05$) increase shoot or root weights of the trees (Fig. 2A, Table 1). The trees growing with P added to the agar showed significantly higher shoot weight ($P = 0.04$, Table 1), but root weight did not differ from those of plants provided with apatite as sole P source (Tukey test $P > 0.05$, Fig. 2A). Root weight, expressed as proportion of total biomass (plant + external mycelium), did not differ significantly between the treatments, but the proportion of the biomass in external fungal mycelium progressively declined along the gradient of increasing P supply from −P quartz to +P apatite treatments (Fig. 3A), an effect

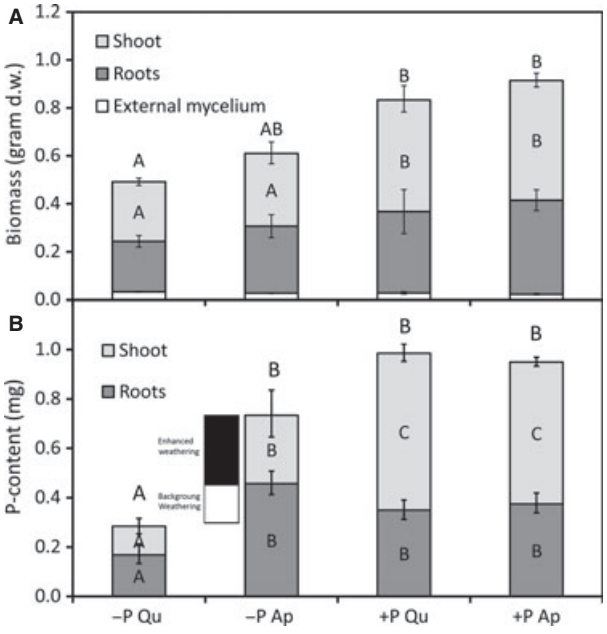

Fig. 2 Results of the *weathering experiment*: (A) biomass of *Pinus sylvestris* shoots and roots and of external mycelium of *Paxillus involutus*. Different capital letters denote significant difference ($P < 0.05$) based on Tukey post hoc test for comparisons between mean shoot and total plant biomass across the four treatment combinations: −P = nutrient solution without orthophosphate, +P = nutrient solution with orthophosphate, Qu = quartz only, Ap = 5% apatite mixed with quartz. Standard errors of the means of roots and shoots are shown. There were no significant differences in the biomass of external mycelium or roots across the four treatments ($P > 0.05$). (B) P-content in the shoot, roots and total plant biomass in response to orthophosphate and apatite treatments, with Tukey post hoc test results indicated as in (A), with ±1 standard error indicated for roots and shoots. The white bar shows the amount of P released from the apatite by rinsing well contents of control microcosms containing non-mycorrhizal *Pinus sylvestris*, and the black bar shows the inferred net weathering by the fungus.

that was significant ($P = 0.02$) for comparisons between overall effects of −P and +P treatments (Table 1).

The proportion of ^{14}C allocation to roots, as with biomass, showed no significant effects of the P treatments, whereas the proportion allocated to external mycelium showed an even stronger and more significant ($P < 0.001$, Table 1) decline with increasing P supply (Fig. 3B) than biomass (Fig. 3A).

The biomass of external mycelium of *Paxillus involutus* was produced in inverse proportion to the biomass of *Pinus sylvestris* roots ($R^2 = 0.55$, $P = 0.02$, Fig. 4). This indicates that there are important trade-offs between plant carbon investment into root biomass (including mycorrhizal root tips) and mycorrhizal external mycelium, the greatest investment in the latter tending to occur most frequently when P is most limiting, as in the −P quartz treatment (Fig. 4). Interestingly, microcosms in which apatite was the sole P source showed the widest range of root and external fungal biomass of all the treatments.

Table 1 ANOVA results for main effects on *Pinus sylvestris* ectomycorrhizal with *Paxillus involutus* of supplying wells containing apatite mixed with quartz vs. quartz only, in microcosms with agar nutrient solutions lacking or containing orthophosphate, and the interaction between the mineral and nutrient solution treatments

			P value		
	Degrees of freedom	*F* ratio	Apatite	Ortho phosphate	Apatite × ortho phosphate interaction
Biomass shoot	1, 13	10.3	0.23	**<0.001**	0.55
Biomass roots	1, 13	2.6	0.25	**0.04**	0.70
Biomass external mycelium	1, 10	1.8	0.12	0.15	0.86
Biomass total plant	1, 13	6.03	1.82	**<0.001**	0.56
P-content shoot	1, 13	36.2	**0.01**	**<0.001**	**<0.001**
P-content roots	1, 13	6.6	**0.01**	0.13	**0.02**
P-content total plant	1, 13	24.7	**<0.001**	**<0.001**	**<0.001**
Root/total plant biomass (w/w)	1, 10	0.7	0.49	0.31	0.96
External mycelium/total plant biomass (w/w)	1, 10	3.1	0.27	**0.02**	0.64
^{14}C root/^{14}C total plant plus fungus	1, 10	0.4	0.96	0.30	0.90
^{14}C external mycelium/^{14}C total plant plus fungus	1, 9	4.4	0.44	**0.01**	0.80

Values in bold typeface are significant at ($P < 0.05$).

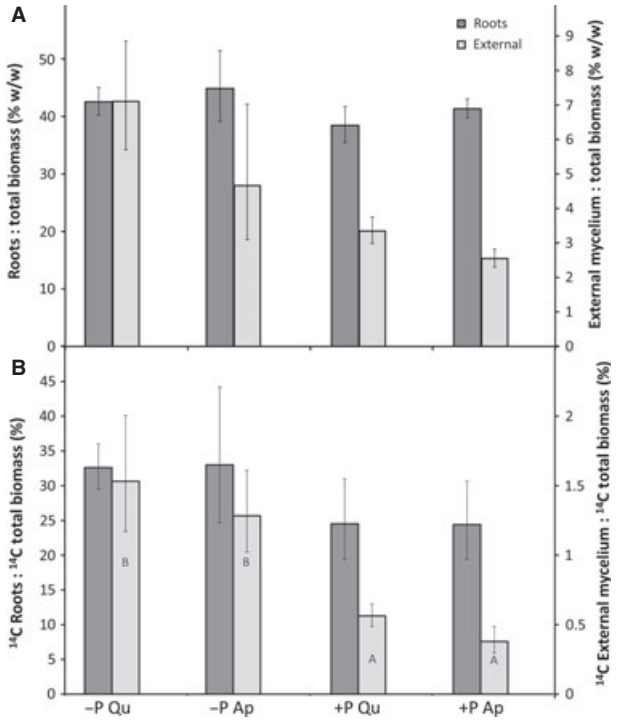

Fig. 3 Results of the *Weathering experiment*: (A) root biomass (dark grey bars and axis on the left) and external mycelium biomass (light grey bars and axis on the right) both as a percentage of the total plant and fungal biomass. *X*-axis treatment codes as in Fig. 2, and error bars denote ±1 standard error. No significant differences were found between treatments. (B) Allocation of ^{14}C to roots (dark grey bars and axis on the left) and external mycelium (light grey bars and axis on the right) both as a percentage of the ^{14}C in the combined plant and fungal biomass. Error bars denote ±1 standard error. Where there are significant differences between treatments ($P < 0.05$), these are shown by bars not sharing the same letter code based on Tukey post hoc test.

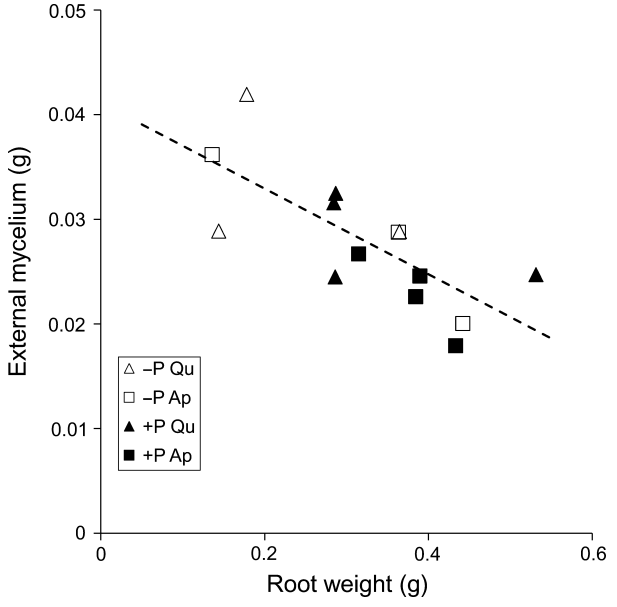

Fig. 4 Results of the *Weathering experiment*: the relationship between biomass of roots and external ectomycorrhizal mycelium ($y = -0.041x + 0.011$, $R^2 = 0.55$, df = 12, $P = 0.002$). Mineral treatment codes as in Fig. 2.

The P-content of the mycorrhizal trees more than doubled in the presence of apatite as sole source of the element, compared with trees grown under −P conditions, supplied with quartz (Fig. 2B). P release from apatite in non-mycorrhizal controls was 0.14 (±0.02) mg per microcosm, based on water rinsates of these wells, that is, approximately one-third of the total uptake of P in the plants with fungal access to apatite (0.41 mg, see also the black/white bar in Fig. 2B). This corre-

sponds to an average weathering rate over the duration of the experiment (20 weeks) of 7.5 (±1.1) × 10^{-10} mol P m^{-2} s^{-1} in control wells and 2.2 (±0.52) × 10^{-9} mol P m^{-2} s^{-1} in wells with fungal access. Access to apatite as sole P source increased the total P-content of plants to attain values not significantly different to those in the plants grown with orthophosphate supplied in the agar medium (Fig. 2B). However, the shoot P concentrations in the plants supplied with apatite only were significantly lower (Tukey test, $P < 0.05$), than in plants provided with orthophosphate, and the proportion of P retained in roots (which will include mycorrhizal fungus) appeared especially high (Fig. 2B).

C-allocation experiment

In the C-allocation experiment, each microcosm contained one pair of wells with apatite and another pair with only quartz, which showed much less intensive fungal colonization. The fungal hyphae in the apatite wells were covered with white crystal deposits, visible under dissecting microscope, that resemble the calcium oxalate deposits confirmed by spectroscopic analyses to occur in similar microcosm studies with the same host plant and fungus, but with wells containing 100% (1 g) apatite grains (Schmalenberger *et al.*, 2009). P concentrations in the shoots were in the same range as found in the *weathering experiment* (0.90 and 0.98 μg g^{-1}, respectively). Again, most P (68%) was found in the belowground parts of the plant with more than 50% in the EcM root tips (Fig. S2).

The shoots adsorbed more than 90% of the added $^{14}CO_2$ within 4 h. Owing to technical problems, only one of the four replicate seedlings could be used for autoradiographic studies of the dynamic carbon flux from the plant to the mycorrhizal fungal partner *in situ*, but all microcosms were suitable for ^{14}C budgeting via the oxidation–scintillation counting method. The microcosm that could be used for autoradiography showed a much stronger allocation of ^{14}C into apatite wells (Fig. 5A top left and bottom right; Fig. 5B), compared with quartz wells (Fig. 5A, top right and bottom left; Fig. 5B), 24 h after supply of $^{14}CO_2$ to the plant. From 24 to 45 h, the ^{14}C activity in the apatite wells decreased substantially, indicating respiratory losses faster than allocation of further ^{14}C assimilates (Fig. 5B). However, from 45 to 49 h, the ^{14}C counts in these wells increased again, coinciding with a strong increase in the total belowground ^{14}C signal (Fig. 5B), almost certainly reflecting mobilization during the previous night of ^{14}C-labelled starch in the plant shoots accumulated during the initial exposure to $^{14}CO_2$ the two days earlier. This mobilization of stored carbohydrates maintains assimilate supply to roots and fungal partner through the daily light–dark cycle that drives alternation of photosynthesis of new carbohydrates and reliance on reserves. Both quartz wells did not show such an increase (see inset Fig. 5B), suggesting that the very small quantities of ^{14}C allocated to these wells are less influenced by the temporal dynamics of plant and fungal recent assimilate pools.

These findings were further corroborated by the other microcosms in which ^{14}C was preferentially allocated into the apatite rather than quartz-only wells, by a factor of 17, based on oxidation–scintillation measurements (Fig. 6A). This apatite effect was highly significant ($P < 0.001$) in two-way ANOVA, in which microcosm was included as a random factor to account for the nested design (Table 2). Analysis of $^{14}CO_2$ released from the wells after they removed from the

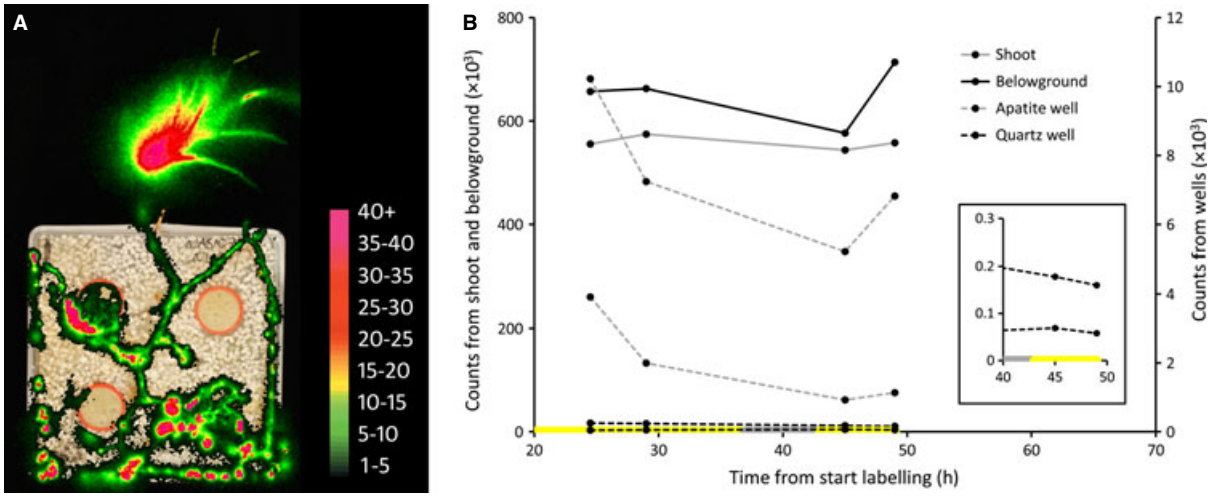

Fig. 5 Results of the *C-allocation experiment*: (A) digital autoradiograph showing ^{14}C distribution 24 h after commencing labelling of shoots on a false colour scale of counts per 0.25 mm^2 pixel after imaging for 15 min, (B) counts of ^{14}C detected in different components of the plant–fungus autoradiograph over time. The yellow shading along the *X*-axis denotes day and the black shading shows night. Inset shows data for the two quartz wells on an expanded *Y*-axis scale for the final two time intervals of measurement.

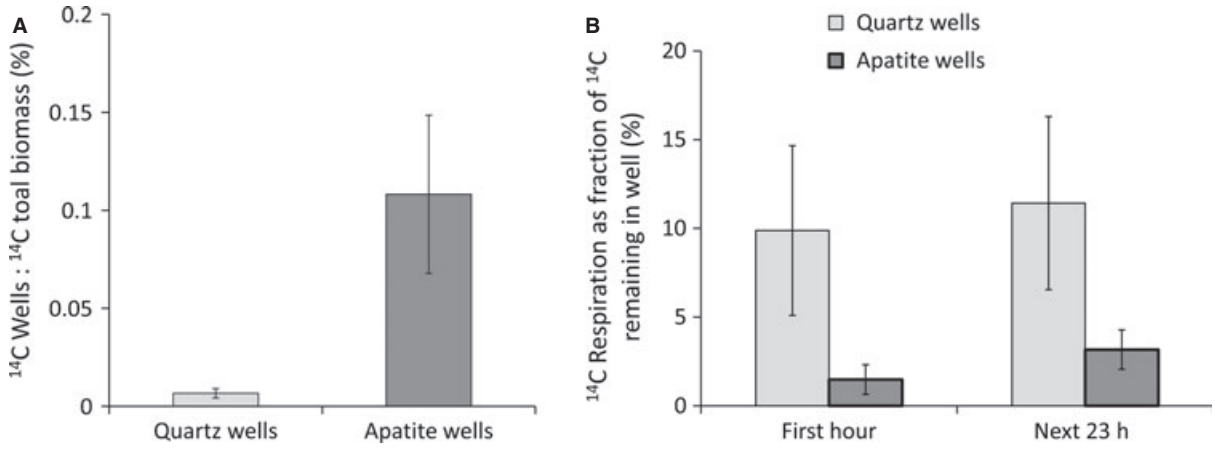

Fig. 6 Results of the *C-allocation experiment*: (A) comparison of total ^{14}C content of wells as a percentage of total ^{14}C remaining in plant and fungus at harvest, measured after sample oxidation, $P < 0.001$ for effect of apatite vs. quartz, with microcosm included as a random factor to account for the nested design of the experiment (see Table 2). (B) The proportion of ^{14}C in wells released as $^{14}CO_2$ after 0- to 1-h incubation and 1- to 23-h incubation after removal from the microcosms, the latter indicative of fungal respiration. Means are shown of the four replicated microcosms. Error bars denote ±1 standard error. Apatite and quartz wells did no differ significantly in first-hour respiration ($P > 0.05$), but did differ in the next 23 h ($P < 0.01$), based on ANOVA (Table 2), including microcosm as a random factor to account for the nested design of the experiment.

Table 2 ANOVA results for ^{14}C content of wells as a percentage of total ^{14}C remaining in plant and fungus at harvest and $^{14}CO_2$ released from wells after 1 h and 23 h as a percentage of ^{14}C remaining in wells, in all cases in relation to mineral type (apatite vs. quartz-only wells), with microcosm ($n = 4$) as a random factor to account for the nested design

Variable	Factors	Degrees of freedom	*F* ratio	*P* value
^{14}C content of wells, as a percentage of total ^{14}C in biomass*	Mineral (Ap vs. Qu)	1,11	46.21	**<0.001**
	Microcosm (as random factor)	3,11	0.50	0.692
$^{14}CO_2$ released from wells after 1 h as a proportion of ^{14}C in wells†	Mineral (Ap vs. Qu)	1,11	0.13	0.723
	Microcosm (as random factor)	3,11	0.18	0.905
$^{14}CO_2$ released from wells 1–23 h, as a proportion of ^{14}C in wells†	Mineral (Ap vs. Qu)	1,11	11.60	**0.006**
	Microcosm (as random factor)	3,11	3.36	0.056

Values in bold indicate $P < 0.05$.
*Natural-log-transformed to correct for heteroscedasticity.
†Arcsine square root-transformed.

microcosms, using the same approach (Table 2), revealed no significant difference between the proportion of the ^{14}C in the wells that was released CO_2 from apatite and quartz-only wells incubated for the first 1 h ($P > 0.05$), but found a significantly higher proportional release from quartz-only wells in the subsequent 23 h (Fig. 6B), consistent with the greater ^{14}C retention in wells containing apatite (Figs 5B and 6A).

DISCUSSION

Apatite weathering rate

In the *weathering experiment*, an average of 110 µg P was removed from each apatite well, corresponding to a rate of $2.2{\cdot}(\pm 0.52) \times 10^{-9}$ mol m^{-2} s^{-1}, or expressed as dissolved apatite $[Ca_{10}(PO_4)_6F_2]$: 3.7×10^{-10} mol m^{-2} s^{-1}. This mycorrhizal weathering rate is in the range of reported abiotic dissolution rate for fluorapatite at pH ±5.5, whilst the dissolution rate in the control microcosms was similar to reported fluorapatite dissolution rates at neutral pH (Guidry & MacKenzie, 2003; Chaïrat *et al.*, 2007; Welch *et al.*, 2002; see Fig. S3). Fungal activity could have caused a drop in pH, as seen in similar microcosm experiments where *Paxillus involutus*, again mycorrhizal with *Pinus sylvestris*, caused significant acidification around its hyphae on biotite surfaces (Bonneville *et al.*, 2011). Such acidification would increase apatite dissolution, but as we did not measure pH in the wells, we cannot verify that acidification was the driving factor in fungal weathering in the present study.

Alternatively, fungal exudation of organic chelators could be responsible for the increase in apatite dissolution rate, alone or in combination with acidification. We found crystal deposits, most likely calcium oxalate, on the fungal hyphae and only in the apatite wells. Previous studies have established that the

crystals formed on *Paxillus involutus* growing in symbiosis with *Pinus sylvestris* on apatite are calcium oxalate (Schmalenberger *et al.*, 2009). Oxalate is proposed as an important fungal weathering agent (e.g. Graustein *et al.*, 1977; Landeweert *et al.*, 2001). We are aware of only one study showing a mineral-specific response in EcM fungal oxalate production (Schmalenberger *et al.*, 2009), but, as in our experiment, it could not be excluded that oxalate production was upregulated by high calcium concentrations (Jackson & Heath, 1993) rather than a unique and specific response to apatite grains. For oxalate exudation to be responsible for significantly enhancing the apatite dissolution rate, the oxalate concentration should be relatively high (>1 mM, see Fig. S3).

Wallander (2000) studied apatite weathering in a pot experiment in which soil solutions could migrate between fungal and root compartments. He found that apatite weathering increased (by a factor of 1.5–2.8) when EcM fungi were present, but, as these experiments were carried out in non-sterile soils, a direct role of EcM in this weathering scenario could not be unequivocally demonstrated because the effects of other micro-organisms and products of living and dead roots could not be excluded. Interestingly, Wallander (2000) found a positive relationship between phosphate and oxalate concentrations in the fungal weathering compartment, indicating that oxalate (<0.6 mM) could play a role in fungal-mediated apatite dissolution. Such a low oxalate concentration may suggest a minor, direct dissolution effect; however, if the concentrations reach locally much higher values at the surface of apatite grains through intense and targeted fungal–mineral interactions, as indicated by Leake *et al.* (2008), Schmalenberger *et al.* (2009) and in the present study, it seems likely that oxalate plays a more significant role in weathering.

Carbon allocation

The ^{14}C labelling experiment (Fig. 5A) permitted the visualization of the role of EcM fungi in connecting plant photosynthetic energy to the weathering of nutrient-rich minerals. The presence of apatite wells not only shaped the fungal biomass distribution and activity (Figs 1 and 5A), but also the EcM root tip distribution and the whole root architecture as well. Localized patches of apatite grains supplying P to the fungal partner stimulate the proliferation of EcM root tips near to the resource, shortening the distance required for fungal transport of P to the plant in return for photosynthate-derived carbon. Such increased EcM root tip growth has also been observed on the surface of mesh bags containing apatite buried in forest soils (Hagerberg *et al.*, 2003).

At the end of our experiments, the mineral wells were colonized for, on average, 10 weeks, and the colonizing fungus was still active as shown with the ^{14}C labelling. This suggests that there may be a continuous resupply of nutrients detected by the fungus. It is likely that the durability of the nutrient source permits the build-up of a long-term nutrient and carbon exchange structure of roots and EcM root tips around the mineral wells. In contrast, exploitation of labile nutrients in litter patches by EcM may be so fast (Leake *et al.*, 2001) that there may be less advantage to allocate root biomass close to such resources as these may be very transient. Exploitation of nutrients in localized litter patches can give rise to rapid expansion of the whole EcM mycelia network (Leake *et al.*, 2004), whilst in other cases, there is persistent intensive proliferation in the depleted resource (Leake *et al.*, 2001).

In the ^{14}C labelling experiment, the apatite wells received 17 times more ^{14}C than the quartz wells, which is the same magnitude of response reported by Rosling *et al.* (2004) for ^{14}C allocation to patches of potassium feldspar vs. quartz in non-sterile peat microcosms. The proportion of ^{14}C allocated by the fungus into wells that was subsequently respired was seven times lower in the apatite wells, compared with the quartz wells (Fig. 6). The higher proportion of ^{14}C allocation by *Paxillus involutus* retained in apatite rather than quartz wells may reflect active secretion of oxalic acid by the fungus and its accumulation, as suggested by the observed crystals on hyphae, and previous studies confirming calcium oxalate crystals on the fungus grown under similar conditions (Schmalenberger *et al.*, 2009). Bidartondo *et al.* (2001) reported a 17% reduction in respiration rate, normalized to fungal biomass, for *Paxillus involutus* in contact with apatite for 10 days, but a strong increase for two *Rhizopogon* isolates, compared with control compartments without apatite. In our experiment, the fungus was in contact with apatite for an average of 10 weeks, which is likely to have affected the quantities of dissolved calcium, oxalate secretion and fungal morphology including the extent of rhizomorph production. Together, these factors will affect respiration rates of the fungi relative to biomass.

The absence of readily available P stimulated the growth of external mycelium (Fig. 3A), at the cost of root biomass (Fig. 4). Also the ^{14}C labelling showed an increased carbon allocation into the external mycelium. This is in line with earlier findings with other EcM fungi (Wallander & Nylund, 1992; Ekblad *et al.*, 1995). Increased proportional energy investment in the fungal partner under low P conditions highlights the central role of the mycorrhizal partnership in plant P nutrition (Smith & Read, 2008).

Ecological relevance

In our experiments, we demonstrated a preferential flow of photosynthates through EcM mycelia from *Pinus sylvestris* roots towards apatite, coinciding with a threefold increase in the apatite dissolution rate. The design of the microcosms supported the development of a mycelium network, resembling the natural growth in soils. A potential disadvantage of the use of small microcosms could be the enormous difference in size between the tree seedlings used in this study and the average size of forest trees. However, we found that the bio-

mass of external mycelium in the microcosms was between 2 and 7% of the tree biomass (Fig. 3A), which is similar to the proportion of net primary production (4%) allocated to external mycelium in the top 30 cm of a Swedish spruce forest (Wallander *et al.*, 2004). Both the close-to-nature fungus–plant biomass distribution and external mycelium architecture underpin the relevance of this type of microcosm for the studies of EcM ecology. It allows the actions of EcM fungi to be isolated from effects of other micro-organisms, tree roots and decaying organic matter, whilst maintaining the integrity of photosynthate energy fluxes, plant transpiration pathways and nutrient source-sink relations between the fungus and the plant as found in nature.

The concentration of apatite (5 or 10% w/w) in the wells was high relative to most soils (typically 0–1%, Deer *et al.*, 1997). However, given that 6 g of perlite grains was added to each microcosm, the percentage apatite in the total mass of minerals (quartz, perlite and apatite) was only 2% w/w. Furthermore, its location in patches reflects the kinds of heterogeneity found in poorly sorted glacial deposits that are widespread in the Boreal forest region in which EcM trees such as *Pinus sylvestris* are dominant. One important difference to soil was locating the apatite in plastic wells that isolated these grains from mycorrhizosphere bulk fluids (in this case provided in agar). As a consequence, weathering agents and dissolution products that are excess to nutrient requirements of the fungi and plants are likely to accumulate in this restricted area, potentially slowing the rate of apatite dissolution compared with soil conditions where these products may be washed away from weathering sites maintaining undersaturation. However, in seasonally dry forests, localized proliferation of some EcM fungi is associated with the creation of soil patches seasonally enriched in oxalic acid to concentrations of 12–68 mM in which the availability of phosphorus and other essential nutrients was increased, presumably by weathering (Griffiths *et al.*, 1994). Under such conditions where the upper soil horizons become dry but the trees have access to deeper supplies of water (see Brantley *et al.*, 2011), specialized EcM fungi are kept metabolically active by host photosynthate, able to secrete organic acids and absorb nutrients released, whilst the activities of saprotrophic microbiota that degrade oxalic acid in moist soil may be restricted. Our microcosm experiments provide some obvious parallels to these conditions, with the agar acting as 'ground water' and the mineral weathering occurring in patches of relatively dry mineral grains in the absence of saprotrophic microbial activities.

As illustrated in our present experiments in which 0.05–0.1 g of apatite was supplied in each plastic well, EcM fungi are adapted to locate and colonize specific locations from which nutrient elements can be extracted, even to the scale of individual apatite grains of 1 mm diameter (Smits *et al.*, 2008), which will have a mass <0.002 g. The importance of such intimate contact interactions between EcM fungi and apatite has been suggested by Blum *et al.* (2002), on the basis of direct access to apatite-derived Ca in temperate forest ecosystems in which there was a disparity between weathering rates and export of dissolved Ca in drainage waters. A field study in a Michigan Podzol under EcM forest revealed a non-random distribution of fungal hyphae over different mineral surfaces, with preferential colonization of feldspars over quartz (Smits, 2009). These lines of evidence, both from the field and from our microcosm studies, demonstrate the existence of a preferential flow of photosynthetic energy through the EcM mycelium network towards specific nutrient-rich mineral surfaces in the soil, facilitating weathering and biogeochemical cycling of nutrient elements required to maintain ecosystem productivity.

CONCLUSIONS

In this study, we demonstrated an integrated chain of tree and fungal physiological mechanisms involved in apatite weathering. First, the trees invested proportionally more photosynthate energy into the EcM fungal partner under low P conditions. Second, the EcM fungus preferentially allocated the assimilate received from the plant towards interaction with patches of apatite grains. And third, the fungal colonization of apatite increased its dissolution rate three times. The interdependence of these chains of events affirms the importance of studying EcM fungal weathering with the plant and fungus in symbiosis and confirms the ability of the fungal partners to direct their growth and allocation of photosynthates received from the plant to intensively interact with small patches of specific minerals. These findings highlight the importance of scale in EcM fungal–mineral interactions for our understanding and modelling of biotic weathering.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Results of the *weathering experiment*: the mean number of wells (of a total of 4, in each of four replicate microcosms) colonized by *Paxillus involutus* over 20 weeks.

Fig. S2 Results of the *C-allocation experiment*: shoot, root and EcM root tip fraction of total biomass, ^{14}C, and P.

Fig. S3 Published apatite dissolution rates in relation to pH, (Welch *et al.*, 2002; Guidry & MacKenzie, 2003; Chaïrat *et al.*, 2007), compared to the rates reported in the present study.

Table S1. Nutrient solutions.

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