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Ectomycorrhizal fungal biomass in roots and uptake of P from apatite by *Pinus sylvestris* seedlings growing in forest soil with and without wood ash amendment

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Abstract Forest soil from an experimental Norway spruce forest with four levels of wood ash addition (0, 1, 3 and 6 tonnes ha^{-1}) was used to inoculate pine (*Pinus sylvestris*) seedlings with indigenous ectomycorrhizal (EM) fungi. Uptake of ^{32}P and ^{86}Rb in a root bioassay was used to estimate the demand for P and K by seedlings grown in the different soils. Utilisation of P from apatite was tested in a laboratory system where uptake by the ectomycorrhizal mycelium was separated from uptake by roots. The demand for P and K in the seedlings was similar regardless of the ash treatment. Variation in EM levels, estimated as fungal biomass (ergosterol) in roots, was large in the different soils, but not related to ash addition. Uptake of P from apatite was, on average, 23% of total seedling P and was not related to EM levels. It was concluded that the improved P uptake from apatite by EM fungi found in earlier studies is probably not a general phenomenon among EM fungi. The small effect of ash addition on EM levels and P uptake suggests that addition of granulated wood ash is a forest management treatment that will have only minor influence on ectomycorrhizal symbiosis.

Keywords Apatite · Ectomycorrhiza · Root bioassay · Wood ash

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Introduction

The use of logging residues as an energy source in Sweden is expected to increase in the future. Recycling the ash after burning can compensate for mineral nutrients lost from the forest with increased harvest (Egnell et al. 1998). However, it is necessary to evaluate the ecological consequences of such wood ash amendments to forests soils before this management can be used on a larger scale. Forest trees are largely dependent on ectomycorrhizal (EM) fungi for the uptake of mineral nutrients (Smith and Read 1997). The extramatrical mycelium extending from EM roots exploits the soil for nutrients such as N and P (Smith and Read 1997), and also K (Marschner and Dell 1994) and Mg (Jentschke et al. 2000). However, the EM community may be affected by different forest management practices. For instance, N fertilisation (Kårén and Nylund 1997; Wallenda and Kottke 1998; Jonsson et al. 2000; Taylor et al. 2000; Erland and Taylor 2002), fire (Jonsson et al. 1999), and clear-cutting (Dahlberg and Stenström 1991) have all been shown to result in changes in the composition of the EM community.

Different EM fungal species appear to play different roles in the soil. Some fungi can produce enzymes involved in the uptake of organic nutrients from the soil (Colpaert and van Tichelen 1996; Smith and Read 1997), while some seem to be important in weathering of primary minerals in the soil (Griffiths et al. 1994; Jongmans et al. 1997; Wallander et al. 1997; Wallander 2000a, 2000b; Landerweert et al. 2001). Wallander (2000a) showed that some EM fungi stimulated P uptake from apatite by exuding organic acids into the soil solution. Griffiths et al. (1994) found large amounts of oxalic acid in soil solution collected from soil colonised by the EM fungus *Gautieria monticola*. Furthermore, the concentration of oxalic acid was positively related to the concentration of P in the soil solution, suggesting that oxalic acid was involved in releasing P from Al and Fe phosphate in the soil. Wood ash is another potential source of P for EM fungi although the P content in wood ash is much lower than that in apatite. Mahmood et al. (2002) recently investigated the

influence of wood ash amendment on the EM community of a Norway spruce forest. Although no significant influence on any individual species was reported, the occurrence of two EM species on the root tips tended to increase in plots with ash addition, and many of the ash granules were colonised by the same fungi. In axenic cultures these isolates produced large amounts of calcium oxalate crystals in response to ash addition and it was suggested that these fungi were involved in the dissolution of the ash granules, presumably to obtain P (Mahmood et al. 2001).

Different methods are used to measure effects of nutrient additions on the nutrient status of plants. The most widely used are nutrient concentration in the foliage and, of course, plant biomass. However, nutrients in foliage and biomass represent the integrated effect of earlier environmental conditions. Another way to measure tree nutrient status is to estimate the nutrient demand of the plant instantaneously by using root bioassays. Such root bioassays, developed by Harrison and Helliwell (1979) and Jones et al. (1987), provide an integrated measurement of the balance between the demand of the plant for P and K and the available supply of these elements in the soil. A combination of methods to estimate plant nutrient status would allow a deeper understanding of the effects of the nutrient addition to the plant-soil system.

The aims of the present study were to investigate if (1) the demand for K and P differed in Scots pine seedlings grown in forest soils with different additions of wood ash, (2) addition of wood ash to forest soil influences the amount of EM fungal biomass on roots of pine seedlings, (3) ectomycorrhizal pine seedlings grown in these different soils differed in their ability to utilise P from apatite. The seedlings were grown in soil collected from an experimental Norway spruce forest with four levels of wood ash application (0, 1, 3 and 6 tonnes ha^{-1}).

Materials and methods

Forest soil was collected from the humus layer (top 5 cm) of an experimental forest located at Torup (experimental site no. 241) in the southwest of Sweden (56° 55′ N, 13° 05′ E). The soil type and texture has been classified as podzol and sandy-till (Nohrstedt et al. 1996). The present forest was planted in 1957 with Norway spruce [*Picea abies* (L.) Karst] after clear-cutting of Scots pine (*Pinus sylvestris* L.). The experiment was a randomised block design with three blocks per treatment. The treatments used in the present study were control and fertilisation with 1, 3 and 6 tonnes ha^{-1} granulated wood ash applied 6 years before sampling of the soil. The ash granules contained 140 mg Ca g^{-1}, 15 mg Mg g^{-1}, 41 mg K $g^{-1}$and 8 mg P g^{-1} (Eriksson 1998).

Soil from the humus layer was taken from each plot (4×3=12 different soils) and sieved through a 5 mm mesh. Four replicate pots (7×7×7 cm) were filled with each soil (making a total of 48 pots). Five seedlings of *P. sylvestris* (5 weeks old) were planted in each pot, making a total of 20 seedlings planted in each soil type and a total of 240 planted seedlings (Fig. 1). The seedlings were watered with distilled water and grown in a greenhouse for 4 months. After this time, six to nine seedlings of equal size were randomly harvested from each ash treatment (2–3 seedlings were randomly taken from all three blocks of each treatment). The soil was gently removed from the roots and the seedlings were placed between dampened towels and transported to the Merlewood Research Station (Centre of Ecology and Hydrology, Cumbria, UK). These seedlings were used in the root bioassay for ^{32}P and ^{86}Rb to estimate the demand for P and K, respectively, by the seedlings (see below). The seedlings were analysed within 3 days of sampling, this period of time having been found not to influence uptake rates significantly in other studies (e.g. Jones et al. 1992; Dighton et al. 1993).

Two seedlings of equal size from each pot (except for the 1 tonne ash ha^{-1} treatment, which was omitted from this part of the experiment) were replanted in bags of approximately 150 cm^3 constructed of a nylon mesh (100 µm mesh size). The bags were filled with the forest soil taken from the original pot. One of the bags was placed in a larger pot (9×9×9 cm), which was filled with acid-washed sea sand (0.6–2 mm; Ahlsell, Sweden) and the other in a similar pot with the addition of apatite (ground to particles of between 50 µm and 250 µm) added at a concentration of 1% (w/w). This made a total of 4×3×3×2=72 larger pots (Fig. 1). Seedlings from the original pots not used for root bioassays or the apatite experiment were discarded. Some of the seedlings died during the experiment and were omitted from the analysis. The apatite used came from Siilinjärvi, Finland, and contained 160 mg P g^{-1}. The quartz sand contained less than 0.13 mg P g^{-1}. The design of the pots ensured that the outer compartment of the pot consisted of sand only accessible to fungal mycelium growing through the nylon mesh, while both roots and EM fungi could grow in the inner compartment. The pots were placed on a capillary mat on a tray according to a method developed by Sen (1990). The capillary mat transported distilled water from a reservoir at a distance of 5 cm below the seedlings. The volume of the reservoir (6 l) was adjusted every 2 weeks.

The seedlings in the apatite experiment were grown in the pots for 220 days. At harvest, the soil was gently shaken from the roots and the root systems were washed under tap water. The seedlings were freeze-dried. After freeze-drying, the dry weights of the shoots and roots were recorded.

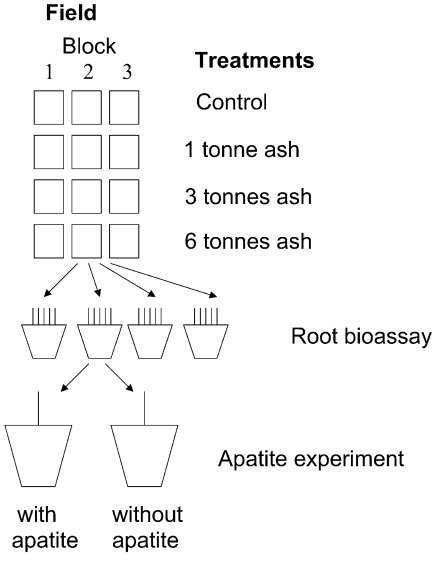

Fig. 1 Study design. Soil was collected from field plots (three blocks per treatment) with four treatments: control, addition of 1, 3, or 6 tonnes ash. Four pots were filled with soil from each plot and planted with five seedlings each; six to nine seedlings from each treatment were used for the root bioassay. Two seedlings from each pot were used for the apatite experiment; planted in pots with and without added apatite. The 1 tonne ash treatment soil was not included in the apatite experiment. In total, 72 pots were used for the apatite experiment, three replicate blocks in the field experiment, three ash addition treatments (0, 3, 6 tonnes ash), two apatite addition treatments (+/−) and four replicate pots (3×3×2×4=72)

Analyses

Root bioassay of ^{32}P and ^{86}Rb

On the 3rd day after sampling, the roots of intact seedlings, with attached mycorrhizas, were carefully washed free of soil and adhering litter, before carrying out bioassays for P and K (using ^{86}Rb as an analogue for K) as described in Harrison and Helliwell (1979) and Jones et al. (1987). In the P bioassay, the roots were first immersed in a solution of 5×10^{-4} M $CaSO_4$, and for the K bioassay in 5×10^{-4} M $CaCl_2$, both treatments lasting 30 min to maintain root cell membrane integrity and to remove ions from the apoplast. The roots were then transferred to the uptake solution for 15 min. For P, this was a solution consisting of 1 l $CaSO_4$ at 5×10^{-4} M and 1 l KH_2PO_4 at 5×10^{-6} M; for K it was 1 l each of the same strengths of calcium chloride and rubidium chloride. The solutions were labelled with ^{32}P or ^{86}Rb at approximately 2 MBq. The roots were removed from the solution after exactly 15 min and washed for 5 min under running water to remove surface-adsorbed ions. A subsample (20–200 mg fresh weight) was taken from each root and placed in 15 ml distilled water in a separate counting vial. ^{32}P or ^{86}Rb activity was counted by Cerenkov light in a Packard 2000 CA liquid scintillation counter. After this, each root subsample was removed, blotted and weighed, and the vial recounted for ^{32}P or ^{86}Rb diffused from the free space of the root subsample, i.e. for P or Rb, which had not been metabolically adsorbed. Both sets of results were corrected for background counts, isotopic decay and counting efficiency. The samples were corrected for physical quenching of Cerenkov light, due to the root biomass in the vials, by acid-digesting a stratified subset of the root subsamples. For more details on the method see Harrison and Helliwell (1979) and Jones et al. (1987).

Ergosterol

Freeze-dried roots were analysed for ergosterol as an estimate of ectomycorrhizal fungal biomass (Wallander and Nylund 1992). The roots were milled in a ball mill to a fine powder, and 20–30 mg roots was extracted with 2 ml 10% KOH in methanol, and 0.5 ml cyclohexane. The samples were sonicated for 15 min, extracted overnight and then refluxed at 70°C for 30 min. After cooling, 0.5 ml H_2O and 1.5 ml cyclohexane were added. The samples were mixed in a vortex apparatus for 20 s, centrifuged for 5 min at 3,000 rpm and the cyclohexane phase was transferred to another test tube. The methanol was extracted with a further 1.5 ml volume of cyclohexane. The cyclohexane was evaporated under N_2 and the samples were dissolved in methanol. Prior to quantification of ergosterol, the samples were filtered through a 0.5 μm Teflon syringe filter (Millex LCR-4; Millipore, Milford, Mass.). The chromatographic system consisted of a HPLC (Pharmacia-LKB model 2248), UV detector (Pharmacia model 2141) and a C_{18} reverse-phase column (Nova-Pak 0.39 cm ×7.5 cm) preceded by a C_{18} reverse phase guard column (Waters). Extracts were eluted with methanol at a flow rate of 1 ml min^{-1} and monitored at 282 nm.

Elemental analysis of plant material

Freeze-dried shoots and roots of pine seedlings were weighed and digested in 5 ml concentrated HNO_3. Ca, K and P analysis was performed by atomic emission spectrometry (ICP Jobin Yvon Sequential Analyser). The total amount of P in seedlings grown with and without apatite addition was calculated from the P concentration and the biomass, to estimate the amount of P originating from the apatite.

Statistics

Effect of wood ash addition on ^{32}P and ^{86}Rb was analysed with one-way ANOVA (n=6–9). The effect of wood ash addition and apatite addition on ergosterol concentration of the roots and P uptake from apatite were analysed with two-way ANOVA using ash treatment and apatite addition as fixed effects and blocks used as replicates (randomised complete block design). A mean from the four seedlings used for each block was calculated and used in the ANOVA analysis. Least significant differences (LSD) were used to evaluate differences between the treatments when the ANOVA analysis was significant (P<0.05). Correlation analysis was performed to estimate if there was a relationship between ergosterol concentration in roots and total P content. In this analysis all individual seedlings were included (n=60).

Results

All seedlings grown in the different forest soils were well colonized by EM fungi although no attempts were made to quantify the levels of EM biomass in the roots before they were used in the ^{32}P and ^{86}Rb uptake experiment or in the apatite experiment. There was no significant difference in seedling biomass or uptake of ^{32}P and ^{86}Rb of seedlings grown in soils with different ash additions, although there was a non-significant trend suggesting that increasing amounts of ash resulted in lower P demand (Table 1).

No significant effect of ash addition or apatite addition on the amount of EM biomass (ergosterol) in the roots was found, although there was a tendency for it to increase when apatite and ash were added together (Table 2). Seedlings differed considerably in the levels of EM fungal biomass in the roots, as seen by the large range of ergosterol values in Fig. 2.

The concentrations of P in shoots and roots did not differ between the different ash treatments (Table 3). There was a tendency for seedlings grown with apatite to take up

Table 1 Biomass (g) and uptake of ^{32}P and ^{86}Rb [mean ±SE; pg (mg root)$^{-1}$ 15 min^{-1}] of *Pinus sylvestris* seedlings grown in soils amended with different amounts of granulated wood ash. One-way ANOVA (n=6–9) was used to test differences between treatments

Ash treatment (tonnes ha^{-1})	Biomass (g)	^{32}P uptake [pg (mg root)$^{-1}$]	^{86} Rb uptake [pg (mg root)$^{-1}$]
0	0.29±0.07	1,063±258	1,089±123
1	0.22±0.05	1,093±91	1,560±313
3	0.33±0.08	872±152	1,531±116
6	0.16±0.04	789±156	1,687±319
ANOVA (P-value)	ns[a]	ns	ns

[a]Not significant

Table 2 Biomass and ergosterol concentration (mean ±SE,*n*=4) in roots of seedlings grown in different ash amended soils with or without addition of apatite in a fungal compartment separated from the roots by a mesh. Differences between treatments were tested with two-way ANOVA (using ash treatment and apatite addition as factors) and LSD to separate the means. Different letters indicate statistically different values ($P<0.05$)

Ash addition (tonnes ha^{-1})	Apatite addition	Biomass (g)	Ergosterol [µg (g root)$^{-1}$]
0	+	0.85±0.11 ab	68.9±3
3	+	0.80±0.02 ab	142.7±84
6	+	1.09±0.23 a	240.6±118
0	−	0.61±0.06 b	186.2±21
3	−	0.71±0.12 ab	106.2±51
6	−	0.79±0.13 ab	180.8±74
ANOVA ash		ns[c]	ns
Apatite		0.05	ns
Ash × Apatite		ns	ns

[c]Not significant

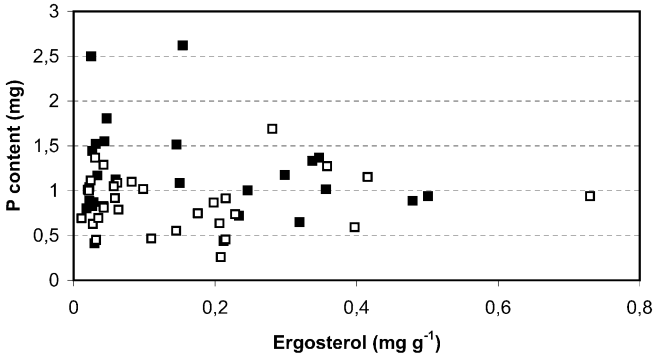

Fig. 2 Correlation between ergosterol concentration of the roots and total plant P content in seedlings grown with (*filled squares*) and without (*open squares*) apatite

more P than seedlings grown without apatite, although this was only statistically different at the 6 tonnes ash treatment (P=0.05) (Fig. 3). The amount of P in seedlings grown without apatite (all P taken up from the forest humus) was 0.88 mg P and the mean amount of P in seedlings grown with apatite was 1.14 mg P. This means that, on average, 0.26 mg P per seedling was taken up from the apatite, which is 23% of the total P in the seedlings. The amount of weathered apatite was calculated to be 3.3 mg apatite per pot, which is approximately 0.3% of the added apatite. The concentration of Ca and K in shoots and roots did not differ between treatments. Average shoot Ca concentration among all seedlings was 5.1±0.3 mg g^{-1} and shoot K concentration was 4.0 ±0.2 mg g^{-1}. Average root Ca concentration was 2.4 ±0.1 mg g^{-1} and root K concentration 6.4±0.2 mg g^{-1}.

There was no correlation between ergosterol in the roots and P content of seedlings grown either with or without apatite (Fig. 2).

Table 3 Shoot and root P concentrations (mean ±SE, *n*=4) in seedlings grown in different ash amended soils with or without addition of apatite in a fungal compartment separated from the roots by a mesh. Differences between treatments were tested with two-way ANOVA (using ash treatment and apatite addition as factors

Ash addition (tonnes ha^{-1})	Apatite addition	Shoot P (mg g^{-1})	Root P (mg g^{-1})
0	+	1.3±0.2	1.6±0.1
3	+	1.6±0.1	1.3±0.09
6	+	1.4±0.1	1.4±0.2
0	−	1.5±0.1	1.3±0.04
3	−	1.4±0.1	1.1±0.03
6	−	1.3±0.2	1.3±0.15
ANOVA			
Ash treatment		ns[a]	ns
Apatite (*P*-value)		ns	ns (P=0.07)
Ash × Apatite		ns	ns

[a]Not significant

Discussion

Scots pine seedlings grown in the different ash-treated soils did not differ in the demand for P and K as measured by root bioassay. This suggests that the nutrients added with the ash were already present in sufficient amounts in the soil at the time of our sampling. This is also supported by the fact that the seedlings did not respond to the added ash with increased growth. Mahmood et al. (2003), on the other hand, demonstrated a strong growth response by adding wood ash to mycorrhizal Norway spruce seedlings grown in pots. However, in their study, seedlings were grown in nutrient poor peat/s and substrate with slow release N added as a fertiliser, which probably resulted in enhanced demand of nutrients from the wood ash. In our study it is likely that the original soil contained enough nutrients except for N, which probably resulted in N limiting growth. In a full-scale experimental forest, ash addition also did not influence growth of Norway spruce

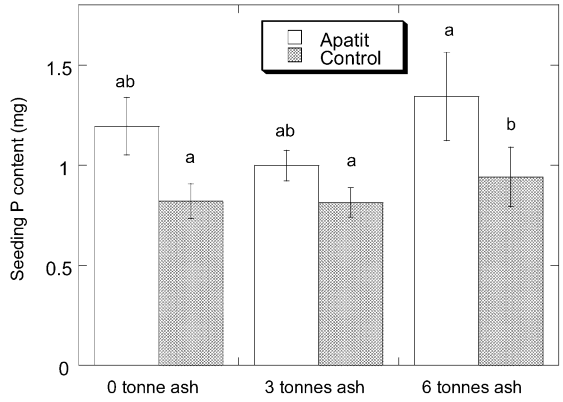

Fig. 3 Total content of P in seedlings grown in soil with different ash additions and with or without addition of apatite in a fungal compartment separated from the roots by a mesh. Ash amendments are 0, 3 or 6 tonnes ha^{-1}. The addition of apatite had a significant positive influence on total P content at the 6 tonnes ash addition (ANOVA, P=0.05,n=4) while ash addition did not influence P content significantly. Different letters indicates significant different values (P<0.05). *Bars*SE

significantly, and it was concluded that N most likely limited growth (Jacobsson and Ring 1995). Clarholm (1998) used the same root bioassay to study the effect of wood ash addition on P and K demand in another experimental Norway spruce forest in southwest Sweden. Just as in our study, ash addition had no influence on uptake of ^{32}P and ^{86}Rb by the roots. According to Harrison et al. (1995), this suggests that P and K did not limit the seedlings grown in the different soils.

The soils used in the present experiment were sampled 5 years after the addition of the wood ash. The immediate effect of the ash treatment could be seen as elevated P concentration in needles 1 year after addition of the highest dose of wood ash (Jacobsson and Ring 1995) but this effect diminished after 2 years. In a laboratory experiment, Eriksson (1998) found that most Ca and K were lost from hardened wood ash during 1 year of simulated precipitation while P was released much more slowly. The same was found by Hagerberg and Wallander (2002), who buried mesh bags with wood ash in forest soil.

Total P content was higher in Scots pine seedlings grown with apatite compared to seedlings grown without apatite, although the influence of apatite addition varied among the treatments (Fig. 2). No relationship was found between P uptake and EM fungal biomass on the roots, as estimated from ergosterol content (Fig. 2), which suggests that a change in EM levels may occur without having a significant influence on the P-absorbing capacity of mycorrhizal seedlings. Thus, we could not detect any significant influence of ash addition on the capacity of the seedlings to take up P from sparingly soluble sources. The calculated weathering of the apatite used in the present experiment was rather low (0.3% of added apatite). In a similar experimental system (Wallander 2000b), up to 0.9% of the added apatite was weathered in the same period of time. In that study,*Suillus variegatus* or an unidentified white EM fungus was used as the mycorrhizal symbiont. Non-mycorrhizal seedlings and seedlings colonised by less efficient EM fungi in the same experiment resulted in a weathering rate of 0.3% of added apatite, which is a rate similar to that reported here. The EM community that colonised the seedlings in the present study was not characterised, which makes it impossible to speculate about possible roles of individual EM species.

Another reason for the low P uptake from the apatite is probably that the seedlings were not limited by P since shoot P concentrations were rather high and well above the deficiency levels reported by Linder (1995). Hagerberg et al. (2003) demonstrated recently in a field experiment that EM-induced dissolution of apatite is greatly promoted by P deficiency of the associated Norway spruce trees, and Wallander and Hagerberg (2004) argue that host nutrient status may be the key factor determining the outcome of interactions between EM fungi and minerals in soil.

Exudation of organic acids (especially oxalic acid) may be the key mechanism by which some species of EM fungi release P from apatite or from Al and Fe phosphates in the soil (Griffiths et al. 1994; Wallander et al. 1997; Ahonen-Jonnarth et al. 2000; Wallander 2000a). The release of P from ash may, however, not always be accompanied by better P uptake by the seedlings. Mahmood et al. (2003) found earlier that P concentration in soil solution increased in response to an oxalic-acid-producing EM fungus, but P content in Norway spruce seedlings were similar regardless of whether they were colonized or not. In the present study it was not possible to estimate if P release from the apatite varied dependent on the ash treatment, but P uptake into the seedlings was similar in all cases and values were low compared to earlier studies with more efficient EM species.

In conclusion, in this study we found no influence of ash addition on biomass or nutrient uptake of pine seedlings. The large variation found in soil samples taken from replicate blocks in the field appeared to be more important for the formation of EM biomass on the roots, or the P-uptake from apatite capability of *P. sylvestris*seedlings, than addition of up to 6 tonnes ha^{-1} granulated wood ash. We thus found no negative consequences of the ash additions in the present study.

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