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ORIGINAL PAPER

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The elemental content in the mycelium of the ectomycorrhizal fungus *Piloderma* sp. during the colonization of hardened wood ash

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Abstract *Piloderma* sp., a wood ash-colonizing ectomycorrhizal (EM) fungus, was grown symbiotically with Norway spruce in microcosms which contained granules of hardened wood ash. Mycelium close to the granules was sampled 3 times over a period of 11 weeks and the elemental content was investigated with particle induced X-ray emission. Mycelium from microcosms without wood ash was used as controls. The contents of P and K were similar in mycelium growing close to wood ash granules to those in control mycelium, while the Ca content increased from 23±21 mg g^{-1} in controls to 63±8 mg g^{-1} in mycelium growing close to wood ash granules. The Ca content was also increased in other parts of the mycelium more distant from the wood ash. *Piloderma* sp. may have a role in the short-term storage of Ca released from wood ash, rather than in releasing and storing P.

Keywords Ectomycorrhizal fungus · Wood ash · Particle-induced X-ray emission · Phosphorus · Calcium

Introduction

Fertilization with hardened wood ash, where elements are bound to carbonates, has been suggested as a means of ameliorating possible shortages of mineral nutrients other than N induced by anthropogenic N deposition (Lundborg 1997). The application of hardened wood ash results in an increased content of extractable P (Fransson et al. 1999) and Ca (Eriksson 1998; Eriksson et al. 1998) in the organic horizon as well as an increase in pH (Eriksson 1998; Eriksson et al. 1998; Staples and van Rees 2001). After wood ash fertilization of a Norway spruce forest the foliage content of P, K and Ca increased (Staples and van Rees 2001; Arvidsson and Lundkvist 2002).

Field and laboratory studies on the dissolution of hardened wood ash have shown that up to 30% of the Ca is lost from the wood ash within 2 years (Eriksson 1998; Hagerberg and Wallander 2002; Steenari et al. 1998). K on the other hand, is more rapidly lost from granulated wood ash—as much as 50–80% during the first few months (Hagerberg and Wallander 2002; Steenari et al. 1998). The release of other mineral nutrients is generally slower than that of Ca and K (Eriksson 1998; Hagerberg and Wallander 2002; Steenari et al. 1998).

Ectomycorrhizal (EM) fungi take up and translocate NO_3^-, NH_4^+, K and P (review by Marschner and Dell 1994), Mg (Jentschke et al. 2000) and possibly Ca (Blum et al. 2002) into host plants. Some EM fungi colonize granules of hardened wood ash in the field (Mahmood et al. 2002) and wood ash also stimulates the production of mycelium in some EM species (Hagerberg and Wallander 2002; Mahmood et al. 2002; Mahmood et al. 2001).

Piloderma sp. (Tor-35) is an EM species that readily colonizes wood ash granules and was the EM species which colonized spruce roots most frequently in a wood ash-fertilized forest (Mahmood et al. 2002). *Piloderma* sp. has the ability to increase the dissolution rate of hardened wood ash when growing in pure culture and when grown symbiotically with Norway spruce seedlings (Mahmood et al. 2003). The mycelial content of P was higher than in other EM fungi, when growing in pure culture with wood ash. When grown on substrates of low nutrient content, it was found that *Piloderma* sp. translocated more Ca and K to host plants than other EM fungi (Mahmood et al. 2003). However, this difference between *Piloderma* sp. and other EM fungi diminished when wood ash was added to the pots. Translocation of Ca, K or P from hardened wood ash to host plants was similar in *Piloderma* sp.-colonized plants and plants colonized by other EM fungi, although *Piloderma* sp. increased the content of soluble

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P in the substrate (Mahmood et al. 2003). It is possible that *Piloderma* sp. stores mineral nutrients obtained from the wood ash in its mycelium rather than translocating them to the host. In this case mineral nutrients would accumulate in the fungal mycelium after colonizing wood ash.

Since *Piloderma* sp. increases the solubilization of P from wood ash, but does not seem to increase the transport of P to the host plant, we wanted to determine whether P and other elements accumulated in the mycelium when *Piloderma* sp. was grown on granules of hardened wood ash.

Materials and methods

Microcosms and sampling

EM associations were synthesized (Finlay et al. 1988; Mahmood et al. 2002; Duddridge 1986) between 3- to 7-week-old Norway spruce [*Picea abies* (L.) Karst.] seedlings and *Piloderma* sp. (strain Tor-35; Mahmood et al. 2002; kept at the Department of Microbial Ecology, Lund University). The root systems of the aseptically grown seedlings were placed in a Petri dish which contained sterile peat–vermiculite (1:4) moistened with half strength MMN solution. *Piloderma* sp. was inoculated together with the root systems in the Petri dishes, which were then sealed with tape. The Petri dishes were kept in a constant-temperature chamber at 18°C, with approximately 200 µmol m^2 s^{-1} photosynthetically active radiation and a 14/10 h day/night cycle. After 3 months seedlings with well-established mycorrhizal roots were transferred to eight observation microcosms (24.5×24.5×2.5 cm; Nunc, Denmark) containing a 5- to 6-mm-thick layer of non-sterile peat as a substrate (Mahmood et al. 2001). The microcosms were kept in the constant-temperature chambers under the same conditions as the synthesis Petri dishes. After 4 months, when the external mycelium had begun to colonize the peat, three granules of hardened wood ash (0.3–0.7 cm diameter; Ljungbyverket, Sydkraft Sverige Syd, Sweden; Table 1.) were placed in front of the mycelium in each of four microcosms (Fig. 1). Three pieces of granite of the same size as the wood ash granules, were also inserted with the wood ash close to the mycelial front, in order to see if the fungus responded to an inert object of similar shape. The granite grits were acid washed and could be regarded as inert as the short experimental time makes it unlikely that any substantial amounts of elements would be released from the grits. Mycelium was sampled beside a wood ash granule (1 mm distance) from each microcosm after 27, 54 and 76 days and control mycelium from microcosms without wood ash was taken at the same times. The mycelia were immediately freeze-dried after sampling and were kept at −20°C until particle induced X-ray emission (PIXE) analysis. The sampled portion of mycelium constituted

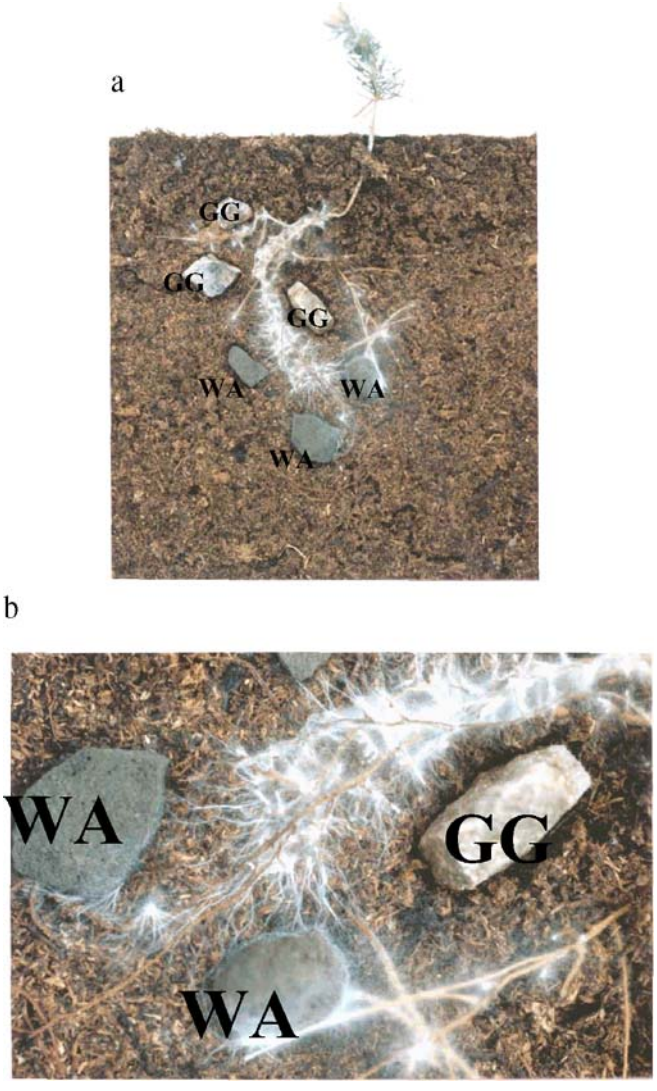

Fig. 1 **a** Photograph of the microcosms showing the spruce seedlings in symbiosis with *Piloderma* sp. and the wood ash granules (WA) and granite grit (GG), which were placed into the microcosms. **b** Detail showing the colonization of WA by dense mycelium

Table 1 Contents of the elements in peat and ash used in this study as revealed by the lithium borate method (ASTM method D3682) estimated from single samples. n.d. Not determined

Element	Peat	Wood ash
Ca (mg g^{-1})	2	110
K (mg g^{-1})	0.2	36
P (mg g^{-1})	0.2	6.6
Mn (mg g^{-1})	0.03	5.4
S (mg g^{-1})	1	4.2
Ti (mg g^{-1})	n.d.	2.4
Zn (mg g^{-1})	0.01	1.5
Cu (µg g^{-1})	1	71
Pb (µg g^{-1})	10	64
Cr (µg g^{-1})	1	63

approximately <1% of the total mycelium in each microcosm. Mycelial samples could not be retrieved successfully from all four microcosms at each sampling occasion, but at least three replicates were collected per treatment and sampling occasion. At the last harvest, mycelia were also sampled from beside the granite grit in three microcosms.

Analysis of elemental content

Samples of wood ash and peat were taken and the elemental contents of each were determined by the lithium borate method (ASTM method D3682).

The mycelial samples were subjected to PIXE analysis (Johansson and Campbell 1988; Wallander et al. 2002). The analyses were performed with the Lund nuclear microprobe. Protons with an energy of 2.5 MeV were focussed with magnetic lenses to a micrometre-sized beam, which was scanned across the samples. In each spot an elemental analysis was performed and from the data collected elemental maps of sample composition were produced using the software GeoPIXE II (CSIRO Exploration and Mining, Clayton, Australia). The detection limit in each analysis was calculated from the peak-to-background ratio in the X-ray spectrum. The region of analysis was selected in the centre of the mycelial samples as the risk that elements would leak out was regarded as lower there compared to the cut ends. Simultaneously with PIXE, scanning transmission ion microscopy (STIM) was performed on the samples in order to determine the mass density of each spot (Lefevre et al. 1987). STIM is based on the detection of energy loss when protons pass through the sample.

Statistical evaluation

All the statistical analyses were performed using the software SPSS 11.0 (SPSS, Chicago, Ill.). Differences in the elemental contents of mycelia sampled from microcosms with and without hardened wood ash were evaluated with one-way ANOVA. Heterogeneity of variance was detected when evaluating the effects on Ca content and the logarithmic values were used in the ANOVA (Sokal and Rohlf 1995).

Results

Element contents of the hardened wood ash and peat used in this experiment are given in Table 1. After 27 days the wood ash granules were colonized by the mycelium of *Piloderma* sp. (Fig. 1). At the end of experiment the granite grit was also colonized.

The contents of P, Ca and K in the mycelia sampled from the microcosms with or without wood ash granules are shown in Fig. 2. The content of P at the first sampling occasion was 2.1±0.9 mg g^{-1} in mycelium close to wood ash granules and 1.2±0.3 mg g^{-1} in microcosms without wood ash, but the difference was not statistically different (Fig. 2a). Mycelia sampled close to wood ash granules had a significantly higher Ca content (P<0.001) than that sampled from microcosms without wood ash on all sampling occasions; 63±8 mg g^{-1} and 23±21 mg g^{-1}, respectively, at the first sampling occasion (Fig. 2b). The Ca content of mycelia close to the granite grit was similar to that of mycelia close to the wood ash granules. The K content of the mycelia was also similar in both microcosms with and without wood ash granules (Fig. 2c; 9±2 mg g^{-1} and 6±2 mg g^{-1}, respectively, at the first sampling occasion). The

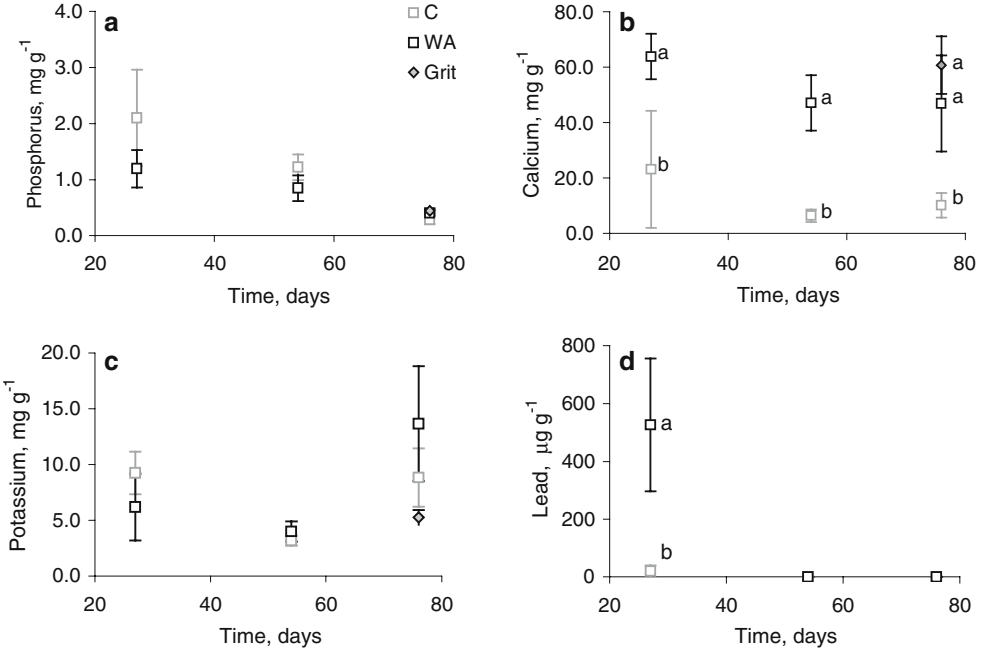

Fig. 2a–d Elemental content estimated by particle induced X-ray emission (PIXE) analysis over time in mycelia of *Piloderma* sp. sampled close to WA and GG and from control microcosms to which no wood ash had been added. Mycelia were sampled from beside the grit only at the last sampling occasion. **a** P, **b** Ca, **c** K and **d** Pb. The error bars represent the SEM (n=3, apart from day 54 for the WA and day 76 in the control microcosms, where n=4). Significant differences (P<0.05) of treatment are shown by different letters. *C* Control microcosms; for other abbreviations, see Fig. 1

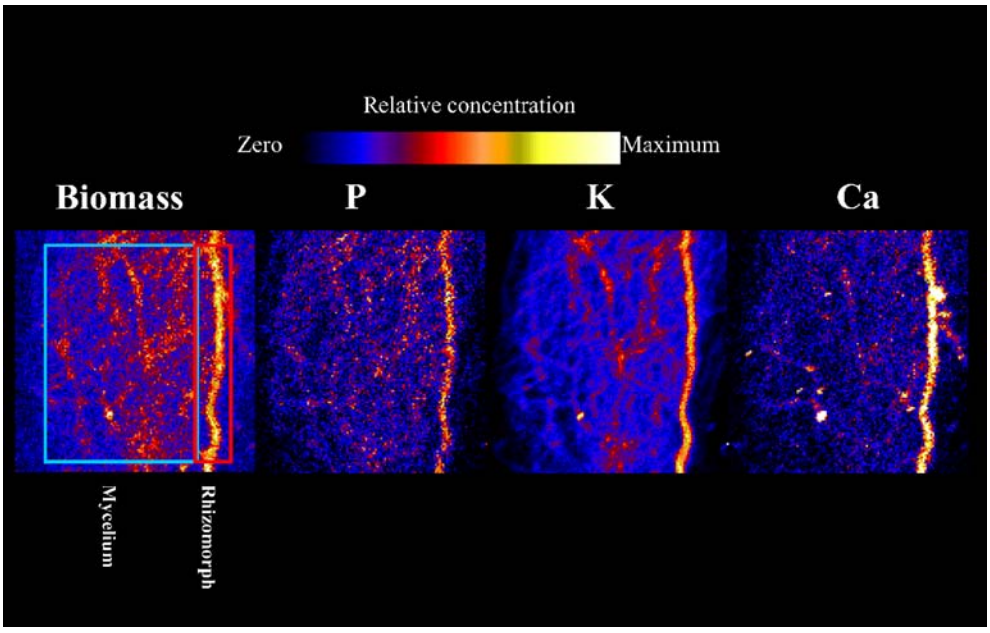

Fig. 3 PIXE analysis of rhizomorph and connected mycelium close to a WA on the first sampling occasion. The biomass and elemental content could be estimated inside selected areas, for example the box outlined in red (rhizomorph) or the box outlined in blue (mycelium) or for both

average K content in mycelium sampled from granite grit was lower than in samples from wood ash granules; however, there was a large variation and the difference was not statistically significant (Fig. 2c). The detection limit did not exceed 0.3 mg g^{-1} for any of the samples.

Amongst the heavy metals analysed only the average Pb content in mycelium sampled close to the wood ash granules was higher (530±230 μg g^{-1}; Fig. 2d) than that in mycelium from microcosms without wood ash (20±20 μg g^{-1}) on the first sampling occasion. However, due to the large variation the difference was not significant (P=0.09). The presence of wood ash had no effect on the mycelial contents of Cr, Cu, Fe, Mn, S, Ti and Zn, neither at the wood ash nor at the granite grit (data not shown).

The PIXE analysis made it possible to determine the elemental composition of the rhizomorphs individually as well as the more diffuse mycelium (Fig. 3). However, the values for the rhizomorphs did not differ from the values for the mycelium as a whole. Rhizomorphs growing close to wood ash granules contained 1.2±0.5 mg P g^{-1}, 10±2 mg K g^{-1} and 44±6 mg Ca g^{-1}.

Discussion

Mycelium growing close to wood ash granules had a similar P content to mycelium in microcosms without wood ash. Since the fungus does not appear to accumulate P in the mycelium close to wood ash granules and does not increase the translocation of P from wood ash into the host plant (Mahmood et al. 2003), this indicates that *Piloderma* sp. does not increase the plant-available P from wood ash through uptake and translocation.

In this study the mycelium of *Piloderma* sp. was found to have a high Ca content compared to field samples of other EM fungi in earlier investigations (Kottke 1998; Wallander et al. 2002; Wallander et al. 2003). The Ca content in mycelium sampled from microcosms without wood ash in this study (23 mg g^{-1}) was for example 10 times higher than the Ca content of *Paxillus involutus* mycelia sampled in the field (2 mg g^{-1}; Wallander et al. 2003). The Ca content in mycelium close to wood ash granules (63 mg g^{-1}) was comparable to the highest contents found in rhizomorphs and mycelia of different EM fungi sampled from local sources of mineral Ca (up to 67 mg g^{-1}; Wallander et al. 2002). Turnau et al. (2001) found using PIXE analysis that rhizomorphs of *Suillus luteus* growing in soils of high Ca content had a similar content of Ca (38.6 mg g^{-1}).

Mahmood et al. (2003) found that the ratio of the Ca content in the shoots to the Ca content in the roots was lower in spruce seedlings growing in substrate amended with wood ash and colonized by *Piloderma* sp. than in seedlings colonized by other EM fungi. The high accumulation of Ca in fungal mycelium observed in this study suggests that an explanation of the low shoot:root Ca content might be that *Piloderma* sp. takes up Ca from the wood ash but retains it in the fungal mantle of the EM roots. The Ca content was also high in mycelia collected around the granite grit, which indicates that Ca is translocated from the wood ash to the rest of the mycelium. Species of the genus *Piloderma* are known to precipitate Ca in the cell walls in the form of calcium oxalate crystals (Arocena et al. 2001) and calcium oxalate crystals accumulate in the medium when *Piloderma* sp. is grown in pure culture with wood ash or calcium phosphate (Mahmood et al. 2001). It is believed that depositing calcium oxalate is a way of removing excess Ca from the cytoplasm (Snetselaar and Whitney 1990) transported from a source by a pressure-driven flow through the mycelium (Jennings 1994). Coating of the mycelium by calcium oxalate makes the hyphae more hydrophobic, which has been suggested to work as a protection against microbial attacks and grazing soil animals (Whitney and Arnott 1987).

The K content in the mycelium was also similar in both microcosms with and without wood ash granules. The average K content in mycelium sampled from granite grit was lower than in samples from wood ash granules, although the difference was not significant. Similar to Ca, the mycelial content of K in this study was much higher than the contents found in rhizomorphs and mycelia of EM fungi sampled from mesh bags with sand–wood ash mixtures in the field (0.3 mg g^{-1}; Wallander et al. 2003), but in the same range as K contents in *Suillus luteus* rhizomorphs sampled from polluted sites (6.5 mg g^{-1}, Turnau et al. 2001). Differences in K retention are likely to be species related, since certain EM species are able to contain higher contents of different elements compared to other EM species (Wallander et al. 2003). Rather than being important for P nutrition, *Piloderma* sp. might be an important fungus for Ca and K nutrition in forests where cations are depleted, like in Ca-poor forests where EM fungi seem to contribute to the nutrition of conifers by the uptake of Ca, released by weathering in the B horizon of the soil (Blum et al. 2002). The presence of EM fungi with a good capacity to take up K is especially important in forests with poor K status, since trees with poor K status do not seem to be able to utilize K from minerals (Hagerberg et al. 2002) in order to increase their K uptake. When fertilizing with wood ash a large amount of K is released rapidly and the presence of EM fungi with a good K uptake capacity is also important here to prevent leakage of K from the soil.

This study and the study of Mahmood et al. (2003) have shown that *Piloderma* sp. acquires primarily Ca, but also K to some extent, from wood ash. Ca is taken up and distributed throughout the fungal mycelium. The ecological role of a high Ca content in the mycelium is poorly understood and this study shows that Ca accumulation could be an important feature for some fungi. The ability of *Piloderma* sp. to take up and maintain higher contents of Ca and K than other EM fungi suggests that this fungus is more important in the Ca and K nutrition of its host trees than in their P nutrition. P did not accumulate in the mycelium, and since Mahmood et al. (2003) have shown that there was no increased transport of P from wood ash by *Piloderma* sp., it seems that *Piloderma* sp. does not colonize wood ash in order to obtain P.

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References

Arocena JM, Glowa KR, Massicotte HB (2001) Calcium-rich hypha encrustations on *Piloderma*. Mycorrhiza 10:209–215

Arvidsson H, Lundkvist H (2002) Needle chemistry in young Norway spruce stands after application of crushed wood ash. Plant Soil 238:159–174

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

Duddridge JA (1986) The development and ultrastructure of ectomycorrhizas. III. Compatible and incompatible interactions between *Suillus grevillei* (Klotzch) Sing., and 11 species of ectomycorrhizal hosts in vitro in the absence of exogenous carbohydrate. New Phytol 103:457–464

Eriksson HM (1998) Short-term effects of granulated wood ash on forest soil chemistry in SW and NE Sweden. Scand J For Res [Suppl] 2:43–55

Eriksson HM, Nilsson T, Nordin A (1998) Early effects of lime and hardened and non-hardened ashes on pH and electrical conductivity of the forest floor, and relations to some ash and lime qualities. Scand J For Res [Suppl] 2:56–66

Finlay RD, Ek H, Odham G, Söderström B (1988) Mycelial uptake, translocation and assimilation of nitrogen from ^{15}N-labelled ammonium by *Pinus sylvestris* plants inected with four different ectomycorrhizal fungi. New Phytol 110:59–66

Fransson A-M, Bergkvist B, Tyler G (1999) Phosphorus solubility in an acid forest soil as influenced by form of applied phosphorus and liming. Scand J For Res 14:538–544

Hagerberg D, Wallander H (2002) The impact of forest residue removal and wood ash amendment on the growth of the ectomycorrhizal external mycelium. FEMS Microbiol Ecol 39:139–146

Hagerberg D, Thelin G, Wallander H (2002) The production of ectomycorrhizal mycelium in forests: Relation between forest nutrient status and local mineral sources. Plant Soil 252:279–290

Jennings DH (1994) Translocation in mycelia. In: Wessels JGH, Meinhardt F (eds) The Mycota. I. Growth differentiation and sexuality. Springer, Berlin Heidelberg New York, pp 163–173

Jentschke G, Brandes B, Kuhn AJ, Schroeder WH, Becker JS, Godbold DL (2000) The mycorrhizal fungus *Paxillus involutus* transports magnesium to Norway spruce seedlings. Evidence from stable isotope labeling. Plant Soil 220:243–246

Johansson SAE, Campbell JL (1988) PIXE: a novel technique for elemental analysis. Wiley, Chichester

Kottke I (1998) *Xerocomus badius-Picea abies*, an ectomycorrhiza of high activity and element storage capacity in acidic soil. Mycorrhiza 7:267–275

Lefevre HW, Schofield RMS, Overlay JC, MacDonald JD (1987) Scanning-transmission ion microscopy as it complements particle induced X-ray-emission microanalysis. Scanning Microsc 1:879–889

Lundborg A (1997) Reducing the nitrogen load: whole-tree harvesting. Ambio 26:387–393

Mahmood S, Finlay RD, Erland S, Wallander H (2001) Solubilisation and colonisation of wood ash by ectomycorrhizal fungi isolated from a wood ash fertilised spruce forest. FEMS Microbiol Ecol 35:151–161

Mahmood S, Finlay RD, Wallander H, Erland S (2002) Ectomycorrhizal colonisation of roots and ash granules in a spruce forest treated with granulated wood ash. For Ecol Manage 160:65–74

Mahmood S, Finlay RD, Fransson A-M, Wallander H (2003) Effects of hardened wood ash on microbial activity, plant growth and nutrient uptake by ectomycorrhizal spruce seedlings. FEMS Microbiol Ecol 43:121–131

Marschner H, Dell B (1994) Nutrient uptake in mycorrhizal symbiosis. Plant Soil 159:89–102

Snetselaar KM Whitney KD (1990) Fungal calcium oxalate in mycorrhiza of *Monotropa uniflora*. Can J Bot 68:533–543

Sokal RR, Rohlf FJ (1995) Biometry. 3rd edn. Freeman, New York

Staples TE, van Rees KCJ (2001) Wood/sludge ash effects on white spruce seedling growth. Can J Soil Sci 81:85–92

Steenari BM, Marsic N, Karlsson LG, Tomsic A Lindqvist O (1998) Long-term leaching of stabilized wood ash. Scand J For Res [Suppl] 2:3–16

Steenari BM, Karlsson LG, Lindqvist O (1999) Evaluation of the leaching characteristics of wood ash and the influence of ash agglomeration. Biomass Bioeng 16:119–136

Turnau K, Przybylowicz WJ Mesjasz-Przybylowicz J (2001) Heavy metal distribution in Suillus luteus mycorrhizas as revealed by micro-PIXE analysis. Nucl Instr Meth Phys Res B 81:649–658

Wallander H, Johansson L, Pallon J (2002) PIXE analysis to estimate the elemental composition of ectomycorrhizal rhizomorphs grown in contact with different minerals in forest soil. FEMS Microbiol Ecol 39:147–156

Wallander H, Mahmood S, Hagerberg D, Johansson L, Pallon J (2003) Elemental composition of ectomycorrhizal mycelia identified by PCR-RFLP analysis and grown in contact with apatite or wood ash in forest soil. FEMS Microbiol Ecol 44:47–65

Whitney KD, Arnott HJ (1987) Calcium oxalate crystal morphology and development in *Agaricus bisporus*. Mycologia 79:180–187