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SHORT NOTE

Michael D. Rumberger · Peter Lentzsch · Babette Münzenberger · Reinhard F. Hüttl

Nutrient amounts of ectomycorrhizae analysed by EDX using ESEM and ICP

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Abstract Energy-dispersive X-ray (EDX) analysis coupled with an environmental scanning electron microscope (ESEM) was tested as an alternative to the inductively coupled argon plasma (ICP) spectrometer method for nutrient analyses of ectomycorrhizae. The results of EDX–ESEM and ICP were compared for 12 ectomycorrhizal morphotypes collected in beech and Scots pine forests in northern Brandenburg. The amounts of Al, Ca, Mg and S analysed in the outer hyphal layers of the sheath with the EDX–ESEM technique correlated well with the amounts of these elements in the whole mycorrhiza as assessed by ICP. For the elements P and K, no such correlation existed, indicating an uneven distribution of these elements in the ectomycorrhiza. It is concluded that the EDX–ESEM technique could be a useful and reliable tool for the analysis of nutrient elements in ectomycorrhizae, especially for studies focussing on small-scale soil heterogeneity or on infrequent morphotypes.

Keywords Ectomycorrhiza · Energy-dispersive X-ray · Environmental scanning electron microscope · Inductively coupled argon plasma spectrometer · Nutrient amounts

Introduction

Tree species of the temperate and boreal zone profit from the symbiosis with ectomycorrhizal fungi. The mycobionts improve the water supply of the host (Coleman et al. 1989; Duddridge et al. 1980; Guehl and Garbaye 1990), improve the resistance of the host tree against root pathogens (Haug and Oberwinkler 1987; Sen 2001), strengthen their tolerance to heavy metals in the soil (Hartley et al. 1999; Leyval et al. 1997) and are of great importance in nutrient mobilisation and uptake (e.g. Finlay 1989; Marschner and Dell 1994; Read and Perez-Moreno 2003). Although there are laboratory-based studies on the underlying mechanism of improved nutrient absorption rate (e.g. Bücking and Heyser 2003), so far only few investigations have been carried out on the nutrient storage of the ectomycorrhizal community (Kottke et al. 1998). Wallander et al. (2003) suggest that high concentrations of K in the rhizomorphs of *Suillus granulatus* (L. ex Fr.) Kuntze are a sign that this fungus is a good accumulator of K. However, the amounts of stored elements do not themselves reflect nutrient uptake rates (Kottke et al. 1998). Additionally, the accumulation of nutrients can have other functions than the nutritional supply of the host trees, e.g. formation of crystals for the decontamination of products of the metabolism and as protection against desiccation of the hyphae (Arocena et al. 2001; Casarin et al. 2003). Therefore, it is difficult to interpret the nutrient amounts of ectomycorrhizae as a sign of the nutritional importance of the ectomycorrhiza for the host trees. However, Bücking and Heyser (2001) argue that the accumulation of nutrients in ectomycorrhizae can moderate nutritional fluctuations in the soil. They analysed the accumulation of ^{33}P in the mantle and the Hartig net of mycorrhiza of *Pinus sylvestris* L. by microautoradiographic studies. A major problem for more comprehensive studies is the lack of a method which allows a high throughput of small samples.

The inductively coupled argon plasma (ICP) spectrometer method used by Haug et al. (1992) and Kottke et al. (1998) to analyse nutrient amounts in mycorrhiza needs 35–120 mg of dried ectomycorrhizae, equivalent to 500–1,000 ectomycorrhizal root tips, for each measurement. Thus, using ICP, an estimation of average element values of the ectomycorrhiza according to the proportional composition of the ectomycorrhizal community is very diffi-

M. D. Rumberger · R. F. Hüttl
Chair of Soil Protection and Recultivation,
Brandenburg University of Technology,
P.O. Box 101344, 03013 Cottbus, Germany

M. D. Rumberger · P. Lentzsch · B. Münzenberger (✉)
Institute of Primary Production and Microbial Ecology, Leibniz Centre for Agricultural Landscape and Land Use Research (ZALF) e.V.,
Eberswalder Strasse 84,
15374 Müncheberg, Germany
e-mail: bmuenzenberger@zalf.de
Tel.: +49-33432-82153
Fax: +49-33432-82330

cult, especially when the frequency of ectomycorrhiza is low, e.g. due to climatic influences like drought, or when studying small morphotypes that prevail, for example, on deciduous trees (Rumberger et al. 2004).

Rapp (1991) used the energy-dispersive X-ray (EDX) technique to study the influence of liming and ammonium sulphate fertilisation on nutrient amounts in ectomycorrhizae and fine roots in an old-growth beech stand. The investigations were carried out on coal-sputtered cross-sections with a transmission electron microscope. More recently, this method has been used by Bücking et al. (1998, 2002), Bücking and Heyser (1999, 2000a,b, 2003) and Winn-Börner (1991). They investigated the subcellular distribution of Ca, K, S and especially P. Bücking and Heyser (2003) found that the P uptake and translocation by the ectomycorrhizal fungus is related to the carbohydrate supply of its host plant. Lussenhop and Fogel (1999) were able to quantify the Ca, K and P amounts in the hyphal sheaths of frozen, planed, etched and Al-coated ectomycorrhizae by comparing the collected quanta of Ca, K and P of the sample with the collected quanta derived from filter paper soaked with calibrated solutions. Recently, Lux et al. (2002) employed the EDX technique coupled with environmental scanning electron microscopy (ESEM) to study the formation of silicate in leaves and roots of millet [*Sorghum bicolor* (L.) Moench]. The steam-saturated atmosphere in the ESEM ensures that the samples cannot dry out during the measurement (Danilatos 1988). The ESEM technique does not require elaborate sample preparation and fixation. Thus, artefacts due to dispersion of nutritional elements during fixation can be avoided (Bonanomi et al. 2001).

The EDX technique is limited to the detection of elements with an ordinal number between 10 and 25, such as Al, Ca, K, Mg, P and S (Leapman and Hunt 1991). However, peak values of micronutrients cannot be clearly distinguished from the background using EDX and lighter elements, such as N, are very difficult to analyse, because they only emit X-rays with a low energy level (Bücking et al. 1998). Another analytic method, electron energy-loss spectroscopy (EELS), permits precise subcellular localisation and measurement of N amounts, which is not possible either by ICP or EDX (Kottke et al. 1998). On the other hand, disadvantages of EELS lie in the embedding procedure and the very small measurable volume.

The aim of the present study was to develop a method that allows analysis of a large number of small ectomycorrhiza samples in a relatively short time. This is necessary to investigate small-scale heterogeneity of ectomycorrhiza in the soil and to study ectomycorrhizal morphotypes with a low frequency. EDX coupled with ESEM has been used to investigate amounts of nutrient elements in ectomycorrhizae (Rumberger et al. 2004), and data obtained by analysing the approximate outer 10 μm of the hyphal sheath of ectomycorrhizae have been correlated with data from the conventional ICP method that analyses whole ectomycorrhizae.

Materials and methods

The three study sites are located in the northeastern lowlands of Germany. Stand p/b 57 is a mixed forest formed by 114-year-old Scots pine (*P. sylvestris* L.) and 57-year-old European beech (*Fagus sylvatica* L.) trees. Stands b 91 and b 140 are pure beech forests with 91- and 140-year-old trees, respectively. For a detailed site description, see Rumberger et al. (2004).

Samples were taken with a soil corer (8 cm in diameter) in April 2002. Ectomycorrhizae were removed from the soil, rinsed gently with tap water, cleaned with tweezers and sorted according to the different morphotypes. The ectomycorrhizae of each sample were pooled (Table 1) and a part was utilised for investigations with the ICP method. For this, mycorrhizae were dried to constant weight at 65°C. Ectomycorrhizae (5–10 mg) were digested under pressure with 65% HNO_3 (suprapur) and analysed with ICP-OES (Unicam 701). The remaining ectomycorrhizae in each pooled sample were frozen in dH_2O and stored at −20°C until analysis by EDX. After thawing, the unfixed ectomycorrhizae were analysed individually on a slide, coated with Ramson fat for better adhesion, in the ESEM (Philips XL 30) with the integrated DX4 detector system (EDAX Inc.). The Si(Li) X-ray detector with an ultrathin beryllium window collected the X-ray quanta between 0 and 10 keV. The distance between the detector and the

Table 1 List of the samples of the analysed ectomycorrhizal morphotypes with specification of the sample stand, sampled horizon, tree and fungal species and number of measurements by ICP and EDX

Sample	Fungi	Tree species	Sample stand	Sample horizon	ICP	EDX
Genea	*Genea hispidula*	Beech	b 91	min	1	81
Type 1	Unknown species	Beech	b 91	min	1	75
Type 2	Unknown species	Beech	b 91	min	1	15
Type 3 (org)	Unknown species	Scots pine	p/b 57	org	1	258
Type 3 (min)	Unknown species	Scots pine	p/b 57	min	1	99
Lact. (org)	*Lactarius subdulcis*	Beech	b 91	org	1	165
Lact. (min)	*Lactarius subdulcis*	Beech	b 91	min	1	76
Russ. (org)	*Russula ochroleuca*	Beech	b 91	org	1	15
Russ. (min)	*Russula ochroleuca*	Beech	b 91	min	1	36
X. bad.	*Xerocomus badius*	Scots pine	p/b 57	min	1	72
X. chr. (org)	*X. chrysenteron*	Beech	b 140	org	1	75
X. chr. (min)	*X. chrysenteron*	Beech	p/b 57	min	2	132

org Organic layer, *min* mineral soil

object was geared to 10 mm. The pressure of the specimen chamber was 3.9 Torr, the temperature 1–2°C and the relative humidity 100%. The acceleration voltage of 20 kV was used for the measurements taking 60 s. Elements (Al, Ca, K, Mg, S, P) were analysed in the outer hyphal sheath of mycorrhizae up to an assumed depth of 10 μm, which is equivalent to two to three hyphal layers. For each sample, between 5 and 86 ectomycorrhizae were measured each at their base, their middle part and their tip. The average value for each sample was calculated. Sufficient amounts of ectomycorrhizae for replicate measurements by the ICP method were only available for one sample (Table 1).

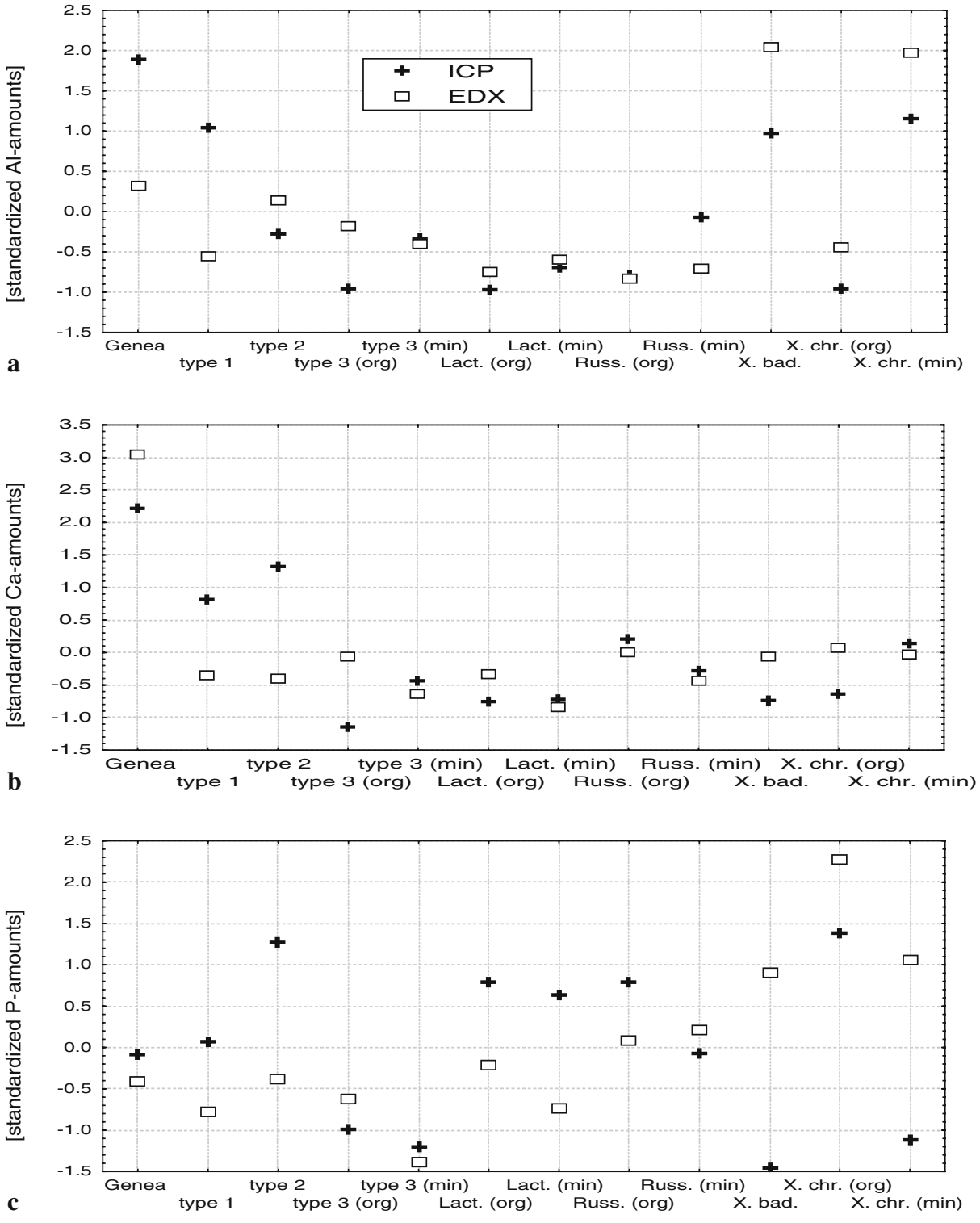

Fig. 1 Relations of aluminium (**a**), calcium (**b**) and phosphorus (**c**) amounts in 12 morphotypes between the standardised measurements of ICP and EDX. For characteristics of the morphotypes, see Table 1

The peak values of the EDX analysis were compared with the results from the ICP analysis. For this purpose, the average values of 12 samples (Table 1) of the ICP and EDX measurements were standardised by Statistica and correlated by Statistica ANOVA/MANOVA (Version 5). To test which nutrient amounts of each morphotype were over- or under-estimated by EDX in comparison to ICP, the standardised element amounts of Al, Ca and P were documented for all analysed elements (Fig. 1).

Results

The results of the EDX and the ICP ectomycorrhiza analyses were significantly correlated for Al and Ca amounts (r=0.66, P=0.02 and r=0.62, P=0.03, respectively). After elimination of runaway values, the S amounts (r=0.75, p=0.008) and the Mg amounts of the ectomycorrhizae were also significantly correlated (r=0.75, P=0.02). In both cases, the runaway value came from site b 140. P (r=0.17, P=0.6) and K (r=0.498, P=0.1) showed no correlation with regard to the two methods.

The standardised nutrient amounts for the ectomycorrhizae of the 12 samples are shown for Al, Ca and P (Fig. 1). For Al, the results of the EDX and the ICP analysis gave nearly similar relations between the amounts for the ectomycorrhizae of the samples (Fig. 1a). In relation to the values obtained with ICP, the EDX technique measured relatively lower values for the ectomycorrhizae of *Genea hispidula* Berk. et Br. and type 1. For the different morphotypes, similar Ca amounts could be obtained with both methods (Fig. 1b).

However, there was a difference between morphotypes: the ratio of Ca amounts detected with EDX compared with the values of the ICP analysis was lower for types 1 and 2 than for the other morphotypes. In contrast, for *G. hispidula* and type 3 org, the Ca amount measured with the EDX technique was higher than the results of the ICP analysis. The P amounts (Fig. 1c) of the outer layers of the hyphal sheath of the ectomycorrhizae differed from the amounts of the whole ectomycorrhiza analysed by ICP. In general, the results of the EDX technique were relatively too high for the ectomycorrhizae of *Xerocomus badius* (Fr.) Kühner ex E. J. Gilbert und *Xerocomus chrysenteron* (Bull.) Quél. and relatively too low for types 1 and 2 and *Lactarius subdulcis* (Bull.:Fr.) Gray, as compared with the results of the ICP analysis.

Discussion

The results of this study indicate that the amounts of Ca and Al of the outer hyphal layers of the ectomycorrhizae analysed by the EDX technique are representative of the element amount in the whole ectomycorrhizae as analysed with ICP. With one or two exceptions, the Mg and S amounts detected with EDX were also significantly correlated with the ICP values for the whole ectomycorrhiza. In contrast, no such correlation existed for K and P. Bücking and Heyser (2000b) observed that the intercellular distribution of the Ca amounts in Scots pine ectomycorrhiza of *Suillus bovinus* (L. ex. Fr.) Kuntze was almost homogeneous. This could explain the good correlation of the Ca amounts of the whole ectomycorrhiza with that in the upper hyphal layers of the ectomycorrhizal sheath. However, the EDX measurements overestimated the Ca amounts for the ectomycorrhizae of the samples *Genea* and type 3 org. The higher Ca amount in the outer sheath of these two morphotypes in relation to Ca amount of the whole ectomycorrhiza may be due to Ca-oxalate accumulation on their surface as found by Kottke et al. (1998) for *Paxillus involutus* (Batsch) Fr.

In general, the EDX and the ICP analyses detected similar amounts of Al in the ectomycorrhizae, except for a few morphotypes. These small differences may be explained by the presence of soil on the ectomycorrhizal surfaces or of crystals or extracellular pigments containing Al and Si in the mantle of certain morphotypes (Hodson and Wilkins 1991; Turnau et al. 1996). Despite thoroughly cleaning the ectomycorrhizae, some soil particles were still detected by ESEM analysis. However, these soil particles and the possible presence of crystals did not affect the significant correlation between measurements from the two methods.

The P amounts of the outer hyphal layers of the ectomycorrhizal sheath measured by EDX were not correlated with that of the whole ectomycorrhiza. P is stored in polyphosphate granules and in smaller amounts as free phosphate (Kottke and Martin 1994). Different fungal species differ in their physiological ability so that storage and translocation of P to the host tree vary between the morphotypes and samples. Presumably, there are some ectomycorrhizae that transfer P quickly to the host tree while others accumulate P strongly. Bücking and Heyser (2000a) found that the distribution of P varies between the ectomycorrhizae of *S. bovinus* (L. ex. Fr.) Kuntze and *Pisolithus tinctorius* (Pers.) Coker & Cough. In the ectomycorrhiza of *S. bovinus*, the P amounts increased slightly from the outer to the central parts of the ectomycorrhiza, whereas in the ectomycorrhiza of *P. tinctorius*, the P amounts decreased strongly from the sheath to the root. In our investigation, EDX overestimated P amounts in the ectomycorrhizae of the genus *Xerocomus*, which might have a similar P translocation pattern as the ectomycorrhizae of *P. tinctorius*. In contrast, the P amounts of other morphotypes such as types 1 and 2, *G. hispidula* and *L. subdulcis* were underestimated by EDX. In some ectomycorrhizae, the distribution of P depends on the P availability in the surrounding soil (Bücking and Heyser 2000a). For example, the P translocation to the host tree by *P. tinctorius* is supply-dependent, but not by *S. bovinus*. Apparently, the ectomycorrhizae of the samples differed in their P distribution so that some morphotypes have a quite homogeneous distribution of P in cross-section and other types have a very heterogeneous one. Thus, EDX results can only provide an adequate estimate of the P amount in morphotypes with a homogeneous P distribution in cross-sections but fail completely in other morphotypes.

The K amounts of the outer hyphal sheath also did not represent the amounts of the whole ectomycorrhiza. The K amounts are linked with the polyphosphate amounts of the ectomycorrhiza (Bücking and Heyser 2000a,b). Consequently, the K amounts are not homogeneously distributed in the cross-sections of the ectomycorrhizae, and therefore the inner parts of the ectomycorrhizae of some morphotypes contain higher K amounts than the outer parts (Bücking and Heyser 2000b). Moreover, K was easily washed out of the outer sheaths of the ectomycorrhizae during the cleaning procedure (Haug et al. 1992).

The function of nutrient accumulation in ectomycorrhizae has been discussed in detail in the literature. Wallander et al. (2003) interpreted ectomycorrhizae with high Ca amounts found in rhizomorphs of the fungus *S. granulatus* (L. Fr.) Kuntze as good accumulators of Ca. In the same way, Kottke et al. (1998) explain the high nutrient amounts of the ectomycorrhizae of *X. badius* as a sign that this fungus has a special ability to accumulate nutrients. Bücking and Heyser (2001) argue that the accumulation of nutrients in ectomycorrhizae can moderate nutritional fluctuations in the soil. However, the data obtained by EDX and by ICP must be interpreted with caution. An estimation of the transfer function of different morphotypes is not possible. Nevertheless, knowing the nutrient amounts in ectomycorrhizae may contribute to understanding their functioning and call for further research.

Although the ectomycorrhizal samples analysed here belonged to different soil horizons, forest stands, tree and fungal species, the amounts of Al, Ca, Mg and S obtained with both methods correlated well. Thus, the EDX technique coupled with ESEM is a suitable method to investigate ecological effects such as forest transformation processes (Rumberger et al. 2004). The great advantage of the EDX technique is that only 20–30 ectomycorrhizal root tips are necessary for measurement. These ectomycorrhizae can be obtained even in small soil volumes and for rare morphotypes. Therefore, this method can be used in combination with rhizotrons and for investigations into the small-scale heterogeneity of soils. Finally, the EDX technique also allows the seasonal variation of nutrient amounts in ectomycorrhizae to be investigated.

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