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ORIGINAL PAPER

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Extraction of extraradical arbuscular mycorrhizal mycelium from compartments filled with soil and glass beads

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Abstract This study presents a novel method for the extraction and quantification of extraradical mycelium (ERM) of arbuscular mycorrhizal fungi (AMF) from a substrate that simulates soil better than previously used artificial growth media. Fungal compartments were constructed from small net pots with a latticed wall and filled with a mixture of glass beads and 40 µm wet sieved soil. The net pots were surrounded by a 30-µm mesh membrane through which hyphae but not roots could grow. They were inserted into soil where a *Glomus intraradices* (BEG 110) colonized potato plant was growing. The ERM that had grown out from roots through the membrane was successfully collected and quantified after harvest by washing out the soil/glass bead mixture through a sieve with a mesh width of 40 µm. Concentrations of P, Zn, Cu and Mn in the AMF ERM were analysed.

Keywords Extraction method · Extraradical mycelium · Fungal compartment

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Introduction

The extraradical mycelium (ERM) of arbuscular mycorrhizal fungi (AMF) mainly consists of AMF hyphae and AMF spores. The extraradical hyphae proliferate in the soil and provide the surface area for fungal uptake of phosphate (Li et al. 1991a; Jansa et al. 2003), copper (Li et al. 1991b), zinc (Chen et al. 2003) or nitrogen (Hawkins and George 2001). The mycelium also transports and transfers these elements to the host. Fungal compartments in soil (Kothari et al. 1991; Caris et al. 1998) can be used to study the mycelium without interference from roots. Previous studies indicated that the amount of hyphae in the soil, estimated as the weight of soil the hyphae had bound into aggregates (Graham et al. 1982), as well as the hyphal spread (Jakobsen et al. 1992) and the hyphal architecture (Bago et al. 1998a,b) have a possible impact on AMF contribution to plant nutrition and plant growth response. Furthermore, the AMF extraradical hyphae contribute to the stabilization of soil aggregates (Miller and Jastrow 1990; Kabir and Koide 2002) or might protect the host plant from toxic levels of deleterious elements in the growth medium (Li and Christie 2001; Rivera-Becerril et al. 2002). Soil-borne spores with their ability to withstand adverse soil conditions for many years are considered to be the major reproductive organs of the AMF. However, under some conditions, the extraradical hyphae also act as an important source of initial root colonisation (Addy et al. 1997).

Information about the extraradical part of the AMF is crucial to predict the capacity of different AMF isolates or species to enhance plant nutrition and plant growth. However, quantitative and qualitative parameters of soil-grown AMF ERM were rarely recorded up to now because it is difficult to extract AMF hyphae from the soil matrix. Several methods were developed to overcome this problem. The membrane filtration technique (Hanssen et al. 1974) to extract minute amounts of AMF hyphae from soil has been used frequently in many modifications (e.g. Schubert et al. 1987; Li et al. 1991c; Jakobsen et al. 1992). It allows an estimation of hyphal length per unit soil, but is inappropriate to extract intact hyphae in amounts large enough to

study ERM architecture or to analyse the material for, e.g. element concentrations. Hydroponic and aeroponic cultures (Hung and Sylvia 1988; Hawkins and George 1997) and simple growth media like sand (Vilariño et al. 1993; Jentschke et al. 1999) or glass bead substrates supplied with nutrient solution (Redecker et al. 1998; Chen et al. 2001) were previously used to grow AMF-colonised plants, or as a filling for fungal compartments. Separate fungal compartments filled with theses simple substrates facilitate the harvest of AMF ERM. However, growth, architecture, composition and microbial associations of ERM in nutrient solution, glass beads or sand might be very different from that in soil. Therefore, we have developed a mixture of 40-µm wet sieved soil and glass beads as a filling for fungal compartments to allow faster sampling of AMF ERM compared with the membrane filtration technique while simulating soil better than previously used artificial growth media. The novel method was used to test the effect of soil P supply on hyphal growth and element concentrations.

Materials and methods

Production of 40-µm wet sieved soil

Subsoil material of a Luvisol from Weihenstephan, southern Germany (48°25′N, 11°50′E), classified as loamy sand (45.2% sand, 42.0% silt, 12.8% clay) was used to produce 40-µm wet sieved soil. Before sieving, the soil contained (mg kg^{-1}) 5.2 and 3.4 $CaCl_2$ (0.0125 M)-extractable NH_4^+ and NO_3^-, respectively, 3.8 $NaHCO_3$-extractable P, 58 calcium acetate lactate-extractable (Schüller 1969) K, and 1.93 (Fe), 1.75 (Mn), 0.10 (Zn) and 0.16 (Cu) DTPA-extractable micronutrients. The soil had a pH (0.01 M $CaCl_2$) of 7.3, a $CaCO_3$ equivalent of 23.3%, and 0.2% organic matter.

Approximately 2 kg of air-dried soil was placed into a 10-L bucket. Between 7 and 8 L tap water were added and the soil was completely brought into suspension by stirring. Coarse particles were allowed to settle for 10–20 s before the soil suspension was poured through a sieve with a mesh width of 40 µm (Retsch Test Sieve 3310-1, Retsch, Germany). This procedure was repeated three times on each 2-kg soil portion. The sieved soil suspension was collected in another bucket and allowed to settle until the water above the soil layer had become clear and could be siphoned off using a flexible tube. The remaining sludge was transferred to a shallow, heat-resistant dish and was dried at 65°C until the material had become solid.

Construction and filling of fungal compartments

Fungal compartments were constructed from 70-ml net pots with a latticed wall (inner diameter=5 cm; Teku G5R, Pöppelmann Teku, Germany). The latticed wall of the pot was treated with a silicone primer (Elch SO 9473001042, Rhodia, Germany) before a nylon membrane (mesh width 30 µm, Sefar Nitex 03-30/18, Sefar, USA) was fixed with silicone (Würth Lebensmittelsilikon, Würth, Germany) to cover the openings. The fine mesh width allowed hyphae but not roots to enter the compartment.

The dried, wet sieved soil was dry heated for 10 h at 90°C to eliminate fungal propagules. After cooling, 200 mg K (K_2SO_4), 200 mg N (NH_4NO_3), 100 mg Mg ($MgSO_4$), 50 mg (low-P compartments) or 200 mg (high-P compartments) P [$Ca(H_2PO_4)_2{\cdot}H_2O$], 10 mg Zn ($ZnSO_4{\cdot}H_2O$), 10 mg Cu ($CuSO_4$), 4 mg Fe ($FeNH_4$ citrate), 400 mL deionised water and 1 kg glass beads (diameter=2 mm) were added per kg dry soil and mixed thoroughly. Each fungal compartment was filled with 110 g (2 g cm^{-3}) of the wet mixture.

Installation of fungal compartments

Axial shoot cuttings of potato (*Solanum tuberosum* L. cv. Désirée) plants were rooted in aerated nutrient solution (concentration of the element/applied form: N: 5 mM/Ca $(NO_3)_2{\cdot}4H_2O$; P: 0.5 mM/KH_2PO_4; K: 1.5 mM/KH_2PO_4 and K_2SO_4; Ca: 3.5 mM/$Ca(NO_3)_2$ and $CaSO_4{\cdot}2H_2O$; Mg: 0.6 mM/$MgCl_2{\cdot}6H_2O$; S: 1.5 mM/$CaSO_4{\cdot}2H_2O$ and K_2SO_4; Fe: 40 µM/Fe-EDTA; B: 56 µM/H_3BO_3; Mn: 3.6 µM/$MnSO_4$; Zn: 1.5 µM/$ZnSO_4$; Cu: 1.6 µm/$CuSO_4$; Mo: 0.7 µM/$Na_2MoO_4{\cdot}2H_2O$) for 3 weeks before they were transferred to 2-L pots (inner diameter=16 cm; Teku TC 16 L, Pöppelmann Teku; one plant per pot), each containing 1.930 g dry, fertilised soil at a bulk density of 1.3 g cm^{-3}. The same soil substrate as for the wet sieving was used for growing the plants. It was fertilised with 50 mg P [$Ca(H_2PO_4)_2{\cdot}H_2O$] per kilogram dry soil and the same amounts of other nutrients as the wet sieved soil. Before fertilisation, the soil was dry heated twice for 12 h at 85°C with an interval of 24 h at room temperature to eliminate fungal propagules, and inoculum of *Glomus intraradices* (BEG 110), consisting of colonised root pieces and adhering air-dried loess soil, was added (10% w/w) to the soil of the mycorrhizal treatments (n=4). Inoculum for nonmycorrhizal controls (n=4) was filtered with deionised water (70 ml per 50 g dry inoculum through Blue Ribbon filter paper, Schleicher & Schüll, Germany) before being autoclaved. The filtrate was added to the soil of the nonmycorrhizal treatments to encourage similar microflora as in the mycorrhizal treatments. Two high-P and two low-P fungal compartments were inserted into each pot. The top openings of the inserted compartments were covered with adhesive tape. Plants were grown in the greenhouse under ambient conditions from May to June 2002. The planting pots were set up completely randomised. Daily water loss from the pots was estimated gravimetrically and replaced by deionised water. The water content of the soil in the plant compartment was 22% (w/w) after watering. Thirty days after plants were transferred to soil, an additional 100 mg K (K_2SO_4), 100 mg N (NH_4NO_3) and 6 mg Fe ($FeNH_4$ citrate) per kilogram dry soil were fertilised to the rooted soil and 70 mg P [$Ca(H_2PO_4)_2{\cdot}H_2O$] per kilogram dry soil was added to the high-P compartments. Plants were harvested 45 days after they were transferred to soil. Roots were washed free

of soil and representative samples (0.8–1.0 g) of the fresh plant roots were taken and stained with trypan blue in lactic acid (Koske and Gemma 1989) to evaluate the AMF-colonised root length by a modified grid line intersection method (Tennant 1975; Kormanik and McGraw 1982).

Harvest of AMF ERM from fungal compartments and analysis of P, Zn, Cu and Mn concentrations

When plants were harvested, one high-P and one low-P fungal compartment were removed from each planting pot and cleaned from adhering soil. Then, the filling of the fungal compartments was transferred to a sieve with a mesh width of 40 μm. The wet sieved soil was carefully washed through the mesh with tap water, leaving ERM and glass beads in the sieve. To separate the ERM from the glass beads and to clean the ERM, the ERM–glass bead mix was then transferred into a 1-L beaker, deionised water was added and the mixture stirred before the water was poured back into the sieve, leaving the glass beads in the beaker. This procedure was repeated three times. The ERM was rinsed with deionised water before it was collected from the sieve using forceps and a Pasteur pipette. The extracted material was transferred into a 2.5-mL Eppendorf tube together with approximately 1 mL deionised water and was dropped into liquid nitrogen before being freeze dried at −30°C and 0.04 mbar for 4 days (freeze dryer P 15 K, Piatkowski GmbH, Munich, Germany).

After the dry weight of the AMF ERM had been determined, the total amounts extracted from each fungal compartment were dry ashed at 500°C, oxidised with 2 mL of 1:3 diluted HNO_3 and taken up into 10 mL of 1:30 diluted HCl. Phosphorus concentrations in the samples were analysed colorimetrically with a spectrophotometer at 436 nm wavelength after staining with ammonium molybdate–vanadate solution (Gericke and Kurmies 1952). Concentrations of Cu, Zn, Fe and Mn were measured by atomic absorption spectrometry (ATI Unicam 939/Solaar, Thermo Electron, USA). A Cs–La buffer was added to the samples before they were analysed for Mn (Schinkel 1984).

Results and discussion

The novel method was less laborious than other techniques to isolate ERM previously used by our research group. Between 1.5 and 2.0 kg of dry wet sieved soil were prepared by one person per day. The extraction of mycelium from one fungal compartment took approximately 20 min.

The AMF-colonised root length in the mycorrhizal treatments was 75.2% (±5.4 SD), whereas no colonisation was detected in the nonmycorrhizal controls. The material extracted from the fungal compartments of the mycorrhizal treatment consisted of both hyphae and spores. No fungal material could be extracted from the compartments of the nonmycorrhizal controls, indicating that only negligible amounts of mycelium from other fungi had grown into the compartments. Extracted amounts of soil-grown AMF ERM were large enough to analyse element concentrations. Significantly larger amounts of AMF ERM were produced in the high-P compartments compared with the low-P compartments (Table 1). In previous studies, no stimulation of hyphal growth by application of inorganic P to fungal compartments was observed (Li et al. 1991b; Olsson and Wilhelmsson 2000) and it has been concluded that AMF do not actively forage for soil P by increasing fungal biomass per unit soil (Olsson et al. 2003). However, there is evidence that AMF species differ considerably in their ability to scavenge a localised P source in the soil (Boddington and Dodd 1998). It is also possible that increased amounts of AMF ERM in high-P compartments were the result of an enhanced root growth in the immediate vicinity of the high-P compartments. Roots connected to AMF ERM within the high-P compartments might have grown at a higher rate due to a better supply with P (Cui and Caldwell 1996). The spatial distribution of roots was not assessed in the present experiment.

In our study, P concentrations in AMF ERM were in a much lower range than values reported by Solaiman and Saito (2001) for ERM of *Gigaspora margarita* associated with *Allium cepa*, which were between 5 and 15 mg g dry weight $(DW)^{-1}$. It has been shown that the accumulation of polyphosphate in AMF hyphae increases with increasing P availability in the growth medium (Ezawa et al. 2003). In our experiment, P concentrations in AMF ERM did not differ significantly between the P supply levels. A possible explanation could be that P taken up by AMF ERM was efficiently translocated and transferred to the host plant.

The concentrations of Zn and Cu were higher in hyphal material obtained from the low-P compartments compared with the high-P compartments. This effect may be related to a dilution effect in the high-P treatment or may be the result

Table 1 Amount of AMF material harvested from the fungal compartments and results of the element analysis

Compartment	AMF material harvested from fungal compartments (mg DW per compartment)	Element concentrations in AMF material harvested from fungal compartments			
		P (mg/g DW)	Zn (μg/g DW)	Cu (μg/g DW)	Mn (μg/g DW)
High P	16.70±6.99	3.62±0.31	342.5±73.8	248.3±37.9	297.8±82.1
Low P	7.73±1.99*	3.35±0.69	604.4±89.4*	361.3±50.6*	450.3±143.3

Shown are the mean values of the high-P and the low-P compartments±SD
DW dry weight
*$P<0.05$, *t* test; mean values for low-P compartments significantly different from the mean values of the high-P compartments

of effective P/micronutrient co-transport in the hyphae to the roots under high-P soil conditions.

In general, the consistency of the results on ERM micronutrient concentrations indicate that soil contaminations did not interfere with the analysis. Small amounts of clay particles were found entangled in the freshly extracted hyphae, but freeze-dried ERM formed a swablike structure, whereas the soil particles accumulated at the bottom of the Eppendorf-tube. Thus, freeze-drying may be the most suitable method to process AMF ERM after extraction from fungal compartments.

Finely branched hyphae were observed in the collected ERM even after freeze-drying, indicating that the processing had left hyphal structures at least partly intact. This shows a potential to adapt this technique to the study of the architecture of soil-grown hyphae. It may be appropriate to soak the contents of the fungal compartments with water and use an ultrasonic treatment as has been described for the extraction of root hairs (Gahoonia et al. 2001). In another application, fungal compartments can be completely inserted into the soil to determine the distribution and mineral nutrient contents of AMF ERM, e.g. in different soil depths or with increasing distance from the root. The suitability of soils to be wet sieved must be tested. Most likely, soils with a relatively high silt content and a low organic matter content are most suitable for this method.

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