Mycorrhiza (2001) 11:291–296
DOI 10.1007/s00572-001-0135-8

ORIGINAL PAPER

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A novel inserted membrane technique for studies of mycorrhizal extraradical mycelium

Accepted: 25 September 2001 / Published online: 13 November 2001

Abstract A simple "inserted membrane technique" (IMT) for sampling mycorrhizal extraradical mycelium (ERM) was developed as an alternative to the commonly used membrane filtration technique (MFT). The ERM of two types of mycorrhiza, orchid and arbuscular, was extracted by insertion of cellulose nitrate or cellulose acetate membrane filters (0.45–0.6 µm pore size) into the mycorrhizosphere of host plants. The membranes with adhered ERM were removed at harvest and stained with trypan blue for estimation of total hyphal length and with enzyme stains to indicate the viability of the ERM. There are two apparent advantages of the IMT over the MFT: (1) Samples were cleaner and easier to observe, particularly when the hyphae were stained for enzyme activities and (2) the ERM remained intact and, thus, was also suitable for observation of ERM morphology. There were statistically significant correlations between the lengths of ERM extracted from clinoptinolite using MFT and IMT for both mycorrhizal types tested, orchid mycorrhiza (r=0.63) and arbuscular mycorrhiza (r=0.80). Linear regression analysis indicated the best fit for the data obtained (P<0.05, n=14 for orchid mycorrhiza and P<0.001, n=26 for arbuscular mycorrhiza). Advantages of the new technique over other techniques for studying ERM are discussed.

Keywords Extraradical mycelium · Vital staining · Orchid mycorrhiza · Arbuscular mycorrhiza

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Introduction

One of the important advances in the last decade of mycorrhizal research has been the increased emphasis on the structure, organisation and function of the extraradical mycelium (ERM) (see Smith and Read 1997 for references). The hyphae forming the ERM of mycorrhizas absorb nutrients from the soil (Smith 1967; Alexander et al. 1984; Sylvia 1990). The length, spatial distribution and viability of the ERM are closely related to symbiosis effectiveness in terms of nutrient uptake and plant growth. Only metabolically active hyphae, for example, can participate in this process (Sylvia 1990). Study of the extraradical phase of the mycorrhizal symbiosis is, therefore, necessary to obtain a more detailed insight into the functioning of the symbiosis.

The first step in ERM assessment, for example determining lengths and metabolic activity, is extraction of the ERM from the soil or growth substrate. One set of ERM extraction techniques is based on vacuum filtration of a soil suspension through a membrane filter. This membrane filtration technique (MFT) was introduced by Hanssen et al. (1974). The technique has been modified recently by several authors and is now widely used for assessment of ERM lengths of arbuscular mycorrhizal fungi (AMF) (Sylvia 1992; Boddington et al. 1999). Vilariño et al. (1993) developed another extraction technique using a rotating wire frame to retrieve the ERM fragments from an agitated soil suspension. A third set of extraction techniques is based on sucrose flotation and centrifugation (Schubert et al. 1987), also combined with sonication (Miller and Jastrow 1998). All these techniques are suitable for quantification of the lengths of ERM with respect to spatial distribution of the hyphae in soil (Sylvia 1990; Dodd 1994). Their disadvantage, however, is that they severely disturb the ERM network during sample processing. Thus, the identification of structures such as branched absorbing structures (BAS) (Bago et al. 1998a, b) or the measurement of morphological parameters such as hyphal branching or anastomoses formation is often difficult. Disturbance of the ERM during extraction can

also lead to loss of and/or underestimation of hyphal viability (Vosátka and Dodd 1998). In an attempt to prevent from the loss of enzyme activity in ERM, Saito et al. (1993) incubated soil cores in nitro blue tetrazolium chloride prior to ERM extraction in order to detect succinate dehydrogenase activity. There are also non-destructive techniques for studying the morphology of hyphae forming the ERM network but, so far, these have been limited to artificial systems without a soil component. Giovannetti et al. (1994) used such a system in which the ERM of AMF grew between two permeable membranes. Another possibility is the cultivation of these fungi on agar plates in association with whole host plants (Alexander and Hadley 1985) or with transformed (Bécard and Fortin 1988; Bécard and Piché 1992) or non-transformed plant roots (Bago et al. 1998a, b).

Here we describe a novel "inserted membrane technique" (IMT) for extraction of ERM from a substrate. We aimed to verify the potential of this technique for assessing the spread, morphology and viability of ERM from fungi forming orchid mycorrhiza (OM) or arbuscular mycorrhiza (AM) and to validate the IMT in comparisons with the MFT.

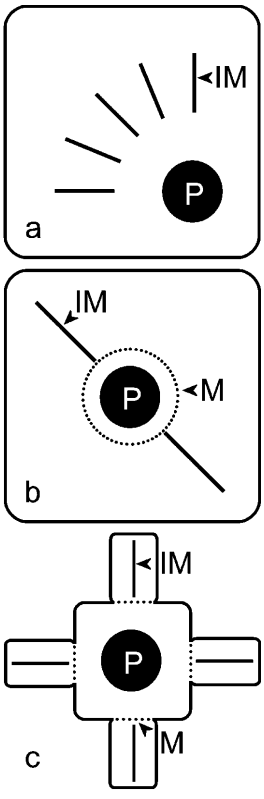

Fig. 1a–c Schematic diagram of the application of the inserted membrane technique (IMT) (top view). Membranes are inserted either into the rhizosphere (**a**), a hyphal compartment (**b**) or hyphoboxes (**c**) separated from roots by a nylon mesh (*M* mesh, *IM* inserted membrane, *P* host plant)

Materials and methods

Inserted membrane technique

Cellulose acetate/cellulose nitrate membrane filters (MF-Millipore, Millipore Corporation, 47 mm diameter, 0.45 µm pore size) or cellulose nitrate membrane filters (Pragopore, Pragochema Ltd., Czech Republic, 0.60 µm pore size) wetted with deionised water were inserted vertically into the substrate at the beginning of each experiment in a position radial to the plant. Membrane filters were inserted into zones where either the mycorrhizosphere (Fig. 1a) or the hyphosphere (Fig. 1b, c) were predicted to develop. After each growth period, the membranes with adhering hyphae were carefully removed using forceps and gently washed with deionised water. All membranes were then placed into small Petri dishes (50 mm diameter), flooded with 5 ml of a solution of either trypan blue or enzyme activity stains and then mounted on microscope slides for observation.

Experiment 1

The aim of this experiment was to test suitability and efficiency of IMT for orchid ERM extraction and to compare various staining techniques applied to orchid ERM extracted using IMT. Adult *Ophrys vernixia* Brot. plants from the Attica peninsula (Greece) were grown under cold glasshouse conditions from May 1990 to August 1997. In September 1997, at the start of the growing period, they were transplanted into a mixture of clinoptinolite (natural zeolite, particle size 2.5–5 mm), quartz sand and perlite (1:1:1, v/v/v). The plants were naturally mycorrhizal and were not inoculated with additional mycorrhizal fungi. At the beginning of the experiment (March 1998), individual plants (seven replicates) were transplanted into 250-ml plastic pots containing clinoptinolite as the growth substrate. Five halves of Millipore membrane filters were placed around each plant, with the upper edge of the filter 5 cm below the substrate surface (Fig. 1a). After 12 weeks, the membrane filters were removed from the mycorrhizosphere and stained with either trypan blue (Phillips and Hayman 1970), iodonitrotetrazolium chloride (INT) for NADH diaphorase activity (Sylvia 1988), nitro blue tetrazolium chloride (NBT) for succinate dehydrogenase activity (Kough and Gianinazzi-Pearson 1986), fluorescein diacetate (FDA) for non-specific esterase activity (Schubert et al. 1987) or fast blue RR for alkaline phosphatase (ALP) activity (Tisserant et al. 1993). After staining, the membranes were soaked overnight in glycerol and then mounted on microscope slides in glycerol, except when using FDA, when the samples were observed directly in the stain solution immediately after staining. The slides were examined using an Olympus BX-60 microscope and photographed with an Olympus PM-10 AK microphotographic system.

Experiment 2

The aim of this experiment was to compare the lengths of the ERM hyphae of orchid mycorrhiza extracted by IMT and MFT. Seven *Serapias lingua* L. plants, as in experiment 1, were grown under the same conditions as in experiment 1. In September 1998, at the start of the experiment, they were transplanted into clinoptinolite. Individual plants were transferred to 3-l rhizoboxes containing a central root-hyphal compartment (40 mm diameter) separated from the hyphal compartment by a nylon mesh (200 µm pore size). At the time of planting, two 15×70 mm pieces of membrane filter (Pragopore) with 0.6 µm pore size were placed radially into the hyphal compartment, with the upper edge of the filter strip 4 cm below the substrate surface (Fig. 1b). The naturally mycorrhizal plants were grown under these conditions for a period of 27 weeks (September 1998–March 1999). The membranes were then removed, stained with trypan blue and mounted on microscope slides in glycerol. ERM lengths were assessed using a grid-line intersect technique (Giovannetti and Mosse 1980). Thirty random microscope fields (each covering 854×633 µm of the membrane area) from each membrane were recorded at a distance of 3–4 cm from the root-hyphal compartment using a colour CCD video camera Hitachi KP-C 550 and Genius HiVideo Pro digitising card. The images on a PC monitor were overlaid with a grid (edge length 33.7 µm) and the number of intersects counted. The results were expressed as ERM lengths per 1 cm^2 of the inserted membrane filter.

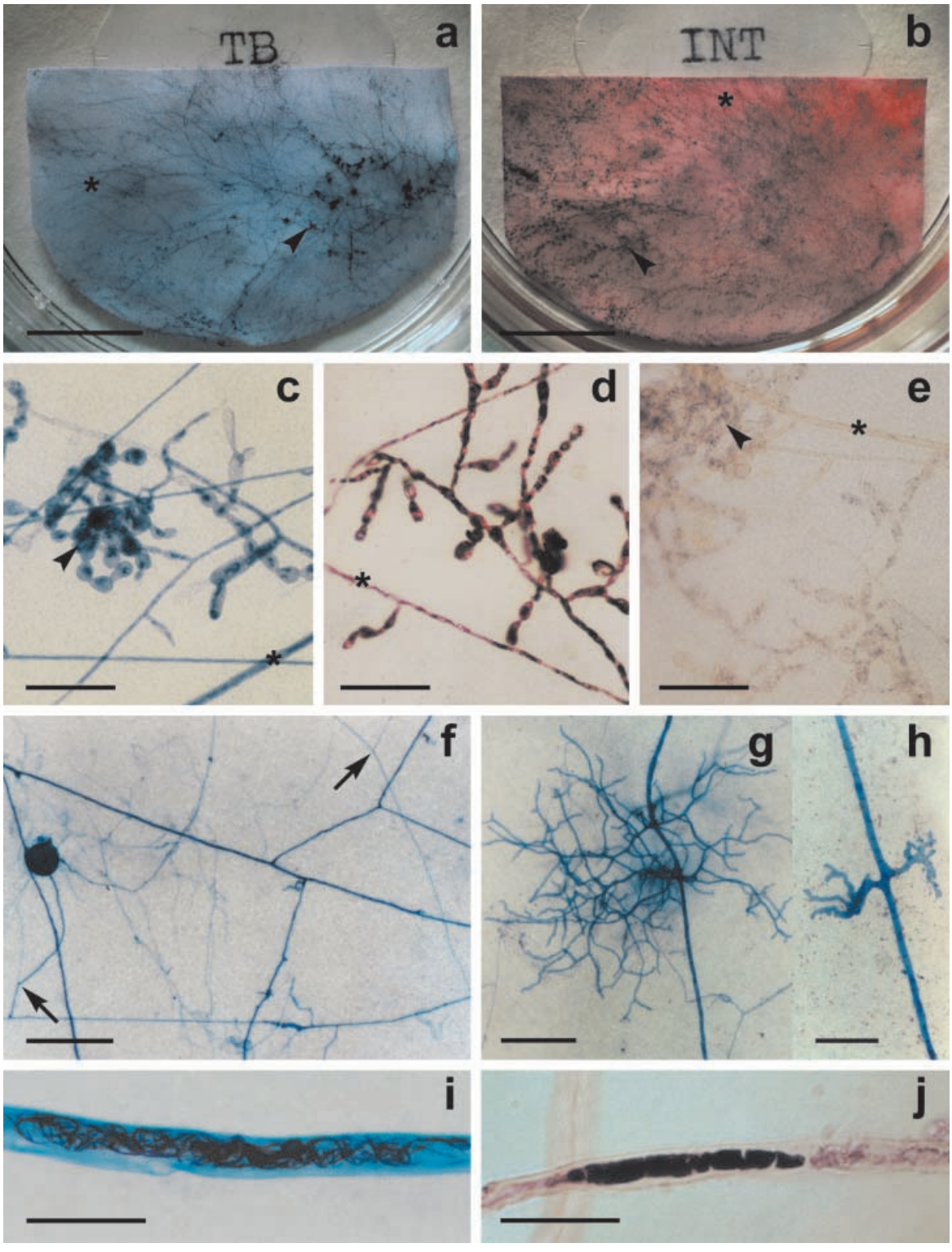

Fig. 2 Extraradical mycelium (ERM) of orchid (OM) (**a**–**e**) and arbuscular (**f**–**j**) mycorrhizas (AM) extracted using the IMT. (**a**, **b**) The whole membrane removed from the mycorrhizosphere of *Ophrys vernixia* plants showing the OM fungus after staining with trypan blue (**a**) or iodonitrotetrazolium chloride (INT) (**b**); *bars* 10 mm. **c**–**e** Comparison of three different staining techniques for orchid mycorrhizal ERM using trypan blue (**c**), INT (**d**) or nitro blue tetrazolium chloride (NBT) (**e**); *bars* 50 µm. **f** ERM of AM fungus *Glomus mosseae* BEG25 shows hyphae with gradually decreasing diameters; *bar* 200 µm. **g** Fully developed branched structure; *bar* 100 µm. **h** Short side branches formed on thick hyphae, probably the first developmental stages of branched structures seen in **g**; *bar* 50 µm. **i**, **j** Dye deposition after staining for NADH diaphorase (**i**) or alkaline phosphatase (ALP) (**j**) activities; *bars* 20 µm (*Asterisk* hyphae of OM ERM, *arrowhead* branching of AM ERM with gradually decreasing hyphal diameter, *arrow* monilioid cells of OM ERM)

For validation of the IMT against the MFT, 2–3 g of clinoptinolite were sampled at a distance of 3–4 cm from the root-hyphal compartment. The ERM was extracted using a modified technique according to Jakobsen et al. (1992). Samples of substrate plus hyphae were stained in 5 ml of 0.05% trypan blue in lactoglycerol (30 min, 90°C), transferred to 25 ml of distilled water, sonicated (5 min) and agitated (1 min) on a magnetic stirrer at full speed. Aliquots (5 ml) were then pipetted into 50 ml of distilled water and the suspension blended using a domestic mixer at high speed (1 min). A 10-ml portion of the suspension was then filtered through a pragopore membrane filter (0.6 µm pore size). The lengths of ERM were assessed using the grid-line intersect technique as for the IMT, except that 40 microscope fields were recorded. The results are expressed as ERM length per 1 g of dry clinoptinolite.

Experiment 3

The aim of this experiment was to test various staining procedures on ERM hyphae of AMF and compare the hyphal lengths extracted using IMT and MFT. Individual *Plantago lanceolata* L. plants were grown under glasshouse conditions in 1-l plastic pots which had four 100-ml hyphoboxes attached to the sides, separated from the pot compartment by a nylon mesh (36 µm pore size) (Fig. 1c). Seven pots, each with four hyphal compartments, were filled with clinoptinolite and inoculated with approximately 200 spores of *Glomus mosseae* (BEG25). One membrane filter (Pragopore 47 mm diameter, 0.6 µm pore size) was placed into each hyphal compartment at the beginning of the experiment. After 12 weeks, the ERM was extracted using both IMT and MFT and stained with 0.05% trypan blue in lactoglycerol for assessment of hyphal length and with INT or fast blue RR salt to determine activities of NADH diaphorase and ALP. Twenty-six replicate membranes were assessed using each technique. The procedures for extraction and measurement of the ERM were as described in experiment 2.

Statistical analysis

Regression analysis of the hyphal lengths of ERM extracted using the IMT and MFT was performed on data from experiments 2 and 3. Data from experiment 3 were $\log_{10}$ transformed before analysis to ensure even distribution of the data throughout the data range. Correlation coefficients indicated best fit. All calculations were performed using the Statgraphics Plus 5.0 software package.

Results

Experiment 1

Abundant levels for ERM, typical of OM fungi, developed on the membrane filters, i.e. narrow septate hyphae with numerous anastomoses and clusters of monilioid cells (Fig. 2a–e). Staining with trypan blue resulted in samples with a high contrast between the hyphae and the membrane filter (Fig. 2c). A high contrast was also achieved after staining with INT for NADH diaphorase activity and resulted in the deposition of formazan in the shape of high-density coiled fibres (Fig. 2d). Staining of the ERM with NBT for succinate dehydrogenase activity resulted in a much lower contrast between active and inactive hyphae and the background (Fig. 2e). The INT staining for NADH diaphorase activity proved to be very stable; colour was maintained in INT samples for up to 2 years, whereas NBT staining considerably decreased (data not shown). Staining with FDA for esterase activity was unsuitable for the samples prepared by the IMT due to high background fluorescence from the filter itself. No ALP activity was detected in the ERM.

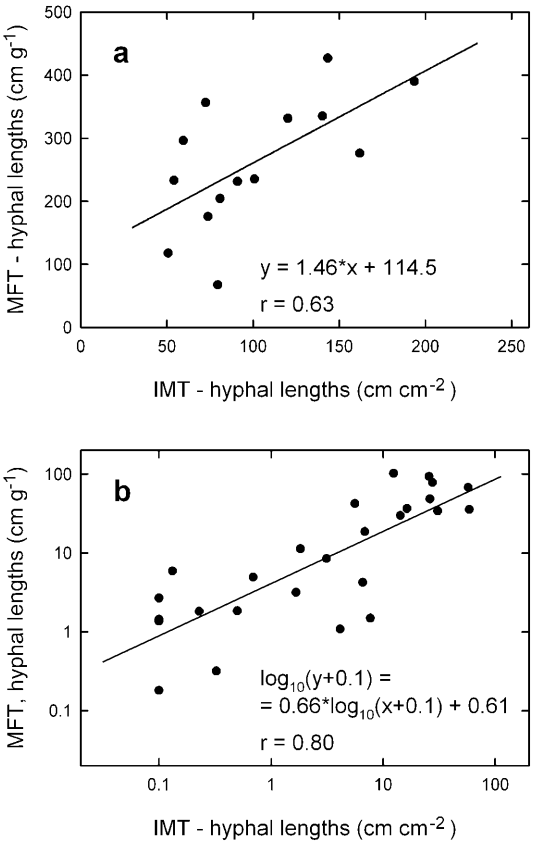

Fig. 3 Correlation between lengths of ERM extracted from the substrate by IMT and the membrane filtration technique (MFT) for OM (**a**) and AM (**b**) after linear regression analysis. Regression equations and correlation coefficients are presented

Experiment 2

The morphological features of ERM observed on membranes were the same as in experiment 1. Lengths of ERM extracted using the IMT or MFT for OM showed significant positive correlation (r=0.63, P<0.05). A linear regression analysis fit the data obtained (Fig. 3a).

Experiment 3

Staining of the ERM of *G. mosseae* with trypan blue resulted in a high contrast between hyphae and the membrane filter. After extraction of the ERM by IMT, thick hyphae with diameters of up to 20 µm were observed, with thinner branches that gradually decreased in diameter (Fig. 2f). Using the IMT, large amounts of fine hyphae were observed, with diameters of approximately 1.5 µm. In contrast, very limited amounts of these fine hyphae were observable after application of the MFT (data not shown). Numerous intact branched structures (Fig. 2g)

were observed after IMT extraction, as well as short lateral branches, which we assume are early developmental stages of the branched structures (Fig. 2h). These structures were not observed after MFT extraction.

The deposition of formazan after staining for NADH diaphorase activity was observed in both thick and fine hyphae of *G. mosseae*. Formazan precipitated as fibres which were coiled at higher densities (Fig. 2i). After staining for ALP activity, a black precipitate was observed within both thick and fine hyphae of *G. mosseae* (Fig. 2j). The proportion of hyphae showing ALP activity was lower than that showing NADH diaphorase activity (data not shown). Lengths of *G. mosseae* ERM extracted using the IMT or MFT were significantly positively correlated (r=0.8, P<0.001). Linear regression analysis was performed on the data (Fig. 3b).

Discussion

Although several techniques for sampling of ERM from soils/substrates have been developed (Dodd 1994), they all result in severe disturbance of the hyphae. The ERM extracted using the IMT in our study was relatively clean and undisturbed. This is important for several reasons. Firstly, the availability of undisturbed ERM may facilitate studies of hyphal morphology, which are still very limited (Dodd et al. 2000). The IMT allowed examination of structures not observable using MFT, for example branched hyphal structures similar to the BAS described by Bago et al. (1998a, b). The BAS we observed were more branched than those described for *G. intraradices* grown on agar plates (Bago et al. 1998a, b) but were very similar to the arbuscule-like structures reported by Mosse and Hepper (1975) for *G. mosseae*. Knowledge of the morphological characteristics of hyphae may even improve distinction between mycorrhizal and non-mycorrhizal hyphae. Such a distinction is often difficult on highly fragmented ERM after blending using the MFT (Abbott and Robson 1985). Secondly, reduced disturbance of the ERM is also advantageous in the assessment of hyphal viability. The IMT greatly reduced loss of hyphal viability due to sample processing. Thirdly, the cleaner samples of ERM extracted using the IMT allowed more precise assessment of hyphal length and viability. The IMT also seems to be appropriate for the preparation of samples for computer-aided hyphal length measurements, for example image analysis (Green et al. 1994). The IMT can be regarded as a technique in which the ERM is partly purified from the substrate or soil, similarly to the wire frame (Vilariño et al. 1993) and sucrose flotation/centrifugation techniques (Schubert et al. 1987).

None of the membranes inserted into the substrate exhibited any signs of decomposition during the growth period, in accordance with the claim by the Millipore Corporation (Anonymous 1989) that they are biologically inert. Thus, no nutritional effects of filter decomposition are to be expected. However, the possibility should be tested that the physical properties at the microsite surface of the membrane filter differ from those within the substrate and may thus influence hyphal spreading and viability.

In the present experiments, the best procedure for assessment of hyphal viability was staining with INT. FDA staining was not suitable for the IMT due to high levels of background fluorescence. Staining with INT gave a high contrast for hyphae of both mycorrhizal types tested. Coiled strings observed within hyphae after INT staining were similar to the patterns of dye deposition reported in spores of the parasitic fungus *Sphaerotheca fuliginea* (Weiersbye-Witkowski and Straker 1997), although we cannot explain this cytochemical localisation of the enzyme reducing INT.

Differences in the properties of various substrates or soils can contribute to very high variability in ERM length (Sylvia 1990). Data on the lengths of the ERM obtained using MFT are conventionally related to one-dimensional weight units, while the data obtained using IMT are related to bi-dimensional area units. The IMT may allow comparison of ERM growth in substrates with different granularity and specific weights because these parameters are closely related to the potential growing space for the ERM. The ERM lengths of *G. mosseae* extracted using the IMT were very similar to the mean values of 30 cm cm^{-2} obtained by Bago et al. (1998a) for *G. intraradices* growing in monoxenic in vitro culture with non-transformed tomato roots, so far the only available value of ERM length in relation to area. The range of ERM lengths observed for OM was much broader and cannot be compared with other results since, to our knowledge, there are no published data on the densities of the ERM of OM.

In our experiments, we found significant positive correlation between the hyphal lengths obtained using the MFT and the IMT. A linear model provided best fit data for both OM and AM. Such a relationship would be expected since the membrane is inserted radially to the main direction of hyphal growth and is not a physical barrier to spread of the ERM.

Preliminary testing of the IMT has shown it to be suitable also for studying of the ERM of an unidentified ericoid mycorrhizal fungus, producing dark sterile mycelia, and of *Oidiodendron* sp. associated with micropropagated *Rhododendron* sp. (Jansa and Vosátka, unpublished results). In addition, the ERM of the ectomycorrhizal fungus *Laccaria laccata* grown in axenic culture on vermiculite and peat has been successfully collected using the IMT (Vosátka, unpublished results).Whilst extraction of ERM by MFT from soils with either high organic matter or high clay contents is difficult (Boddington 1997), the IMT has also been successfully applied to collect ericoid ERM from peat-based substrates containing high amounts of organic matter, while the MFT completely failed to extract the mycelium (Vosátka, unpublished results).

The IMT is labour saving and needs no specialised equipment. This substantial reduction in manipulation steps may facilitate experiments with radioisotopes. The

ERM can then be easily extracted and stained with a minimum of manipulation. It may even be possible to use the IMT for immunodetection of the AM-specific protein glomalin instead of the horticultural plastic strips used by Wright and Upadhaya (1999).

The broader applicability of this new technique can help to collect data about the spreading and viability of ERM in a range of substrates/soils and mycorrhizal associations and so help to improve our understanding of the function of the ERM in mycorrhizal symbioses.

Acknowledgements We would like to thank Martina Zilová for help with the validation of both extraction techniques for AMF and to Jan Jansa for help with ericoid mycorrhiza testing. Funding of the work by the Grant Agency of the Czech Republic (grants 206/98/P281 and 526/99/0865) is greatly appreciated.

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