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ORIGINAL ARTICLE

An in vivo whole-plant experimental system for the analysis of gene expression in extraradical mycorrhizal mycelium

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Abstract Arbuscular mycorrhizal fungi (AMF) establish beneficial mutualistic symbioses with land plants, receiving carbon in exchange for mineral nutrients absorbed by the extraradical mycelium (ERM). With the aim of obtaining in vivo produced ERM for gene expression analyses, a whole-plant bi-dimensional experimental system was devised and tested with three host plants and three fungal symbionts. In such a system, *Funneliformis mosseae* in symbiosis with *Cichorium intybus* var. *foliosum*, *Lactuca sativa*, and *Medicago sativa* produced ERM whose lengths ranged from 9.8 ± 0.8 to 20.8 ± 1.2 m per plant. Since ERM produced in symbiosis with *C. intybus* showed the highest values for the different structural parameters assessed, this host was used to test the whole-plant system with *F. mosseae*, *Rhizoglomus irregulare*, and *Funneliformis coronatus*. The whole-plant system yielded 1–7 mg of ERM fresh biomass per plant per harvest, and continued producing new ERM for 6 months. Variable amounts of high-quality and intact total RNA, ranging from 15 to 65 μg RNA/mg ERM fresh weight, were extracted from the ERM of the three AMF isolates. Ammonium transporter gene expression was successfully determined in the cDNAs obtained from ERM of the three fungal symbionts by RT-qPCR using gene-specific primers designed on available (*R. irregulare*) and new (*F. mosseae* and *F. coronatus*) ammonium transporter gene sequences. The whole-plant experimental system represents a useful research tool for large production and easy collection of ERM for morphological, physiological, and biochemical analyses, suitable for a wide variety of AMF species, for a virtually limitless range of host plants and for studies involving diverse symbiotic interactions.

Keywords Arbuscular mycorrhizal fungi · Extraradical mycelium · Whole-plant experimental system · Ammonium transporters · Gene expression

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Introduction

Arbuscular mycorrhizal (AM) fungi (AMF) are soil microorganisms that establish beneficial mutualistic symbioses with most land plants (Smith and Read 2008). AMF receive up to 20% of plant photosynthetically assimilated carbon (Délano-Frier and Tejeda-Sartorius 2008), in exchange for mineral nutrients, mainly P, N, Cu, Fe, K, and Zn (Smith and Read 2008; Casieri et al. 2013), which are translocated from soil to host plants by means of a large network of extraradical mycelium (ERM). Several works studied the development, functioning, and activity of ERM in vivo, improving knowledge on its fundamental role as an efficient scavenger of soil nutrients and in the establishment of nutrient flows from soil to plants and among different plants (Fellbaum et al. 2014; Giovannetti et al. 2015). Such flows are facilitated by high levels of interconnectedness among hyphae belonging to the same and to

different extraradical networks (Giovannetti et al. 2004; Mikkelsen et al. 2008), possibly affecting symbiotic efficiency and plant nutrition.

ERM gene expression analyses, including targeted and high-throughput expression profiling, have been mostly carried out in vitro, using AMF-inoculated root organ cultures (ROCs) transformed with a root-inducing plasmid derived from *Agrobacterium rhizogenes* (Ri T-DNA) (Table 1). Most targeted approaches have focused on the characterization of nutrient transporter genes of *Rhizoglomus irregulare* or *Rhizoglomus intraradices* (formerly *Rhizophagus irregularis* and *Rhizophagus intraradices*, see Sieverding et al. 2015 and Young 2015) ERM grown in ROCs. These studies revealed that aquaporins (Li et al. 2013), ammonium (López-Pedrosa et al. 2006; Pérez-Tienda et al. 2011; Calabrese et al. 2016), phosphorus (Maldonado-Mendoza et al. 2001; Fiorilli et al. 2013), monosaccharides (Helber et al. 2011), and zinc (González-Guerrero et al. 2005) transporters were differentially regulated in ERM in response to mineral or organic compounds availability.

The roots used in transformed ROCs, compared with those of whole plant, have an altered hormonal balance and a different way of acquiring carbohydrates, which may affect multiple physiological traits of the symbiotic interaction. Indeed, some concerns have been raised as to the lack of extensive cytological, biochemical, and molecular studies, in particular when investigating fungal symbiotic gene expression (Bago and Cano 2005). Non-transformed root cultures were rarely used, e.g., in the study of AM fungal recognition stages in wild type and mutant tomato lines (Bago et al. 2006) and in a fungal phosphate transporter assay (Maldonado-Mendoza et al. 2001). However, the question as to whether such axenic cultures, deprived of plant stems and leaves and of AM fungal-associated microbiota, may successfully mimic whole-plant mycorrhizal symbiosis outcomes remains to be answered. A lower number of studies utilized alternative experimental systems, collecting ERM from plants grown in pots in symbiosis with different AM fungal species (Table 1).

With the aim of obtaining profuse ERM production by whole mycorrhizal plants in vivo, we devised a bi-dimensional experimental system characterized by the physical separation of the roots from the substrate and of ERM from the roots. Such a system allowed us to obtain 1–7 mg of ERM per plant at each mycelial harvest and could be utilized with a wide variety of AMF and plant species. To test whether the system may represent a valuable tool for ERM gene expression studies, the expression of genes encoding for AMTs was analysed in ERM produced by three different AMF, *Funneliformis mosseae*, *R. irregulare*, and *Funneliformis coronatus*, growing in symbiosis with *Cichorium intybus* var. *foliosum*.

Materials and methods

Development of the whole-plant experimental system for in vivo ERM production

Host plant seeds were surface-sterilized, germinated, and grown for 10 days in sterile plastic boxes filled with steam-sterilized quartz grit (aquarium gravel, mean diameter size 2 mm, Fig. 1a) and then inoculated with spores or sporocarps, mycelium, and colonized roots obtained from pot-culture soil after wet sieving through a 100-μm-mesh size sieve (Fig. 1b). Inoculated plants were grown in 10-cm diameter pots disinfected by chlorination, filled with the steam-sterilized quartz grit, placed into sun-transparent bags (Sigma-Aldrich B7026), and maintained in a growth chamber at 25 °C, with 16 h of light per day (photon flux density of 350 μmol m^{-2} s^{-1}). Plants were not fertilized and watered weekly with 3-ml distilled water. After 4 weeks' growth (Fig. 1c), grit was washed from roots, and extraradical hyphae, spores, and sporocarps adhering to plant roots were carefully removed with forceps (Fig. 1d) under a Leica M 205C dissecting microscope (Leica, Milano, Italy) and each root system was wrapped in a nylon net (41 μm mesh, Millipore NY4100010) to obtain a flat mesh pocket (Fig. 1e). Nylon-enclosed root systems were then placed between two semicircular 13-cm diameter membranes (cellulose acetate and cellulose nitrate mixture, 0.45-μm pore diameter size, MF-Millipore™ HAWP14250) to obtain "root sandwiches" (Fig. 1f). By using a hot scalpel blade, a hole was made on the edge of sterile plastic Petri dishes (14-cm diameter), to allow plant shoot growth. Plants were transferred into such Petri dishes containing sterile quartz grit on the bottom (Fig. 1g); the root sandwich was covered with grit and then moistened with 10-ml distilled water (Fig. 1h). After sealing with Parafilm M, the root-containing lower half of plates was wrapped into aluminium foil and the whole-plant system placed into sun-transparent bags. Plants were maintained in the growth chamber and watered weekly with 3 ml of distilled water without fertilization. Water loss was limited by Petri dishes parafilm sealing and bagging.

Experiment 1 was carried out to assess possible changes of ERM structural traits caused by the use of root-enclosing nylon net and whether the system allowed the production of a large biomass of ERM. In this experiment, three different host plant species (*C. intybus* var. *foliosum*, *Lactuca sativa*, and *Medicago sativa*) were inoculated with a pot-culture soil sieving of *F. mosseae* isolate IMA1.

In experiment 2, aimed at testing the whole-plant experimental system for the production of ERM suitable for molecular analyses, the host plant *C. intybus* var. *foliosum* (sugar chicory of Trieste), was inoculated with sievings of three different AMF: *F. mosseae* isolate IMA1, *R. irregulare* isolate IMA6 (formerly referred as *R. intraradices*), and *F. coronatus* isolate IMA3.

Table 1 Experimental systems (*ES*) used in studies of gene expression in extraradical mycelium of arbuscular mycorrhizal fungi: root-organ cultures (*ROCs*), excised non-transformed roots (*ERs*), whole plants (*WPs*)

ES	Plant species	Fungal species/isolate	Analysis target	Reference
ROCs	*Cichorium intybus*	*Rhizoglomus irregulare* DAOM 197198	GintPT (P transporter)	Fiorilli et al. (2013)
ROCs	*Cichorium intybus*	*Rhizoglomus irregulare* DAOM 197198	RiPTR2 (dipeptide transporter)	Belmondo et al. (2014)
ROCs	*Cichorium intybus*	*Rhizoglomus irregulare* DAOM 197198	RiPEIP1 (preferentially expressed in plant)	Fiorilli et al. (2016)
ROCs	*Daucus carota*	*Rhizoglomus intraradices*	GintPT (P transporter)	Maldonado-Mendoza et al. (2001)
ROCs	*Daucus carota*	*Rhizoglomus intraradices*	cDNA library	Sawaki and Saito (2001)
ROCs	*Daucus carota*	*Rhizoglomus intraradices*	Glutamine synthethase	Breuninger et al. (2004)
ROCs	*Daucus carota*	*Rhizoglomus intraradices* AH01	GintAQPF1–2 (aquaporins)	Li et al. (2013)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 181602	Chitin synthase	Ubalijoro et al. (2001)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	GiPT (P transporter)	Olsson et al. (2005)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	GintZnT1 (Zn transporter)	González-Guerrero et al. (2005)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	RiAMT1 (ammonium transporter)	López-Pedrosa et al. (2006)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	P transporter	Olsson et al. (2006)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 181602	Heavy metal-induced genes	Waschke et al. (2006)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	cDNA library	Porcel et al. (2006)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 181602	cDNA library (organic N metabolism)	Cappellazzo et al. (2007)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	GintMT1 (metallothionein)	González-Guerrero et al. (2007)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	GintAQP1 (aquaporin)	Aroca et al. (2009)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	Sulfur metabolism	Allen and Shachar-Hill (2009)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	GintGRX1 (glutaredoxin)	Benabdellah et al. (2009)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	GintABC1 transporter	González-Guerrero et al. (2010a)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	GintSOD1 (CuZn SOD)	González-Guerrero et al. (2010b)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 181602	N transfer pathway enzymes	Tian et al. (2010)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 181602	As and P transporters	González-Chávez et al. (2011)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	RiAMT2 (ammonium transporter)	Pérez-Tienda et al. (2011)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	N metabolism	Fellbaum et al. (2012)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	cDNA library	Tisserant et al. (2012)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	RiGRX (glutaredoxin)	Tamayo et al. (2014)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	GintPT (P transporter)	Zhang et al. (2016)
ROCs	*Daucus carota*	*Rhizoglomus irregulare* DAOM 197198	RiAMT3 (ammonium transporter)	Calabrese et al. (2016)
ROCs	*Medicago truncatula*	*Rhizoglomus irregulare* DAOM 181602	Heavy metal-induced genes	Waschke et al. (2006)
ROCs	*Solanum tuberosum*	*Rhizoglomus irregulare* DAOM 197198	RiMst2 (monosaccharide transporter)	Helber et al. (2011)
ERs	*Medicago truncatula*	*Rhizoglomus intraradices*	GintPT (P transporter)	Maldonado-Mendoza et al. (2001)
WPs	*Allium cepa*	*Gigaspora margarita*	Alkaline phosphatase	Aono et al. (2004)
WPs	*Allium cepa*	*Rhizoglomus irregulare* DAOM 181602	Alkaline phosphatase	Aono et al. (2004)
WPs	*Cucumis sativus*	*Funneliformis mosseae* BEG12	GmPT (P transporter)	Benedetto et al. (2005)
WPs	*Cucumis sativus*	*Funneliformis mosseae*	GmosAAP1 (aminoacid permease)	Cappellazzo et al. (2008)
WPs	*Hordeum vulgare*	*Rhizoglomus irregulare* DAOM 181602	Ginmyc1–2, Ginhb1	Delp et al. (2003)
WPs	*Lolium perenne*	*Funneliformis mosseae* UK115	FmALP (alkaline phosphatase)	Liu et al. (2013)
WPs	*Lotus japonicus*	*Gigaspora margarita*	CuZn SOD	Lanfranco et al. (2005)
WPs	*Medicago truncatula*	*Funneliformis mosseae*	GmTOR2 (putative cell cycle checkpoint)	Requena et al. (2000)

Table 1 (continued)

ES	Plant species	Fungal species/isolate	Analysis target	Reference
WPs	*Medicago truncatula*	*Gigaspora margarita*	CuZn SOD	Lanfranco et al. (2005)
WPs	*Medicago truncatula*	*Rhizoglomus intraradices* BEG141	PEPISOM, DESAT, and SOD	Seddas et al. (2008)
WPs	*Medicago truncatula*	*Rhizoglomus irregulare* DAOM 197198	cDNA library/oligoarrays	Tisserant et al. (2012)
WPs	*Petroselinum crispum*	*Funneliformis mosseae*	β-tubulin gene	Bütehorn et al. (1999)
WPs	*Petroselinum crispum*	*Funneliformis mosseae* BEG12	cDNA library	Requena et al. (2002)
WPs	*Petroselinum crispum*	*Funneliformis mosseae* BEG12	Glutamine synthethase	Breuninger et al. (2004)
WPs	*Solanum lycopersicum*	*Rhizoglomus irregulare* DAOM 181602	Ginmyc1–2, Ginhb1	Delp et al. (2003)

Five replicate plates for each plant species were prepared in exp. 1, whereas 40 to 70 replicate plates for each fungal species were prepared in exp. 2. Petri plates of exp. 1 were opened 2 weeks after preparing root sandwiches, and ERM spreading from the nylon net onto the membranes (Fig. 1i) was stained for the localization of succinate dehydrogenase activity and used for the assessment of ERM density, explored area, length, and anastomosis frequency, as previously described (Pepe et al. 2016). Briefly, hyphal density was measured by using a microscope eyepiece grid and expressed as mm hyphal length per mm^2 of membrane surface area, explored area was measured by using a transparent reference grid and expressed as mm^2 and hyphal length was calculated from values of hyphal density and explored area recorded on each membrane. Anastomosis frequency was recorded by counting the proportion of hyphal contacts in ERM leading to hyphal fusion.

The root sandwiches of exp. 2 were opened after 4 weeks' growth, and three membranes for each fungal species were stained and used for the assessment of ERM structural traits as described above. ERM growing on the root-containing surfaces of the remaining membranes from exp. 2 was harvested in ice-cold sterile distilled water using a rubber cell scraper. The root sandwiches from exp. 2 were then reassembled and ERM allowed to regrow for successive harvests. Mycelium collected in exp. 2, originating from the first harvest of each replicate plate, was stored in Eppendorf tubes at −80 °C for molecular analyses, after removing excess water with a filter paper.

Test of the in vivo whole-plant-produced ERM for gene expression analyses

For RNA extraction, two aliquots of ERM for each fungal species (about 30 mg of fresh biomass each) were prepared by combining the mycelium collected from each plate replicate in exp. 2. RNA extraction from ERM of *F. mosseae*, *F. coronatus*, and *R. irregulare* was performed using the Plant RNeasy Kit (Qiagen), according to the manufacturer's instructions. cDNAs were obtained from 1 μg of total DNase-treated RNA in a 10-μl reaction containing 5× PrimeScript RT Master Mix (Takara), according to the manufacturer's instructions. RNA samples were checked for DNA contamination by RT-PCR analyses conducted in the presence or absence of RT with the primers ef1-α F (5′-TTGCTTTCGTCCCAATATCC-3′) and ef1-α R (5′-AGTGGAAGACGAAGGGGTTT-3′), previously checked for the amplification of *elongation factor 1-α* (ef1-α) genes in the three AMF species. These primers were designed based on the partial sequences encoding ef1-α of AMF reported by Sokolski et al. (2010).

Since no sequences for AMTs encoding regions were available for *F. coronatus* and *F. mosseae*, the degenerated primer pairs AMT2.1 (5′-GGIGYIITIGAYTTYGCIGG-3′)–AMT2.2 (5′-CCNARCCANCCRAACCA-3′) and NH4F (5′-

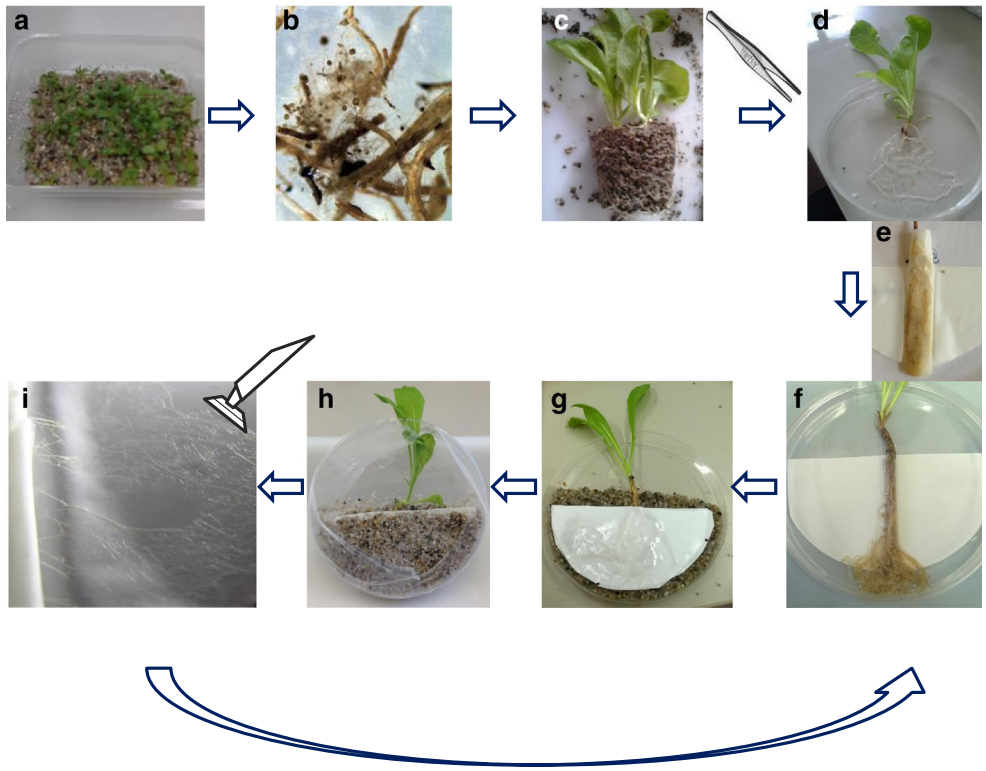

Fig. 1 Experimental model system used for in vivo production of extraradical mycelium for molecular analyses. Germlings grown in sterile quartz grit (**a**) are inoculated with pot-culture soil sieving (**b**) and maintained in pots filled with sterile quartz grit in growth chamber. After 4 weeks' growth (**c**), grit is washed from roots and extraradical hyphae and spores are removed with forceps (**d**), root systems are wrapped in nylon nets (**e**), and placed between two 13-cm diameter membranes (root sandwiches, **f**). Plants are then transferred in Petri dishes with sterile quartz grit on both sides of root sandwich (**g**–**h**) and maintained in the growth chamber for 1 month before opening the sandwiches and harvesting extraradical mycelium with a cell scraper (**i**). The root sandwiches may be reassembled for successive mycelial harvests

CARTGGTWYTTYTGGGG-3′)–NH4R (5′-CCNARCCANCCRAACCA-3′), designed on the basis of conserved motifs present in NH_4^+ transporters of other organisms, were used for PCR amplification of DNA from pot-culture spores of *F. coronatus* and *F. mosseae*, respectively. PCR reaction was carried out in a final volume of 25 μl, containing 5× Green GoTaq® Reaction Buffer (Promega), 1.5-mM $MgCl_2$, 0.2 mM of dNTPs, 2 μM of each primer, 1 unit of GoTaq® DNA Polymerase (Promega), and 50 ng of genomic DNA. The PCR program was as follows: 94 °C for 3 min (1 cycle), 95 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s (35 cycles), and 72 °C for 5 min (1 cycle). The PCR products were cloned in the pGEM®-T Easy Vector (Promega) and the ligation products used to transform XL10-Gold Ultracompetent *Escherichia coli* cells (Stratagene, La Jolla, CA, USA). Plasmids from positive colonies were purified by Wizard® Plus SV Minipreps (Promega) and sequenced using T7 vector primer at Institute of Parasitology and Biomedicine López Neyra, Armilla-Granada (Spain). New AMT primers for *F. mosseae* and *F. coronatus* mycelial samples were designed on the basis of such sequences, while those for *R. irregulare* mycelial samples were designed on the basis of *R. irregulare* AMT2 sequence (accession number FM993985), by using Primer3 software (http://bioinfo.ut.ee/

Table 2 AMT gene-specific primers designed in this study

AMF isolate	Primer	Sequences (5′–3′)	Tm (°C)
F. mosseae IMA1	*Fm*AMT F	ATGAATGTCGGAGGAGAACC	62.9
F. mosseae IMA1	*Fm*AMT R	ACCAAATCCAGCAAGCAATC	63.9
F. coronatus IMA3	*Fc*AMT F	GGTCTGCTAAGATGGGAGGA	63.1
F. coronatus IMA3	*Fc*AMT R	CCTAACCAACCGAACCAAAG	63.1
R. irregulare IMA6	*Ri*AMT F	AGGGGGTTGGTTAGATGGTC	63.8
R. irregulare IMA6	*Ri*AMT R	AGCCAATTCACCCAATTCAG	63.8

primer3-0.4.0/) (Table 2). PCR amplification of cDNAs obtained from *R. irregulare*, *F. mosseae*, and *F. coronatus* ERM RNAs was performed in a final volume of 25 μl, containing 5× GoTaq G2 Flexi® Reaction Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.2 μM of each primer, 1 unit of GoTaq G2 Flexi® DNA Polymerase (Promega), and 1-μl 1:10 diluted cDNA. The PCR thermal program consisted in an initial incubation at 95 °C for 3 min, followed by 36 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The SV Wizard® (Promega) PCR-purified amplicons were ligated into the pGEM®-T Easy vector (Promega) and the ligation products used to transform XL10-Gold Ultracompetent *E. coli* cells (Stratagene, La Jolla, CA, USA). Plasmids from positive clones were purified by Wizard® Plus SV Minipreps (Promega) and sequenced using T7 vector primer at GATC Biotech AG (Constance, Germany). Sequences were deposited in EMBL Nucleotide Sequence Database (www.ebi.ac.uk/embl/) under the accession numbers LT614808-LT614819 and LT615270-LT615277.

Preliminary real-time RT-qPCR AMT expression analyses were carried out at the Department of Soil Microbiology and Symbiotic Systems of the EEZ of Granada, on an iQ™5 Multicolor real-time PCR Detection System (Bio-Rad), to test the performance of designed primers on RNA extracted from the whole-plant system ERM (data not shown). Analyses of samples obtained in exp. 2 were carried out at the Department of Agriculture, Food, and Environment of the University of Pisa, on a CFX Connect™ real-time PCR Detection System (Bio-Rad). RT-qPCR reactions were carried out in a final volume of 20 μl containing 10 μl of iQ™ SYBR Green Supermix 2× (Bio-Rad), 0.2 μM of each primer and 1 μl of a 1:10 diluted cDNA template. The PCR thermal program consisted in an initial incubation at 95 °C for 3 min, followed by 36 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, where the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked with a heat-dissociation protocol (from 58 to 95 °C) after the final cycle of the PCR. The efficiency of the primer sets was evaluated by performing real-time RT-qPCR on several dilutions of DNA. Gene expression data were normalized to the expression of the *elongation factor 1-α* (ef1-α) using the ef1-α F and ef1-α R primers. Real-time RT-qPCR determinations were performed on at least three replicate samples for each AMF cDNA, and at least two independent cDNA samples for each AMF were analysed. The relative levels of transcription were calculated by using the $2^{-\Delta CT}$ method (ratio (reference / target) = $2^{CT(reference) - CT(target)}$), a variation of $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Our method produces results similar to those obtained by the Livak method, in which one of the samples is chosen as the calibrator and the expression of the gene of interest in all other samples is expressed as an increase or decrease relative to the calibrator. In our study, we decided to use the $2^{-\Delta CT}$ method because it was more convenient to present individual ERM gene expression data for each AMF, rather than as a fold change relative to the expression level in the ERM of one of the species tested, arbitrarily chosen as a calibrator sample.

Data analysis

Percentage data were subjected to arcsine transformation before statistical analyses. One-way ANOVA was used to detect significant differences between data obtained for different plant species (exp. 1) or different fungal species (exp. 2). Analyses were performed using the software SPSS (version 23).

Results

Development of the whole-plant experimental system for in vivo ERM production

In exp. 1, hyphal density, explored area, mycelial length, and interconnectedness were assessed on 2-weeks old *F. mosseae* ERM produced in symbiosis with host plants (52 days' old) belonging to three different species. Mean hyphal density (hyphal length per unit of membrane surface area), length, and explored area of *F. mosseae* ERM were significantly larger when *C. intybus* was used as host plant compared with *L. sativa* and *M. sativa* ($F_{2,12} = 4.76$, $P = 0.03$ for density and length; $F_{2,12} = 33.7$, $P < 0.01$ for explored area) (Table 3). Interconnectedness of ERM (the proportion of contacts leading to hyphal fusion), ranged between 39.4 ± 2.5 (in *L. sativa*, mean ± standard error of mean) and 47.6 ± 3.0% (in *M. sativa*), with no significant differences among host plants. Since ERM produced in symbiosis with *C. intybus* showed the highest values for the different structural parameters assessed, this host plant was used in exp. 2 to perform quantitative and qualitative evaluations of ERM obtained with the whole-plant in vivo system.

In exp. 2, structural traits of ERM produced by *F. mosseae* and *R. irregulare* in symbiosis with 66 days' old *C. intybus* plants showed significant differences, compared with those of *F. coronatus*, with mean hyphal densities of 9.5 ± 0.2, 8.6 ± 0.5, and 6.7 ± 0.4 mm of hyphal length per mm^2 of membrane surface, respectively ($F_{2,24} = 7.71$, $P = 0.003$) and explored areas of 5762 ± 110, 5445 ± 272, and 4536 ± 489 mm^2 per plant, respectively ($F_{2,24} = 14.92$, $P < 0.001$). Also, values of ERM hyphal lengths and interconnectedness were significantly lower in *F. coronatus*, compared with those formed by the other two AMF isolates (Table 4). Overall, in the first harvest, each replicate whole-plant system inoculated with *F. coronatus* produced lower amounts of ERM fresh biomass (ranging from 1 to 3.5 mg), compared

Table 3 Structural traits of extraradical mycelium (*ERM*) (mean ± standard error of mean) produced in the whole-plant in vivo system by *Funneliformis mosseae* IMA1, in symbiosis with different host plant species, after 2 weeks' growth

Plant species	ERM density (mm/mm^2)	ERM length (m/plant)	ERM explored area (mm^2)	ERM fusion frequency (%)
L. sativa	2.9 ± 0.1 b[a]	9.8 ± 0.8 b	3320 ± 232 c	39.4 ± 2.5 a
C. intybus	3.7 ± 0.1 a	20.8 ± 1.2 a	5655 ± 140 a	40.7 ± 4.7 a
M. sativa	2.9 ± 0.3 ab	14.0 ± 1.7 ab	4750 ± 223 b	47.6 ± 2.9 a

[a] Means followed by the same letter do not differ significantly at $P \leq 0.05$ by Tukey's tests

with those inoculated with *F. mosseae* and *R. irregulare* (ranging from 4 to 7 and from 3 to 6 mg of ERM fresh biomass, respectively) (Table 4).

It is interesting to note that the whole-plant experimental system continued producing new ERM, allowing further mycelial harvests, up to a maximum plant lifespan of 6 months. Successive harvests from 100 and 135 days' old *C. intybus* plants produced lower ERM amounts per plant (1–2 mg), which were not used in this work.

Test of the in vivo whole-plant produced ERM for gene expression analyses

For each RNA extraction trial, 30 mg of ERM biomass was prepared, by combining the mycelium collected from each plant replicate of the first harvest. RNA extractions showed variable RNA yield, depending on AMF isolates and extraction replicates, and showed 260/280 ratios (ranging between 2.07 and 2.2) and 260/230 ratios (ranging between 2.0 and 2.3) within the acceptable range for "pure" RNA (Table 4).

As a first step to test these RNAs and since no sequences of protein-encoding genes were available for *F. coronatus* and *F. mosseae*, partial genes encoding ammonium transporters were obtained from these fungal species using a PCR strategy based on the use of degenerate primers and spore genomic DNA. *F. coronatus* DNA was successfully amplified with the primer pairs AMT2.1 and AMT2.2 while *F. mosseae* DNA was amplified with the primer pairs NH4F and NH4R. The sequences showed good similarity to characterized NH_4^+ transporter genes of *R. irregularis* (81% identity with GintAMT3 sequence KU933909 for *F. mosseae* and 80% identity with GintAMT1 sequence AJ880327 for *F. coronatus*) and were used to design AMT-specific primers for RT-PCR analyses.

Synthesis of cDNA from the RNA extracted from ERM of the three fungal species and PCR amplification of cDNA regions including putative AMT sequences were successful, producing fragments of 193 (*R. irregulare*), 192–193 (*F. mosseae*), and 187 bp (*F. coronatus*) in length. Analysis of PCR products showed that the primers designed for each AM fungal species amplified a single AMT gene: mRNA sequences obtained from *R. irregulare* showed 95% identity with the *R. irregulare* AMT2 mRNA sequence FM998935 (94% similarity to the ERZ95095 NH_4^+ transporter from *R. irregulare* genome); mRNA sequences obtained from *F. mosseae* showed 79–80% identity with the *R. irregulare* GintAMT3 sequence KU933909 (85–87% identity with the ESA07081 NH_4^+ transporter from *R. irregulare* genome), and mRNA sequences obtained from *F. coronatus* showed 81% identity with the *R. irregulare* AMT1 mRNA sequence AJ880327 (89% identity with the ESA03014 NH_4^+ transporter from *R. irregulare* genome).

Real-time RT-qPCR analyses of putative AMT sequences from ERM of the three fungal species, showed high relative transcription levels for *R. irregulare* and *F. mosseae* AMTs, whereas expression of the *F. coronatus* AMT was low (Table 4).

Table 4 Biomass and structural traits of extraradical mycelium (*ERM*) and amount of RNA extracted from ERM (mean ± standard error of mean) produced by *Funneliformis mosseae* IMA1, *Rhizoglomus irregulare* IMA6, and *Funneliformis coronatus* IMA3 growing outside nylon nets enclosing roots of *Cichorium intybus*, after 4 weeks' growth

Fungal species	Sampled plants	ERM length (m/plant)	ERM fusion frequency (%)	ERM fresh weight (mg/plant)	Extracted RNA (μg/mg ERM fresh weight)	Relative expression levels of putative AMTs
F. mosseae	39	54.86 ± 19 a[a]	39.7 ± 1.0 a	5.3 ± 0.6 a	15.7 ± 8.7	0.0569 ± 0.0074[b]
F. coronatus	67	30.47 ± 38 c	6.1 ± 0.6 b	2.7 ± 0.6 b	87 ± 21	0.0024 ± 0.0008
R. irregulare	63	47.12 ± 52 b	41.3 ± 2.6 a	4.4 ± 0.4 a	65.5 ± 0.8	0.2730 ± 0.0348

[a] Means followed by different letters differ significantly for $P < 0.05$ by Tukey's tests.

[b] Data were calibrated by the expression values obtained for the gene encoding the elongation factor 1-α. Relative expression levels were calculated by the $2^{-\Delta CT}$ method

Discussion

In this work, an in vivo whole-plant bi-dimensional experimental system allowed the production of viable, intact, and free from roots/substrate debris ERM by *F. mosseae*, *R. irregulare*, and *F. coronatus*, which met the quantitative and qualitative criteria for obtaining RNA to be used for gene expression analyses.

The experimental system may allow the study of ERM structure and function, of its sporulation dynamics and spore ontogeny in any isolate of AMF which has been cultured and maintained in pot cultures. Most fast-growing in vitro cultures currently used for research are limited to AMF isolates belonging to the genus *Rhizoglomus* (formerly *Rhizophagus*), with 12 available strains in the Glomeromycota in vitro collection (GINCO) and 24 strains in the Banco de Glomeromycota in vitro (BGIV). A significantly lower number of isolates belonging to other species (e.g., *Dentiscutata reticulata*, *Gigaspora* spp., *Claroideoglomus claroideum*) were established in monoxenic cultures, and limited information is available on their long-term maintenance, availability, and physiological traits (Becard and Fortin 1988; de Souza and Declerck 2017; Bidondo et al. 2012).

The whole-plant system used in this work is applicable to any plant-fungus combination and allows fast collection of ERM due to its physical separation from both roots and substrates. ERM harvest appears easier, compared with other experimental systems previously used for molecular analyses, where ERM had to be collected after solubilizing the solid ROC medium or by plucking hyphae from plant roots removed from pots filled with glass beads or quartz sand (references in Table 1). In a modification of the ROC system used to study N translocation by AMF, ERM was easily collected after colonization of mycelial compartments where phytagel was replaced by liquid medium without sucrose (Cruz et al. 2007). A common limit of the ROC systems is that, even in the absence of sucrose, transformed roots grow and contaminate with root tissue the ERM compartment, forcing the operators to periodically trim and remove overgrowing roots. A recently proposed ROC modification may allow the collection of root-free ERM, reducing lab work and the risk of microbial contamination due to periodical root trimming (Rosikiewicz et al. 2017).

Our in vivo whole-plant experimental system does not exclude AM fungal-associated microbiota, successfully mimicking mycorrhizal symbioses occurring in natural conditions (Battini et al. 2016). In addition, such a system may actually manage the concerns raised about the lack of extensive cytological, biochemical, and molecular studies in the physiologically altered transformed roots used in ROCs (Bago and Cano 2005).

The use of quartz grit as the substrate in the whole-plant system is not mandatory, since other matrices (e.g., sand, glass beads, granular clays) may be applicable. In this work, as only distilled water was added to the substrate, a limited plant biomass and lifespan were achieved. Plant nutrient solutions could be utilized during culture to test whether increased host growth and lifespan possibly leads to enhanced ERM production.

ERM emerging from root-enclosing nylon net was characterized by values of hyphal density and interconnectedness which were consistent with data obtained in works carried out using the same in vivo experimental system without the nylon net (Pepe et al. 2016), showing that it did not alter ERM growth and structure. The amount of ERM obtained varied depending on the fungal symbiont: the minimum ERM yields (ranging from 1 to 3.5 mg per plant) were obtained from *F. coronatus*, characterized by low ERM interconnectedness and extent, whereas 3 to 7 mg of ERM fresh biomass per plant was produced by *F. mosseae* and *R. irregulare*. Successive harvests of ERM produced by a single whole-plant system were feasible, although ERM could be collected only twice in our non-fertilized system, where plant growth was sustained by seed/cotyledon reserves and/or by minimal mineral nutrients possibly released by the grit/water system. Indeed, a maximum experimental plant lifespan of 6 months was achieved; since at that date, hosts became chlorotic and died. In this work, chicory was selected as the plant species better adapted to the experimental growth conditions, compared with lucerne and lettuce. Different plant species could be tested for their ability to maintain viability and ERM production in the experimental system.

Maximum values of RNA obtained from ERM varied among extraction replicates and fungal isolates (ranging from 21 to 87 μg per mg of ERM fresh biomass). By using the extracted RNA, AMT expression was detected in ERM produced in vivo by *R. irregulare*, *F. mosseae*, and *F. coronatus*, using real-time RT-qPCR. The relative transcription levels of putative AMT genes differed among the AMF tested, possibly representing the expression of different NH_4^+ transporters in the three isolates, as suggested by the analysis of sequences. Further analyses are needed to reveal which NH_4^+ transporters are active in ERM produced in the whole-plant experimental system by the three isolates tested. Indeed, previous works reported that the expression of *R. irregulare* genes encoding two high-affinity NH_4^+ transporters in ERM, RiAMT2, and RiAMT1 (López-Pedrosa et al. 2006; Pérez-Tienda et al. 2011) and the low-affinity NH_4^+ transporter RiAMT3 (Calabrese et al. 2016) is modulated by environmental conditions and nutrient levels.

In conclusion, the whole-plant system devised may allow in vivo studies of ERM morphological and physiological traits and gene expression, and may be particularly useful to assay genes involved in nutrient uptake. The system may be utilized to test many different AMF/plant species combinations at variable nutritional levels, or in the absence of exogenous nutrients. The system represents a useful research tool for large

production and easy collection of ERM, suitable for any isolate of AMF which has been so far maintained in pot cultures, for a virtually limitless range of host plants and also for studies involving mycorrhizal symbioses other than the arbuscular ones.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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