Journal of Experimental Botany, Vol. 58, No. 6, pp. 1473–1483, 2007
doi:10.1093/jxb/erm009 Advance Access publication 8 March, 2007

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Common mycorrhizal networks provide a potential pathway for the transfer of hydraulically lifted water between plants

Louise M. Egerton-Warburton[1,2,3,*,†], José Ignacio Querejeta[1,4,*] and Michael F. Allen[1]

[1] *Centre for Conservation Biology, The University of California, Riverside, CA 92521, USA*

[2] *Chicago Botanic Garden, 1000 Lake Cook Road, Glencoe, IL 60022, USA*

[3] *Weinberg College of Arts and Sciences, Northwestern University, Evanston, IL 60201, USA*

[4] *Departamento de Conservación de Suelos y Aguas, Centro de Edafología y Biología Aplicada del Segura-CSIC, Campus de Espinardo, PO Box 4195, E-30100 Murcia, Spain*

Received 14 December 2006; Revised 3 January 2007; Accepted 8 January 2007

Abstract

Plant roots may be linked by shared or common mycorrhizal networks (CMNs) that constitute pathways for the transfer of resources among plants. The potential for water transfer by such networks was examined by manipulating CMNs independently of plant roots in order to isolate the role(s) of ectomycorrhizal (EM) and arbuscular mycorrhizal fungal (AMF) networks in the plant water balance during drought (soil water potential –5.9 MPa). Fluorescent tracer dyes and deuterium-enriched water were used to follow the pathways of water transfer from coastal live oak seedlings (*Quercus agrifolia* Nee; colonized by EM and AMF) conducting hydraulic lift (HL) into the roots of water-stressed seedlings connected only by EM (*Q. agrifolia*) or AMF networks (*Q. agrifolia*, *Eriogonum fasciculatum* Benth., *Salvia mellifera* Greene, *Keckiella antirrhinoides* Benth). When connected to donor plants by hyphal linkages, deuterium was detected in the transpiration flux of receiver oak plants, and dye-labelled extraradical hyphae, rhizomorphs, mantles, and Hartig nets were observed in receiver EM oak roots, and in AMF hyphae of *Salvia*. Hyphal labelling was scarce in *Eriogonum* and *Keckiella* since these species are less dependent on AMF. The observed patterns of dye distribution also indicated that only a small percentage of mycorrhizal roots and extraradical hyphae were involved with water transfer among plants. Our results suggest that the movement of water by CMNs is potentially important to plant survival during drought, and that the functional ecophysiological traits of individual mycorrhizal fungi may be a component of this mechanism.

Key words: Common mycorrhizal networks, deuterium, drought, fluorescent tracers, hydraulic lift, rhizomorph, *Quercus agrifolia*.

Introduction

Plant roots in natural communities are often linked by shared or common mycorrhizal networks (CMNs) that constitute pathways for the transfer of resources among plants. Such networks may form 'guilds of mutual aid' among neighbouring plants of the same or different species (Perry *et al*., 1989), or redistribute resources along classical source–sink gradients from resource-rich (source) to resource-poor plants (sink; He *et al*., 2004). Net translocations of carbon, nitrogen, and phosphorus between plants connected by CMNs have been documented (Simard *et al*., 1997; He *et al*., 2004; Leake *et al*., 2004). However, the transfer of water by CMNs has received remarkably little attention. In this study, the potential for water transfer by CMNs during drought was examined.

Mycorrhizal fungi play a fundamental role in the uptake and transport of water from the bulk soil to the host plant. Soil water uptake and transport to the host plant (positive flow) by mycorrhizal hyphae has been widely demonstrated in both ectomycorrhizal (EM: Duddridge *et al*., 1980; Brownlee *et al*., 1983) and arbuscular mycorrhizal

* The first two authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: l-egerton@northwestern.edu

fungal (AMF) associations (Ruiz-Lozano and Azcon, 1995; Augé, 2001). Mycorrhizae may also contribute to water redistribution within the rhizosphere through reverse flow, or the transfer of water from the root to the mycorrhizal fungus. Specifically, oaks conducting hydraulic lift (HL) were found to transfer this hydraulically lifted water directly to their mycorrhizal fungi and associated extraradical hyphae (Querejeta *et al*., 2003).

HL, or the passive transport of water from deep moist soils to upper soil layers by roots, helps maintain a shallow soil–water reservoir that sustains root growth and longevity, and plant physiological activity (e.g. transpiration) in the deep-rooted 'lifter' plant and shallow-rooted co-occurring plant species (Horton and Hart, 2004, and references therein). The roots of neighbouring plants may either directly capture HL efflux, or receive HL water through mycorrhizal hyphae (Caldwell and Richards, 1989; Horton and Hart, 2004). However, these two processes are not mutually exclusive: plant roots may exude HL water into the rhizosphere where it is taken up again, either by the same or another plant, with or without mycorrhizal fungi.

To differentiate between these possibilities, CMNs were manipulated independently of plant roots to isolate the role of mycorrhizal hyphal networks in plant water balance during soil drying. Fluorescent tracer dyes and deuterium-enriched water were used to follow the pathways of water transfer from *Quercus agrifolia* plants conducting HL to adjacent oaks or shrub plants linked by EM or AMF networks. It was predicted that plants sharing CMNs, and with one individual conducting HL, could redistribute HL water to all neighbouring seedlings equally during drought, as long as mycorrhizal linkages were maintained (the 'mutual aid' hypothesis).

Materials and methods

Experimental systems

The pathways of water transfer were evaluated in experimental mesocosms (detailed in Querejeta *et al*., 2003). Briefly, each mesocosm comprised two adjacent upper (plant) chambers, and a single taproot chamber attached to one of the upper chambers. The plant compartments were separated by a 2 mm perforated stainless steel plate sandwiched between two stainless steel mesh screens (35 μm pore), to prevent the bulk flow of water and solutes between compartments. The taproot compartment was separated by a 20 mm air gap from the plant chamber to prevent hyphae bridging the gap in any of the mesocosms. All compartments in the mesocosm were filled with a steam-sterilized mixture of a loamy soil, coarse sand, and fine sand (1:1:1 v/v/v) with pH 6.8, KCl-extractable N (NO_3+NH_4) 4 μg g^{-1}, and HCO_3-extractable P 9 μg g^{-1}. Mycorrhizal colonization of the taproots was, on average, less than 2% because of waterlogged conditions in the taproot compartment. Root segments bridging the air gap between upper plant and taproot compartments were woody and non-mycorrhizal throughout the study.

Experiment 1: conspecific transport of HL water

California coast live oak seedlings were established in six mesocosm systems. Germinating acorns were planted into the two upper root chambers of six mesocosms and inoculated with plugs of culture of *Cenococcum geophilum* Fr. (Ascomycota), or a species of *Cortinarius* subgenus *Telamonia* (Basidiomycota; *Cortinarius evernius* Fries, AJ236077, 98% over 534 bp), each supplemented with slurry comprising *Boletus amygdalinus* Thiers (Basidiomycota) and *Boletus dryophilus* Thiers (Basidiomycota) that was prepared by blending sporocarps in deionized water (Parladé *et al*., 1996), and a spore suspension of *Pisolithus tinctorius* (Pers.) Coker & Couch (Basidiomycota; Mycorrhizal Applications Inc., Grants Pass, OR, USA) in deionized water (total 15×10^8 spores per mesocosm). Seedlings acquired AMF from corn plants grown in the same greenhouse. The mesocosms were irrigated to field capacity twice a week with deionized water for 8 months.

To simulate Mediterranean-type summer drought conditions, irrigation to the two upper compartments was terminated. Soil in the taproot chamber was maintained near saturation by keeping half of the compartment submerged in water. Oaks accessing taproot compartments (hereafter called donor oaks) therefore had an unlimited supply of moisture during drought, whereas the oaks in the adjacent compartment (hereafter referred to as receiver oaks) became increasingly water-stressed; drought conditions were maintained for 12 d. Three of the six mesocosms were used as controls in which the hyphal connections between donor and receiver compartments were severed at the onset of the drought treatment: the two upper plant compartments were separated, and all hyphae and rhizomorphs wiped from the mesh surfaces.

The pathways of HL water transfer from donor to receiver oaks were evaluated using deuterium-labelled water and fluorescent tracer dyes. In order to distinguish HL water from existing soil moisture, irrigation water (δD –60‰) was enriched with deuterium (D_2O, 99.9 atom% D) to obtain a δD value of +160‰. To minimize any dilution effects caused by irrigation water in plant tissues or soil, deuterium-enriched water was supplied 4 d prior to destructive sampling of the mesocosms. Three hundred ml of D_2O-enriched water was injected into the taproot compartment of each mesocosm at dusk. The fluorescent tracer dye was only introduced into these mesocosms on the day before sampling since dye transfers from plants to their mycorrhizal fungi have been shown to occur overnight (Querejeta *et al*., 2003). Fifty ml of 2 mM Lucifer Yellow carbohydrazide solution (LYCH, $C_{13}H_9K_2N_5O_9S_2$ dipotassium salt, pH 6.9; Molecular Probes Inc., Eugene, OR, USA) was injected into the taproot compartment of all mesocosms at dusk (Querejeta *et al*., 2003). At dawn the following day, the mesocosms were destructively sampled. Samples of oak stem and rhizosphere soil were immediately placed into individual sealed glass vials, wrapped in Parafilm®, and frozen (–20 °C) until water was extracted for δD analysis. Water extractions were undertaken using a cryogenic vacuum distillation line (Ehleringer and Osmond, 1989). Soil and root samples were also collected, and viewed and scored for the presence of LYCH.

A second set of mesocosms (n=3) was used to trace the movement of HL water into the transpirational flux of receiver seedlings over 12 d of drought. These mesocosms were identical in mycorrhizal fungal composition and oak species identity and age to the mesocosms described above. In two mesocosms, the hyphal connections between donor and receiver compartments were severed at the onset of the drought treatment (controls). In the third mesocosm, the hyphal connections were maintained between donor and receiver plants. A 4.5 ml aliquot of D_2O (99.9 atom% D) was injected into the taproot compartment of each mesocosm at the onset of the imposed drought. A high label concentration was used to ensure that the deuterium could be detected in the transpiration

flux throughout the drought period. Each day, transpiration water was collected shortly after dawn using a cryogenic method for sampling atmospheric water vapour (Helliker *et al*., 2002). A removable acrylic chamber (15×15×60 cm) was placed on top of the receiver compartment and tightly clamped using a rubber gasket. The acrylic chamber was fitted with an inlet spigot (4 mm internal diameter) connected to a column packed with Drierite to minimize the input of exogenous water vapour into the system, and an outlet spigot (4 mm internal diameter) connected to a glass vapour sampling apparatus and, in turn, an air pump. The glass tubing of the vapour sampling apparatus was placed in a Dewar containing crushed dry ice. The pump was used to pull air through the system at a constant flow rate of 300 ml min^{-1} for 20–30 min so that water vapour condensed within the glass tubing. After sampling, the pump was turned off, and the apparatus was removed from the Dewar and allowed to reach ambient temperature to avoid any sample contamination caused by the condensation of outside moisture within the glass tubing. Once at room temperature, the glass tubing was removed from the apparatus, covered with a rubber stopper and wrapped in Parafilm®, and stored in the freezer (–20 °C) until stable isotope analyses were undertaken.

All water samples were analysed for hydrogen isotope ratios using a continuous flow, high temperature reduction technique at the Stable Isotope Laboratory in the Department of Earth and Planetary Sciences, the University of New Mexico. Briefly, 1 µl aliquots of water were injected into a helium stream, vaporized, and then reduced to CO and H_2 by passing the sample through a graphite column heated to 1450 °C. The resultant gases were purified by gas chromatography (5A mol sieve, 100 °C), and then analysed using a Finnigan MAT CONFLO II interface/open split for helium dilution interfaced to a Finnigan MAT Delta XL Plus mass spectrometer. All δD values are expressed relative to Vienna-standard mean ocean water (V-SMOW) and defined as:

$$\delta D\,(‰) = (R_{SAMPLE} - R_{V\text{-}SMOW})/R_{V\text{-}SMOW} \times 1000$$

where R_{SAMPLE} is the ratio of deuterium to hydrogen atoms in the sample. The precision of measurement was ±2‰.

Experiment 2: interspecific transport of HL water

California coast live oak seedlings were established in four mesocosms. Four germinating acorns were planted into one of the upper plant chambers of each mesocosm, and inoculated with plugs of *Cenococcum geophilum* or *Cortinarius* subgenus *Telamonia*, as outlined in Experiment 1. These plants similarly acquired AMF from adjacent corn plants. The mycorrhizal oak seedlings established mycelial networks that spread into the adjacent (empty) compartment within 8 months. Fourteen months after establishment, seedlings of coastal live oak, *Salvia mellifera* (black sage, Lamiaceae), *Eriogonum fasciculatum* (buckwheat; Polygonaceae), and *Keckiella antirrhinoides* (bush snapdragon; Scrophulariaceae) were planted in the adjacent upper compartment of all mesocosms. *Salvia*, *Eriogonum*, and *Keckiella* are co-dominant species in the coastal sage scrub and chaparral communities that border oak woodlands in southern California. In addition, coastal live oak hosts both AMF and EM fungi, and *Salvia* is highly dependent on AMF for plant growth and survival, whereas species of *Eriogonum* and *Keckiella* are rarely dependent on AMF (Titus and Tsuyuzaki, 2002). Four germinated acorns, and seed of *Salvia*, *Eriogonum*, and *Keckiella*, were sown into the soil at 10 mm depth. Seedlings were thinned to two plants of each species per mesocosm after the first true leaves had fully expanded. All mesocosms were irrigated to field capacity twice a week with deionized water.

Drought was initiated 4 months after the seedlings were planted. In the control mesocosms (n=2), all hyphal connections between the donor oak and receiver plant compartments were severed at the onset of the drought treatment by separating the two upper plant compartments, and wiping all hyphae and rhizomorphs from the mesh surfaces. The two remaining mesocosms were undisturbed so that the hyphal linkages between donor and recipient plants remained intact. Soil in the taproot chamber was maintained near saturation throughout the imposed drought by maintaining the lower half of the compartment submerged in water. The older and larger oak seedlings accessing taproot compartments (donor oaks) therefore had unlimited supply of moisture during drought, while the younger oak and shrub seedlings in adjacent compartments (receiver plants) became increasingly water-stressed. Mesocosms inoculated with *Cenococcum* were destructively sampled after 6 d of drought, while mesocosms inoculated with *Cortinarius* subgenus *Telamonia* were sampled after 11 d of drought.

The pathways of water transfer were evaluated using Alexa Fluor 568 ($C_{33}H_{31}N_4NaO_{10}S_2$, sodium salt; Molecular Probes Inc., Eugene, OR, USA). Similar to LYCH, Alexa Fluor 568 is a low molecular weight, and cell membrane-impermeant molecule. This dye is also highly water-soluble, and produces a strong, photostable yellow-orange to orange-red fluorescence that contrasts markedly with the yellow-green fluorescence of LYCH and the pale green autofluorescence of fungal hyphae. As a result, neither fluorescent tracer can be confused with organic substrates, or autofluorescence from root or fungal tissues. Fifty ml of 5 mM Alexa Fluor 568 (pH 6.8 in deionized water) was injected into the taproot compartments of all mesocoms at dusk the day before sampling. At dawn the following day, the mesocosms were disassembled, and soil and hyphal samples from donor and receiver compartments collected for analysis.

Plant and soil water potentials

Plant pre-dawn xylem water potential measurements were made on freshly clipped stems of 8-month-old oak seedlings using a Scholander-type pressure bomb. Soil water potential measurements were conducted on freshly collected soil samples using the chilled mirror dewpoint method (CX-2, Decagon Devices, Pullman, WA, USA). In this method, a small sample of fresh soil (4 cm diameter×0.5 cm thick) is equilibrated within the headspace of a sealed chamber containing a mirror, an optical sensor, an internal fan, and an infrared temperature sensor. The internal fan is for air circulation, which reduces vapour equilibrium time and controls the boundary layer conductance of the mirror surface. At equilibrium, the relative humidity of the air in the chamber is the same as the water potential of the sample. A thermoelectric (Peltier) cooler precisely controls the mirror temperature, and an optical reflectance sensor detects the exact point at which condensation first appears on the mirror. The accuracy of this technique is ±0.1 MPa in the 0 MPa to –60 MPa range.

Root colonization

Root colonization by EM and AMF was evaluated in all species. Freshly harvested oak root samples from each chamber were washed with distilled water and EM colonization expressed as the percentage root tips colonized. Samples of fine roots in oaks, and in *Salvia*, *Eriogonum*, and *Keckiella* were stained with Trypan blue (Koske and Gemma, 1989), and evaluated for percentage colonization by AMF using the modified line intersect method (McGonigle *et al*., 1990).

Fluorescent dye transfer and distribution in roots and extraradical hyphae

Ectomycorrhizal root tips collected from oak seedlings were sorted into morphological types based on colour, texture, and branching

pattern. A subsample was retained for molecular analysis (see below), and the remainder transversely sectioned by hand using a two-edged razor blade. A series of thin sections—four successive sections at each EM root tip, and another four sections mid-way along the root—were mounted in glycerol, and then examined on a Zeiss Axioskop 2 microscope using fluorescence and filter combinations suitable for LYCH (blue/violet; excitation 400–425 nm, dichroic mirror 460 nm, emission 475 nm), or Alexa Fluor 568 (FITC filter set; excitation 475–490 nm, mirror 505 nm, emission 503–535 nm). An individual root tip was scored as positive when LYCH or Alexa Fluor fluorescence was detected in all the sections (tip, mid-way). In general, it was found that a positive fluorescent signal in the first tip section was followed by positive signals in the remaining sections. A root tip was classed as negatively labelled when no fluorescent signal was observed in any section. Whole fine roots of *Salvia*, *Keckiella*, and *Eriogonum* were also mounted in glycerol, and viewed and scored for the presence or absence of label in each root tip.

External hyphae were extracted from replicate 10 g (fresh weight) soil samples. Each soil sample was suspended in 100 ml of deionized water plus 4 ml of sodium hexametaphosphate solution (39 g l^{-1}). Soil suspensions were agitated for 30 s, left to settle on the bench for 60 min, and then decanted through a 37 μm sieve. The hyphal material on the sieve was rinsed with deionized water, suspended in 12 ml of deionized water in a centrifuge tube, and centrifuged for 10 min (3600 *g*). The resulting pellet was rinsed in deionized water, and centrifuged for 10 min (twice), after which, the supernatant was decanted and the hyphal pellet resuspended in 10 ml of fresh deionized water. A 2 ml aliquot of the hyphal suspension was filtered over a membrane, and mounted on glass microscope slides in glycerol. Hyphae were viewed and the presence or absence of dye-labelled hyphae scored using the same fluorescence microscopy conditions as root samples. Hyphal counts were taken in at least 100 fields of view per slide (×400), and the length of hyphae was calculated and converted to m hyphae per gram soil (Tennant, 1975). The remaining hyphae were analysed for mycorrhizal community composition.

Ectomycorrhizal community composition

Genomic DNA was extracted in replicate samples of each EM morphotype (2–3 tips per sample), as well as hyphae and rhizomorphs recovered from the screens separating the donor and receiver compartments. Extraction followed the methods described in Gardes and Bruns (1996), except that a glassmilk slurry was used for DNA binding and purification (GeneClean Spin Kit, Q-Biogene, Irvine, CA, USA). Each sample of genomic DNA was used as a template PCR using the primer pairs ITS1f/ITS4 (fungal nuclear ribosomal internal transcribed spacer, nrITS) or ITS1f/ITS4b (Basidiomycete-specific nrITS). Positive controls using *Agaricus bisporus*, and controls without DNA were included to check for reaction efficacy and contamination, respectively. All positive PCR products were screened by restriction fragment length polymorphisms (RFLPs) using the restriction endonucleases *Hin*fI and *Alu*I. The amplified products of each unique RFLP type were then sequenced.

PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Mississauga, Ontario, Canada), and prepared using ITS1f and/or ITS4 primers and the Research Genetics QuickStart dye terminator sequencing kit (ResGen/Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's specifications. The primer pair ML5/ML6 was used for samples that failed to amplify with ITS1f/ITS4 primers. Amplified products were screened using automated nucleotide sequencing dye terminator chemistry on a Beckman CEQ 8000 sequencer (Beckman-Coulter Genetic Analysis Systems, Fullerton, CA, USA). Root or hyphal sample sequences were aligned automatically using Clustal X against reference sequences obtained from the best BLAST hits for mycorrhizal root or fungi (http://www.ncbi.nlm.nih.gov/BLAST), as well as GenBank accessions of fungi known to form mycorrhizal associations with oaks.

Arbuscular mycorrhizal community composition

Direct immunofluorescence was used to evaluate the composition of the AMF community. For each soil sample, 400 μl aliquots of hyphal suspension were placed into each of five microfuge tubes followed by 100 μl of an individual antiserum of the four major AM genera (*Scutellospora*, *Gigaspora*, *Acaulospora*, *Glomus*) conjugated to fluorescein isothiocyanate (FITC); 100 μl of deionized water was added to the fifth tube as a control for fungal autofluorescence. Samples were incubated overnight at room temperature, and then filtered and rinsed with deionized water over a membrane, and mounted in glycerol. All samples were viewed under fluorescence microscopy using the FITC filter combination. FITC-labelled AM hyphae exhibit a brilliant white-green fluorescence compared with the orange-red signal typical of Alexa Fluor-labelled hyphae. LYCH does not produce a detectable signal under FITC filter conditions. Counts were taken on FITC-reactive (fluorescent) and non-reactive hyphae, and hyphal length calculated as per the LYCH-labelled samples. Since immunological methods detect live hyphae, the AMF data were also used to estimate hyphal viability.

Statistics

Differences in soil and plant water potentials, δD, the total and dye-labelled hyphal lengths were analysed between donor and receiver compartments in Experiment 1 using *t* tests. To test for significant differences in total hyphal and labelled hyphal lengths among EM species used for inoculation (*Cenococcum*, *Cortinarius*) and receiver plants (oak, *Salvia*, *Eriogonum*, *Keckiella*) in Experiment 2, data were analysed using two-way analysis of variance (ANOVA), with *post hoc* tests using Fisher's Least Significant Difference for significant variables.

Results

Experiment 1: conspecific water transfer

Receiver oak plants were significantly water stressed after 11 d of drought. Pre-dawn xylem water potential values in 8-month-old receiver plants averaged –4.5±0.5 MPa (n=5), in contrast to –0.3±0.1 MPa (n=4) in donor oaks in all the mesocosms. Measurements of soil water potentials also showed similar trends. In receiver compartments, soil water potential ranged from –4.9 MPa to –7.4 MPa, with mean –5.9±0.8 MPa (n=6) compared with –3.7±0.5 MPa (n=6) for the donor oaks. Thus, direct root access to the taproot chamber adequately maintained the water status of donor oaks throughout the imposed drought even though the soil in the upper root compartment demonstrated a significant depletion of moisture.

The root systems of both donor and receiver oak plants were heavily mycorrhizal (Table 1), and the extent of EM and AMF colonization did not differ significantly among compartments (Table 1). Within the EM community, the percentage of root tips colonized by species of *Boletus*,

Table 1. *Percentage root colonization by ecto- and arbuscular mycorrhizal fungi, and the percentage of root tips colonized by ectomycorrhizal fungal species in mesocosms after 11 d of drought (Experiment 1)*

Data reported as mean values, mean ±SE.

Ectomycorrhizal fungus	Donor oak	Receiver oak	
Boletaceae (AY534211, 92%, 505 bp)[a]	18.8 (4.8)	16.2 (13.9)	NS[b]
Boletus dryophilus (AY372286, 99%, 436 bp)	31.8 (8.5)	25.7 (6.5)	NS
Cenococcum geophilum[c]	7.4 (7.4)	3.4 (3.3)	NS
Cortinarius subg. *Telamonia* (*C. evernius*, AJ236077, 98%, 534 bp)	2.7 (2.7)	0.3 (0.1)	*
Lactarius sp. (VT2407, AF349717, 99%, 208 bp)	5.2 (2.1)	13.2 (0.9)	*
Pisolithus tinctorius[c]	5.8 (5.8)	13.6 (12.1)	NS
Thelephoraceae (Taylor#9, U83471, 99%, 191 bp)	0.2 (0.2)	3.2 (2.6)	*
Total % EM colonization	72.0 (2.2)	74.7 (2.4)	NS
Total % AMF colonization	23.4 (8.8)	19.7 (7.8)	NS
% Root tips labelled	17.6 (3.9)	18.6 (7.3)	NS

[a] GenBank accession number, and % sequence similarity over *n* base pairs.
[b] NS, not significant; * donor and recipient plants differ significantly at $P < 0.05$.
[c] Match by mycorrhizal root morphology.

Cenococcum, and *Pisolithus* were similar between donor and receiver plants. However, root tips colonized by *Cortinarius* were significantly more abundant in donor oaks whereas *Lactarius* was abundant in receiver oaks. Both *Lactarius* and the Thelephoraceae were detected on roots, but not used to inoculate plants. Spores or material of both taxa may have been unwittingly transported on the *Boletus* sporocarps collected from the field, and subsequently incorporated during inoculation. Hyphae of *Cenococcum,* and hyphae and rhizomorphs of *Boletus* species, *Cortinarius* sp, and *Pisolithus* were also recovered from the mesh and air gap between donor and receiver compartments (Fig. 1).

Oak roots were also well colonized by AMF (Table 1). DNA sequence data identified some of these fungi as a species of *Glomus (*AY236262, 95% over 191 bp). Arbuscular mycorrhizal hyphae were also detected on the mesh screens and in the air gaps between donor and receiver compartments. Within the soil, hyphae of *Glomus* and *Scutellospora* were the most abundant in both compartments (*Glomus* 0.69±0.1 m g^{-1} soil; *Scutellospora* 0.73±0.1 m g^{-1} soil). The immunofluorescent data also revealed that 43–47% of AMF hyphae were viable in donor compartments, and 44% were viable in receiver compartments with intact hyphal linkages. Breaking the hyphal linkages between donor and receiver compartments resulted in a reduction of AMF hyphal viability for receiver plants (33% viable hyphae).

The LYCH tracer dye was readily detected in the hyphae (Fig. 2A, B) and vesicles of root segments

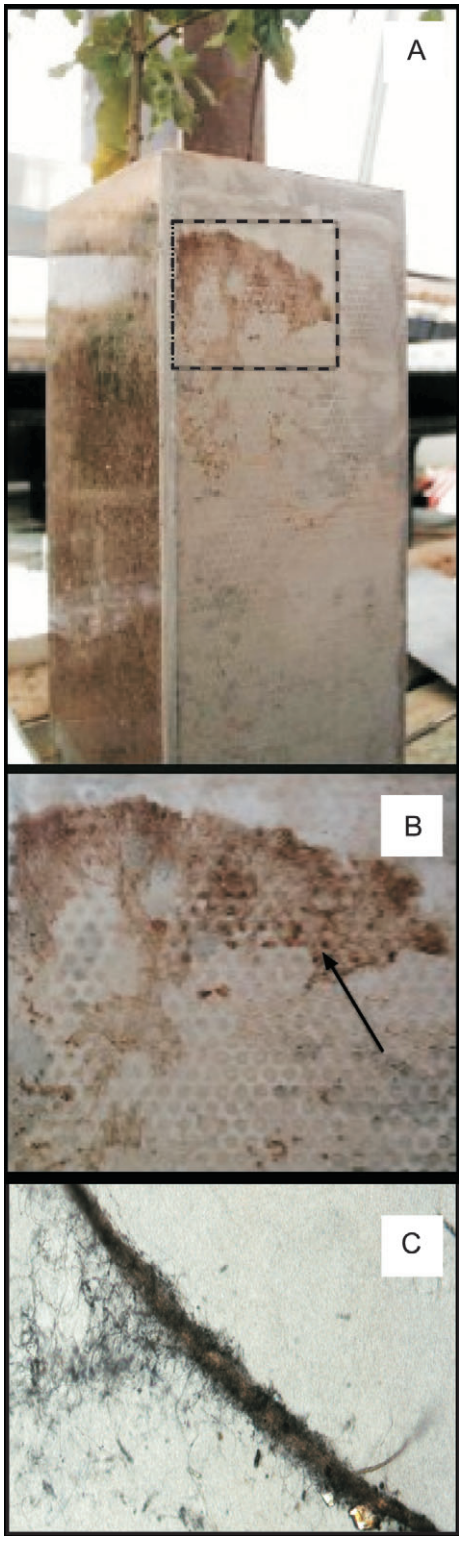

Fig. 1. Experimental mesocosms showing mesh screen (A, B) with hyphal fan of *Cortinarius* sp., and a rhizomorph retrieved from the air gap between the mesh screens (C).

colonized by AMF (not shown; Querejeta *et al*., 2003), as well as EM roots, hyphae, and rhizomorphs in receiver oaks (Fig. 2C). Dye transfer also occurred at night in

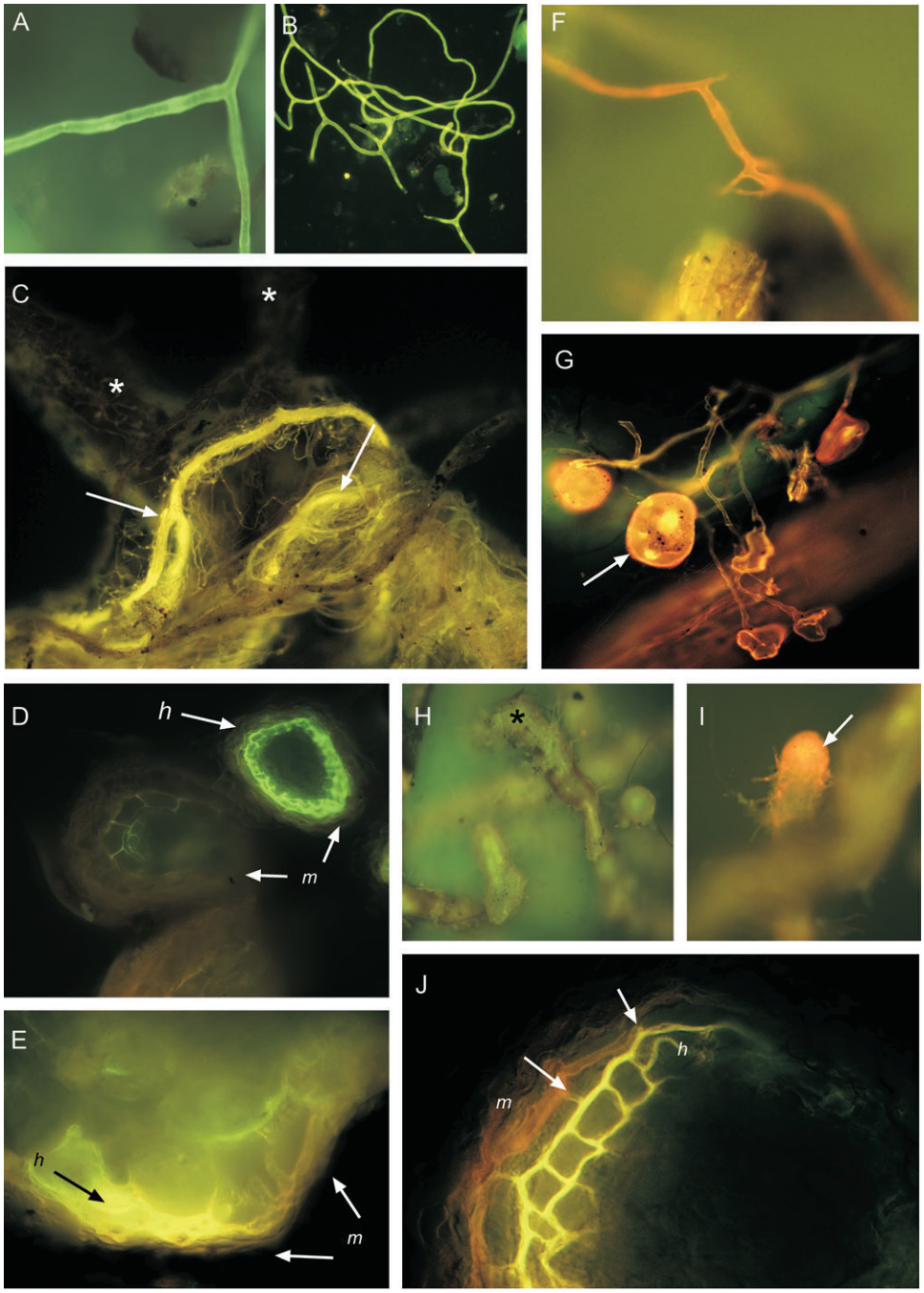

Fig. 2. Autofluorescence in mycorrhizal fungal hyphae from the mesocosms (A); Lucifer yellow CH labelling of AMF hyphae (B), and EM rhizomorphs (C), but not any of the adjacent hyphae or roots. Transverse sections of a cluster of *Quercus* roots colonized by *Boletus (*D), and a root tip colonized by *Cenococcum* (E), and demonstrating locations indicating the localization of tracer dye in the Hartig net and mantle. Localization of Alexa Fluor 568 in AMF hyphae and spores (F, G), and EM root tips (H) in comparison with autofluorescent (non-labelled) roots (I). Transverse section of a *Quercus* root tip colonized by *Cortinarius*, and demonstrating the localization of Lucifer yellow CH within the Hartig net (J). Within an individual image, arrows denote the localization of LYCH or Alexa Fluor dye; asterisks denote non-labelled roots or hyphae; h, denotes Hartig net; m, denotes mantle.

association with HL, as has also been noted previously (Querejeta *et al*., 2003). Within the EM root tips of *Quercus* colonized by *Cenococcum*, *Boletus*, or *Cortinarius*, the tracer dye was detected within the mantle and/or the Hartig net (e.g. *Cenococcum*; Fig. 2D, J), but not the root vasculature. This pattern of dye distribution suggests the direct transfer of water from donor to receiver plants via fungal hyphae. Nevertheless, the external hyphae

(Fig. 2C), and EM root tips, even those in conspecific clusters (Fig. 2D), were not uniformly labelled with dye. That is, not all root tips constituted entry or exit points for water, and not all hyphae were equally associated with the transfer of water (Fig. 3).

Despite low soil water potentials in the receiver compartment, the standing biomass of mycorrhizal hyphae, and the length of LYCH-labelled hyphae recovered from mesocosms with intact hyphal linkages did not differ significantly between donor and receiver compartments (Fig. 3). However, only a small proportion of the total hyphal length was labelled with LYCH (Figs 2C, 3). In mesocosms with severed hyphal linkages (controls), the standing biomass of mycorrhizal hyphae did not differ significantly between donor and receiver compartments either, but hyphae containing LYCH were not recovered from the receiver compartment.

In oak plants with intact or severed hyphal linkages, water extracted from the stem xylem or rhizosphere soil in donor plants was enriched with deuterium relative to the irrigation water (δD –60‰; Table 2). These data further support the presence of HL in the donor oak plants. In addition, a comparison of relative deuterium enrichment shows a small but statistically significant gain in δD in plants with intact hyphal linkages over those with no hyphal connections to the donor plant (stem xylem 11‰, rhizosphere soil 31‰; Table 2). These data indicate that tracer water with a higher concentration of deuterium may be required to quantify the transfer of HL water from donor to receiver seedlings. The δD of the rhizosphere soil water also suggests that small amounts of deuterium-enriched tracer water may have leaked from the roots of the receiver plant into the rhizosphere soil when hyphal linkages were maintained. In contrast, the small deuterium enrichment detected in the rhizosphere soil of receiver oak plants with severed hyphal links was consistent with the evaporation of moisture from upper soil layers.

At the onset of drought, the δD values in the transpiration flux in receiver plants with intact or severed hyphal linkages were also similar to the signature for the irrigation water (Fig. 4). As drought progressed, the deuterium enrichment in the transpiration stream increased linearly (R^2=0.984, P <0.05) in receiver plants with intact hyphal linkages. As a result, plants connected to donor oaks conducting HL showed up to 136‰ enrichment in the transpiration flux in comparison with plants with severed hyphal links. Transpiration, rather than soil evaporation, probably accounted for the bulk of water vapour collected from receiver compartments because canopy leaf area was much larger than the area of soil exposed to direct evaporation. A small amount of deuterium enrichment was also detected in the transpiration flux of receiver oaks with severed hyphal links (R^2=0.712, P <0.05; Fig. 4). This enrichment indicates that soil

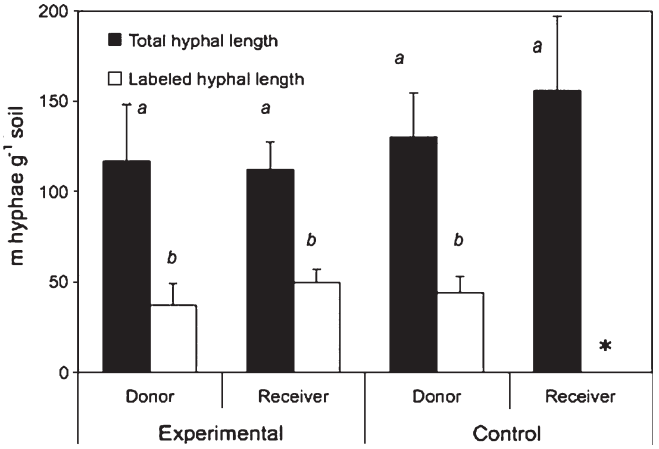

Fig. 3. Total length of mycorrhizal hyphae (EM+AMF), and LYCH-labelled hyphae recovered in oak mesocosms with intact or severed hyphal links (Experiment 1). Error bars indicate the standard error of the means. For each response variable, bars with the same letter do not differ significantly at P <0.05 (Fisher's LSD).

Table 2. *Mean δD values of water extracted from rhizosphere soil and stem xylem in donor or receiver oak seedlings with intact or severed hyphal linkages*

Hyphal links	Stem xylem (‰)		Rhizosphere soil (‰)	
	Donor	Receiver	Donor	Receiver
Intact	481 (65)	–33.1 (3.2)	36.7 (6.8)	–12.8 (6.3)
Severed	450 (32)	–44.2 (2.6)	31.2 (8.8)	–44.0 (2.6)
Significance[a]	NS	*	NS	*
(t test, P)	(0.098)	(0.036)	(0.261)	(0.039)

[a] NS, not significant (P >0.05); * δD in plants with intact or severed hyphal linkages differs significantly at P <0.05.

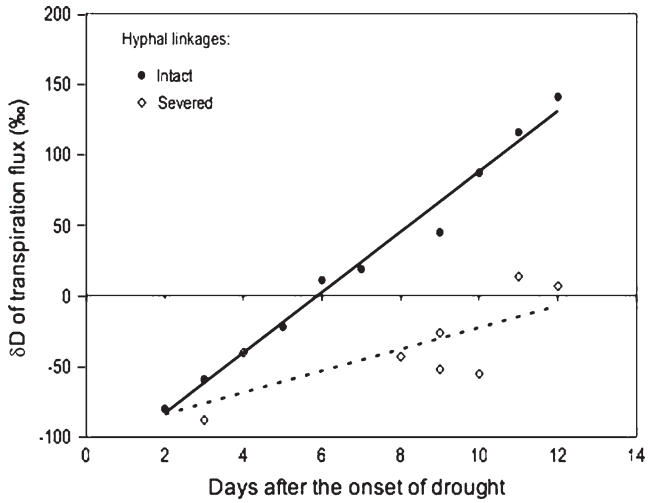

Fig. 4. Hydrogen isotopic composition of water vapour transpired by receiver mycorrhizal oaks during a 12-d drought period in mesocosms with intact (n=1) or severed (n=2) hyphal linkages. The deuterium-enriched tracer water was injected into the taproot compartment of donor oaks on day 2.

moisture may have evaporated during the drought, or that deuterium-enriched water vapour may have diffused between compartments. Overall, however, deuterium enrichment was consistently and significantly higher in the transpiration flux of receiver seedlings sharing intact hyphal connections with donor plants than in unlinked plants.

Experiment 2: interspecific transfer of HL water

Soil water potential in the receiver compartment averaged –4.1 MPa, but was found to reach –5.9 MPa in the upper soil layers.

Root colonization was not significantly different among donor and receiver oak plants inoculated with *Cortinarius* (Fig. 5B). In *Cenococcum* mesocosms, however, colonization was significantly lower in receiver plants grown in the mesocosm where hyphal linkages had been severed (control; Fig. 5A). In both *Cenococcum* and *Cortinarius* mesocosms, AMF root colonization was generally higher in receiver than donor oak plants because of the proximity of *Salvia* plants with extensive AMF root colonization. Root colonization by AMF was low in both *Eriogonum* (4–10%) and *Keckiella* (2–5%).

The presence of the Alexa Fluor tracer in AMF hyphae and spores (Fig. 2E) and EM root tips (Fig. 2G) in receiver plants confirmed the transport of HL water between compartments. Regardless of the mycorrhizal inoculum (*Cortinarius* versus *Cenococcum*), there was no significant difference in the percentage of root tips labelled with the Alexa Fluor tracer dye in donor plants (mean 43%; Fig. 5). In *Cenococcum* mesocosms with intact hyphal linkages, similar percentages of root tips were labelled in receiver oaks and *Salvia*; dye was not detected in *Eriogonum*. In intact *Cortinarius* mesocosms, a greater percentage of oak than *Salvia* roots was labelled,

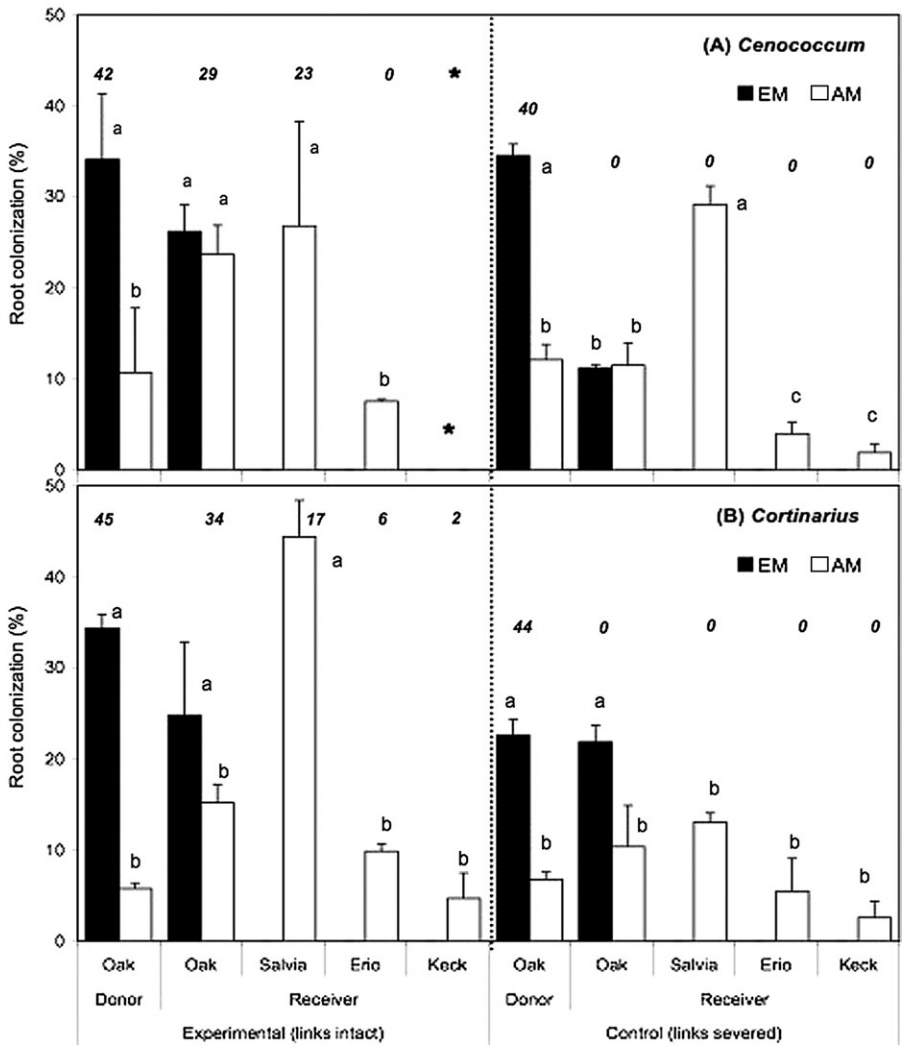

Fig. 5. Percentage root colonization by EM and AMF in donor oaks, and receiver oaks, *Salvia*, *Eriogonum*, and *Keckiella* (Experiment 2). Number in bold over each column denotes the percentage of root tips in which Alexa Fluor dye was recovered. Erio, *Eriogonum*; Keck, *Keckiella*. Error bars indicate the standard error of the means. In each of the *Cortinarius*- or *Cenococcum*-inoculated mesocosms, bars with the same letter do not differ significantly at $P < 0.05$ (Fisher's LSD). Asterisk denotes no surviving seedlings of *Keckiella* in the *Cenococcum* mesocosms.

and the few root tips of *Eriogonum* and *Keckiella* containing tracer showed only patchy labelling within the external hyphae. The tracer was not detected in mesocosms where hyphal links between compartments were severed (control). Across species, the percentage of labelled roots covaried with mycorrhizal colonization in receiver plants (Pearson's product moment correlation coefficient=0.858; P <0.05). Nevertheless, only a small percentage of the total number of roots was labelled with dye.

In all mesocosms, the total length of mycorrhizal hyphae was significantly higher in receiver than donor compartments (Fig. 6). In addition, the length of labelled hyphae was directly proportional to the total hyphal length in mesocosms with intact hyphal links, so that a higher percentage of hyphae in the receiver compartments contained Alexa Fluor than the donor compartments. Only a small percentage of hyphae were labelled with the tracer (2–13% of hyphal length).

Discussion

By experimentally manipulating CMNs independently of plant roots and using fluorescent dye and isotopic tracers to detect the pathways of water flow, these experiments have demonstrated the transfer of water between plants connected only by mycorrhizal hyphae. Further, these transfers occurred in association with HL, and were expressed whether donor oaks were involved in conspecific or interspecific transfers of water, or grown with plants hosting EM or AMF. These findings, therefore, extend the functional role of HL to include the contribution of lifted water to neighbouring plants through EM and AMF networks.

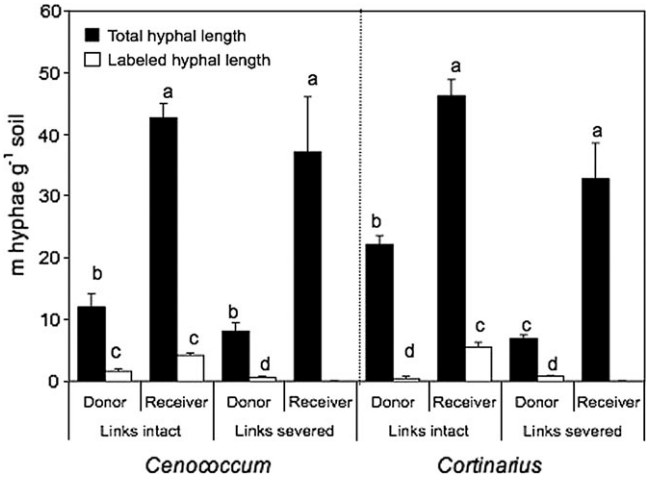

Fig. 6. Total hyphal length (EM+AMF), and length of hyphae containing Alexa Fluor tracer dye in *Cenococcum* and *Cortinarius* mesocosms with intact and severed hyphal linkages (Experiment 2). Error bars indicate the standard error of the means. In each of *Cortinarius*- or *Cenococcum*-inoculated mesocosms, bars with the same letter do not differ significantly at P <0.05 (Fisher's LSD).

Our conclusions are based on two main lines of evidence: first, the soil water potentials and donor plant δD signatures indicated the recharge of shallow soil layers by deep-water sources (HL). This increase in soil moisture and δD was the result of HL by plant roots rather than capillary rise from the taproot chamber because the upper plant and lower taproot compartments were spatially segregated such that the taproot represented the only hydraulically intact pathway. The occurrence and magnitude of this mechanism under drought conditions were also consistent with previous observations from greenhouse and field studies of HL in *Quercus* (Millikin-Ishikawa and Bledsoe, 1999; Querejeta *et al*., 2003).

A second line of evidence comes from the deuterium enrichment data and patterns of dye localization. Plants that were not linked by mycorrhizal hyphae to a donor plant conducting HL showed no evidence of dye accumulation within root or mycorrhizal tissues, and deuterium enrichment within the transpiration flux consistent with soil moisture evaporation. Conversely, the fluorescent dye and deuterium tracers strongly suggest the transfer or movement of small amounts of HL water between plants connected by mycorrhizal hyphae. Deuterium enrichment within the transpiration flux, stem xylem, and rhizosphere soil clearly demonstrated that receiver plants had access to HL water during the imposed drought. In addition, the consistent localization of tracer dyes in EM rhizomorphs, mantle and Hartig net, and EM and AMF hyphae in receiver plants, but not the root vasculature, implies that water was transferred between mycorrhizal root tips by hyphae. Such patterns provide support for earlier studies of carbon and nutrient transfers among plants by CMNs (Simard *et al*., 1997). Based on the rhizosphere δD enrichment in receiver plants, however, the possibility cannot be ruled out that hyphae are 'leaky' (Sun *et al*., 1999), i.e. HL water may be exuded from hyphae into the surrounding soil and, in turn, reabsorbed by hyphal tips during plant–plant transfer.

The use of fluorescent dye and isotopic tracers can also be used to answer an essential question: do the results produced by a tracer dye adequately represent the movement of water in transfer experiments? The most obvious concern is the degree to which the movement and diffusivity of a dye flow differs from that of water owing to their differences in size and charge (Kuhn *et al*., 2000). Because these results show reasonable agreement between the fluorescent dye and isotopic tracers, this potential problem may be relatively minor.

The strength of the fluorescent tracer dyes is in visualizing the transfer pathway. For instance, the dye tracer studies shed light on some of the controls over water transfer. Only a small percentage of EM and AMF roots and hyphae in donor and receiver plants contained

tracers and, even within EM root clusters, only certain root tips were labelled (Fig. 2). This imbalance may have been influenced by plant traits such as phenology, water uptake per unit root biomass, and root morphology (Jackson *et al*., 2000), or indicate that HL water was lost as vapour water flux from the soil. Alternatively, the labelling pattern may indicate that not all fungal species transport water. Fungal ecophysiological strategies may influence water transfer. For example, the dominant taxa within the mesocosms, i.e. *Boletus*, *Cortinarius*, and *Pisolithus*, produce hydrophobic mantles and well-differentiated rhizomorphs, two traits considered typical of drought-resistant EM (Agerer, 2001). These well-differentiated rhizomorphs transport and hold significant amounts of water in the large diameter vascular vessels (Duddridge *et al*., 1980; Brownlee *et al*., 1983; Agerer, 2001, see also Fig. 2). *Lactarius* produces smooth, undifferentiated rhizomorphs, whereas *Cenococcum* mycorrhizae form envelopes of external hyphae rather than rhizomorphs (Agerer, 2001) that promote more localized distributions of water (Fig. 4). Further, hyphal anastamosis by AMF may create large interconnected networks with low resistance to solute flow (Giovanetti *et al*., 2004). Along with the large diameter in *Glomus* and *Scutellospora* hyphae (Hart and Reader, 2002), the dye data suggest that these networks may have transferred water, especially in AMF-dependent plants such as *Salvia*.

Even so, the percentage of hyphal and root tips labelled was, at best, weakly correlated with the percentage of root tips colonized (EM) or hyphal lengths (AMF) of these taxa. One explanation is that the allocation of plant photosynthates differed significantly among fungal species, but these differences were not reflected in any variations in water transfer among fungal species or functional groups. This independence of carbon allocation is verified by the similar percentages of root and hyphae that were labelled with dye in mesocosms with single or multiple mycorrhizal fungal species, or conspecific or interspecific plant assemblages. As a result, it can be hypothesized that plants acquire and transfer water from a number of fungal partners.

These results also provide support for the 'guild of mutual aid' prediction made by Perry *et al*. (1989), in which resources should be available to all plants as long as they are connected by CMNs. It is noted that fungal and plant dye labelling was high in roots of receiver *Quercus* and *Salvia* plants connected by CMNs to donor *Quercus* conducting HL, but not in the roots of *Eriogonum* and *Keckiella*. Both *Eriogonum* and *Keckiella* seldom rely on AMF for growth, whereas *Quercus* and *Salvia* are highly dependent on EM and AMF, respectively, for plant survival and growth. Consequently, host plant dependence on mycorrhizae will play a role in determining if a plant species joins a CMN and receives HL water through hyphae. Plant species with lower dependence on mycorrhizal fungi may still acquire water from fluxes within the rhizosphere of adjacent mycorrhizal plants (Fig. 4), especially if water is made available through hyphal tip exudation (Sun *et al*., 1999). However, these conclusions must be viewed with caution because of the limited number of mesocosms of each treatment that were examined in this study.

Scaling the night-time flux of HL water into CMNs up to the level of plant communities could result in substantial benefits to adjacent plants during drought, and, especially, where receiver plants provide a strong sink for resources (e.g. hyphal abundance). Studies suggest the quantities of water redistributed through HL may be significant, *c*. 25–33% of daily evapotranspiration (Richards and Caldwell, 1987; Dawson, 1996). Further experiments using deuterium-labelled water are currently underway to quantify the amount of water that can be translocated through CMNs. Even if this flux is insufficient to influence the water balance of receiver plants, other benefits of this process may include preventing the senescence of mycorrhizae in receiver plants during severe drought. Receiver plants could also derive multiple benefits from maintaining the mycorrhizal status and connection to CMNs during drought, such as enhanced nutrient uptake and/or access to resources from other plants (Simard *et al*., 1997; Leake *et al*., 2004), protection against pathogens (Marx, 1969), or a rapid recovery from episodes of severe water stress (Augé, 2001).

Acknowledgements

This work was supported by the National Science Foundation (DEB 9981548, MFA), and a MEC/Fulbright postdoctoral fellowship (JIQ). We are grateful to Roberto Lepe, Angela López, Antonio Celis, Van Stout, Elías Serna, and Mike Kienitz for help in the field, greenhouse, and laboratory.

References

Agerer R. 2001. Exploration types of ectomycorrhizae. A proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. *Mycorrhiza* **11,** 107–114.

Augé RM. 2001. Water relations, drought and vesicular–arbuscular mycorrhizal symbiosis. *Mycorrhiza* **11,** 3–42.

Brownlee C, Duddridge JA, Malibari A, Read DJ. 1983. The structure and function of mycelial systems of ectomycorrhizal roots with special reference to their role in forming inter-plant connections and providing pathways for assimilate and water transport. *Plant and Soil* **71,** 433–443.

Caldwell MM, Richards JH. 1989. Hydraulic lift: water efflux from upper roots improves effectiveness of water supply by deep roots. *Oecologia* **79,** 1–5.

Dawson TE. 1996. Determining water use by trees and forests from isotopic, energy balance and transpiration analyses: the roles of tree size and hydraulic lift. *Tree Physiology* **16,** 263–272.

Duddridge JA, Malibari A, Read DJ. 1980. Structure and function of mycorrhizal rhizomorphs with special reference to their role in water transport. *Nature* **287,** 834–836.

Ehleringer J, Osmond CB. 1989. Stable isotopes. In: Pearcy RW, Ehleringer J, Mooney HA, Rundel PW, eds. *Plant physiological ecology. Field methods and instrumentation*. New York: Chapman and Hall, 281–290.

Gardes M, Bruns TD. 1996. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. *Canadian Journal of Botany* **74,** 1572–1583.

Giovanetti M, Sbrana C, Avio L, Strani P. 2004. Patterns of belowground plant interconnections established by means of arbuscular mycorrhizal networks. *New Phytologist* **164,** 175–181.

Hart MM, Reader RJ. 2002. Host plant benefit from association with arbuscular mycorrhizal fungi: variation due to differences in size of mycelium. *Biology and Fertility of Soils* **36,** 357–366.

He X, Critchley C, Hock N, Bledsoe C. 2004. Reciprocal N ($^{15}NH_4^+$ or $^{15}NO_3^-$) transfer between non N_2-fixing *Eucalyptus maculata* and N_2-fixing *Casuarina cunninghamiana* linked by the ectomycorrhizal fungus *Pisolithus* sp. *New Phytologist* **163,** 629–640.

Helliker BR, Roden J, Cook C, Ehleringer JR. 2002. A rapid and precise method for sampling and determining the oxygen isotope ratio of atmospheric water vapor. *Rapid Communications in Mass Spectrometry* **16,** 929–932.

Horton JL, Hart SC. 2004. Hydraulic lift: a potentially important ecosystem process. *Trends in Ecology Evolution* **13,** 232–235.

Jackson RB, Sperry JS, Dawson TE. 2000. Root water uptake and transport: using physiological processes in global predictions. *Trends in Plant Science* **5,** 482–488.

Koske RE, Gemma JN. 1989. A modified procedure for staining roots to detect VA mycorrhizas. *Mycological Research* **92,** 486–505.

Kuhn AJ, Schröder WH, Bauch J. 2000. The kinetics of calcium and magnesium entry into mycorrhizal spruce roots. *Planta* **210,** 488–496.

Leake JR, Johnson D, Donnelly DP, Muckle GE, Boddy L, Read DJ. 2004. Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. *Canadian Journal of Botany* **82,** 1016–1045.

Marx DH. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* **59,** 153–163.

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA. 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **115,** 495–501.

Millikin-Ishikawa K, Bledsoe CS. 1999. Seasonal and diurnal patterns of soil water potential in the rhizosphere of blue oaks: evidence for hydraulic lift. *Oecologia* **125,** 459–465.

Parladé J, Pera J, Alvarez IF. 1996. Inoculation of containerized *Pseudotsuga menziesii* and *Pinus pinaster* seedlings with spores of five species of ectomycorrhizal fungi. *Mycorrhiza* **6,** 237–245.

Perry DA, Amaranthus MP, Borchers S, Brainerd R. 1989. Bootstrapping in ecosystems. *BioScience* **39,** 230–237.

Querejeta JI, Egerton-Warburton LM, Allen MF. 2003. Direct nocturnal water transfer from oaks to their mycorrhizal symbionts during severe soil drying. *Oecologia* **134,** 55–64.

Richards JH, Caldwell MM. 1987. Hydraulic lift: substantial nocturnal water transport between soil layers by *Artemisia tridentata* roots. *Oecologia* **73,** 486–489.

Ruiz-Lozano JM, Azcón R. 1995. Hyphal contribution to water uptake in mycorrhizal plants as affected by the fungal species and water status. *Physiologia Plantarum* **95,** 472–478.

Simard SW, Perry DA, Jones MD, Myrolds DD, Durall DM, Molina R. 1997. Net transfer of carbon between ectomycorrhizal tree species in the field. *Nature* **388,** 579–582.

Sun YP, Unestam T, Lucas SD, Johanson KJ, Kenne L, Finley R. 1999. Exudation-reabsorption in a mycorrhizal fungus, the dynamic interface for interaction with soil and soil microorganisms. *Mycorrhiza* **9,** 137–144.

Tennant D. 1975. A test of a modified line intersect method of estimating root length. *Journal of Ecology* **63,** 995–1001.

Titus JH, Tsuyuzaki S. 2002. Arbuscular mycorrhizal distribution in relation to microsites on recent volcanic substrates of Mt. Koma, Hokkaido, Japan. *Mycorrhiza* **12,** 271–275.