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Hydraulic redistribution of water from *Pinus ponderosa* trees to seedlings: evidence for an ectomycorrhizal pathway

Jeffrey M. Warren[1,2], J. Renée Brooks[3], Frederick C. Meinzer[1] and Joyce L. Eberhart[4]

[1]USDA Forest Service, Pacific Northwest Research Station, Corvallis, OR 97331, USA; [2]Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA; [3]Western Ecology Division, US Environmental Protection Agency/National Health and Environmental Effects Research Laboratory, Corvallis, OR 97333, USA; [4]Department of Forest Science, Oregon State University, Corvallis, OR 97331, USA

Author for correspondence:
Jeffrey M. Warren
Tel: +1 (865) 576 3918
Fax: +1 (865) 574 0133
Email: warrenjm@ornl.gov

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Summary

• While there is strong evidence for hydraulic redistribution (HR) of soil water by trees, it is not known if common mycorrhizal networks (CMN) can facilitate HR from mature trees to seedlings under field conditions.

• Ponderosa pine (*Pinus ponderosa*) seedlings were planted into root-excluding 61-μm mesh barrier chambers buried in an old-growth pine forest. After 2 yr, several mature trees were cut and water enriched in D_2O and acid fuchsin dye was applied to the stumps.

• Fine roots and mycorrhizal root tips of source trees became heavily dyed, indicating reverse sap flow in root xylem transported water from stems throughout root systems to the root hyphal mantle that interfaces with CMN. Within 3 d, D_2O was found in mesh-chamber seedling foliage > 1 m from source trees; after 3 wk, eight of 10 mesh-chamber seedling stem samples were significantly enriched above background levels. Average mesh-chamber enrichment was 1.8× greater than that for two seedlings for which the connections to CMN were broken by trenching before D_2O application.

• Even small amounts of water provided to mycorrhizas by HR may maintain hyphal viability and facilitate nutrient uptake under drying conditions, which may provide an advantage to seedlings hydraulically linked by CMN to large trees.

Key words: common mycorrhizal network (CMN), ectomycorrhizal fungi, hydraulic lift, ponderosa pine (*Pinus ponderosa*), water transport.

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Introduction

Hydraulic redistribution of water (HR) in which roots passively transfer water from moist to drier regions of the soil profile along a water potential (Ψ) gradient has been reported to occur in more than 50 woody and herbaceous species (Caldwell *et al.*, 1998; Jackson *et al.*, 2000; Espeleta *et al.*, 2004; Meinzer *et al.*, 2004). While recent laboratory studies illustrate the potential for HR between plants through a common mycorrhizal network (CMN) (Querejeta *et al.*, 2003; Egerton-Warburton *et al.*, 2007; Plamboeck *et al.*, 2007), the role of mycorrhizal fungal symbionts in facilitating HR under natural conditions has not been documented previously.

The transport and efflux of HR water by root systems is supported by multiple, independent lines of evidence including diel (usually nocturnal) localized increases in soil water content (θ) and concurrent recovery of soil water potential (Ψ_{soil}) (Richards & Caldwell, 1987; Brooks *et al.*, 2002; Warren *et al.*, 2007), reversal of xylem sap flow in roots away from the main stem (Burgess *et al.*, 2000; Brooks *et al.*, 2006), and studies that quantify and track source water movement within

the soil–plant system based on natural or enriched abundances of stable isotopes (Dawson, 1993; Brooks *et al*., 2006). Although the occurrence of HR suggests water efflux from roots, little is known about the importance of root water transport directly into the CMN that are prevalent in many terrestrial plant ecosystems. Recent laboratory studies using mesocosms have shown that deuterated water and fluorescent dye can move via HR from donor roots through mycorrhizal fungi into the soil (Querejeta *et al*., 2003) and from donor roots, through a CMN, into interconnected receiver plants through both ectomycorrhizal (EM) and arbuscular mycorrhizal (AM) hyphal linkages (Egerton-Warburton *et al*., 2007). However, evidence of passive water transport between plants via CMN in a field setting has not been demonstrated previously. Fungal transport of water between plants has implications across trophic levels, impacting soil–plant water relations under drought conditions, and interacting with or enabling concurrent transfer of various solutes including carbon, nutrients (Jennings, 1987; Cairney, 1992) and potentially regulatory compounds (Ebel *et al*., 1996; Karabaghli-Degron *et al*., 1998) or signals (Meinzer, 2002).

Many studies have shown a role of mycorrhizal fungi in facilitating soil water uptake and transport (Read & Boyd, 1986; Augé, 2001). Some studies have illustrated direct water transport through hyphal strands in both AM (Faber *et al*., 1991; Ruiz-Lozano & Azcón, 1995) and EM fungi (Brownlee *et al*., 1983), but other studies have not found evidence for fungal water transport (George *et al*., 1992). A recent laboratory study has demonstrated water transport through hyphal structures of some, but not other EM species associated with a CMN shared by several different tree species, illustrating the complexity of potential transport pathways (Plamboeck *et al*., 2007). Fungal water transport may be particularly prevalent for species that form vessel-like rhizomorphic hyphal structures, which can lose septations (cross-walls) between individual cells and potentially transport water at rates equivalent to that of root xylem (Duddridge *et al*., 1980).

Water transported via mass flow through vessel hyphae or symplastic/apoplastic flow through smaller septate hyphae is regulated by Ψ gradients in the system. Plant transpiration induces a large Ψ gradient between soil and foliage that drives soil/fungal/root water towards foliage during the day. However, when transpiration is limited by stomatal closure or low vapor pressure deficit, hydraulically redistributed water can be driven out of the roots and into the surface soil or into the network of hyphal and rhizomorphic structures that constitute the CMN. There is some evidence of hyphal water loss into soil driven by Ψ gradients (Querejeta *et al*., 2003) and evidence of hyphal exudates of carbon and ions (Unestam & Sun, 1995; Sun *et al*., 1999), which suggests concurrent water loss. Source–sink relationships of trees and fungi have been shown to drive phosphorus, nitrogen and carbon fluxes between trees through hyphae of the CMN (Simard *et al*., 1997, 2002), suggesting concurrent water transport, but evidence for direct fungal water transport between trees has been limited to the laboratory setting (Egerton-Warburton *et al*., 2007).

This study was designed to determine whether native ectomycorrhizal fungi of ponderosa pine could transfer water from large trees to seedlings under field conditions. Seedlings were planted in mesh chambers that excluded roots and mass flow of water, but allowed passage of fungal hyphae and rhizomorphs. Isotopically labeled water was applied to cut stumps of large trees nearby to create a Ψ gradient that could drive HR towards the seedlings. We hypothesized that subsequent detection of the isotope in mesh-chamber seedlings would provide substantial evidence for water transport between trees and seedlings via mycorrhizae. We also measured soil and plant water dynamics, and monitored the isotopic signal to determine transport throughout the broader environment. We hypothesized significant spatial variation in response to the treatments based on our past work at the site describing variability in HR partially attributed to root distribution patterns.

Materials and Methods

Site description

The field site was an old-growth ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) stand located within the Metolius Research Natural Area within the Deschutes National Forest in central Oregon, USA (44°30′ N, 121°37′ W) at an elevation of 915 m. The stand consisted primarily of ponderosa pine, and contained 250–300-yr-old dominant trees, with groups of suppressed trees ranging from 50 to ≥ 100 yr old (Ryan *et al*., 2000; Law *et al*., 2001) and small groups of pine regeneration < 20 cm tall. Other woody components included bitterbrush (*Purshia tridentata* (Pursh) DC), Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), grand fir (*Abies grandis* (Dougl. ex D. Don) Lindl.) and manzanita (*Arctostaphylos* sp.).

The soil was a loamy sand with particle size distribution approx. 77% sand, 19% silt and 4% clay in the upper 40 cm (Warren *et al*., 2005). Pine root surface area in the upper 1 m declines with depth, with 60% of measured surface area occurring in the upper 40 cm (Warren *et al*., 2005). Mean annual precipitation is 550 mm, with a prolonged seasonal drought between late spring and early to late autumn when < 10% of annual precipitation occurs. Past work at this site has quantified HR and its importance for maintaining tree root hydraulic conductivity in the upper soil (Brooks *et al*., 2002; Domec *et al*., 2004; Meinzer *et al*., 2004; Warren *et al*., 2007).

Chamber design

To separate potential water transport by fungi from soil or root water transport, it was necessary to isolate pine seedlings in mesh chambers that excluded ingrowth or outgrowth of pine roots, but allowed unimpeded passage of associated

mycorrhizal hyphae and rhizomorphs. Cylindrical chambers were constructed using sections of PVC pipe (20.7 cm inside diameter, 21.8 cm outside diameter, 43 cm height). A 5.4-cm hole-saw was used to cut 55 holes into each chamber, which removed approx. 50% of the wall surface area. Multiple layers of fine (61-μm aperture) and/or coarse (2.46-mm aperture) stainless steel mesh screen (TWP Inc., Berkeley, CA, USA) were wrapped around the outside of each chamber and secured with hot glue (Bostik, dual-temperature), silicone sealant (nonfungicidal; General Electric, Waterford, NY, USA) and metal straps. A similar mesh screen bottom was also secured. For the no-mesh chambers, only a single layer of the coarse screen was used, which gave seedlings full exposure to the bulk soil, external roots and larger soil organisms. For the mesh chambers, four layers of screen were used, ordered from the chamber wall as fine, coarse, fine, coarse. The inner coarse screen provided an approx. 2-mm-wide air gap between the two 61-μm screens to prevent mass flow of soil water, while the outer coarse screen acted as a protective covering for the more delicate fine screen (Fig. S1 in Supplementary material). Ectomycorrhizal hyphae and many rhizomorphs are smaller than 61 μm and thus should readily penetrate the mesh chambers. In contrast, even the smallest woody roots are larger than 61 μm; for example, 95–100% of the finest woody roots of 11 temperate tree species are > 100 μm (Withington *et al.*, 2006). Extensive analysis of minirhizotron images from this old-growth ponderosa pine site has described minimum root diameters > 0.5 mm, and all roots found across different-aged pine stands nearby were > 0.2 mm, with the exception of a single 100-μm root, which was probably not a pine root (Andersen *et al.*, in press; C. Andersen, pers. comm.). Thus roots of seedlings planted within mesh chambers would be limited to the chamber, and external roots from surrounding trees would be excluded from the chamber. Final chamber volume was approx. 13 l.

Study design

The primary goal of the study was to test if D_2O added to cut tree stumps could be detected subsequently in seedlings enclosed by root-excluding mesh that were accessible only through hyphae and possibly vapor transport. As this technique was new, and had not been carried out previously in a field setting, it was uncertain if the label would be transported from stump to roots, then into the broader environment. Thus three no-mesh chambers were included to verify if the label was even being released from the cut tree-root systems. The mesh chamber design restricts liquid water flux from soil into the chamber, but does not address potential vapor water flux, which may confound evidence of hyphal water transport. To test potential vapor flux into the chambers, hyphal connections to the CMN were broken in two of the 12 replicate mesh chambers by trenching (trenched chambers) 5 d before D_2O application (see Isotope application). A narrow drain spade was inserted concentrically around the chamber, then the chamber was pried upward, breaking all connections to the bulk soil. These chambers were subsequently lifted and turned, then replaced in the soil. While it was useful to test for vapor flux in this manner, we wanted to retain most mesh chambers undisturbed to maximize the potential to detect any D_2O signal in any mesh-chamber seedling. Thus only two chambers were selected for trenching, which resulted in an unbalanced design that limits the statistical significance of results with regard to vapor phase movement.

Chamber and seedling installation

In mid-September 2003, 15 holes were excavated at the field site within 2 m of seven 65 to ≥ 100-yr-old ponderosa pine trees, ranging in size from 6 to 17 cm diameter at 1.4 m. There were four additional mature trees located 2–6 m from the excavated holes. Twelve mesh-barrier and three no-mesh chambers were randomly installed into the holes, extending 1 cm above the soil surface. Soil removed from the holes was transported to Oregon State University, where it was steam pasteurized to reduce viable fungal inoculum. Pasteurized soil was placed into and around the chambers, and two ponderosa pine seedlings were planted in each chamber. The pine seedlings were 2-yr-old, bare-root nursery stock from a 1066-m-elevation seed source on the nearby Warm Springs Reservation. The seedlings were maintained for 2 yr, during which time it was expected that native EM fungi at the site would grow through the mesh screen layers and into the chamber, colonizing seedling roots and potentially displacing any pre-existing EM fungi.

Soil and plant water potential

Ψ_{soil} was measured using thermocouple psychrometers (PST-55, Wescor, Logan, UT, USA) installed at depths of 30 and 60 cm. Before installation, the psychrometers were calibrated individually in the laboratory against salt solutions of known osmolality (Brown & Bartos, 1982). A soil auger was used to excavate 60-cm-deep holes, psychrometers were placed into the intact soil profile in the side of the hole at each depth, then holes were repacked with the excavated soil by layer. Sensors were positioned at each depth at four locations along a transect through the study area within 1 m of source trees. In addition, a psychrometer was inserted vertically into a 30-cm-deep hole produced with a tile probe within two of the mesh chambers. Water potentials were measured every 30 min with a 30-s cooling time to accommodate the Peltier effect, and data were recorded using a datalogger (CR-7, Campbell Scientific, Logan, UT, USA). Reflective insulation was secured around exposed psychrometer cables and the datalogger to minimize confounding temperature gradients. Ψ_{leaf} was measured on foliar samples collected before dawn from chamber seedlings or surrounding large trees before, during and after tree cutting and D_2O application. Ψ_{leaf} was determined for individual pine

fascicles using a Scholander-type pressure chamber (PMS, Corvallis, OR, USA).

Soil water content

Soil volumetric water content (θ) was quantified using multisensor, frequency domain capacitance probes that contained up to eight annular capacitance sensors (EnviroSCAN, Sentek Pty Ltd, Adelaide, Australia) capable of quantifying minute changes in θ (±0.003%). Each probe was installed vertically into a 5.65-cm-diameter PVC access tube within 1 m of source trees, with sensors spaced at 20, 30, 40, 50 and 60 cm deep ($n = 4$) (Brooks *et al.*, 2002). Capacitance sensors were frequency normalized by calibration against air and water in the laboratory for precision, and a custom field-based calibration was used to improve accuracy (Morgan *et al.*, 1999; Warren *et al.*, 2005). θ was measured during the study period every 30–60 min and recorded using a datalogger (model RT6, Sentek Pty Ltd).

Isotope application

On 25 August 2005, five trees ranging in diameter from 10 to 17 cm in the center of the study area were cut and removed, and D_2O containing acid fuchsin dye was applied to the stumps. The trees were prepared by complete removal of bark and phloem tissue between approx. 0.25–1.5 m from the base of the trees using a two-handled wood shaver. Each tree was cut at approx. 1.5 m height, then an approx. 0.75-m-long, tight-fitting rubber sleeve (cut from vehicle tire inner tubes) was slid over the exposed xylem tissue and secured near the base using closed cell foam 'weather stripping' and stainless steel hose clamps. Next, the rubber sleeve was briefly compressed down to the clamps, the tree was cut again just above the compressed sleeve, then the sleeve was pulled up and water was quickly (< 20 s) applied into the sleeve, creating a reservoir (approx. 2–4 l) secured above the cut stump with stakes (Fig. S1). Paper towel was taped below the sleeve to absorb any water leaked from the system. Over the next 24 h, 20 l water enriched with 1.9 l 99.9% deuterium oxide and containing 2 l 1% (w/v) acid fuchsin dye (filtered to 0.22 μm) was applied to the reservoir systems incrementally as the water was absorbed (total 2–6 l per tree). The bright red dye was used to identify quickly progression of the isotopic signal into the root system, and also provided a highly visible sign of potential leaks. Strict D_2O application and sampling protocols were used to ensure the deuterium label did not contaminate the site. Over the next 18 d, an additional 106 l nonlabeled 'chase' water was applied to the system to facilitate isotope transport from roots into the surrounding environment. Water application to the smallest tree was suspended after only 2 l had been applied when a small leak was detected (but absorbed by the paper towel); while the remaining larger ≥ 90-yr-old trees continued to receive the chase water (25–35 l applied to each tree). Quantity of label and water application to individual trees was determined indirectly by their uptake strength; water was added to maintain the cut surface under water to prevent loss of conductivity by embolism and resin occlusion. Tree stumps initially absorbed up to 3 l d^{-1} applied water after cutting, declining to < 1 l d^{-1} by 18 d of application.

Isotope analysis

To assess potential transport of deuterium by mycorrhizae into the seedlings and into the broader environment, plant and soil water samples were collected for δD and $\delta^{18}O$ analysis both before and after D_2O application to the cut trees. One day before trees were cut and the label introduced, we sampled plants and soils to establish the background isotopic signature of the environment. We sampled a bitterbrush stem ($n = 1$), large ponderosa pine tree stem and foliage ($n = 6$), chamber seedling foliage ($n = 4$) and soil from one soil profile (10, 20, 30, 50, 100 cm).

Once the label was introduced, we sampled soils at 10 cm ($n = 2$) within 7 m of source trees, foliage and/or suberized stem tissue from naturally established understory plants, and foliage from all chambered seedlings at days 3 (six chambers), 5 (no chambers), 8 and 15 postcutting to identify timing of label transport. Natural plant samples were primarily mature bitterbrush and small groups of *P. ponderosa* seedlings <15 cm high ($n = 1, 7, 4, 7$, respectively). Foliage sample sizes from the chambered seedlings were minimal (one to two needles) to prevent potential reduction in the transpirational driving force that could affect root and hyphal water transport. In addition, lower canopy foliage (2–5 m high) from the six large residual trees 0.5–5 m from source trees was sampled on days 3, 5, 8, 11 and 15 postcutting ($n = 3$–6).

After 21 d, we ended the experiment by again extensively sampling soils, chambered seedlings and naturally established plants in the general vicinity. Eighteen natural plant samples and 12 soil samples at 10 cm depth were collected at random locations within 13 m of source trees. Each chambered seedling was harvested, and the entire suberized stem tissue was collected for a much larger, more robust and integrative water sample to determine the presence of label within the seedlings. Samples were collected in glass vials with Polyseal cone inserts in the cap and sealed with Parafilm to prevent evaporation.

Water was extracted from the plant and soil samples using cryogenic vacuum distillation (Ehleringer & Osmond, 1989; Dawson, 1993). Water samples were analysed for δD and $\delta^{18}O$ on an isotope ratio mass spectrometer (Delta plus, Finnigan, Bremen, Germany) interfaced with a high-temperature conversion/elemental analyser (TC/EA ThermoQuest, Finnigan) located at the Integrated Stable Isotope Research Facility at the Western Ecology Division of the Environmental Protection Agency, Corvallis, OR, USA. All δD and $\delta^{18}O$ values are expressed relative to Vienna-standard mean ocean water (V-SMOW) in ‰.

$$\delta D \quad \text{or} \quad \delta^{18}O = \left(\frac{R_{sample}}{R_{standard}} - 1 \right) 1000 \qquad \text{Eqn 1}$$

where R is the ratio of deuterium to hydrogen atoms or ^{18}O to ^{16}O atoms of the sample and the standard V-SMOW. Measurement precision was 2‰ for δD and 0.3‰ for $\delta^{18}O$, as determined from replicates and standards run with the study samples.

Measurements of $\delta^{18}O$ were used so that δD values enriched from the tracer could be distinguished from δD values enriched from evaporation. Both δD and $\delta^{18}O$ of water become enriched through evaporation, whereas only δD increases with additions of D_2O. The pretreatment natural abundance variation in δD and $\delta^{18}O$ of water from various plant and soil samples collected before isotope application were related linearly along a local evaporation line ($\delta D = -80.8 + 2.68\ \delta^{18}O$, $R^2_{adj} = 0.97$, $n = 16$). Subsequent samples were determined to be enriched if they fell above the 99% prediction interval of this evaporation range, approx. mean + 3 SD.

Chamber root and mycorrhiza analysis

After seedling stems were harvested for isotopic analysis, chambers were removed from the soil and inspected for damage and the presence and penetration of roots, hyphae and rhizomorphs (Fig. S1). Seedling root systems were removed from the chambers, placed in plastic bags with a moist paper towel, and kept at 5°C until they were analysed for ectomycorrhizas (EM). Root tips were inspected under a stereomicroscope to separate different EM morphotypes using the characteristics described by Goodman *et al.* (1996). Characterization of EM is labor-intensive, therefore seedlings from only six chambers were examined for EM. Three root samples were also collected from the upper 20 cm in the bulk soil outside chambers to assess source root characteristics and to note some of the dominant EM types present at the site. Morphotypes were described briefly and photographed, and representative root tips were placed in 1.5-ml microcentrifuge tubes containing 300 µl CTAB buffer and stored at 5°C for later detailed morphological work or DNA extraction.

For the molecular analyses, DNA was extracted from replicate samples of EM root tips from each morphotype per seedling using a modification of Gardes & Bruns (1993), by purifying the DNA using the GENECLEAN extraction kit (Qbiogene, Irvine, CA, USA) following removal of the aqueous phase after the chloroform step. The internal transcribed spacer (ITS) region, located between the nuclear small and nuclear large rDNA, was amplified using PCR with the standard fungal specific primers ITS-1F and ITS-4 (Gardes & Bruns, 1993). Cycling conditions included an initial denaturation at 94°C for 30 s followed by 35 PCR cycles (93°C, 35 s; 55°C, 53 s; 72°C, 30 + 05 s per cycle). Restriction digests were performed on the amplified PCR products with the enzymes *Hin*f1, *Dpn*II and *Hae*III (Promega, Madison, WI, USA), followed by separation on agarose electrophoresis gels stained with ethidium bromide. The resulting RFLP patterns allowed us to assess the accuracy of morphotyping, compare samples with one another, and identify appropriate samples for sequencing (Horton & Bruns, 2001). Sequencing was performed by the Center for Genome Research and Biocomputing Core Laboratory at Oregon State University using an ABI Prism 3730 genetic analyser (Applied Biosystems, Foster City, CA, USA). Successful DNA sequences were identified to genus or species level by querying the GenBank database using the nucleotide–nucleotide (blastn) BLAST search option on the National Center for Biotechnology Information website (Altschul *et al.*, 1997). Sequence similarities ≥ 98% were considered to be a match with our unknown EM root tips.

Statistical design and analyses

To address our primary goal, ANOVA and *post hoc* tests were used to test if mesh-chamber seedling foliage or stem tissue was significantly enriched above pretreatment background samples (SAS ver. 9.1, SAS Institute, Cary, NC, USA). In addition, differences between the mesh, no-mesh and trenched treatments were tested with ANOVA with the assumption of homogeneity of variances among the treatments. Tests comparing mesh or trenched to background samples do not include the no-mesh treatment (which had a confounding outlier) and are identifiable in the results by reduced degrees of freedom (df; 27 instead of 30). Differences between mesh and trenched chambers were also examined using *t*-tests, which can test for equality of variance and can be applied to groups of equal or unequal variance. In order to describe the progression of label movement into the chambers, a repeated-measures mixed model was used to test for an interaction between treatment and timing of D_2O detection. It should be noted that all statistical tests that included no-mesh or trenched seedlings had low replication ($n = 3$ or $n = 2$) in comparison with mesh seedlings ($n = 10$) or background values ($n = 16$), which limited the rigor of tests for equality of variance between comparable groups and the statistical power of those analyses.

Results

Fine roots and mycorrhizal root tips of the source trees were heavily dyed with the acid fuchsin dye, indicating that reverse sap flow in root xylem transported water from the stem throughout the root systems to the root hyphal mantle that interfaces with the CMN. No roots were observed passing into any of the mesh chambers, however some roots approached chambers and grew along the outer mesh surface. In contrast, there was an abundance of hyphal strands, rhizomorphs and tissue-like hyphal structures readily visible on the surface of the chambers, between mesh layers, and inside the chambers

Table 1 Dominant ectomycorrhizal (EM) fungal species present on root tips of six mesh-chamber seedlings, including morphotypic description of fungal mantle, extraradical hyphae and rhizomorphs, results of DNA amplification and sequencing, and reference to Fig. S2 (Supplementary material) of specific EM root tips

RFLP type	PCR reps	Fig. S2	Seedling number	Morphotype description	GenBank accession number	Sequence identification
		b	11	Very fuzzy, white emanating hyphae and rhizomorphs – acid fuchsin tracer present		
			11	Dense, peg-like clusters, gelatinized tips – acid fuchsin present		
A	2		11	Truffle primordium, red-brown matted emanating hyphae with gelatinized rhizomorphs	EF458015	*Rhizopogon ochraceorubens* 99% match
B	5		2, 5, 8	Smooth, thin type, long tips, few emanating hyphae and no rhizomorphs		
C	2	f	2	Copious white to pinkish rhizomorphs with crystals	EF458016	*Rhizopogon vulgaris* 99% match
D	2	e	11	White, smooth clusters with white rhizomorphs	EF458014	Uncultured EM clone *Lactarius* sp. 98% match
E	4	c	2, 8	White turning violet, blue gelatinized rhizomorphs	EF458011	*Rhizopogon* aff. *salebrosus* 99% match
F	6	d	5, 8, 12	Fuzzy, white clusters with pinkish extraradical hyphae and rhizomorphs	EF458012	Uncultured EM isolate Suillaceae 99% match
G	5		10	Variable		
H	1		5	Smooth, thin	EF458013	*Wilcoxina* (99% match)
J	2		12	Translucent, loosely clustered tips	EU442595	Cortinariaceae

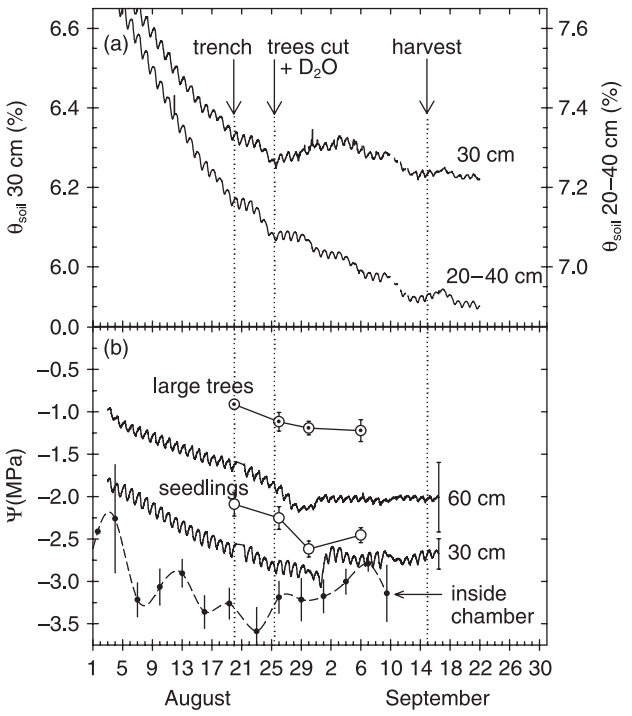

Fig. 1 Soil and plant water dynamics during the study period showing the impact of manipulations on (a) soil water content (θ) at one specific location (30 cm, probe 2) or throughout the upper soil (20–40 cm, $n = 16$, mean SE = 0.46); (b) soil water potential (Ψ) outside chambers at 30 or 60 cm ($n = 3$–4) or inside chambers (approx. 30 cm; $n = 2$; 3-d mean values) in relation to predawn foliar Ψ for large trees ($n = 3$–5) or chamber seedlings ($n = 15$), ±1 SE.

(Fig. S1). Using a hand lens, hyphae and rhizomorphs could easily be seen penetrating unimpeded through the mesh screen. Some rhizomorphs were about the same size as the mesh opening, but there was no evidence that they were constricted as they passed through the mesh layers. Microscopic inspection of root tips from mesh chambers during morphotyping revealed visible evidence of acid fuchsin dye at the hyphal mantle in two of the four EM types present on roots of one seedling (no. 11; Table 1), however, no dye was readily visible in other seedlings sampled. Strong dye absorption by the external large root systems, dilution, and the light reddish-brown coloring of seedling roots limited other visual evidence of the dye. There were also two cases where rhizomorphs from the bulk soil appeared to contain acid fuchsin dye, lending support to the potential for direct rhizomorphic water transport from source trees. All the chamber seedlings sampled were associated with some EM types characterized by large, vessel-like rhizomorphs (Table 1). Images of representative root tips and hyphal structures from mesh seedlings are available in Fig. S2.

Hydraulic redistribution of soil water by roots of large trees was evident in the upper soil based on diel oscillations in both θ and Ψ_{soil} (Fig. 1), and reflects the strong influence of the six residual trees and various shrubs within the study area (Brooks *et al.*, 2002; Warren *et al.*, 2005, 2007). Chamber trenching, tree cutting and application of 126 l water to the system each had significant impacts on soil and plant water dynamics, including slowing the seasonal decline in soil moisture content, although effects were spatially variable. Upper soil θ increased at half the sensor locations after large trees were cut and water applied (Fig. 1a), probably caused by a combination of reduced

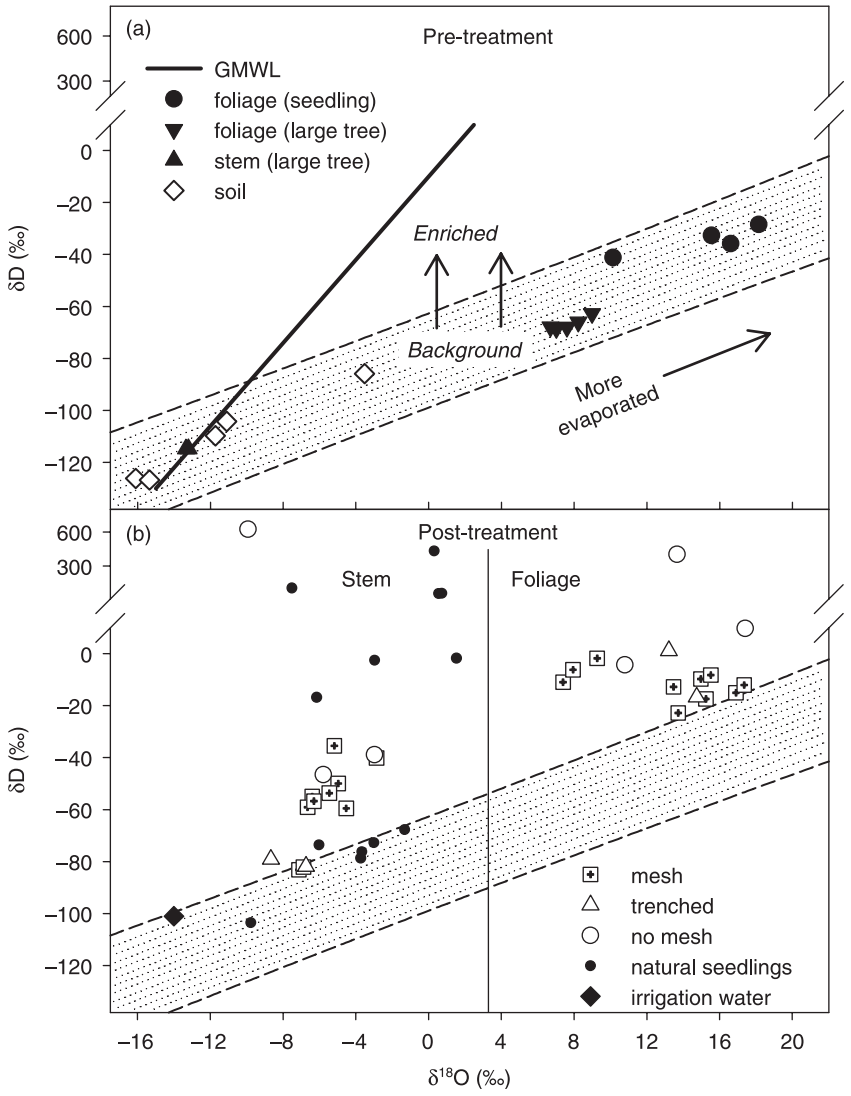

Fig. 2 Stable isotope ratios of water extracted from soil, woody stems and foliage in an old-growth ponderosa pine (*Pinus ponderosa*) ecosystem collected (a) before; (b) after research manipulations. Post-treatment data include chamber seedling stem (left side) and foliar (right side) samples collected 21 d after large tree cutting and application of water highly enriched in deuterium to the cut surfaces of the stumps. Irrigation chase water and natural seedling stem samples collected 8–22 d post-treatment are shown for reference. The local evaporation range (shaded area between dashed lines) is based on the 99% prediction interval (approx. ±3 SD) of the pretreatment regression line ($R^2 = 0.96$; $n = 16$) and represents ambient background values of the two isotopes. As precipitation did not occur during this experiment, isotope values above this range could exist only if water enriched with deuterium were applied to the system. The global meteoric water line represents the isotopic composition of precipitation.

evapotranspiration, increased HR of residual root systems (Brooks *et al.*, 2006), and the input of irrigation water. Ψ_{soil} was lower (more negative) in the upper soil (30 cm) than in deeper soil (60 cm) during the experiment (Fig. 1b). Patterns of soil Ψ inside the chambers (approx. 30 cm) were partially confounded by temperature effects on the insulated, but necessarily exposed psychrometer cables; however, maximum daily Ψ values were available and suggested slightly drier conditions inside the chambers in comparison with the bulk soil. While the psychrometer installation in the chambers was not ideal, the relative temporal values remain useful for following trends in chamber Ψ_{soil}. Tree cutting and addition of water stopped the seasonal decline in Ψ_{soil}, and in some locations Ψ_{soil} increased, including inside the chambers. Similarly, predawn Ψ_{leaf} of seedlings and large trees stopped declining towards the end of the experiment. Seedlings in chambers buried to approx. 40 cm depth displayed Ψ_{leaf} in between that of the bulk soil at 30 and 60 cm depth. Larger trees had access to deeper water sources with Ψ_{soil} closer to zero, as evidenced by higher Ψ_{leaf} than seedlings.

Isotopic analyses of δD and $\delta^{18}O$ were used to test for the presence of HR water from the cut trees and the timing of water transport into mesh-chamber seedlings via mycorrhizae. Pretreatment samples displayed a wide range in both δD and $\delta^{18}O$ (−127 to −28‰ for δD; −16 to 18‰ for $\delta^{18}O$), but the variation was highly correlated between the two isotopes reflecting local evaporation, which influenced both isotopes. We used this relationship to establish the local background evaporation range (Fig. 2a). Deep soil (50 and 100 cm) and stem tissue of deeply rooted shrubs and trees had isotopic signatures that fell at the intersection of the local evaporation line and the global meteoric waterline, indicating that they were not influenced by local evaporation and probably reflected local precipitation inputs. Soil water showed increasing evaporative enrichment with decreasing depth (30, 20, 10 cm). Because of evaporative enrichment from transpiration, foliar

water showed the greatest evaporative enrichment, especially from seedlings, which had shallow roots and depended largely on uptake of water subjected to evaporative enrichment near the soil surface. As there were no precipitation inputs during the experimental labeling, any sample falling above the shaded background area indicated label enrichment, as only elevated levels of deuterium, not ^{18}O, were added to the cut trees. After deuterium was added to the system, foliage samples from all treatments became enriched with the label within 3–15 d post-treatment, and maintained enrichment through day 21 (Fig. 2b). Foliar tissue from the no-mesh-chamber seedlings was enriched significantly above background, mesh and trenched samples (day 21, $P < 0.01$, df = 30), driven by one seedling displaying much higher enrichment that reflects its unimpeded access to the bulk soil and to source tree roots. The mesh and trenched seedling foliar samples were also enriched above pretreatment background levels (day 21, $P < 0.001$, df = 27), but were not different from one another (day 21, $P = 0.87$, *t*-test, df = 10). Foliar samples from the mesh chambers became more enriched above background than trenched samples by day 3 ($P = 0.12$, $F = 4.3$, df = 4), but the trend of higher enrichment in mesh chambers declined throughout the study as the nonlabeled chase water diluted the label within the system (day 8, 15, 21; $P = 0.23$, 0.66, 0.87; df = 11) and mesh δD – trench δD declined from 19 to 8‰.

The magnitude of label enrichment varied across sampling dates, probably because of individual variation in the degree of foliar transpiration, seedling vigor, connectivity to labeled roots, and dilution of the label by chase water. For some seedlings, foliage became more enriched through evaporation, as indicated by an increase in $\delta^{18}O$. However, later in the study, some seedling foliage displayed a reduced evaporative signal, probably caused by seedling uptake of water from a less enriched source – either deep soil water or chase water added to the large tree stumps. Nevertheless, the seedling foliage clearly obtained the labeled water that was applied to the stumps of the large cut trees, which could be used to monitor timing of label transport (see below).

The seedling stem tissue collected 3 wk after the D_2O pulse application was not affected by the confounding influence of evaporative enrichment from transpiration and other processes, or by the variability caused by subsampling of a few needles, and integrated the label enrichment of the entire soil area where the seedlings take up water. Stem tissue from the no-mesh-chamber seedlings was significantly enriched above background, mesh and trenched samples ($P < 0.01$, df = 30), reflecting large amounts of deuterium uptake (Fig. 2b). The mesh seedlings were significantly enriched above background levels ($P < 0.0001$, df = 27), clearly indicating hyphal uptake of hydraulically redistributed water from the cut trees. Trenched seedlings had lower stem enrichment than mesh seedlings ($P < 0.11$, *t*-test, df = 10), but were statistically higher than background values ($P < 0.05$, df = 27). The trenched seedling with high foliar enrichment did show slight stem enrichment, but was < 25% of mean stem enrichment of the enriched mesh seedlings. The two trenched seedlings and two of the mesh seedlings (nos 5 and 10) showed little to no stem δD enrichment with similar values (approx. −82‰ for δD, −6.9‰ for $\delta^{18}O$) (Fig. 2b).

The deuterium label was found in seedlings in two of the mesh chambers within 3 d, then spread progressively further into other planted and naturally occurring seedlings and the broader environment over the next 3 wk (Fig. 3). There was a significant treatment × timing interaction that described a delay in label arrival into the trenched chambers in comparison with the mesh chambers. This interaction was apparent between days 3–15 following D_2O treatment ($P = 0.12$, $F = 2.5$), with the highest significance displayed between sampling days 8 and 15 ($P = 0.044$, $t = 2.23$) when the foliage became clearly enriched in both trenched chambers. There was no correlation between seedling chamber distance from source trees (0.9–1.9 m) and degree of δD enrichment, probably because of chase water effects and rooting distribution of the source trees. Enriched stem tissue from mesh-chamber seedlings had much lower δD values than nearby enriched natural seedlings (Figs 2b, 4). Stem tissue from nonenriched mesh and trenched chambers had δD values very similar to nonenriched natural seedlings (Figs 2b, 4). There was an exponential decline in stem δD enrichment of natural seedlings with distance sampled up to 13 m from source trees (Fig. 4). Evidence of δD enrichment in other natural woody plants and soils also declined with distance, such that no significant deuterium enrichment was found in the environment beyond 6.5 m from source trees. There was no evidence of δD enrichment in foliage of large trees 1.8–6.5 m from the source, probably because of signal dilution in the transpiration stream from their access to abundant deep water sources relative to the amount of HR water they might acquire.

There were two to four dominant EM morphotypes detected per chamber seedling, and at least eight EM morphotypes detected on roots of large trees sampled from the bulk soil. Three of the chamber seedlings had only one dominant type (≥ 75% of the tips), and all these types were in the Suillaceae with well developed rhizomorphs. Rhizomorphic EM types were present on more than 50% of the tips of all chambered seedlings, but only three seedlings had any non-rhizomorphic EM types. There were more EM types present at lower abundances on the roots outside the chambers, with only one type on one root displaying > 25% relative abundance. Of the 26 types described from the three exterior root samples, 12 had no rhizomorphs, including *Cenococcum* sp., which was visibly identified by morphotyping in all three exterior root samples. A total of 29 successful DNA extractions were completed on 14 of the seedling morphotypes. In all but one case, replicate samples taken from a given morphotype on a seedling matched one another, indicating that the morphotyping was able accurately to group EM types

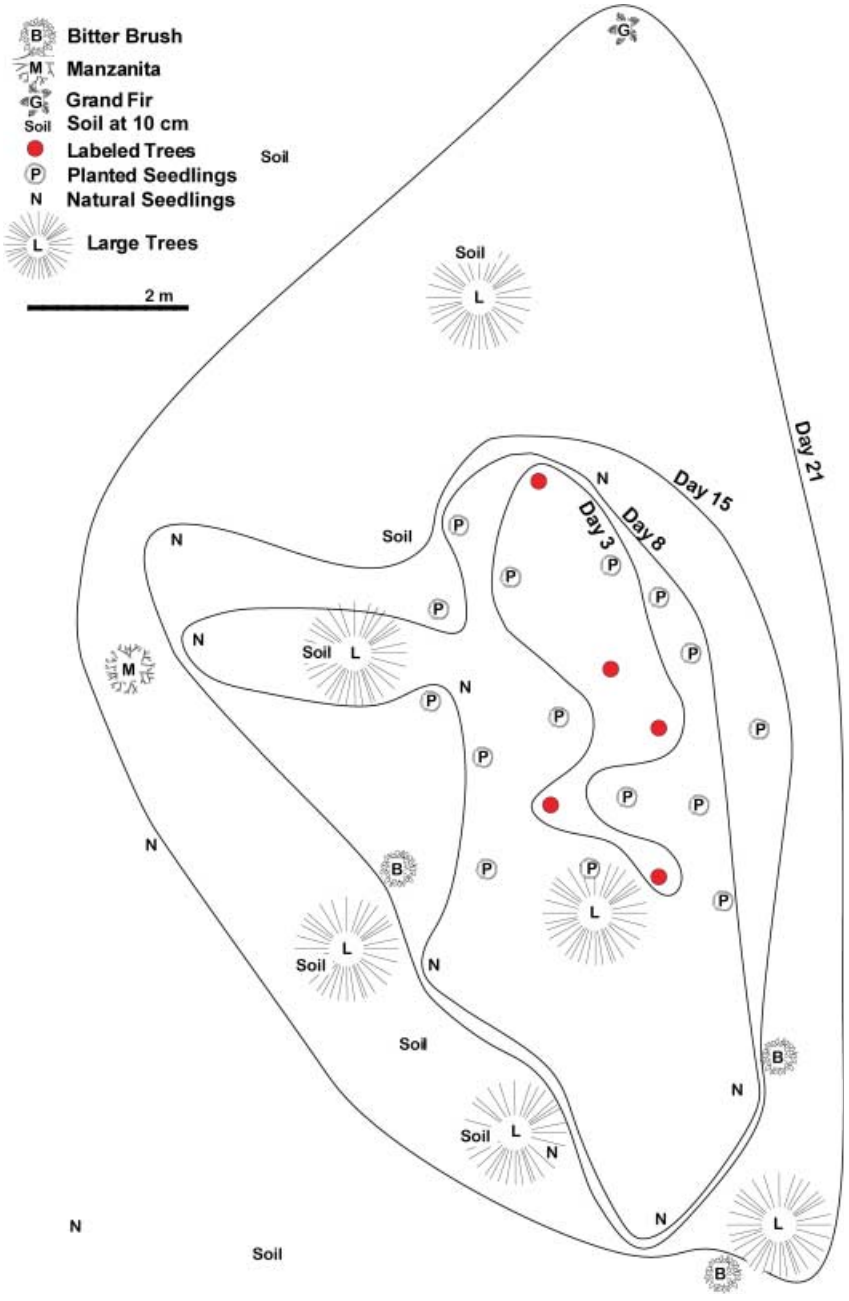

Fig. 3 Progression of lateral spread of the deuterium label applied to cut stems of five labeled ponderosa pine (*Pinus ponderosa*) trees (closed circles) by ectomycorrhizal water transport into mesh-chamber seedlings, and root/hyphal/soil water transport into the broader environment.

potentially involved in fungal HR. On one seedling, four morphotypes were identified but amplification revealed identical types, probably a decomposer fungus that had colonized the seedling rather than the targeted EM fungi. Repeated amplification of another morphotype was not successful. The RFLP analysis identified 10 unique EM types, some of which were successfully sequenced, confirming the presence of three *Rhizopogon* spp., a *Suillus* sp., a *Wilcoxina* sp. and a *Lactarius* sp. (Table 1). Another sequence appeared to be that of a fungus that is not currently in the BLAST search database, but its closest affinities are with species in the Cortinariaceae. While some EM types were unique for individual seedlings, other types were seen on multiple seedlings and on roots from the larger trees.

Discussion

Hydraulic redistribution is known to result in localized increases in upper soil water content, but the pathways of water transfer from deeply rooted plants to other plants and heterotrophic soil organisms that do not have direct access to deep water sources have not been clearly identified. This study

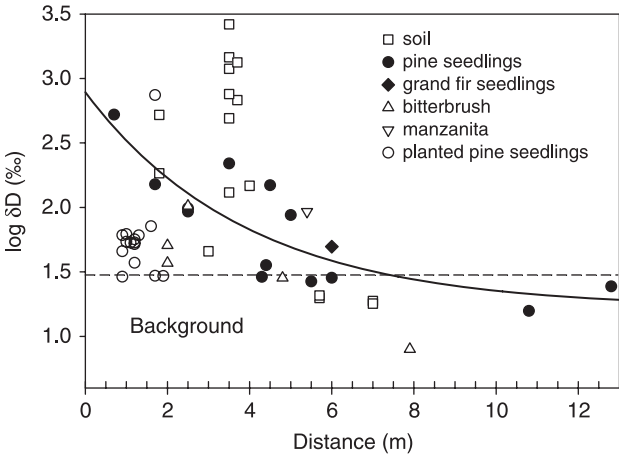

Fig. 4 Relative magnitude and distance from source that deuterium label was detected in upper soil (10–20 cm) or woody stem tissue from various naturally occurring plants and planted chamber seedlings 1–3 wk after isotope application. Samples were considered labeled if values were above the +3 SD background threshold (dashed line). An exponential decay curve was fitted to data from natural seedlings ($R^2 = 0.71$; $n = 14$; $P = 0.001$), which did not have direct access to groundwater.

provides clear evidence that EM fungi associated with shallow-rooted seedlings can readily access HR water from roots of large trees, and possibly facilitate transport directly between tree and seedling through a CMN. The seedlings may thus maintain a direct hydraulic link to source water at depth through EM hyphae, a pathway that may be more efficient for conserving and utilizing hydraulically redistributed water than its release to the soil and reabsorption by shallow roots and/or hyphae. Nevertheless, conclusions concerning direct transfer of water between plants via a CMN must be tempered by the low replication of trenched control chambers in the present study, which were used to control for possible soil water vapor transport to seedlings.

Liquid water flux into the chambers was unlikely because of the approx. 2-mm air-gap barrier built into the chambers, which was necessary to separate hyphal from soil water transport. Under the drought conditions in this study, liquid flux was also limited by low unsaturated soil hydraulic conductivity (K). K declined exponentially with soil drying such that when trees were cut at soil θ approx. 7.1% in the upper 20–40 cm, K would have been $< 10^{-6}$ mm h^{-1} based on the equations of Brooks & Corey (1964) using soil hydraulic parameters derived from soil water-release curves, soil physical characteristics (Campbell, 1985; Warren *et al.*, 2005), and estimates of saturated soil hydraulic conductivity ($K_s = 16$–54 cm h^{-1}; J.M.W., unpublished data). There was probably some vapor water flux into and out of the chambers caused by depth-dependent diel temperature gradients, although site-specific calculations of vapor flux suggest that this component was minimal and limited primarily to vertical transport between soil layers (J.M.W., unpublished data). Significant vapor flux into chambers was also unlikely, as chamber enrichment was not uniform: two mesh-barrier and both trenched chambers lacked stem isotopic enrichment. Vapor flux into the chambers would also involve evaporation and condensation of water, which would result in substantial isotopic depletion of both δD and $\delta^{18}O$ inside the chamber – a pattern we did not detect. As liquid or vapor flux of water into the chambers was unlikely, the significant enrichment of mesh-chamber seedlings above pretreatment values indicated that EM hyphal structures could readily access and transport HR water through the mesh barriers, to be taken up by the seedlings either directly through a CMN or from the soil in the chamber.

Trenching disconnected chamber hyphae from the CMN, thereby preventing direct fungal water transport from trees to seedlings while continuing to permit hyphal water uptake from the bulk soil. The seedling stem tissue of the two trenched chambers showed little evidence of the deuterated water, unlike eight of the 10 mesh chambers, which showed high label accumulation. Lower label enrichment of trenched seedlings was consistent with the loss of a direct link to source water through CMN. In addition, there was acid fuchsin dye visible at the root hyphal mantle in root tips of one of the mesh-chamber seedlings, which could only have been transported directly from source roots via hyphae into the chamber seedling.

Both mesh and trenched chambers displayed foliar label enrichment; however, in trenched chambers enrichment was delayed, possibly attributable to hyphal label uptake from the soil, rather than through a CMN. Hyphal structures protruded from the mesh chamber walls after trenching, which could have continued to provide a pathway for water uptake and transport from the bulk soil following source water release. Actively growing residual hyphae could eventually anastomose with the CMN, thereby re-establishing a direct link to labeled water, although little is known about regrowth of severed EM hyphae.

While the study design was unbalanced (mesh $n = 10$, trenched $n = 2$), the above-mentioned points all support EM hyphal water transport and provide some evidence for direct hyphal HR between trees and seedlings. Nonetheless, future studies should include additional trenched control chambers with total removal of residual hyphae in some chambers (Egerton-Warburton *et al.*, 2007) to minimize a potential confounding effect of hyphal water uptake from the bulk soil.

Several of the dominant EM types described on seedling roots and in the bulk soil were characterized by rhizomorphic structures that theoretically have the capacity to transport significant amounts of water across appreciable distances. Identifying the relative importance of these EM types for water transport was not possible in this study because of sample limitations compounded by high EM species diversity, spatial distribution of roots and CMN, and relative sink strengths in the system, including the strong sinks from the remaining six

noncut large trees connected to the same or different CMN. The root distribution of HR source trees was also probably nonuniform, which could lead to patchiness of label release into the CMN, and thus differences in label proximity to the individual EM colonies and CMN associated with any specific chamber seedling root tip.

To be physiologically relevant, hyphal HR must transport enough water from source roots to elicit impacts on hyphal and/or recipient root water relations. Water transport within central vessel hyphae of *Suillus bovinus* rhizomorphs attached to *Pinus sylvestris* seedlings has been estimated at approx. 0.27 m h^{-1} (Duddridge *et al.*, 1980; Brownlee *et al.*, 1983), or potentially approx. 2.2 m d^{-1} assuming an 8-h period during which the transpirational driving force is near its maximum. This is similar to maximum reported rates of water transport in stem xylem of two temperate conifers, *Tsuga heterophylla* and *Pseudotsuga menziesii* (approx. 2.4–5.4 m d^{-1}) (Meinzer *et al.*, 2006). In this study, the rate of label transport from large trees and into mesh-barrier seedlings (0.13–0.53 m d^{-1}) was lower than maximum rates through rhizomorphs or tree xylem, but similar to pathway-integrated rates of label movement into natural seedlings (0.16–0.63 m d^{-1}) and soils (0.09–0.44 m d^{-1}) located > 2 m from the source trees. Tree cutting and water application certainly would enhance water transport through a CMN if the pathway does exist, but transport would also be moderated by the competing driving forces of residual trees and various pathway resistances. Resistance is related to the fourth power of the conduit radius, such that smaller conduits increasingly limit the distance of potential water transport. As HR generally occurs for < 12 h each day, time as well as conduit resistance would limit the transport distance of hydraulically redistributed water into a CMN, particularly for EM fungal species that lack the large central vessel hyphae of some rhizomorphs. However, fungal conduit diameter did not appear to limit hyphal water transport in this study, as D enrichment developed in most chambers at rates similar to those found in soil and plant samples taken from the wider environment.

While the magnitude of total HR in the upper soil was relatively small at this site (< 0.15 mm d^{-1} or up to 15% of total site water use; Warren *et al.*, 2007), HR water is important for delaying and moderating seasonal loss of root conductivity in drying soil (Domec *et al.*, 2004), which should also prolong resource uptake from EM fungi. Past studies of California oak savanna species have demonstrated that hydraulically redistributed water can move from roots into fungal hyphae following a Ψ gradient under laboratory conditions (Querejeta *et al.*, 2003), which may partially explain lower-than-expected EM hyphal desiccation and turnover rates in drying soil under field conditions (Querejeta *et al.*, 2007). Mycorrhizal HR could help ensure that the belowground carbon investment necessary for plant nutrient and water acquisition remains in viable structures, thereby allowing rapid responses to drought-ending precipitation events, as we have documented previously for this system (Warren *et al.*, 2005). If tree-root water is released directly to EM fungi, then daily hydration of the CMN should facilitate transport of resources to host seedlings, and concurrently enhance resource availability if HR water is also released to the resident rhizosphere saprotrophs associated with nutrient cycling.

While HR directly and/or indirectly benefited hyphae of the CMN and the interconnected seedlings, hydraulically redistributed water was also released into the broader environment, as evidenced by the appearance of label in soils and other plant species. The influence of HR from the ≥ 90-yr-old source trees was detected at distances up to approx. 7 m. The larger ≥ 250-yr-old trees at the site potentially have an even greater area of influence. The HR fluxes into the broader environment could be enhanced following events such as defoliation, wildfire or logging that reduce or limit transpirational sink strength without directly disrupting soil water transport pathways. While HR through root systems of cut trees potentially could function for years in the absence of phloem transport from the aboveground portion of the tree (Bhupinderpal-Singh *et al.*, 2003), it is likely that any role of the CMN in facilitating HR would decline rapidly as source tree carbohydrates became limited and hyphal root tips senesced.

The impact and process of mycorrhizal transfer of C or nutrients between trees in natural ecosystems is dependent on interactions between specific species, source and sink strengths, and prevailing environmental conditions (Simard *et al.*, 2002). Small mycorrhizal fluxes of water from roots and between trees may facilitate C or nutrient transfer, but are more likely to be directly significant for root and associated EM survival in ecosystems that experience annual or seasonal drought, because the water fluxes may be sufficient to prevent embolism-induced catastrophic hydraulic failure. This research investigated how a significant ecological process, hydraulic redistribution of soil water by plant roots, may also be associated with and affect symbiotic fungi and their extraradical hyphae that link plants together through a common mycorrhizal network. Hypha-facilitated transfer of redistributed water from deeply rooted source tree roots through to interconnected seedlings could have important ecological implications, which could be applicable to modeling plant responses to drought stress.

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Images of the field study (a) 61-μm mesh chamber (left) and control chamber; (b) seedlings planted within chambers near source trees; (c) initial source tree cutting and reservoir sleeve attachment (wet paper towel was wrapped over exposed xylem to limit water loss, isotope-labeling pathway is indicated by pink flagging); (d) two source trees after final tree cutting with reservoir system in place; (e–h) 61-μm mesh chambers with abundant hyphal structures visibly penetrating through the mesh layers.

Fig. S2 Examples of dominant ectomycorrhizal (EM) fungal morphotypes on source trees (a) or seedlings (b–f) that received deuterated water via mycorrhizal linkages to source trees. (a) Unidentified *Piloderma*-like EM type with acid fuchsin dye tracer visible at hyphal root tip of source tree; (b) unidentified EM type with acid fuchsin visible on target mesh-chamber seedling; (c) *Rhizopogon* sp.; (d) *Suillus* sp.; (e) *Lactarius* sp.; (f) unidentified EM rhizomorph.

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