Title: Agnostic fungi: plant functional types and tissue stoichiometry explain nutrient transfer in common arbuscular mycorrhizal networks of temperate grasslands

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Abstract:

Plants and mycorrhizal fungi form mutualistic relationships that affect how resources flow between organisms and within ecosystems. Common mycorrhizal networks (CMNs) could facilitate preferential transfer of carbon and limiting nutrients but this remains difficult to predict. Do CMNs favor fungal growth at the expense of plant resource demands (a fungi-centric view), or are they passive channels through which plants regulate resource fluxes (a plant-centric view)? We used stable isotope tracers ($^{13}CO_2$ and $^{15}NH_3$), plant traits, and mycorrhizal DNA to quantify above- and belowground carbon and nitrogen transfer between 18 plant species along a 520-km latitudinal gradient in the Pacific Northwest, USA. Plant functional type and tissue stoichiometry were the most important predictors of interspecific resource transfer. Of "donor" plants, 98% were ^{13}C enriched, but we detected transfer in only 2% of "receiver" plants. However, all donors were ^{15}N enriched and we detected transfer in 81% of receivers. Nitrogen was preferentially transferred to annuals (0.26 ± 0.50 mg N per g leaf mass) compared to perennials (0.13 ± 0.30 mg N per g leaf mass). This corresponded with tissue stoichiometry differences. Our findings point to a simple mechanistic answer for long-standing questions regarding transfer of resources between plants via mycorrhizal networks.

Keywords: arbuscular mycorrhizal fungi, carbon, common mycorrhizal networks, ecological stoichiometry, grasslands, nitrogen, plant functional type

Introduction:

Plant-mycorrhizal associations are thought to have emerged as rudimentary root systems over 400 million years ago, facilitating the expansion of terrestrial life that followed[1]. The transformative power of early fungal symbioses is still evident today in all major plant lineages, from bryophytes to angiosperms [2]. Over 85% of all contemporary flowering plant species form symbioses with fungi, with arbuscular mycorrhizal (AM) associations being the most common [3]. Today, the relationships between plants and AM fungi dominates both managed and unmanaged landscapes and are estimated to be responsible for up to 80% of global primary productivity [2]. Fungi are gregarious and form symbioses with more than one individual plant [4]. By extension, it has been widely hypothesized that the multi-plant-fungal relationships form "common mycorrhizal networks" (CMNs) which facilitate carbon and nutrient transfer between organisms, beyond the immediate plant-fungus mutualism formed by individuals. This relationship is often simplified to the mutual exchange of carbon-based photosynthates for limiting soil nutrients that are more readily bioavailable to fungi [5], but is known to exist along a continuum from parasitic to mutualistic [6–8]. A synthesis of empirical research at macro- and micro-levels of ecological organization reveals a gap in understanding of structural and functional properties that could predict how carbon and nutrients are transferred in CMNs [9].

The literature holds myriad and often complimentary, but sometimes contradictory, hypotheses that could explain the CMN mutualism as a key structural and functional component of ecosystems. For example, the "Wood Wide Web" hypothesis emerged from the analysis of isotopically labeled carbon transferred between plants, presumably through fungal mycorrhizae. Simard et al. [10] concluded that plants that allocate carbon to sustain common fungal symbionts also benefit from shared nutrients, while plants associating with mycorrhizal fungi outside that

network cannot. The “economics” hypothesis [11] proposes that plants and fungi engage in “trades” of nutrients mined by fungi in exchange for plant photosynthates [12–14]. In this hypothesis, the terms of trade between plant and fungi are mediated by supply and demand for limiting resources, which could create a dynamic market emerging from interactions between environmental, biochemical, and biophysical variables. Complementing the economic analogy, the “kinship” hypothesis proposes that phylogenetic distance within or across species promotes functional gradients and preferential flow of resources in CMNs [15], in which transfer of carbon and nutrients are predicted to be more frequent and abundant between related individuals (e.g., seedlings and trees of the same species) than between unrelated individuals [16].

The past two decades have seen extensive but inconclusive research on these hypotheses and how they relate to empirical measurements of CMN structure and function. On the one hand, economic analogies suggest that the reciprocally regulated exchange of resources between plants and fungi in CMNs should favor the most beneficial cooperative partnerships [11,12]. On the other hand, reciprocal transfer is only found in a subset of symbionts under specific conditions, while increased competition in CMNs is a more common observation [17,18]. At the core of this controversy is whether CMNs actively support fungal growth at the expense of plant resource demands (i.e., a fungi-centric view) or function as passive channels through which plants regulate resource fluxes (i.e., a plant-centric view). If plant-centric, we expect to find that the structure and functioning of CMNs give rise to consistent spatiotemporal patterns of resource allocation akin to those predicted by the kinship hypothesis. If fungi-centric, we expect to find that spatiotemporal patterns of resource allocation reflect the composition and functioning of the fungal community regardless of the connecting plant nodes in CMNs. Data exist to support both opposing views [see 9,19]; therefore, we posit that CMNs are neither plant- nor fungi-centric.

Instead, perhaps rather than gregarious, AM fungi are “agnostic” with respect to plant species composition and relatedness, and yet affected by major plant functional traits that are known to influence resource use and allocation. There is no clear evidence to suggest that AM fungi exhibit specificity for plant species as do host-specific pathogens [20]. Even in the systems dominated by ectomycorrhizal (EM) associations where most previous research into CMN structure and function was developed, fungi tend to be generalists, although plant taxa-specific EM fungi occur at relatively high proportion, especially in early successional temperate plant communities [21,22]. Building on previous research, our hypothesis represents a first-principles simplification in which gradients in resource limitation, rather than species-specific plant-fungi interactions, might allow us to better understand and predict the rates and direction of resource transfer in CMNs.

To test the “agnostic fungi” hypothesis, we asked if interactions among biophysical and biogeochemical processes could explain resource transfer in CMNs with more accuracy than previous plant- or fungi-centric analogies. We studied how soil resource-use efficiency and plant traits that are known to regulate physiological performance [23] and that are expected to affect the transfer of carbon and nitrogen [24] in paired experiments including restored prairie and pasture experimental sites under ambient conditions and rain exclusion shelters (designed to affect soil water and nutrient mass flow). We replicated our paired experimental setting at three different locations, with study sites distributed across a 520 km latitudinal gradient. Due to the climatically driven differences in communities across sites, not all species were present at all sites (Table S1); however, all functional groups were present at all sites and most species were present at more than one site. In this experiment, we also sequenced strain-level variation in root fungal DNA, plant functional types, and leaf stoichiometric traits to test if relatedness explained

difference in resource transfer. This allowed us to generalize our inferences about the structure and function of arbuscular CMNs in temperate grasslands. The diversity of plants growth forms included in our study also affect CMN composition and function. For example, Davison et al. [25] found plant growth form altered AM fungal community and functional diversity. Plant total biomass can also increase nitrogen received through a CMN, an effect that may be compounded by whether plants are woody or herbaceous [26]. This could be explained by larger or denser plants providing more carbon. Both plants and fungi have economic spectra characterized by fast or slow traits and nutrient strategies which together form an interacting continuum potentially driven by N availability [27]. It is unclear to what extent plant or fungi characteristics drive these plant-fungal interactions. Therefore, we designed an experiment to quantify how plant-fungal interactions influence the structure and functioning of CMNs across broad environmental gradients and resource constraints.

Materials and Methods:

We conducted our experiment at three sites situated on a 520 km latitudinal transect that spans three Mediterranean climates: cool, moist (northern site; Tenino, WA) to warm, moist (central site; Eugene, OR) to warm, dry (southern site; Selma, OR). Each circular plot was 3 m in diameter. Half of our plots were restored prairie systems (n = 10 per site) while the other half of the plots had introduced pasture grasses established prior to restoration (n = 10 per site). Restored prairie plots were mowed, raked, received herbicide, and seeded in 2014-2015, followed by seeding in fall 2015, 2016, and 2017 [28]. We erected rainout shelters that excluded 40% of the rainfall on half the plots at each site (n = 10 rain exclusion, 10 control per site; Fig. 1).

All plants in our system have the potential to form associations with AM fungi (Table S1) [29–31]. Previous work demonstrated that the rainout shelters had minimal effects on aboveground community structure or function [23], possibly due to the shoulder season effect of the Mediterranean rain seasonality. This network of experimental sites was established in 2010 and has been extensively studied since then [28,32–35] including work on mycorrhizal fungi [36,37]. Treatments had marginal effects on the soil water potential (especially during the early growing season). Pasture plots were dominated by one to a few species of introduced perennial grasses which we targeted as donors in these plots. We used different species of perennial grass at each site. For restored prairie plots, we targeted the native perennial forb *Sidalcea malviflora* ssp. *virgata* common to ambient and drought treatments at all sites. Despite those differences, we did not find significant changes in the average plant community composition or productivity under rain exclusion, which also did not affect morphological and functional traits (e.g., specific leaf area, iWUE, and C:N ratios) of the functional groups we selected for this experiment [23,32].

To test our new hypothesis, we built upon those preliminary data with a dual isotope label approach as follows. We labeled perennial plants central to each plot (hereafter, ‘donors’) and monitored leaf ^{15}N and ^{13}C for the surrounding plants (‘receivers’) for up to 21 days. In addition, we sampled roots and soils at 21 days post-labelling. We characterized AM fungal DNA isolated from the root samples, as well as stable isotopes in all leaf and root samples. Our experimental design was nested in a multi-year experiment where data loggers were used to continuously measure temperature and moisture in all the manipulated plots.

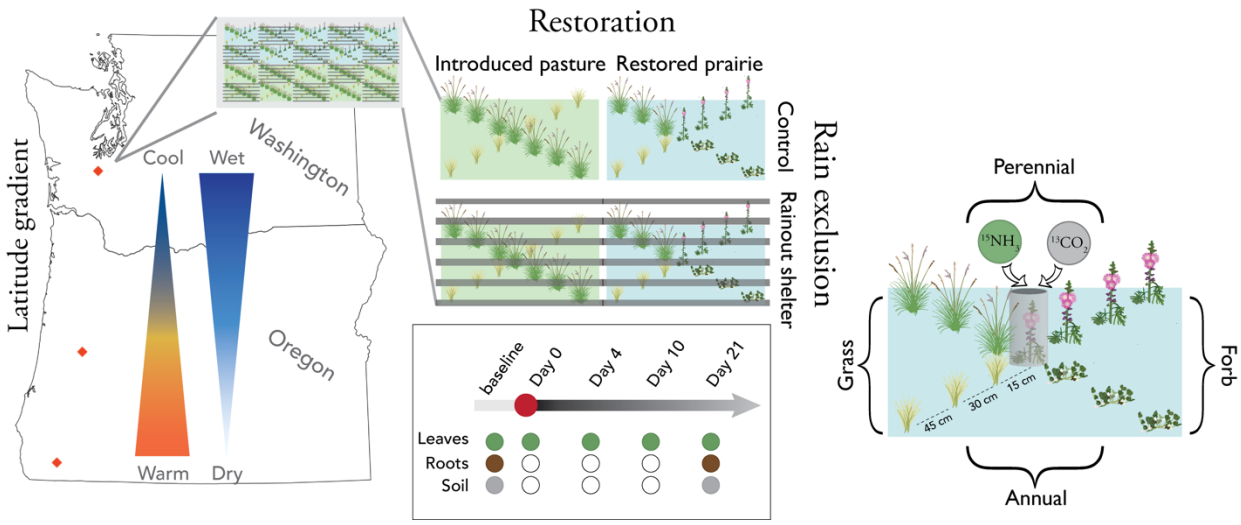

Figure 1. Schema of experimental set up, sampling, and effects of rainout shelters.

Isotopic labelling

At each site, we selected a healthy perennial forb (*Sidalcea malviflora* ssp. *virgata* in restored prairie plots [except in one plot where we used *Eriophyllum lanatum* due to a lack of *S. malviflora ssp. virgata*]), or a perennial grass (*Alopecurus pratensis*, *Schedonorus arundinaceus*, or *Agrostis capillaris*) in pasture plots at the center of each plot to receive the isotopic labels. On sunny days between 11AM and 3PM at peak productivity for each site, we applied isotopically enriched carbon (^{13}C) and nitrogen (^{15}N) as a pulse of carbon dioxide (CO_2) and ammonia (NH_3) to the leaves of target "donor" species common across experimental sites. We performed the labeling experiment using custom-made field-deployable clear chambers with internal fans built to allow gas mixing and fast in situ assimilation of isotopic labels, following established protocols developed in previous isotopically labeling studies [38,39,e.g., 40]. Before performing the experiment, we tested our approach in the field to optimize gas exposure and labeling amounts, which included checking for leaks and contamination outside of the chamber. We covered the donor plant with a clear plastic cylinder and injected gas in sequence at 20-minute

intervals. For $^{13}CO_2$, we made three injections of 2 mL pure CO_2 (enriched at 98 atm % ^{13}C) to double the amount of CO_2 in the chamber each time. For NH_3, we made two injections of 10 mL pure NH_3 (98 atm% ^{15}N) at 20 minute intervals. The dates of application were based on peak productivity estimated from Normalized Different Vegetation Index (NDVI) at each site [see 28 for details]. We sampled leaves from each donor plant immediately after labeling (time point 0) as well as from all plants approximately 4 days (time point 1), 10 days (time point 2), and 21 days (time point 3) post-labelling as logistics permitted (Fig. 1, Table S2). We also collected leaves at time points 1, 2, and 3 from up to twelve plants in each plot representing three replicates of factorial grass/forb structural groups and annual/perennial life history strategies (Table S1). The number of plants and represented groups depended on which plants were growing in each plot. For example, pasture plots were limited to only grasses and the northern pasture plots had only perennial grasses.

By applying enriched gases only to the aboveground portions of established perennial donor plants, we introduced the isotopic tracer to both the plant and mycorrhizal fungal tissues with minimal exposure of the soil to the tracers. At the end of the experiment, we harvested entire plants and the soil surrounding the roots at time point 3 and kept them in cool conditions until processing. We separated the roots and rhizospheres, and collected roots from each plant, selecting approximately ten ~3 cm fine root fragments per sample (i.e., third order or finer, where available) for DNA extraction and identification. All roots and rhizosphere soils were stored at -80º C until processing.

Baseline and Resource Transfer Calculations

Before isotopic labeling, we collected soil, leaves, and roots from each site. We collected soils in late spring and early summer 2019 to 20 cm depth in each plot. From these soil samples, we removed root fragments that represented the typical roots seen in each plot. We collected leaves for each species in each plot; however, these leaves were contaminated with ^{15}N during transport. To replace contaminated samples, we separately sampled leaves from biomass samplings collected in late spring and early summer 2019, ensuring that annual and perennial grasses and forbs were represented at each site.

We oven-dried all samples at 65º C to constant mass and encapsulated them for stable isotope analysis. All stable isotope analysis was done at UC Davis Stable Isotope Facilities using an Elementar Vario EL Cube or Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). We calculated the amount of carbon and nitrogen in each plant compartment (leaves and roots) using standard label recovery equations [40], using baseline values measured before application of the labelled gases to capture background variations in isotopic composition of unenriched leaves, roots, and soil samples.

We designated all samples with greater than two standard deviations above baseline samples as “enriched” in a particular isotope. Baseline values were calculated on a site by rain exclusion treatment basis by plant functional type basis (Table S3). Site-specific baseline soil and root isotope ratios represent the whole community because of interconnected rhizospheres where is it was not possible to identify specific species. In all cases, baseline values fell within the expected range for our region (Fig. 2). For each enriched sample, we calculated isotope excess as

Equation 1

$$atm\%_{excess} = atm\%_{post\ label} - atm\%_{baseline}$$

For enriched donor plants, we calculated % derived from label immediately following label application as

Equation 2

$$\%DFL_{donor} = \frac{atm\%_{excess}}{atm\%_{labelling\ gas} - atm\%_{baseline}} * 100$$

For enriched receiver plants, we calculated % derived from label (%NDFL and %CDFL, respectively) for each relevant point in space and time as [40]

Equation 3

$$\%DFL_{receiver} = \frac{atm\%_{receiver\ excess}}{atm\%_{\ donor\ excess}} * 100$$

When we calculated %DFL in roots, we used donor leaves as the source (atm% donor excess). We then calculated the amount derived from label on a per mass basis as

Equation 4

$$DFL\ (\text{mg N / g leaf}) = 1000 * 1g * \left(\frac{N\ or\ C\%}{100}\right) * \left(\frac{DFL\%}{100}\right)$$

We calculated intrinsic water-use efficiency following Farquhar and Richards [41] using the baseline ^{13}C values from the original samples because the contamination only occurred with ^{15}N.

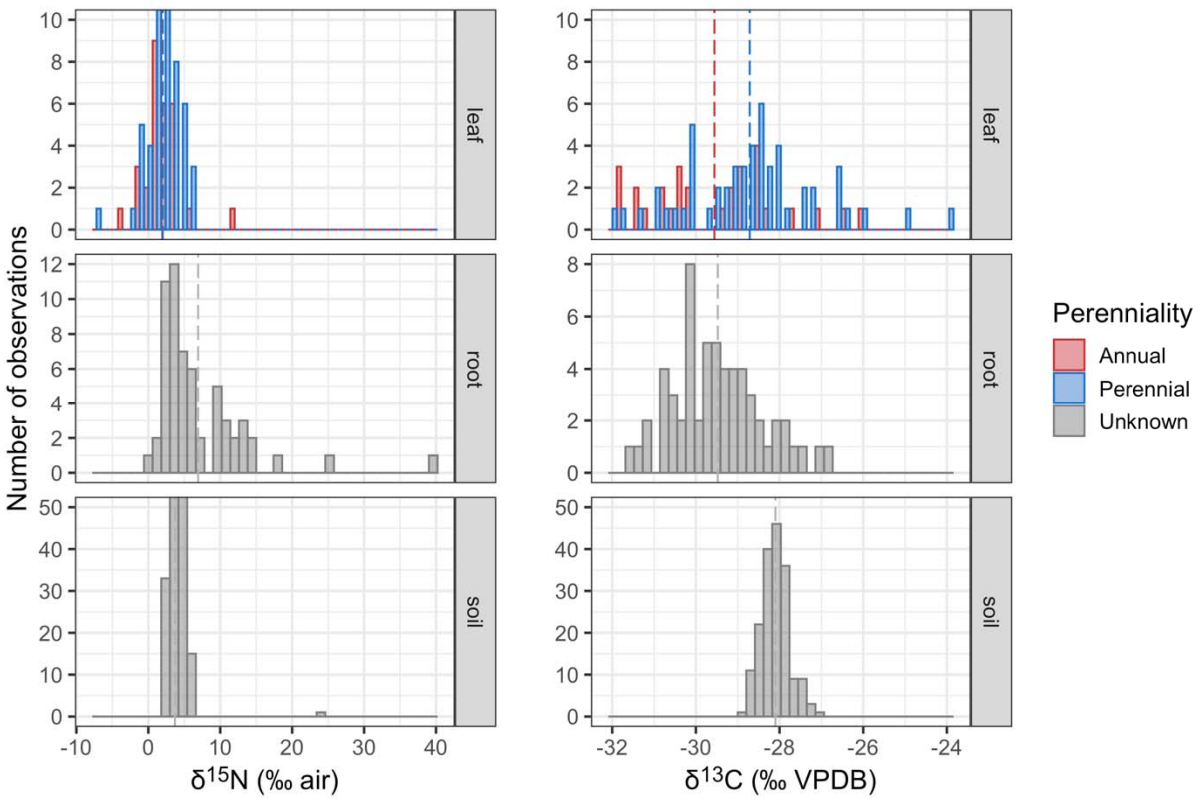

Figure 2. Natural abundance of stable isotopes in leaves, roots, and soil before labelling. Dashed lines indicate mean value. Exact values used to calculate derivation from label (Eq. 1 – Eq. 4) are available in Table S3.

To better understand post-labelling soil enrichment, we selected a subset of rhizosphere soils that represented six donor plants at each site divided equally between restored prairie and pasture plots, and selected the three most highly ^{15}N enriched interspecific receivers in each plot across the sites. In addition, we sampled three most highly enriched interspecific receivers at each site and restored prairie-introduced pasture combination (if they had not been sampled previously). Because pasture plots were sometimes monodominant grasses, we sampled the top three enriched intraspecific receivers at each site and treatment. In total, this came to 48 post-labelling soil samples in 29 plots.

Fungal DNA analysis

We extracted DNA from roots of 450 plants harvested at time point 3 (21 days post-label) using Qiagen DNeasy Powersoil HTP kits (Qiagen, Hilden, Germany). We only analyzed DNA from roots, not from the soils collected from each plant's rhizosphere. We characterized each sample's AM fungal composition with a two-step PCR protocol that amplified a ~550bp fragment of the SSU rRNA gene (the most well-supported region for AM fungal taxonomic resolution [42]. We used WANDA (5'- CAGCCGCGGTAATTCCAGCT- 3') and AML2 (5'-GAACCCAAACACTTTGGTTTCC-3') primers [43,44]. We used primers with unique indices so we could multiplex several projects on a single run. We quantified successful PCR amplicons with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Waltham, MA, USA) on a SpectraMax M5E Microplate Reader (Molecular Devices, San Jose, CA, USA) before purifying with QIAquick PCR Purification kits (Qiagen). We sequenced the purified pools on the Illumina MiSeq platform (paired-end 300bp, Illumina Inc., San Diego, CA, USA) at the University of Oregon Genomics and Cell Characterization Core Facility (Eugene, OR, USA). Reads were deduplicated with UMI-tools using unique molecular identifiers (UMIs) inserted during PCR [45].

We assigned amplicon sequence variants (ASVs) using the dada2 pipeline with standard quality filtering and denoising parameters [46]. The dada2 pipeline maintains strain-level diversity at the scale of individual sequence variants rather than clustering sequences into OTUs. This fine-scale measure of fungal sequence diversity was particularly important for our analyses to maintain the greatest chance of detecting a single AM fungal 'individual' in multiple plant root samples. Taxonomy was assigned to ASVs using the MaarjAM database (2019 release)

[47]. We used a Bayesian mixture model in the DESeq2 package [48] to scale ASV counts within and across samples to avoid artificial taxon abundance biases [49].

Data analysis

We performed all analyses in R ver. 4.0.4 [50]. We removed one plant with ^{15}N outlier data. We also removed five mislabeled samples. To meet statistical assumptions, we only included data from enriched plants with successful root fungal DNA extraction in our analyses and figures. We limited receivers to those for which we also had sufficient root fungal DNA. In total, from 1762 leaves measured for isotopic content, we analyzed data from 366 unique plants: 60 donors and 306 receivers.

We tested the relationship between receiver leaf %DFL and plant traits (grass/forb, annual/perennial, iWUE, C:N, degrees of connectivity, interaction term between grass/forb and annual/perennial) and site conditions (position on latitude gradient, pasture/restored, rain exclusion treatment, distance from donor, time from labelling) with a mixed-effect ANOVA (plot as random effect; Table 1). We constructed a phyloseq object using the ASV table with normalized counts (McMurdie and Holmes 2013), and used iGraph, metagMisc, and RCy3 (Nepusz and Csardi 2006, Mikryukov 2017, Gustavsen et al. 2019) to create networks for each plot. In each network, nodes represented individual plants and edges between nodes represent plants sharing at least one fungal DNA sequence variant. The weighted edges are based on how many fungal ASVs were shared among plants. We calculated degrees of connectivity with tidygraph [55] to examine how many plants each individual plant was ‘connected’ to (by means of shared fungal ASVs) in each plot (Fig. 3). We also calculated whether each receiver plant shared fungal ASVs with the central donor plant in each plot. We visualized individual plot

networks in CytoScape. We visualized shifts in AM fungal community composition using non-metric multi-dimensional scaling (NMDS) in the vegan package, demonstrating the AM fungal community similarity across plants [56].

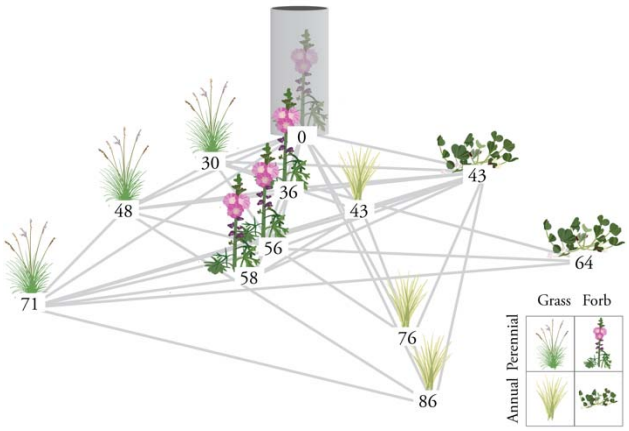

Figure 3. Example of how networks were constructed for each plot. The grey cylinder indicates the donor plant for the plot. Numbers beneath the receivers are the distance (in centimeters) from the donor. Degrees were calculated as how many plants each individual plant was connected to by shared fungal ASVs; for example, the perennial grass at 71 cm has 7 degrees.

Results:

All donors were ^{15}N enriched in their leaves at time of labelling. Two donors were not ^{13}C enriched in their leaves at time of labelling. We recovered DNA data from the roots of 88.3% of donors. We sampled 1,444 leaves from 434 receiver plants at three time points. Of these leaves, 81.0% were ^{15}N enriched and 2.4% were ^{13}C enriched. We recovered DNA from the roots of 77.9% of the receivers. At time point 3 (roughly 21 days post-labelling), we collected roots from 46 of the initial 60 donors and 306 of the initial 434 receivers. Of the roots of the collected donors, 97.8% were still 15N-enriched and 23.9% were still ^{13}C-enriched at time point

3. Of the roots of the collected receivers, 33.0% were still ^{15}N enriched and 10.5% were still ^{13}C enriched at time point 3.

Assimilation of isotopic tracers was similar between labeled “donor” plants with no significant differences on average between sites or experimental treatments within sites, including rainfall exclusion or restored status (Fig. S1). At all sites, foliar assimilation of ^{15}N and ^{13}C by donor plants led to enrichment levels ranging from approximately 5-10 fold higher than baselines. Foliar enrichment levels decreased consistently at all sites and treatments over the 21 day sampling period. Annual forbs had the greatest enrichment level and perennial forbs the lowest enrichment level (Fig. 4). We found significant spatial and temporal differences in foliar and root isotope ratios in donors and receivers resulting from interspecific transfer of carbon and nitrogen (Fig. S2; Table 1). Receiver foliar enrichment levels did not correlate with donor foliar enrichment levels within the same plot (Fig. S3).

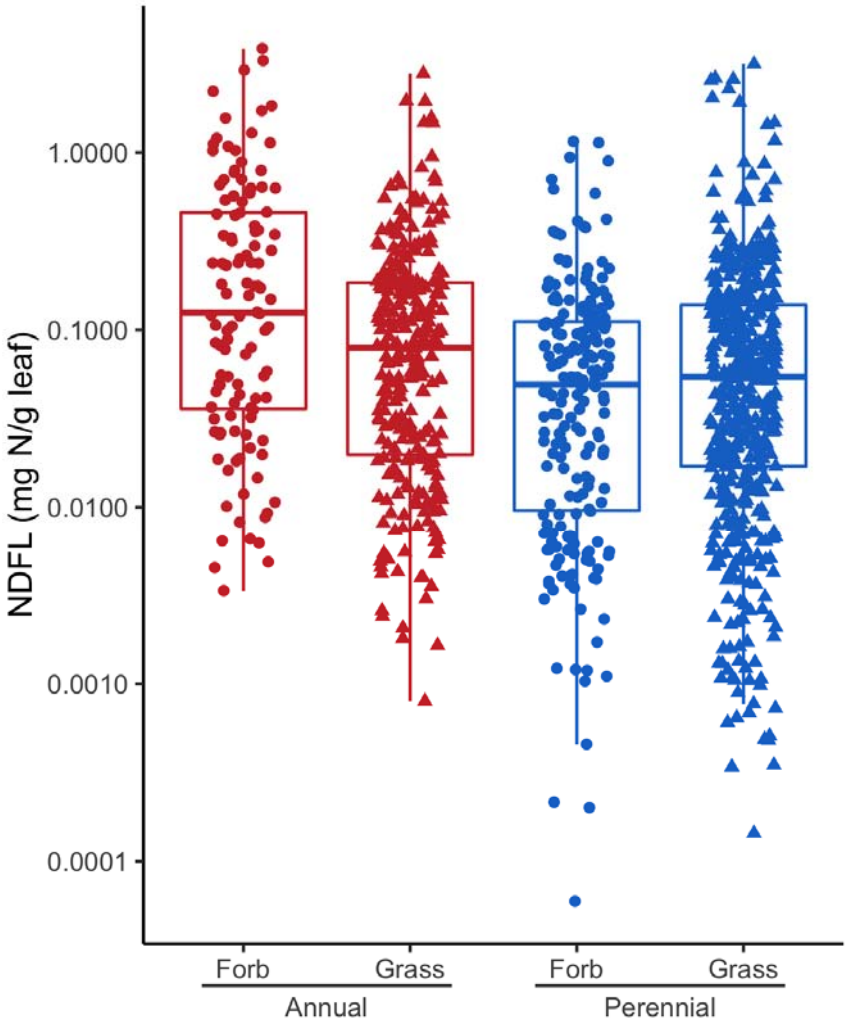

Figure 4. Annuals had greater leaf nitrogen derived from label (NDFL) scaled by mass than perennials. Points represent enriched individual receiver plants with associated DNA data at one time point of sampling. All three post-enrichment time points are shown. Y-axis is log10 scale. See Fig. S2 for values over distance and Fig. S4 for NDFL in roots as well as CDFL in roots and leaves.

Table 1. ANOVA results effects on leaf nitrogen derived from label (%NDFL). Only enriched receiver leaves with associated DNA data were included in the analysis (n = 1094). Leaf %CDFL results available in Table S4.

	DF	χ^2	P-value
Fixed effects			
Annual/perennial	**1**	**23.84**	**<0.001**
Grass/forb	**1**	**13.08**	**<0.001**
iWUE	1	0.08	0.78
Degree of connectivity	1	0.13	0.72
C:N	**1**	**37.56**	**<0.001**
Site	2	0.83	0.66
Drought treatment	1	0.54	0.46
Restoration treatment	1	3.45	0.06
Distance from donor	**1**	**9.71**	**<0.001**
Time from labelling	**1**	**84.47**	**<0.001**
Annual/perennial:Grass/forb interaction	**1**	**8.21**	**<0.001**
Random effect			
Plot			**<0.001**

Allocation of ^{13}C and ^{15}N tracers to roots and subsequent transfer to "receiver" species did not follow expected patterns (e.g., despite donors being perennial, annual receivers were more enriched, indicating that relatedness did not drive the transfer, Fig. 4, Table 1). Allocation and transfer varied significantly between functional groups due to their intrinsic differences in tissue stoichiometry (Table 1). We selected 18 common annual/perennial and grass/forb species of receiver plants, which revealed significant differences between functional types for NDFL

(Fig. 4) but little detectable CDFL relative to baseline (Fig. S2). Rain exclusion treatment, restoration treatment, and site did not affect interspecific transfer of nitrogen (Fig. S5; ANOVA, $P > 0.05$, Table 1). We observed very low carbon enrichment, but of the 2.4% of leaves that were enriched in carbon, plant functional type and site affected carbon transfer (Table S4). We did, however, observe significant differences in nitrogen transfer by plant functional type (Table 1), mirroring intrinsic differences in tissue stoichiometry and iWUE (Fig. 5), despite no significant enrichment in soils collected from the rhizosphere of those same plants (Fig. S6). We also found that C:N affected NDFL, although not in a simple linear manner and with no apparent correlation between NDFL and iWUE (Fig. 5). We did detect a low level of soil enrichment in 4 out of 23 donor soil samples (ranging from 0.382 to 0.479 atm% ^{15}N).

Annuals had greater ^{15}N foliar enrichment compared to perennials (ANOVA, $P < 0.001$, Tables 1 and 2). Foliar enrichment decreased over both time and space (ANOVA $P < 0.001$; Fig. S1, Fig. S2). On average, annuals had a lower leaf nitrogen content and higher C:N than perennials (Table 2, Fig. 5). Forbs had higher NDFL than grasses (ANOVA, $P < 0.001$, Table 1) as well as a lower C:N. There was a significant interaction between annual/perennial and grass/forb form (ANOVA, $P = 0.003$, Table 1).

Table 2. Leaf nitrogen derived from label (NDFL) and leaf tissue nitrogen (N%) four days after labeling.

			%NDFL				**NDFL (mg N/g leaf)**				**N (%)**			
		n	*Mean*	*SD*	*Min*	*Max*	*Mean*	*SD*	*Min*	*Max*	*Mean*	*SD*	*Min*	*Max*
Annual	**Forb**	54	6.528	9.769	0.101	56.005	0.749	0.819	0.041	3.858	0.749	0.819	0.041	3.858
	Grass	92	4.847	9.032	0.098	63.884	0.34	0.463	0.011	2.79	0.340	0.463	0.011	2.790
Perennial	**Forb**	67	0.952	1.069	0.090	5.582	0.214	0.228	0.021	1.156	0.214	0.228	0.021	1.156
	Grass	148	1.632	3.066	0.088	22.434	0.252	0.484	0.013	2.948	0.252	0.484	0.013	2.948

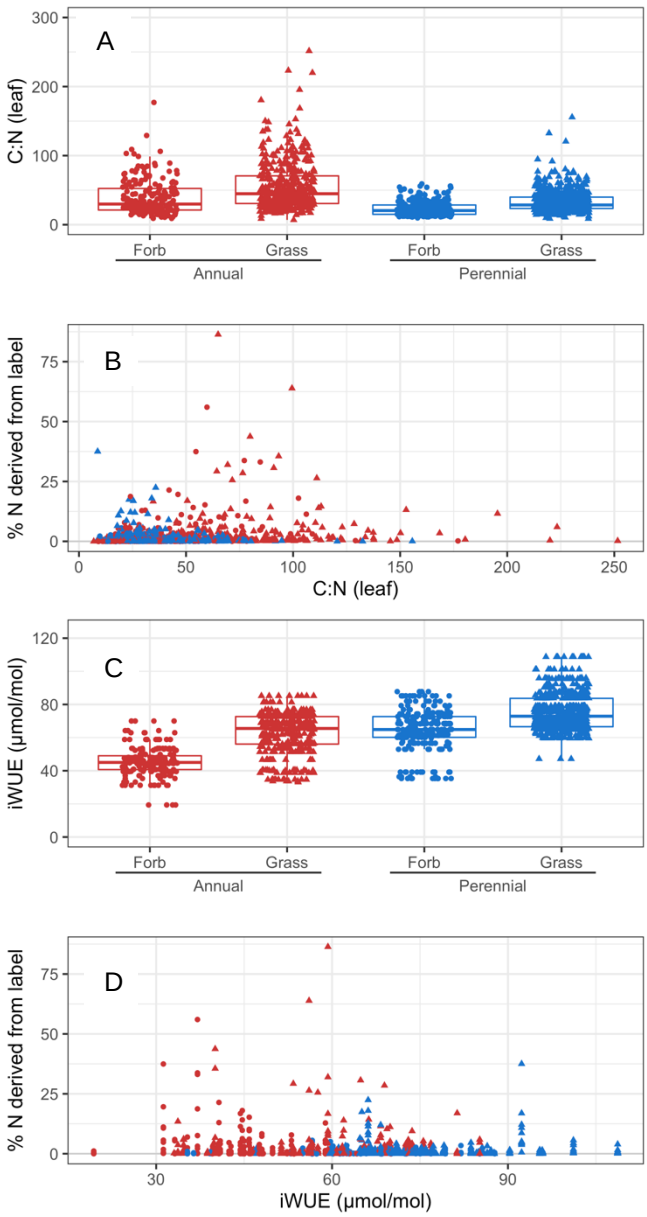

Figure 5. Stoichiometric and functional traits compared to nitrogen derived from transfer. A) Leaf C:N by annual/perennial. B) Percent ^{15}N derived from label (DFL) compared to C:N in leaves. C) iWUE by annual/perennial as measured before labelling. D) Percent ^{15}N DFL compared to iWUE in leaves at all time points. Letters above boxplots indicate significant differences.

Our analysis of fungal community composition shows a high degree of connectivity between plants of different species but no obvious pattern of connectedness that could explain preferential nutrient transfer by plant functional groups. We found that 97.25% ± 8.01 (SD) of all plants roots within each experimental plot shared at least one fungal DNA sequence variant (ASV) with another plant of the same plot. Fungal community composition was similar across plant functional groups (Fig. 6, PERMANOVA pseudo-F statistic = 2.269, R^2 = 0.005, p = 0.001). Annual plants shared fungi with more plants in the same plot (4.74 plants ± 2.62 SD) compared to perennials (4.04 plants ± 2.49 SD ; t-test, P = 0.019; Table S5), but degrees of connectivity did not predict nitrogen transfer (Table 1). Seventy-three percent of plants were colonized by four or fewer fungi and shared fungi with five or fewer other plants in the plot, making it difficult to determine if strength of connectivity altered nitrogen transfer (Fig. S7).

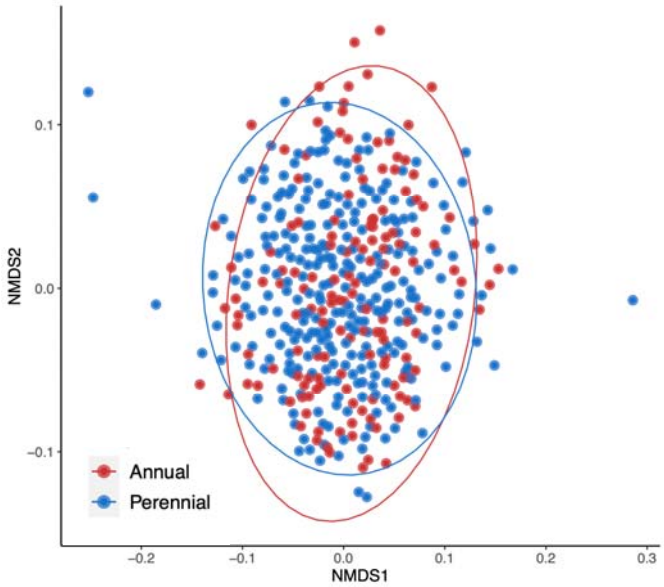

Figure 6. NMDS of fungal communities. Each point represents an individual plant ordinated by the Bray Curtis dissimilarities for AM fungal composition. PERMANOVA pseudo-F statistic = 2.269, R^2 = 0.005, p = 0.001.

Discussion:

In our extensively replicated spatiotemporal isotopic tracer experiment, the assimilation and allocation of limiting resources in CMNs was neither plant- nor fungi-centric. Our data show that AM fungi are "agnostic" with respect to plant species composition and relatedness, and that the transfer of nitrogen (an important nutrient that can limit plant growth in our system) is regulated by major functional traits that are known to influence resource use and allocation in plant communities. The rates and direction of resource transfer in CMNs, inferred from pulse labeling and recovery of ^{15}N in leaves and rhizospheres, can be predicted from leaf C:N and distance from donor species (Table 1). Across all sites and treatments, we observed a stronger sink for ^{15}N in annual plants (Table 2), indicating preferential transfer of limiting resources to that functional group of plants. This observation is contrary to the expectation of the kinship hypothesis that preferential transfer would be expected to occur from a perennial donor to a perennial receiver. Although plants shared ~97% fungal ASVs in their roots (Fig. 6), connectivity did not predict ^{15}N transfer (Table 1), and thus we found no evidence for a fungi-centric hypothesis based on which all connected plants would have been expected to be equally enriched in ^{15}N. Our data suggest that rates and direction of resource transfer in CMNs reflect plant nutrient requirements and spatial proximity.

We designed this experiment to be informed by previous hypotheses on how carbon and nitrogen transfer occurs in highly connected CMNs, where a dilution of the applied ^{13}C label is to be expected given the vast amounts of carbon in the soil and plant biomass. We conducted repeated spatiotemporal sampling of isotopic enrichment levels at increasing distances from donor species, days to weeks after labelling, and in well-established communities exposed to multiple years of experimental treatments, expecting to find evidence of kinship (i.e., greatest

resource transfer in related plants), driven by CMN economics (i.e., ^{15}N transfer rates coinciding with ^{13}C investment in root and fungal mass). Our donor plants were perennials while annuals received, on average, an order of magnitude higher enrichment. Thus, we discarded kinship as a possible driver of transfer in this system. We also did not find evidence of a relationship between ^{15}N in leaves and ^{13}C in roots because we did detect ^{15}N enrichment in leaves but no ^{13}C enrichment in roots. We did, however, find significant ^{13}C enrichment in the donor plants. Therefore, our data do not support either hypothesis, and instead suggest AM fungi are "agnostic" with respect to plant species partnerships, forming CMNs where the rates and direction of resource transfer ultimately reflects a sink-source strength effects, consistent with previous observations of stoichiometric source-sink manipulations of carbon and nitrogen within plants [57–59], but in our case observed at the community scale.

Nitrogen enrichment levels remained high in leaves and many roots at the end of the experiment, allowing us to measure NDFL across the community and infer the main drivers of N transfer. However, carbon enrichment levels faded before plants were harvested approximately 21 days post-labelling, either due to losses via respiration or redistribution in CMNs where it became undetectable in all but a few cases. After controlling for variation in assimilation rates by calculating NDFL, we found that annual plants received greater ^{15}N enrichment than perennial plants. Plants closest to the donor were most enriched, and ^{15}N enrichment decreased over time (Fig. S2, Fig. S3). Plant level of connectivity through an assumed CMN did not predict ^{15}N enrichment. Although the rainout shelters had limited effect, there were major differences across the latitudinal gradient represented by the sites in temperature and soil moisture availability [23,28]; however, neither treatment nor site affected our results.

The major drivers of differences in allocation of ^{13}C and ^{15}N to roots and subsequent transfer to "receiver" species were the intrinsic difference in plant functional types and correlated traits, including measured leaf C:N. Previous studies in northern California under environmental conditions similar to those found in our southernmost experimental site showed rapid (days to weeks) transfer of ^{15}N applied to the leaves of ectomycorrhizal pines to surrounding annual AM plant receivers [60]. Those results were interpreted as evidence for agnostic CMNs because "direct fungal connections are not necessary for N transfer among plants" and "leaves of the annual plants had greater ^{15}N derived from source and were more enriched (^{15}N at % excess and $\delta^{15}N$ values) than perennial receivers, irrespective of the mycorrhizal type." Similarly, as proposed by He et al. [60], our observation of agnostic ^{15}N transfer from donor to receivers suggests that annual plants were a strong sink for N which could be explained by stoichiometric gradients that affect root exudation and recapture of N-containing materials from rhizodeposition [61,62]. Our data corroborate rapid agnostic transfer among AM plants, with no detectable enrichment in root or soil ^{13}C near roots 21 days post-labelling, but do not allow us to determine general mechanisms that are responsible for the ^{15}N transfers.

We inferred a high connectivity between plants within each given treatment and site given the highly similar fungal composition in the root systems of both perennial and annual plants (Fig. 6). Given the constraints of ASV-identified data, we did this analysis on a strain-level scale and it is possible that separate spores of the same ASV separately infected plants within the same plot. However, we are reasonably confident in our use of fungal ASVs as a proxy for connectivity given the strong overlap in our community and because individuals of one ASV can anastomose in the soil [63]. This overlap could explain the lack of support for the kinship hypothesis in our dataset and offers further support for stoichiometric gradients in

general, and C:N gradients in particular, as a principal control of terms of trade in CMNs [11]. Because we observed such high rates of shared ASVs (~97%) and we observed high levels of N enrichment in receivers at our first post-label sampling point four days after application, we could not test whether receivers connected to donors or whether plants were connected to the network affected 15N transfer. We found unexpectedly low soil isotopic enrichment (fig. S6) which suggested that the labels did not remain in the fungal network. This low level was likely driven by the fact that we did not sample soils until 21 days after labeling and hyphal turnover for grass-associated AM fungi can be less than one month [64]. Because N is a major limiting nutrient in this system, according to the current paradigm of CMNs, it would be quickly taken up end recycled or transferred rather than accumulating in the soil.

We found that plant-soil stoichiometric gradients and functional traits were the strongest drivers of resource sharing in grassland CMNs. We interpret this finding as evidence of biochemical and biophysical sinks, in which nutrients are allocated to plants with the greatest need for those nutrients, either through a 'passive' mycorrhizal network or direct uptake from soil, or that nutrients should be allocated through water flow. Expanding on previous studies, we propose that agnostic AM fungi facilitate spatiotemporal dynamics of carbon and nitrogen transfer through CMNs in ways that are neither plant- nor fungi-centric. That is, plants and fungi that are located closer together in space and with stronger demand for resources over time are more likely to receive larger amounts of those limiting resources.

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Competing interests:

The authors have no competing interests to declare.

Author contributions:

L.C.R.S, S.D.B., and B.J.M.B. designed the research; H.R.D., K.L.S., T.M.M., B.B., and P.B.R. performed the research; H.R.D. led the data analysis and manuscript preparation with K.L.S. assisting; all authors contributed to revising the manuscript and approved the publication.

Data availability:

Data used in these analyses are available online at the Data Dryad repository (https://doi.org/10.5061/dryad.7pvmcvdxt). While this paper is under review, data can be accessed using the following unpublished link: https://datadryad.org/stash/share/bz0dQHa9xnzgOqwsF_3N0TSx3Oh6OWmElTExtAjyGUc

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Supporting information

Table S1. List of species at each site with number of leaf samples per species.

Table S2. Collection dates for each time point at each site.

Table S3. Baseline values used to calculate amount derived from label (DFL).

Table S4. ANOVA results effects on leaf carbon derived from label (%CDFL).

Table S5. List of fungal ASVs by annuals and perennials.

Figure S1. Enrichment decreases over time in labelled plants.

Figure S2. Decreased receiver enrichment with distance from donor in leaves and roots.

Figure S3. Receiver enrichment did not correlate with donor enrichment.

Figure S4: Enrichment derived from label for A) CDFL in leaves, B) NDFL in roots, and C) CDFL in roots.

Figure S5. No trend in ^{15}N enrichment across site and rain exclusion treatment.

Figure S6. Limited soil enrichment 21 days after labelling.

Figure S7. Histogram of connectivity metrics showing left skew.