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Measuring carbon gains from fungal networks in understory plants from the tribe Pyroleae (Ericaceae): a field manipulation and stable isotope approach

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Abstract Partial mycoheterotrophy, a newly discovered form of mixotrophy in plants, has been described in at least two major lineages of angiosperms, the orchids and ericaceous plants in the tribe Pyroleae. Partial mycoheterotrophy entails carbon gains both directly from photosynthesis and via symbiotic mycorrhizal fungi, but determining the degree of plant dependence on fungal carbon is challenging. The purpose of this study was to determine if two chlorophyllous species of Pyroleae, *Chimaphila umbellata* and *Pyrola picta*, were receiving carbon via mycorrhizal networks and, if so, if their proportional dependency on fungal carbon gains increased under reduced light conditions. This was accomplished by a field experiment that manipulated light and plants' access to mycorrhizal networks, and by using the stable carbon isotope composition ($\delta^{13}C$) of leaf soluble sugars as a marker for the level of mycoheterotrophy. Based on leaf soluble sugars $\delta^{13}C$ values, we calculated a site-independent isotope enrichment factor as a measure of fungal contributions to plant C. We found that, under each treatment and over time, the two test species demonstrated different isotopic responses caused by their different intrinsic physiologies. Our data, along with previously published studies, suggest that *Chimaphila umbellata* is primarily an autotrophic understory plant, while *Pyrola picta* may be capable of partial mycoheterotrophy. However, in this study, a 50% decrease in light availability did not significantly change the relative dependency of *P. picta* on carbon gains via mycoheterotrophy.

Keywords Mycoheterotrophy · Mixotrophy · Soluble sugars · ^{13}C · Pyroleae · Mycorrhizae

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Introduction

Recent interest has turned to examining an alternative pathway to photosynthesis for supplying the necessary carbon in chlorophyllous (photosynthetically active) understory plants (Selosse and Roy 2009). This pathway, where plants acquire carbon via a symbiotic relationship with fungi, is referred to as mycoheterotrophy (Leake 1994). The majority of mycoheterotrophic plants studied thus far associate strictly with either arbuscular mycorrhizal (AM) or ectomycorrhizal (EM) fungi, while a few species of mycoheterotrophic orchids have been found to associate with saprotrophic fungi (Merckx et al. 2009; Selosse et al. 2010). The most striking examples of mycoheterotrophy are plants that have lost the ability to photosynthesize (full mycoheterotrophy), such as leafless species in the subfamily Monotropoideae (Bidartondo 2005). Fully mycoheterotrophic plants are presumably completely dependent on fungi to meet all of their carbon and nutrient demands, and only produce aboveground structures for reproduction and dispersal (Leake 1994; Merckx et al. 2009). However, recent research on leafy-green plants that are close relatives to full mycoheterotrophs has revealed that many of these

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taxa are mixotrophic, or more specifically, partially mycoheterotrophic, gaining carbon both from photosynthesis and from their symbiotic mycorrhizal fungi (Selosse and Roy 2009). All partially mycoheterotrophic species examined thus far access fungal carbon via EM fungi that are simultaneously associated with surrounding trees (Selosse and Roy 2009). Therefore, partially mycoheterotrophic plants are tapping into carbon from surrounding autotrophic trees that is transferred to these plants by shared EM fungal symbionts.

A carbon isotope model for plant mycoheterotrophy

Partial mycoheterotrophy was confirmed when stable carbon isotope analyses of leafy orchid species that associate with EM fungi were found to have bulk leaf $^{13}C/^{12}C$ ratios intermediate to full mycoheterotrophs and autotrophs inhabiting the same site and living under similar microclimatic conditions (Gebauer and Meyer 2003; Bidartondo et al. 2004; Julou et al. 2005; Abadie et al. 2006). Additional examples of partial mycoheterotrophy outside the orchid family have been found in green Pyroleae (Ericaceae) species that also have stable carbon isotope ratios ($\delta^{13}C$) inbetween full mycoheterotrophs and surrounding autotrophs (Fig. 1; Tedersoo et al. 2007; Zimmer et al. 2007). The carbon isotope model presented in Fig. 1 aims to highlight the distinct $\delta^{13}C$ values of autotrophic and mycoheterotrophic plants. The cause of such differences is inferred to exist because of discrepancies in the mode of plant C acquisition. Autotrophic plants that fix CO_2 into organic carbon via C3 photosynthesis are more depleted in ^{13}C than atmospheric CO_2, due to both physical and chemical influences on leaf carbon metabolism and the microenvironments in which they live (Fig. 1). For example, stomata can influence the concentration of CO_2 inside the leaf compared to the external atmosphere, and this can in turn influence the availability of $^{12}CO_2$ and $^{13}CO_2$ for assimilation. Furthermore, the greatest isotope discrimination takes place because the primary carboxylating enzyme for C3 photosynthesis, RuBisCO, preferentially reacts with $^{12}CO_2$ relative to $^{13}CO_2$ (Farquhar et al. 1989). Newly fixed C is in the form of sugars which are, on average, slightly enriched in ^{13}C compared to secondary metabolites and structural carbon that make up the largest proportion of bulk leaf tissues (Brugnoli and Farquhar 2000). Once a plant has fixed carbon from the atmosphere and transformed it into organic carbohydrates, some portion of this carbon is commonly transferred to the plant's mycorrhizal fungi (Smith and Read 2008). Adopting the isotope food-chain model proposed by Fry (2006), but in the context of plant–fungal systems, we see that EM fungi are enriched in ^{13}C by an average of 2‰ compared to their hosts (Fig. 1; Taylor et al. 2003; Högberg et al. 1999; Gleixner et al. 1993). Because fully mycoheterotrophic plants are receiving all their C via EM fungi, it follows that they are also enriched in ^{13}C compared both to their fungal hosts and surrounding autotrophic plants (Fig. 1; Gebauer and Meyer 2003; Trudell et al. 2003).

A challenge for researchers has been determining the degree of dependence on fungal carbon gains in partially mycoheterotrophic plants. For example, in orchids capable of partial mycoheterotrophy, there is some evidence that as

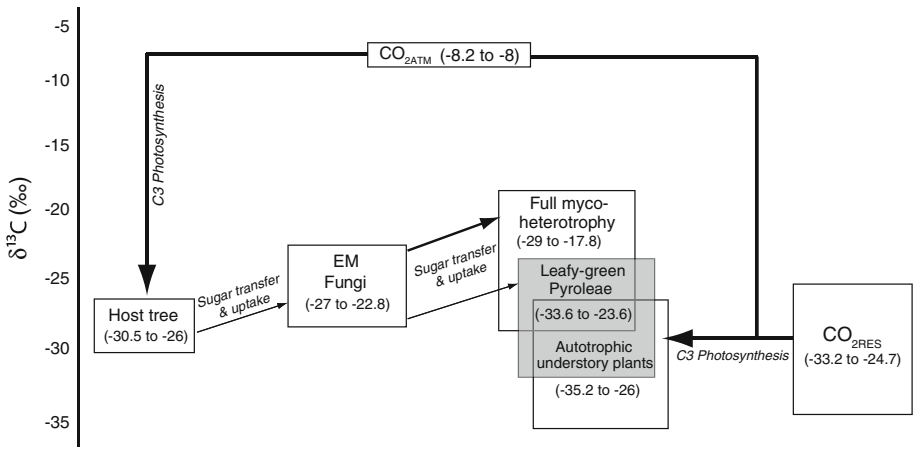

Fig. 1 Schematic diagram of carbon exchange from autotrophic plants to their associated ectomycorrhizal (EM) fungi, and from EM fungi to partially and fully mycoheterotrophic plants. The height of each *box* represents the known ranges of $\delta^{13}C$ for the various carbon pools, and the actual known ranges of $\delta^{13}C$ values of each pool are in *parentheses* below each carbon source or sink; CO_{2ATM} atmospheric CO_2, CO_{2RES} CO_2 respired by soil microbes and understory foliage which is used by understory plants for photosynthesis. Processes in *italics* are those known to cause isotope discrimination (Högberg et al. 1999; Farquhar et al. 1989; Trudell et al. 2003; Preiss and Gebauer 2008). Literature references used for $\delta^{13}C$ values include: Wingate et al. (2010), Hynson et al. (2009), Mayor et al. (2009), Tedersoo et al. (2007), Zimmer et al. (2007), Mortazavi et al. (2005), Taylor et al. (2003), Dawson et al. (2002), Buchmann et al. (1997), and Pearcy and Pfitsch 1991). Weight of *arrows* represents the degree of isotopic enrichment or depletion from source to sink

light becomes a limiting factor, the proportion of photosynthetic C fixation decreases while the proportion of C gains via fungi increases. This results in orchid leaf $\delta^{13}C$ that is enriched compared to leaf $\delta^{13}C$ of surrounding autotrophic plants (Liebel et al. 2010; Preiss et al. 2010). Autotrophic plants in the understory would theoretically become more depleted in ^{13}C than those in the forest canopy owing to a combination of biochemical and leaf-level processes, such as an increase in C_i/C_a (the ratio of the CO_2 concentration inside the leaf to the atmosphere outside the leaf), which would lead to greater discrimination against $^{13}CO_2$ by RuBisCO (Farquhar et al. 1989). Accumulated seasonal changes in bulk tissue $\delta^{13}C$ values of plants may mask the degree of fungal C dependency in partial mycoheterotrophs. This may be especially true for long-lived evergreen species such Pyroleae species where their bulk leaf tissue $\delta^{13}C$ values reflect an integrated isotopic composition from a mixing of all carbon gains throughout the life span of a leaf (Dawson et al. 2002). Thus, if a plant is primarily dependent on C gains via photosynthesis, any evidence of ephemeral partial mycoheterotrophy may be masked in an analysis of bulk leaf $\delta^{13}C$ alone.

Study system for the present investigation

Here, we report the results of a field manipulation experiment that employed the use of carbon stable isotope abundances of leaf soluble sugars as a marker for plant dependency on fungal C. Soluble sugars are the initial products of photosynthesis and have an estimated latency time within leaves of only 1–2 days (Brugnoli et al. 1988). Sugars are the dominant forms of carbon that are passed from autotrophic trees to their EM fungi, which is reflected in the bulk tissue ^{13}C enrichment of EM fungi compared to their host trees (Fig. 1; Smith and Read 2008; Högberg et al. 1999). Carbon transfer from EM fungi to mycoheterotrophs has been demonstrated using a $^{14}CO_2$ tracer (McKendrick et al. 2000), but the specific carbon sources and transfer pathways are still unknown (Leake and Cameron 2010). However, since sugars are the major transport materials in biological systems due to their high energy and chemical stability, they are likely to be among the forms of carbon that are transported to, or digested by, mycoheterotrophic plants (Smith and Read 2008). In this study, we assumed that (1) soluble sugars are the dominant compounds transferred from fungi to mycoheterotrophic plants, (2) leaves of partially mycoheterotrophic plants are sinks for the soluble sugars of fungal origin, (3) soluble sugars from photosynthesis are isotopically distinguishable from soluble sugars obtained from EM fungi, and (4) leaf soluble sugars $\delta^{13}C$ represents a far more sensitive assay for carbon exchange within mycorrhizal networks than bulk leaf carbon $\delta^{13}C$. Based on these assumptions, we hypothesized that, under decreased light availability, plants capable of partial mycoheterotrophy should increase their proportional dependency on fungal C gains, and this should be recorded as an enrichment of leaf soluble $\delta^{13}C$. The two test species compared in this study belong to sister genera in the tribe Pyroleae (Ericaceae), *Pyrola picta* and *Chimaphila umbellata*. They were chosen based on the results of prior studies that showed indications of partial mycoheterotrophy in both *P. picta* and *C. umbellata* (Tedersoo et al. 2007; Zimmer et al. 2007; Hynson et al. 2009). To address our hypothesis, we designed and executed a field experiment where light levels and access to a mycorrhizal network were manipulated, then leaf soluble sugars $\delta^{13}C$ were analyzed from each treatment over the course of a single growing season and compared to the $\delta^{13}C$ values of bulk leaf tissue. To our knowledge, this is the first full factorial field manipulation experiment that examines mycoheterotrophic networks in forest understory plants.

Materials and methods

Field sites and experimental treatments

In the spring of 2008, ten plots each for the two test species of Pyroleae, *Pyrola picta* (PY1–PY10) and *Chimaphila umbellata* (CH1–CH10) were established. Plots were located in University of California's blodgett experimental forest in the foothills of the Sierra Nevada mountains (38°54′N, 120°39′W) at an elevation of about 1,340 m, where there is a mean annual precipitation of 1,600 mm and mean annual temperature of 11.2°C (Battles et al. 2001). At this site, the forests are second growth Sierran mixed conifer dominated by *Abies concolor, Pinus lambertiana* and *Pseudotsuga menziesii* with sparse understories and midstories composed mainly of *Quercus kelloggii* and *Abies concolor* saplings (Battles et al. 2001). Plots were chosen based on the presence of at least four colonies of either *P. picta* or *C. umbellata*. To minimize the effects of environmental stochasticity on leaf carbon isotope signatures, plots were limited to ≤ 2 m^2. Each plot also contained a seedling of *P. menziesii*, *P. lambertiana*, *A. concolor*, or in one plot *Taxus brevifolia*, that were used as a reference of a fully autotrophic understory plant. Average understory light environment across all plots was calculated based on instantaneous 15 s averages of light measurements made with a Li-Cor 250-A (Li-Cor Biosciences, Lincoln, NE, USA) held in every plot at midday on a single sampling day. To make each measurement, the Li-Cor light sensor was held level on the forest floor next to each Pyroleae colony or reference plant. The average light

across all plots and treatments was 62 μmol m^{-2} s^{-1}, but due to the heterogeneity of light reaching the forest understory, values ranged from 5 to 386 μmol m^{-2} s^{-1}.

In early May, directly after snowmelt, leaf samples from each Pyroleae colony as well as conifer seedlings from every plot were collected at midday for baseline carbon isotope data (time = 0). Leaves were put immediately on dry ice for transport back to UC Berkeley where they were freeze-dried and kept dry for soluble sugar extraction and stable carbon isotope analysis (see below). After the first leaf samples were collected, four treatments manipulating light and/or access to mycorrhizal networks (MNs) were applied to Pyroleae colonies in each plot, and an additional light manipulation treatment was applied to the conifer seedlings. In total, there were 10 replicates of four treatments per Pyroleae species, plus 20 replicates for conifer seedlings for a grand total of 100 treatments (Table 1).

The treatments were applied in full factorial design on a per plot basis in the following way: (A) Pyroleae colonies of both species were covered with 50% shade cloth to alter the light environment, (B) colonies of both species were covered with 50% shade cloth and a 0.7-m-deep trench was dug 0.5 m around colonies to exclude any connections with mycorrhizal networks, (C) a 0.7-m-deep trench was dug around colonies of both species of Pyroleae, (D) an additional colony per plot was left un-shaded with no trench (control), and (E) 50% shade cloth was applied to the conifer seedling in each plot as an autotrophic reference plant. Approximately 2 weeks after the treatments were in place, leaf samples were collected at midday from each Pyroleae treatment and every reference plant (time 1, day 16) and, as with the baseline samples, leaves were placed immediately on dry ice and freeze-dried back at the laboratory. After samples were collected, treatments B and C were retrenched to sever any possible newly formed hyphal bridges between Pyroleae colonies and surrounding roots (Simard et al. 1997; Teste et al. 2006). The sampling scheme was repeated approximately every 2 weeks throughout the course of the growing season, resulting in a total of four sampling periods from 15 May to 26 June 2008; here-on referred to as time 0 to time 3. In total, the experiment ran for 44 days (Fig. 2 and Supplementary Fig. 1). Due to the limited number of leaves on our test species and reference plants, samples from some treatments were not collected at sampling time 3 and one *Chimaphilia umbellata* plot's sample was lost from treatment C, time 1.

Leaf soluble sugar extraction

In the laboratory, freeze-dried leaf samples were ground to a fine powder. About 150 mg of leaf material was used for soluble sugar extraction, following the protocol of Brugnoli et al. (1988) with a few modifications. Briefly, leaf soluble

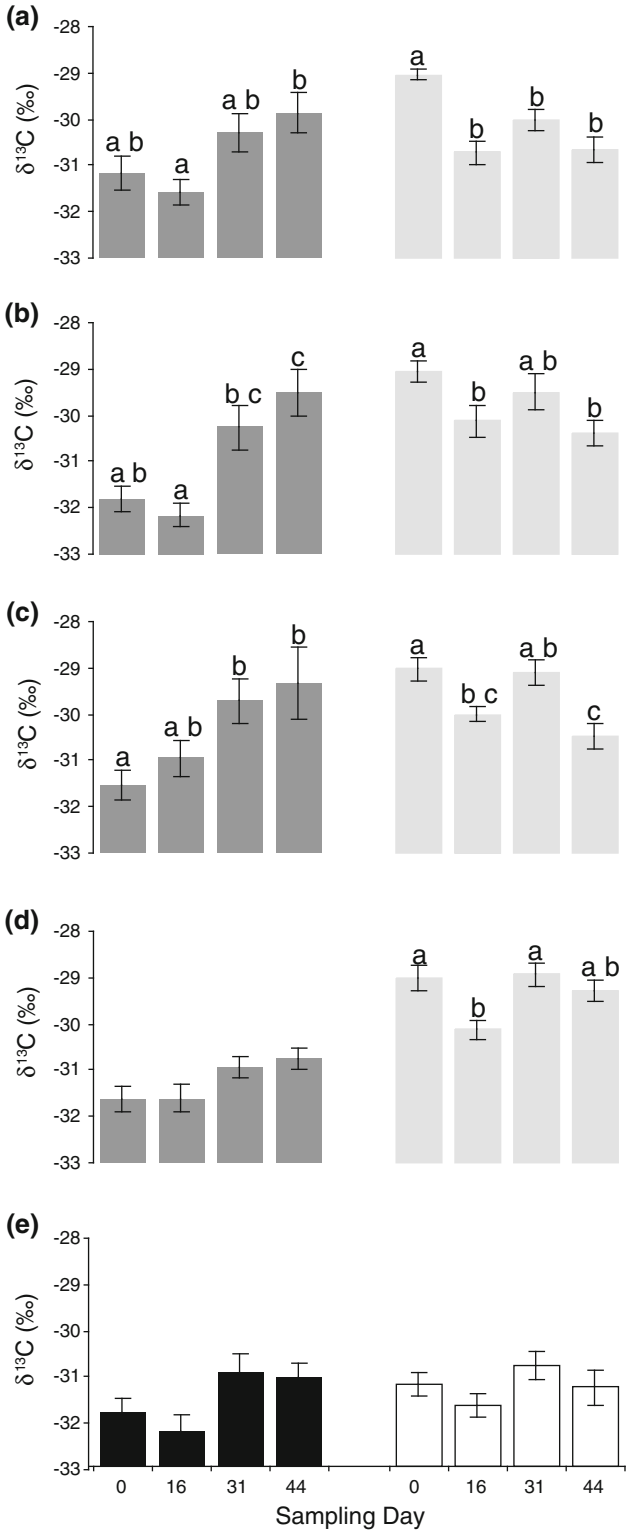

Fig. 2 Mean $\delta^{13}C$ values from the leaf soluble sugars of *Pyrola picta* (*dark gray bars*), *Chimaphila umbellata* (*light gray bars*), reference plants from *P. picta* plots (*black bars*) and *C. umbellata* plots (*open bars*) under treatments A–E over time: **a** treatment A (shade); **b** treatment B (shade + trench); **c** treatment C (trench); **d** control; **e** treatment E (shaded reference plant). *Error bars* represent one standard error. *Letters* that are different per species and treatment are significant at $\propto \leq 0.05$

sugars were extracted in water by lysing the cells with equal weight of polyvinylpolypyrrolidone (PVPP 1:1, w/w) and shaking at room temperature for 45 min. Samples were then centrifuged at 12,000 rpm for 20 min and the supernatants containing the soluble sugars were purified with Dowex-50 (H^+) resin (Sigma-Aldrich, St. Louis, MO, USA) for the separation of amino acids from organic acids and sugars, and Dowex-1 (Cl^-) resin (Sigma-Aldrich) for the separation of organic acids from sugars. The purified sugars of each sample from every sampling and treatment were freeze-dried for carbon stable isotope analysis. Sugar standards at 25 and 100 mM concentrations were regularly processed by the method outlined above and analyzed for their $\delta^{13}C$ values as a quality control for the extraction and purification steps.

Stable isotope analysis

Freeze-dried leaf soluble sugars were analyzed for carbon stable isotope ratios via elemental analyzer/continuous flow isotope ratio mass spectrometry (ANCA/SL elemental analyzer coupled with a Thermo Finnigan Delta^{Plus} XL, gas phase isotope ratio mass spectrometer; Bremen, Germany). Carbon isotope ratios are expressed in the common δ notation as:

$$\delta^{13}C = \left(R_{sample}/R_{\text{standard}} - 1\right) 1,000\,[‰] \quad (1)$$

where R_{sample} and R_{standard} are the ratios of heavy isotope to light isotope of a sample and the standard respectively. Standards included sucrose, NIST 1577 bovine liver, and NIST 1547 peach leaf. As an additional quality control measure; randomly selected leaf soluble sugar samples from previous runs were re-run to check for precision in their $\delta^{13}C$ values. When there was enough leaf material remaining after sugar extraction, leaf tissue from sampling time 0 (prior to treatment) and sampling time 3 (44 days of treatment) from each plot, and all five treatments were analyzed for bulk $\delta^{13}C$ and percent N. The total number of samples analyzed for each species, sampling time, and treatment are summarized in Table 1. All isotope analyses were conducted at the Center for Stable Isotope Biogeochemistry at the University of California, Berkeley. Long-term (5+ year) external precision for C isotope analyses is 0.1‰.

Calculation of fungal carbon contribution to leaf soluble sugars' $\delta^{13}C$ and statistics

To isolate the contribution of ^{13}C enriched fungal derived carbon on the $\delta^{13}C$ of Pyroleae species' leaf soluble sugars, we have developed the following enrichment factor, $\varepsilon_{\text{fungal}}$, as:

$$(\delta^{13}C_A - (\delta^{13}C_B - \delta^{13}C_C)) - \delta^{13}C_D = \varepsilon_{fungal} \quad (2)$$

where $\delta^{13}C$ refers to the delta ^{13}C value of the soluble sugars of either *P. picta* or *C. umbellata*, the subscript refers to the treatment (A–D), and $\varepsilon_{\text{fungal}}$ is a site-independent enrichment factor reflecting the influence of decreased light availability on test species' C acquisition strategy. Equation 2 normalizes $\delta^{13}C$ for the impacts of both trenching and shading that are independent from any fungal effects. For each plot, the $\delta^{13}C$ values of treatment C are subtracted from treatment B to isolate the effect of shade on Pyroleae species leaf soluble sugars $\delta^{13}C$ that is independent of mycorrhizal networks (MNs). Then, this value is subtracted from treatment A to isolate the signature of ^{13}C-enriched fungal carbon transferred through MNs to Pyroleae species. Lastly, treatment D (control) $\delta^{13}C$ values are subtracted from the other treatments to normalize $\delta^{13}C$ for environmental effects, such as differences in humidity, temperature, and CO_2 concentrations throughout the growing season. This final step normalizes

Table 1 Number of leaf samples collected for either soluble sugar and bulk leaf tissue stable carbon isotope analysis and percent leaf nitrogen at four sampling times (t = 0–3) from *Pyrola picta* (*PYPI*) and *Chimaphila umbellata* (*CHUM*) plots under treatments A–E

Plot	Treatment (A–E)	Sugars				Bulk/*n*	
		$t = 0$	$t = 1$	$t = 2$	$t = 3$	$t = 0$	$t = 3$
PYPI	A = shade	10	10	10	8	6	5
	B = shade + trench	10	10	10	9	6	3
	C = trench	10	10	10	9	6	4
	D = control	10	10	10	10	4	6
	E = shaded reference	10	10	10	9	5	6
CHUM	A	10	10	10	10	2	6
	B	10	10	10	10	1	6
	C	10	9	10	10	1	6
	D	10	10	10	9	3	6
	E	10	10	10	9	3	6

for differences in $\delta^{13}C$ across plots and over time (Preiss and Gebauer 2008; Dawson et al. 2002). ε_{fungal} was calculated per Pyroleae species, per plot, and per sampling. The ε_{fungal} values from time 0 represent the baseline isotope signatures for each test species to which ε_{fungal} values post-treatment were compared. Enrichment factors were averaged across plots for each sampling interval per species, and standard errors around the means were calculated (Fig. 3). Any deviations from 0‰ in ε_{fungal} values from the two test species at time 0 indicate the slight intraspecific differences in leaf soluble sugars $\delta^{13}C$. ε_{fungal} values from sampling times 0–3 were tested for normality using a Shapiro–Wilks test and compared to each other using a multi-way ANOVA and post hoc Tukey's HSD. For a plant capable of mycoheterotrophy, the expected output of Eq. 2 is that, as light availability decreases, ε_{fungal} increases (becomes more enriched in ^{13}C), whereas for a primarily autotrophic plant, ε_{fungal} should decrease with decreasing light availability.

Due to the non-normal distribution of $\delta^{13}C$ values of the leaf soluble sugars for both species, the effect of time and treatment were determined using non-parametric Mann–Whitney *U* tests with sequential Bonferroni corrected *P* values (Fig. 2; Supplementary Fig. 1). For samples where bulk leaf tissue $\delta^{13}C$ was analyzed, these values were compared to the same sample's soluble sugars $\delta^{13}C$ values at each sampling time via independent Student's *t* tests (Fig. 4). Change in percent leaf nitrogen from sampling times 0 and 3 within every treatment for Pyroleae species and reference plants were compared via independent Student's *t* tests (Supplementary Fig. 2). All *P* values were considered statistically significant at $\alpha \leq 0.05$.

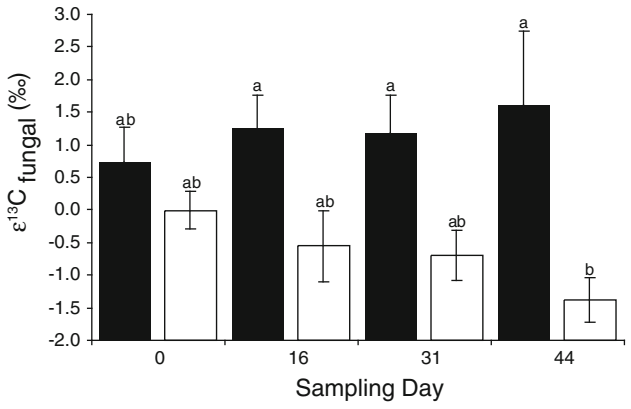

Fig. 3 Mean enrichment in ^{13}C per species and per sampling time based on the output of the isotope enrichment factor calculation ($\varepsilon^{13}C_{fungal}$), *Pyrola picta* (*black bars*) and *Chimaphila umbellata* (*open bars*), *error bars* represent one standard error. Calculation of $\varepsilon^{13}C_{fungal} = (\delta^{13}C_{shade} - (\delta^{13}C_{shade\&trench} - \delta^{13}C_{trench}) - \delta^{13}C_{control}$. Different *letters* are significant at $\propto \leq 0.05$

Results

$\delta^{13}C$ values of leaf soluble sugars by treatment over sampling time

In the *Pyrola picta* plots, the $\delta^{13}C$ values from treatment A (shading) were significantly more enriched in ^{13}C at time 3 compared to time 1 ($P = 0.006$), but were not significantly different at sampling time 0 and time 3 ($P = 0.101$; Fig. 2). In contrast, the $\delta^{13}C$ values of *Chimaphila umbellata* under treatment A became significantly more depleted in ^{13}C over the 44-day sampling period (time 0 vs.

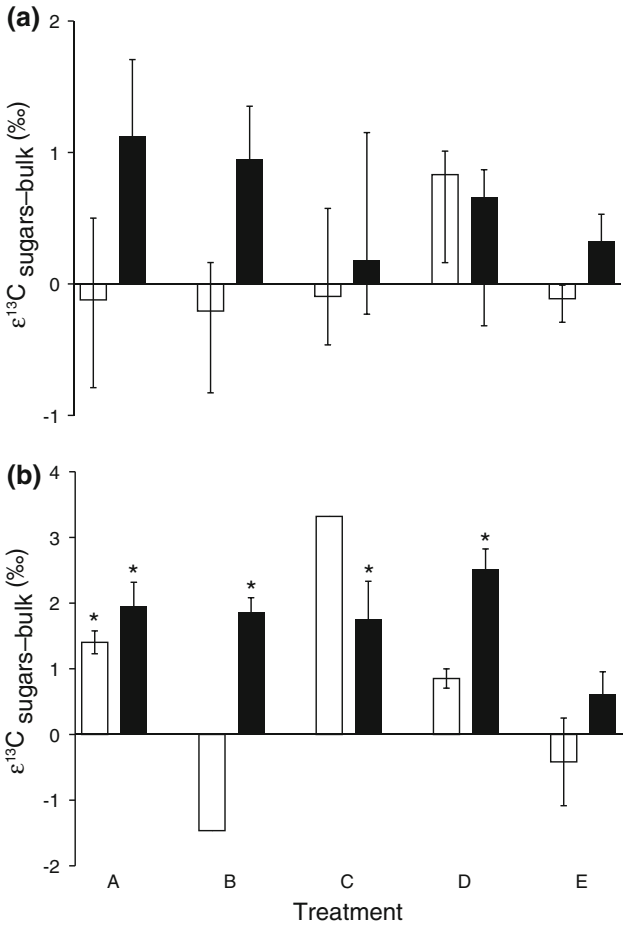

Fig. 4 Mean differences in the $\delta^{13}C$ of leaf soluble sugars and bulk leaf tissue of **a** *Pyrola picta* and **b** *Chimaphila umbellata* from sampling time 0, prior to treatment (open bars) to sampling time 3, 44 days of treatment (*black bars*). Treatment $A = 50\%$ shade screen, treatment $B = 50\%$ shade screen and 0.7 m deep trench, treatment $C = 0.7$ m deep trench, treatment $D =$ control, treatment $E = 50\%$ shade screen applied to a neighboring conifer seedling. Statistical comparisons were made between the mean $\delta^{13}C$ values of bulk leaf tissue and leaf soluble sugars at sampling time 0 or time 3 per treatment and per test species. *Asterisks* represent significant differences between the $\delta^{13}C$ values of bulk leaf tissue versus soluble sugars at $\propto \leq 0.05$. *Error bars* represent one standard error

1 $P < 0.001$, time 0 vs. 2 $P = 0.008$, time 0 vs. 3 $P < 0.001$). For treatment B (shading and trenching), both species showed trends similar to treatment A. *Pyrola picta* was significantly more enriched in ^{13}C at time 3 compared to time 0 ($P < 0.001$; Fig. 2), while *C. umbellata* became significantly more depleted in ^{13}C from time 0 to 3 ($P = 0.025$). The effects of treatment C (trenching) on the $\delta^{13}C$ values of both species was again similar to the other two treatments where *P. picta* became significantly more enriched in ^{13}C over the course of the sample period (time 0 vs. 2 $P = 0.045$, time 0 vs. 3 $P = 0.006$; Fig. 2) while *C. umbellata* became significantly more depleted in ^{13}C (time 0 vs.1 $P = 0.024$, time 0 vs. 3 $P = 0.006$; Fig. 2). In the control treatments (treatment D), the $\delta^{13}C$ values of *P. picta* did not change significantly over the course of the growing season, while at sampling time 1 (day 16), the *C. umbellata* controls were significantly more depleted in ^{13}C than at the sampling prior to treatment ($P = 0.02$) and 31 days into the experiment (time 1 vs. 2 $P = 0.012$; Fig. 2). For the shaded autotrophic reference plants, no statistically significant differences in $\delta^{13}C$ among sampling periods for all 20 plots were detected (Fig. 2). Overall, at the end of the sampling time (day 44), the mean $\delta^{13}C$ values for every *C. umbellata* treatment except the controls showed significant ^{13}C depletion (Fig. 2). There was also a trend toward ^{13}C enrichment in the average $\delta^{13}C$ values of *P. picta* leaf soluble sugars that was significant for all treatments except A (shaded) and treatment D (controls) which did not vary significantly.

$\delta^{13}C$ values of leaf soluble sugars by sampling time over treatments

At time 0, there were no significant differences between the $\delta^{13}C$ values of the leaf soluble sugars from *P. picta* and the reference plants (Supplementary Fig. 1). After 44 days of manipulation, the $\delta^{13}C$ of leaf soluble sugars from *P. picta* under each treatment could not be differentiated from each other or from the shaded reference plants (Supplementary Fig. 1). Conversely, *C. umbellata* individuals were significantly more enriched in ^{13}C prior to treatment than surrounding references (treatments A–D time 0 vs. treatment E time 0, $P < 0.001$). Over time, $\delta^{13}C$ values for all treatments, except the controls (treatment D time 3 vs. treatment E time 3, $P = 0.02$), became more depleted in ^{13}C, and were statistically indistinguishable from the $\delta^{13}C$ values of the shaded references (Supplementary Fig. 1). Also, *Chimaphila umbellata* treatments A (shaded), and C (trenched) were significantly different from the controls at the end of the sampling time (treatment A time 3 vs. treatment D time 3, $P = 0.027$, and treatment C time 3 vs. treatment D time 3, $P = 0.032$; Supplementary Fig. 1).

Influence of fungal carbon contributions to Pyroleae species leaf soluble sugars $\delta^{13}C$

Isotope enrichment factor calculations (see "Materials and methods") showed that the ε_{fungal} values of *P. picta* and *C. umbellata* responded differently over time (Fig. 3). Though both species started at time 0 (prior to treatment) with ε_{fungal} values that were not significantly different from one another ($P = 0.233$), by the end of the experiment, *C. umbellata* was significantly more depleted in ^{13}C compared to the time prior to treatment (time 0 vs. time 3 $P = 0.008$) and compared to *P. picta* at sampling times 1, 2 and 3 ($P = 0.016$, 0.02 and 0.016, respectively; Fig. 3). The ε_{fungal} values of *P. picta* were not significantly different from each other over the course of the sampling period, although the mean ε_{fungal} at time 3 was over twice that observed at time 0 (Fig. 3).

$\delta^{13}C$ values of bulk leaf tissue compared to leaf soluble sugars within treatments and over sampling time

For *P. picta* and *C. umbellata* plots, the bulk leaf tissue $\delta^{13}C$ values within a treatment and between sampling times 0 and 3 were not significantly different from one another. In the *P. picta* plots under all four treatment types (A–D) and throughout the course of the experiment, there were no significant differences in bulk leaf tissue $\delta^{13}C$ compared to the leaf soluble sugars. This was also true for the $\delta^{13}C$ values of the reference plants from *P. picta* plots (Fig. 4). For *C. umbellata* treatments A, B, C and D at time 3, and treatment A at time 0, $\delta^{13}C$ values of the leaf soluble sugars were consistently, and significantly, more enriched in ^{13}C compared to respective bulk leaf tissue (all P values <0.02; Fig. 4). However, this was not the case for $\delta^{13}C$ values from the leaf soluble sugars and bulk leaf tissue of the reference plants, which were not significantly different from each other at either sampling time (Fig. 4).

Percent leaf N in treatments and over sampling time

Percent leaf nitrogen decreased in *C. umbellata* under all treatments from the time prior to the beginning of the treatments to 44 days after treatments were in place. Differences in percent leaf nitrogen in *C. umbellata* were statistically significant from sampling time 0 to time 3 in treatments A–C (A time 0 vs. 3 $P = 0.013$, B time 0 vs. 3 $P = 0.035$, C time 0 vs. 3 $P = 0.041$; Supplementary Fig. 2), but not in the controls. Analytical power for these tests was diminished by a limited number of samples of *C. umbellata* plants at time 0 under treatments A–C (Table 1). Percent leaf nitrogen did not change significantly under treatments or in controls of *P. picta* plants nor did it change in the reference plants from the *P. picta* and *C. umbellata* plots (Supplementary Fig. 2).

Overall, *C. umbellata* plants prior to treatment application, and *P. picta* plants both before, and 44 days after, treatment had higher percentages of leaf nitrogen than reference plants (Supplementary Fig. 2).

Discussion

Field manipulations and leaf soluble sugar $\delta^{13}C$ to detect mycoheterotrophy

In this study, we manipulated light and plant access to mycorrhizal networks and used leaf soluble sugars $\delta^{13}C$ to track changes in a plant's dependency on carbon gains mediated by mycorrhizal fungi. We relied on changes in $\delta^{13}C$ of each test species in the absence of MNs (treatments B and C) to assess the contribution of fungal carbon under increased light limitation when MNs were left in tact (treatment A). By targeting the $\delta^{13}C$ of leaf soluble sugars, we were able to detect significant effects of the treatments over the course of the sampling period that would have gone undetected if only bulk leaf $\delta^{13}C$ was analyzed (Fig. 4). This is especially clear from treatment A (shading) in *P. picta* plots, where the average $\delta^{13}C$ of leaf soluble sugars became more enriched in ^{13}C by approximately 2‰, whereas average bulk $\delta^{13}C$ remained relatively constant over time (Fig. 4). Based on leaf soluble sugar $\delta^{13}C$, our two test species showed different responses to the treatments that we suggest are caused by their differing physiologies and their distinct C sources.

Dependency of Pyroleae species on fungal derived C under increased shade

To determine the level of mycoheterotrophy under reduced light conditions, we developed and applied a carbon isotope model based on an isotope enrichment factor approach similar to that reported by Preiss and Gebauer (2008) and Emmett et al. (1998). The model uses the $\delta^{13}C$ data from leaf soluble sugars instead of $\delta^{13}C$ of bulk leaf tissue. The model also adjusts $\delta^{13}C$ to compensate for the impacts of our treatments on $\delta^{13}C$ that are independent from the influence of MNs. For the purposes of this study, we assumed that the leaves of our test species were sinks for either autotrophically or mycoheterotrophically derived carbon. We believe that there are three lines of support for this assumption: (1) Pyroleae species have evergreen leaves that are known to maintain year-round growth (Whigham 2004), (2) we conducted our experiment during the beginning of the growing season when leaves are well known to have very high (and perhaps the highest) demand for carbon (Wardlaw 1990), and (3) even though plants in dark understories often operate at or near the compensation point between being a source versus a sink for carbon, they can be effective carbon sinks during periods where photosynthetic carbon gains are greater than respiratory losses (Chazdon 1988). At the first sampling prior to treatment (time 0), ε_{fungal} values for *P. picta* and *C. umbellata* were similar and close to 0‰ (Fig. 3). Our result that the two test species began at essentially the same ε_{fungal} values validates the efficacy of Eq. 2 in normalizing $\delta^{13}C$ between species and across plots that may be experiencing different environmental conditions. Interestingly, *P. picta* ε_{fungal} values at time 0 were on average slightly more enriched than those of *C. umbellata*, indicating that some carbon gains via fungi may be intrinsic to the physiology of *P. picta* even under ambient light conditions (Fig. 3). The most important model expectation for plants capable of mycoheterotrophy is that, under increased light limitation, ε_{fungal} will increase over time (plants will become more enriched in ^{13}C). Conversely, in a plant species that is incapable of mycoheterotrophy and relies primarily on C gains via photosynthesis, under decreased light availability, ε_{fungal} will decrease. Despite the parallel isotope effects of each treatment on our test species, the aforementioned patterns were partially shown. Over time, *C. umbellata*'s ε_{fungal} values became significantly more depleted (time 0 vs. 3 $P = 0.008$; Fig. 3), indicating that this species is primarily autotrophic. However, even though *P. picta*'s mean ε_{fungal} at the end of the experiment had more than doubled from the time prior to treatment, based on the enrichment calculation this species did not become significantly more enriched in ^{13}C over time (time 0 vs. 3 $P = 0.44$). Therefore, *P. picta* does not appear to be fully autotrophic and may indeed be partially mycoheterotrophic, but other factors besides light are important in determining its ability to tap into mycorrhizal networks for carbon. Our findings, as well as previous investigations of green Pyroleae species bulk leaf carbon and nitrogen stable isotope values, give indications that some species in this tribe are capable of partial mycoheterotrophy (Tedersoo et al. 2007; Zimmer et al. 2007; Hynson et al. 2009). However, in general, Pyroleae species investigated so far do not appear to be as dependent on mycoheterotrophic C gains as some green terrestrial orchids (Bidartondo ert al. 2004; Julou et al. 2005; Abadie et al. 2006).

Ecophysiological considerations on Pyroleae species $\delta^{13}C$ leaf soluble sugars under shading and trenching treatments

Our field manipulations allowed us to detect some common patterns in the $\delta^{13}C$ values of the leaf soluble sugars for our two test species, as well as for the reference plants. For example, the controls of our two test species showed no significant change in $\delta^{13}C$ over time (Fig. 2), indicating

that the changes in $\delta^{13}C$ of our two test species while under treatments A–C were due to primarily a treatment effect rather than changes in the environment that could influence carbon isotope discrimination.

Even though *P. picta* and *C. umbellata* are within the same tribe (Liu et al. 2010; Freudenstein 1999), have similar mycorrhizal fungal associates (Massicotte et al. 2008), similar phenologies and geographic ranges in the western U.S.A., and are often found in the same habitats, the results of this study show that they seem to have significantly different physiological responses to environmental and biological variables. From the first sampling prior to treatment application, over the 44-day course of the experiment, average $\delta^{13}C$ of leaf soluble sugars from *P. picta* plants were not statistically differentiated under decreased light availability and access to MNs, or both (Supplementary Fig. 1). Conversely, the $\delta^{13}C$ leaf soluble sugars of *C. umbellata* became more depleted under the same treatments (Supplementary Fig. 1). If we focus just on changes in $\delta^{13}C$ under treatment A (shade) over time, the leaf soluble sugars of *P. picta* became increasingly enriched in ^{13}C (Fig. 2). Without determining the effect of shade in the absence of MNs, this ^{13}C enrichment could be due either to an increased import of ^{13}C enriched carbon from heterotrophic tissues, such as the starchy rhizomes of the plants (Cernusak et al. 2009 and references therein), or to a proportional increase in the import of ^{13}C enriched carbon from MNs relative to photosynthetically derived C (Preiss et al. 2010). Under the same shade treatment, *C. umbellata* became increasingly more depleted in ^{13}C over the course of the experiment (Fig. 2). A depletion in ^{13}C under increased shade is a common trend among autotrophic C3 plants (Michelsen et al. 1996; Göttlicher et al. 2006) due either to an increase in stomatal conductance, and increase in C_i/C_a leading to greater discrimination by RuBisCO against ^{13}C (Farquhar et al. 1989; Lloyd et al. 1992; Berry et al. 1997), or to an overall decrease in photosynthetic rate which would also lead to an increase in C_i/C_a (Farquhar et al. 1989; Zimmerman and Ehleringer 1990).

We found that *P. picta* and *C. umbellata* responded differently to cutting off MNs through trenching (treatment C). Trenching and severing hyphal networks and roots may have affected the water availability in our *P. picta* plots and the water status of *P. picta* plants. A decrease in water availability could lead to an increase in ^{13}C enrichment over time as previous research has shown (Ehleringer et al. 1986, 1991; Farquhar et al. 1989). However, most previous trenching studies that have focused on understory plants found that trenching actually significantly increases water availability due to the release of water from severed roots (Teste et al. 2006; Coomes and Grubb 2000; Simard et al. 1997). This release of water would lead to a decrease in $\delta^{13}C$ due to an increase in stomatal conductance and increased discrimination by RuBisCO against ^{13}C (Farquhar et al. 1989; Zimmerman and Ehleringer 1990). These contrasting results suggest that future studies need to consider physiological measurements of plant–water relationships to assess if in fact they do experience water deficits. Finally, we found a significant depletion in $\delta^{13}C$ for trenched *C. umbellata* plants over time (Fig. 2). The causes for this response are not clear, but may be explained by an overall decrease in photosynthetic rate, an increase in the availability of water, or ^{13}C depleted soil-respired CO_2 (Ehleringer et al. 1986; 1991; Coomes and Grubb 2000). Therefore, while the stable isotope approach used here provides new insights into the physiology of Pyroleae species, measurements of gas exchange and soil respiration $\delta^{13}C$ among our treatments over time would have provided additional useful information on the carbon balances of our test species.

In treatment B, where both light and access to MNs were manipulated by shading and trenching, the effects on leaf soluble sugars $\delta^{13}C$ were similar to those of treatments A and C on each test species. The observed increasing trend of ^{13}C enrichment in *P. picta*, and decreasing trend of ^{13}C enrichment in *C. umbellata* for treatment B were intermediate to that of treatments A and C except at sampling time 1 in *P. picta* plots (Supplementary Fig. 1). The intermediate $\delta^{13}C$ values after treatment B indicate that there was an averaging effect of the two treatments on the leaf soluble sugars $\delta^{13}C$, where the $\delta^{13}C$ values of each test species under treatment B represent a mixture of the influences of both shading and trenching.

Final remarks

To date, studying mycoheterotrophy has led researchers to new understandings of the prevalence of mycorrhizal networks. In this investigation, using field manipulations and a stable isotope approach based on $\delta^{13}C$ of leaf soluble sugars, we have demonstrated that two closely related understory species differ in their degree of dependency on mycorrhizal networks to meet their carbon demands. It is important to note that, though soluble sugars were targeted as the transfer compound from EM fungi to our test species, there is currently little known on the actual biochemical pathways and compounds involved in mycoheterotrophic systems. Quantifying complete carbon and nitrogen budgets for mycoheterotrophs would provide much needed constraints to create more realistic models of plant–fungal interactions as well as provide new insights into the economy of below ground networking.

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