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RESEARCH ARTICLE

Stable isotope analysis indicates partial mycoheterotrophy in arbuscular mycorrhizal woody seedlings in tropical forests

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Abstract

1. Chlorophyllous plants exhibiting partial mycoheterotrophy obtain carbon through mycorrhizal interactions in addition to photosynthesis. In arbuscular mycorrhizal (AM) plants, the *Paris*-morphotype (i.e. hyphal coils) is considered essential for mycoheterotrophic carbon gains. Numerous tree species in tropical lowland forests form this morphotype, and under light- and nutrient-limitation, additional carbon gain would be beneficial. However, if seedlings of woody species in the understory of tropical lowland forests exhibit partial mycoheterotrophy remains unexplored.
2. Here we (a) examined the AM morphotype (*Paris*- or *Arum*-type) in seedlings of 41 tropical woody species, and (b) to determine if any of the target *Paris*-type species are partially mycoheterotrophic, we compared their multi-element stable isotope natural abundance (^{13}C, ^{2}H, ^{18}O, ^{15}N) with neighbouring autotrophic non-*Paris*-type reference seedlings.
3. About 50% of the investigated species (and 80% of the genera) exhibited the *Paris*-type, expanding the number of tropical plant genera with *Paris*-type AM. Enrichment in ^{13}C, but not in ^{18}O in target compared with neighbouring reference plants indicated partial mycoheterotrophy in seedlings of 6 of the 21 investigated *Paris*-type AM species.
4. Our results indicate for the first time that carbon gain through mycoheterotrophy occurs in seedlings of AM tropical tree species. In tropical forests, partial mycoheterotrophy during seedling establishment may confer so far unrecognised ecological advantages influencing seedling recruitment and ecosystem dynamics.

KEYWORDS
C transfer, endomycorrhiza, lowland forest, *Paris*-type, saplings, stable isotope natural abundance, trees

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1 | INTRODUCTION

The vast majority of plants are associated with mycorrhizal fungi, which optimise their nutrient and water supply from the soil. In return, mycorrhizal fungi receive organic carbon (C) compounds from the plant associates (Smith & Read, 2008). This mutualistic interaction, however, can shift to an exploitive disparity, where plants often referred to as 'cheaters' gain C from their fungal partners—a nutritional mode called mycoheterotrophy (Leake, 1994; Merckx, 2013). Some plants use fungal-derived C (either from photosynthesising plants or from saprotrophic nutrition) to such an extent that they lost their photosynthetic ability and have become achlorophyllous. This phenomenon, which is known as full mycoheterotrophy, occurs in several hundred endo-mycorrhizal plant species (Merckx, 2013).

Some chlorophyllous mycorrhizal plant species gain C from their associated fungi in addition to their own photosynthetic C gain, that is, they exhibit partial mycoheterotrophy. This nutritional mode has been recognised in herbaceous plants, mostly from the Orchidaceae, Ericaceae, and some Gentianaceae. It is considered, *inter alias*, a strategy to gain additional C where photosynthesis is severely light-limited (Cameron & Bolin, 2010; Gebauer & Meyer, 2003; Hynson et al., 2013; Preiss et al., 2010; Zimmer et al., 2007). Recent evidence from stable isotope analyses shows that in herbaceous species additional C gain from fungal partners (i.e. partial mycoheterotrophy) is considerably more widespread than previously assumed (Gebauer et al., 2016; Giesemann et al., 2021).

Over 70% of flowering plant species are associated with endosymbiotic arbuscular mycorrhiza (AM; Brundrett, 2017). The AM fungal partners belong to the subphylum Glomeromycotina within the phylum Mucoromycota (Spatafora et al., 2016). AM is typical for most herbaceous (i.e. non-woody) plant species, and has been especially well studied for agricultural plants (Genre et al., 2020). In forest ecosystems, AM associations dominate in tropical lowland trees (e.g. Alexander, 1989; Mangan et al., 2004), whilst ectomycorrhizal associations prevail in boreal and temperate biomes (Baldrian et al., 2023). Studies of C transfer between woody plant species have mainly focussed on ectomycorrhizal plants (e.g. Avital et al., 2022; Cahanovitc et al., 2022; Klein et al., 2023; Simard et al., 1997), but rarely AM plants (e.g. Avital et al., 2022; Lerat et al., 2002), and these studies did not focus on partial mycoheterotrophy.

With about 43,000 tree species, tropical forests contain by far the highest tree diversity of any biome, yet, they belong to the most threatened terrestrial systems (Wright, 2010). Intact tropical forests provide important ecosystem services, acting as tremendous C pools and sinks (Brockerhoff et al., 2017; Harris et al., 2021; Pan et al., 2011). Vegetation-climate feedbacks with tropical forests are crucial for global climate change projections, and their C relations have thus received considerable attention (Hubau et al., 2020; Strengers et al., 2010). Yet, if woody plants in tropical forests supplement C from fungi through partial mycoheterotrophy has not been addressed (Kuyper & Jansa, 2023).

Seedlings in the shaded understory of tropical forests are severely light-limited (Holste et al., 2011; Pearcy, 1987), with light conditions often below the light compensation point (Baltzer & Thomas, 2007). Yet, seedlings of many species can survive, that is, maintain a positive C balance, for many years (Hubbell et al., 1999; Rüger et al., 2009). Additionally, soil phosphorus (P) availability to plants is low in many tropical forests (Turner et al., 2007; Turner & Engelbrecht, 2011; Vitousek, 1984), with nutrient limitation restricting plant growth (Holste et al., 2011; Santiago et al., 2012). During the dry season water availability to plants is reduced, also limiting C gains from photosynthesis (Comita & Engelbrecht, 2009; Engelbrecht et al., 2002; Santiago et al., 2017). Under these conditions, an ability for additional C gain through mycoheterotrophy might provide a significant ecological and evolutionary advantage. In fact, mycoheterotrophic AM plant lineages probably evolved in tropical forests (Merckx & Bidartondo, 2008), where light and P (but not N) are limiting factors. At the same time, AM allowed various achlorophyllous fully mycoheterotrophic herbacous taxa to diversify in the tropics (Gomes et al., 2019; Merckx, 2013).

A specific AM morphotype, the *Paris*-morphotype (named after its occurrence in *Paris quadrifolia*), occurs in all achlorophyllous fully mycoheterotrophic plants and is thus considered indispensable for their fungal C gain (Imhof, 1999). This morphotype is characterised by intracellular hyphal coils that colonise the plant root cells (Gallaud, 1905). In contrast, a second AM morphotype, the *Arum*-type (named after its occurrence in *Arum maculatum*), has intercellular hyphae with emerging branched fungal structures (i.e. arbuscules). Chlorophyllous herbaceous partially mycoheterotrophic AM plant species in the understory of temperate forests also all exhibited the *Paris*-type AM, whilst plants with *Arum*-type AM exhibited autotrophy (Giesemann et al., 2021; Giesemann, Rasmussen, et al., 2020). *Paris*-type AM is thus considered to be a prerequisite for fungal C gain in both achlorophyllous (fully mycoheterotrophic) and chlorophyllous (partially mycoheterotrophic) AM plant species, yet not all plant species with *Paris*-type AM gain C from fungi (Giesemann et al., 2021; Murata-Kato et al., 2022).

The *Paris*-morphotype occurs in a significant proportion (≈40%) of all AM plants (Dickson et al., 2007). Thus, a large share of AM plant species globally may potentially exhibit partially mycoheterotrophic C gain (Giesemann et al., 2021). So far, research on PHM has focussed on herbaceous understory plants (Giesemann et al., 2021; Giesemann, Rasmussen, et al., 2020; Kusakabe et al., 2024; Murata-Kato et al., 2022).

Quite a number of common tropical tree species are in genera that form *Paris*-type AM, indicated for example by a comparison of a comprehensive review of AM morphotypes (Dickson et al., 2007) with tree species occurring in forests of Central Panama (Condit et al., 2013). About 31 plant genera included in the review contain woody species that occur in the Panama Canal area (Condit et al., 2013). Thereof, 18 genera (which include 64 woody species in Central Panama) included species characterised as *Paris*-type (and 13 genera with 51 species as *Arum*-type, Dickson et al., 2007). Combined with the potential ecological advantages that partial mycoheterotrophy might confer to plants in the understory of tropical

forests, we expect that mycoheterotrophy might occur, or even be widespread amongst seedlings of tropical tree species. Since all AM fungi are obligate biotrophic, that is, gain their C from living host plants (Trépanier et al., 2005), and lack saprotrophic capability (Tisserant et al., 2013), that would imply C sharing amongst trees via common mycorrhizal networks (Horton, 2015; Merckx et al., 2024). This in turn may have pervasive, yet entirely unrecognised implications for the composition, productivity, and C cycle of tropical forests. However, investigations on partial mycoheterotrophy in AM woody species are currently lacking in tropical forest ecosystems as well as in other ecosystems.

Mycoheterotrophic understory plants that utilise AM fungi as a C source can be identified by comparing their natural stable isotope signature to autotrophic plants co-occurring under the same environmental conditions. Tissues of mycoheterotrophic plants are enriched in ^{13}C, reflecting a relative ^{13}C enrichment of their fungal C source, that is, the AM fungal hyphae (Courty et al., 2011; Gomes et al., 2020, 2023; Klink et al., 2020, 2022), which in turn is due to the fact that the fungi take up ^{13}C-enriched carbohydrates from their photosynthesising host plants (Gleixner et al., 1993). Significant ^{2}H enrichment of leaves can serve as an additional and independent indication for fungus-to-plant C transfer (Cormier et al., 2018; Gebauer et al., 2016), because secondary heterotrophic organic compounds originating from fungi are often enriched in ^{2}H compared with primary photosynthetic compounds (Yakir, 1992). However, ^{13}C and ^{2}H abundances of leaves are also strongly influenced by stomatal regulation. Leaf ^{18}O isotope abundances, which are affected by transpiration but not by mycoheterotrophic C exchange, can be used to separate the effect of mycoheterotrophic C gains on ^{13}C or ^{2}H abundance from a transpiration effect (Barbour, 2007; Cernusak et al., 2004; da Silveira Lobo Sternberg, 1988; Farquhar et al., 1982): ^{13}C enrichment compared with neighbouring autotrophic plants without parallel ^{18}O enrichment is indicative of heterotrophic C gain rather than lower transpiration and higher water use efficiency. Additionally, mycoheterotrophic plants tend to be generally enriched in ^{15}N and display higher total N concentrations than autotrophic reference plants, although these parameters are highly variable (Giesemann, Eichenberg, et al., 2020; Gomes et al., 2020), and thus not necessarily diagnostic of partial mycoheterotrophy in plants associated with AM.

In this study, we evaluated if seedlings of (at least some) common tropical woody species (i.e. trees or shrubs) in the understory of tropical forests in Central Panama exhibit partial mycoheterotrophy, that is, gain organic C from fungal AM partners in addition to their own photosynthesis. To this end, we (a) assessed the AM morphological type (*Paris*- or *Arum*-type) for tropical woody species from genera that include species with known AM morphotype (based on Dickson et al., 2007), and (b) compared the multi-element stable isotope natural abundance (^{13}C, ^{2}H, ^{18}O, ^{15}N) of potentially mycoheterotrophic target woody seedlings with *Paris*-type AM with neighbouring autotrophic reference plants (i.e. without *Paris*-type AM) to determine if they exhibit partially mycoheterotrophic C gain. Specifically, we tested if any *Paris*-type AM target species showed enrichment in the heavy isotopes ^{13}C without simultaneous enrichment in ^{18}O relative to neighbouring non-*Paris*-type reference plants, that is, the isotopic signature indicative of partial mycoheterotrophy.

2 | MATERIALS AND METHODS

2.1 | Study area

This study was conducted in semi-deciduous lowland forests in Central Panama, in the Soberania National Park and on Barro Colorado Island, that is, within an area of about 20 km × 3.5 km along the Panama Canal (approx. 9.07°–9.16° N, 79.65°–79.85° W). The climate is moist tropical with mean annual precipitation of about 2100–2600 mm, a distinct dry season from January to April (Engelbrecht et al., 2007), and a mean annual temperature of ~25°C (Autoridad Nacional del Ambiente Panamá, 2010). Soils formed on volcanic rocks or marine sediments include Oxisols and Alfisols (Baillie et al., 2007; Turner & Engelbrecht, 2011). Sampling took place in the understory of old secondary forests along established trails. Research and collection permission was granted by the Panamanian Ministry of the Environment (MiAmbiente, SE/AP-23-19).

2.2 | Study species and sampling design

For this study, we selected woody species (all C3-photosynthesis and non-succulent) from genera for which information on the AM type (*Paris*- or *Arum*-type) was available in the comprehensive review by Dickson et al. (2007). From these species, we *a priori* selected species from *Paris*- and *Arum*-type genera that are relatively frequent and abundant in Central Panama (Condit et al., 2013), and that are shade tolerant (Rüger et al., 2009). We included two of the most speciose and common shrub genera in the understory of Central Panama, *Piper* and *Psychotria* (Foster & Hubbell, 1990), since they promised to allow for numerous and easily identifiable *Arum*-type reference plants (based on Dickson et al., 2007). During sampling (see below), we opportunistically included additional woody species from genera for which AM morphotype information was available in Dickson et al. (2007). Overall, we collected samples from seedlings of 41 woody species within 20 genera and 15 families (see Table S1, including 8 *Psychotria* and 14 *Piper* species) with a wide range of abundances and light requirements. For comparison, we also collected the fully mycoheterotrophic, achlorophyllous herbaceous species *Voyria tenella* (Gentianaceae), which has *Paris*-type AM (Imhof, 1997).

We focussed on juvenile plants 30–80 cm in height, referred to as seedlings in the following (consistent with the tropical forest literature, e.g. Comita et al., 2007). Sampling of tree and shrub seedlings took place during the dry season (in March and April, Table S1), whilst the full mycoheterotroph *Voyria tenella*, which only emerges in

the wet season, was sampled in September (together with its respective reference plants).

For *a posteriori* microscopic determination of the species' AM morphotype in our study area, we collected roots of two to three individuals per plant species. Roots were mostly collected from the same individuals as for leaf sampling (below) or occasionally from separate plants along forest trails. Sampled roots were washed with tap water and fixed in ethanol (60%) until further processing for microscopic observations (see below).

The sampling design for leaf stable isotope analysis followed the approach of Gebauer and Meyer (2003), with few modifications. We aimed to compare the isotopic signature of potential partially mycoheterotrophic target plants (i.e. species with *Paris*-type AM) with autotrophic reference species (i.e. without *Paris*-type AM) growing under similar environmental conditions in their direct vicinity (up to 2 m distance, mostly considerably closer). This design accounts for variations in environmental factors (e.g. nutrient availability, microclimate, light conditions) and sampling time, which are known to affect stable isotope signatures (Dawson et al., 2002). Specifically, walking along established trails, we first searched for *a priori* target plants (*Paris*-type genus according to Dickson et al., 2007). If at least three (up to 6) *a priori* reference plants ('*Arum*-type AM' or 'non-mycorrhizal') grew in the immediate vicinity, we sampled leaves for analyses (see below). For each *a priori* target species, we sampled a minimum of five such sampling plots (total 105) with the plots separated by at least 30–50 m.

For stable isotope analyses, from each plant individual, at least one healthy, fully developed leaf was collected, carefully cleaned with a moist paper towel, and kept in a paper bag. If necessary, the number of leaves per sample was increased to ensure sufficient material for analyses. For *Voyria tenella*, we sampled the entire above-ground parts.

2.3 | AM morphotype determination in roots

For microscopic determination of the AM morphological type, an arbitrary subsample of the fine roots of each plant species was prepared and stained according to a modified staining protocol based on Phillips and Hayman (1970) and Vierheilig et al. (2005) following recommendations of T. Camenzind (pers. comm.). 1–2 cm root segments were cleared in 10% KOH (w/v) at 60°C (water bath: W760, Memmert GmbH, Schwabach, Germany) for 1.5 h to 1.5 days (depending on hardness), bleached in 3% (v/v) H_2O_2 at room temperature for 30 min to 4 h (depending on pigmentation), acidified in 1% (v/v) HCl and stained for 0.5–1 h in 0.05% (w/v) Trypan Blue lactoglycerol solution at 60°C. Stained roots were mounted onto slides with lactoglycerol for assessment. We examined the mounted root samples with a compound microscope at 100x magnification (Motic BA210, Motic Instruments Inc., Richmond, Canada).

Based on the presence of fungal structures in the roots and their morphological characteristics, we *a posteriori* classified the investigated plant species into (1) species with *Paris*-type AM hyphal coil structures in their roots. They either had exclusively *Paris*-type AM or exhibited both *Paris*-type and *Arum*-type AM structures. In the following, we refer to these species, which were potentially partially mycoheterotrophic, as '*Paris*-type' plants. (2) species without any *Paris*-type AM structures. They had either only *Arum*-type AM or were non-mycorrhizal. In the following, we refer to these species as 'non-*Paris*-type' plants and consider them as autotrophic reference plants. Microscopically unclear plant species were excluded from further analyses (not available, 'NA', Table S1).

2.4 | Multi-element stable isotope analyses of leaves

Leaf samples (or entire above-ground plant samples in the case of *Voyria tenella*) were oven-dried to constant weight at 105°C, ground to a homogenous, fine powder using a ball mill (Retsch Schwingmühle MM2, Haan, Germany), and weighed into tin and annealed silver capsules (micro balances: Sartorius CPA2P & MSE3.6P-000-DM, Göttingen, Germany). Relative nitrogen and C isotope natural abundances ($\delta^{15}N$, $\delta^{13}C$) were determined simultaneously using an EA-IRMS coupling, and relative natural abundances of hydrogen ($\delta^{2}H$) and oxygen isotopes ($\delta^{18}O$) were measured using a TC-IRMS coupling as specified in Zahn et al. (2023). Relative isotope abundances were denoted as δ values relative to their respective standards: $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ or $\delta^{18}O = (R_{sample}/R_{standard} - 1) \times 1000‰$, where R_{sample} and $R_{standard}$ are the ratios of heavy to the light isotope of the samples and the respective standard (for utilised standards refer to Zahn et al., 2023).

2.5 | Replication statement

Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriate scale
Species	Plots	Minimum of 5, Mean of 14
Arbuscular mycorrhiza type	Pooled species	21 '*Paris*-type' species 12 'non-*Paris*-type' reference species

2.6 | Data analysis

To normalise for environmental and temporal variation of the isotopic signature (see above), we compared within each plot the δ of the potentially partially mycoheterotrophic '*Paris*-type' target plant individuals to the average δ of the neighbouring autotrophic 'non-*Paris*-type' reference plants (classification based on our own *a posteriori*

microscopic observations, see Table S1). Additionally, we also compared within each plot the δ of each individual 'non-*Paris*-type' reference plant to the average δ of the 'non-*Paris*-type' reference plants. To that end, we calculated the comparative enrichment, that is, their enrichment factors ε according to Preiss and Gebauer (2008): $\varepsilon = \delta S - \delta REF$, where δS is a single $\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$ or $\delta^{15}N$ value of an individual plant ('*Paris*-type' target or 'non-*Paris*-type' reference), and δREF is the mean value across all 'non-*Paris*-type' reference plants in the respective plot. An analogous standardisation was carried out for Total N concentrations.

After the *a posteriori* reclassification of the species' AM morphotype, 79 plots contained a minimum of one '*Paris*-type' target individual and one 'non-*Paris*-type' reference individual and were thus suitable for the calculation of ε. The mean sample size (i.e. number of plots) for each of the 21 a posteriori '*Paris*-type' target species was 14 (minimum 5, see Table S2 for details).

We initially strived to include a diverse range of co-occurring reference taxa to account for a potentially wide variation of physiological signals inherent in the isotopic signature. After reclassification, each target plant had on average two reference individuals from two reference species in the same plot (Figure S2), with almost all reference plants belonging to the genus *Piper* (Piperaceae, plus one species in the genus *Acalypha*, Euphorbiaceae). Limited phylogenetic representation introduces the risk that the isotopic range of autotrophic plants is not fully represented and leads to false-positive results. Yet, *Piper* is one of the most speciose genera with >>30 species in the area, with a wide range of habitats and accordingly high diversity of ecological and physiological strategies (Foster & Hubbell, 1990; Fredeen & Field, 1996; Kyllo et al., 2003; Lasso & Jaramillo, in press). We included 11 different *Piper* species, which exhibit pronounced variation of habitat and functional traits, so a systematic bias of isotopic signatures between target and reference plants is rather unlikely.

We first tested for overall differences amongst the three groups ('*Paris*-type' target, 'non-*Paris*-type' reference, fully mycoheterotrophic *Voyria*) in their isotopic enrichment compared with their respective neighbouring 'non-*Paris*-type' reference plants ($\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, $\varepsilon^{15}N$) and N concentrations (εTotalN). We fitted separate linear mixed models ('lmer', package lme4, version 1.1.29; Bates et al., 2015) for each variable, including 'Species' and 'Plot' as random effects. And 95% confidence intervals (CIs) and *p*-values were computed using a Wald *t*-distribution approximation. For *post hoc* pairwise comparison between groups, we used the 'emmeans' and 'contrast' functions (package emmeans, version 1.7.5; Russell et al., 2022) with Bonferroni-Holm *p*-value adjustment. Excluding *Voyria* as a group in these analyses did not qualitatively change the results.

Subsequently, in order to identify individual '*Paris*-type' target species with an isotopic signature consistent with partial mycoheterotrophy, we then ran separate linear mixed models for each parameter, which compared individual '*Paris*-type' target species to all pooled neighbouring 'non-*Paris*-type' reference plant species, including 'Plot' as random effect.

Calculations and statistics were conducted in R version 4.2.3 (R Core Team, 2023).

3 | RESULTS

3.1 | AM morphotypes

Overall, about half of the investigated species (21 out of 41 species, in 16 out of 20 genera, Table S1) exhibited *Paris*-type AM, that is, we detected coil structures in the roots. Of those, most (81%) had only *Paris*-type AM, whilst the others showed both *Paris*- and *Arum*-type structures (i.e. also had arbuscules). About 12 of the species we examined (in 2 genera) did not show any *Paris*-type structures: they exhibited only arbuscules characteristic for the *Arum*-type AM (4 species) or had no visible AM structures (8 species). In additional 8 species, the AM morphotype could not be conclusively determined.

Comparing our microscopic root assessments of tropical woody species in Central Panama with the previous literature-based classification (Dickson et al., 2007; Table S1), all genera which had been known to contain *Paris*-type AM species consistently exhibited *Paris*-type AM in our study, except for two species, which remained unclear. In our study, species in six additional genera exhibited *Paris*-type structures, whilst species in the same genera exhibited *Arum*-type structures in Dickson et al. (2007). For instance, in *Psychotria*, which we had *a priori* considered to be *Arum*-type, six of the species showed *Paris*-type structures (whilst two remained unclear). Several of the genera including *Arum*-type AM species according to Dickson et al. (2007) exhibited no visible AM structures in our study (e.g. most *Piper* species), or the morphotype could not be determined.

From the *a posteriori* AM morphotype determination, 21 of the investigated 41 species were considered as potentially partially mycoheterotrophic ('*Paris*-type' target species), and 12 species were regarded as autotrophic 'non-*Paris*-type' reference plants ('*Arum*-type' or 'non-mycorrhizal') (Tables S1 and S2).

3.2 | Comparison of isotopic signatures amongst plants with different AM morphotypes

Overall, '*Paris*-type' woody target plants did not show significantly higher isotope enrichment (ε) of ^{13}C or ^{2}H than 'non-*Paris*-type' reference plants, but instead, they were depleted in ^{2}H and ^{18}O (Table 1). Thus, not surprisingly, across species, no general trend to partial mycoheterotrophy in the investigated '*Paris*-type' plants was indicated by the stable isotopes. In contrast, as expected, the fully mycoheterotrophic *Voyria* showed highly significantly stronger enrichment in both ^{13}C and ^{2}H relative to reference plants and showed no significant difference in ^{18}O and ^{15}N enrichment (Table 1). Enrichment of ^{15}N and TotalN did not differ across groups (Table 1). We will not further elaborate on the N data due to their limited diagnostic power for partial mycoheterotrophy in AM plants (full results are available in Table S5 and Figure S1).

TABLE 1 Comparison of stable isotope enrichment and total nitrogen concentrations (ε, i.e. the difference between neighbouring plants within a plot) in tropical woody seedlings without '*Paris*-type' AM, with '*Paris*-type' AM, and in the fully mycoheterotrophic herbaceous species *Voyria tenella*. Shown are mean $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, $\varepsilon^{15}N$ (‰) and εTotalN (mmol gdw^{-1}) and standard deviations (SD) across the respective plots and species (see Tables S3 and S4 for detailed results). Different letters indicate significant differences between groups ($p < 0.05$, within each parameter) based on linear mixed-effect models and *post hoc* tests for pairwise comparisons. All models included 'Species' and 'Plot' as random effect.

	Woody seedlings without *Paris*-type AM	Woody seedlings with *Paris*-type AM	*Voyria tenella*
$\varepsilon^{13}C$	0.00 ± 1.02^{a}	0.57 ± 2.36^{a}	6.11 ± 1.37^{b}
$\varepsilon^{2}H$	0.00 ± 4.97^{a}	-5.27 ± 9.52^{b}	22.57 ± 9.16^{c}
$\varepsilon^{18}O$	0.00 ± 0.98^{a}	$-0.93 \pm 2.07^{b,*}$	0.39 ± 0.69^{a}
$\varepsilon^{15}N$	0.00 ± 0.71^{a}	-0.12 ± 1.87^{a}	1.50 ± 1.84^{a}
εTotalN	0.00 ± 0.25^{a}	-0.06 ± 0.53^{a}	0.23 ± 0.26^{a}

Note: Standard deviation is given in "italic".

*Difference not significant after Bonferroni-Holm correction.

3.3 | Isotopic signatures of individual '*Paris*-type' target species compared with 'non-*Paris*-type' reference plants

At the level of individual species, seedlings of six '*Paris*-type' target species (28.6%) showed significant enrichment in ^{13}C compared with the neighbouring autotrophic 'non-*Paris*-type' reference plants, whilst at the same time not showing an ^{18}O enrichment (Figure 1; Table S5). Their combined ^{13}C and ^{18}O isotopic signature thus supports partial mycoheterotrophy. The significance of ^{13}C enrichment in each of these species was based on at least six intra-plot comparisons (see Figure S3). One of these species, *Anacardium excelsum*, was additionally significantly enriched in ^{2}H compared with the 'non-*Paris*-type' reference plants and thus fulfilled a second criterion for partial mycoheterotrophy. In contrast, the remaining five species with ^{13}C enrichment were depleted in ^{2}H (Figure 1b; Table S5).

Whilst no '*Paris*-type' target species showed significant ^{18}O enrichment, seven species were significantly depleted in ^{18}O relative to reference plants (Figure 1c; Table S5). This complicates interpretation with regard to partial mycoheterotrophy (see below for discussion).

Only one target species was significantly enriched in ^{2}H, but 18 target species were significantly depleted in ^{2}H compared with the 'non-*Paris*-type' reference plants (Figure 1b; Table S5).

Seedlings of nine of the target species were not enriched in ^{13}C and ^{2}H, and indistinct in $\varepsilon^{18}O$ from reference plants, that is, these species showed isotopic signatures that clearly do not support partial mycoheterotrophy.

4 | DISCUSSION

To the best of our knowledge, this is the first study that investigated stable isotope natural abundances of tropical woody species with respect to partial mycoheterotrophy. Seedlings of six species exhibited isotopic signatures indicative of partial mycoheterotrophy, that is, ^{13}C enrichment compared with neighbouring autotrophic species without a parallel ^{18}O enrichment. For one of our study species, namely, *Anacardium excelsum*, ^{2}H enrichment provides additional evidence for partial mycoheterotrophy.

4.1 | AM morphotypes

The *Paris*-type AM is considered to be a prerequisite for a fungus-to-plant C transfer in both achlorophyllous fully mycoheterotrophic and chlorophyllous partially mycoheterotrophic plants with AM (Giesemann et al., 2021; Imhof, 1999, but see Murata-Kato et al., 2022). We showed that *Paris*-type AM is indeed rather widespread in seedlings of tropical woody species, occurring in 50% of the investigated species (21 of 41) and in 80% of the genera (16 of 20) in lowland moist forests in Panama.

Several tropical woody plant genera contain both *Paris*- and *Arum*-type species (based on comparison with Dickson et al., 2007) and we occasionally observed two morphological structures in the same plant species, even within the same root system (e.g. *Anacardium excelsum*, *Cecropia insignis*, *Croton billbergianus*, *Acalypha macrostachya*). In general, plant and AM fungal identity as well as the environment are considered to impact the formation of *Arum*- or *Paris*-type structures along a continuum (e.g. Ahulu et al., 2006, 2007; Kubota et al., 2005; Lovelock et al., 2003). It remains poorly understood what exactly determines the formation of specific AM morphotype structures (Bennett & Groten, 2022; Dickson et al., 2007), and consequently if a plant individual, species or genus may be capable of partial mycoheterotrophy. Thus, *in situ* AM morphotype evaluation is pivotal. AM fungal communities in tropical forests are recognised as highly diverse (Alexander & Lee, 2005; Kottke et al., 2006; Muthukumar et al., 2003; Zhang et al., 2021) with considerable spatial (Mangan et al., 2004) and temporal (Herre et al., 2009; Husband et al., 2002) variation. Further investigations combing morphotype determination and fungal associate identification in different woody species along environmental gradients may help to better understand the formation of *Paris*-type AM structures.

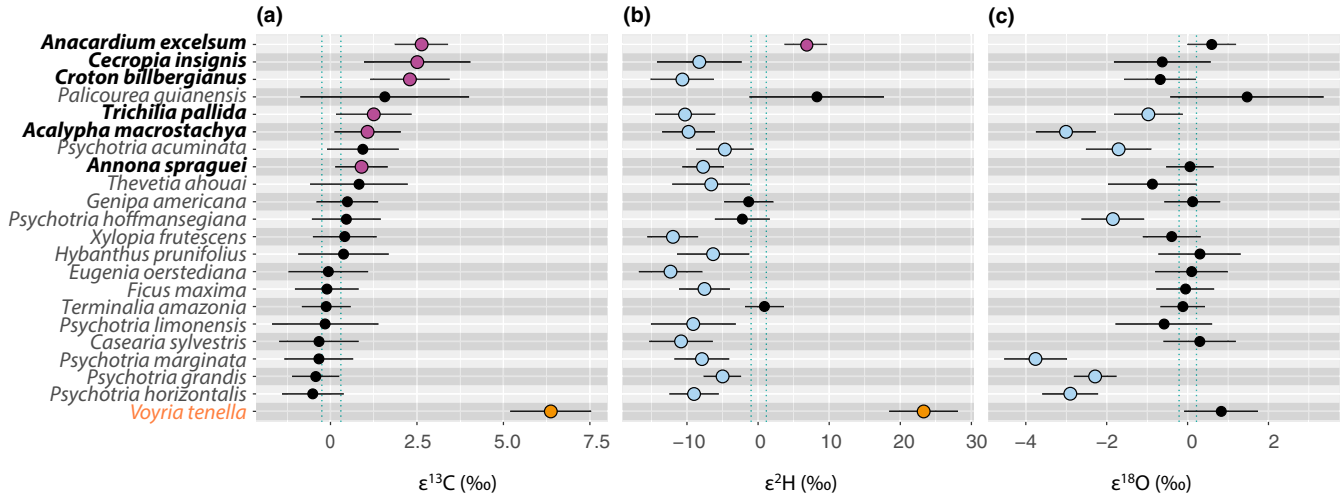

FIGURE 1 Enrichment factors $\varepsilon^{13}C$ (a), $\varepsilon^{2}H$ (b), and $\varepsilon^{18}O$ (c) and 95% CI of individual seedlings in '*Paris*-type' woody species in comparison to pooled neighbouring seedlings in 'non-*Paris*-type' reference species (95% CI displayed by dotted vertical lines) based on linear mixed models (Table S5). Purple and blue symbols indicate significant enrichment or depletion of '*Paris*-type' species in comparison to 'non-*Paris*-type' reference plants, respectively. Orange symbols indicate significant enrichment for fully mycoheterotrophic *Voyria tenella*. The six species that exhibit isotopic signatures indicative of partial mycoheterotrophy are highlighted in bold.

4.2 | Isotopic evidence for partial mycoheterotrophy in woody tropical seedlings

Seedlings of six species with *Paris*-type AM (*Anacardium excelsum*, *Annona spraguei*, *Cecropia insignis*, *Croton billbergianus*, *Acalypha macrostachya* and *Trichilia pallida*) displayed significant ^{13}C enrichment compared with the neighbouring autotrophic 'non-*Paris*-type' reference plants, and none of these species exhibited a parallel enrichment of ^{18}O. We can thus exclude that enrichment in ^{13}C in these species was caused by lower transpiration and a higher water use efficiency of the target relative to reference plants (and all woody species in the study system have C3 photosynthesis). The isotopic signature thus provides a strong indication for supplementary gain of enriched ^{13}C through fungus-to-plant C transfer, that is, that these species exhibit partial mycoheterotrophy. The ^{13}C-enriched seedlings exhibited a similar isotope pattern to fully mycoheterotrophic *Voyria* in our study area (Figure 1; Table S5)—of course with a smaller ^{13}C (and ^{2}H) enrichment—and to other fully (Courty et al., 2011; Gomes et al., 2020; Merckx et al., 2010) or partially (Giesemann et al., 2021; Giesemann, Rasmussen, et al., 2020; Murata-Kato et al., 2022) mycoheterotrophic herbaceous AM plants. Leaf ^{13}C enrichment of mycoheterotrophic plants in the forest understory compared with autotrophic ones reflects a relative ^{13}C enrichment of their biogenic fungal C source, that is, the AM fungal hyphae (Courty et al., 2011; Gomes et al., 2020, 2023), which in turn is mainly due to the supply of ^{13}C enriched carbohydrates from canopy trees to the AM fungi (Courty et al., 2011; Gleixner et al., 1993). Leaf exposure to high irradiance leads to ^{13}C enrichment during photosynthesis in canopy trees (and thus AM fungi) compared with plants in the shaded understory (Courty et al., 2011). Additionally, the C that plants in the forest understory assimilate through photosynthesis is ^{13}C-depleted due to CO_2 originating from soil respiration (Gebauer & Schulze, 1991). This enhances the contrast in the ^{13}C signature between photosynthetic and biogenic C sources in understory plants. Such effects are especially pronounced in tropical forests, due to higher rates of respiratory CO_2 released from the soil, leading to a higher vertical profile of air $\delta^{13}C$ in tropical forests, compared with temperate ones (Courty et al., 2011; Hanba et al., 1997; Quay et al., 1989).

Recently, some concerns have been raised that ^{13}C enrichment (without parallel ^{18}O enrichment) may not always be the result of mycoheterotrophy (Murata-Kato et al., 2022). They argued that in herbaceous species in temperate forests, differences in plant phenology could also explain ^{13}C enrichment, with species that photosynthesise early before canopy closure being relatively ^{13}C enriched because they grow under higher light levels (limited ^{13}C discrimination) and lower soil respiration (less ^{13}C-depletion of ambient CO_2 at the forest floor) conditions than species that continue to photosynthesis throughout the growing season (Murata-Kato et al., 2022). Similarly, more light is available and soil respiration is lower in the understory of tropical lowland forests in the dry season compared with the wet season (Cusack et al., 2019; Newell et al., 2002). However, photosynthetic CO_2 uptake is limited in the dry season (Comita & Engelbrecht, 2014; Restrepo-Coupe et al., 2013), and seasonal light differences in the understory of semi-deciduous tropical forests are small compared with temperate forests (Gaviria et al., 2017; Matsuo et al., 2021). In addition, our investigated species all have long-lived leaves, which most likely developed in the wet season (Kitajima et al., 2013; Kursar & Coley, 1993) and there is no evidence that our '*Paris*-type' species with ^{13}C enrichment would have different leaf phenology. Partial mycoheterotrophy therefore is the most parsimonious explanation for their significant ^{13}C-enrichment (without ^{18}O enrichment) compared with neighbouring 'non-*Paris*-type' species.

In our study, we considered 'non-*Paris*-type' species (i.e. with *Arum*-type AM or non-mycorrhizal) as autotrophic (Giesemann et al., 2021). If this is indeed a general pattern, or if fungus-to-plant C transfer can also occur in *Arum*-type species, has been questioned (Murata-Kato et al., 2022). However, in our study, none of the 'non-*Paris*-type' species showed conspicuous ^{13}C enrichment (all $\varepsilon^{13}C < 0.4$ ‰, except two species in which only one individual was sampled, see Table S2). Even if *Arum*-type plants in our study would indeed exhibit some ^{13}C enrichment due to partial mycoheterotrophy, this would lead to under- rather than overestimating the occurrence of mycoheterotrophy in the investigated *Paris*-type AM plants. The finding of an apparent ^{13}C enrichment in some *Arum*-type species by Murata-Kato et al. (2022) may be traced back to a different sampling design. We collected leaves from closely neighbouring target and reference plants (within max. 2 m^2) fulfilling the requirement of growth under similar light conditions, whilst Murata-Kato et al. (2022) collected their target and reference plants within a much larger area (25 m^2). At this scale, light conditions can already vary considerably on the forest floor (Nicotra et al., 1999).

In two of the emerging partially mycoheterotrophic species, *Acalypha macrostachya* and *Trichilia pallida*, the ^{13}C enrichment signature may indeed even underestimate fungus-to-plant C transfer. In these species, ^{18}O was depleted relative to 'non-*Paris*-type' reference plants, indicating higher transpiration and lower water use efficiency (Barbour, 2007). This counteracts any ^{13}C enrichment by heterotrophic nutrition. Higher transpiration and lower water use efficiency indicated by ^{18}O depletion may also explain their ^{2}H depletion (see below).

Five additional '*Paris*-type' species (all in the genus *Psychotria*) exhibited higher transpiration than their respective reference plants (indicated by ^{18}O depletion). In these species, which did not differ in $\varepsilon^{13}C$ from 'non-*Paris*-type' reference plants, the transpiration effect leading to ^{13}C depletion may have completely masked possible ^{13}C enrichment through biogenic C gain. Thus, it is possible that these species also exhibit partial mycoheterotrophy, but currently, we cannot conclusively resolve their nutritional mode based on the stable isotope signatures.

Our finding of stable isotope signatures indicative of partial mycoheterotrophy in seedlings of nearly 30% of the studied *Paris*-type AM tropical woody species, provides for the first time evidence that supplementary C gain through partial mycoheterotrophy exists in seedlings of tropical tree species with AM associations.

4.3 | ^{2}H depletion amongst partially mycoheterotrophic seedlings

A ^{2}H enrichment relative to 'non-*Paris*-type' reference plants, which has been observed in fully and partially mycoheterotrophic herbaceous AM species (Giesemann et al., 2021; Giesemann, Eichenberg, et al., 2020; Gomes et al., 2020) and is considered an additional independent indication for biogenic organic matter gain from fungi, occurred only in seedlings of one '*Paris*-type' tree species (*Anacardium excelsum*).

Instead, five out of the six ^{13}C-enriched '*Paris*-type' species exhibited even significant depletion of ^{2}H. This was unexpected and has not been described before, neither for fully nor partially mycoheterotrophic AM plants (Giesemann et al., 2021; Giesemann, Eichenberg, et al., 2020; Gomes et al., 2020). In those species that also exhibit depletion of ^{18}O compared with the reference plants, higher transpiration may explain the observed ^{2}H depletion (i.e. for *Acalypha macrostachya* and *Trichilia pallida*). Yet, in the species where no ^{18}O depletion emerged, such a transpiration effect can be excluded (for *Annona spraguei*, *Cecropia insignis*, and *Croton billbergianus*). A major fungus-to-seedling C supply in the form of ^{2}H-depleted lipids (higher lipid-to-carbohydrate gain ratio) might potentially explain ^{2}H-depleted leaf tissues in these species: AM fungi receive substantial amounts of lipids (i.e. fatty acids) from their 'C donor plant' partner (Jiang et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017) and lipids represent the major C-storage compounds in AM fungi (Wipf et al., 2019). Lipids have been recognised to be ^{2}H-depleted compared with cellulose due to ^{2}H-fractionation during the biosynthesis of plant organic compounds (Cormier et al., 2018).

4.4 | Possible ecological implications of partial mycoheterotrophy for tropical woody seedlings

Our results indicate for the first time that supplementary C gain through partial mycoheterotrophy exists in seedlings of tropical tree species with AM associations. They imply that plant–fungus–plant C transfer occurs in some tropical tree species with AM via common mycorrhizal networks (Merckx et al., 2024). Additional C gain and redistribution of C through partial mycoheterotrophy may have pervasive ecological implications considering the severe light, and frequently also P and water limitation to tree regeneration in the understory of tropical lowland forests (Comita & Engelbrecht, 2014; Holste et al., 2011; Santiago et al., 2017).

Partial mycoheterotrophy is considered a strategy to allow for seedling establishment, survival, and growth under C-limited conditions, especially light limitation (e.g. Preiss et al., 2010). Seedlings of shade-tolerant species can survive for decades in the deep shade of the tropical forest understory (Hubbell et al., 1999; Rüger et al., 2009). Biogenic C supply through partial mycoheterotrophy in addition to photosynthetic C gain may lead to a lower whole-plant light compensation point in some species, and help to maintain a positive C balance under these conditions. Additional C gain through partial mycoheterotrophy may also allow to alleviate the trait-based trade-off between survival in the shade and growth rates in seedlings of the respective species (Ellers et al., 2012). Yet, those species, for which we found evidence for partial mycoheterotrophy in seedlings, were not the most shade-tolerant ones in the system (Rüger et al., 2018) and even included pronounced light-requiring pioneers (*Cecropia insignis* and *Croton billbergianus*). These results imply that partial mycoheterotrophy

may provide additional or alternative advantages to woody seedlings (particularly of pioneer species) beyond survival and growth under light-limited conditions.

Partial mycoheterotrophy may additionally enable plants to cope with P limitation and/or drought during the dry season by providing organic C when photosynthesis is limited (Comita & Engelbrecht, 2014; Holste et al., 2011; Santiago et al., 2017). Fully mycoheterotrophic plants in tropical forests are associated with low-fertility sites (Gomes et al., 2019; Sheldrake et al., 2017) and the dependency of partially mycoheterotrophic plants on fungal-derived carbon can increase under drought (McCormick et al., 2022). The distribution of the woody species, with evidence for partial mycoheterotrophy in seedlings, was associated with dry sites (with the exception of *Croton billbergianus*), whilst no pattern emerged for the association with soil P availability (Condit et al., 2013). C gains from fungi can also be crucial for germination in plants with tiny seeds that provide only limited C resources (Eriksson & Kainulainen, 2011). In our study, the species with evidence for partial mycoheterotrophy exhibited a wide range of seed sizes. Seedlings of pioneer species with small seeds and thus low C reserves might benefit from additional biogenic C supply through partial mycoheterotrophy during initial survival and growth. Detecting partial mycoheterotrophy amongst seedlings of species with variable light, P and water requirements and different seed sizes suggests that ecological advantages differ across species and possibly growing conditions.

The seedling stage comprises a bottleneck in population dynamics (Harper, 1977) and the foundation for future forest composition. Even small C gains through partial mycoheterotrophy may confer seedlings of the respective species a performance advantage in the forest understory and expand their niches (Merckx et al., 2024; Tedersoo et al., 2020). It may thus impact species' relative regeneration success, and subsequently forest community composition (Mangan et al., 2010; Parihar et al., 2020). Although any direct contribution from fungi to overall C cycling processes in tropical forests is likely insignificant, effects on forest composition mediated by AM may have pervasive indirect effects on ecosystem function, specifically for C uptake and storage (Merckx et al., 2024).

Partial mycoheterotrophy amongst AM plants requires at least a tripartite association interconnecting a mycoheterotrophic 'C receiver plant' via an AM fungus to a photosynthetic 'C donor plant', because AM fungi are obligate biotrophic, that is, they entirely depend on organic C supplied by a living plant partner (Tisserant et al., 2013; Trépanier et al., 2005). Our results therefore imply for the first time that C transfer through common mycorrhizal networks must occur amongst woody tropical AM species, which are dominant in lowland tropical forests. Thus, C transfer via AM in forests is not restricted to herbaceous species (e.g. Giesemann et al., 2021; Sheldrake et al., 2017), where it has been suggested to be prevalent (Merckx et al., 2024). Plant–fungus–plant C exchange dynamics amongst woody plants within common mycorrhizal networks have been primarily studied in ectomycorrhizal systems in temperate and boreal climates (e.g. Avital et al., 2022; Cahanovitc et al., 2022; Klein et al., 2023; Simard et al., 1997) and only few studies consider woody AM species (e.g. Avital et al., 2022; Lerat et al., 2002). However, direct C transfer and resource transfer within such common mycorrhizal networks remains highly debated (Figueiredo et al., 2021; Henriksson et al., 2023; Karst et al., 2023; Klein et al., 2023; Luo et al., 2023), because it is challenging to firmly establish a fungal connection between plants (see Rillig et al. (2024) for a critical evaluation of the definition of common mycorrhizal networks). Achlorophyllous fully mycoheterotrophic AM herbaceous plants provide conclusive examples for plant–fungus–plant C transfer, and thus for common mycorrhizal networks (Merckx et al., 2024). Shared mycorrhizal fungi in common mycorrhizal networks have been suggested to link over- and understorey C and nutrient exchanges (Balandier et al., 2022), and to play a fundamental role in balancing plant interactions within communities harbouring mycoheterotrophs (Selosse et al., 2006; Tedersoo et al., 2020). To confirm the indication for a plant–fungus–plant C transfer in seedlings of woody AM species based on our results, valuable insights could be gained from tracer experiments (Klein et al., 2016) and natural abundance stable isotope signatures of fungal hyphae extracted from plant roots (Gomes et al., 2023; Klink et al., 2020; Zahn et al., 2023) together with DNA sequencing for fungal partner identification.

Additional studies are also called for to explore how widespread partial mycoheterotrophy is in (tropical) woody species, and across forests under different environmental conditions (e.g. different soils, seasonality) and to quantify the extent of transfer of biogenic C though partial mycoheterotrophy. The ecological advantages that partial mycoheterotrophy provides for woody species in tropical forests remain to be investigated, as well as the consequences for community composition and ecosystem function.

5 | CONCLUSIONS

We detected stable isotope signatures indicative of partial mycoheterotrophic nutrition in seedlings of almost 30% of the investigated *Paris*-type AM woody species in the understory of neo-tropical lowland forests and thus provide the first evidence for additional C gain through fungal interactions in tropical woody AM species. *Paris*-type AM, considered a prerequisite for biogenic C gain, occurred in seedlings of about half of the investigated species, suggesting that partial mycoheterotrophy might actually be rather widespread in tropical woody plants.

Given the far-reaching possible ecological implications of partial mycoheterotrophy in tree seedlings of tropical (as well as other) forests, we urgently need to gain further insights.

AUTHOR CONTRIBUTIONS

Bettina M. J. Engelbrecht conceived the idea for the study. Bettina M. J. Engelbrecht, Gerhard Gebauer, and Franziska E. Zahn developed

the research design. All authors were involved in the sample collection. Species identification was done by Blexein Contreras. The sample preparation and analyses were conducted by Franziska E. Zahn. Gerhard Gebauer supervised and quality-controlled the isotope abundance analyses. Franziska E. Zahn analysed the data and wrote the first manuscript draft with inputs from Bettina M. J. Engelbrecht and Gerhard Gebauer. All authors commented and approved the final version of the manuscript. This work was supported by the German Research Foundation (DFG GE 565/9-1). Our study brings together authors from different countries, including scientists based in the country where the study was carried out. All authors were engaged early on with the research and study design to ensure that the diverse sets of perspectives they represent were considered from the onset. Whenever relevant, literature published by scientists from the region was cited.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Additional data are available in the Supporting Information and deposited in Dryad Digital Repository: https://doi.org/10.5061/dryad.kprr4xhf2 (Zahn et al., 2024).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Estimated enrichment factors $\varepsilon^{15}N$ (a) and εTotalN concentration (b) with 95% CI of individual seedlings in '*Paris*-type' species in comparison to pooled neighbouring seedlings in 'non-*Paris*-type' reference species (95% CI displayed by dotted vertical lines).

Figure S2. Histogram of the number of 'non-*Paris*-type' reference seedlings across plots.

Figure S3. Comparison of the $\delta^{13}C$ values of the individuals of the six '*Paris*-type' target species with significant ^{13}C enrichment compared with the mean $\delta^{13}C$ of all 'non-*Paris*-type' reference plants in the respective plot.

Table S1. Morphotype determination based on literature and microscopic observations.

Table S2. Isotopic signature for all study species.

Table S3. Summaries of linear mixed models (estimated using REML and nloptwrap optimiser, R function: lmer) predicting $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, $\varepsilon^{15}N$ and εTotalN, respectively with *Group* ('*Paris*-type', 'non-*Paris*-type', 'FMH' (fully mycoheterotrophic)) for woody tree and shrub species (and *Voyria*).

Table S4. Pairwise comparisons of *Group* effect ('*Paris*-type', 'non-*Paris*-type', 'FMH' (fully mycoheterotrophic)) on $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, $\varepsilon^{15}N$ and εTotalN of woody tree and shrub species obtained from linear mixed models' post hoc tests comparing differences in means.

Table S5. Summaries of linear mixed models (estimated using REML and nloptwrap optimiser, R function: lmer) comparing $\varepsilon^{13}C$, $\varepsilon^{2}H$, $\varepsilon^{18}O$, $\varepsilon^{15}N$ and εTotalN, respectively of individual potentially partially mycoheterotrophic '*Paris*-type' target species (and fully mycoheterotrophic *Voyria*) to pooled 'non-*Paris*-type' reference plants.

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