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Carbon and Nitrogen Metabolism in Mycorrhizal Networks and Mycoheterotrophic Plants of Tropical Forests: A Stable Isotope Analysis[1][W]

Pierre-Emmanuel Courty[2], Florian Walder[2], Thomas Boller, Kurt Ineichen, Andres Wiemken, Alain Rousseau, and Marc-André Selosse*

Botanical Institute, University of Basel, CH–4056 Basel, Switzerland (P.-E.C., F.W., T.B., K.I., A.W.); Laboratoire de Biologie et de Physiologie Végétales, EA 926 (DYNECAR), Université des Antilles et de la Guyane, 97159 Pointe-a-Pitre, France (A.R.); and Centre d'Ecologie Fonctionnelle et Evolutive (UMR 5175), 34293 Montpellier cedex 5, France (M.-A.S.)

Most achlorophyllous mycoheterotrophic (MH) plants obtain carbon (C) from mycorrhizal networks and indirectly exploit nearby autotrophic plants. We compared overlooked tropical rainforest MH plants associating with arbuscular mycorrhizal fungi (AMF) to well-reported temperate MH plants associating with ectomycorrhizal basidiomycetes. We investigated ^{13}C and ^{15}N abundances of MH plants, green plants, and AMF spores in Caribbean rainforests. Whereas temperate MH plants and fungi have higher $\delta^{13}C$ than canopy trees, these organisms displayed similar $\delta^{13}C$ values in rainforests, suggesting differences in C exchanges. Although temperate green and MH plants differ in $\delta^{15}N$, they display similar ^{15}N abundances, and likely nitrogen (N) sources, in rainforests. Contrasting with the high N concentrations shared by temperate MH plants and their fungi, rainforest MH plants had lower N concentrations than AMF, suggesting differences in C/N of exchanged nutrients. We provide a framework for isotopic studies on AMF networks and suggest that MH plants in tropical and temperate regions evolved different physiologies to adapt in diverging environments.

Mycorrhizal fungi symbiotically associate with plant roots and form networks between nearby plants through hyphal connections (Smith and Read, 2008). Although there is increasing evidence for such links between plants of different species (Selosse et al., 2006), functional roles of mycorrhizal networks in plant physiology or ecosystem functioning remain debated. Whether carbon (C) transfer between plants occurs by way of mycorrhizal networks is particularly controversial (Bever et al., 2010; Courty et al., 2010). Nevertheless, mycoheterotrophic (MH) species are extreme and relevant models of plants that in most cases receive all their C through mycorrhizal networks (Leake, 1994, 2004; Selosse and Roy, 2009). These achlorophyllous plants live in the forest understory and exploit mycorrhizal fungi associated with surrounding autotrophic trees as a C and energy source

(Taylor and Bruns, 1997; Selosse et al., 2002; Bidartondo, 2005; Roy et al., 2009; see Martos et al., 2009 and Ogura-Tsujita et al., 2009 for exceptions to this rule). These MH plants fully depend on the mycorrhizal network supported by autotrophic plants, and mycoheterotrophy opens a window to study of the properties of mycorrhizal networks. This work describes features of green plant—fungus—MH plant networks in overlooked examples from tropical ecosystems.

MH nutrition evolved many times independently in land plants, involving diverse plant and fungal taxa (Leake, 2004). In temperate ecosystems, MH plants mostly associate with basidiomycetes, as exemplified by orchids (Taylor et al., 2002) and Ericaceae (Bidartondo, 2005). MH models from temperate regions were investigated using three major tools. First, molecular barcoding of mycorrhizal fungi demonstrated their association, often with high specificity, with fungi that are ectomycorrhizal on surrounding trees (Leake, 2004; Bidartondo, 2005; Roy et al., 2009). Second, ^{14}C labeling of tree photosynthates demonstrated C transfer to MH plants (Björkman, 1960; McKendrick et al., 2000). Third, stable isotope abundances were used as a major indicator to indirectly support the role of ectomycorrhizal fungi as a food source (Gebauer and Meyer, 2003). Compared with their host trees, ectomycorrhizal fungi are enriched in ^{13}C and ^{15}N (Gebauer and Dietrich, 1993; Hobbie et al., 2001; Mayor et al., 2009). In contrast, transfer from fungi to MH plants does not modify ^{13}C and ^{15}N abundances (or only

slightly increases ^{15}N abundance; Trudell et al., 2003). In consequence, abundances of heavy isotopes are higher in MH plants than in cooccurring green plants (Trudell et al., 2003). This pattern is also reported in some tropical forests where MH plants associate with ectomycorrhizal fungi (Roy et al., 2009). Isotopic abundances were also instrumental in the discovery that several green plants, phylogenetically close to MH species, obtain part of their C from ectomycorrhizal networks, since their ^{13}C and ^{15}N abundances are intermediate between autotrophic and MH plants (Gebauer and Meyer, 2003; Julou et al., 2005; Tedersoo et al., 2006; Roy et al., 2009). This nutrition is called partial mycoheterotrophy, a kind of mixotrophy that mixes photosynthetic and MH nutrition (for review, see Selosse and Roy, 2009).

However, most MH species live in tropical forests devoid of ectomycorrhizal fungi (Leake, 1994; Smith and Read, 2008). In these forests, some MH orchids associate with saprotrophic basidiomycetes (e.g. Martos et al., 2009; Ogura-Tsujita et al., 2009), but most MH lineages connect to another kind of mycorrhizal network, formed by arbuscular mycorrhizal fungi (AMF; Imhof, 2009). AMF are obligate biotrophic fungi that belong to Glomeromycota and evolved mycorrhizal symbioses independently from Basidiomycetes (Smith and Read, 2008; Sanders and Croll, 2010). Few AMF-associated MH plants (AMF-MH) have been investigated so far, and studies have focused on mycorrhizal morphology (Imhof, 2009) and identification of AMF partners (Bidartondo et al., 2002; Franke et al., 2006; Merckx and Bidartondo, 2008). Two features limit the use of stable isotopes in AMF-MH. First, AMF can associate with C_3, C_4, or CAM plants, and may thus mix C sources that differ in ^{13}C abundance (Allen and Allen, 1990), whereas ectomycorrhizal fungi only associate with C_3 plants. Second, AMF produce spores without conspicuous organs such as the fruitbodies of basidiomycetes, and extraradical mycelium and spores of AMF are not easy to sample for isotopic measurements. Allen and Allen (1990) and Nakano et al. (1999) analyzed ^{13}C abundances in spores of AMF associated with herbs: The ^{13}C abundance followed that of their C_3 or C_4 hosts, with some depletion ($-2‰$ to $-4‰$ in δ^{13}C). Isotopic abundances were recently measured in two tropical rainforest AMF-MH, *Burmannia capitata* (Burmanniaceae) and *Voyria aphylla* (Gentianaceae): They showed higher ^{13}C abundance ($+5.5‰$ in δ^{13}C), but similar ^{15}N abundance compared with understory green plants (Merckx et al., 2010). Enrichment in ^{13}C for AMF-MH is paradoxical, since depletion is expected in AMF (Allen and Allen, 1990; Nakano et al., 1999). However, isotopic abundances in AMF and canopy trees, likely the main C donors to forest mycorrhizal networks (Högberg et al., 1999), have not been investigated up to now. Our aim was to provide isotopic data for several points along the continuum between tropical plant (canopy and understory), AMF fungus, and AMF-MH. A precise understanding of this continuum is required before predictions can be made on partially MH species associated with AMF.

We focused on four AMF-MH species at five sites of a Caribbean rainforest harboring C_3 trees (Rousteau, 1996; Bonal et al., 2000; see Supplemental Table S1). They belong to two families that independently evolved MH nutrition (two species each of Gentianaceae and Burmanniaceae; see Supplemental Fig. S1), allowing a phylogenetic replication of our results. We extracted AMF spores from the soil to measure their ^{13}C and ^{15}N abundances, and we verified that they included the species colonizing AMF-MH. We provide here, to our knowledge, the first report of ^{13}C and ^{15}N abundances along an AMF network in situ, including MH plants. We did not detect significant differences in isotopic abundances between canopy leaves, AMF mycorrhizal roots, AMF, and AMF-MH; however, due to specific photosynthetic conditions, green understory leaves are considerably depleted in ^{13}C. We propose a general framework for the interpretation of isotopic studies on AMF-MH, including predictions of isotopic abundances to be expected in partially MH species associated with AMF. Moreover, AMF-MH unexpectedly showed lower nitrogen (N) contents than MH plants associated with basidiomycetes, suggesting some physiological differences in N nutrition. Thus, we speculate that, beyond superficial similarities, tropical AMF-MH and—mostly temperate—MH taxa associated with basidiomycetes have different nutritional physiologies that evolved in adaption to their respective environments.

RESULTS AND DISCUSSION

^{13}C Abundances Differ in Canopy versus Understory Green Leaves

Since isotopic abundances significantly differed at the five study sites for each sample category ($H = 15.706$, degrees of freedom [df] = 4, $P < 0.001$ and $H = 27.143$, df = 4, $P < 0.001$ for δ^{13}C and δ^{15}N, respectively; Kruskal-Wallis test, see Supplemental Table S1), data were analyzed separately (Fig. 1, A–E). Values for δ^{13}C were variable for canopy leaves, due to interspecific differences (see Supplemental Table S1 for detailed sampling at Sofaïa 1), as expected in tropical rainforests (Bonal et al., 2000; Buchmann et al., 2004), and values for δ^{15}N were in the usual range for rainforests (e.g. Högberg and Alexander, 1995). Leaves of understory plants were significantly depleted in ^{13}C compared with canopy leaves at all sites (on average by 5.3‰), but there was no significant difference in δ^{15}N (Fig. 1, A–E). An approximately 5‰ difference in ^{13}C between canopy trees and understory plants is common in rainforests, as well as in any dense forest, due to several factors (for review, see Buchmann et al., 2004; Lüttge, 2008). First, high light conditions enhance photosynthesis and drier conditions reduce stomatal conductance: These two factors limit equili-
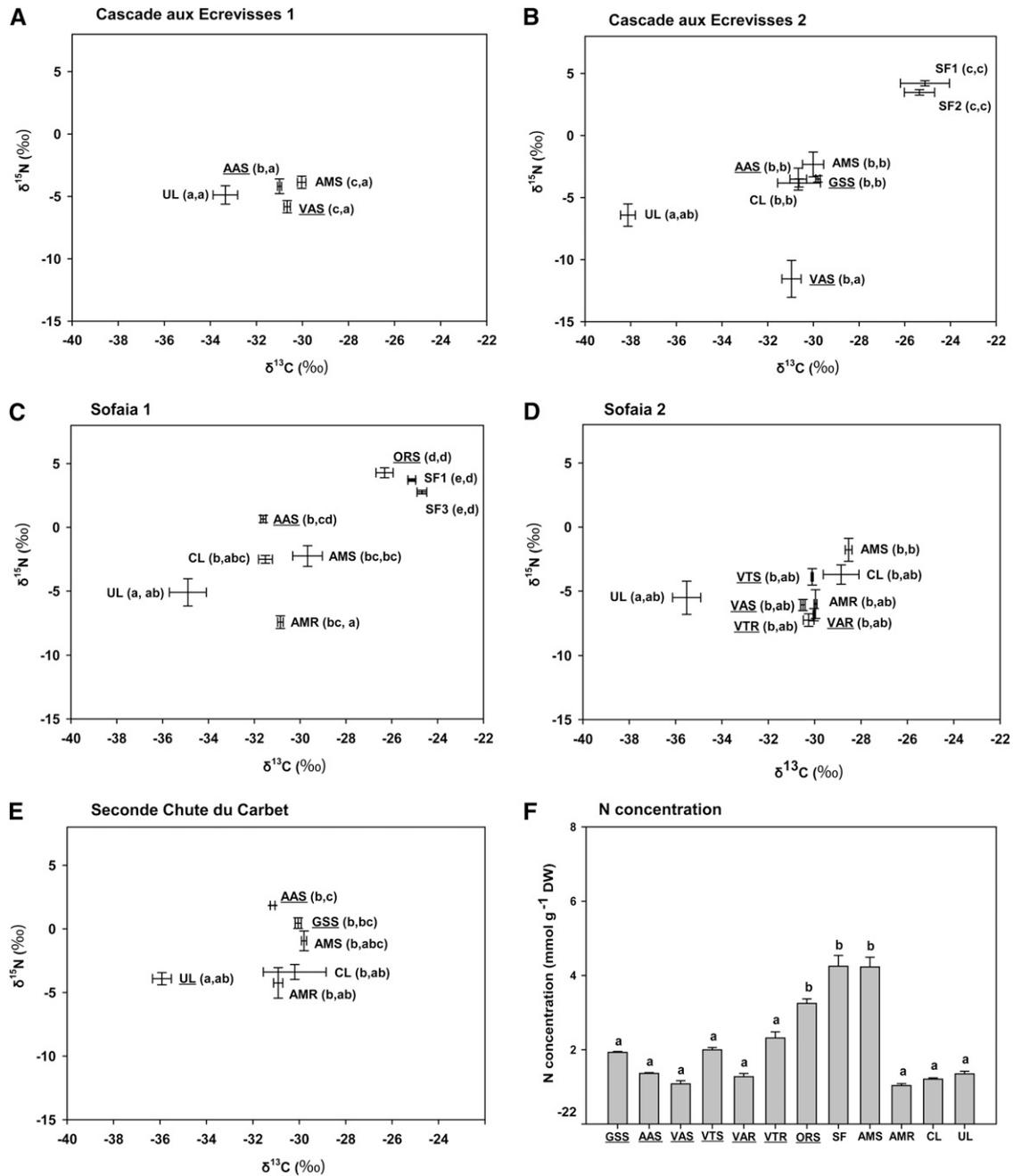
Figure 1. C versus N isotope abundance of plants and fungi at the five investigated sites (A–E) and N concentration (pooled for all sites; F), means ± SE. Labels of AMF-MH organs are underlined. Abbreviations: AAS, shoots of the MH Burmanniaceae *A. aphylla*; AMR, fine roots of canopy trees mycorrhizal with AMF; AMS, spores of AMF; CL, green canopy leaves (see Supplemental Table S1 for species names); GSS, shoots of the MH Burmanniaceae *Gymnosiphon* sp.; SF, saprotrophic fungi (1: *Lycogalopsis solmsii*; 2: *Collybia* sp.; 3: *Marasmius* sp.); UL, green understory leaves (see Supplemental Table S1 for species names); VAR, roots of the MH Gentianaceae *V. aphylla*; VAS, shoots of *V. aphylla*; VTR, roots of the MH Gentianaceae *V. tenella*; VTS, shoots of *V. tenella*; ORS, shoots of the MH orchid *W. aphylla*. Different letters denote significant differences according to pairwise *t* tests (Bonferroni corrected, $P < 0.01$; in A–E, the first letter is for δ^{13}C and the second one for δ^{15}N).

bration between leaf intercellular and atmospheric $^{13}CO_2$ and $^{12}CO_2$ concentrations in canopy leaves, thereby reducing the isotopic discrimination. Second, the depletion in understory plants is enhanced by incorporation of ^{13}C-depleted CO_2 originating from soil respiration. To summarize, measured isotopic abundances in autotrophic plant leaves indicated a canonical tropical rainforest pattern.

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AMF Negligibly Differ in Isotopic Abundances from Canopy Trees

AMF spores isolated from soil contained mixes of several species, as showed by barcoding on the internal transcribed spacer (ITS) of nuclear ribosomal DNA that provided several operational taxonomic units (OTUs; see Supplemental Table S3; GenBank accession numbers HQ857159–91 and JF276256–74). Despite probable differences in species composition and proportions, independent replicates showed quite similar isotopic abundances at each site (differences between replicates <0.5‰ in $\delta^{13}C$ and <1.1‰ in $\delta^{15}N$, see Supplemental Table S1). Thus, we make the approximation, in this discussion, that AMF locally share a common isotopic abundance. AMF hyphae, which are the active transport pathways, may differ isotopically from AMF spores that are enriched in C reserves. However, in natural ecosystems, it is difficult to recover clean hyphae without cytoplasm loss by hyphal breakage. In addition, our results in simpler systems (sorghum [*Sorghum bicolor*] and flax [*Linum usitatissimum*] cultures in terragreen-sand mixture; F. Walder, unpublished data) have shown similar $\delta^{13}C$ values in AMF spores and hyphal networks. Thus, we assume here that isotopic abundances in spores are a good proxy for these in AMF mycelia.

Spores were higher in $\delta^{13}C$ than both understory and canopy leaves (Fig. 1, A–E), but this difference was only significant for understory leaves. When they were available for analysis, mycorrhizal fine roots of canopy trees were also slightly, but nonsignificantly, more depleted in ^{13}C than AMF spores (by 0.96‰; Fig. 1, A, B, and E). The observed ^{13}C enrichment in spores contrasts with the 2‰ to 4‰ depletion reported for spores of AMF colonizing temperate herbs, as compared with their host's roots (Nakano et al., 1999, on *Gigaspora margarita*) or roots and shoots (Allen and Allen, 1990, on mixed AMF); a similar approximately 2‰ depletion as compared to host was obtained in our sorghum (C_4) and flax (C_3) cultures mentioned above (F. Walder, unpublished data). A contribution of C_4 or CAM host plants could enrich AMF in ^{13}C (Nakano et al., 1999), but such plants were absent at our study sites. Since AMF displayed similar $\delta^{13}C$ differences with leaves and roots at all sites (Fig. 1, A–E), this ^{13}C enrichment might be an idiosyncrasy of rainforest ecosystems. Indeed, as compared with previously studied temperate herbs, rainforest trees may differ by a higher flux of photosynthates to AMF (a higher flux would reduce the intensity of fractionation; Dawson et al., 2002); alternatively, tropical AMF may have lower levels of anaplerotic C fixation: This pathway, which provides carboxylic acids to control cellular pH and/or assimilate N, often refixes respiratory CO_2 depleted in ^{13}C (Raven and Farquhar, 1990).

Can a contribution of understory plants explain AMF $\delta^{13}C$? On the one hand, mixing canopy and depleted understory sources could lead to the observed $\delta^{13}C$ value in AMF if the latter receive compounds enriched in ^{13}C from all sources (see Supplemental Materials and Methods S1). Actually, carbohydrates transferred in

plants from leaves to roots and likely to AMF are enriched in ^{13}C by approximately 3‰ in comparison with leaf bulk C (Gleixner et al., 1993). Detailed calculation (see Supplemental Materials and Methods S1) nevertheless shows that for enrichments up to 3‰, the major contribution (55%–100%) is of canopy origin. On the other hand, available data showing depletion in ^{13}C in AMF spores (Allen and Allen, 1990; Nakano et al., 1999; see above) is incompatible with this calculation, which assumes enrichment as compared with the plant source (see Supplemental Materials and Methods S1). If little or no depletion occurs, then the use of compounds from understory plants would reduce the $\delta^{13}C$ value in AMF as compared with canopy tree leaves and roots. Although the exact fractionation at the plant/AMF interface deserves further study, we thus conclude that, in all scenarios, canopy trees constitute the major C source for AMF. This is congruent with the higher photosynthetic activity of canopy leaves (Lüttge, 2008), and with the situation reported in temperate regions, where forest ectomycorrhizal basidiomycetes receive 57% to 100% of their C from canopy trees (Högberg et al., 1999). Nevertheless, the observed isotopic pattern contrasts with ectomycorrhizal basidiomycetes from temperate forests, which are 1.2‰ to 2.9‰ enriched compared with canopy leaves (Högberg et al., 1999), and this suggests a different fractionation, or the transfer of isotopically distinct C compounds, during C exchange in AMF mycorrhizae and ectomycorrhizae.

AMF spores were significantly less enriched in ^{15}N than saprotrophic fungi (Fig. 1, A and D). Even if considerable variation may occur depending on the N source (Gebauer and Taylor, 1999), this contrasted with the ^{15}N enrichment usually reported in ectomycorrhizal fungi (Hobbie et al., 2001; Mayor et al., 2009). AMF were enriched in ^{15}N as compared with tree mycorrhizal roots (+3.01‰ on average) and all green leaves (+1.02‰), but often not significantly (Fig. 1, A–E). This is less than the >5‰ difference in $\delta^{15}N$ between ectomycorrhizal fungi and host plant leaves in both temperate forests (Gebauer and Taylor, 1999; Trudell et al., 2003; Tedersoo et al., 2006; Selosse and Roy, 2009) and tropical rainforests (Roy et al., 2009; Diédhiou et al., 2010). We think that these differences do not only result from tropical conditions, but mainly from physiological differences between AMF and ectomycorrhizal fungi. Although the pathways for N transfer to the plant are subject to debate (Chalot et al., 2006), they may not be fully identical in these two symbioses, leading to different fractionations. To summarize, to our knowledge, we provide the first measurements of $\delta^{13}C$ and $\delta^{15}N$ values for rainforest AMF in situ, and reveal negligible differences between AMF and canopy tree leaves. The reasons for these negligible differences deserve further physiological studies.

AMF-MH Negligibly Differ in Isotopic Abundances from AMF Spores

ITS barcoding of mycorrhizal fungi revealed OTUs from a single *Glomus* group A clade in *Gymnosiphon* sp.

at Seconde Chute Carbet (SCC) and *Apteria aphylla* at Cascade aux Ecrevisses 2 (CE2), as well as another clade in *V. aphylla* at Sofaïa 2 and CE2 (see Supplemental Table S2; Fig. 2; *Voyria tenella* was not investigated). At Sofaïa 2, *V. aphylla* displayed most of the OTUs from *Glomus* group A found at CE2 (11 out of 13), plus OTUs from four additional Diversisporales clades (Fig. 2). Although barcoding on a single, highly polymorphic locus such as ITS has some limitations (Sanders and Croll, 2010), our data are congruent with published evidence, based on ribosomal DNA coding sequences, that different AMF clades cooccur in *Voyria* species (Bidartondo et al., 2002; Merckx et al., 2010), and that several *Glomus* species occur in Burmanniaceae (Merckx and Bidartondo, 2008; Merckx et al., 2010). Fungal specificity is often relaxed in tropical MH species (Martos et al., 2009; Roy et al., 2009), although the absence of fungal overlap between coexisting AMF-MH at CE2 is congruent with some partner preference. Beyond these corroborating results, AMF diversity in *V. aphylla* roots overlapped with that of AMF sporulating in soil at CE2 and Sofaïa 2: At least one OTU from each *V. aphylla* mycorrhizal clade was found, among other OTUs, in spores pooled from surrounding soil (Fig. 2; see Supplemental Table S3). Thus, ITS barcoding confirms that AMF colonize our AMF-MH and that investigated soil spores partly originate from the mycorrhizal network linked to our AMF-MH. We did not further test whether the same OTUs occurred on the nearby tree roots, since it would be difficult to investigate the dense cluster of surrounding roots, but we consider that mycelia sporulating or providing C to AMF-MH are necessarily connected with surrounding plants to support their C needs.

Shoots of cooccurring AMF-MH species did not significantly differ in $\delta^{13}C$, except at CE1, nor in $\delta^{15}N$, except at CE2 (Fig. 1, A–E); reasons for these exceptions are unclear. Conversely, at Sofaïa 1, *A. aphylla* had sharply lower $\delta^{13}C$ and $\delta^{15}N$ values than the MH orchid *Wullschlaegelia aphylla*, which associates with saprotrophic basidiomycetes (Martos et al., 2009; Fig. 1A). This supports the hypothesis of a similar nutrition for all AMF-MH, differing from the nutrition in other MH plants. AMF-MH shoots did not significantly differ from canopy leaves, AMF spores, and AMF roots for both $\delta^{13}C$ and $\delta^{15}N$ (with few exceptions, showing diverging trends; Fig. 1, A–E). *A. aphylla*, *V. aphylla*, and *V. tenella* are on average somewhat depleted in ^{13}C compared to AMF spores, but this is not statistically significant. This situation resembles that for MH plants associated with basidiomycetes that share similar ^{13}C and ^{15}N abundances with their fungi (Gebauer and Meyer, 2003; Trudell et al., 2003; Roy et al., 2009; Selosse and Roy, 2009), as exemplified here by the MH *W. aphylla* and saprotrophic fungi.

At Sofaïa 2, the only site where enough material was recovered, AMF-MH roots did not differ isotopically from AMF-MH shoots and AMF spores (Fig. 1B). As a result, AMF-MH shoots were significantly higher in $\delta^{13}C$ than understory leaves (on average by 4.8‰), but

similar in $\delta^{15}N$ (or slightly higher at Sofaïa 1; Fig. 1, A–E). Our finding corroborates data from a French Guiana rainforest, where shoots of the AMF-MH *Dictyostega orobanchoides* (Burmanniaceae) and *V. aphylla* were enriched in ^{13}C (by 5.5‰ and 5.4‰, respectively) but not in ^{15}N as compared with understory leaves (Merckx et al., 2010). However, having access to AMF spores, we demonstrate here that the difference in $\delta^{13}C$ is not driven by the AMF-MH metabolism or by $\delta^{13}C$ values in AMF (Fig. 3), but that it likely arises from the photosynthetic conditions in canopy and understory trees, added to negligible changes in $\delta^{13}C$ along AMF networks. In this respect, AMF-MH plants contrast with MH plants associated with basidiomycetes, whose high $\delta^{13}C$ is driven by the ^{13}C enrichment of their mycorrhizal fungi.

Unexpectedly Low N Contents in AMF-MH

C and N concentrations did not differ among sites for each sample category ($H = 2.157$, df = 4, $P = 0.707$ for C and $H = 2.327$, df = 4, $P = 0.676$ for N; Kruskal-Wallis test, see Supplemental Table S1), and analyses at each site or on pooled data were congruent. C concentrations did not significantly differ (see Supplemental Fig. S2), while N concentrations revealed some differences (Fig. 1F): Together with saprotrophic fungi, AMF spores had significantly higher N concentrations than all other samples, except the MH orchid *W. aphylla* (see below). AMF-MH shoots showed nonsignificant differences in N concentrations between species (Fig. 1F), which did not correlate with their systematic position, i.e. Gentianaceae versus Burmanniaceae. In *V. aphylla* and *V. tenella*, N concentrations were higher in roots than in shoots (Fig. 1F), as expected since N-rich AMF colonize their roots.

N and C concentrations of AMF-MH were in the range typical for all green leaves (Fig. 1F). Conversely, MH plants associated with basidiomycetes always have a higher N concentration than green plants (Gebauer and Meyer, 2003; Julou et al., 2005; Roy et al., 2009; Selosse and Roy, 2009), and tend to have reduced C concentrations (Stöckel et al., 2011), as exemplified here by the MH orchid *W. aphylla* (Martos et al., 2009; Fig. 1F). The current interpretation for these MH plants is that they recover biomass from fungi with low C/N, and, by partly respiring it, further concentrate N (Abadie et al., 2006; Tedersoo et al., 2006; Stöckel et al., 2011). Moreover, high ^{15}N enrichment in these MH plants supports the hypothesis that they obtain fungal N through pathways differing from those in autotrophic plants (Gebauer and Meyer, 2003; Selosse and Roy, 2009). AMF-MH contrast with this scenario: First, to account for their moderate N enrichment after respiration, we have to suppose that they receive a fungal material with higher C/N than the average fungal biomass; second, as already stated by Merckx et al. (2010), similar ^{15}N enrichments in AMF-MH and green plants suggest that all tap isotopically similar N sources. To summarize, AMF-MH plants from two independent families, Gentianaceae and Burmannia-
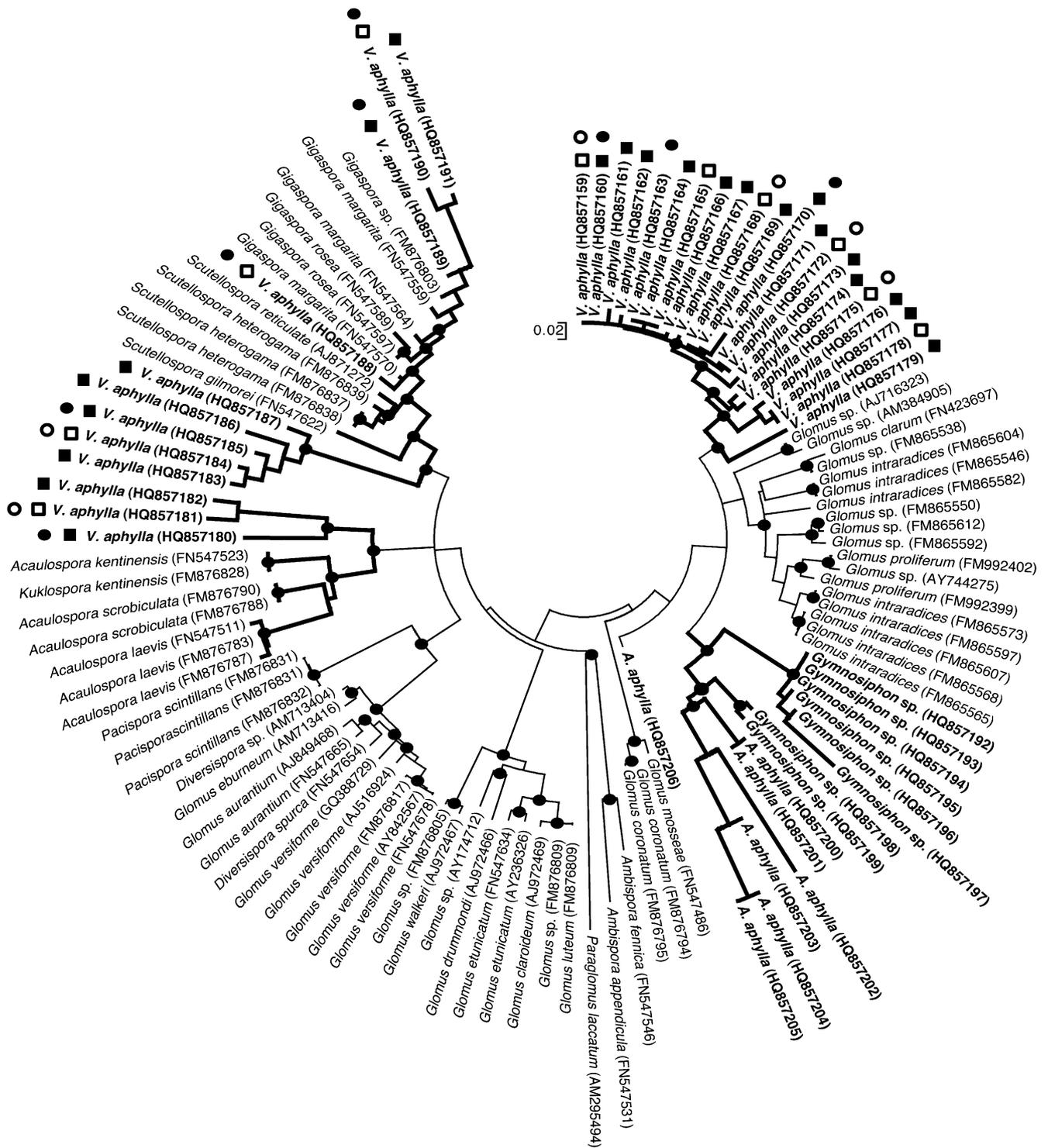
Figure 2. Neighbor-joining tree of AMF OTUs found in the roots of *A. aphylla* at CE2, *Gymnosiphon* sp. at SCC, and *V. aphylla* at Sofaïa 2 (squares) and CE2 (circles), based on an alignment of ITS sequences. For the later species, labels are white whenever the OTU was also found in spore pools from the soil surrounding *V. aphylla* (Supplemental Table S3) and black otherwise. Nodes marked with a circle have bootstrap values >95% (from 5,000 replications); the bar represents 0.02 base substitutions per site.

ceae, differ from MH plants associated with basidio-mycetes for mycorrhizal exchanges and N physiology, but are similar to green plants for N nutrition.

Beyond superficial similarities, such as reduced leaves or absence of stomata (Leake, 1994), the different MH lineages evolved independently, each within dif-

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Figure 3. Diagrammatic differences (mean ± SD) in ^{13}C between plant and fungal compartments: plain lines indicate nutrient flux; dotted lines indicate differences between compartments that are not directly linked. Abbreviation: AM roots, arbuscular mycorrhizal roots.
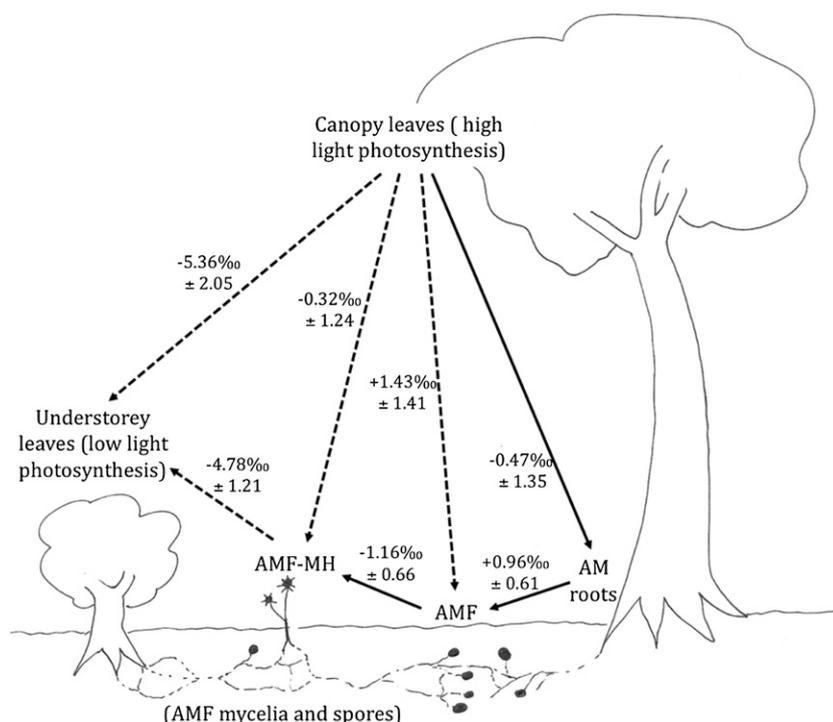
ferent ecological contexts. It was suggested that MH plants associated with basidiomycetes, mostly from temperate regions, might have arisen from a situation where C was simply hitchhiking with organic N and phosphorus (Selosse and Roy, 2009). Mixotrophic plants phylogenetically related to MH support this hypothesis: these partially MH plants are considered to retain the ancestral situation predisposing to MH evolution. In temperate regions, some of them, such as the orchid *Limodorum abortivum* (Girlanda et al., 2006) or ericaceous relatives of *Pyrola* (Tedersoo et al., 2006; Zimmer et al., 2007), occur in environments where N is limiting, but not light. These plants may recover fungal compounds mainly as a source of mineral nutrients, including N (Selosse and Roy, 2009). Even if C acquisition now plays the major role in MH plants associated with basidiomycetes, their high N content may have been inherited from this past functioning, persisting as an abuse of fungal N. Conversely, AMF-MH lineages probably arose in tropical forests (Merckx and Bidartondo, 2008), where light is limiting, but not N (Hedin et al., 2009; Huston and Wolverton, 2009). Thus, we speculate that tropical AMF-MH could have been selected directly for C acquisition, explaining why their N concentration and N nutrition are similar to those of surrounding plants. Thus, the convergence toward a similar MH nutrition may mask divergent histories and physiologies.

Implications for Future Research

We currently have only two isotopic studies on AMF-MH, all from South American rain forests (Merckx

et al., 2010; this study); more broadly, research on tropical mycorrhizal symbioses should be encouraged (Alexander and Selosse, 2009). More direct assessments of the fractionation at the interface between AMF and AMF-MH or autotrophic hosts are required, including analyses of transferred carbohydrates. Especially, the physiology of AMF-MH associations deserves the development of experimentally tractable AMF-MH models. Moreover, population-level, small-scale studies, using more marker loci than here, could rigorously demonstrate that AMF genets link AMF-MH to autotrophic hosts. In the future, investigations on the few available temperate AMF-MH (Bidartondo et al., 2002) could allow us to delineate which of the previous features are tropical, or strictly AMF dependent.

An exciting perspective is that some green species related to AMF-MH may be partially MH (Selosse and Roy, 2009; Cameron and Bolin, 2010; Merckx et al., 2010). Since AMF-MH are similar in ^{15}N and ^{13}C to their fungal sources, which are also similar to their own food source, isotopic enrichment is not a general fingerprint for MH nutrition. However, we predict that in dense forests at least, photosynthetic differences between canopy and understory plants (Fig. 3) may result in ^{13}C differences between partially MH plants and surrounding understory plants. This may not apply to open forest or grassland ecosystems where all leaves receive similar light levels, entailing similar ^{13}C abundances, and where higher frequency of C_4 plants (Bonal et al., 2000; Lüttge, 2008) complicates the use of ^{13}C abundances. Published investigations on candidate partially MH (=mixotrophic) plants associated with AMF showed that five green *Burmannia*

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species from French Guiana grassland (Merckx et al., 2010) and two Gentianaceae species from North American hardwood forest (Cameron and Bolin, 2010) were not enriched in ^{13}C, or in ^{15}N (except for one Gentianaceae), as compared with surrounding ground plants. Given the higher light level in these ecosystems, this finding does not necessarily disprove partially MH nutrition in itself, and, indeed, Merckx et al. (2010) conclude compellingly that the ability of the *Burmannia* species to grow in pots, isolated from any mycelial network, demonstrates the possibility of full autotrophy. In any case, we recommend (1) joint analysis of isotopic abundances in AMF spores and leaves from understory and canopy (the most likely C providers), and (2) the use, in dense C$_3$ forests only, of ^{13}C abundance for detecting partially MH plants.

CONCLUSION

This isotopic abundance analysis of AMF symbioses from rainforests offers a framework for understanding previous studies and planning future studies on AMF-MH and AMF networks. There is negligible isotopic change along the investigated points of the continuum between AMF (N providers to all partners), AMF-MH, and canopy trees (main C providers to all partners; Fig. 3). Thus, all MH plants, associated with AMF or basidiomycetes, share similar ^{13}C and ^{15}N abundances with their mycorrhizal fungi, but high ^{13}C abundance as compared with green plants is not per se a general property of all MH plants. AMF networks differ from ectomycorrhizal ones by (1) a negligible difference in δ^{13}C between green plants (canopy trees) and fungi, (2) the use of isotopically indistinct N sources by green and MH plants associated with AMF (Merckx et al., 2010), and (3) a low N concentration in AMF-MH. The latter trend, found in two families that independently evolved AMF-MH species, suggests a different metabolism in AMF-MH as compared with MH plants associated with basidiomycetes.

MATERIALS AND METHODS

Study Sites and Sampling

Study sites are tropical rainforests along the wet eastern coast of the La Guadeloupe Caribbean Island. The forest harbors C$_3$ shrubs and canopy trees (Rousteau, 1996; Sage, 2001) and rare CAM epiphytes, likely not connected to AMF networks. Five sites (2 × 2 m; including three in the La Guadeloupe National Park) were sampled in the last week of December 2009 (late rainy season; see Supplemental Table S1 for samples and location). At these sites, the average total rainfall per year is >2.33 m, and the average temperature is 24.7°C (ranging from 22.8°C–26.2°C in the coldest and hottest months). Near the Sofaïa spring (lowland tropical rainforest dominated by *Pouteria pallida*, *Guatteria caribaea*, and *Dacryodes excelsa*), we sampled two sites: Sofaïa 1, with the Burmanniaceae *Apteria aphylla* and the MH orchid *Wullschlaegelia aphylla* (associated with saprotrophic fungi; Martos et al., 2009), and Sofaïa 2 (200 m from Sofaïa 1), with the Gentianaceae *Voyria tenella* and *Voyria aphylla*. Near CE2 (tropical rainforest dominated by *Amanoa caribaea*, *D. excelsa*, and *Tapura antillana*), we sampled two sites: CE1, with *V. aphylla* and *A. aphylla*, and CE2 (150 m from CE1), with *V. aphylla*, *A. aphylla*, and the Burmanniaceae *Gymnosiphon* sp. (likely *Gymnosiphon sphaerocarpus*, endemic to the Caribbean islands)

At SCC (transition between lower montane rainforest and montane thicket, dominated by *Amanoa caribaea*, *Richeria grandis*, and *T. antillana*), we sampled the same *Gymnosiphon* sp. and *A. aphylla* (see Supplemental Fig. S1 for these AMF-MH). At all sites, we sampled soil, surrounding understory green plants (taking leaves at the height as from AMF-MH plants and avoiding young seedlings surviving on seed reserves). Whenever these were available, we collected fruitbodies of saprotrophic fungi and green canopy leaves mechanically torn off by heavy rains at the time of sampling (avoiding senescing leaves, and with a special sampling effort at Sofaïa 1, see Supplemental Table S1). Whenever roots of AMF-MH and/or fine roots of canopy trees mycorrhizal with AMF were found, they were carefully washed and kept separately. See Supplemental Table S1 for numbers of sampling repetitions. Samples were dried at 45°C for 48 h, except parts of AMF-MH roots that were kept at −80°C for molecular analyses.

AMF Spore Isolation

For each soil sample (see Supplemental Table S1), 30 g of dried soil was soaked and mixed for 30 min, and passed through 1,000-, 500-, 125-, and 32-μm sieves on a sieve shaker (AS 200 Digit; Retsch) with continuous shaking. The contents of the 125- and 32-μm sieves were resuspended in 20 mL of distilled water, and layered onto a LUDOX (HS-40 colloidal silica, 40 weight % suspension in water, Sigma-Aldrich) solution gradient. After centrifugation (2,000 rpm for 2 min), spores were removed with a syringe, passed through a 32-μm sieve, and washed with tap water. Spores were then observed under a dissecting microscope, picked one by one, and transferred to a petri dish. A minimum of 200 spores were transferred into a zinc capsule and dried at 50°C for 24 h before isotopic analysis and a minimum of 100 spores were pooled for molecular analysis.

Molecular Barcoding of AMF

We used the ITS of nuclear ribosomal DNA that is highly polymorphic, even within isolate (for review, see Sanders and Croll, 2010): Although this may be a limitation for fungal identification, ITS is thus a sensitive marker for comparing AMF diversity in spores and AMF-MH roots. DNA was extracted from spore pools sampled under *V. aphylla* as well as roots of *V. aphylla*, *A. aphylla*, and *Gymnosiphon* sp. (n = 3–4 replicates per sample; see Supplemental Table S2 for sampling sites) using the NucleoSpin tissue KS kit (Macherey-Nagel) and amplified on a T3 thermocycler (Biometra). A first amplification was performed using SSUmAf and LSUmAr primer pairs as in Krüger et al. (2009) on 1 μL of DNA extract in 12.5 μL final reaction volume. A nested PCR was then performed on 1 μL from the first amplicon, using the AMF-specific primers SSUmCf and LSUmBr as in Krüger et al. (2009). For both PCR reactions, the FirePol DNA polymerase (Solis Biodyne; 0.5 units), 2 mM MgCl$_2$, 0.5 μM each primer, and 0.25 mM each deoxynucleotide were used. Negative controls consisted of 1 μL of molecular grade water in all sets of PCR reaction to check for contamination and, in nested PCR, the second control consisted of a reamplified negative control from the first PCR round. Amplified fragments were subcloned into pCR2.1-TOPO and transformed into TOP10 *Escherichia coli* using the TOPO-TA cloning kit (Invitrogen). Individual colonies (24 per cloning) were screened for insert size by PCR amplification using M13 forward (5'-GTAAAACGACGGCCAGTG) and reverse (5'-GGAAACAGC-TATGACCATG) primers. Amplified products were purified with ExoSAP treatment (USB) and DNA sequencing was performed on a 3500 genetic analyzer (Applied Biosystems), using the M13 forward and reverse primers, and ITS-3 (5'-GCATCGATGAAGAACGCAGC). Sequences were manually corrected using Sequencher 4.2 (Gene Codes). We applied a 3% divergence threshold to circumscribe OTUs, and consensus sequences were deposited in GenBank (accession nos. HQ857159–HQ857191 and JF276256–JF276274; see Supplemental Tables S2 and S3). To identify fungal species, BLASTN searches were carried out against the sequence databases at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Consensus sequences were aligned with additional GenBank sequences and a phylogenetic analysis was conducted under MEGA 4 (Tamura et al., 2007). A neighbor-joining tree was created for each genus from the alignment file using the Kimura 2-parameter method and bootstrapping of 5,000 replicates.

Isotopic Analyses

All 184 investigated samples were ground in 1.5-mL Eppendorf tubes using 1.1-mm diameter tungsten carbide balls (Biospec Products, Inc.) in a Retsch MM301 vortexer (Retch GmbH and Co.). Total N and C concentrations

and abundances of ^{13}C and ^{15}N were measured using an online continuous flow CN analyzer (NA 1500; Carlo Erba) coupled with an isotope ratio mass spectrometer (Delta S; Finnigan). Isotope abundances are expressed in $\delta^{13}C$ and $\delta^{15}N$ values in parts per thousand relative to international standards V-PDB and atmospheric N_2: $\delta^{13}C$ or $\delta^{15}n = (R_{sample}/R_{standard} - 1) \times 1,000$, where R is the isotope ratio, i.e. $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The SD of the replicated standard samples ($n = 13$ per each 100 samples) was 0.027‰ for ^{13}C and 0.245‰ for ^{15}N.

Statistics

N and C concentrations, as well as $\delta^{13}C$ and $\delta^{15}N$ values were tested for normality and homogeneity of variances using a nonparametric test (the Kruskal-Wallis test) as the distribution of replicates is not homogenous between sites. One-way ANOVAs were performed for each variable and each site, followed by a pairwise t test (Bonferroni corrected) to calculate pairwise comparisons between group levels at $\alpha = 0.01$. All values were estimated by mean values followed by 95% confidence intervals. A general linear model procedure was used to calculate univariate ANOVA and to evaluate site effects on $\delta^{13}C$, $\delta^{15}N$, and C and N concentrations. Statistical analyses were computed using SPSS.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers HQ857159 to HQ857191 and JF276256 to JF276274.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The four AMF-MH species under study.

Supplemental Figure S2. C concentrations (pooled for all sites), means ± SE.

Supplemental Table S1. The five study sites and samples investigated for isotopic abundances and N concentrations.

Supplemental Table S2. GenBank accession numbers of ITS sequences for the OTUs recovered from AMF-MH roots fungi at Sofaïa 2, SCC, and CE2.

Supplemental Table S3. GenBank accession numbers of ITS sequences for the OTUs recovered from *V. aphylla* roots fungi and/or from AMF spore pools from the soil surrounding *V. aphylla* roots at Sofaïa 2 and CE2.

Supplemental Materials and Methods S1. Mixing model for $\delta^{13}C$ of AMF biomass, assuming a contribution of canopy and understory sources and an isotopic enrichment as compared with these plant sources.

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