Oecologia (2020) 192:929–937
https://doi.org/10.1007/s00442-020-04631-x

PHYSIOLOGICAL ECOLOGY – ORIGINAL RESEARCH

Isotopic evidence of arbuscular mycorrhizal cheating in a grassland gentian species

Kenji Suetsugu[1] · Jun Matsubayashi[2] · Nanako O. Ogawa[2] · Satoe Murata[3] · Risa Sato[3] · Hiroshi Tomimatsu[3]

Received: 4 December 2019 / Accepted: 5 March 2020 / Published online: 14 March 2020

Abstract

All orchids and pyroloids are mycoheterotrophic at least in the early stage. Many species are predisposed to mycoheterotrophic nutrition even in the adult stage, due to the initial mycoheterotrophy during germination. Although other green plants, such as gentian species, also produce numerous minute seeds, whose germination may depend on fungal associations to meet C demands, physiological evidence for partial mycoheterotrophy in the adult stage is lacking for most candidate taxa. Here, we compared the natural abundances of ^{13}C and ^{15}N isotopes in the AM-associated gentian species *Pterygocalyx volubilis* growing in high-light-intensity habitats with those of co-occurring autotrophic C_3 and C_4 plants and AM fungal spores. We found that *P. volubilis* was significantly enriched in ^{13}C compared with the surrounding C_3 plants, which suggests the transfer of some C from the surrounding autotrophic plants through shared AM networks. In addition, the intermediate $\delta^{15}N$ values of *P. volubilis*, between those of autotrophic plants and AM fungal spores, provide further evidence for partial mycoheterotrophy in *P. volubilis*. Although it is often considered that light deficiency selects partial mycoheterotrophy, we show that partial mycoheterotrophy in AM-forming plants can evolve even under light-saturated conditions. The fact that there have been relatively few descriptions of partial mycoheterotrophy in AM plants may not necessarily reflect the rarity of such associations. In conclusion, partial mycoheterotrophy in AM plants may be more common than hitherto believed.

Keywords Gentianaceae · Stable isotope · Symbiosis · Mycorrhiza · Mixotrophy · Mycoheterotrophy

Introduction

Mycorrhizas represent a form of diffuse symbiosis, wherein a single plant simultaneously associates with multiple fungi and each fungus simultaneously associates with multiple plants (Smith and Read 2008). Mycorrhizas typically exhibit mutualistic relationships in which there is an exchange of plant-generated photosynthates and soil-derived mineral nutrients acquired by fungi (Smith and Read 2008). Most terrestrial plants, from liverworts to angiosperms, form such mutualistic arbuscular mycorrhizal (AM) symbioses with fungi belonging to Glomeromycotina.

However, many achlorophyllous (i.e., fully mycoheterotrophic) plants, including species belonging to families Burmanniaceae, Corsiaceae, Gentianaceae, Polygalaceae, Petrosaviaceae, Iridaceae, Thismiaceae, and Triuridaceae, associate with AM fungi, indicating that the transfer of C from AM fungi to plants should certainly occur (Leake 1994; Yamato 2001; Yamato et al. 2011; Bidartondo et al. 2002; Merckx and Bidartondo 2008; Merckx et al. 2010; Courty et al. 2011; Suetsugu et al. 2014; Gomes et al. 2017). Given that AM fungi are obligately biotrophic, these mycoheterotrophic plants indirectly obtain C from surrounding green plants through shared fungi (Bidartondo et al. 2002; Yamato et al. 2016; Gomes et al. 2017).

It is generally assumed that the transition from autotrophy to full mycoheterotrophy have occurred via an intermediate stage that is referred to as partial mycoheterotrophy or

Communicated by Susana Rodriguez Echeverria.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00442-020-04631-x) contains supplementary material, which is available to authorized users.

✉ Kenji Suetsugu
kenji.suetsugu@gmail.com

[1] Department of Biology, Graduate School of Science, Kobe University, Kobe, Japan

[2] Department of Biogeochemistry, Japan Agency for Marine-Earth Science and Technology, Yokosuka, Kanagawa, Japan

[3] Faculty of Science, Yamagata University, Yamagata, Japan

mixotrophy (Gebauer and Meyer 2003; Selosse and Roy 2009; Hynson et al. 2013; Lallemand et al. 2016). Indeed, partial mycoheterotrophy has been detected in many orchids and pyroloids that associate with ectomycorrhizal fungi. The abundance of natural stable isotopes has provided a useful means for assessing the degree of mycoheterotrophy in these taxa (Gebauer and Meyer 2003; Tedersoo et al. 2007; Hynson et al. 2013; Selosse et al. 2017b; Suetsugu et al. 2017, 2018, 2019, 2020), as the ^{13}C and ^{15}N abundances in ectomycorrhizal fungi are significantly higher than those in the surrounding plants (Gebauer and Meyer 2003; Hynson et al. 2013). For example, some green orchids that are closely related to mycoheterotrophic species show ^{13}C and ^{15}N abundances that are intermediate to that of autotrophic plants and full mycoheterotrophs, suggesting partial mycoheterotrophy (Selosse and Roy 2009; Hynson et al. 2013). Besides, deuterium (^{2}H) abundance has been employed as an additional tool to identify partially mycoheterotrophic plants specifically in cases where ^{13}C abundance could not be sufficiently used to do so (Gebauer et al. 2016; Schiebold et al. 2018; Schweiger et al. 2018, 2019). These studies have found that partially mycoheterotrophic species are widespread among forests and meadow orchids with the saprotrophic fungi, the rhizoctonia (Gebauer et al. 2016; Schiebold et al. 2018; Schweiger et al. 2019). Thus, while all the orchids and pyroloids produce minute seeds and rely on fungi for germination, adults of some species can still obtain C from these fungal partners in addition to that from photosynthesis.

In contrast, evidence of partial mycoheterotrophy is largely lacking for AM plants (Giesemann et al. 2020), even though fully mycoheterotrophic plants from diverse lineages exploit AM fungi. Several studies utilizing ^{14}C labeling showed that neighboring plants facilitate interplant C transfer by shared AM fungi, but the transferred C often remains in the roots of the recipient plants, likely within the fungal mycelium (Lerat et al. 2002; Pfeffer et al. 2004; Nakano-Hylander and Olsson 2007; Lekberg et al. 2010). In addition, natural stable isotopic approaches often provide inconclusive evidence for partial mycoheterotrophy in AM plants (Cameron and Bolin 2010; Merckx et al. 2010; Hynson et al. 2013; Bolin et al. 2017; Selosse et al. 2017b). It is known that AM fungal spores have ^{13}C and ^{15}N isotopic signatures similar to those of their host plants (Nakano et al. 1999; Merckx et al. 2010; Courty et al. 2011). Consequently, fully mycoheterotrophic plants can have isotopic signatures similar to those of the surrounding autotrophic plants (Merckx et al. 2010), while ^{13}C, ^{15}N, and ^{2}H isotopes in many forest understory plants have been found to be enriched (Courty et al. 2011; Gomes et al. 2020). Courty et al. (2011) demonstrated that AM-forming fully mycoheterotrophic plants display ^{13}C enrichment compared with that of surrounding understory autotrophic plants in tropical rainforests, the isotopic signature of both the AM fungi and the mycoheterotrophs was similar to that of canopy trees. The ^{13}C enrichment in AM-forming mycoheterotrophs, thus, appears to reflect the $\delta^{13}C$ values of canopy trees (plausible C sources of AM-forming mycoheterotrophs) that have higher $\delta^{13}C$ values than understory plants due to differential stomatal opening, humidity, and photosynthetic rates (Gebauer and Schulze 1991; Courty et al. 2011; Selosse et al. 2017b). Therefore, the ^{13}C signatures of AM-forming partial mycoheterotrophs should be much more similar to those of the surrounding autotrophic plants in open environments, such as fields or open-canopy forests (Hynson et al. 2013). Consistent with this assumption, most studies that have evaluated whether green plants that are phylogenetically close to mycoheterotrophic plants exploiting AM fungi have found negligible or non-existent isotopic differences between the candidate plants and surrounding autotrophs (Cameron and Bolin 2010; Merckx et al. 2010; Field et al. 2015; Bolin et al. 2017). The recent findings that some green understory plants that develop *Paris*-type AM show a significant enrichment in stable isotopes, compared with other autotrophic understory plants (Cameron and Bolin 2010; Giesemann et al. 2020), is possibly due to the same mechanism as those of AM-forming full mycoheterotrophs.

Significant ^{13}C enrichment in mycoheterotrophic plants may be detected even in open habitats if both C_3 and C_4 plants associate with AM fungi, as C_4 plants have significantly higher ^{13}C abundances than C_3 plants. This is owing to differences in isotopic fractionations related to primary CO_2 fixation, a process that is catalyzed by Rubisco in C_3 plants and PEPCase in C_4 plants (O'Leary 1988; Těšitel et al. 2010, 2011). Considering that the ^{13}C abundances in fungal spores mirror those in the surrounding plants that act as C donors (Nakano et al. 1999; Courty et al. 2011, 2015), assessment of ^{13}C abundance can still provide an effective approach for detecting partial mycoheterotrophy in AM plants (Bolin et al. 2017). Indeed, Bolin et al. (2017) showed that the bluethread plants (*Burmannia coelestis*) were significantly enriched in ^{13}C compared with that in the surrounding C_3 reference plants in the population that also contains C_4 plants. Therefore, even though *B. coelestis* can be cultivated without AM fungi or surrounding AM-forming plants (Merckx et al. 2010), this species could at least be facultatively partially mycoheterotrophic.

Here, we focused on the putatively partially mycoheterotrophic plant *Pterygocalyx volubilis* (Gentianaceae; Fig. 1) in fields that also contain C_4 plants (*Miscanthus sinensis* and *Microstegium vimineum*). This gentian species has reduced stubby roots lacking root hairs that are the hallmarks of mycoheterotrophic plants (Leake 1994; Imhof et al. 2013). In addition, *P. volubilis* produces numerous minute dust seeds that are likely dependent on associations with AM fungi for their supply of C during germination (Murata

Fig. 1 *Pterygocalyx volubilis* **a** flowering plant entwining a stem of *Miscanthus sinensis.* **b** Close-up view of a flower. **c** Reduced roots. **d** Dust seeds. Bar = 1 mm

2003; Eriksson and Kainulainen 2011). Consistent with the previous observations (Fujiyoshi et al. 2005), our preliminary morphological investigations revealed that mycorrhizal colonization of *P. volubilis* is characterized by intracellular cell-to-cell spread and is categorized as a *Paris*-type AM that is a common morphological type of mycorrhizal associations in AM-forming mycoheterotrophs (Imhof et al. 2013). Therefore, we assume the likelihood of C transfer from C_4 plants through shared AM networks. Based on these characteristics, partial dependence on fungal-derived C has been suspected (Murata 2003).

In this study, we evaluated the physiological ecology of *P. volubilis*. Specifically, we investigated whether the ^{13}C signature of this AM-associated grassland gentian species differs sufficiently from that of the surrounding C_3 and C_4 plants, to determine whether *P. volubilis* is characterized by partial mycoheterotrophy. AM fungi are difficult to sample since they do not produce conspicuous organs such as fruitbodies, and sampling of extraradical mycelia in the field is technically unrealistic (Courty et al. 2011, 2015). This in turn hampers isotope analysis of AM fungi. We thus isolated AM fungal spores from soil surrounding the roots of *P. volubilis* individuals and determined the ^{13}C and ^{15}N abundances in these spores, by mass spectrometry optimized for microscale analysis (Ogawa et al. 2010). The ^{13}C and ^{15}N abundances in these spores obtained by microscale isotope analysis helped us to confirm the hypothesis that *P. volubilis* actually uses the carbon originating from AM fungi as a part of their energy sources.

Materials and methods

Study site and sampling

The field study was conducted in a warm and temperate grassland in Ikawa, Shizuoka Prefecture, Japan. The population contained ca. 30 flowering individuals of *P. volubilis* (Fig. 1). The samples for isotopic analysis were collected on 20 May 2017. The investigated population was dominated by *Miscanthus sinensis.* The C_4 grasses including *Miscanthus sinensis* accounted for ca. 30% of total vegetation cover.

Six sampling plots of 1 m^2 were selected in the investigated population. In each plot, we collected the leaves of a *P. volubilis* individual and an individual of each co-occurring C_3 and C_4 autotrophic reference species. To limit the influence of environmental factors on stable isotope ratios, such as atmospheric CO_2 isotope composition, microscale light climate, and soil type (Gebauer and Schulze 1991), the leaves of reference plants were collected at the same

height of the focal *P. volubilis* individual. This criterion led us to sample *Miscanthus sinensis* (C_4 plant), *Microstegium vimineum* (C_4 plant), *Deutzia crenata* (C_3 plant) and *Geranium thunbergii* (C_3 plant), since no other plants were present in more than two plots. In addition, we collected the soil around *P. volubilis* roots at each plot to obtain AM fungal spores.

Isotopic analysis of plants

The collected leaves were dried at 60 °C for 4 days and then cut into fine pieces using small scissors and a surgical knife. The abundances of stable C and N isotopes and total N concentrations of samples were measured at the Research Institute for Humanity and Nature (Kyoto, Japan) using a Delta XP mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) that are connected to a Flash EA 2000 elemental analyser (Thermo Fisher Scientific). The relative abundances of the stable isotopes were calculated as $\delta^{15}N$ or $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000$ (‰), where R_{sample} represents the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the sample, respectively, and $R_{standard}$ represents the $^{13}C/^{12}C$ ratio of Vienna Pee Dee Belemnite (VPDB) or the $^{15}N/^{14}N$ ratio of atmospheric nitrogen. Both the isotope ratios of C and N were calibrated using the laboratory standards: glycine ($\delta^{13}C = -34.92$‰) and L-threonine ($\delta^{13}C = -9.45$‰) for C, and DL-alanine ($\delta^{15}N = -2.89$‰) and L-alanine ($\delta^{15}N = 22.71$‰) for N, which are traceable back to international standards (Tayasu et al. 2011). The analytical standard deviations (SD) of these standards were 0.05‰ ($\delta^{13}C$, $n = 8$) for glycine, 0.05‰ ($\delta^{13}C$, $n = 3$) for L-threonine, 0.04‰ ($\delta^{15}N$, $n = 6$) for DL-alanine and 0.14‰ ($\delta^{15}N$, $n = 17$) for L-alanine. The total N concentrations in the leaf samples were calculated using sample weights and the volume of CO_2 and N_2 gas of the laboratory standards (Tayasu et al. 2011).

Isolation of AM fungal spores

Spores of AM fungi were isolated from soil using a method similar to those described in Brundrett et al. (1996) and Courty et al. (2011). The soil of all the plots were pooled, mixed, and sifted (with a 4-mm sieve) before analysis. Each of four subsamples of 10 g soil was soaked and mixed in a substantial volume of tap water, and passed through 500-, 125-, and 53-µm sieves. The contents of the 125- and 53-µm sieves were resuspended in distilled water and centrifuged at 2000 rpm for 5 min. The resulting pellet was again resuspended with 15 mL of distilled water and layered onto 15-mL LUDOX HS-40 (colloidal silica with a density of 1.3 g/mL, Sigma-Aldrich) solution. After centrifugation at 2000 rpm for 5 min, opaque band containing spores was collected with a pipette, passed through a 53-mm sieve, and washed with distilled water. Spores were collected from the material on the sieve transferred to a Petri dish under a dissecting microscope. Approximately 100 spores were dried at 60 °C for 24 h.

Isotopic analysis of AM fungal spores

Our spore samples were too small in volume (< 0.1 mg) to measure using a standard EA-IRMS system. For this reason, $\delta^{13}C$ and $\delta^{15}N$ values of combined spores (10–12 µg dry weight, $n = 5$) were measured at the Japan Agency for Marine-Earth Science and Technology (JAMSTEC), using a Flash EA 1112 coupled to a Deltaplus XP IRMS (Thermo Fisher Scientific, Bremen, Germany) instrument, which is optimized for microscale isotope analysis (Ogawa et al. 2010). The total N concentrations and isotope ratios of C and N were calibrated using the laboratory standards: L-tyrosine ($\delta^{13}C = -20.83$‰, $\delta^{15}N = 8.74$‰), DL-alanine ($\delta^{13}C = -25.36$‰, $\delta^{15}N = -2.89$‰) and glycine ($\delta^{13}C = -34.92$‰, $\delta^{15}N = 2.18$‰) (Tayasu et al. 2011). The analytical precisions of the method determined by repeated analysis of L-tyrosine standards are ± 0.07‰ (1σ, $n = 9$) for $\delta^{13}C$ and ± 0.18‰ (1σ, $n = 9$) for $\delta^{15}N$.

Statistics

After confirming that the datasets were normally distributed using Bartlett's test, differences in the $\delta^{15}N$ values were analyzed using one-way ANOVA, followed by Fisher's multiple comparison test. Since the $\delta^{13}C$ values and the total N concentrations values were not normally distributed, a Kruskal–Wallis nonparametric test and a sequential Mann–Whitney *U* test were used.

In addition, enrichment factors (ε) were calculated from the δ values of each plant group based on the definition by Gebauer and Meyer (2003): $\varepsilon = \delta_S - \delta_{REF}$, where δ_S represents a single $\delta^{13}C$ value of a *P. volubilis* individual and δ_{REF} represents the mean value of all autotrophic C_3 reference plants from a specific sampling plot (Preiss and Gebauer 2008). When we calculated the enrichment factors (ε) of AM spores, we used a simplified site-wise calculation, since spores could not be investigated plot-wise, due to their minute status (Hynson et al. 2013; Preiss et al. 2010). The percentage of C derived from fungi (% C_{df}) in *P. volubilis* was estimated using the linear two-source mixing model: % $C_{df} = (\varepsilon PC/\varepsilon SC) \times 100$, where εPC represents the enrichment factor of *P. volubilis* individuals, and εSC is the mean enrichment factor of AM spores as the endpoint. This model is based on the previous knowledge that AM-forming fully mycoheterotrophs and AM fungal spores have similar ^{13}C isotopic signatures (Courty et al. 2011).

Results

The $\delta^{13}C$ values of *P. volubilis* (− 30.8 ± 1.3‰; mean ± SD) were significantly higher than those of autotrophic C_3 reference plants (− 32.5 ± 1.5‰; $P < 0.05$; Fig. 2, Table S1). The $\delta^{13}C$ values of AM fungal spores (− 25.9 ± 3.0‰) were significantly higher than those of not only C_3 plants but also *P. volubilis* ($P < 0.001$ in both comparisons). Using the mean $\varepsilon^{13}C$ values of AM fungal spores as the fully mycoheterotrophic endpoint, the percentage of C derived from AM fungi was estimated as 23.0 ± 15.3% in *P. volubilis*.

The $\delta^{15}N$ values of AM fungal spores (− 1.1 ± 1.0‰) were significantly higher than that of autotrophic reference plants (− 3.0 ± 1.1‰; $P < 0.01$), while there was no significant difference in the $\delta^{15}N$ values between AM fungal spores and *P. volubilis* (− 1.7 ± 1.5‰; $P = 0.41$). The $\delta^{15}N$ values of *P. volubilis* were also significantly higher than those of autotrophic reference plants ($P < 0.05$). The total N concentrations in *P. volubilis* (3.6 ± 0.9 mmol/g) were also significantly higher than those in reference plants (2.4 ± 0.6 mmol/g; $P < 0.01$). In addition, the N concentrations of AM fungal spores (5.6 ± 1.6 mmol/g) were significantly higher than those of not only autotrophic plants but also *P. volubilis* ($P < 0.01$ in both comparisons).

Discussion

Partial mycoheterotrophy in *Pterygocalyx volubilis*

We found that the $\delta^{13}C$ values of both AM fungal spores and *P. volubilis* were significantly enriched compared with those in co-occurring C_3 plants. Slightly higher $\delta^{13}C$ values have been reported for AM fungal spores than for the AM roots of C_3 plants (the C donors) (mean difference 0.72‰, maximum difference 4.1‰) (Courty et al. 2015). Nonetheless, this cannot explain the difference in $\delta^{13}C$ values between the C_3 plants and AM fungal spores observed in this study (mean difference 6.6‰, maximum difference 10.2‰). This provides evidence that the large degree of enrichment that we observed in the AM spores reflects the influence of the nearby C_4 plants, and that the mixed C isotope signature of the AM fungi originates from both C_3 and C_4 hosts. Consequently, our stable isotope results strongly suggest that C had been transferred from C_4 plants to *P. volubilis* via epiparasitism since the $\delta^{13}C$ values of *P. volubilis* were intermediate between those of AM fungal spores and co-occurring C_3 plant. The $\delta^{13}C$ values of *P. volubilis* provide the evidence for partial mycoheterotrophy in *P. volubilis*. In addition, we found that the $\delta^{15}N$ values of both AM fungal spores and *P. volubilis* were significantly higher than those in the surrounding plants. The intermediate $\delta^{15}N$ values of *P. volubilis* between those of autotrophic reference plants and AM fungal spores provide further evidence for partial mycoheterotrophy in *P. volubilis*.

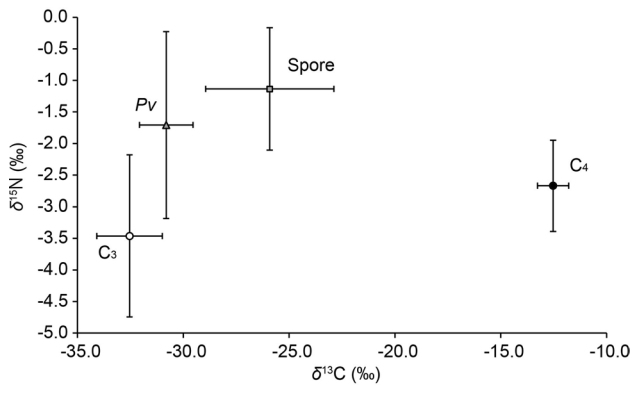

Fig. 2 Values of $\delta^{13}C$ and $\delta^{15}N$ (mean ± 1 SD) in the leaves of *Pterygocalyx volubilis* (*Pv*, gray triangle), neighboring C_3 (open circle) and C_4 (filled circle) autotrophic plants, and AM spores (gray square)

Using the enrichment factors of AM spores as a fully mycoheterotrophic endpoint, we estimated that *P. volubilis* obtained 23 ± 15% of its total C demand from its fungal partners. Approximately 20% of organic matter derived from a fungal source falls within the lower range of the values previously reported for partially mycoheterotrophic species (Gebauer and Meyer 2003; Bidartondo et al. 2004; Julou et al. 2005; Abadie et al. 2006; Stöckel et al. 2011, 2014; Suetsugu et al. 2017; Schweiger et al. 2018). However, our estimation may not be precise due to several limitations. First, AM fungal spores may have $\delta^{13}C$ values different from those of AM hyphae (the active transport pathways), suggesting that it is difficult to use AM spores to accurately estimate the fungal contribution to mycoheterotrophic plants. However, Walder et al. (2013) have shown similar $\delta^{13}C$ values in AM spores and hyphal networks in microcosms containing both C_3 and C_4 species. In addition, since it is technically difficult to recover clean hyphae without losing any cytoplasm by breaking them (Courty et al. 2011), the isotopic abundances of spores are the best proxies for those of AM hyphae since one can obtain spores from the natural field (Courty et al. 2011; Walder et al. 2013). Second, it remains unknown whether these spores are mycobionts that link *P. volubilis* to the surrounding vegetation. However, apart from highly specialized interaction found in fully mycoheterotrophic plants, green plants including putatively partially mycoheterotrophic ones are associated with very diverse AM mycobionts (Merckx et al. 2010; Suetsugu et al. 2014; Yamato et al. 2014; Gomes et al. 2017). In fact, approximately 80% of all angiosperms are associated with AM fungi that contain less than 300 species (Öpik et al. 2013). Given that these spores include multiple morphological species such as *Septoglomus* and *Rhizophagus*, these

spores will include at least some mycobionts in *P. volubilis* and the surrounding vegetation.

Despite several limitations for precise estimation in the degree of mycoheterotrophy, the assumed relatively low dependence of *P. volubilis* on fungal-derived C may be linked to the high levels of irradiation that typify this plant's grassland habitats. Accordingly, we speculate that the partially mycoheterotrophic nutritional mode of *P. volubilis* may have evolved as a byproduct of the acquisition of minerals, such as N, rather than C, as suggested for some partially mycoheterotrophic plants (Selosse and Roy 2009; Selosse et al. 2017a). The difference in both ^{15}N natural abundance and N concentrations between *P. volubilis* and autotrophic reference plants could provide some evidence to support this hypothesis (see also next section).

Evolution of partial mycoheterotrophy in open grasslands

It is of interest to note that, while most of the partially and fully mycoheterotrophic plants examined to date grow in forests with dense overstories that produce deep shade (Merckx et al. 2013a), *P. volubilis*, in contrast, grows in open habitats exposed to high irradiance levels. It has been suggested that an evolutionary switch from autotrophy to full mycoheterotrophy may be accompanied by a shift towards growth in more shaded habitats (Merckx et al. 2013a). However, several recent studies have also shown that some rhizoctonia-associated orchids that grow in open habitats are partially mycoheterotrophic (Girlanda et al. 2011; Schiebold et al. 2018). These studies suggest that the C demand cannot be the sole driver of this nutritional strategy. In a broader context, mixotrophy may have frequently evolved in not only mycoheterotrophic but also parasitic and carnivorous plants that grow in nutrient-poor open habitats to meet the mineral demands of these plants (Selosse and Roy 2009; Selosse et al. 2017a).

In this regard, although mixotrophy is generally considered in the context of C acquisition (Press 1989), mixotrophy sensu lato has long been recognized in hemiparasitic and carnivorous plants, which often grow in open grasslands (Selosse and Roy 2009; Selosse et al. 2017a). Hemiparasitic and carnivorous plants typically obtain their requisite non-C nutrients by parasitizing other plants or digesting animals (Selosse and Roy 2009; Selosse et al. 2017a), and their efficiency in this respect contributes to the success of these plants in nutrient-poor environments (Quested 2008). In addition, certain hemiparasitic and carnivorous plants gain a proportion of their organic C from their host plants (Klink et al. 2019; Press et al. 1987; Schulze et al. 1991). This suggests a scenario wherein C flow occurs due to meeting mineral needs (Hynson et al. 2013; Selosse and Roy 2009), with the resulting mixotrophy in turn facilitating the emergence of further C dependence.

Accordingly, partial mycoheterotrophy might be expected to evolve under light-saturated conditions to compensate for deficiencies associated with growth in mineral nutrient-poor environments. Indeed, *P. volubilis* is often found growing in nutrient-poor grasslands. In this respect, it is notable that both δ^{15}N values and N concentrations in *P. volubilis* were significantly higher than those in the surrounding vegetation. In contrast, a previous study has demonstrated that both values of AM-forming fully mycoheterotrophs are not always higher than those of co-occurring autotrophic plants (Courty et al. 2011; Gomes et al. 2020; Merckx et al. 2010), while mycoheterotrophic plants associated with ectomycorrhizal fungi are always characterized by higher δ^{15}N values and N concentrations than those of autotrophic plants (Gebauer and Meyer 2003; Julou et al. 2005; Stöckel et al. 2011). The discrepancy of δ^{15}N values and N concentrations among AM-forming mycoheterotrophs including *P. volubilis* may suggest that AM-forming mycoheterotrophs utilize nitrogen from different sources, such as from their associated fungal partners or from the substrate by themselves, as discussed by Gomes et al. (2020).

Evolution of partial mycoheterotrophy in a lineage without fully mycoheterotrophic relatives

Similar to the majority of angiosperms, members of Gentianaceae family are primarily autotrophic plants that form AM associations (Leake 1994). Nonetheless, a small percentage (less than 2%) of Gentianaceae species, such as those in the genera *Voyria* and *Voyriella*, are fully mycoheterotrophic (Cameron and Bolin 2010). Phylogenetic analyses have shown that full mycoheterotrophy has appeared independently at four different times during the evolutionary history of Gentianaceae: once in both *Voyria* and *Voyriella*, once in *Exochaenium*, and at least once in *Exacum*. However, these genera are only distantly related to *P. volubilis* (Merckx et al. 2013b).

Green photosynthetic orchids and pyroloids that are closely related to mycoheterotrophic orchids tend to be partially mycoheterotrophic (Hynson et al. 2013; Selosse and Roy 2009). Given that the evolution from autotrophy to full mycoheterotrophy is a gradual process, both initial and partial mycoheterotrophy can be considered intermediate steps in this process. In this respect, the evolution of initial mycoheterotrophy is the critical step for the release from full autotrophy (Eriksson and Kainulainen 2011; Jacquemyn and Merckx 2019). Therefore, it can be speculated that partial mycoheterotrophy may have evolved in plant groups that contain examples of initial mycoheterotrophy but do not include any fully mycoheterotrophic species. Partial mycoheterotrophy in *P. volubilis* would be one such example, given that

species of Gentianaceae with dust seeds have been suspected to be initially mycoheterotrophic (Eriksson and Kainulainen 2011; Murata 2003). Similarly, species of *Obolaria* (Gentianaceae), which do not have fully mycoheterotrophic relatives, have been suspected of being partial mycoheterotrophs (Cameron and Bolin 2010; Leake 1994). Indeed, Cameron and Bolin (2010) found that *Obolaria virginica* specimens they examined were significantly enriched in ^{13}C compared with that of the surrounding vegetation. Considering that this species is a forest understory plant, C transfer from canopy leaves to *Obolaria* individuals is strongly suggested.

Furthermore, it is highly likely that many partially mycoheterotrophic species remain undiscovered, in view of the fact that AM associations have been shown to be the most common type of mycorrhizal association (Leake 1994; Merckx et al. 2013a). It is worth noting that *P. volubilis* also forms *Paris*-type AM association. Only very recently, Giesemann et al. (2020) showed that ^{2}H abundance can be used to identify partially mycoheterotrophic AM plants specifically in cases where ^{13}C abundance does not provide unequivocal evidence of such. They also suggested that partial mycoheterotrophy could potentially be widespread among ca. 100,000 plant species that develop *Paris*-type AM associations since (i) all AM-forming full mycoheterotrophic plants reported so far show *Paris*-type AM associations, and (ii) the seemingly autotrophic plant *Paris quadrifolia* without any known (even initially) mycoheterotrophic relatives appears to obtain nearly 50% carbon from its fungal partners. The daring hypothesis by Giesemann et al. (2020) warrants further investigation.

Conclusions

The present study is one of the few studies to provide convincing evidence that partial mycoheterotrophy can arise in plants forming associations with AM fungi (Giesemann et al. 2020). To our knowledge, this study is the first to report ^{13}C and ^{15}N abundances in both an AM-forming partially mycoheterotrophic plant and the spores of surrounding AM fungi. Nevertheless, the fact that, to date, there have been relatively few descriptions of partial mycoheterotrophy in AM plants may not necessarily be a reflection of the rarity of such associations. Instead it rather suggests the difficulty in demonstrating such relationships based on ^{13}C and ^{15}N abundance analyses, which have facilitated the discovery of mixotrophy in orchids and pyroloids (Selosse et al. 2017b). The transfer of C from AM fungi to putative partially mycoheterotrophic plants is difficult to detect based on ^{13}C abundance, particularly under conditions where the surrounding vegetation primarily comprises species characterized by C_3 photosynthesis.

Nonetheless, determining the $\delta^{13}C$ can represent a powerful approach for detecting partial mycoheterotrophy in plants that form associations with AM fungi when these plants co-occur with C_4 plants, as significantly more enriched $\delta^{13}C$ values are expected in mycoheterotrophic plants under these circumstances than when C_3 plants predominate in the surrounding vegetation, as previously suggested (Bolin et al. 2017). In addition, when the $\delta^{15}N$ values are higher than those in the surrounding vegetation, the $\delta^{15}N$ values could be an additional tool to detect partially mycoheterotrophic plants (Giesemann et al. 2020). The findings of the present study and Giesemann et al. (2020) led us to consider that partial mycoheterotrophy with AM associations (particularly *Paris*-type ones) could be considerably more common than hitherto believed.

Acknowledgements We thank Mr. Masayuki Sato for help with the field study. We also thank Dr. Naohiko Ohkouchi and Dr. Hidetaka Nomaki for helping us to perform microscale isotope analysis. We also thank Dr. Masanori Saito for advice on the identification of AM fungal spores.

Author contribution statement KS planned and designed the research, collected the materials, carried out laboratory work and analyses, and wrote the initial draft. MJ, NOO, and HT conducted laboratory work and carried out analyses. SM and RS conducted the laboratory work. All authors contributed to the manuscript.

Funding This work was financially supported by the JSPS KAKENHI Grant nos. 17H05016 (KS), 17J04991 (JM), and 18K19356 (HT).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Abadie JC, Püttsepp Ü, Gebauer G, Faccio A, Bonfante P, Selosse MA (2006) *Cephalanthera longifolia* (Neottieae, Orchidaceae) is mixotrophic: a comparative study between green and nonphotosynthetic individuals. Can J Bot 84:1462–1477

Bidartondo MI, Redecker D, Hijri I, Wiemken A, Bruns TD, Domínguez L, Sérsic A, Leake JR, Read DJ (2002) Epiparasitic plants specialized on arbuscular mycorrhizal fungi. Nature 419:389–392

Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. Proc R Soc B 271:1799–1806

Bolin JF, Tennakoon KU, Majid MBA, Cameron DD (2017) Isotopic evidence of partial mycoheterotrophy in *Burmannia coelestis* (Burmanniaceae). Plant Spec Biol 32:74–80

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996) Working with mycorrhizas in forestry and agriculture. ACIAR Monogr 32:155–161

Cameron DD, Bolin JF (2010) Isotopic evidence of partial mycoheterotrophy in the Gentianaceae: *Bartonia virginica* and *Obolaria virginica* as case studies. Am J Bot 97:1272–1277

Chalot M, Blaudez D, Brun A (2006) Ammonia: a candidate for nitrogen transfer at the mycorrhizal interface. Trends Plant Sci 11:263–266

Coplen TB (2011) Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. Rapid Commun Mass Spectrom 25:2538–2560

Courty PE, Walder F, Boller T, Ineichen K, Wiemken A, Rousteau A, Selosse MA (2011) Carbon and nitrogen metabolism in mycorrhizal networks and mycoheterotrophic plants of tropical forests: a stable isotope analysis. Plant Physiol 156:952–961

Courty PE, Doubková P, Calabrese S, Niemann H, Lehmann MF, Vosátka M, Selosse MA (2015) Species-dependent partitioning of C and N stable isotopes between arbuscular mycorrhizal fungi and their C_3 and C_4 hosts. Soil Biol Biochem 82:52–61

Eriksson O, Kainulainen K (2011) The evolutionary ecology of dust seeds. Perspect Plant Ecol Evol Syst 13:73–87

Field KJ, Leake JR, Tille S, Allinson KE, Rimington WR, Bidartondo MI, Beerling DJ, Cameron DD (2015) From mycoheterotrophy to mutualism: mycorrhizal specificity and functioning in *Ophioglossum vulgatum* sporophytes. New Phytol 205:1492–1502

Fujiyoshi M, Kagawa A, Nakatsubo T, Masuzawa T (2005) Successional changes in mycorrhizal type in the pioneer plant communities of a subalpine volcanic desert on Mt. Fuji, Japan. Polar Biosci 18:60–72

Gebauer G, Meyer M (2003) ^{15}N and ^{13}C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. New Phytol 160:209–223

Gebauer G, Schulze ED (1991) Carbon and nitrogen isotope ratios in different compartments of a healthy and a declining *Picea abies* forest in the Fichtelgebirge, NE Bavaria. Oecologia 87:198–207

Gebauer G, Preiss K, Gebauer AC (2016) Partial mycoheterotrophy is more widespread among orchids than previously assumed. New Phytol 211:11–15

Giesemann P, Rasmussen HN, Liebel HT, Gebauer G (2020) Discreet heterotrophs: green plants that receive fungal carbon through Paris-type arbuscular mycorrhiza. New Phytol. https://doi.org/10.1111/nph.16367

Girlanda M, Segreto R, Cafasso D, Liebel HT, Rodda M, Ercole E, Cozzolino S, Gebauer G, Perotto S (2011) Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations. Am J Bot 98:1148–1163

Gomes SIF, Aguirre-Gutierrez J, Bidartondo MI, Merckx V (2017) Arbuscular mycorrhizal interactions of mycoheterotrophic *Thismia* are more specialized than in autotrophic plants. New Phytol 213:1418–1427

Gomes SIF, Merckx VSFT, Kehl J, Gebauer G (2020) Mycoheterotrophic plants living on arbuscular mycorrhizal fungi are generally enriched in 13C, 15N, and 2H isotopes. J Ecol. https://doi.org/10.1111/1365-2745.13381

Hynson NA, Madsen TP, Selosse MA, Adam IKU, Ogura-Tsujita Y, Roy M, Gebauer G (2013) The physiological ecology of mycoheterotrophy. In: Merckx V (ed) Mycoheterotrophy: the biology of plants living on fungi. Springer, Berlin, pp 297–342

Imhof S, Massicotte HB, Melville LH, Peterson RL (2013) Subterranean morphology and mycorrhizal structures. In: Merckx VSFT (ed) Mycoheterotrophy: the biology of plants living on fungi. Springer, Berlin, pp 157–214

Jacquemyn H, Merckx VS (2019) Mycorrhizal symbioses and the evolution of trophic modes in plants. J Ecol 107:1567–1581

Julou T, Burghardt B, Gebauer G, Berveiller D, Damesin C, Selosse MA (2005) Mixotrophy in orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals of *Cephalanthera damasonium*. New Phytol 166:639–653

Klink S, Giesemann P, Gebauer G (2019) Picky carnivorous plants? Investigating preferences for preys' trophic levels—a stable isotope natural abundance approach with two terrestrial and two aquatic Lentibulariaceae tested in Central Europe. Ann Bot 123:1167–1177

Lallemand F, Gaudeul M, Lambourdière J, Matsuda Y, Hashimoto Y, Selosse M (2016) The elusive predisposition to mycoheterotrophy in Ericaceae. New Phytol 212:314–319

Leake JR (1994) The biology of myco-heterotrophic ('saprophytic') plants. New Phytol 127:171–216

Lekberg Y, Hammer EC, Olsson PA (2010) Plants as resource islands and storage units—adopting the mycocentric view of arbuscular mycorrhizal networks. FEMS Microbiol Ecol 74:336–345

Lerat S, Gauci R, Catford JG, Vierheilig H, Piché Y, Lapointe L (2002) 14 C transfer between the spring ephemeral *Erythronium americanum* and sugar maple saplings via arbuscular mycorrhizal fungi in natural stands. Oecologia 132:181–187

Merckx V, Bidartondo MI (2008) Breakdown and delayed cospeciation in the arbuscular mycorrhizal mutualism. Proc R Soc B 275:1029–1035

Merckx V, Stöckel M, Fleischmann A, Bruns TD, Gebauer G (2010) ^{15}N and ^{13}C natural abundance of two mycoheterotrophic and a putative partially mycoheterotrophic species associated with arbuscular mycorrhizal fungi. New Phytol 188:590–596

Merckx V, Mennes CB, Peay KG, Geml J (2013a) Evolution and diversification. In: Merckx V (ed) Mycoheterotrophy: the biology of plants living on fungi. Springer, Berlin, pp 215–244

Merckx V, Kissling J, Hentrich H, Janssens SB, Mennes CB, Specht CD, Smets EF (2013b) Phylogenetic relationships of the mycoheterotrophic genus *Voyria* and the implications for the biogeographic history of Gentianaceae. Am J Bot 100:712–721

Murata J (2003) *Pterygocalyx volubilis*. In: Yahara T, Nagata Y (eds) Red data plants. Yamakei, Tokyo, p 156

Nakano A, Takahashi K, Kimura M (1999) The carbon origin of arbuscular mycorrhizal fungi estimated from $\delta^{13}C$ values of individual spores. Mycorrhiza 9:41–47

Nakano-Hylander A, Olsson PA (2007) Carbon allocation in mycelia of arbuscular mycorrhizal fungi during colonisation of plant seedlings. Soil Biol Biochem 39:1450–1458

Ogawa NO, Nagata T, Kitazato H, Ohkouchi N (2010) Ultra sensitive elemental analyzer/isotope ratio mass spectrometer for stable nitrogen and carbon isotope analyses. Earth life Isot 21:339–353

O'Leary MH (1988) Carbon isotopes in photosynthesis. Bioscience 38:328–336

Öpik M, Zobel M, Cantero JJ, Davison J, Facelli JM, Hiiesalu I, Jairus T, Kalwij JM, Koorem K, Leal ME (2013) Global sampling of plant roots expands the described molecular diversity of arbuscular mycorrhizal fungi. Mycorrhiza 23:411–430

Pfeffer PE, Douds DD Jr, Bücking H, Schwartz DP, Shachar-Hill Y (2004) The fungus does not transfer carbon to or between roots in an arbuscular mycorrhizal symbiosis. New Phytol 163:617–627

Preiss K, Gebauer G (2008) A methodological approach to improve estimates of nutrient gains by partially myco-heterotrophic plants. Isot Environ Health Stud 44:393–401

Preiss K, Adam IK, Gebauer G (2010) Irradiance governs exploitation of fungi: fine-tuning of carbon gain by two partially mycoheterotrophic orchids. Proc R Soc B 277:1333–1336

Press MC (1989) Autotrophy and heterotrophy in root herniparasites. Trends Ecol Evol 4:258–263

Press MC, Shah N, Tuohy JM, Stewart GR (1987) Carbon isotope ratios demonstrate carbon flux from C_4 host to C_3 parasite. Plant Physiol 85:1143–1145

Quested HM (2008) Parasitic plants—impacts on nutrient cycling. Plant Soil 311:269–272

Schiebold JMI, Bidartondo MI, Lenhard F, Makiola A, Gebauer G (2018) Exploiting mycorrhizas in broad daylight: partial mycoheterotrophy is a common nutritional strategy in meadow orchids. J Ecol 106:168–178

Schulze ED, Lange OL, Ziegler H, Gebauer G (1991) Carbon and nitrogen isotope ratios of mistletoes growing on nitrogen and non-nitrogen fixing hosts and on CAM plants in the Namib desert confirm partial heterotrophy. Oecologia 88:457–462

Schweiger JMI, Bidartondo MI, Gebauer G (2018) Stable isotope signatures of underground seedlings reveal the organic matter gained by adult orchids from mycorrhizal fungi. Funct Ecol 32:870–881

Schweiger JMI, Kemnade C, Bidartondo MI, Gebauer G (2019) Light limitation and partial mycoheterotrophy in rhizoctonia-associated orchids. Oecologia 189:375–383

Selosse MA, Roy M (2009) Green plants that feed on fungi: facts and questions about mixotrophy. Trends Plant Sci 14:64–70

Selosse MA, Charpin M, Not F (2017a) Mixotrophy everywhere on land and in water: the grand écart hypothesis. Ecol Lett 20:246–263

Selosse MA, Bocayuva MF, Kasuya MCM, Courty PE (2017b) Mixotrophy in mycorrhizal plants: extracting C from mycorrhizal networks. In: Martin F (ed) Molecular mycorrhizal symbiosis. Springer, Berlin, pp 451–471

Smith S, Read D (2008) Mycorrhizal symbiosis. Academic Press, London

Stöckel M, Meyer C, Gebauer G (2011) The degree of mycoheterotrophic carbon gain in green, variegated and vegetative albino individuals of *Cephalanthera damasonium* is related to leaf chlorophyll concentrations. New Phytol 189:790–796

Stöckel M, Tešitelová T, Jersáková J, Bidartondo MI, Gebauer G (2014) Carbon and nitrogen gain during the growth of orchid seedlings in nature. New Phytol 202:606–615

Suetsugu K, Kawakita A, Kato M (2014) Evidence for specificity to *Glomus* group Ab in two Asian mycoheterotrophic *Burmannia* species. Plant Spec Biol 29:57–64

Suetsugu K, Yamato M, Miura C, Yamaguchi K, Takahashi K, Ida Y, Shigenobu S, Kaminaka H (2017) Comparison of green and albino individuals of the partially mycoheterotrophic orchid *Epipactis helleborine* on molecular identities of mycorrhizal fungi, nutritional modes and gene expression in mycorrhizal roots. Mol Ecol 26:1652–1669

Suetsugu K, Ohta T, Tayasu I (2018) Partial mycoheterotrophy in the leafless orchid *Cymbidium macrorhizon*. Am J Bot 105:1595–1600

Suetsugu K, Yamato M, Matsubayashi J, Tayasu I (2019) Comparative study of nutritional mode and mycorrhizal fungi in green and albino variants of *Goodyera velutina*, an orchid mainly utilizing saprotrophic rhizoctonia. Mol Ecol 28:4290–4299

Suetsugu K, Matsubayashi J, Tayasu I (2020) Some mycoheterotrophic orchids depend on carbon from dead wood: novel evidence from a radiocarbon approach. New Phytol. https://doi.org/10.1111/nph.16409

Tayasu I, Hirasawa R, Ogawa NO, Ohkouchi N, Yamada K (2011) New organic reference materials for carbon-and nitrogen-stable isotope ratio measurements provided by Center for Ecological Research, Kyoto University, and Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology. Limnology 12:261–266

Tedersoo L, Pellet P, Kõljalg U, Selosse MA (2007) Parallel evolutionary paths to mycoheterotrophy in understorey Ericaceae and Orchidaceae: ecological evidence for mixotrophy in Pyroleae. Oecologia 151:206–217

Těšitel J, Plavcová L, Cameron DD (2010) Heterotrophic carbon gain by the root hemiparasites, *Rhinanthus minor* and *Euphrasia rostkoviana* (Orobanchaceae). Planta 231:1137–1144

Těšitel J, Lepš J, Vráblová M, Cameron DD (2011) The role of heterotrophic carbon acquisition by the hemiparasitic plant *Rhinanthus alectorolophus* in seedling establishment in natural communities: a physiological perspective. New Phytol 192:188–199

Walder F, Niemann H, Lehmann MF, Boller T, Wiemken A, Courty P (2013) Tracking the carbon source of arbuscular mycorrhizal fungi colonizing C_3 and C_4 plants using carbon isotope ratios ($\delta^{13}C$). Soil Biol Biochem 58:341–344

Yamato M (2001) Identification of a mycorrhizal fungus in the roots of achlorophyllous *Sciaphila tosaensis* Makino (Triuridaceae). Mycorrhiza 11:83–88

Yamato M, Yagame T, Shimomura N, Iwase K, Takahashi H, Ogura-Tsujita Y, Yukawa T (2011) Specific arbuscular mycorrhizal fungi associated with non-photosynthetic *Petrosavia sakuraii* (Petrosaviaceae). Mycorrhiza 21:631–639

Yamato M, Ogura-Tsujita Y, Takahashi H, Yukawa T (2014) Significant difference in mycorrhizal specificity between an autotrophic and its sister mycoheterotrophic plant species of Petrosaviaceae. J Plant Res 127:685–693

Yamato M, Takahashi H, Shimono A, Kusakabe R, Yukawa T (2016) Distribution of *Petrosavia sakuraii* (Petrosaviaceae), a rare mycoheterotrophic plant, may be determined by the abundance of its mycobionts. Mycorrhiza 26:417–427