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## Viewpoint

## Mixotrophy in orchids: facts, questions, and perspectives

## Summary

While orchids germinate thanks to carbon from their symbiotic fungi, variable carbon exchanges exist between adult orchids and their mycorrhizal fungi. Although some truly autotrophic orchids reward their fungi with carbon at adulthood, some species remain achlorophyllous and fully dependent on fungal carbon (mycoheterotrophy). Others are photosynthetic but also import fungal carbon: The so-called mixotrophic (MX) orchids rely on fungi of diverse taxonomy and ecology. Here, we classify MX nutrition of orchids into three types. Type I mixotrophy associates with diverse Asco- and Basidiomycota that are either saprotrophic or ectomycorrhizal, entailing enrichment of the orchids in  $^2\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$ . The two other types associate with rhizoctonias, a polyphyletic assemblage of Basidiomycotas that is ancestrally mycorrhizal in orchids. Type II mixotrophy associates with rhizoctonias that secondarily evolved into saprotrophic or ectomycorrhizal ecology, and thus enrich the orchid in  $^2\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$ . Type III mixotrophy, which remains debated, associates with rhizoctonias that have retained their ancestral lifestyle, that is saprotrophic and/or endophytic in nonorchids, and only entail orchid enrichment in  $^2\text{H}$  and  $^{15}\text{N}$ . Based on a case study of achlorophyllous variants in Mediterranean *Ophrys* and on published data, we discuss the distinct nature and research perspectives of type III mixotrophy.

Terrestrial orchids germinate with the help of fungi providing them with carbon, since their seeds are devoid of reserves (Rasmussen, 1995; van der Heijden *et al.*, 2015; Dearnaley *et al.*, 2016). This fungus-mediated heterotrophic nutrition is called mycoheterotrophy. Most orchids later develop green organs and become

photosynthetic, while the fungus colonizes the roots of the adult plant, in which it forms true mycorrhizal symbioses. However, various species from several independently arisen lineages remain nongreen and mycoheterotrophic (MH) at adulthood (Merckx, 2013). Moreover, two decades ago, some species were found to mix photosynthetic and MH nutrition: the mixotrophic (MX or partially MH) orchids (Selosse & Roy, 2009; Merckx, 2013).

Fungi associated with most orchid species, the so-called rhizoctonias, belong to a polyphyletic aggregate of Basidiomycota, including Serendipitaceae, Ceratobasidiaceae, and Tulasnellaceae (Dearnaley *et al.*, 2012; Selosse *et al.*, 2022). Strikingly, most MH orchids and the first MX orchids discovered mostly associate with nonrhizoctonia mycorrhizal partners, thus questioning whether rhizoctonias can feed MH adult plants (Merckx, 2013; Selosse *et al.*, 2022). Yet, recent publications suggest that even some orchids associated with rhizoctonias could be MX (Selosse & Martos, 2014; Gebauer *et al.*, 2016). In this article, we propose to delineate three types and ecological meanings of MX nutrition in orchids (Table 1). We also offer perspectives for future research on the evolution and physiology of these orchid–fungal interactions.

## Type I mixotrophy is associated with nonrhizoctonia fungi

This nutrition of MX orchids relies on mycorrhizal association with nonrhizoctonias, either Basidiomycota or Ascomycota, which are either ectomycorrhizal on surrounding trees (type Ia, the most frequently described case; Selosse & Roy, 2009; Merckx, 2013) or leaf- or wood-decaying saprotrophs (type Ib; Table 1; such as *Cremastra* or *Calypso* spp.; Suetsugu & Matsubayashi, 2021b; Yagame *et al.*, 2021; Suetsugu *et al.*, 2022). Few rhizoctonias can also occur on the roots (e.g. Julou *et al.*, 2005; Abadie *et al.*, 2006). The MX status of orchids with type I nutrition is supported by four main features.

(1) They are often closely phylogenetically related to fully MH species, sometimes even in the same genus, for example in the Neottieae tribe (Selosse & Roy, 2009) or within the genus *Cremastra* (Suetsugu *et al.*, 2022).

**Table 1** Three types of mixotrophy in orchids, numbered by order of discovery.

Type	Fungal partner	Isotopic enrichment	Evolutionary origin	Main habitat	Albinos
I a	Nonrhizoctonias, ectomycorrhizal <sup>1</sup>	For $^{13}\text{C}$ , $^{15}\text{N}$ and $^2\text{H}$	Derived: shift of fungal partner	Forest	Infrequent, but can be perennial
I b	Nonrhizoctonias, saprotrophic <sup>1</sup>				
II a	Rhizoctonias, ectomycorrhizal <sup>1</sup>	For $^{13}\text{C}$ , $^{15}\text{N}$ and $^2\text{H}$	Derived: shift of ecology of the fungal partner	Forest	Infrequent, but can be perennial
II b	Rhizoctonias, saprotrophic <sup>1</sup>				
III	AL rhizoctonias	For $^2\text{H}$ and $^{15}\text{N}$	Plesiomorphic: ancestral symbiosis	Open places	Rare, survival questionable

Ancestral lifestyle (AL) in rhizoctonias qualifies their ancestral, poorly characterized saprotrophic and/or endophytic lifestyle resulting in limited  $^{13}\text{C}$  enrichment as compared to autotrophic plants (Selosse *et al.*, 2022).

<sup>1</sup>In some cases, a marginal, additional presence of AL rhizoctonias can be observed.

(2) They tend to inhabit forests where canopy cover reduces light availability and limits photosynthesis (e.g. Julou *et al.*, 2005). Moreover, some species have reduced photosynthetic surfaces and/or impaired photosynthetic abilities (e.g. Girlanda *et al.*, 2006).

(3) Their isotopic content differs from that of autotrophic plants for  $^{13}\text{C}$  and  $^{15}\text{N}$  (Gebauer & Meyer, 2003; Julou *et al.*, 2005), as well as  $^2\text{H}$  (Gebauer *et al.*, 2016; Yagi *et al.*, 2024). This aligns with the isotopic enrichments of their associated fungi, which can be estimated from fungal fruitbodies (Mayor *et al.*, 2009) or from intracellular hyphal coils extracted from the mycorrhizas (e.g. Zahn *et al.*, 2023). Whenever MH and autotrophic species co-occur, the  $^{13}\text{C}$  isotopic content of these MX orchids is intermediate between that of MH and autotrophic species growing nearby, and the value of their relative enrichment allows calculation of the percentage of biomass derived from fungal and photosynthetic sources, using a linear two-source isotopic mixing model (Gebauer & Meyer, 2003). The fungal carbon contributions range from undetectable (May *et al.*, 2020) to c. 90% (Hynson *et al.*, 2013) and increase when plants receive less light (e.g. Gonneau *et al.*, 2014). Furthermore, orchids with type I mixotrophy are often richer in total N and  $^{15}\text{N}$  isotope as compared to autotrophic plants, a feature that reflects the composition of their fungal food source (Abadie *et al.*, 2006; Minasiewicz *et al.*, 2023). The latter N-related traits are indirectly linked to mixotrophy, and they do not always follow the  $^{13}\text{C}$  enrichment trends (e.g. Roy *et al.*, 2013), since they also depend on other factors such as the identity of the mycorrhizal fungi (Schiebold *et al.*, 2017).

(4) Albinos, that is fully achlorophyllous individuals, occur sometimes in these otherwise green species (Selosse *et al.*, 2004). Their MH-like isotopic enrichments demonstrate that they are fully supported by fungal resources (Julou *et al.*, 2005; Abadie *et al.*, 2006). In green individuals, isotopic enrichments of above- versus belowground organs (Roy *et al.*, 2013) and allocation of  $^{13}\text{C}$ -labeled photosynthates (Lallemand *et al.*, 2019a) support different uses of carbon resources: fungal carbon supports belowground perennial rhizomes and early growth of the annual shoot, while later in the season, photosynthetic carbon supports aerial parts and fruiting. Thus, albinos survive well thanks to fungal carbon (Shefferson *et al.*, 2016), but their shoots soon rely on autophagy (Lallemand *et al.*, 2019b) and produce seeds more rarely and less abundantly due to restricted carbon supply (Roy *et al.*, 2013), making the albino phenotype rare.

The observation of albinos in orchids with type I mixotrophy, even poorly fitted, supports the MX nutrition. Along with Features #1–#3, this suggests that such MX species may eventually evolve into purely MH species. However, more than just a loss of photosynthesis is required for a successful transition to MH nutrition (Jąkalski *et al.*, 2021).

### Type II mixotrophy is associated with rhizoctonia fungi of unusual ecology

Mixotrophy in orchids associated with rhizoctonias is more difficult to demonstrate using  $^{13}\text{C}$  content for two reasons. First, rhizoctonias do not form conspicuous fruitbodies, making

it difficult to sample fungal material for isotopic analyses. Second, whenever fungal material was obtained by extracting hyphal coils from the mycorrhizas (Gomes *et al.*, 2023; Zahn *et al.*, 2023; Suetsugu *et al.*, 2024a), its  $^{13}\text{C}$  content was only slightly enriched compared with that of autotrophic plants, in accordance with results from isotopic analyses of subterranean MH orchid seedlings (Stöckel *et al.*, 2014). Thus,  $^{13}\text{C}$  analyses cannot detect or quantify fungal carbon in the biomass of rhizoctonia-associated orchids.

The  $^{13}\text{C}$  enrichment in rhizoctonias is much lower than that of ectomycorrhizal or saprotrophic Basidiomycota, likely due to their different nutritional strategy, which remains unclear. Indeed, the ecology of rhizoctonias outside orchid roots is largely hypothetical: they have clear abilities for saprotrophy, as evidenced by their *in vitro* growth (Rasmussen, 1995) and by analysis of their genomes (Miyachi *et al.*, 2020), but they turn out to be also endophytic in nonorchid roots (Selosse & Martos, 2014; evidence reviewed in Selosse *et al.*, 2022). Moreover, their exact net carbon exchanges with orchids are still debated, as discussed later. For ease of reading, this poorly understood ecological niche, saprotrophic and/or endophytic, of rhizoctonias will hereafter be referred to as ‘ancestral lifestyle’ (AL) because it is likely plesiomorphic (= phylogenetically ancestral) in the different rhizoctonia lineages. This rhizoctonia ecology sometimes evolved into other, secondarily derived ecologies. Ectomycorrhizal associations arose at least twice in Tulasnellaceae (Tedersoo & Smith, 2013), twice in Ceratobasidiaceae, which often shifted to pure saprotrophy too (Veldre *et al.*, 2013), and at least once in Serendipitaceae, which also evolved mycorrhizal associations with Ericaceae (Selosse *et al.*, 2007; Weiß *et al.*, 2016).

Such rhizoctonias with secondary ecology happen to support type II MX nutrition in orchids. Some of these MX species rely on ectomycorrhizal rhizoctonias (type IIa; Table 1), for example ectomycorrhizal Ceratobasidiaceae species in *Platanthera minor* (Yagame *et al.*, 2012) or *Apostasia nipponica* (Suetsugu & Matsubayashi, 2021a). Others associate with purely saprotrophic rhizoctonias (type IIb; Table 1), for example *Stigmatodactylus sikokianus* with strictly saprotrophic Serendipitaceae showing  $^{13}\text{C}$  and  $^{15}\text{N}$  enrichment (Suetsugu *et al.*, 2021a, 2024a). As for type I, these orchids can harbor a few AL rhizoctonias as well (Selosse *et al.*, 2022). Although only a limited number of studies have reported them, MX species with type II nutrition exhibit the same four features as these with type I:

(1) Some species are phylogenetically close to MH species (e.g. within the genus *Platanthera*; Yagame *et al.*, 2012) and, indeed, some purely MH orchids also rely on rhizoctonias with secondary ecology, such as *Rhizanthella gardneri* (Bougoure *et al.*, 2010) or *Chamaegastrodia shikokiana* (Yagame *et al.*, 2008) with ectomycorrhizal Ceratobasidiaceae.

(2) They have a forest ecology, entailing low illumination and limited photosynthetic ability.

(3) Their isotopic enrichments are similar to those of type I because the ecology of their rhizoctonias is similar (Yagame *et al.*, 2012; Suetsugu *et al.*, 2021a, 2021b, 2024a), and fungal contributions inferred from  $^{13}\text{C}$  enrichments can be high (> 50% for *P. minor* – Yagame *et al.*, 2012; c. 90% for *A. nipponica* and

*S. sikokianus* – Suetsugu & Matsubayashi, 2021a; Suetsugu *et al.*, 2024a).

(4) Some species display albinos that can survive over several years (e.g. Suetsugu *et al.*, 2019).

Affiliation of MX nutrition to type I or II may seem uncertain in some cases, *Goodyera velutina* (Suetsugu *et al.*, 2019) and *Cypripedium debile* (Suetsugu *et al.*, 2021b), which display a characteristic  $^{13}\text{C}$  enrichment, harboring few ectomycorrhizal nonrhizoctonias and mostly rhizoctonias from clades for which AL is not sure. In both species, albinos survive over several years and display marked  $^{13}\text{C}$  enrichment (Suetsugu *et al.*, 2019, 2021a, 2021b, 2024b; Suetsugu & Matsubayashi, 2022), clearly affiliating their MX nutrition to type I or II.

### Type III mixotrophy gains carbon from AL rhizoctonias

Mixotrophy of types I and II involves evolutionary shifts (Table 1), either a shift of fungal partners (type I) or a shift of the ecology of the involved rhizoctonias (type II). Yet, most orchids associate with AL rhizoctonias, which is the ancestral state in orchids (Wang *et al.*, 2021; Selsos *et al.*, 2022). It was initially suspected that at least some of these orchids could be MX, based on their small deviations in  $^{13}\text{C}$  content, slightly higher or lower than autotrophic plants ('cryptic MH' in Hynson *et al.*, 2013; Selsos & Martos, 2014; Supporting Information Fig. S1). Most of the time, however, their  $^{13}\text{C}$  content falls in the range of autotrophic values (Liebel *et al.*, 2010; Girlanda *et al.*, 2011). This does not disprove MX nutrition per se, since AL rhizoctonias are not strongly enriched in  $^{13}\text{C}$  compared with autotrophs as mentioned previously.

A decisive step was recently taken when  $^2\text{H}$  enrichment as compared to autotrophic plants was observed, not only in type I and II orchids but also in some orchids mycorrhizal with AL rhizoctonias (Gebauer *et al.*, 2016; Schiebold *et al.*, 2017; Schweiger *et al.*, 2018; Yagi *et al.*, 2024). Such an enrichment could result from elevated transpiration, but it would be

accompanied by a corresponding  $^{18}\text{O}$  enrichment, which does not occur. Instead, a gain of  $^2\text{H}$ -enriched fungal resources was hypothesized to cause this. Fungal resources are likely flowing to  $^2\text{H}$ -enriched orchids, as also indirectly supported by their high  $^{15}\text{N}$  and N content (e.g. Schweiger *et al.*, 2018).

One should note that uncertainties persist in interpreting  $^2\text{H}$  enrichments, which have complex origins. Indeed,  $^2\text{H}$  fractionation varies during the synthesis of organic compounds (Holloway-Phillips *et al.*, 2022; Baan *et al.*, 2023a; Lehmann *et al.*, 2024). This variation can be significant even among autotrophic species grown at the same location (e.g. Chikaraishi *et al.*, 2004; He *et al.*, 2020; Holloway-Phillips *et al.*, 2022; Schuler *et al.*, 2023; Baan *et al.*, 2023b). Thus, disentangling the  $^2\text{H}$  enrichment of mycoheterotrophy from species-specific biochemical fractionation remains somewhat challenging. Consequently, caution is still required when interpreting  $^2\text{H}$  enrichment as sole evidence of gain of fungal resources.

Mixotrophic orchids associated with AL rhizoctonias and displaying  $^2\text{H}$  enrichment represent our type III mixotrophy (Table 2). On the one hand, their carbon gain is somewhat expected since AL rhizoctonias do feed orchids at germination so that there is a physiological mechanism for the plant to harvest and utilize fungal C. On the other hand, type III orchids do not really fit three of the four features presented by type I and II orchids: Indeed, while isotopic enrichments for  $^2\text{H}$  and often  $^{15}\text{N}$  (Feature #3) are shared, the other features (Features #1, #2, and #4) differ somewhat, as follows.

(1) They are often not closely related to fully MH species (e.g. in the genera *Orchis* or *Ophrys*). Moreover, as stated in the Introduction section, we do not know any fully MH orchids associated with AL rhizoctonias. Accordingly, linear two-source isotopic mixing models of  $^2\text{H}$  enrichments estimate that, among cases studied so far, the fungal contribution reaches at most 20% (Schweiger *et al.*, 2018), that is much less than the gains for type I and II mixotrophs so that the available carbon may be insufficient to reach full MH nutrition.

**Table 2** Comparison of albinos vs green individuals in three *Ophrys* populations from meadows (see raw data in Supporting Information Tables S1 and S2).

	1. Samos Island (Greece)	2. Pas de l'Escalette (France)	3. Cirque de Navacelles (France)
Geocodes	37°39'51"N, 26°50'38"E	43°54'03"N, 03°30'43"E	43°49'53"N, 03°39'50"E
<i>Ophrys</i> species	<i>Ophrys pelinaea</i>	<i>Ophrys litigiosa</i>	<i>Ophrys apifera</i>
and sampling	April 23, 2015	May 5, 2016	May 28, 2016
Albinos/green <sup>1</sup>	4/20	5/14	6/18
With new bulb <sup>2,3</sup>	0/19*	0/ni	0/18*
Flower number <sup>3</sup>	3.75 ± 0.96/3.85 ± 1.23 ns	2.80 ± 0.84/3.07 ± 0.92 ns	4.33 ± 1.63/4.44 ± 1.65 ns
Height (cm) <sup>3</sup>	30.35 ± 4.79/21.90 ± 3.99*	21.00 ± 3.61/19.49 ± 3.84 ns	48.83 ± 2.64/36.00 ± 6.29*
Leaf $\delta^{13}\text{C}$ (‰) <sup>4</sup>	-30.60 ± 0.10/-31.61 ± 0.09*	-30.03 ± 0.42/-31.86 ± 0.44*	-26.30 ± 0.48/-28.72 ± 0.25*
New bulb $\delta^{13}\text{C}$	-29.80 ± 0.28‰ (n = 4)	-30.22 ± 0.16 (n = 6)	ni
Leaf $\delta^{15}\text{N}$ (‰) <sup>4</sup>	-2.46 ± 0.46/-2.13 ± 0.27 ns	-0.36 ± 0.27/-1.60 ± 0.05*	-0.05 ± 0.15/v1.01 ± 0.17*
New bulb $\delta^{15}\text{N}$	-1.91 ± 0.92 (n = 4)	-1.32 ± 0.21 (n = 6)	ni
Leaf %N	1.38 ± 0.03/2.47 ± 0.07*	4.12 ± 0.34/2.37 ± 0.28*	4.34 ± 0.31/2.67 ± 0.04*
New bulb %N	1.10 ± 0.22 (n = 4)	4.25 ± 0.34 (n = 6)	ni

ni, not investigated; ns, not significant. Numbers and means (±SD) for albinos are italicized while those for green individuals are not.

\*Significant ( $P < 0.05$ ; see notes for statistics).

<sup>1</sup>Number of albinos observed and number of green individuals studied (among a larger number) – this is the number of replicates for the values below.

<sup>2</sup>Presence, for each phenotype, of the newly formed bulb with reserves for the growth in year  $n + 1$ .

<sup>3</sup>Significance according to the Tukey's HSD test.

<sup>4</sup>Significance according to Student's  $t$ -test; Fig. S1.

**Box 1** Albinos in orchids with ancestral lifestyle (AL) rhizoctonias: case studies of three Mediterranean *Ophrys* species

*Ophrys* species are perennial orchids that form a subterranean bulb with reserves for next year, every year after the emergence of their first leaf (Rasmussen, 1995). With the exception of *Ophrys insectifera* (which also lives in forest conditions with type I mixotrophy; Schweiger *et al.*, 2018), *Ophrys* species mostly grow in open places (Rasmussen, 1995; Giralanda *et al.*, 2011) where they display no  $^{13}\text{C}$  enrichment (Liebel *et al.*, 2010; Gomes *et al.*, 2023) but are enriched in  $^{15}\text{N}$  and/or  $^2\text{H}$  (Giralanda *et al.*, 2011; Gebauer *et al.*, 2016). Moreover, *Ophrys* species from meadows mostly associate with ancestral lifestyle rhizoctonias (Tulasnellaceae and Ceratobasidiaceae; Rasmussen, 1995; Schatz *et al.*, 2010; Liebel *et al.*, 2010; Giralanda *et al.*, 2011), although other fungi can be present (Gomes *et al.*, 2023). We thus consider meadow *Ophrys* species as potential models for type III mixotrophy.

Here, we investigate meadow populations of three *Ophrys* species displaying in all 15 albinos (Tables 2, S1; Fig. 1a,b), with the closest ectomycorrhizal trees located > 45 m away. Fungal typing performed by PCR on the roots of the investigated individuals ( $n = 4$  per phenotype and population) provided positive results for Basidiomycota (using general primers ITS86-F and ITS4; 79% positive PCR) and Tulasnellaceae (using 5.8S-OF and ITS4-Tul; 83% positive PCR; all primers and molecular methods as in Petrolli *et al.*, 2022). Underground observations (Table S1) and samplings were performed by lateral digging to allow survival, and whole plants were only harvested for isotopic analyses.

All examined individuals had bulbs from the previous year (Fig. 1c), suggesting that they had already sprouted in previous years. However, while all green individuals initiated any new bulb with reserves for the following year (but one in Samos), no albino displayed a new bulb (Table 2). Thus, they may turn albino before death, in agreement with the fact that no albino survival was observed over 2010–2011 ( $n = 2$  albinos), 2011–2012 ( $n = 6$ ), and 2013–2014 ( $n = 2$ ) at Navacelles and 2013–2014 ( $n = 2$ ) at Escalette (M.-A. Sellose, pers. obs.). The absence of a new bulb is at odds with the fact that underground part type I mixotrophs, albino or green, are mainly sustained by fungal C, and not by photosynthesis (Roy *et al.*, 2013; Gonneau *et al.*, 2014; Lallemand *et al.*, 2019a).

Shoots had similar flower numbers in both phenotypes, but albinos tended to be taller than green individuals in all populations (Table 2), significantly in Samos ( $1.38\times$  taller;  $P < 0.001$ ) and Navacelles ( $1.36\times$ ,  $P < 0.0001$ ). This contrasts with albinos of type I mixotrophs, which tend to be smaller than green individuals (Julou *et al.*, 2005; Abadie *et al.*, 2006; Roy *et al.*, 2013). This is reminiscent of shade-induced stem elongation, a response well-known in monocots (Liu *et al.*, 2016), which may be induced by impaired light perception and/or reduced carbon nutrition in *Ophrys*, while it does not affect albinos of type I mixotrophs.

Isotopic analyses were carried out as in Yagame *et al.* (2021) (Table S2; Fig. S1). The  $^{13}\text{C}$  enrichments of albinos and green individuals did not significantly differ from surrounding autotrophic plants (Fig. S1), except for green *Ophrys pelinaea* at Samos which, together with the other orchid *Orchis anatolica*, was significantly depleted in  $^{13}\text{C}$ . Thus, *Ophrys* mycorrhizal rhizoctonias are likely AL (i.e. not ectomycorrhizal nor truly saprotrophic). In the three populations, albinos were  $^{13}\text{C}$ -enriched compared with green individuals (Table 2), from 1 to 2.4‰. On the one hand, such an enrichment is expected if albinos gain carbon from slightly  $^{13}\text{C}$ -enriched rhizoctonias (Stöckel *et al.*, 2014; Gomes *et al.*, 2023; Zahn *et al.*, 2023). On the other hand, new bulbs were enriched in  $^{13}\text{C}$  (Table 2), not significantly differently from albino leaves at Escalette ( $P = 0.75$  according to the Tukey's HSD test), but significantly more at Samos ( $P < 0.0001$ ). Indeed, heterotrophic organs are enriched in  $^{13}\text{C}$  (Cernusak *et al.*, 2009) so that the use of bulb reserves from previous years by albinos may explain the  $^{13}\text{C}$  enrichment. We cannot distinguish whether albinos grow on fungal and/or bulb carbon.

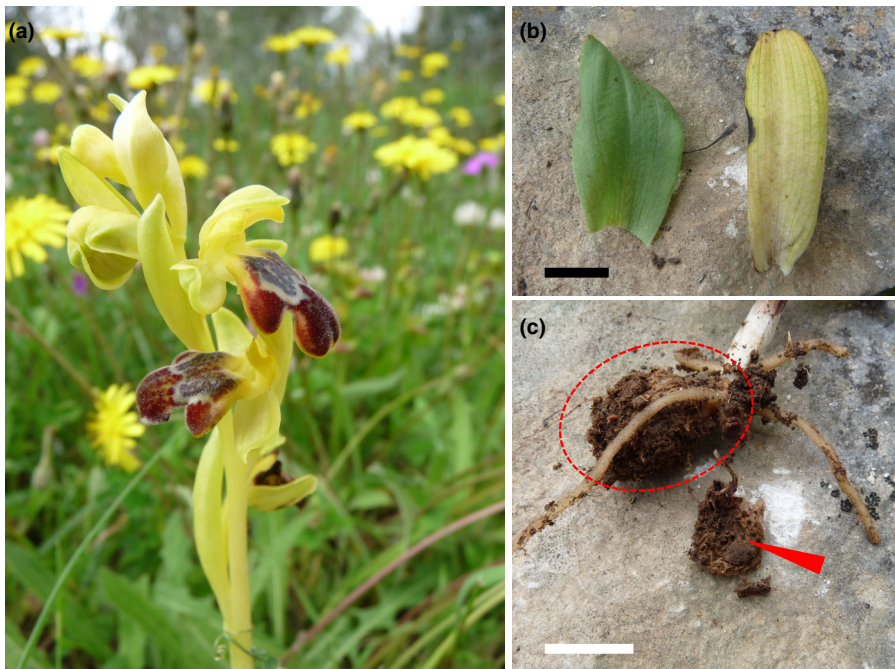
Considering  $^{15}\text{N}$ , *Ophrys* species were significantly enriched compared with autotrophic controls (but N-fixing species; Table S1; Fig. S1), suggesting that they display type III mixotrophy. Compared with green individuals, albinos of Escalette and Navacelles were significantly enriched in  $^{15}\text{N}$  by c. 1.7‰, and in total N by more than 60% (Table 2), which could mean a higher use of fungal resources (see main text). Yet, in Samos, an opposite trend was observed (albeit not significant; Table 2). Whenever new bulbs were examined (Table 2), their  $^{15}\text{N}$  content was always indistinguishable from that of the green leaves of the year that support them (Escalette:  $P = 0.18$ ; Samos  $P = 0.88$ ). Bulb N content was close to that of albino leaves ( $P > 0.05$  for both site), but their  $^{15}\text{N}$  enrichment was either similar (at Samos;  $P = 0.45$ ) or depleted (at Escalette;  $P < 0.001$ ). Nitrogen parameters provide no clear support for increased use of fungal resources in albinos.

Unfortunately, no  $^2\text{H}$  enrichment was measured since the method was not used in orchid research at the time of analyses and evidence for type III mixotrophy in these *Ophrys* ssp. remains here limited to  $^{15}\text{N}$  enrichment. Moreover, measuring  $^2\text{H}$  enrichment would not distinguish between fungal and bulb resources in albinos, as both are likely enriched in  $^2\text{H}$ . To conclude, the observed *Ophrys* albinos are unlikely to survive to the next year, their higher shoots contrasts with the phenotype of albinos in type I mixotrophs, and their isotopic enrichments are congruent with reserves either from their old bulb or from the fungus.

(2) Their ecological niche, mostly in open habitats, exerts less selective pressure for nonphotosynthetic carbon sources than the forest ecology of most type I and II species. One should note, however, that few species with type III mixotrophy in meadows that also thrive in ectomycorrhizal forests may shift to type I mixotrophy: *Ophrys insectifera* (Schweiger *et al.*, 2018) and *Neottia ovata* (Wang *et al.*, 2021) not only associate with AL rhizoctonias but also harbor ectomycorrhizal fungi when growing in forest environments, where alteration of their isotope signatures (Schweiger *et al.*, 2018) suggests that these partners can contribute to their resources.

(4) Albinos are much rarer in orchids with type III mixotrophy, and more generally in orchids mycorrhizal with AL rhizoctonias, than in species with type I and II mixotrophy. To the best of our knowledge, no study has been published

about such albinos. To document their characteristics, we analyzed three meadow *Ophrys* populations associated with rhizoctonias, whose  $^{13}\text{C}$  and  $^{15}\text{N}$  enrichments supported type III mixotrophy, and which displayed a total of 15 albinos (Box 1; Fig. 1). This case study did not evidence long-term survival of albinos, which did not form bulbs with reserves for the next year. They display an etiolated phenotype and represent a fatal shift to albinism, for unknown reasons, rather than a perennial phenotype. Isotopic analyses cannot rule out that they survive on reserves accumulated in bulbs during the previous year, in which they were probably green (Box 1), rather than on fungal resources. Our results are consistent with a poor ability of AL rhizoctonias to support MH growth, and the limited carbon transfer from AL rhizoctonias reported previously.



**Fig. 1** Albino of *Ophrys pelinaea* pictured at Samos in 2015. (a) Albino shoot and flowers. (b) Green and albino leaves. (c) Underground part of a green individual with remains of the bulb of next year (red dotted circle) and remain of the old bulb (red arrow). Bars, 1 cm.

The type III exploitation of fungal carbon thus differs from that of types I and II, on the plant side and/or on the AL rhizoctonia side.

### How type III challenges our assumptions on mixotrophy?

Strikingly, the few available studies of simplified *in vitro* design have reported net carbon transfer from orchids to their mycorrhizal AL rhizoctonias. First, *Serapias strictiflora* displayed similar  $^{13}\text{C}$  enrichment when linked to a Tulasnellaceae cultivated either on substrate devoid of organic compounds or on dead maize roots, which are naturally enriched in  $^{13}\text{C}$  (Látalová & Baláz, 2010), so that no carbon flux from the fungus to the plant occurred. Yet, in the second condition, the fungus displayed  $^{13}\text{C}$  enrichment intermediate between its dead maize substrate and the orchid, suggesting that the plant provided 69% of its carbon needs. In a second experiment, carbon labeling of *Goodyera repens* and its Ceratobasidiaceae partner showed reciprocal carbon transfer, with a net resulting flow to the fungus (Cameron *et al.*, 2006, 2008). In a third experiment in which another Ceratobasidiaceae species connected adult *Dactylorhiza fuchsii* with developing MH seedlings, labeling of adults revealed a transfer of carbon to the fungus and to the seedlings (Read *et al.*, 2024). Unfortunately,  $^2\text{H}$  enrichments were not estimated in these experiments (indeed, the first two were designed before this tool was introduced into orchid research), but such a measurement is clearly required in future similar research.

That said, how can we reconcile these *in vitro* data with the existence of type III mixotrophy in orchids associated with AL rhizoctonias? Of course, it can be argued that the orchids studied *in vitro* do not display type III mixotrophy and/or that the experimental conditions did not favor a carbon flow from AL rhizoctonias. But, most importantly,  $^2\text{H}$  enrichment of orchids is not inconsistent with a net carbon flow to the fungus (by net flow,

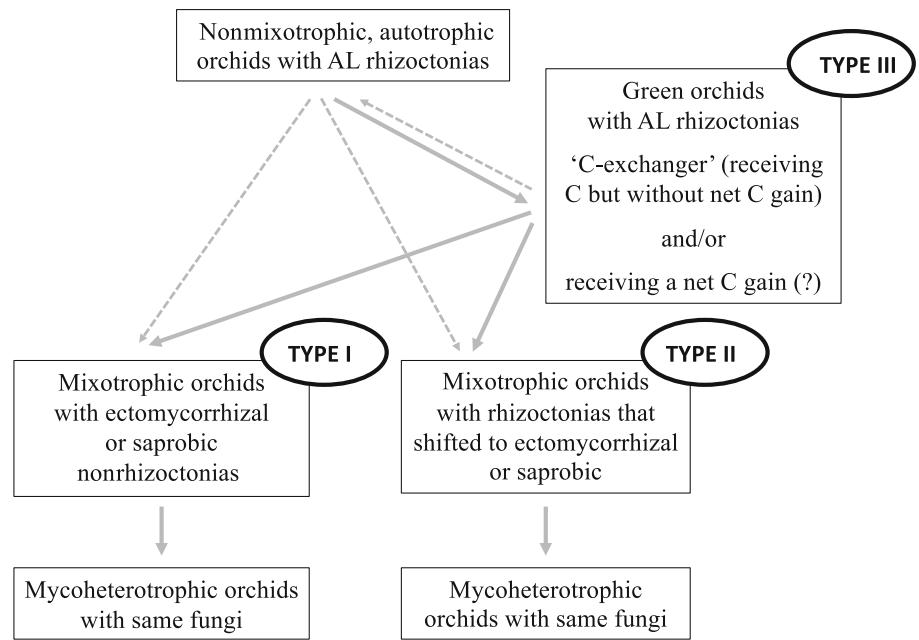
we mean the difference between the orchid-to-fungus and fungus-to-orchids raw flows). We propose an interpretation of these seemingly contradictory data by considering that isotopic content demonstrates a raw, but not a net flow from fungus to orchid. Thus, if the orchid provides more carbon on its side, as seen in the experiments of Cameron *et al.* (2006, 2008), the net flow occurs in the direction of the AL rhizoctonias. This would explain the absence (1) of MH species based on such fungi and (2) of perennial albinos in type III mixotrophy (Box 1). One should not mistake the main carbon source in this exchange: Just because a baker gives change to a customer does not mean that the customer depends on the baker for their money.

We expand here the hypothesis of ‘C-exchangers’ proposed by Lallemand *et al.* (2018) for Ericaceae from the Pyroleae tribe. This group displays a range of situations, including MH species (Hynson *et al.*, 2009), MX species with type I nutrition (Matsuda *et al.*, 2012; Sakae *et al.*, 2024), and species receiving carbon from their fungi but devoid of albino variants and unable to use more fungal carbon in the shade (Lallemand *et al.*, 2018). For the latter type, Lallemand *et al.* (2018) suggested that they gain fungal carbon due to their specific mycorrhizal mechanisms and reward the fungi with even more photosynthetic carbon. Such C-exchangers transfer significant amounts of carbon in both directions so that the plant displays isotopic enrichment, but no net dependence on fungal carbon.

This interpretation is open to falsification and experimental testing. It does not exclude that some type III orchids get net carbon gain from the interaction, but this remains to be demonstrated to assess whether type III mixotrophy is distinct or not from types I and II in which the MX orchids receive a net gain.

If type III nutrition corresponds to that of such C-exchangers, is it truly a mixotrophy? This is a matter of definition. On the one hand, it fits the definition of Merckx (2013), referring to ‘the ability of a plant to obtain carbon simultaneously through autotrophy and

**Fig. 2** Evolutionary pathway linking mixotrophic nutrition types I to III, according to Table 1, and mycoheterotrophic nutrition in orchids. Ancestral lifestyle (AL) rhizoctonia: rhizoctonias with ancestral lifestyle, that is saprotrophic and/or endophytic (see main text). Continuous arrows in gray display the most likely evolutions between the nutrition types, which occurred repeatedly, while thinner dotted arrows display possible, uncertain evolutionary pathways; some species may even display two different types depending on the ecological conditions. Yet, there are no necessary trends, so species can stay at any stage, revert to a previous one or proceed further. Although we cannot rule out direct evolution to types I and II, type III may represent a predisposition for it.



mycoheterotrophy’, and indeed type III nutrition has been considered MX since the very first evidence of  $^2\text{H}$  enrichment (‘partially MH’ in Gebauer *et al.*, 2016). Thus, it may seem too late to adopt any alternative. On the other hand, one would have preferred to keep the word mixotrophy strictly for orchids having a net carbon gain from their fungi, that is a carbon budget requiring the fungus, although this is less easy to demonstrate. This option would have caused less overlap with autotrophy. Indeed, C-exchangers cover their net energetic needs thanks to photosynthesis and are net carbon donors: They can thus be considered autotrophic. It remains unclear whether type III orchids are C-exchangers or low level, but net carbon receivers – and both cases may even exist (Fig. 2). However, their C-exchanger status would explain some features of type III mixotrophy, including the discrepancies with several *in vitro* experiments and the instability of their albino phenotype (Box 1).

To summarize,  $^2\text{H}$  enrichments do not provide direct evidence that type III orchids are not autotrophic, at least based on current knowledge. This also applies to the discovery of  $^2\text{H}$  enrichments in arbuscular mycorrhizal (AM) plants: Some AM plants with the so-called Paris-type mycorrhizas display isotopic  $^2\text{H}$  enrichments, which were claimed to provide evidence for carbon gain from AM fungi (Giesemann *et al.*, 2020, 2021; Gomes *et al.*, 2023). AM symbiosis is the main mycorrhizal association in land ecosystems (van der Heijden *et al.*, 2015), and AM fungi are shared by many neighboring plants (common mycorrhizal networks; Selosse *et al.*, 2006; van der Heijden *et al.*, 2015; Magkourilou *et al.*, 2024). Whether the fungus gains or provides carbon is of major importance for, respectively, the mutualistic or parasitic outcome of the mycorrhizal association. Indeed, the existence of common mycorrhizal networks would become highly unstable if many plants acted as carbon sinks. If  $^2\text{H}$ -enriched AM plants are C-exchangers, then they do not entail any cost to the network nor to the plants linked by shared fungi. Here again, more

research is urgently required to understand the exact carbon budget and impact on the fungus of  $^2\text{H}$ -enriched AM plants.

Be it proven that orchids (and nonorchid plants) with typ III mixotrophy are C-exchangers, and thus mostly feed on their photosynthesis, the debate on whether or not to qualify them as ‘mixotrophs’ should be re-opened. Until then, the use of the term ‘type III mixotrophy’ in the future literature would encapsulate their specific relation to their mycorrhizal fungi and the questions raised previously.

## Research perspectives

In the future, type III mixotrophy evidently deserves more investigation, some of which will be valuable for AM research too. First, the ecology of AL rhizoctonias needs to be clarified, between saprotrophy and/or endophytism in nonorchid roots, or even other trophic niches. It should be clarified in a phylogenetic framework for rhizoctonia families since extant attempts (Veldre *et al.*, 2013 for Ceratobasidiaceae; Weiss *et al.*, 2016 for Serendipitaceae) provide too preliminary a view of the ecological diversifications. Moreover, these taxa should be more carefully surveyed in the environment, especially the Tulasnellaceae that escape the universal primers used for barcoding of fungal communities (Vogt-Schilb *et al.*, 2020; Petrolli *et al.*, 2022). Thanks to the available genomes (e.g. Miyauchi *et al.*, 2020), gene expression in their diverse environmental microsites, from soil or dead organic matter to orchid mycorrhizas and nonorchid roots, could be analyzed.

Second, a budget of the orchid–fungus carbon exchange should be achieved in type III mixotrophy. With better knowledge of AL ecology at hand, reciprocal labeling of orchids and fungus *in situ* could test the relevance and generalize the conclusions of the *in vitro* experiments mentioned previously (Cameron *et al.*, 2008; Látalová & Baláž, 2010; Read *et al.*, 2024). By any means of investigation, a

full carbon budget of some model orchids with type III mixotrophy is required. Finally, detection of the use of fungal carbon in type III mixotrophy urgently needs a more routinely tractable, more direct and less expensive proxy than  $^2\text{H}$  enrichment, which cannot be obtained in most laboratories (unlike  $^{13}\text{C}$  enrichment for types I and II): Perhaps, thanks to comparative transcriptomic and metabolomic research (as used for MH orchids; Jąkowski *et al.*, 2021), some metabolites or specific genes can be found as fingerprints.

Finally, experiments and studies have largely focused on terrestrial and temperate orchids, although most species are tropical. Among these, 80% are epiphytic. Interestingly, some of the latter have a crassulacean acid metabolism (CAM) photosynthesis adapting them to the dry epiphytic conditions (Givnish *et al.*, 2015): CAM metabolism entails a higher  $^{13}\text{C}$  enrichment than in (sometimes coexisting) orchids performing C3 photosynthesis. This feature offers a natural labeling to investigate the carbon flux from CAM orchids to their AL mycorrhizal rhizoctonias.

## Outlook

The three MX nutrition types presented in Table 1 represent distinguishable stages from evolutionary pathways between full autotrophy and MH life in orchids, which may apply in other plant families (Merckx, 2013). In orchids, the identity of the mycorrhizal fungus itself displays stages in this pathway, as examined by Selosse *et al.* (2022). For both MX nutrition and mycorrhizal fungi, shifts (Fig. 2) are multiple, at any taxonomic level, as expected from permanent evolution of the symbiosis. Plastic species that shift mixotrophy from type III in meadows to type I in forest environments, such as *O. insectifera* or *N. ovata* (Schweiger *et al.*, 2018; Wang *et al.*, 2023), potentially represent ongoing evolutionary transitions between types. We also know some species devoid of isotopic enrichment that thus receive nothing from their AL rhizoctonias, such as *Pseudorchis albida* (Schiebold *et al.*, 2017): whether such true autotrophs are ancestral and/or due to reversions remains unclear (Fig. 2).

Orchids exhibit a fascinating evolutionary versatility in their fungal associates and mycorrhizal exchanges. This makes them excellent models not only for studying the diversity of full or partial MH nutrition but also for examining the dynamics of plant–fungus mutualism at the border of exploitation.

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## Competing interests

None declared.

## Author contributions








M-AS and P-LA wrote the paper. M-AS and ST designed the study of Box 1 (sampled with TF and analyzed with the help of LD). All other authors, including ED, JM and FM, contributed to preliminary discussions of typology, and they edited and revised the manuscript. M-AS and P-LA contributed equally to this work.

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## Data availability

All raw data for Box 1 report (isotopic and phenotypic measurements) are available in Supporting Information (respectively, in Tables S2 and S1).

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of plants sampled at Samos, Escalette, and Navacelle.

**Table S1** Phenotypic raw data for investigated *Ophrys* species.

**Table S2** Stable isotopic ( $^{13}\text{C}$  and  $^{15}\text{N}$ ) and nitrogen content raw data for investigated *Ophrys* species.

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