La découverte de la mixotrophie chez les plantes à mycorhizes

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Abstract – The discovery of mixotrophy in mycorrhizal plants. In the typical mycorrhizal symbiosis, which links soil fungi with the roots of ~90% of plant species, fungi exploit soil mineral nutrients in return for plant produced carbon. In contrast, mycoheterotrophy, in which plants instead receive carbon from their associated fungi, has been found in several forest-understorey achlorophyllous plants from various families, including orchids. Moreover, green forest-understorey plants, phylogenetically close to mycoheterotrophs, were recently shown to be mixotrophic, i.e. they receive carbon from both their fungi and photosynthesis. Phylogenetic analyses suggest that in orchids and pyroloids (Ericaceae) at least, mixotrophy preceded the evolution of mycoheterotrophy. In some mixotrophic orchid species, achlorophyllous plants (albinos) can even be found rarely in natural populations. Here, we review the available tools and data on mixotrophic plants, and their associated fungi, and point out open questions and future research perspectives.

Résumé – Dans les symbioses mycorhiziennes typiques, qui lient les racines de près de 90% des plantes à des champignons du sol, ces derniers exploitent des sels minéraux du sol en échange du carbone (sucres) produit par la plante. A l'inverse, dans les cas de mycohétérotrophie, la plante reçoit du carbone des champignons associés : ces plantes non-chlorophylliennes vivent en sous-bois et appartiennent à diverses familles, mais surtout les orchidées dans l'évolution desquelles ce type de nutrition est apparu une trentaine de fois. Bien plus : des plantes chlorophylliennes, elles aussi forestières, et proches évolutivement d'espèces mycohétérotrophes, ont récemment révélé une nutrition mixotrophe, c'est-à-dire qu'elles obtiennent du carbone à la fois de leurs champignons mycorhiziens et de leur photosynthèse. Des analyses phylogénétiques suggèrent que, chez les Orchidées et les Ericacées au moins, la mixotrophie aurait précédé, dans l'évolution, la mycohétérotrophie. Chez certaines espèces d'orchidées mixotrophes, des individus sans chlorophylle, appelés albinos, existent parfois dans les populations naturelles. Dans cet article, nous passons en revue les outils d'étude et les données actuelles sur les plantes mixotrophes et leurs champignons associés. Nous en définissons, enfin, des questions et des recherches pour les années à venir. (Une version simplifiée en français est disponible dans le *Dossier Pour la Science* « Les végétaux insolites » (2012), pages 102 à 107).

Mots-clés : Mycohétérotrophie, Mycorhize, Orchidées

INTRODUCTION

The roots of 90% of plants associate with soil fungi, forming a dual organ called mycorrhiza (Smith & Read, 2008). Here, the fungus usually exploits plant photosynthates and provides mineral resources as a reward, such as nitrogen (N), phosphorous or water collected in the soil by its extraradical mycelium. Plants strongly depend on this symbiosis, especially at low fertility levels, and some plant clades even reversed the exchange. The existence of heterotrophic plants relying on their mycorrhizal fungi for

carbon (C) nutrition is reported since the XIXth century – where for example *Monotropa hypopitys* (now *Hypopitys monotropa*) was thoroughly investigated in the 40's (see review in Bidartondo, 2005). These plants, arisen several times independently in land plant evolution, were once considered as 'saprophytic', but the fact that (i) they feed on their mycorrhizal fungi and (ii) these fungi are not saprophytic suggested that they should be best called 'mycoheterotrophic' (Leake, 1994; see Merckx, 2013 for review). Indeed, these fungi are, most of the time, mycorrhizal on nearby plants, yet with some exceptions in tropical regions (e.g., Martos *et al.*, 2009). A similar case where plants later shift to autotrophy was also known in orchids, whose minute seeds are reserveless and require fungal C to germinate fungi into an underground, mycoheterotrophic' mass of cells (the protocorm, Selosse *et al.* 2011; reviewed in Rasmussen, 1995; Smith & Read, 2008; Dearnaley *et al.*, 2012).

More recently, adult green plants were discovered to remain partially mycoheterotrophic, i.e. to maintain a C flow from the fungus to the plant over their whole lifespan (Selosse & Roy, 2009; Hynson *et al.*, 2013b). This nutritional strategy, where green adult plants obtain C from its the mycorrhizal fungi and its photosynthesis, was discovered in the last decade, mainly thanks to isotopic methods (Gebauer & Meyer, 2003; Bidartondo *et al.*, 2004; Julou *et al.*, 2005). It is a kind of mixotrophy (i.e. the mix of two trophic strategies, namely photosynthesis and mycoheterotrophy) and, although orchids were instrumental in the emergence of this concept, the phenomenon is now suspected, and partly demonstrated, to be more widespread (Selosse & Roy, 2009). In this contribution, we review the history of the discovery of mixotrophy in mycorrhizal plants.

DISCOVERY OF PARTIAL MYCOHETEROTROPHY IN ADULT ORCHIDS

The suspicion of a partial mycoheterotrophy in orchids comes from two lines of observations in species of the Neottieae orchid tribe: isotopic anomalies and existence of achlorophyllous individuals. Gebauer & Meyer (2003) discovered an isotopic anomaly in some forest orchids, with ^{13}C and ^{15}N abundances intermediate between these of autotrophic plants and full mycoheterotrophic plants from the same site (Figure 1). This was confirmed from additional European sites by several studies (Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Tedersoo *et al.*, 2007; Abadie *et al.*, 2006). In these investigations, sampling was performed to avoid factors likely to bias the comparison. First, only leaves were considered to avoid the ^{13}C enrichment of heterotrophic or cellulose-rich organs (Cernusak *et al.*, 2009). Samples were collected at same distance above soil to avoid variable contribution of ^{13}C-impoverished CO_2 from soil respiration. Samples were from same light environments to avoid differences in photosynthesis rates: equilibration of isotopic concentration between environmental air and stomatal chamber is lower at higher photosynthetic rates, and forces increased ^{13}C assimilation. Last, autotrophic and mycoheterotrophic references to which partially mycoheterotrophs were compared were often chosen in the same phylogenetic background. Isotopic approaches also allowed quantification of the heterotrophy level, as we will see below.

Independently, achlorophyllous (= albino, white to pinkish due to anthocyanins, Figure 2) individuals are anciently reported in some Neottieae species. Albinos occur especially in the genera *Epipactis* (Salmia, 1986, 1989; Selosse *et al.*, 2004) and *Cephalanthera* (Julou *et al.*, 2005; Abadie *et al.*, 2006; Roy *et al.*, 2013). In many populations, this phenotype remains stable for green individuals and nearby albinos over years (Renner, 1938; Tranchido-Lombardo *et al.*, 2009; Roy *et al.*, 2013), up to 14 years for albinos (Abadie *et al.*, 2006). Although they tend to perform less well than green individuals (see below), some albinos do form flowers and fruits (Salmia, 1986, 1989; Julou *et al.*, 2005; Tranchido-Lombardo *et al.*, 2009). Albinos were thus anciently suggested to depend on their mycorrhizal fungi for C nutrition (e.g., Renner, 1938). Albinos' mycoheterotrophy is now further corroborated by their low chlorophyll content and lack of CO_2 absorption in the light (Figure 3; Julou *et al.*, 2005); congruently, they display ^{13}C enrichment similar to that of mycoheterotrophic plants (Julou *et al.*, 2005; Abadie *et al.*, 2006). This supported the likelihood of a partial mycoheterotrophy in green conspecifics (Selosse *et al.*, 2004 ; Julou
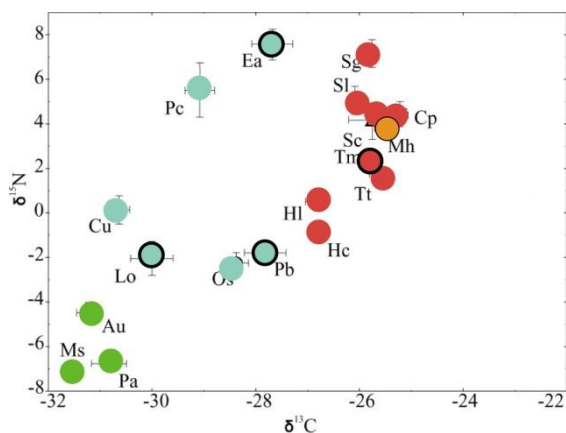
Figure 1. – Isotopes in mixotrophic and mycoheterotrophic plants. A diagrammatic presentation of ^{13}C and ^{15}N abundances (in delta values) of co-occurring plant species from a boreal forest (redrawn from Tedersoo *et al.*, 2007; means ± SE). Autotrophic plants: *Picea abies* (Pa), *Arctostaphylos uva-ursi* (Au), *Melampyrum sylvaticum* (Ms); mixotrophic orchids: *Listera ovata* (Lo), *Platanthera bifolia* (Pb), *Epipactis atrorubens* (Ea); mixotrophic pyroloids: *Orthilia secunda* (Os), *Chimaphila umbellata* (Cu), *Pyrola chlorantha* (Pc); mycoheterotrophic plant: *Monotropa hypopithys* (Mh); ectomycorrhizal fungi: *Suillus granulatus* (Sg), *Suillus luteus* (Sl), *Coltricia perennis* (Cp), *Sarcosphaera coronaria* (Sc), *Tricholoma myomyces* (Tm), *Thelephora terrestris* (Tt), *Helvella lacunosa* (Hl), *Helvella crispa* (Hc).

et al., 2005), especially considering that they often have small leaves and/or inhabit shaded forest sites. Accordingly, survival of albinos is also reported from green parasitic plants such as *Striga hermonthica* (Press *et al.*, 1991) that use other plants' sap to support part of their C needs (see below and Table X1).

Moreover, anomalies in ^{13}C abundance correlate with absence of rhizoctonias, the usual mycorrhizal partners of orchids, the rhizoctonias, a polyphyletic group of
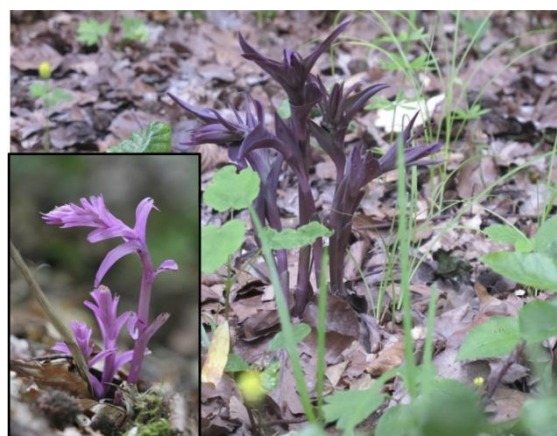

Figure 2. – Albino plants in mixotrophic orchids. An albino (inset) coloured in pink by anthocyanins in the normally green Neottieae (orchid) species *Epipactis purpurata* (main picture). Photo A. Hasenfratz.
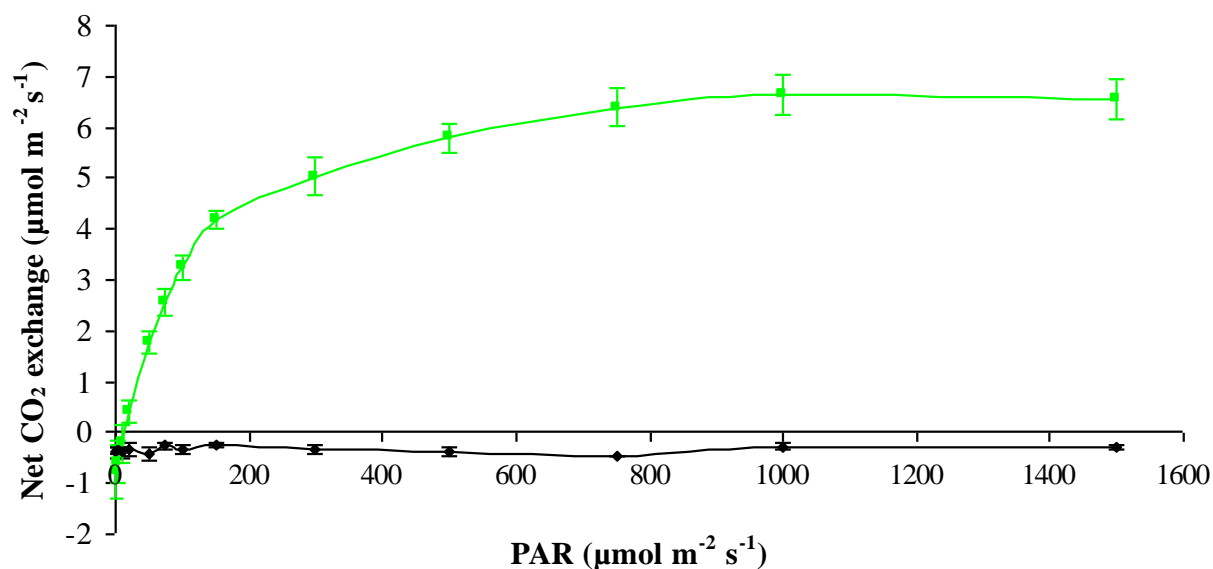

Figure 3. – Heterotrophy and lower basal metabolism (lower CO_2 production in the dark) of albinos. Gas exchanges for *Cephalanthera damasonium* green and albino as a reply to photosynthetic active radiations (PAR).

saprophytic and parasitic basidiomycetes (namely Tulasnellaceae, Ceratobasidiaceae and some Sebacinales; Dearnaley *et al.*, 2012). Neottieae display various levels of specificity to non-rhizoctonias fungi that are known to form ectomycorrhizae on forest trees: most *Epipactis* species show a preference for Pezizomycetes related to truffles, sometimes with additional fungi (Bidartondo *et al.*, 2004; Selosse *et al.*, 2004; Ouanphanivanh *et al.*, 2008; Ogura-Tsujita & Yukawa, 2008; Shefferson *et al.*, 2008; Liebel *et al.*, 2010); *Cephalanthera* species display a large fungal spectrum including Cortinariaceae, Hymenogastraceae and mainly Thelephoraceae (Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Matsuda *et al.*, 2008; Yamato & Iwase, 2008); *Russula* are specific associates to *Limodorum* species (Girlanda *et al.*, 2006; Paduano *et al.*, 2010; Liebel *et al.*, 2010). This is much reminiscent of the fungal associates in fully mycoheterotrophic orchids, which are also ectomycorrhizal on nearby trees (see Dearnaley *et al.*, 2012 for review). A similar feature was reported in Japanese *Cymbidium* species: the green *C. lancifolium* and *C. goeringii* had ^{13}C abundances intermediate between autotrophs and the mycoheterotrophic *C. macrorhizon* and *C. aberrans* (Motomura *et al.*, 2010); at the same time, they displayed Tulasnellaceae and ectomycorrhizal taxa (Russulaceae, Thelephoraceae and Sebacinaceae) that are exclusive partners of mycoheterotrophic *Cymbidium* species (Ogura-Tsujita *et al.*, 2012). In all, surrounding trees are thus likely to be the ultimate C source of partial mycoheterotrophs, and it is even considered that availability of ectomycorrhizal fungi could be a limitation for partial mycoheterotrophy (Liebel *et al.*, 2010). The association of mixotrophic orchids to ectomycorrhizal fungi starts at seeds germination (Bidartondo & Read, 2008; Tesitelova *et al.*, 2012).

These data give an *a posteriori* meaning for experiences carried out by Sadovsky (1965) at a time European protection laws allowed destructive manipulation of native orchids. In repeated attempts to transplant various orchids, he listed some species that did not survive the process: the list interestingly mixes full mycoheterotrophs (*Corallorhiza trifida*, *Neottia nidus-avis*…) and partial mycoheterotrophic species (*Cephalanthera*, *Limodorum* and *Epipactis* spp.), suggesting

that in both cases, disconnecting the fungus from its resources (= nearby tree roots) entailed plant death.

As stated above for the genus *Cymbidium* (Tribe Cymbidieae), partial mycoheterotrophy is not limited to Neottieae. An interesting debate exists over *Corallorhiza trifida* (tribe Maxillarieae): whereas other *Corallorhiza* spp. show deleted plastidial genomes (Freudenstein & Doyle, 1994) and are fully mycoheterotrophic, *C. trifida* has an intact plastidial genome and is somewhat greenish. It is sometime considered as mycoheterotrophic (e.g. McKendrick *et al.*, 2000; Cameron *et al.*, 2009), but is partial mycoheterotrophic based on its ^{13}C natural abundance (one quarter of its C originating from photosynthesis; Zimmer *et al.*, 2008; see next section). A trend to partial mycoheterotrophy was found in Mediterranean and Macaronesian species (*Barlia robertsiana* (tribe Ophrydeae), *Genneria diphylla*, *Habenaria tridactylites* and *Orchis purpurea* (all from tribe Orchidoideae); Liebel *et al.*, 2010: interestingly, *G. diphylla* at least is associated to ectomycorrhizal fungi). The possibility that rhizoctonias-associated orchids also display some mixotrophy is currently limited. Partial mycoheterotrophy is reported in the tropical *Cheirostylis montana* (tribe Cranichideae) from Thailand (Roy *et al.*, 2009) based on ^{13}C isotopic abundance, suggesting the need for more investigations out of Northern America and Eurasia. Thus, many of not all orchid taxa may be predisposed to partial mycoheterotrophy (as well as to full mycoheterotrophy) by their mycoheterotrophic germination.

CARBON SOURCES IN PARTIAL MYCOHETEROTROPHIC ORCHIDS

Investigations on photosynthesis apparatus and *in situ* gas exchange support a partial heterotrophy for some green orchids. CO_2 exchanges in *Cephalantera damasonium* revealed that, as expected from their phenotype, albinos were full heterotrophs, while green individuals exhibited a normal photosynthetic response to light (fig. 3; Julou *et al.*, 2005). After *in situ* ^{13}C labelling, *C. damasonium* showed sub-normal but reduced CO_2 assimilation, while *C. trifida* showed nearly no assimilation, close to the level of the mycoheterotrophic control (Cameron *et al.*, 2009). This was in good agreement with

chlorophyll content and fluorescence values (i.e. proper assembly of photosystems), respectively suggesting sub-normal (but reduced) and very reduced potential for photosynthesis in *C. trifida* (Julou *et al.*, 2005; Zimmer *et al.*, 2008; Cameron *et al.*, 2009). In *C. trifida*, the chlorophyll content is 1% of that of *C. damasonium* (Zimmer *et al.*, 2008). However, Montfort & Küsters (1940), measuring CO_2 exchange in inflorescences and maturing fruits, found that CO_2 assimilation was 2.2 times higher than respiration – whether differences in technology or plant origin explains this remain unclear. Intrinsic photosynthetic limitations also exist in *Limodorum abortivum*, where photosynthetic abilities do not compensate respiration even in full light (Girlanda *et al.*, 2006). In some *C. damasonium* populations however, there is evidence that low light conditions enforce the plant to survive near its compensation point (where respiration equals photosynthesis; Julou *et al.*, 2005) and drive partial mycoheterotrophy. Thus, both intrinsic and environmental factors drive partial mycoheterotrophy, depending on the species and site.

Measurement of photosynthetic activity approaches and stable isotope natural abundance are both powerful tools for investigating partial mycoheterotrophy. However, the information gained from these techniques differs. Gas exchange measurements provide snapshot information about photosynthetic activity, while stable isotope natural abundance data integrate the sources of C gain over the entire life history of a plant or plant organ. This difference may explain the diverging results for *C. trifida*, where isotopic data suggest partial mycoheterotrophy (Zimmer *et al.*, 2008) whereas CO_2 fixation was detected (Montfort & Küsters, 1940) or not (Cameron *et al.*, 2009). However, the major interest of stable isotopes is to allow a quantitative and comparative approach of heterotrophy level (Fig. 1): the percent of C derived from the fungi can be estimated from a linear mixing model. Briefly, the ^{13}C abundance in mixotrophs can be viewed as a mixing a proportion p of fungal C and $(1-p)$ of photosynthetic C. Reference values can be derived from ^{13}C abundance in surrounding full autotrophs and full mycoheterotrophs, although some discrepancies may exist

between ^{13}C abundances of co-occurring MH species (Zimmer *et al.*, 2007; Motomura *et al.*, 2010), so that albinos (Fig. 2) are the ideal conspecific reference for mycoheterotrophic ^{13}C abundance whenever available. Not unexpectedly, linear mixing models reveal a continuum from autotrophy to full mycoheterotrophy in mixotrophs.

Moreover, a given species shows variable heterotrophy level. First, from one site to another: for the well-studied *C. damasonium*, this level ranges from $p = 20$ to $p = 85\%$ (Gebauer & Meyer, 2003); in this species, a comparison in various light environments over Europe showed that low light levels result in strong, mycoheterotrophy, while higher irradiances drive the plant towards autotrophy (Preiss *et al.*, 2010). Yet, since we have no C budget for mixotrophs, we ignore where ^{13}C enrichment in shaded plants reflects a compensation of the reduced photosynthesis by an increased flow of fungal C. Alternatively, this enrichment may simply result from a lower dilution of a fixed amount of fungal C in a lower input of photosynthetic C, as suggested in albinos (Roy *et al.*, 2013). Second, mixotrophy may vary over the season in shoots, at least at moderate to high light levels: p increases from 80% at shoot emergence, where fungal C is thus the main resource, to 20% at fruiting time (Roy *et al.*, 2013). At this time, the photosynthesis of expanded leaves, whose efficiency looks to improve along the growth season, takes over the C furniture, partially explaining albinos' lower fitness (see below). Thus, partial mycoheterotrophy in orchids is a flexible nutritional mode, driven by light, C availability and development.

There are, however, four main caveats when using stable isotopes: First, analysis of tissues does not take into account the anabolic C that may not mix heterotrophic and autotrophic C sources in the same ratio. In *C. damasonium* at least, ^{13}C abundances do not significantly differ in catabolic CO_2 *versus* biomass C (Roy *et al.*, 2013). Second, the relative contribution of respiratory CO_2 may be enhanced in mixotrophs since they have lower need for external CO_2, leading to higher ^{13}C abundance that biases estimates of p values. Third, beyond problems about the mycoheterotrophic baseline stated above, the autotrophic baseline for orchids remains questionable: *Goodyera repens*, a species provide C to its fungus (Cameron *et al.*, 2008),

is less enriched in ^{13}C than nearby autotrophs (Hynson *et al.*, 2009a; Motomura *et al.*, 2010), and indeed several green orchids show the same trend (Liebler *et al.*, 2010). Fourth, partial mycoheterotrophic species likely have lower photosynthesis rates, resulting, as stated above, in better equilibration of [13]CO$_2$ concentration between environmental air and stomatal chamber and therefore, in increased ^{13}C discrimination (= lower ^{13}C abundance in photosynthetic C). The two latter caveats may result in under-estimation of heterotrophy level in many orchids when using non-orchid autotrophs as references. As a result, several species with normal ^{13}C abundance may be 'cryptic' partial mycoheterotrophs, with heterotrophy levels not detectable by isotopic methods. Indeed, many orchids from more or less open environments associate with rhizoctonias mainly but occasionally display ectomycorrhizal fungi, e.g. *Cypripedium* (Shefferson *et al.*, 2005, 2007), *Gymnadenia* (Stark *et al.*, 2009), *Orchis* (Lievens *et al.*, 2010), or *Cryptostylis ovata* (Sommer *et al.*, 2012). These associates that were likely hidden in studies based on fungal cultivation, since ectomycorrhizal fungi do not grow easily, may allow a C flow to the plant, at least in some light environments. Moreover, a few rhizoctonias are overlooked ectomycorrhizal fungi, such as some species in Tulasnellaceae (Bidartondo *et al.*, 2003) and Ceratobasidiaceae (Bougoure *et al.*, 2009; Veldre *et al.*, 2013) that could provide C.

One may finally deplore the absence of labelling experiments directly demonstrating the C transfer from plant to (i) fungus and (ii) partial mycoheterotrophic orchid: thus, the fact that nearby trees are the ultimate C source still requires rigorous demonstration. However, there are two indirect additional evidences for the use of mycorrhizal fungi as trophic source: both ^{15}N abundance and N/C ratio are high in partial mycoheterotrophic orchids as compared to surrounding autotrophs, a trend reported for mycoheterotrophs (Gebauer & Meyer, 2003; Abadie *et al.*, 2006; Tedersoo *et al.*, 2007; Selosse & Roy, 2009; Liebel *et al.*, 2010; Roy *et al.*, 2013); these values are lower than in partial than in full mycoheterotrophs, again supporting a mixed tropic strategy. The studies of rhizoctonias orchids mentioned above also demonstrate that some putatively autotrophic orchids exhibit higher (although less extreme) ^{15}N abundance and N/C ratio than other autotrophs, again raising the possibility of a cryptic partial mycoheterotrophy.

Recently, Yagame *et al.* (2013) opened the possibility that the green orchid *Cremastra appendiculata* (tribe Cypripedieae), which associate to with fungi belonging to saprophytic *Coprinellus* (Psathyrellaceae) could be mixotrophic. This would make sense, as some fully mycoheterotrophic tropical orchids associate to saprophytic fungi (see Selosse *et al.*, 2010, for a review). Yet, the demonstration that saprophytic can support mixotrophic orchids remains pending until isotopic data are available.

PARTIAL MYCOHETEROTROPHY IN ADULT PYROLOIDS

Orchids have been up to now major models for investigations on partial mycoheterotrophy, but this nutritional mode is more widespread. Species from the Pyroleae sub-tribe (Ericaceae), also called pyroloids, are small shade-tolerant perennial shrubs with impressive underground rhizome network that grow in more or less low light temperate, alpine and boreal forests. They are candidates for partial mycoheterotrophy for three reasons: First, a close, although not fully resolved, phylogenetic relationship links with the mycoheterotrophic Monotropeae and Pterosporeae (all members of the Monotropoideae; Kron *et al.*, 2002; Tedersoo *et al.*, 2007; Matsuda *et al.*, 2012). A pyroloid, *Pyrola aphylla*, is even mycoheterotrophic (Zimmer *et al.*, 2007; Hynson & Bruns, 2009), although its recognition as a separate species, or a species complex (Jolles & Wolfe, 2012), or simply an albino variant of the green *P. picta*, remains unclear (Camp, 1940; Haber, 1987). Second, as in orchids, seeds are very small and pyroloids undergo a mycoheterotrophic subterranean germination (Christoph, 1921; Lihnell, 1942) where fungi related to the *Sebacinales* and various ectomycorrhizal taxa provide C (Smith & Read, 2008; Hashimoto *et al.*, 2012; Hynson *et al.*, 2013a). Third, as many other basal Ericaceae (Selosse *et al.*, 2007), pyroloids form ectomycorrhiza-like associations: the fungus forms a sheath around the root but in addition, hyphal coils penetrating root cells (Khan, 1972; Robertson & Robertson, 1985; Vincenot *et al.*, 2008). The fungal taxa involved are typical ectomycorrhizal fungi that also colonize

surrounding tree roots (Robertson & Robertson 1985; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007; Vincenot *et al.*, 2008; Hynson & Bruns, 2009; Matsuda *et al.*, 2012), thus allowing a mycelial link to surrounding autotrophic trees.

Investigation on stable isotopes provided variable results. A first investigation in two boreal Estonian forest revealed that some pyroloids had higher ^{13}C and ^{15}N abundances and N/C than surrounding autotrophs, including plants from the Ericaceae family (Tedersoo *et al.*, 2007; Fig. 1). Based on a linear mixing model (using *Monotropa hypopytis* as baseline), 10 to 68% of C was of fungal origin in *Orthilia secunda*, *Pyrola chlorantha, P. rotundifolia* and *Chimaphila umbellata*. The latter species did not differ from autotrophs on one of the investigated sites, and heterotrophy level did not correlate among sites, indicating a complex regulation of this parameter among species. In a second set of investigations in more southern sites from Germany and California, (Zimmer *et al.*, 2007; Hynson *et al.*, 2009b), ^{15}N abundance and N/C values were intermediate between autotrophic and mycoheterotrophic plants for *O. secunda*, *C. umbellata*, *P. chlorantha*, *P. minor* and *P. picta*; however, based on ^{13}C abundance, only *P. chlorantha* showed significant C gain at a single low irradiance site. Given the broad range of irradiances in investigated sites, light availability is unlikely to be the only driver of the heterotrophy level (Zimmer *et al.*, 2007). Alternatively, it was suggested that the C gain was not solely based on C needs, but could arise as a 'side-product' of the nitrogen and phosphorus nutrition (Selosse & Roy, 2009) as is known in mixotrophic algae (see below); indeed, a specific strategy to obtain fungal N in pyroloids could explain their unusual ^{15}N abundance and their disappearance after anthropogenic N deposition (Allen *et al.*, 2007), and some fungal C may move with the nitrogen. In Japanese *Pyrola japonica*, an endemic mixotrophic pyroloid that has unusual mycorrhizae devoid of fungal mantles, 84% of which are formed by *Russula* spp., isotopic values negatively correlated with light availability (Matsuda *et al.*, 2012), suggesting higher heterotrophy levels in shaded conditions. Concomitantly, the rate of mycorrhizal colonization and *Russula* frequency was higher in shaded conditions, perhaps compensating for low light levels. In

an experimental shading of *C. umbellata* and *P. picta* (the only experimental studies hitherto carried out on mixotrophs; Hynson *et al.*, 2012) leaf sugars reflecting the immediate C source supported a decrease of fungal C contribution over the growth season, in the first species, but not the second. At the same time, bulk C did not change in *C. umbellata*, suggesting that isotopic contents inherited from previous physiological status can dissimulate instantaneous trends (see above).

It is thus possible that mixotrophy implies very variable features among pyroloid species, and that in some species at least, mycorrhizal symbionts and C nutrition change in accordance to light availability and growth stage, in a flexibility similar to that reported above for orchids. To further support physiological variations among pyroloids, the phylogenetic analysis by Matsuda *et al.* (2012) suggested that the *Pyrola + Orthilia* clade may have evolved higher mycoheterotrophic abilities, as further supported by the existence of the mycoheterotrophic *P. aphylla*, while the *Moneses + Chimaphila* sister clade would be less prone to mixotrophy. Interestingly, no evidence for lysis or degradation of intracellular hyphae is reported in living pyroloid root cells of pyroloids (Tedersoo *et al.*, 2007; Vincenot *et al.*, 2008), adding to the idea that such a process is not necessary for C transfer, in contrast to a lasting view issuing from early works on mycoheterotrophic orchid seedlings (Rasmussen, 1995).

[14]C labelling was claimed to show transfer from autotrophs to pyroloids, in dual pot cultures of *Larix kaempferi* seedlings and *Pyrola incarnata* from Japan (Kunishi *et al.* 2004; Hashimoto *et al.*, 2005), but this experiment and its controls is still not published in details. A C gain from fungi may explain some ecophysiological features of pyroloids, such as the few reserves in winter (the fungal C may contribute to development of new organs in spring for *P. incarnata*; Isogai *et al.*, 2003), the low capacity for adjusting vegetative growth and leaf area after shading (in *P. rotundifolia* at least; Hunt & Hope-Simpson, 1990), or the sensibility to forest disturbances that affect mycorrhizal networks (logging, burning; Zimmer *et al.*, 2007). However, at least some green pyroloids can be outplanted (e.g. Hunt & Hope-Simpson, 1990): contrasting with partial mycoheterotrophic orchids, the use of fungal C

in pyroloids may be thus more facultative, allowing disconnections from mycorrhizal network in some conditions. This may also resolve the discrepancies between studies reporting variable levels of heterotrophy (Tedersoo *et al.*, 2007, Zimmer *et al.*, 2007; Hynson *et al.*, 2009b), if one assumes variable levels of dependency on mycorrhizal network from one population to another.

EVOLUTION OF PARTIAL MYCOHETEROTROPHY AND MYCOHETEROTROPHY

Partial mycoheterotrophic lineages add to a long list of taxa where partial heterotrophy exists in autotrophic organisms. In the general case, such a mixed strategy is called mixotrophy. This word was sometimes used as a synonym for partial mycoheterotrophy (Selosse & Roy, 2009): however, mixotrophy is larger than partial mycoheterotrophy, since mixotrophic strategies are very diverse, as exemplified below. Many independent phyla of planktonic eukaryotes are mixotrophic, either by uptake of dissolved organic matter (Kamjunke & Tittel, 2009), or by phagotrophy on unicellular preys (Jones, 2000). The latter process is a plesiomorphic condition, since ancestors of plastid-bearing taxa are considered to be phagotrophs (a process through which plastids were indeed acquired). Mixotrophy is ecologically relevant in oceans, where planktonic algae achieve up to 95% of the bacterivory in the superficial layer (Zubkov & Tarran, 2008; Moorti *et al.*, 2009). In contrast, mixotrophy in land plant is secondarily evolved, i.e. represents a reversion from full autotrophy, and its ecological relevance is still to be estimated in terrestrial ecosystems. Mixotrophy is mainly found in plants displaying close interactions with surrounding organisms: beyond partial mycoheterotrophy, making use of the widespread mycorrhizal symbiosis, mixotrophy occurs in carnivorous (Adamec, 1997) and hemiparasitic plants (Press & Graves, 1995). The later are green plants that obtain some nourishment, especially mineral nutrient and sometimes C, by parasitizing other plants. For example, despite being photosynthetic, mistletoes derive up to 63% of their C from their host (Bannister & Strong, 2001) and *Striga hermonthica* can survive as albino (Press *et al.*, 1991).

When considering phylogenetic frameworks, partial mycoheterotrophy appear as a step toward evolution of the full mycoheterotrophy (Bidartondo *et al.*, 2004; Selosse *et al.*, 2004). Mycoheterotrophic species are nested within partial mycoheterotrophic lineages in Neottiae (Figure 4; Abadie *et al.*, 2006; Selosse & Roy, 2009) and in the genus *Cymbidium* (Motomura *et al.*, 2010; Ogura-Tsujita *et al.*, 2012). Pyroloids are close to the mycoheterotrophic Monotropoideae (Monotropeae and Pterosporeae; Kron *et al.*, 2002), suggesting a similar scenario (Tedersoo *et al.*, 2007); however, the relationships within Monotropoideae deserve new analyses and a basal position of pyroloids remains uncertain (K. Kron, comm. pers.). Undoubtedly, in orchids and pyroloids at least, the initial mycoheterotrophic germination of seedlings (likely plesiomorphic in these taxa) was an additional predisposition to mixotrophy.
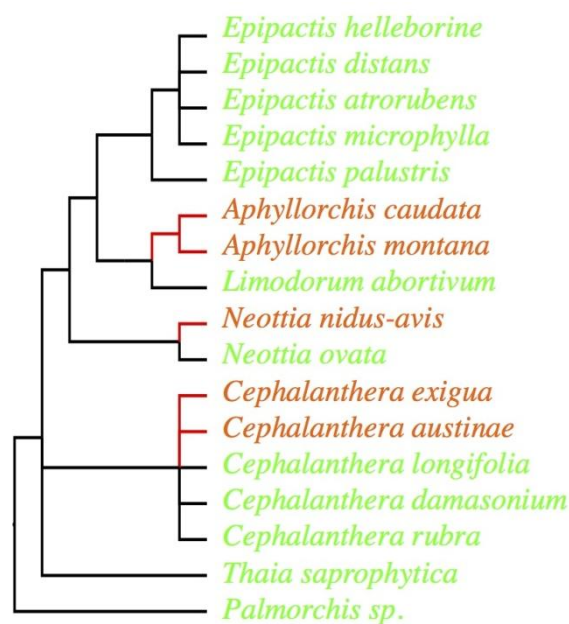

Figure 4. – Polyphyly of mycoheterotrophs. A phylogeny of Neottieae with branches with >80% bootstrap values. Green species have chlorophyll, while brown ones are mycoheterotrophic.

Evolutionary transition to mycoheterotrophy can be expected to occur frequently, since partial mycoheterotrophs often grow in young forests, with loose canopy that tend to close due to ongoing plant

ecological succession. Thus, they undergo increasing shading that selects for more light-independent C supply (Selosse & Roy, 2009); indeed, this may explain the convergent evolution to full mycoheterotrophy observed within the Neottieae (Figure 4; Roy *et al.*, 2009). Full mycoheterotrophy is never shown to be evolutionary reversible, maybe due to irreversible loss of photosynthetic genes both in the plastid and nucleus. However, there are possible reversions from partial mycoheterotrophy to full autotrophy. For example, the *Epipactis palustris-gigantea* clade (Figure 4) is autotrophic, as shown by survival upon transplantation (Sadovsky, 1965), autotrophic ^{13}C values, and association to rhizoctonias only (Bidartondo *et al.*, 2004; Zimmer *et al.*, 2007; Illyes *et al.*, 2009). Although Neottieae phylogeny (Fig. 4) and evolution of nutritional traits remain poorly resolved, the genus *Epipactis* is unlikely to be basal (Pridgeon *et al.*, 2008; Roy *et al.*, 2009), making reversion from partial mycoheterotrophic to autotrophy parsimonious scenario for the *Epipactis palustris-gigantea* clade. Biologically, a reversion is not unexpected, as long as photosynthesis genes are not impaired in partial mycoheterotrophs.

In Neottieae, albinos suggest that the transition from partial mycoheterotrophy to mycoheterotrophy cannot be sudden. In a comparison between albinos and co-occurring green individuals *in situ*, albinos displayed (1) more frequent shoot drying at fruiting, possibly due to stomatal dysfunctions, (2) lower basal metabolism (Figure 3), (3) increased sensitivity to pathogens and herbivores, (4) higher dormancy and maladapted sprouting, and, probably due to the previous differences, (5) fewer seeds, with lower germination capacity (Roy *et al.*, 2013; Figure 5). Over the growing season, fungal colonization reached its minimum in both phenotypes at time of fruiting, so that the lack of photosynthesis in fruiting albinos may contribute to C limitation, and to the above-mentioned trends. With a 10^3-reduction of their fitness, albinos failed a successful transition to mycoheterotrophy because some traits inherited from their green ancestors were maladaptive (Roy *et al.*, 2013). Conversely, mycoheterotrophy requires at least degeneration of leaves and stomata, optimization of the temporal pattern of fungal colonization and shoot sprouting, and new defences against pathogens and herbivores.

Transition to mycoheterotrophy thus likely requires progressive and joint evolution of these traits, while a sudden loss of photosynthesis leads to unfit plants. This supports an evolutionary metastability of mixotrophy, and what prevents the emergence of fully heterotrophic taxa in mixotrophic lineages. In spite of their survival and successful fruiting in some cases, albinos likely represent snapshots of failed transitions from mixotrophy to mycoheterotrophy; noteworthy, they are not reported from pyroloids. They are ecological equivalents to mutants in genetics, *i.e.* their dysfunctions may suggest what makes mycoheterotrophy successful. Although their determinism remains unknown, they offer fascinating models for comparing the physiology of mixo- and mycoheterotrophs within similar genetic backgrounds.
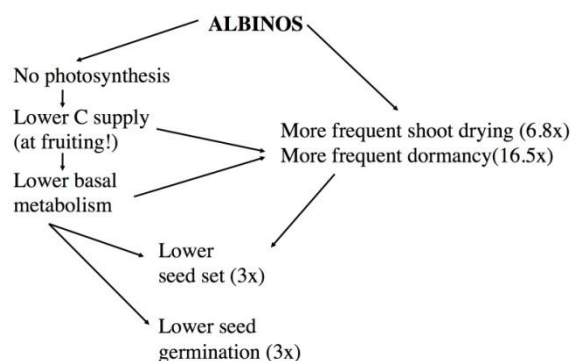

Figure 5. – Lower fitness of albino individuals. A summary of maladaptations in albinos compared to green individuals (Roy *et al*., 2013) that result in a *ca.* 1000x lower fitness in *Cephalanthera damasonium* populations.

PERSPECTIVES

The existence of mixotrophy awaits confirmation from other taxa displaying mycoheterotrophic lineages (e.g., Burmanniaceae, Gentianaceae, Dioscoreales, Polygalaceae, Iridaceae, Pandanales and Petrosaviaceae; Selosse & Roy, 2009). However, these taxa associate with arbuscular mycorrhizal (AM) fungi. Specific ^{13}C and ^{15}N abundances in ectomycorrhizal fungi were instrumental in discovering partial mycoheterotrophy in orchids and pyroloids. We know few of isotopic abundances values in

AM fungi, or even in the associated mycoheterotroph, making it difficult to predict isotopic abundances in candidate AM-associated mixotrophs. Existing works suggest that fractionation is low, or even absent (Cameron & Bolin, 2010; Merckx *et al.*, 2010; Courty *et al.*, 2011), but these works are blurred by (i) the potential occurrence of C4 plants in the system (Cameron and Bolin, 2010) and (ii) the mix of over- and understorey photosynthetic C that differ in ^{13}C abundances (Merckx *et al.*, 2010; Courty *et al.*, 2011). Moreover, the fact that green Burmanniaceae can be cultivated alone (Merckx *et al.*, 2010) is not in favour of a mixotrophy on AM fungal links to surrounding plants these plants at least.

Moreover, lineages devoid of mycoheterotrophs or minute seeds with mycoheterotrophic germination may display mixotrophy, but this remains to demonstrate. There is some evidence from isotopic labelling C transfer can occur between various plants, e.g. between tree seedlings through shared ectomycorrhizal fungi (Simard *et al.*, 1997) or between understorey herbs and overstorey tree, through shared AM fungi (Lerat *et al.*, 2004). However, this method does not evaluate the impact on the long-term C budget of receiving plants. Since no major effect on spontaneous ^{13}C abundance is reported in ectomycorrhizal trees (e.g., Diédhiou *et al.*, 2010), assuming that the same fractionation would occur as in orchids and pyroloid systems, the impact on C budget may be limited. Thus, it remains unsure that mycorrhizal linking plants can significantly contribute to their C nutrition in "normal" green plant. But, once again, decisive tests are still lacking.

Furthermore, the ecological relevance of partial mycoheterotrophs on plant communities (as demonstrated for hemiparasitic plants, e.g. Watson 2009) and on their associated fungi (exact load of the C loss, existence of a reward such as vitamins and/or protection, etc.) is still not known. These open questions are relevant, at least in boreal forest where partial mycoheterotrophs dominate the understorey (Tedersoo *et al.*, 2007), and are not assessed for full mycoheterotrophs either. If one assumes, as suggested by C flow, that the plant is parasitic on the fungus, then some conflict may occur and fungi may undergo selection to avoid this parasitism. In seedlings mycoheterotrophy that give rise to autotrophic adults, the interaction maybe positively selected on the fungal side, since investment in a future C-producing plant can compensate the C flow to seedlings.

Mixo- and mycoheterotrophy plants remain a fascinating research area, demonstrating the power of fungi and microbial interactions in shaping plant evolution. At the epistemological level, the many cited works show how these newly discovered plant trophic strategies have enhanced researches on mycorrhizal fungi, mycorrhizal networks between plants, and metabolism of plant-fungal symbioses.

Acknowledgements

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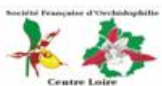
1ᵉʳ et 2
MARS 2014

16ᵉ Colloque
SFO

BLOIS
Halle aux grains

Orchidées

Actes du 16e colloque sur les Orchidées de la Société Française d'Orchidophilie

Quel avenir pour les orchidées dans leur milieu ?

1er et 2 mars 2014
Blois, La Halle aux Grains

Avec le soutien de la Société botanique de France

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L'Orchidophile
200 fascicules publiés depuis 1970
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N° 1 (1993) : *Synopsis des orchidées européennes, par Pierre Quentin*
N° 2 (1995) : *Synopsis des orchidées européennes, deuxième édition, par Pierre Quentin*
N° 3 (1996) : *Actes du 13ème colloque de la SFO, Grenoble, 29 juin – 2 juillet 1995*
N° 4 (1999) : *Compte-rendu des premières journées rencontres orchidophiles Rhône-Alpes, Lyon, 30 mai-1er juin 1998*
N° 5 (1999) : *Les hybrides des genres* Nigritella *et/ou* Pseudorchis, *par O. Gerbaud et W. Schmid (coédition SFO-AHO)*
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N° 7 (2010) : *Actes du 15ᵉ colloque sur les orchidées de la Société Française d'Orchidophilie, Montpellier, 30 mai - 1er juin 2010*
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Ouvrages
Divers ouvrages sur les orchidées tempérées et tropicales, de France, d'Europe et du monde, dont :
Les orchidées de France, Belgique et Luxembourg. 2005. (M. Bournérias et D. Prat, coordinateurs)
Atlas des orchidées de France. 2010. (F. Dusak et D. Prat, coordinateurs)
Sabots de Vénus, orchidées fascinantes. 2013. (Collectif SFO, supplément à l'Orchidophile)

La Société Française d'Orchidophilie, fondée en 1969, a pour objectifs majeurs :
- d'étudier la répartition et l'écologie des Orchidées en France et dans d'autres pays ;
- de protéger les espèces sauvages les plus menacées ;
- de favoriser la culture des espèces horticoles ;
- d'encourager les études sur la biologie des orchidées.

Ces objectifs sont atteints grâce :
- à des réunions et colloques ;
- à des voyages d'étude ;
- au réseau de cartographes ;
- aux activités régionales menées dans les associations locales affiliées ;
- aux publications (bulletin, cartographies, ouvrages).

The "Société Française d'Orchidophilie" (French Orchid Society), formed in 1969, aims the main following activities:
- studying orchid distribution and ecology in France and everywhere else;
- protecting most endangered wild species;
- promoting cultivation of horticultural species;
- encouraging studies on orchid biology.

These goals are reached through:
- meetings and symposiums;
- field trips;
- network of cartographers;
- local activities of regional affiliated associations;
- publications (bulletin, cartographies, books).