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ORIGINAL ARTICLE

Three-year pot culture of *Epipactis helleborine* reveals autotrophic survival, without mycorrhizal networks, in a mixotrophic species

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Abstract

Some mixotrophic plants from temperate forests use the mycorrhizal fungi colonizing their roots as a carbon source to supplement their photosynthesis. These fungi are also mycorrhizal on surrounding trees, from which they transfer carbon to mixotrophic plants. These plants are thus reputed difficult to transplant, even when their protection requires it. Here, we take profit of a successful ex situ pot cultivation over 1 to 3 years of the mixotrophic orchid *Epipacis helleborine* to investigate its mycorrhizal and nutrition status. Firstly, compared with surrounding autotrophic plants, it did not display the higher N content and higher isotopic (^{13}C and ^{15}N) abundance that normally feature mixotrophic orchids because they incorporate N-, ^{13}C-, and ^{15}N-rich fungal biomass. Second, fungal barcoding by next-generation sequencing revealed that the proportion of ectomycorrhizal fungi (expressed as percentage of the total number of either reads or operational taxonomic units) was unusually low compared with *E. helleborine* growing in situ: instead, we found a high percentage of rhizoctonias, the usual mycorrhizal partners of autotrophic orchids. Altogether, this supports autotrophic survival. Added to the recently published evidence that plastid genomes of mixotrophic orchids have intact photosynthetic genes, this suggests that at least some of them have abilities for autotrophy. This adds to the ecological plasticity of mixotrophic plants, and may allow some reversion to autotrophy in their evolution.

Keywords Mycoheterotrophy · Mycorrhizae · Orchid transplantation · Rhizoctonia · Stable isotopes · ^{13}C · ^{15}N

Introduction

Some plants from temperate forests display a mixotrophic nutrition that relies on both their photosynthates and resources extracted from the mycorrhizal fungi colonizing their roots (for reviews, see (Selosse and Roy 2009; Selosse et al. 2016; Jacquemyn and Merckx 2019). These plants belong to the orchid (e.g., from the Neottieae tribe) and *Ericaceae* families and are also called partial mycoheterotrophs, because the heterotrophic nutrition using carbon from mycorrhizal fungi is called mycoheterotrophy (Hynson et al. 2013). Such mixotrophic plants rely on the ability of mycorrhizal fungi to establish networks between plants of different species, due to a low mycorrhizal specificity: the so-called mycorrhizal networks (Selosse et al. 2006; Simard et al. 2012) allow fungi to gain nutrients on some plants and to deliver part of it to others. Mixotrophic temperate plants rely on the network formed by ectomycorrhizal fungi, which also associate with surrounding trees (Selosse and Roy 2009).

Five lines of evidence demonstrated this mixotrophic status. Firstly, the mixotrophic plants shifted from the usual mycorrhizal fungi of their respective family (e.g., the rhizoctonias in orchids; (Dearnaley et al. 2012)) to ectomycorrhizal taxa, establishing mycorrhizal networks with nearby trees (Hynson et al. 2013). Secondly, the (hitherto elusive) compounds provided by ectomycorrhizal fungi to mixotrophs is naturally

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enriched in ^{13}C as compared with photosynthates, and the biomass of mixotrophic plants is thus enriched in ^{13}C compared with that of autotrophic plants (Gebauer and Meyer 2003; Bidartondo et al. 2004; Julou et al. 2005; Hynson et al. 2013); note that the biomass acquired by mycoheterotrophy is often also enriched in ^{15}N). Importantly, ^{13}C abundance in mixotrophs can be used to calculate the percentage of mycoheterotrophy in each organ and season (Gebauer and Meyer 2003; Gonneau et al. 2014) or in different light environments (Preiss et al. 2010; Gonneau et al. 2014). Thirdly, beyond isotopic evidence of a raw C flow from the fungus to the orchid, the survival of rare achlorophyllous variants, in mixotrophic *Neottieae* orchids at least, demonstrates a net flow in favor of the plant: these achlorophyllous variants survive well (Selosse et al. 2004; Julou et al. 2005; Lewis 2015; Shefferson et al. 2016), but produce a limited fruit set (Salmia 1989; Roy et al. 2013; Gonneau et al. 2014). Fourthly, green mixotrophic individuals display a limited photosynthesis rate, even under the compensation point (where photosynthesis equals respiration), due to the shade of the tree canopy (Julou et al. 2005) or to limited intrinsic photosynthetic abilities (Girlanda et al. 2006). Fifthly, mixotrophs often contain more nitrogen (N) as compared with autotrophic, non-N fixing plants, probably because (i) plant respiration eliminates C and thus concentrates N from the biomass received from fungi, as described in heterotrophs, and/or (ii) the fungal biomass is likely already N-rich (Abadie et al. 2006; Hynson et al. 2013).

Recently, a split use of the two (photosynthetic and mycoheterotrophic) resources by mixotrophic *Neottieae* orchids was revealed, based on the variations of ^{13}C abundance in various organs and labeling of photosynthates in situ. Photosynthates produced after leaf expansion are mostly used for aerial parts, leaves, and fruits which are green before ripening (Roy et al. 2013; Bellino et al. 2014). Mycoheterotrophic resources are mostly used for underground roots and rhizomes (Gonneau et al. 2014): see Fig. 5 therein), as well as for elaboration of starch reserves (Lallemand et al. 2019a). This explains why achlorophyllous variants produce fewer seeds (Roy et al. 2013), but have good rhizome survival (Shefferson et al. 2016).

Mixotrophic orchids thus strongly depend on mycorrhizal networks for survival, and these results in some difficulties in transplantation attempts, as reported by Sadovsky (1965) for mixotrophic *Neottieae*. This puts constraints on protection and transplantations to save populations menaced by changes in land use. Yet, there are seldom examples of successful transplantation: for example, Delforge (2016, 2017) reports that two *Epipactis helleborine* individuals (*Neottieae* tribe) survived transplantation to a forest edge environment the first year and even flowered. While one individual then disappeared, the other one persisted over 6 years at least (Delforge 2016, 2017). Furthermore, some commercial nurseries sell mixotrophic *Epipactis* spp. grown in pots, such as *E. helleborine*, although every time we accessed these products ($n = 2$) they turned out to belong to *E. palustris*, a related but autotrophic species (Lallemand et al. 2018). The *Epipactis helleborine* orchid species usually harbors a dominance of ectomycorrhizal fungi in its roots (Bidartondo et al. 2004; Ogura-Tsujita and Yukawa 2008; Těšitelová et al. 2012; Jacquemyn et al. 2016; Jacquemyn and Merckx 2019) and, from many isotopic data, largely relies on mycoheterotrophy for its rhizome survival and growth of young shoots (Gebauer and Meyer 2003; Gonneau et al. 2014; Schiebold et al. 2017; Lallemand et al. 2019a); Xing et al. 2019), so that successful transplantation and pot culture appear unexpected.

Here, we used a common garden growth experiment to investigate the stability of the phenotype of various morphologically distinct subspecies of *E. helleborine* after transplantation (see Delforge 2016, for a review of these subspecies; the taxonomic outcome of this experiment will be reported elsewhere). Transplanted *E. helleborine* were successfully grown in pots placed in a common garden, where the absence of a mycorrhizal network prompted the questions of (1) their mycorrhizal associates after transplantation, and (2) their level of autotrophy. Using, respectively, metabarcoding methods to identify the fungal community in roots and isotopic and N abundance to characterize the autotrophy level, we evidence here autotrophic survival of the mixotrophic *E. helleborine*.

Material and methods

E. helleborine culture ex situ

The investigated plants were harvested with 2 L of undisturbed soil surrounding each plant, in 2013 or 2015 (Table 1). Their forests of origin were mixed but dominated by *Fagus sylvatica*, with a dense canopy as is typical for the ecology of this species in Central Europe (Těšitelová et al. 2012). The plants belonged to three different subspecies of *E. helleborine* (see Table 1 and Delforge 2016, for a review of these subspecies and their debated taxonomic status). After cutting two long roots, the plants were individually placed in square pots (18 × 18 cm, height 20 cm; Fig. 1) filled with the soil collected at the same time as the orchids. After potting, orchids were deposited at a propagation bed of the Botanical Garden of the University of Ulm on a 5-cm layer of sand in order to prevent waterlogging. Pots were moved weekly to limit the ability of local soil fungi to establish permanent links with the plants (indeed, some ectomycorrhizal trees grow at a distance > 5 m in the garden). They were watered daily and put below a large grid covered with an aluminum top in order to provide shadier conditions (distance to the top of highest orchids was 75 cm). Despite shading, plants grew in a luminous conditions based on Ellenberg's indicator value (7.0 ±

Table 1 Origin and sampling of the *E. helleborine* individuals from the Botanical Garden of the University of Ulm investigated in this study (sampling in 2016)

Transplantation to the Botanical Garden Ulm	Sub-species	Sampled individuals*	Forest of origin	Geocodes
June 2015	*E. helleborine* type	0/1	Ulm	48° 24′ 05″ N 09° 55′ 05″ E
July 2013	*E. helleborine minor*	5/1	Königsbronn	48° 44′ 10″ N 10° 05′ 31″ E
July 2013	*E. helleborine* type	5/1	Königsbronn	48° 44′ 10″ N 10° 05′ 31″ E
July 2013	*E. helleborine moratoria*	5/1	Ulm	48° 24′ 05″ N 09° 55′ 05″ E

*Number of individuals sampled for leaf isotopic abundances/number of individuals sacrificed for fungal barcoding of root fungi and mycorrhiza isotopic abundances

0.63; mean ± confidence interval) calculated on the basis of the plants spontaneously growing at the same place and in the same conditions (*Brassica napus*, *Sinapis arvensis*, *Echinacea purpurea*, *E. pallida*, *Heracleum sphondylium*, *Lythrum salicaria*, *Agrimonia eupatoria*, *Campanula trachelium*, *C. persicifolia*, *C. medium*, *Taraxacum officinale*, *Leontodon autumnalis*, *Malva sylvestris*, *M. moschata*, *Verbascum densiflorum*, *V. phlomoides*, *Geranium sanguineum*, *Plantago major*). Ellenberg's indicator value represents the preference of individual species, based on empirical field observations in Central Europe, ranging from 1 (deep shade) to 9 (full sunlight; Ellenberg et al. 1991).

Fig. 1 Pot cultures of *E. helleborine* individuals investigated in this study (*E. helleborine* sampled in 2015 near Ulm, pictured July 2017)

Survival rate after transplantation in the garden was ca. 60%, but surviving plants flowered in all years after sampling (vegetative and reproductive descriptions will be reported in a separate paper investigating the three different subspecies of *E. helleborine*).

Stable isotope analyses

We sampled about 1 g (fresh weight) of leaves from each *E. helleborine* individual in July 2016, 3 years after their transplantation to pot culture (Table 1). We also added for these analyses mycorrhizal roots of the four individuals sampled for fungal barcoding (see below and Table 1; two root pieces per individual). As a reference for autotrophic plants, we used weeds growing in the same pots, i.e., an unidentified *Arabidopsis* species, *Plantago major*, and *Taraxacum sp.* The two latter species form arbuscular mycorrhizas while the first one is not mycorrhizal but harbors various root endophytes as do other Brassicaceae (Almario et al. 2017). Six replicates from independent plants were sampled for each species. To minimize environmental variations influencing ^{13}C abundance, all leaf samples were collected at a similar light level and at a similar distance from the pot soil, i.e., under the grid covered with aluminum. Samples were ground in 2-mL Eppendorf tubes in a ball mill MM200 (Retsch Gmbh, Haan, Germany) and analyzed for total N concentration, as well as $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios using an elemental analyzer (EA) coupled to a ThermoFinnigan DeltaV Advantage Continuous-Flow Isotope-ratio mass spectrometer, and expressed as δ-values (Hynson et al. 2013). Isotope values were calibrated using internal calibrated standards (EDTA and ammonium oxalate). The standard deviations of the replicated standard samples were 0.024‰ for ^{13}C and 0.199‰ for ^{15}N. Statistical analyses were performed using R environment for statistical computing (R Core Team 2015). Analysis of variance (ANOVA) was used to evaluate differences in mean $\delta^{13}C$, $\delta^{15}N$, and % N among species from a given site

(function aov {stats}). The alpha type I error threshold was set at 0.05.

Identification of *E. helleborine* root fungi

For metabarcoding of root fungi by next-generation sequencing (NGS), roots of four plants (see Table 1; i.e., two plants from each site of origin) were harvested in 2016, i.e., 1 or 3 years after transplantation to pot culture (Table 1). This sampling was intended to limit damage in the common garden growth experiment. Roots were screened for mycorrhizal colonization, and 4 independent colonized root pieces were submitted to DNA extraction for the subsequent assessment of fungal communities as in Schneider-Maunoury et al. (2018, 2019). We amplified the ITS2 region by using the two general primer pairs ITS3/ITS4-OF and ITS86-F/ITS4, as such a choice of primers covers a large fungal set, including orchid mycorrhizal fungi (Waud et al. 2014; Jacquemyn et al. 2016), but not necessarily arbuscular mycorrhizal ones, known to be underrepresented in orchids. The four pooled amplicon libraries, generated in a PCR reaction (see Schneider-Maunoury et al. (2018) for detailed parameters), were sequenced with an Ion Torrent sequencer (Life Technologies, Carlsbad, USA). In brief, the downstream analyses included at first a R-OTU (Reference Operational Taxonomic Unit) database generation utilizing the full-length amplicons only, i.e., reads containing both the ITS86-F and ITS4 primers, in both types of amplicons (the primer pair ITS86-F/ITS4 resides within the stretch amplified by the ITS3/ITS4-OF pair), allowing a 25% error rate for primer recognition and a minimum required length of 200 bp. Trimmed sequences were clustered into R-OTUs with SWARM (Mahé et al. 2014), followed by removal of singletons, as well as chimera removal with UCHIME (Edgar et al. 2011) against the UNITE database (Kõljalg et al. 2013). Next, reads from the original amplicon sets were extracted and trimmed if they contained the ITS86F and ITS4 primers, and assigned to R-OTUs using BLASTN (Altschul et al. 1990) with a 97% similarity threshold. Assigning a taxonomy to each R-OTU was finally accomplished by comparing the representative sequences of each R-OTU to the UNITE reference database using BLASTN with a 90% similarity threshold. The above-described steps were carried out using selected scripts from the QIIME package v1.9.1 (Caporaso et al. 2010), as well as home-made scripts. Representative sequences for each mycorrhizal OTU found in this study were submitted to GenBank under accession numbers MN459665-MN459894. OTUs were manually screened for possible orchid mycorrhizal families based on Dearnaley et al. (2012) and information of previously detected mycorrhizal fungi from the roots, germinating seeds and protocorms of various *Epipactis* species (Bidartondo et al. 2004; Selosse et al. 2004; Ogura-Tsujita and Yukawa 2008; Těšitelová et al. 2012; Jacquemyn et al. 2016; Jacquemyn and Merckx 2019); we also included all potentially ectomycorrhizal fungi according to (Tedersoo et al. 2010; 2013). Analysis was restricted to these taxa. We tested the null hypothesis of no difference in the proportions of rhizoctonia and non-rhizoctonia fungi sequences and OTUs number among four groups represented by data from the present study, these from Těšitelová et al. (2012) and these from Jacquemyn et al. (2016 and 2019), i.e., whenever format of published data allowed respective comparisons: for this, we performed the chi-square test with Yate's correction followed by a pairwise proportional test with the Bonferroni correction. Proportions instead of raw sequences and OTU number data were applied in the calculation to normalize results between the studies.

Results

Stable isotope analyses

The leaf isotopic abundance in ^{13}C and ^{15}N of the three subspecies of *E. helleborine* (Fig. 2a) did not differ significantly from those of the reference autotrophic *Arabidopsis* sp., *Plantago major*, and *Taraxacum* sp. growing in the same pots and conditions, whatever the sub-species (see statistics in caption of Fig. 2a). Mycorrhizal roots displayed the same ^{13}C abundance as leaves (Fig. 2b). The average total N content of *E. helleborine* was lower than that of the reference autotrophic species, significantly for *Arabidopsis* sp. and *Plantago major* (Fig. 3). Thus, neither N content nor isotopic abundances indicated any contribution of N-rich, $^{13}C/^{15}N$-enriched biomass originating from ectomycorrhizal fungi in the aerial and root biomass of pot-cultivated *E. helleborine* individuals.

Identification of *E. helleborine* root fungi

The quality-filtered pyrosequencing data set comprised 584 OTUs represented by 613,118 sequences. After analysis, 88.9% of the total number of sequences (544,929 sequences, 454 OTUs) could be assigned to Ascomycota and Basidiomycota, and a relatively large representation of Glomeromycota (arbuscular mycorrhizal fungi; 11,646 sequences, 63 OTUs, 10.7% of all fungal OTUs) were recovered. Putatively orchid mycorrhizal according to Dearnaley et al. (2012) and/or ectomycorrhizal taxa covered 167 OTUs (402,272 sequences, 65.6%). Among these, we found the usual fungal associates of autotrophic orchids, the so-called rhizoctonias: from the three rhizoctonia families, Ceratobasidiaceae were ubiquitous and highly abundant (198,383 sequences, 49.3% of all sequences in this category; in all, 49 OTUs; Fig. 4); Serendipitaceae (1 OTU) occurred in 3 plants (but reached high abundance in only one of these); no Tulasnellaceae was found. Several ectomycorrhizal clades potentially mycorrhizal on

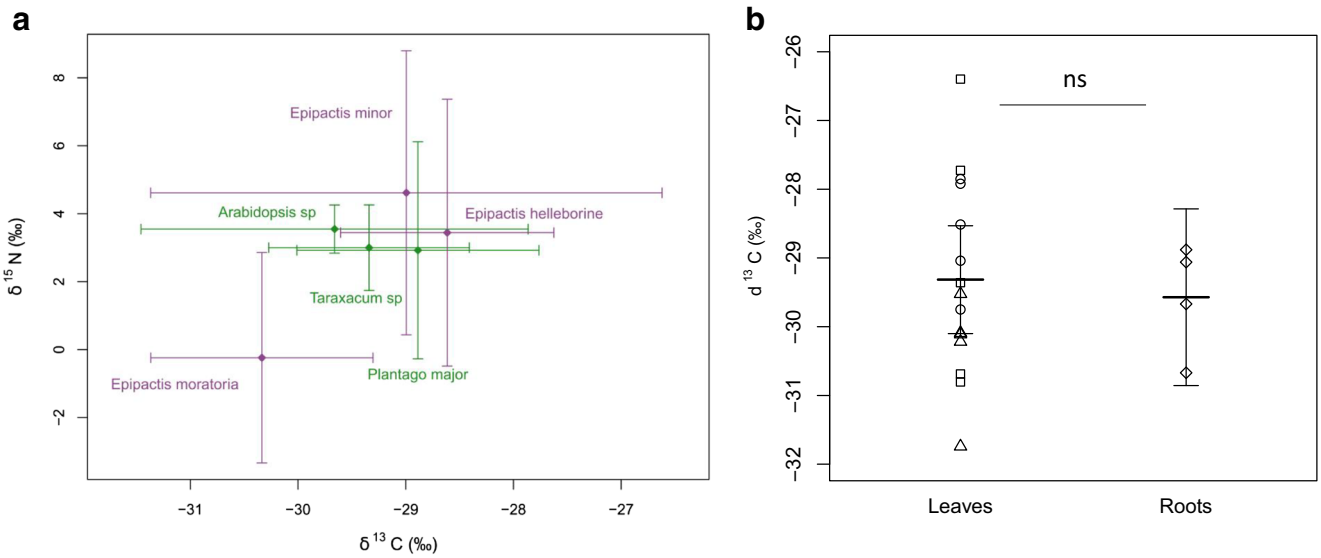

Fig. 2 Isotopic abundance of *E. helleborine* individuals (violet; $n = 5$ replicates for leaves and $n = 4$ for roots) and surrounding autotrophic weeds (green; $n = 6$ replicates). **a** $\delta^{13}C$ and $\delta^{15}N$ values of leaves (distinguishing the 3 sub-species). **b** $\delta^{13}C$ of roots versus leaves of *E. helleborine* (all sub-species pooled). Bars represent the 95% confidence intervals of the mean for each species. No significant difference was found between species for $\delta^{13}C$ nor $\delta^{15}N$ (ANOVA; $F = 1.50$ and $p = 0.232$ for ^{13}C; $F = 2.26$ and $p = 0.086$ for ^{15}N), or between *E. helleborine* organs (to a Tukey honest significant difference test; $p = 0.65$)

E. helleborine were recorded. Helotiales (76,164 sequences, 18.9%) and Herpotrichellaceae (33,708 sequences, 8.4%) were ubiquitous, although with different abundances from one plant to another, while other taxa occurred on some plants only (Fig. 4a), namely Tuberaceae (*Tuber anniae*, 14,380 sequences, 3.6%, from 2 plants), Pyronemataceae (67,960 sequences, 16.9%, from 4 plants), Sebacinaceae, *Inocybe* and *Cortinarius* (for these three taxa: 64 sequences, 0.01%, from 3 plants; Fig. 4). The individuals transplanted to pot more recently (1 year of cultivation; column 1 in Fig. 4a) revealed more abundant ectomycorrhizal fungi than the ones cultivated for 3 years (columns 2–4; respectively 70.65% of all sequences versus 48.18% on average): *Pyronemataceae* dominated in its fungal community (12 OTUs and 24,681 sequences).

On average, the proportion of rhizoctonias found in this study was significantly higher than that of other available studies (Table 2) calculated as sequence proportions ($\chi^2(0.05,3) = 161.07$; $p < 0.0001$) or as OTU proportions ($\chi^2(0.05,2) = 39.37$ $p < 0.001$).

Discussion

We observed 1- to 3-year survival in pots for *E. helleborine*, with normal development and flowering (Fig. 1; developmental traits will be reported later in a comparative study of *E. helleborine* subspecies) that correlates with (i) an unusually high abundance of rhizoctonias and (ii) isotopic and N signatures that do not differ from nearby autotrophic plants.

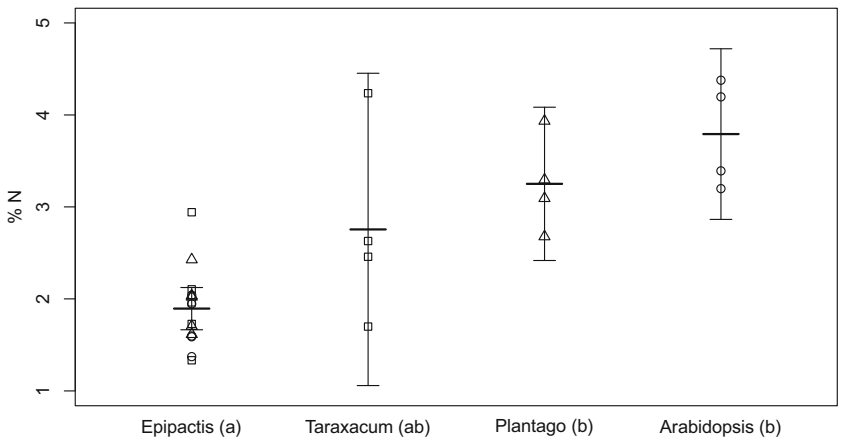

Fig. 3 Total N content of leaves of *E. helleborine* individuals ($n = 5$ replicates) and surrounding autotrophic weeds ($n = 6$). Different letters in brackets after the plant names indicate different content according to a Tukey honest significant difference test

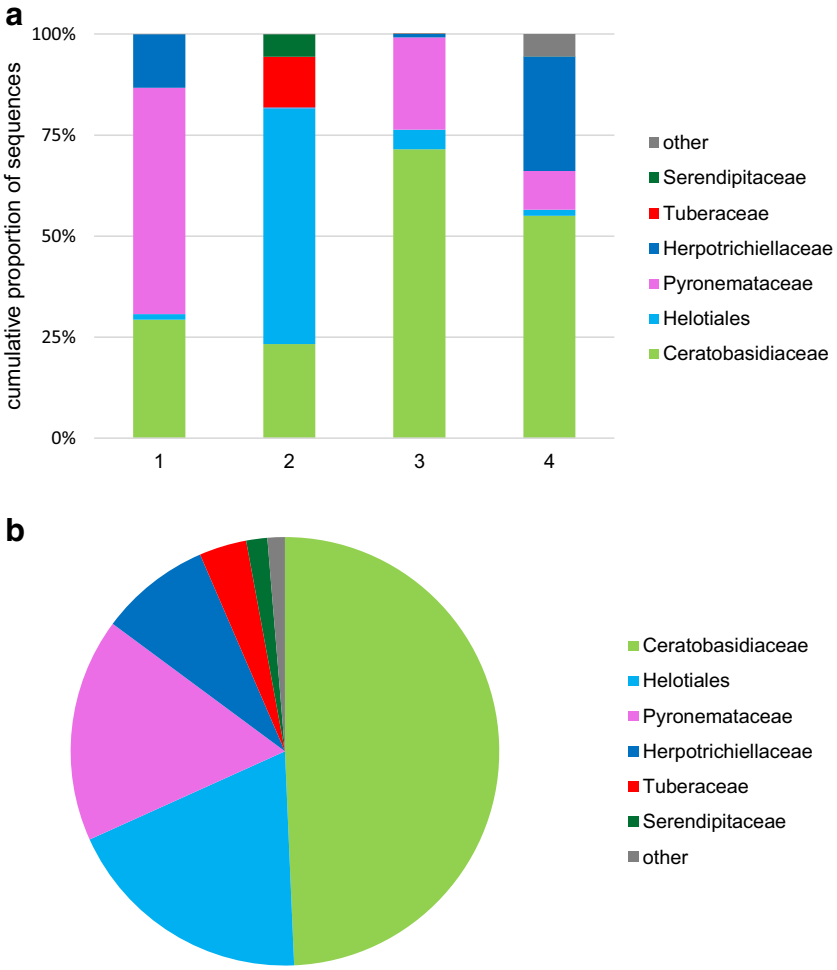

Fig. 4 Putative mycorrhizal fungal families (i.e., orchid mycorrhizal or ectomycorrhizal) detected in *E. helleborine* individuals from pot culture. Relative abundance of each mycorrhizal fungal family (i.e., orchid mycorrhizal or ectomycorrhizal) is calculated as the proportion of DNA sequences. **a** Communities for the four individuals (Table 1), namely: 1, *E. helleborine* (type) from Ulm after 1 year in pots; 2, *E. helleborine minor* from Königsbronn; 3, *E. helleborine* (type) from Königsbronn; *E. helleborine moratoria* from Ulm; the latter three were kept for 3 years in pot culture. **b** Total community found in the four barcoded *E. helleborine* individuals. The category "other" includes Sebacinaceae and unidentified Sebacinales, *Inocybe* and *Cortinarius*

Although this short-term experiment may reflect survival rather than a sustainable niche (i.e., we do not assess average life expectancy), we discuss these observations in terms of mycorrhizal interaction and physiology of *E. helleborine*, in the general framework of evolution and biological conservation of mixotrophic plants.

Mycorrhizal fungi are dominated by rhizoctonias

Pot-cultivated *E. helleborine* revealed a community of root mycorrhizal fungi (including some endophytes sensu Wilson 1995, i.e., fungi colonizing the roots loosely without forming true mycorrhizas) qualitatively (= taxonomically) close to that

Table 2 Percentage of rhizoctonias versus non-rhizoctonia ectomycorrhizal taxa including species whose exact status, endophytic or ectomycorrhizal, is debated, such as Helotiales or *Herpotrichiellaceae*. The value is calculated as percentage of reads (in italics) or as percentage of OTUs (bold, in brackets), which was not calculable for the work by Jacquemyn et al. (2016)

Study:	Current study	Xing et al., 2019	Jacquemyn et al. 2016	Těšitelová et al. 2012
Growth conditions:	Pot culture	*In natura*	*In natura*	*In natura*
Barcoding method:	NGS	NGS	NGS	Sanger (cloning)
Rhizoctonia taxa	*50.87%* **[29.9%]**	*3.30%* **[10.6%]**	*0.03%*	*0%* **[0%]**
Non-rhizoctonia ecto-mycorrhizal taxa	*49.13%* **[49.1%]**	*96.70%* **[89.4]**	*99.97%*	*100%* **[100%]**
Statistics*	*a* **[a]**	*b* **[b]**	*b*	*b* **[b]**

*Column with different letters differ significantly according to the chi-square test with Yate's correction (χ^2 *(0.05,3) = 161.07, p < 0.0001* for percentage of reads, χ^2 **(0.05,3) = 39.37, p < 0.001** for percentage of OTUs), followed by pairwise proportional test with the Bonferroni correction

reported from natural sites, but with striking quantitative differences. The finding of *Glomeromycota*, which normally form arbuscular mycorrhizae and are poorly targeted by the primers we used, was unexpected (but see e.g. Abadie et al. 2006): although their interaction with orchid roots is unexpected and unclear, we cannot exclude an asymptomatic colonization resulting from culture in the company of some arbuscular mycorrhizal weeds such as *Taraxacum* and *Plantago* spp. (a non-mycorrhizal colonization described in non-arbuscular mycorrhizal; Cosme et al. 2018).

On the one hand, the main taxa found here were also reported in situ for *E. helleborine* (see Bidartondo et al. 2004; Ogura-Tsujita and Yukawa 2008; Těšitelová et al. 2012; Jacquemyn et al. 2016; 2019). This includes rhizoctonias, which occur in many autotrophic orchids (Dearnaley et al. 2012): *Ceratobasidiaceae* that abound here are also reported *in natura* in *E. helleborine*. Ectomycorrhizal ascomycetes with possible endophytic abilities are also reported *in natura* in *E. helleborine*: taxa with this dual ecology include *Tuber* spp. (Schneider-Maunoury et al. 2018, 2019), *Pyronemataceae* (Hansen et al. 2013), Helotiales (Wang et al. 2006), and *Herpotrichellaceae* (such as *Exophiala*, which include the so-called dark septate endophytes; Jumpponen 2001). Ectomycorrhizal basidiomycota, finally, are usually much more diverse *in natura* than the few taxa found in this study (e.g., Těšitelová et al. 2012; Jacquemyn et al. 2016; 2019).

On the other hand, not only the diversity but also the abundance of ectomycorrhizal and/or endophytic taxa (asco- or basidiomycetes) is very low in pot-cultivated *E. helleborine*. A comparison with samplings *in natura*, barcoded by NGS or cloning, clearly supports this (Table 2). Of course, differences in methods and choice of primers for NGS may affect this comparison: however, our unpublished data set on *E. helleborine* from four forests sites in Europe (M.-A. Selosse, E. Delannoy & J. Minasiewicz, unpubl; 15 individuals barcoded with exactly the same methods of analysis as in this study) revealed 99.1% of ectomycorrhizal/endophytic fungal sequences (difference with the current data significant, χ^2 (0.05,1) = 64.9, $p < 0.05$), representing 93.27% of the relative number of OTUs (χ^2 (0.05,1) = 16.4, $p < 0.001$). Thus, the abundance of rhizoctonias, especially *Ceratobasidiaceae*, is unusually high in pot cultures (Table 2). Unfortunately, the composition of the root mycorrhizal community of the *E. helleborine* populations of origin (Table 1) at the time of sampling, which may predispose to this composition, remains unknown.

The finding of few potentially ectomycorrhizal taxa is unexpected in these pots where C-providing plant hosts are lacking. Indeed, mixotrophic orchids are unlikely to give them carbon, and instead even exploit them (see discussion below). We do not believe that contamination explains our data, but we consider three non-exclusive possibilities. Firstly, ectomycorrhizal taxa may be surviving here, perhaps declining over time: indeed, they are more numerous in the plants transplanted 1 year before (especially *Pyronemataceae*), but a firm conclusion cannot be drawn from this single plant. Secondly, ectomycorrhizal taxa may colonize the pot from the soil, since some ectomycorrhizal trees exist at some distance in the surroundings: however, we did not see any direct contact of pots with the soil, which was limited by (i) a layer of sand and (ii) weekly moving of the pots. Yet, we cannot exclude colonization by transient contacts reaching the orchid roots. Thirdly, there is increasing evidence that several ectomycorrhizal fungi, beyond the ascomycetous taxa mentioned above, also have endophytic abilities, i.e., colonize the roots of non-ectomycorrhizal plants in a loose way (for indirect evidence and a review on this, see Schneider-Maunoury et al. 2018, 2019; Selosse et al. 2018). This especially applies to the genus *Tuber* (Gryndler et al. 2014; Schneider-Maunoury et al. 2018, 2019), although this is not demonstrated for *Tuber anniae*, the North American species recently found to occur also in Europe (Wang et al. 2013) that was detected here. This is also demonstrated for *Sebacinaceae* (Selosse et al. 2009; Weiß et al. 2016), and remains pending for other ectomycorrhizal basidiomycetes (including *Inocybe*: Schneider-Maunoury et al. 2018). In this explanation, ectomycorrhizal mycelia may survive in pots by colonizing endophytically the roots of co-occurring weeds (Figs. 1 and 3) and/or *E. helleborine*.

Isotopic and N signatures of autotrophy

Whatever the reason for their presence, these root fungi did not provide detectable contribution to the biomass of the orchid since isotopic ^{13}C and ^{15}N abundances as well as total N content were similar to those of surrounding autotrophic references. A very small flow, which would not entail significant deviations in isotopic abundances, may of course occur, but the N content, lower than that of autotrophic references, argues against this. This lack of apparent mycoheterotrophy is in good agreement with the paucity of ectomycorrhizal fungi in roots and the lack of nutritional resources for the few detected, because links to surrounding ectomycorrhizal hosts are regularly disturbed.

Mixotrophy in *Neottiae* (the orchid tribe encompassing *Epipactis*) may thus display some plasticity. The ratio of aerial biomass acquired by photosynthesis and mycoheterotrophy is reported to vary with the light level in mixotrophs: increasing light positively correlates with higher contribution of photosynthesis and, thus, a lower ^{13}C content (Preiss et al. 2010; Gonneau et al. 2014). Indeed, the site of pot cultivation is rather sunny, as shown by a relatively high Ellenberg's indicator value for light (value of 7 for a maximum of 9), and there is no competition for diffuse light with similarly high plants. A similar situation is sometimes reported for *E. helleborine* in

full light, in open fields quite far away from nearest ectomycorrhizal trees (e.g., Rydlo 2008). We may have here an extreme of the continuum from mycohetero- to autotrophy, leading to undetectable mycoheterotrophy because of light level and lack of fungal resources. The possibility of autotrophic survival in *E. helleborine* is also congruent with the recent report that its plastid genome retains a full set of photosynthetic genes without any evidence of selective relaxation (Lallemand et al. 2019b) and has intact photosynthetic abilities. Moreover, the phylogenetically related mixotrophic *Limodorum abortivum* displayed some photosynthetic compensation (higher chlorophyll content and possibly higher photosynthetic activity) after experimental eradication of its fungal partners in situ (Bellino et al. 2014). Here, the lack of fungal resources in pots may have entailed a similar compensation.

In this framework, the observation of similar ^{13}C abundance in mycorrhizas and leaves is very relevant. As exemplified in the related mixotrophic *Cephalanthera damasonium* (Lallemand et al. 2019a), ^{13}C-enrichment of mycorrhizal fungi and derived plant starch normally induce higher ^{13}C abundance in mycorrhizas than in leaves. In pot-cultivated *E. helleborine*, the two organs displayed similar abundances, which can be explained if (i) the underground carbon is photosynthetic in origin and (ii) there is little biomass of ectomycorrhizal fungi, either as hyphae or delivered to the root cells. Alternatively, but less likely, we cannot exclude that root fungi provide resources from a different nutrition, e.g., saprotrophic or endophytic, which would not entail isotopic differences as compared with photosynthetic resources (see below). Finally, this autotrophic signature for underground parts is somewhat unexpected in the current model of nutrition in mixotrophic *Neottieae*, where photosynthates are mostly used for aboveground parts (Bellino et al. 2014; Gonneau et al. 2014) and migrate poorly underground (Lallemand et al. 2019a). Yet, a small flow to underground parts exists in labeling experiments (Lallemand et al. 2019a) and we speculate that, in pots, the absence (or limited presence) of fungi entails a stronger sink that directs more plant C to roots than when nutrients flow from the mycorrhizal network. This may also mean that autotrophic life in *E. helleborine* requires quite high light levels, as in this study.

Autotrophic survival in *E. helleborine* in evolution of mixotrophy

Pot-cultivated *E. helleborine* displays three features that contrast with those of mixotrophy (as described in Section 1): (i) rhizoctonias, not ectomycorrhizal fungi, dominate in their roots and since an endophytic, non-ectomycorrhizal niche can be proposed for the few existing ones, there is likely no mycorrhizal network with surrounding plants; (ii) neither ^{13}C nor ^{15}N abundances offer significant evidence for gain from the few available ectomycorrhizal fungi; (iii) their N content is not higher than that of surrounding autotrophs. This supports autotrophy in these specific conditions, for a species otherwise reported as mixotrophic in its natural environments.

There is currently an open question on autotrophy in rhizoctonia-associated orchids: their slight isotopic difference with surrounding autotrophs (Selosse and Martos 2014) as well as their ^{2}H abundance (Gebauer et al. 2016; Schiebold et al. 2018) suggests that they recover some fungal biomass. Yet, the net flow, i.e., when also considering the potential reverse flow from orchid to fungus, is unknown. One ex situ experiment involving *Ceratobasidiaceae* reveals a net flow in favor of the fungus (Cameron et al. 2008), and no achlorophyllous variants of rhizoctonia-associated orchid survive in nature. Thus, the question of the net contribution of rhizoctonias remains open (see discussion in Lallemand et al. 2017) and, to facilitate reading, we provisionally consider below rhizoctonia-associated orchids as "autotrophic." Pot-cultivated *E. helleborine* displays all the features of such orchids; even the presence of a few ectomycorrhizal fungi in the roots is reported from rhizoctonia-associated orchids (e.g., Jacquemyn et al. 2017). Our observations have consequences for plant protection and for the evolution of mixotrophy.

In terms of plant protection, ex situ conservation and transplantation is thus possible. The successful *E. helleborine* transplantation by Delforge, (2016, 2017), which this author explains by a reconnection to the mycorrhizal network, may have been helped by autotrophic survival, at least transiently. We show here that absence of mycorrhizal network may even not impair survival, at least transiently. The failed transplantations by Sadovsky (1965) may be due to two factors. Firstly, not all plants, and even not all populations, may have a physiological status allowing transplantation, as shown by the 60% survival in our study (see Section 2): more on the physiology and autotrophy level of the population of origin could help, but this was overlooked in this study, which was initially designed as a taxonomic study. Secondly, not all receiving sites may be suitable, e.g., in terms of light (see above): sunnier sites may be targeted to enhance photosynthesis, although a negative trade-off is possible with desiccation in the absence of an artificial cover, as in our garden. We are far from predicting the factors allowing transplantation of mixotrophs, and more data on transplantation of various mixotrophic species (which often stay in the "gray literature") are required to help save threatened populations.

In terms of evolution, one should remember that similar mycorrhizal, nitrogen, and isotopic features occur in closely related *Epipactis* species, such as *E. palustris* (Lallemand et al. 2018) and *E. gigantea* (Schiebold et al. 2017), which associate with *Ceratobasidiaceae* (Bidartondo et al. 2004; Jacquemyn et al. 2016, 2017). These *Epipactis* species belong to rhizoctonia-associated, putatively autotrophic orchids, and thus, an autotrophy-to-mixotrophy transition (or vice-versa)

occurred in the evolution of the genus *Epipactis*. In the framework of *Neottieae* evolution as a whole, it is still unclear in which direction this transition occurred, and a reversion from mixo- to autotrophy is also possible (Lallemand et al. 2019b, c). Autotrophic survival in *E. helleborine*, combining the intact plastid genomes of mixotrophic orchids (retaining all photosynthetic genes; (Feng et al. 2016; Lallemand et al. 2019b), makes a reversion possible, even if the physiology of mixotrophs is deeply rooted in their dependence on two carbon sources, as mentioned above (Gonneau et al. 2014; Lallemand et al. 2019a).

Our findings add to the reported plasticity of mixotrophs: they are known to survive the disappearance of chlorophyll (achlorophyllous variants; e.g., Julou et al. 2005; Gonneau et al. 2014) and to adapt to light level (Preiss et al. 2010; Gonneau et al. 2014); they now turn out to display nearly autotrophic survival in some environments. Such nutritional plasticity is a major attribute for successional species, such as *Epipactis* species, which colonize early-stage forests with variable access to light and ectomycorrhizal networks. The mycorrhizal interaction with rhizoctonias in autotrophic orchids versus ectomycorrhizal fungi in mixotrophs recently turned out to be a continuum rather than an alternative (Jacquemyn et al. 2017, and references therein); autotrophic versus mixotrophic nutrition also turns out to be a continuum, even within a given species. A quite similar statement was made in the mixotrophic *Pyrola japonica* under different light environments (Matsuda et al. 2012), in the *Ericaceae* family where mixotrophs are considered transplantable (Figura et al. 2019). Thus, autotrophic survival of mixotrophs may be sought in various phylogenetic backgrounds, even beyond orchids.

Conclusion

Some mixotrophic *E. helleborine* can be cultivated in pots, where they behave as autotrophs. Many other mixotrophic orchids may have a high trophic plasticity that was overlooked till now. They display reduced mycorrhizal colonization by the fungi that usually link them to surrounding trees, and from which they indirectly extract part of their carbon resources in forest (mixotrophy). Instead, they associate with rhizoctonia taxa that normally colonize autotrophy orchids. This further suggests that mixotrophy is ecologically, if not evolutionarily, plastic.

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