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Letters

Mixotrophic orchids do not use photosynthates for perennial underground organs

Most plants are autotrophic and interact with soil fungi, forming mycorrhizal symbioses (van der Heijden *et al.*, 2015) where plants gain mineral nutrients and provide photosynthates to fungi. Yet, plants repeatedly evolve heterotrophy (Těšitel *et al.*, 2018), and several lineages, especially in orchids, import carbon from their mycorrhizal fungi, a strategy called mycoheterotrophy (Merckx, 2013). Green plants that are photosynthetic but also import carbon from their mycorrhizal fungi have raised considerable interest over the past two decades (Julou *et al.*, 2005; Selosse & Roy, 2009). These plants with two carbon sources are termed mixotrophic, and pave the evolutionary way to full mycoheterotrophy (Selosse & Roy, 2009). Mixotrophy enables them to adapt to shaded conditions (Julou *et al.*, 2005; Preiss *et al.*, 2010; with some exceptions: Girlanda *et al.*, 2011; Schiebold *et al.*, 2017) and sometimes drives a reduction of their photosynthetic abilities (Girlanda *et al.*, 2006). Their study is therefore of crucial interest to an understanding of the evolution to full mycoheterotrophy.

Some temperate forest orchids, for example from the genera *Epipactis* and *Cephalanthera*, are remarkable models of mixotrophy. Mixotrophy may be a common feature of all orchids (see discussions in Selosse & Martos, 2014; Stöckel *et al.*, 2014), but these forest orchids specifically display two strong lines of evidence for mixotrophy. First, their roots associate with ectomycorrhizal fungi that themselves associate with the surrounding trees, which are the ultimate carbon source of the whole consortium. Since the carbon provided by such fungi is enriched in ^{13}C compared to that fixed by photosynthesis (Hynson *et al.*, 2013), the ^{13}C enrichment in the orchid biomass allows us to estimate the proportion of carbon issued from mycorrhizal fungi. In aerial parts, the contribution of fungal carbon ranges from a few percent to > 90%, depending on species (Hynson *et al.*, 2013, 2016), light level (Preiss *et al.*, 2010; Gonneau *et al.*, 2014) and also the period of the growing season, over which photosynthates increasingly contribute to aerial biomass (Roy *et al.*, 2013). A second feature supporting the importance of fungal carbon exploitation is the survival in some species of achlorophyllous variants called 'albinos' (Salmia, 1989; Selosse *et al.*, 2004; Suetsugu *et al.*, 2017), which behave as full mycoheterotrophs and, accordingly, show a high ^{13}C enrichment in their biomass (e.g. Julou *et al.*, 2005).

A model for mixotrophic nutrition was proposed for these perennial orchids (Gonneau *et al.*, 2014; see their Fig. 5) based on isotopic enrichments. The contribution of mycoheterotrophy and photosynthesis to the biomass of a given organ can be calculated thanks to ^{13}C enrichment, using surrounding autotrophs and albinos as references, respectively, for the enrichment of fully autotrophic and fungal-derived biomasses. This approach has shown that photosynthates are the main carbon source of shoots and fruits late in the growing season (Roy *et al.*, 2013; Gonneau *et al.*, 2014). Accordingly, albinos, which lack photosynthesis, undergo physiological problems late in the season, which in particular prevents successful fruiting and seed ripening probably due to carbon limitation (Roy *et al.*, 2013). Moreover, the application of fungicide to soils eliminates mycorrhizal fungi without impairing fruit maturation (Bellino *et al.*, 2014). Less is known about the underground parts of the plants. When they start sprouting, the young green shoots display a strong contribution (*c.* 80%) of fungal carbon. Moreover, rhizomes of albino and green *Cephalanthera damasonium* revealed similar ^{13}C enrichment, which did not differ from the aerial parts of albinos, pointing to a mycoheterotrophic nutrition for underground organs (Gonneau *et al.*, 2014; see their Supporting Information Table S1).

This carbon allocation model for mixotrophic *Epipactis* and *Cephalanthera* thus states that aerial parts and fruits rely on photosynthesis, while underground parts mostly rely on fungal carbon. Suetsugu *et al.* (2018) recently described a similar trend in an unrelated mixtrophic orchid, *Cymbidium macrorhizon*. However, this remains poorly supported by belowground data, and the model is mostly based on natural isotopic enrichments, which represent indirect evidence (Lallemand *et al.*, 2017). In this paper, we report two attempts to test this model in *C. damasonium*. First, we extracted root starch from green and albino individuals. Roots store most starch during winter (Rasmussen, 1995): the above-mentioned model predicts that ^{13}C enrichments should be identical if fungi indeed support root nutrition and reserves (Prediction 1). Second, we monitored photosynthetic carbon allocation by the *in situ* ^{13}C-labelling of photosynthates: following this model, the labelling should be abundantly directed to aboveground parts, but in lower amounts to belowground ones (Prediction 2). In this second experiment, the default expectation for autotrophic plants would be a substantial migration belowground of photosynthates to contribute to reserves (e.g. Harris & Jeffcoat, 1972; Major *et al.*, 1978; Kandiah, 1979).

Similar starch origin in photosynthetic individuals and nonphotosynthetic albinos

Prediction 1 was tested on *C. damasonium* roots, by analyzing winter starch from three green individuals and three albinos at Boigneville (France). In this population, studied by Julou *et al.* (2005) and Roy *et al.* (2013; see location in this reference), the two phenotypes are mycorrhizal with ectomycorrhizal Thelephoraceae and Cortinariaceae (Julou *et al.*, 2005). Different roots were chosen as intra-individual replicates to account for between-root

variabilities. Underground sampling was carried out on 15 January 2015, that is, during the winter resting stage, together with the roots of four autotrophic species for comparison: *Dioscorea communis*, *Hedera helix*, *Neottia ovata* (an autotrophic orchid; Těšitelová *et al.*, 2015), *Rubia tinctorum* (four individuals each). Leaves from five individuals of each phenotype were also collected in the previous growing season for reference. Levels of mycorrhizal colonization in *C. damasonium* (expressed as mean percentage of root section colonized by fungi) did not differ between green individuals and albinos (Supporting Information Fig. S1a; Kruskal–Wallis test: $\chi^2_{(1)} = 2.25$; $P = 0.13$), whose root sections were colonized at 41% and 50% on average, respectively.

Root starch was extracted as explained in Damesin & Lelarge (2003) from the roots of *C. damasonium* and the autotrophic controls. In brief, 50 mg of tissue powder was suspended in 1 ml of distilled water. After centrifugation, starch was extracted from the pellet by HCl solubilisation. The starch powder obtained after desiccation of the precipitate was used for weighing and carbon isotope analysis. Although the roots were mycorrhizal, the absence of starch in fungi allows us to access the orchid's reserve exclusively. No significant difference was observed for starch content in green compared to albino roots (4.4 ± 0.6 vs 5.4 ± 1.0%; mean percent of root dry weight ± 95% confidence interval; $P = 0.07$, Student's *t*-test).

Isotopic enrichments were estimated as was done in Lallemand *et al.* (2017). Considering the leaves collected in the previous growth season (10 July 2014), albino leaves were enriched in ^{13}C as compared to green ones (Fig. 1), as was expected due to the use of fungal resources. For the underground material, bulk root material did not differ in ^{13}C enrichment between albino and green *C. damasonium* (Fig. 1). It was, however, much higher than in roots from autotrophic controls (−23.8 ± 0.3 vs −32 ± 0.6‰; mean $\delta^{13}C \pm SD$; Fig. 1), as was expected from both the presence of fungal hyphae and the use of fungal resources. Starch extracts were richer in ^{13}C than the corresponding bulk root material, but again the material did not differ in ^{13}C enrichment between albino and green *C. damasonium* (Fig. 1). In comparison, in autotrophic controls, starch displayed similar or higher ^{13}C enrichment as compared to bulk root materials (Fig. 1), confirming previous results (Bathellier *et al.*, 2008; Göttlicher *et al.*, 2006). Moreover, starch in the autotrophs showed a significantly lower ^{13}C enrichment than that of albino and green *C. damasonium*, congruently with the use of a carbon source derived from photosynthesis in the roots of autotrophs. The presence of photosynthesis did not change the high ^{13}C enrichment in *C. damasonium*, suggesting that the production of ^{13}C-depleted photosynthates did not detectably contribute to starch production.

The fact that neither the quantity nor the ^{13}C enrichment of starch depended on the photosynthetic phenotype thus verifies Prediction 1 in *C. damasonium*, that is, that carbon reserves in green individuals are built on the same fungal source as in albinos.

Photosynthates contribute massively to aerial parts, but much less to underground organs

Prediction 2, that leaf photosynthates migrate poorly underground was tested in June, during a period of starch accumulation in *C. damasonium* roots (Rasmussen, 1995): the percentage of root cortical cells containing starch shifts from < 40% in late May to > 50% in mid-July (M. Roy, C. Gonneau & M-A. Selosse, unpublished data from Boigneville). We pulse-labelled green *C. damasonium* individuals as described in Le Tacon *et al.* (2013),

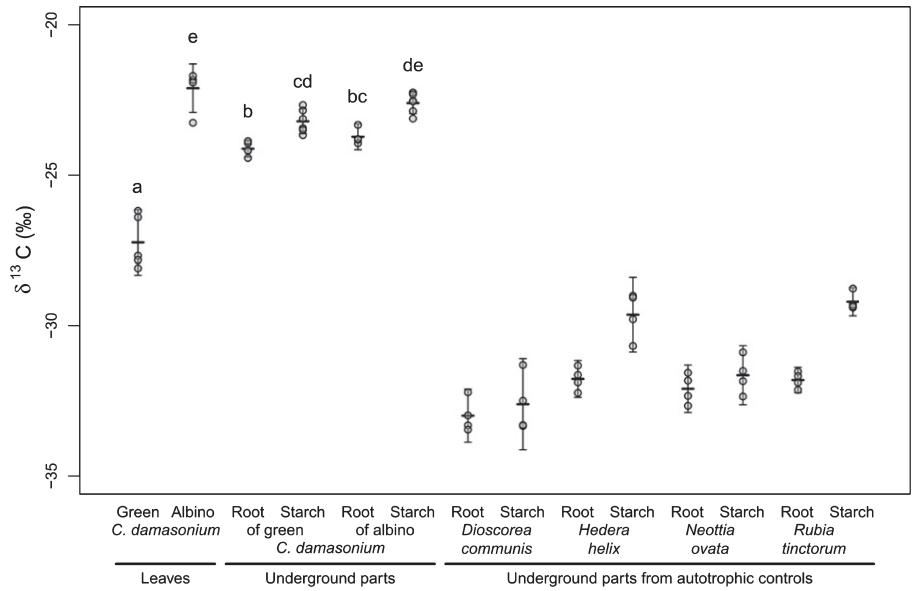

Fig. 1 ^{13}C enrichments in leaves, roots (bulk material) and root starch extracts of green and albino *Cephalanthera damasonium* ($n = 5$ individuals for leaves, $n = 3$ individuals for roots and starch) and autotrophic controls (*Dioscorea communis*, *Hedera helix*, *Neottia ovata* and *Rubia tinctorum*; $n = 4$ each) collected at Boigneville. Leaves were sampled on 10 July 2014, and underground parts on 15 January 2015. For underground parts, analyses were replicated twice, except for bulk roots in one green and two albino individuals due to insufficient material, so the number of replicates is five and four, respectively. Mean $\delta^{13}C \pm 95\%$ confidence intervals; individual values are shown with small transparent dots. Different letters indicate $P < 0.05$ according to pairwise comparisons of means from Tukey's HSD test; material from autotrophic controls showed obvious differences with other samples and were therefore not included in the test.

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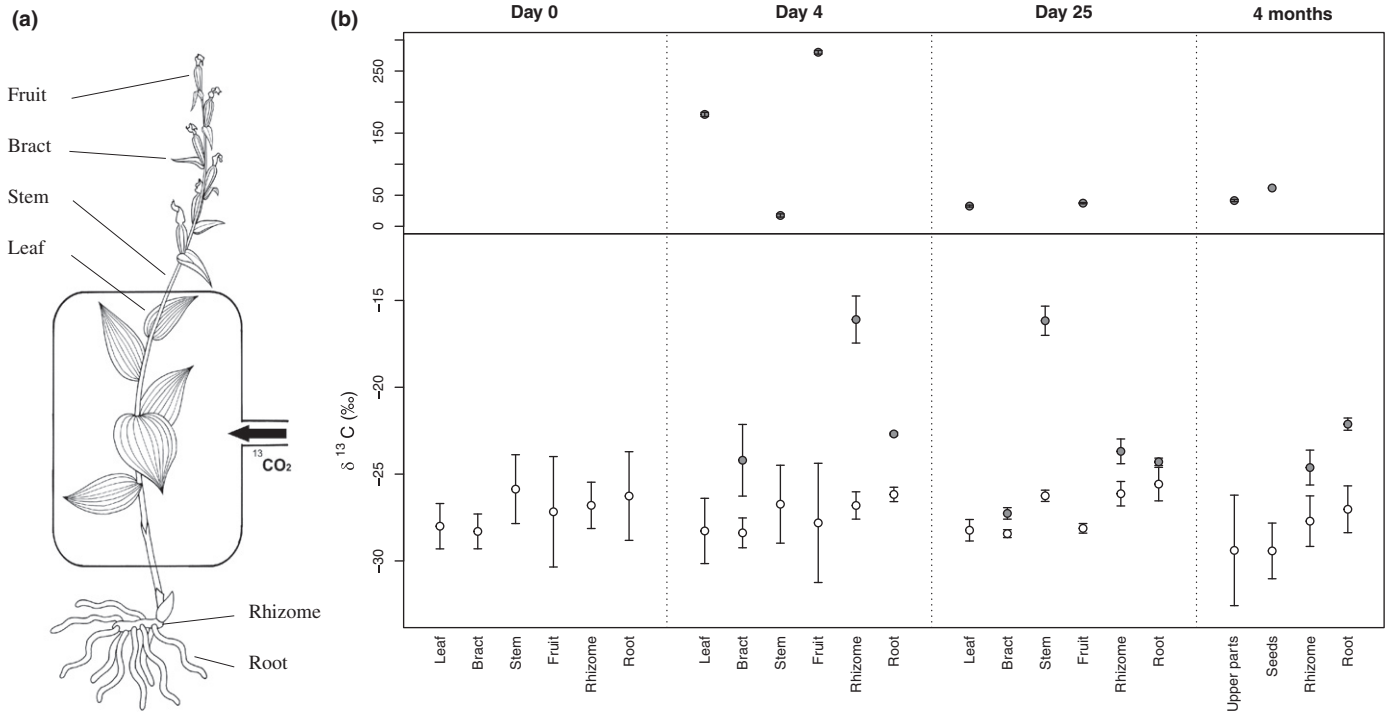

Fig. 2 Labelling experiment carried out in a population of green *Cephalanthera damasonium* at Le Vésinet. (a) Labelling experiment: only one shoot section and leaves (in the bag; see also Supporting Information Fig. S2b) were labelled (drawing courtesy of Alain Jouy). (b) ^{13}C enrichments in different organs and at various times after labelling of nonlabelled controls (open circles, mean of three plants) or labelled plants (closed circles, mean of three measures for one labelled plant). Day 0 is 8 June 2017. At day 126, all aerial parts were fully dry and sometimes lost, and remnants pooled together after separating seeds. For each organ at each sampling time, labelled and control values always significantly differed according to a Student's *t*-test at $P < 0.05$. Bars indicate 95% confidence intervals.

on 8 June 2017 (early fruiting stage), in a population at Le Vésinet (France; 48°54′45″N, 2°06′27″E; of the same phenology as Boigneville; Fig. S2a). Fifteen early fruiting individuals of similar shoot size were selected, with a minimum separation of 1.5 m. Each plant was partly enclosed (part of the stem and all leaves, but not the fruits and bracts; Fig. 2a) in a chamber made from gas-tight polyethylene film (200 μm thick). The bottom and top of the stem were sealed with Terostat (a solvent-free, elastic silicone sealant; Henkel France SAS, Boulogne-Billancourt, France) in order to make a tight connection between the stem and the chamber (Fig. S2b). Pure $^{13}CO_2$ gas was then injected into each chamber for 1 min at a rate of 0.01 l min^{-1} (0.01 l total) as was done in Le Tacon *et al.* (2013). Each plant was labelled for 6 h (from 11:00 to 16:00 h), after which each labelling chamber was carefully opened and removed.

We harvested three control plants just before labelling, and initially planned to harvest three labelled and three nonlabelled plants at three dates after labelling, namely at 4, 25 and 126 d. Unfortunately, heavy grazing after the labelling only allowed us to harvest a single intact labelled plant per date. Moreover, at day 126, the senescent stems had dry and destroyed leaves (Fig. S2c). Total dry weights for all organs were recorded, and ^{13}C enrichments were estimated as in Lallemand *et al.* (2017). Considering all plants sampled at days 0 and 4 ($n = 7$), above and belowground biomasses were roughly equal (11.7 (6.4–23.2) vs 9.9 (5.7–19.8) g; mean dry weight (range)). At day 126 however, the aerial parts had experienced considerable senescence and material loss, with 77% mass loss (compared to only 29% for roots; see note in Table 1).

Throughout all samplings, the ^{13}C enrichment in all the organs of control plants remained in a range expected from for *C. damasonium* (e.g. Julou *et al.*, 2005; Roy *et al.*, 2013; Fig. 2b), that is, higher than for C_3 reference plants (as measured at day 0; Fig. S3). Organs from labelled plants were always enriched in ^{13}C compared to control ones ($P < 0.05$; Student's *t*-tests). At days 4 and 25, leaves (labelled), stems and fruits (immediately above the labelled zone; Fig. 2a) displayed a very strong increase in ^{13}C enrichment ($\delta^{13}C > -20‰$). Bracts were mildly labelled, as expected for source organs. The rhizomes and roots of labelled plants displayed limited ^{13}C enrichment (Fig. 2b), with the exception of the rhizome of plant from day 4 (note that the plant on that day had the highest systemic labelling level). At day 126, dried upper parts and seeds were still labelled, whereas rhizomes and roots displayed only limited ^{13}C enrichment.

Based on the weight of the various plant parts, the distribution of labelled photosynthates to underground organs ranged from 1.7% at day 4 to 7.6% at day 126 (Table 1). In all, less than one-tenth of photosynthates produced during the 6-h labelling, at the time of starch accumulation, were later detected in underground parts. This distribution is partly unexpected: on the one hand, labelled photosynthates, especially those from upper leaves, usually migrate to fruits (e.g. Major *et al.*, 1978; Addo-Quaye *et al.*, 1986) and the persistent labelling of fruits and seeds confirms that the same

Table 1 Percentage of the total ^{13}C found in the different organs of ^{13}C-labelled plants at days 4, 25 and 126 after labelling.

Organs	Day 4	Day 25	Day 126*
Fruits†	71.1%	70.2%	0.1%†
Bracts	0.1%	0.3%	
Stem	8.3%	11.4%	92.3%*
Leaves	18.8%	13.7%	
Rhizome	0.1%	0.2%	0.2%
Roots	1.6%	4.2%	7.4%

*Taking into account a mass loss of 77% in aerial parts and 29% in roots by day 126. Large portions of the aerial parts, especially the leaves, were lost by senescence or removed by grazing at day 126 (Supporting Information Fig. S2c). While average root mass per plant showed a mild decrease between fruiting time (days 0–25; 11.6 ± 2.8 g DW, mean ± 95% confidence interval) and fall (day 126; 8.2 ± 3.3 g; Student's *t*-test, $P = 0.085$), aerial parts, which did not differ between days 0, 4 and 25 (10.1 ± 2.8 g on average), experienced an average mass reduction of 77% in fall (2.4 ± 0.9 g; Student's *t*-test, $P < 0.001$). Thus, we applied a correction for biomass assuming that only 23% of the aerial parts and 71% of the root parts remained at day 126.
†Seeds only at day 126.

happens in *C. damasonium*. On the other hand, photosynthates, especially those from the lower leaves, also usually migrate to belowground organs (Harris & Jeffcoat, 1972; Major *et al.*, 1978), especially in perennial species (e.g. Kandiah, 1979) that accumulate reserves such as the starch detected above in winter *C. damasonium*. Such a transfer to the roots has been observed *in vitro* within days for the autotrophic orchid *Goodyera repens* (Cameron *et al.*, 2008): roots received *c.* 20% of ^{14}C-labelled carbon within 4 days (see Fig. 3 in Cameron *et al.*, 2008) instead of just 1.6% in our study.

The very low carbon transfer to the rhizome and roots verifies Prediction 2 in *C. damasonium*, that is, that photosynthates are more abundantly allocated to the aboveground parts than to belowground ones, even at the time of belowground starch accumulation.

Conclusions

We confirm that belowground structures in *C. damasonium* are supported by fungal carbon resources rather than by plant photosynthates since: (1) starch ^{13}C enrichment is identical in albino and green plants; and (2) a direct observation of photosynthate movements supports only a 1.7% allocation to belowground structures in the starch accumulation period (day 4). Some contribution of photosynthates to underground organs is, of course, not excluded: starch in green individuals is 0.6‰ depleted in ^{13}C as compared the albinos (Fig. 1), and although not significant, this value may be biologically relevant evidence for a minor contribution of ^{13}C-depleted photosynthates; indeed, a portion of photosynthates was able to reach rhizomes and roots in the labelling experiment (Table 1). An intriguing question, not addressed here, is that of the nonfruiting shoots occurring in this and other mixotrophic orchids (Salmia, 1989; Roy *et al.*, 2013): in such cases, photosynthates that cannot be allocated to fruits may reach the rhizomes and roots in larger quantities. Indeed, in our data, the percentage of labelled ^{13}C belowground is still rising at day 126, and this may reflect an ability, especially very late in the season, to relocate a few photosynthates belowground. This deserves a separate analysis, as it may demonstrate that shoot formation in plants that do not flower is not only a failed attempt to reproduce, but may be under positive selection due to its contribution to underground organs.

However, in fruiting plants at least, the fungal contribution likely dominates belowground, explaining why starch accumulation is not reduced when photosynthesis is affected (Fig. S1). Fungal resources are thus (at least in part) converted into starch belowground, as observed in germinating orchids that are fully mycoheterotrophic, and accumulate starch after fungal colonization (Richardson *et al.*, 1992; Rasmussen, 1995; Dearnaley *et al.*, 2016). The synthesis of starch from fungal resources was also claimed in the mycorrhizas of another mixotrophic species, *Pyrola japonica* (Ericaceae; Matsuda *et al.*, 2012).

Our observations also explain why the survival of albinos is not impaired compared to green individuals, both in direct observations over a few years (Roy *et al.*, 2013) and in lifespan modelling approaches (Shefferson *et al.*, 2016). Our data provide two additional lines of evidence supporting the model of Gonneau *et al.* (2014) of divergent resources useage: photosynthates are mainly invested in aerial parts (and contribute to fitness by seeding) while fungal resources are mainly invested in underground parts (and contribute to fitness by individual survival). Here, more direct evidence was obtained for underground parts, based on a labelling experiment and on an analysis of accumulated starch, which integrates over longer time periods.

A simple system of source–sink distribution may apply, with C-consuming fruits and underground parts acting as sinks. The fungal resources available and/or the respective strength of the belowground vs aerial demands limit the ability of fungi to rescue the fruits. A partial rescue happens in albinos, allowing some seed production (Roy *et al.*, 2013). Yet, in normal, green individuals, photosynthetic resources are sufficient for fruit formation, and this likely did not select for a stronger fungal exploitation to evolve: in particular, colonization in *C. damasonium* is at its lowest at the time of fruiting (Roy *et al.*, 2013). This explains the persistence of intact photosynthesis in mixotrophic orchids (Bellino *et al.*, 2014; Suetsugu *et al.*, 2018; F. Lallemand & M-A. Selosse, unpublished).

In the Neottieae tribe, to which *C. damasonium* belongs, full mycoheterotrophy evolved multiple times (Selosse & Roy, 2009), and mixotrophy predisposes to an evolution of heterotrophy since underground survival is already largely independent of photosynthesis. Transition to full mycoheterotrophy mainly requires evolutionary novelties to maintain seed production, in order to produce new individuals. Indeed, many mycoheterotrophs, orchids and nonorchids, display active underground asexual reproduction (Klimešová, 2007), for example in orchids by far-reaching stolons bearing bulbils (e.g. in *Epipogium*; Roy *et al.*, 2009) or sprouting roots (e.g. in *Wullschlaegelia*; Martos *et al.*, 2009), or even by roots that develop into new rhizomes after separation from the mother rhizome (in *Neottia*; Champagnat, 1971). These various evolutionary assemblages recruit independent strategies to increase asexual reproduction using belowground organs. They may have

compensated for the reduced seed set at the time of transition to mycoheterotrophy. The underground use of fungal nutrition, already secured by mixotrophic ancestors, may have (at least transiently) allowed them to maintain the production of new individuals.

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Author contributions

M-AS, CD and BZ planned and designed the research. M-AS sampled for starch analyses; FL, NF, BZ and M-AS performed labelling experiments. Starch and isotopic analyses were conducted by FL, TF, CF, CG and NF. M-AS and FL wrote the first version of the manuscript that was later edited by all authors.

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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 Mycorrhizal colonization and starch content in roots of albino and green *C. damasonium* collected at Boigneville on 15 January 2015 ($n = 3$ each).

Fig. S2 Labelling experiment carried out in a population of green *C. damasonium* at Le Vésinet.

Fig. S3 Mean ^{13}C enrichments of *C. damasonium* organs and leaves of reference autotrophic species at day of labelling at Le Vésinet.

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Key words: ^{13}C, carbon allocation, *Cephalanthera damasonium*, isotopic enrichment, Neottieae, partial mycoheterotrophy, photosynthates labelling, starch.

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