Irradiance governs exploitation of fungi: fine-tuning of carbon gain by two partially myco-heterotrophic orchids

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While all members of the Orchidaceae are fully dependent on mycorrhizal fungi during their achorophyllous juvenile stages, mature plants may remain fully myco-heterotrophic, become fully autotrophic or develop a nutritional mode where the carbon gain through photosynthesis is complemented by organic carbon from fungal partners. This so-called partial myco-heterotrophy is intriguingly complex. Current knowledge indicates a large range in the proportion of fungus-derived carbon between and within partially myco-heterotrophic plant species. However, the driving factors for this variation are so far mostly unknown. Here we show for two green species of the orchid genus *Cephalanthera* that light availability is the major determinant of the degree of myco-heterotrophy. Using leaf stable isotope natural abundance analysis together with time-integrated microscale light climate monitoring we could demonstrate that there is a sensitive reaction to varying light availability within forests. Low light levels result in strong myco-heterotrophy while higher irradiances successively drive the orchids towards autotrophy. Our results demonstrate that partial myco-heterotrophy in these species is not a static nutritional mode but a flexible mechanism driven by light availability which allows a balanced usage of carbon resources available in nature.

**Keywords:** partial myco-heterotrophy; Orchidaceae; stable isotopes; carbon gain; irradiance; mycorrhiza

1. INTRODUCTION

Since the recent discovery of a novel nutritional mode in the world’s largest plant family, the Orchidaceae, a dogma in plant sciences meaning that green plants (with the exception of some hemiparasites) are autotrophic is no longer valid (G. Gebauer in Whitfield 2007). Although green plants are able to photosynthesize, some specialized terrestrial orchids have recently been shown to additionally use an underground carbon source—their mycorrhizal fungi (Gebauer & Meyer 2003). A switch of their mycorrhizal associates from typical *Rhizoctonia* species (a polyphyletic group of fungi) to ectomycorrhizal partners that are simultaneously associated with trees enables the looting of organic nutrients (Bidartondo et al. 2004). Analogue mechanisms have in the meantime also been found in some green pyroloids (Ericaceae, Tedersoo et al. 2007; Zimmer et al. 2007; Hynson et al. 2009) and ongoing investigations continually reveal further species that exhibit this complex form of nutrition (Bidartondo et al. 2004; Julou et al. 2005; Abadie et al. 2006; Zimmer et al. 2008), which can be referred to as partial myco-heterotrophy (Gebauer & Meyer 2003). Although it can be hypothesized that many more green plants from diverse taxa may up to now not noticebly gain organic compounds through myco-heterotrophic means, we know little on the factors driving this phenomenon.

Stable isotope natural abundances in organism tissues are a convenient tool to study the use of isotopically distinguished nutrient sources. The incorporation of fungus-derived carbon, for example, is reflected by the green plants’ leaf isotope signature since fungal tissues are enriched in the heavy carbon stable isotope $^{13}$C relative to accompanying fully autotrophic plants (Högberg et al. 1999). Previous studies indicate a large range in the proportion of fungus-derived carbon between and within partially myco-heterotrophic species (Gebauer & Meyer 2003; Bidartondo et al. 2004; Julou et al. 2005; Abadie et al. 2006; Tedersoo et al. 2007; Zimmer et al. 2007, 2008; Cameron et al. 2009; Hynson et al. 2009) but the driving factors for this variation remain mostly unknown.

*Zimmer et al. (2007)* suggested a negative relationship between the quantity of carbon gain from fungi and light availability in the wintergreen *Orthilia secunda* based on results of a previous comparison of three independent investigations on the trophic status of green orchids at different forest types by Gebauer (2005), who raised the hypothesis that the exploitation of mycorrhizal fungi might be affected by the prevalent light climate. To test this hypothesis experimentally, we combined leaf stable isotope natural abundance analysis with time-integrated microscale light climate monitoring and investigated two partially myco-heterotrophic orchid species (*Cephalanthera damasonium* and *C. rubra*) together with 12 fully autotrophic and one fully myco-heterotrophic reference species.

2. MATERIAL AND METHODS

(a) Study sites and investigated species

Plant samples were collected in 2007 and 2008 from three forest sites in northeast Bavaria: an open *Pinus sylvestris*...
stand, a forest dominated by Fagus sylvatica and a mixed stand composed of several conifers (e.g. P. sylvestris, Picea abies) and broadleaf species (e.g. F. sylvatica, Acer campestre). All sites are located at 480–520 m a.s.l. and characterized by mean annual precipitation of 700–1000 mm and mean annual temperatures of 6–9°C. In total, 224 understory plant samples were collected from a fully myco-heterotrophic (Neottia nidus-avis, n = 11), a fully autotrophic (Cypripedium calceolus, n = 9) and two partially myco-heterotrophic orchid species (Cephalanthera damasonium, n = 18; C. rubra, n = 18); and from 11 diverse (monocotyledons/dicotyledons, tree saplings/herbs, evergreen deciduous, ectomycorrhizal/ arbuscular- or non-mycorrhizal) autotrophic non-orchid species (A. campestre, n = 9; A. pseudoplanatus, n = 3; Anthericum ramosum, n = 12; Carex flacca, n = 20; Convallaria majalis, n = 11; Euphorbia cymparissias, n = 20; F. sylvatica, n = 48; Fragaria vesca, n = 3; Galium odoratum, n = 10; G. verum, n = 10; Polygala chamaebuxus, n = 22).

(b) Microscale light climate monitoring
For each of the 56 orchid individuals, a 1 m² plot including two to four autotrophic non-orchids was selected. As soon as the young orchid shoots could be identified, a calibrated light sensor (silicon photodiode BPW 21, Infineon, Germany) connected to a mini data logger (HOBO H8, ONSET, USA) was installed right next to each shoot at about 15 cm height. Irradiance was logged every 15 min from the day of sensor installation until the development of seed capsules (2007: 9 May–20 June; 2008: 18 May–6 July). Measured values were converted into photosynthetic active radiation (μmol photons m⁻² s⁻¹) and averaged as daily means (from sunrise to sunset). Because of the equal global solar radiation during each month from May to July in the two sampling years, the measured relative light availability of understory plants had not to be adjusted (total in 2007: 488 kWh m⁻²; total in 2008: 494 kWh m⁻²; weather station of the Ecological-Botanical Garden Bayreuth, 32–40 km apart from the three sampling sites). Furthermore, potential differences in the orchids’ developmental stage are known to have no significant influence on their leaf δ¹³C values as has been found for four C. damasonium individuals that were analysed every month from June to September 2003 at an F. sylvatica-dominated forest in northeast Bavaria (B. Burghardt 2003, unpublished data).

(c) Carbon stable isotope abundance analysis
Leaf samples (and stem samples of the leafless N. nidus-avis) were taken following the criteria described by Gebauer & Meyer (2003). The plant material was oven-dried at 105°C and ground to a fine powder. Relative C isotope abundances were measured with an elemental analyser coupled to a continuous flow isotope ratio mass spectrometer as described in Bidartondo et al. (2004). Measured abundances are denoted as δ values, which were calculated according to the following equation: δ¹³C = (Rsample/Rstandard – 1) × 1000 [%], where Rsample and Rstandard are the ratios of heavy to light isotope of the samples and the respective standard. Standard gases were calibrated with respect to international standards by using the reference substances ANU sucrose and NBS 19, provided by the International Atomic Energy Agency (Vienna, Austria).

(d) Data preparation and statistics
To facilitate precise data comparisons between sites and plots, δ values were normalized according to Preiss & Gebauer (2008): δ¹³C values of the orchids and the non-orchid autotrophic reference plants were used to calculate δ¹³C enrichment factors (ε) of every plant against the mean of the autotrophic plants for each plot: ε = δ⁻¹³C - δNBS with S as single value of a sample from an autotrophic, partially or fully myco-heterotrophic orchid and REF as the mean value of all autotrophic reference plants from the respective plot.

Differences between δ¹³C values of Cephalanthera individuals and autotrophic reference plants were analysed using Mann–Whitney U-tests at different light levels (below and above 200 μmol m⁻² s⁻¹). To test for significant (α = 0.05) correlations between measured light availability and δ¹³C values or enrichment factors (ε), respectively, regression analyses were performed. All statistical tests were conducted using SigmaPlot v. 11.0 (Systat Software, Inc., USA). Means are given as ± 1 s.d.

3. RESULTS AND DISCUSSION
(a) Responses of δ¹³C to varying irradiance
δ¹³C values in leaves of autotrophic non-orchids ranged from −34.2 to −26.3% (figure 1) and showed a significant, positive correlation with light availability (F₀.001, R² = 0.293, p < 0.001). These δ¹³C values and their dependence on light climate are based on the carbon isotope discrimination during C₃ photosynthesis (fractionation during carboxylation by Rubisco) and on stomatal regulation, which affects the intercellular partial pressure of CO₂ (Farquhar et al. 1989). Leaf isotope signatures of the fully autotrophic orchid C. calceolus responded in the same way as autotrophic non-orchids (F₀.001, 3, R² = 0.518, p < 0.029), demonstrating that members of the Orchidaceae per se do not show any peculiarity in carbon nutrition. This is consistent with the findings of Zimmerman & Ehleringer (1990), who analysed the carbon isotope composition of a Panamanian epiphytic C₃ orchid (Catasetum viridiflavum) and found higher δ¹³C values with increasing irradiance owing to increasing stomatal limitation to photosynthesis.

The achlorophyllous orchid N. nidus-avis showed the highest δ¹³C values of all investigated species (−23.1 ± 1.10% on average). Such a relative ¹³C enrichment is characteristic of all fully myco-heterotrophic plants that associate with ectomycorrhizal fungi (Preiss & Gebauer 2008) and fits the food-chain model (Trudell et al. 2003). Since these plants’ carbon demand is exclusively covered through organic compounds supplied by fungi, δ¹³C values of N. nidus-avis are not correlated with the microscale light climate (F₀.001, 3, R² = 0.011, p = 0.411; figure 1).

A quite interesting pattern turned out for the two Cephalanthera species. Although these green orchids are able to photosynthesize and a positive correlation of their carbon isotope signatures with light availability just as in other green plants could thus be expected, no significant response to varying irradiance was found (figure 1; C. damasonium: F₀.001, 3, R² = 0.149, p = 0.064). Their mean δ¹³C values (C. damasonium: −28.1 ± 1.4‰; C. rubra: −28.6 ± 1.6‰) range between those of fully autotrophic and fully myco-heterotrophic plants as typical for partial myco-heterotrophs. While δ¹³C values of
Cephalanthera individuals and fully autotrophic plants are significantly different at irradiances below 200 μmol m$^{-2}$ s$^{-1}$ ($p < 0.001$), these groups are statistically indistinguishable ($p = 0.059$) at higher light levels indicating a pronounced shift towards autotrophic nutrition at sufficiently light-exposed sites. However, since it has been shown that irradiance-dependent physiological effects can strongly influence leaf δ$^{13}$C values, isotope data have to be related to a fine spatial scale before assessing the question whether partial myco-heterotrophy is a flexible or a static nutritional mode.

**Effects of irradiance on partial myco-heterotrophy**

Figure 2 shows the plants’ δ$^{13}$C enrichment normalized for environmental changes by relating all isotope data to references of the respective plot as described by Preiss & Gebauer (2008). Owing to referencing δ$^{13}$C values of *N. nidus-avis* and the two *Cephalanthera* species against autotrophic species whose δ$^{13}$C values increase with increasing irradiance (cp. figure 1), the enrichment factors (e) of these orchids decrease with increasing light availability. However, there are different physiological processes behind the reactions of *Neottia* and the *Cephalanthera* species. While the non-photosynthetic *N. nidus-avis* completely relies on myco-heterotrophic carbon supplies and constantly gains 100% of its carbon from mycorrhizal fungi, green *Cephalanthera* species are able to use both the fungal and the atmospheric carbon source. Relating the *Cephalanthera*’s δ$^{13}$C enrichment factors to those of *N. nidus-avis* shows that the incorporation of organic carbon from fungi is not a constant parameter in these green orchids. As indicated by the arrows (figure 2), *Cephalanthera* shoots receive about half as much fungus-derived carbon as achlorophyllous plants under low light conditions, while the proportion of heterotrophic nutrition strongly decreases with increasing irradiance. This finding is also supported by previous results of gas-exchange measurements showing that photosynthetic rates in *C. damasonium* can increase when more light is available (Julou et al. 2005). Thus, the constant δ$^{13}$C values in *Cephalanthera* along an irradiance gradient seen in figure 1 are obviously caused by successively lowering the incorporation of δ$^{13}$C-enriched fungal tissue with increasing light availability. During the transfer of organic compounds like amino acids, fully and partially myco-heterotrophic plants are also gaining small amounts of nitrogen affecting their δ$^{15}$N signature. In accordance with the decreasing carbon gain, a trend towards lower δ$^{15}$N enrichment with increasing light availability could be seen in *Cephalanthera* shoots (not shown). Since weak gains of organic nitrogen were still detectable at high irradiances, it seems as if partially myco-heterotrophic plants do not totally give up their fungal nutrient source. However, we were able to demonstrate that, at high irradiances, above ground parts of adult *Cephalanthera* plants can cover almost all of their carbon demands through assimilation of atmospheric CO$_2$.

**4. CONCLUSIONS**

It has been shown that two partially myco-heterotrophic *Cephalanthera* species strongly supplement their carbon gain through photosynthesis by organic carbon from fungal partners under low light conditions but become almost completely autotrophic when they are exposed to sufficiently high irradiances. This demonstrates that partial myco-heterotrophy is not a strictly static nutritional mode but may be a flexible mechanism allowing a balanced use of carbon resources available in nature. The fact that the degree of myco-heterotrophy may successively change—driven by the prevalent microscale light climate—could explain several discrepancies.
between previous studies that investigated the trophic status of numerous green Orchidaceae and Ericaceae.

This work was supported by the German Research Foundation and contributes to the DFG project GE 565/7-1. The authors would like to thank the technical staff of BayCEER–Isotope Biogeochemistry for skilful assistance in mass spectrometry and Michael Gaag (all University of Bayreuth) for innovative ideas and their implementation during light sensor construction. We gratefully acknowledge permission for orchid sampling by the Regierung von Oberfranken and Mittelfranken.

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