

Letters

Is it better to give than to receive? A stable isotope perspective on orchid–fungal carbon transport in the green orchid species *Goodyera repens* and *Goodyera oblongifolia*

In the field of orchid research, species within the tribe Cranichideae have taken center stage as a result of the recent findings of Cameron *et al.* (2006, 2008), which demonstrated carbon transport from adult *Goodyera repens* (L.) R. Br. orchids to their mycorrhizal fungus *Ceratobasidium cornigerum* (Bourdot) D. P. Rogers. The dependence of orchids in their early stages of development on fungi is a long-recognized trait of the family (Bernard, 1909; Dearnaley, 2007). However, there has been much controversy over the potential for carbon ‘repayment’ to the fungi once the orchid has formed leaves and is capable of assimilating its own carbohydrates through photosynthesis (Alexander & Hadley, 1985; McCormick *et al.*, 2006; Smith & Read, 2008).

Using ^{14}C -labeled carbon fed either to the mycelia of the orchid’s fungal symbiont or to the plant as $^{14}\text{CO}_2$, Cameron *et al.* (2008) were able to quantify the carbon transport between the orchid and fungus over an 8-d period. Their findings were that the net transfer of carbon from *G. repens* to *C. cornigerum* was over five times greater than that of carbon transported from the fungus to the plant. While this extremely well-executed study provides the ‘first full bidirectional C budget for any mycorrhizal association’ (Cameron *et al.*, 2008), there are some limitations of their model and methods that must be taken into account. As mentioned in their recent article and the commentary by Johnson (2008), the carbon allocation to fungal biomass within the orchid’s roots cannot be separated from that to the roots alone; nor can carbon respiration from the plant versus that from the fungus. Furthermore, the use of radiocarbon labeling gives measurements of carbon flow within a system for only a relatively short period of time. Also, as many of these labeling experiments are carried out in the laboratory, it is difficult to relate results to any field setting. A complementary method that has been applied to examine carbon and nitrogen gains from fungi by partially and fully myco-heterotrophic plants associated with ectomycorrhizal (ECM) and litter- or wood-decaying

saprotrophic (SAP) fungi is the use of naturally occurring stable isotopes of carbon and nitrogen (^{13}C : ^{12}C and ^{15}N : ^{14}N) (Gebauer & Meyer, 2003; Trudell *et al.*, 2003; Ogura-Tsujita *et al.*, 2009). Measured isotope abundances are denoted as δ values and are calculated according to the equation: $\delta^{15}\text{N}$ or $\delta^{13}\text{C} = (\text{R}_{\text{sample}}/\text{R}_{\text{standard}} - 1) \times 1000$ [‰], where R_{sample} and $\text{R}_{\text{standard}}$ are the ratios of heavy isotope to light isotope of the samples and the respective standard. In contrast to radiocarbon labeling, the analysis of the bulk carbon isotope values of field-collected plants gives an integrated view of carbon assimilation throughout the period during which the tissue was synthesized (Dawson *et al.*, 2002).

While there is a subset of orchid species that remain myco-heterotrophic for their entire life cycle and lack the ability to photosynthesize (Leake, 1994), it was previously believed that green species are completely released from their dependence on heterotrophic carbon gain once leaves are formed (Alexander & Hadley, 1985). However, recent analysis of the carbon and nitrogen isotope signatures of some green orchids has revealed that many of these putative autotrophic orchids that associate with ECM fungi actually still partially rely on these fungi to meet their carbon demands. These orchid species have been referred to as mixotrophs or partial myco-heterotrophs. Unlike obligate myco-heterotrophic orchids, which have $\delta^{13}\text{C}$ signatures most similar to those of their fungal symbionts, mixotrophic orchids tend to have $\delta^{13}\text{C}$ signatures intermediate between those of surrounding autotrophic and myco-heterotrophic plants (Bidartondo *et al.*, 2004; Julou *et al.*, 2005; Abadie *et al.*, 2006; Teder-soo *et al.*, 2007; Zimmer *et al.*, 2007).

Interestingly, an additional category of orchids that are depleted in ^{13}C compared with surrounding autotrophic plants is emerging from recent stable isotope analysis of species in the closely related tribes Orchideae and Cranichideae (H. T. Liebel *et al.*, unpublished), the latter containing the genus *Goodyera* (data herein). We collected leaf samples of *Goodyera oblongifolia* Raf. from four sites in northern California and southern Oregon, USA, and *G. repens* from a single site in the Austrian Alps (Table 1). The site in the Alps was an open rocky outcrop habitat, while all samples collected in the USA were from the deeply shaded understories of mixed conifer forests. Altogether, leaves of 15 *G. oblongifolia* and five *G. repens* individuals were collected. In addition, at each sampling site a minimum of five autotrophic individuals from at least one species were collected for a total of 40 individuals of six species (Table 1). These collections were used as reference plants representative of the autotrophic understory.

The collected plant samples were then analyzed for carbon and nitrogen stable isotope abundances via elemental analyzer/continuous flow isotope ratio mass spectrometry at either the

Table 1 Sampling locations in the USA (CA, OR) and Austria (Vorarlberg) including *Goodyera* and reference species collected (n , number of replicates), and mean (± 1 SD) $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values (‰) in leaves of *Goodyera* and reference species

Location	Species (n)	$\delta^{15}\text{N} \pm 1$ SD	$\delta^{13}\text{C} \pm 1$ SD
El Dorado National Forest, CA 38°54'01.70"N 120°34'26.77"W	<i>Goodyera oblongifolia</i> (4)	-2.4 \pm 0.8	-32.3 \pm 0.8
	<i>Abies concolor</i> (5)	-3.4 \pm 0.9	-31.0 \pm 0.3
El Dorado National Forest, CA 38°54'3.47"N 120°34'28.40"W	<i>G. oblongifolia</i> (5)	-3.2 \pm 0.8	-33.3 \pm 1.2
	<i>A. concolor</i> (5)	-4.0 \pm 0.7	-30.6 \pm 0.7
	<i>Ribes roezlii</i> (5)	-4.3 \pm 1.2	-31.4 \pm 0.6
Plumas National Forest, CA 40°03'36.02"N 120°51'32.99"W	<i>G. oblongifolia</i> (5)	-2.0 \pm 1.1	-33.2 \pm 0.7
	<i>A. concolor</i> (5)	-3.8 \pm 1.1	-30.4 \pm 1.0
Willamette National Forest, OR 44°18'36.00"N 122°00'36.02"W	<i>G. oblongifolia</i> (1)	-1.6	-33.4
	<i>Tsuga heterophylla</i> (5)	-2.2 \pm 0.8	-31.9 \pm 1.9
Marultal, Vorarlberg 47°11'44"N 9°53'57"E	<i>G. repens</i> (5)	-6.2 \pm 1.1	-36.6 \pm 1.9
	<i>Knutia sylvatica</i> (5)	-8.9 \pm 0.8	-32.1 \pm 0.6
	<i>Mercurialis perennis</i> (5)	-7.7 \pm 0.6	-30.3 \pm 0.7
	<i>Vaccinium vitis-idaea</i> (5)	-9.7 \pm 0.9	-30.5 \pm 0.5

Laboratory of Isotope Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), Germany or the Center for Stable Isotope Biogeochemistry at University of California Berkeley, as described in Zimmer *et al.* (2007). Once δ values were obtained for all samples from the USA (Table 1), the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of all reference plants were tested for inter-site variation with a one-way ANOVA and Tukey's Honestly Significant Differences (HSD). Because of significant differences at an α value of 0.05 in the $\delta^{15}\text{N}$ values of the reference plants between two sites in California ($P = 0.007$), the δ values from the USA could not be pooled to make comparisons across sites between *Goodyera* samples and their respective references. To make these comparisons, δ values for both elements and all samples collected in the USA and at the single Austrian site (for consistency) were converted into site-independent enrichment factors (ϵ) and pooled based on species identity and location (USA or Austria). The calculation of ϵ factors systematically eliminates the majority of the influence of spatial variation on δ values resulting from site-specific differences in carbon and nitrogen isotope abundances, thus allowing comparisons of these values across sites (Emmett *et al.*, 1998; Gebauer & Taylor, 1999; Preiss & Gebauer, 2008). First, for each site the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of reference plants were averaged. Then, on a per site basis, these averages were subtracted from the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the *Goodyera* samples and reference plants to create site-independent enrichment factors ($\epsilon = \delta x_S - \delta x_R$) for each sample (where $\delta x_S = \delta^{13}\text{C}$ or $\delta^{15}\text{N}$ of individual samples per site and $\delta x_R = \text{mean } \delta^{13}\text{C}$ or $\delta^{15}\text{N}$ of all reference plants per site). The resulting mean of both ^{13}C and ^{15}N ϵ factors of the autotrophic reference plants was equal to 0‰. However, the enrichment factors of individual reference plants clustered at $c.$ 0‰, reflecting the small inter- and intraspecific variations in their isotope signatures, which were not significantly different between sites. The two *Goodyera* species' ϵ factors species separated as distinct

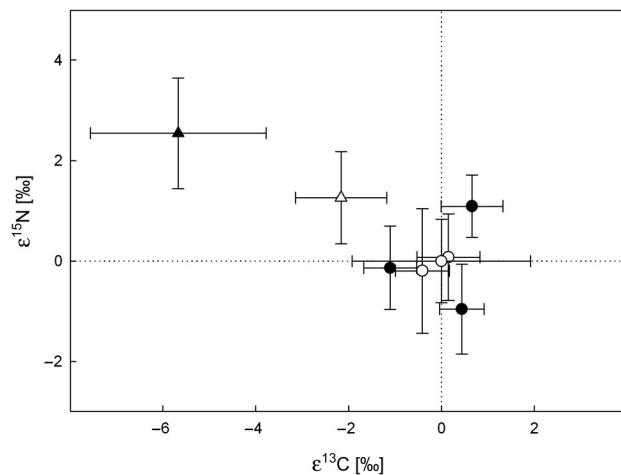


Fig. 1 Mean enrichment factors (ϵ) of ^{13}C and ^{15}N from the leaves of *Goodyera oblongifolia* (open triangle), *Goodyera repens* (closed triangle) and each species of autotrophic reference plants collected in the USA (open circles) and in Austria (closed circles). Error bars indicate 1 SD for each *Goodyera* species and their respective reference plants.

groups for both elements based on the differences of their δ values from the mean of their respective references (Fig. 1). The variance around the mean $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values of reference plants used to calculate $\epsilon^{15}\text{N}$ and $\epsilon^{13}\text{C}$ was retained by calculating ϵ factors for not only both *Goodyera* species, but also reference plants on a site by site basis. Statistical comparisons between the individual enrichment factors of individual *G. repens* and *G. oblongifolia* plants and their respective autotrophic references from either Austria or western USA were made using Mann–Whitney U -tests. Both *G. repens* ($P = 0.002$) and *G. oblongifolia* ($P = 0.008$) were significantly enriched in ^{15}N compared with surrounding autotrophic plants (Fig. 1). By contrast, both *Goodyera* species were significantly depleted

in ^{13}C in comparison to their references ($P < 0.001$; Fig. 1). *Goodyera repens* plants from the open sunny habitat in the Alps were considerably more depleted in ^{13}C compared with *G. oblongifolia* from deeply shaded forests.

Although the sample size of *G. oblongifolia* and *G. repens* individuals collected in this study were relatively small, the stable isotope evidence presented here shows that these orchids do not exhibit any trends toward full or partial myco-heterotrophy. In fact, these orchids' consistent depletion in ^{13}C compared with surrounding autotrophic plants reveals a distinct nutritional strategy. The physiological mechanism leading to this depletion remains unknown, but may be related to the transfer of ^{13}C -enriched carbon compounds from these orchids to their associated fungi (*sensu* Gleixner *et al.*, 1993). This would fit well with Cameron *et al.*'s (2006, 2008) findings of carbon transfer from orchid to fungus, as well as with isotope food-chain models where the source of a nutrient is left depleted in the heavy isotope compared with the sink (Fry, 2006). What is unclear is why *Goodyera* species would be significantly more depleted in ^{13}C than surrounding autotrophic mycorrhizal plants that are transferring substantial amounts of carbon to their fungal symbionts (Smith & Read, 2008). Habitat may also play a key role in determining the ^{13}C enrichment factors of *Goodyera* species. For instance, there exists some evidence that green orchids capable of partial myco-heterotrophy increase their dependence on fungal assimilated carbon when in deeply shaded habitats, leading their leaf $\delta^{13}\text{C}$ values to become more enriched than those of surrounding autotrophic plants (Bidartondo *et al.*, 2004; McCormick *et al.*, 2004; Zimmer *et al.*, 2007). If *G. oblongifolia* individuals from our forested sites were at an earlier stage of seedling development more dependent on heterotrophic carbon gain than *G. repens* from open sites, then this could explain why the former is less depleted in ^{13}C than the latter. The significant enrichment in ^{15}N (a hallmark of all myco-heterotrophic orchids studied to date) found in both *Goodyera* species supports this theory, and Cameron *et al.*'s (2008) statement that these orchids are more parasitic upon their fungal symbionts than other mycorrhizal plants and therefore may govern the amount of nutrient exchange to the fungus. This idea of 'orchid control' over its mycorrhizal associations is further exemplified by the unique morphology of orchid mycorrhizas, where fungi that are known to be saprotrophic or ectomycorrhizal when independent of orchids form intracellular coils when in association with orchids (Rasmussen, 2002).

Cameron *et al.*'s (2006, 2008) work provides the first example, in *G. repens*, of an orchid species that, upon becoming photosynthetically active, can transfer carbon back to its mycorrhizal fungus. Unlike other green orchids studied to date, species within the tribes Orchideae and Cranichideae, including *G. repens* and *G. oblongifolia*, are the first species found to be depleted in ^{13}C compared with surrounding autotrophic plants (H. T. Liebel *et al.*, unpublished and data herein). In summary, based on carbon stable isotope abundances and the identity of their mycorrhizal associates, it is now clear that terrestrial orchids

can utilize at least four nutritional strategies: autotrophy, where green orchids have carbon isotope signatures indistinguishable from those of surrounding autotrophs and mainly associate with *Rhizoctonia* species (a polyphyletic group of fungi); partial myco-heterotrophy, where green orchids have carbon isotope signatures intermediate between those of autotrophs and myco-heterotrophs and associate with ECM fungi; obligate myco-heterotrophy, where orchids have lost the ability to photosynthesize, are specialized on either ECM or SAP fungi, and are enriched in ^{13}C , similar to their host fungi; and an additional strategy found in green orchids from the tribes Orchideae and Cranichideae, which mainly associate with ceratobasidioid and tulasnelloid fungi and are depleted in ^{13}C compared with surrounding autotrophs (Fig. 1 and H. T. Liebel *et al.*, unpublished). The variability of the ecology and physiology of orchids is not surprising, as orchids are the largest plant family, whose evolutionary history potentially stretches back to the late Cretaceous (Ramírez *et al.*, 2007). While there is still much to discover about the intriguing Orchidaceae, combining the use of naturally abundant isotopes and radioactive tracers along with molecular methods, especially those that allow comparisons at the genotype level (Johnson, 2008), will continue to help us understand the links between the evolutionary history of orchids, their physiology and interactions with fungi.

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