Carbon nutrition of mature green orchid *Serapias strictiflora* and its mycorrhizal fungus *Epulorhiza* sp.

K. LÁTALOVÁ and M. BALÁŽ*

Faculty of Science, Masaryk University, Kotlářská 2, CZ-61137 Brno, Czech Republic

Abstract

We studied the nutritional modes of the orchid *Serapias strictiflora* and its mycorrhizal fungus *Epulorhiza* sp. using the differences in carbon isotopic composition ($\delta^{13}C$) of C_3 orchid and C_4 maize tissues. We found that if cultivated in substrate lacking any organic compounds, the mycorrhizal extraradical mycelia ($\delta^{13}C$ = -26.3 ± 0.2 ‰) developed well, despite being fully dependent on nutrition from orchid roots ($\delta^{13}C$ = -28.6 ± 0.1 ‰). If the mycorrhizal fungus had additional access to and colonized decaying maize roots ($\delta^{13}C$ = -14.6 ± 0.1 ‰), its isotopic composition ($\delta^{13}C$ = -21.6 ± 0.4 ‰) reflected a mixture of biotrophy and saprotrophy. No statistically significant differences in $\delta^{13}C$ of new storage tubers were found between *Epulorhiza*-associated orchids with ($\delta^{13}C$ = -28.2 ± 0.1 ‰) and without access to maize roots ($\delta^{13}C$ = -28.6 ± 0.2 ‰). We conclude that autotrophy is the predominant nutritional mode of mature *S. strictiflora* plants and that they supply their mycorrhizal fungus with substantial amount of carbon (69 ± 3 % of the fungus demand), even if the fungus feeds saprotrophically.

Additional key words: $\delta^{13}C$, biotrophy, extraradical mycelium, heterotrophy, saprotrophy.

Introduction

It is widely acknowledged that orchids in early developmental stages are fully dependent on associated mycorrhizal fungi for their carbon requirements. This nutritional dependence, called myco-heterotrophy, persists up to maturity in a small portion of orchids (Leake 1994, 2004). However, the majority of orchid species have well-developed leaves at maturity and photoassimilate carbon dioxide like the other green-leaved plants. The actual ratio between the two possible nutritional modes (mixotrophy degree) has been investigated for several orchid species (for review see Selosse and Roy 2009). Mature *Goodyera repens* plants were found to be fully autotrophic, as no substantial radioactivity was detected in their tissues after the associated mycorrhizal fungus was fed with ^{14}C insoluble sugars (Alexander and Hadley 1985). In recent experiments, the same species was shown to obtain small amounts of carbon *via* amino acid transfer from the fungus to the host (Cameron *et al.* 2006, 2008), but the opposite flow of carbon compounds from host to the fungus was over five times higher (Cameron *et al.* 2008). Gebauer and Meyer (2003) compared the carbon isotopic composition ($\delta^{13}C$) of fully myco-heterotrophic orchids, green-leaved photoassimilating orchids and plants from other families considered fully autotrophic. Using a linear two-source isotopic mixing model, they found that one green-leaved orchid species obtained high amount of carbon from the associated mycorrhizal fungus (*Cephalanthera damasonium*, 85 % of carbon demand), while some other green-leaved species under study (*Cephalanthera rubra*, *Dactylorhiza sambucina*, *Ophrys insectifera*, *Neotinea ustulata*, *Orchis mascula* and *Platanthera bifolia*) were only marginally dependent on their mycorrhizal fungi, usually obtaining less than 10 % of their carbon from fungus. In later studies, a similar approach was used to quantify the degree of mixotrophy in other terrestrial orchid species, and molecular tools were used to determine their mycorrhizal fungi (Bidartondo *et al.* 2004, Zimmer *et al.* 2007). It was revealed that orchids associated with fungi simulta-

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Abbreviations: Ben - benomyl; ERM - extraradical mycelium; OM - orchid mycorrhiza.
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* Corresponding author; fax: (+420) 541211214, e-mail: balaz@sci.muni.cz

neously forming ectomycorrhizas with forest trees obtained more carbon compounds *via* myco-heterotrophy compared to the orchids associated with rhizoctonias. A slightly different mixotrophy assessment technique, which compared $\delta^{13}C$ of green-leaved photosynthesizing *C. damasonium* plants with fully myco-heterotrophic albino individuals, revealed that green-leaved individuals obtained almost 50 % of their C demand by means of myco-heterotrophy (Julou *et al.* 2005). Considering that the nutritional status of only a few taxa of the large *Orchidaceae* family has been evaluated so far, the need for extensive investigation of a wide range of species using various approaches is obvious.

Although green-leaved orchids photoassimilate CO_2, it was not clear for a long time whether they could supply their mycorrhizal fungi with carbon compounds. The carbon flow from host to fungus is typical for many mycorrhizal types, *e.g.*, for arbuscular mycorrhiza and ectomycorrhiza (Smith and Read 2008), but has not been confirmed for orchid mycorrhiza (OM) despite a century of research. Cameron *et al*. (2006, 2008) showed recently that $^{14}CO_2$ photoassimilated by the orchid *G. repens* is readily translocated to the mycorrhizal fungus. These authors used the same orchid-mycorrhizal fungus combination previously used by other researchers (Hadley and Purves 1974, Alexander and Hadley 1985). The attempt by Hadley and Purves (1974) to demonstrate carbon transfer from the host to mycorrhizal fungus failed due to negligible translocation of fixed $^{14}CO_2$ to the orchid's underground organs. In a study by Alexander and Hadley (1985), allocation of photosynthetically fixed C to the roots was satisfactory, but again, no evidence of C transfer from the orchid to the mycorrhizal fungus was obtained. The different results obtained by these studies can be attributed to the nature of the substrates and the environmental conditions (Cameron *et al.* 2006, Dearnaley 2007). We therefore argue that carbon nutrition in orchids and their OM fungi requires further research using more orchid species and various experimental approaches.

In this paper we tried *1*) to demonstrate that OM symbiosis occurs even if conditions prevent saprotrophic nutrition of the OM endophyte; *2*) to quantify the relative contribution of biotrophy (associated with live orchid roots) and saprotrophy (decomposing maize roots) in the carbon nutrition of the mycorrhizal fungus; and *3*) to assess the carbon gain of photosynthesizing green-leaved orchid *S. strictiflora* from OM symbiosis if an external carbon source is available to its endophyte. For these purposes we determined $\delta^{13}C$ values of C_3 orchid and C_4 maize tissues from greenhouse experiments and compared these with $\delta^{13}C$ values of mycorrhizal extra-radical mycelia (ERM).

Materials and methods

Ten-day-old maize (*Zea mays* L.) seedlings were planted in 48 polyethylene pots (12 × 12 × 12 cm), filled with 1.2 dm^3 of rinsed and autoclaved clinoptilolite (natural zeolite, particle size 1 - 2.5 mm; *Zeocem*, Bystré, Slovakia). In the central position there was a 200-cm^3 flask buried in the substrate to form a hole for later planting of an orchid. For later ERM extraction, one round membrane filter (5 cm in diameter, pore size 0.65 µm; *Pragochema*, Prague, Czech Republic) was also buried in substrate in a vertical position, with its top edge 2 cm below the substrate surface (Baláž and Vosátka 2001). The pots were covered with polyethylene and aluminum foil to prevent evaporation and light access. The plants were cultivated in a glasshouse under a 16-h photoperiod with photosynthetically active radiation of 250 µmol m^{-2} s^{-1}, temperature of 25/22 °C and approximately 80 % relative humidity. They were watered as needed using deionized water and fertilized once a week with 200 cm^3 of nutrient solution containing [mg dm^{-3}] NH_4NO_3 (48.25), $Ca(NO_3)_2 \cdot 4\,H_2O$ (153.5), KH_2PO_4 (7.75), K_2HPO_4 (7.5), K_2SO_4 (205), $MgSO_4 \cdot 7\,H_2O$ (125), FeNaEDTA (18.35), $MnCl_2 \cdot 4\,H_2O$ (3.75), $ZnSO_4 \cdot 7\,H_2O$ (1.2), H_3BO_3 (0.75), KI (0.375), $Na_2MoO_4 \cdot 2\,H_2O$ (0.0625), $CuSO_4 \cdot 5\,H_2O$ (0.0125), and $CoCl_2 \cdot 6\,H_2O$ (0.006).

Ten weeks thereafter, on February 24, 2004, maize cultivation ceased. Twelve randomly chosen pots were assigned to determine $\delta^{13}C$ values of maize roots and leaves. For the remaining 36 pots, the above-ground maize parts were cut off and the covering foil and buried flask were removed. Intact *Serapias strictiflora* Welwitsch *ex* Veiga plants were planted into the central holes of 24 randomly chosen pots, using rinsed and autoclaved clinoptilolite as the substrate. These were also randomly divided into two equal subsets, from which one was treated 1 and 6 weeks after *S. strictiflora* transplantation with 200 cm^3 of 1 g dm^{-3} benomyl solution (*Fundazol 50 WP*®, *Chinoin*, Budapest, Hungary) (+C_4+Ben) in order to suppress mycorrhizal ERM development and to create a control for possible photosynthetic fixation of $^{13}CO_2$ released by microorganisms decomposing the maize roots in the substrate. The second subset (+C_4–Ben), as well as pots prepared *de novo* (described later), received the same amount of distilled water. In the central hole of the remaining 12 pots, surface-sterilized (3 min in 7% calcium hypochlorite) roots were buried into rinsed and autoclaved clinoptilolite (2 pieces per pot). Twelve pots were simultaneously prepared *de novo*, each with one *S. strictiflora* planted and one membrane filter buried into rinsed and autoclaved clinoptilolite (-C_4-Ben). Each treatment variation was thus represented by 12 replicates.

The plants were kept in semi-controlled glasshouse at irradiance from 200 to 1250 µmol m^{-2} s^{-1}, temperature of 15/25 °C and relative humidity from 70 to 95 % until the experiment was finished. The pots were arranged randomly and the air in the glasshouse was agitated by an electric fan to minimize $^{13}CO_2$ gradients.

The orchids used were collected from a natural site in Portugal in 1996 and prior to experiments were kept in a cold glasshouse under conditions similar to these used during the experiment. During this period, they were never artificially inoculated with any fungus. It is thus highly probable that the mycorrhizal fungus associated with *S. strictiflora* in experiments is its natural endophyte. It was isolated from surface-sterilized (3 min in 7 % calcium hypochlorite) root segments on potato-dextrose agar. The isolated fungal strain was identified by sequencing of ITS regions of its rDNA. Briefly, the mycelium was scraped with a lancet from a margin of a colony actively growing on potato-dextrose agar and processed using the sorbitol method of DNA isolation (Štorchová *et al.* 2000). The isolated DNA was amplified using the ITS1F/ITS4 primer pair (Gardes and Bruns 1993) and sequenced by *GATC Biotech* (Konstanz, Germany). The ITS1-5.8SrDNA-ITS2 sequence obtained was manually edited in *SeqScanner 1.0* (*Applied Biosystems*, Foster City, CA, USA), compared to published sequences using a *BLAST* similarity search (Altschul *et al.* 1997) and deposited in GenBank as EU418851. The sequence (632 bp) showed 96 % similarity to *Epulorhiza* sp. SC 034 (GenBank AB369932) isolated from roots of *Cypripedium macranthos* var. *rebunense*, 96 % similarity to *Epulorhiza* sp. SO 035 (GenBank AB369931) isolated from roots of the same host plant, and 95 % similarity to uncultured *Tulasnellaceae* isolate 968 (GenBank DQ925661) from roots of *Cypripedium macranthos* var. *speciosum* (Shefferson *et al.* 2007). Thus, genotype analysis revealed that the isolate belonged to the genus *Epulorhiza*, known to encompass OM fungi (Ma *et al.* 2003).

The mycorrhizal status of the isolate was confirmed by back inoculation of surface-sterilized (1 min in 70 % ethanol, 20 min in 7 % calcium hypochlorite) seeds of *S. lingua* (species closely related to *S. strictiflora*), which were highly stimulated by the fungus during their early development. The strain Ep/Sst/07 is available from the corresponding author on request.

For $\delta^{13}C$ assessment, maize roots and leaves were harvested on February 24, 2004, whereas *S. strictiflora* organs (leaves, roots and newly developed storage tubers) and mycorrhizal ERM and maize roots for colonization assessment were harvested 106 d thereafter, on June 9, 2004. On that date, *S. strictiflora* plants were approximately in the middle of their flowering period and still showed no visible symptoms of transition into the dormant state. The plant tissue samples were dried at 80 °C to constant mass, homogenized using a ball mill and weighed (approx. 0.8 mg) into tin capsules for $\delta^{13}C$ analysis.

The mycorrhizal ERM for $\delta^{13}C$ assessment was extracted using a wet-sieving procedure. The whole volume of substrate from which all maize roots were carefully removed was stirred for 1 min in 5 dm^3 of distilled water and then left 10 s to allow the substrate to settle. The supernatant was passed through a 200-µm sieve over a 50-µm sieve. The material collected on the lower sieve was transferred into a small amount of distilled water in a 10-cm Petri dish, from which clusters of ERM were picked up under a stereomicroscope, carefully checking their purity, and placed into Eppendorf tubes using tweezers. The ERM collected was lyophilized and than weighed (approx. 0.8 mg) into tin capsules for $\delta^{13}C$ analysis. The carbon isotopic composition of all fungal and plant samples was determined using a *PDZ Europa ANCA-GSL* elemental analyzer interfaced to a *PDZ Europa 20-20* isotope ratio mass spectrometer (*Sercon*, Crewe, UK). The analyses were done at *UC* (Davis, CA, USA) stable isotope facility and results are expressed in standard notation as $\delta^{13}C$ (‰) relative to the *Pee Dee Belemnite* international standard. The measurement precision calculated as standard deviation of $\delta^{13}C_{standard}$ was 0.03 ‰ (n = 26).

Samples of maize roots and one root from each *S. strictiflora* plant were fixed in FAA for several days after harvesting and than transferred to 70 % ethanol, in which they were stored until further processing. Colonization of maize roots by OM fungus was assessed by microscopic examination (300× magnification) of roots cleared in 2.5 % KOH, acidified in 1 % HCl and stained with 0.05 % trypan blue in lactoglycerol (Koske and Gemma 1989). The results are expressed as a percentage of the maize root's length colonized by *Epulorhiza* sp. Orchid roots were processed according to Rasmussen and Whigham (2002); they were fixed in FAA for 3 d, transferred to 70 % ethanol for several days and than hand-sectioned. The slices were stained with 0.01 % acid fuchsin in lactoglycerol overnight at ambient temperature. Three segments of each root were sectioned: a basal segment (5 - 10 mm from the root base), a middle segment (middle of the root) and an apical segment (5 - 10 mm from the root apex). Ten whole thin sections from each root/distance combination were mounted in glycerol and observed under an *Olympus BX-50* microscope at low magnification (40×). Mycorrhizal colonization was estimated as the percentage of mesodermal cells occupied by intact or collapsed pelotons of all mesodermal cells suitable for formation of pelotons (outer cortical layers where the fungus never formed pelotons and inner cortical layers including the endodermis that were never colonized despite massive colonization of middle cortical layers were not considered as layers suitable for formation of pelotons).

The mycelia of fungi grown in the substrate were extracted using the inserted membrane technique (Baláž and Vosátka 2001). They were stained with 0.05 % trypan blue in lactoglycerol and used for quantification of mycorrhizal ERM length. This was assessed from digital images (20 images per sample, each at a resolution of 2048 × 1536 pixels corresponding to 1.74 mm^2 of the filter area) using a computer-aided grid-line intersect method. The results are expressed as hyphal length per unit filter area.

The relative contribution of saprotrophy to C nutrition of mycorrhizal ERM (C_S) was calculated using a linear two-source isotopic mixing model (Gebauer and Meyer 2003). As the first step, ^{13}C enrichment of mycorrhizal ERM biomass relative to orchid root tissues ($\varepsilon_{ERM\text{-}Ser}$) was calculated according to:

$\varepsilon_{ERM\text{-}Ser} = \delta^{13}C_{ERM(-C4\text{-}Ben)} - \delta^{13}C_{Ser.roots(-C4\text{-}Ben)}$

where $\delta^{13}C_{ERM(-C4\text{-}Ben)}$ is carbon isotopic composition of orchid mycorrhizal ERM from -C_4-Ben treatment and $\delta^{13}C_{Ser.roots(-C4\text{-}Ben)}$ is carbon isotopic composition of orchid roots from -C_4-Ben treatment.

The linear two-source isotopic mixing model was then determined as:

$Cs\ [\%] = [(\delta^{13}C_{ERM+C4+Ben} - \varepsilon_{ERM\text{-}Ser}) - \delta^{13}C_{Ser.roots+C4+Ben} / (\delta^{13}C_{Zea.roots} - \delta^{13}C_{Ser.roots+C4+Ben})] \times 100$

where $\delta^{13}C_{ERM+C4+Ben}$ is carbon isotopic composition of orchid mycorrhizal ERM from +C_4+Ben treatment, $\varepsilon_{ERM\text{-}Ser}$ is enrichment of mycorrhizal ERM biomass relative to orchid root tissues and $\delta^{13}C_{Zea roots}$ is carbon isotopic composition of maize roots. This calculation assumes that ^{13}C enrichment of mycorrhizal ERM in comparison to the organic material utilized as its C source would be the same for both biotrophy and saprotrophy.

The δ^{13}C values of plant tissues and mycorrhizal ERM were subjected either to Welch *t*-tests with separate variance estimates and approximate degrees of freedom or to one-way *ANOVA*. The t-test assumptions were checked using an *F*-test (variance homogeneity) and a Shapiro-Wilk test (normality of residuals or differences). The ANOVA assumptions were checked using Cochran, Hartley and Bartlett tests (variance homogeneity), a normal probability plot and a Shapiro-Wilk test (normality of residuals assumption). In all tests calculated, the null hypothesis was rejected if $P < 0.05$. The mean and standard error values were calculated from 12 replicates. All calculations were performed using *Statistica 6* software (*StatSoft*, Tulsa, OK, USA).

Results

The orchids in all treatments grew well, showing no symptoms of root necrosis. Depending on their size, the plants formed three to five healthy roots and two or three new tubers. At the end of the experiment, 21 of 36 plants were flowering, forming one to four flowers. The OM developed well in all treatments, including -C_4-Ben (Fig. 1*A*), in which the fungus fed solely on healthy orchid roots, *i.e.*, by means of biotrophy. In this treatment, the ERM length was 1.73 ± 0.46 m cm^{-2} and mean colonization of orchid roots (calculated as arithmetic mean from colonization of basal, middle and apical root segments; Table 1) was 34 ± 8 % for intact fungal pelotons and 51 ± 9 % for collapsed pelotons. In the +C_4-Ben treatment, a dematiaceous hyphomycete, probably *Ramichloridium* sp. or relative to this genus, was found in the *S. strictiflora* mycorrhizosphere (Fig. 1*B*,*C*). It was distinguishable from the OM *Epulorhiza* by the presence of characteristic dark pigmented conidiophores and conidia (Domsch *et al.* 1993). However, the linear vegetative hyphae of the contaminating fungus and *Epulorhiza* sp. stained with trypan blue were often hardly distinguishable, even though relatively intact mycelia were available using the inserted membrane technique. The length of hyphae in the +C_4-Ben treatment (2.51 ± 0.44 m cm^{-2}) thus refer not only to the mycorrhizal ERM, but also to the contaminating fungus. Moreover, we were not able to separate this contaminating fungus from the mycorrhizal ERM collected using wet sieving, and fungal samples from this treatment were thus excluded from δ^{13}C analysis. However, orchid tissues from the +C_4-Ben treatment were analyzed for C isotopic composition, as no signs of contaminating fungus were found in the orchid roots (Fig. 1*D*); these values were not used for evaluation of the ERM nutrition in any step. Colonization of orchid roots in the +C_4-Ben treatment was 18 ± 4 % for intact pelotons and 58 ± 7 % for collapsed pelotons.

For the +C_4+Ben treatment, OM symbiosis was unexpectedly not completely suppressed by the fungicide application. The mycorrhizal ERM length was 0.35 ± 0.03 m cm^{-2}, which was significantly different from the length for -C_4-Ben treatment (t = 3.0, df = 11.11, P = 0.012). Mycorrhizal fungus occupied 33 ± 5 % of the mesodermal cells in the form of intact pelotons and 42 ± 3 % in the form of collapsed pelotons. One-way *ANOVA* of root colonization data for -C_4-Ben, +C_4-Ben and +C_4+Ben treatments revealed no significant differences for the percentage of cortical cells occupied by intact pelotons (F = 2.53, df_{error} = 31, P = 0.096) or collapsed pelotons (F = 0.87, df_{error} = 31, P = 0.43). No

Table 1. Colonization of basal, middle and apical region of *Serapias strictiflora* roots by mycorrhizal fungus *Epulorhiza* sp. expressed a percentage of cortical cells occupied by intact or collapsed pelotons The plants were cultivated either the presence of decaying maize roots in the mycorrhizosphere (+C_4) or not (-C_4) and with (+Ben) or without not (-Ben) fungicide benomyl. Means ± SE, n = 12.

Treatment	Root segment	Intact pelotons [%]	Collapsed pelotons [%]
+C_4-Ben	basal	20 ± 7	76 ± 6
	middle	19 ± 5	65 ± 9
	apical	14 ± 7	33 ± 12
+C_4+Ben	basal	29 ± 8	44 ± 7
	middle	42 ± 6	45 ± 4
	apical	29 ± 7	37 ± 6
-C_4-Ben	basal	37 ± 10	61 ± 10
	middle	43 ± 12	56 ± 12
	apical	22 ± 7	37 ± 12

Table 2. Carbon isotopic composition ($\delta^{13}C$) of *Zea mays* and *Serapias strictiflora* tissues and of *Epulorhiza* sp. extraradical mycelium (ERM). The treatments were the same as in Table 1. Means ± SE, *n* = 12.

Species	Sample	Treatment	$\delta^{13}C$ [‰]
Zea mays	leaves	-Ben	-15.2 ± 0.1
	roots	-Ben	-14.6 ± 0.1
Serapias strictiflora	leaves	$+C_4$-Ben	-31.5 ± 0.1
		$+C_4$+Ben	-30.4 ± 0.2
		$-C_4$-Ben	-31.1 ± 0.2
	roots	$+C_4$-Ben	-28.6 ± 0.1
		$+C_4$+Ben	-28.2 ± 0.2
		$-C_4$-Ben	-28.6 ± 0.1
	new tubers	$+C_4$-Ben	-28.7 ± 0.1
		$+C_4$+Ben	-28.2 ± 0.1
		$-C_4$-Ben	-28.6 ± 0.2
Epulorhiza sp.	ERM	$-C_4$-Ben	-26.3 ± 0.2
		$+C_4$+Ben	-21.6 ± 0.4

contaminating fungus was observed for this treatment using either inserted membrane extraction or wet sieving.

The inoculation of maize roots with surface-sterilized *S. strictiflora* roots was unsuccessful. No mycorrhizal mycelium was extracted using the buried membrane or by wet sieving, but a large amount of contaminating fungus was recovered. No samples from this treatment were thus analyzed for isotopic composition or hyphal length.

Decaying maize roots were colonized by OM *Epulorhiza* in both treatments in which they occurred simultaneously, *i.e.*, $+C_4$-Ben and $+C_4$+Ben. Characteristic septate hyphae and chains or loose clusters of monilioid cells were observed in peripheral layers or maize roots, *i.e.*, in the rhizodermis and outer cortical layers (Fig. 1*E*,*F*). However, the presence of contaminating fungus precluded proper quantification of the relative length of maize roots colonized by OM fungus in the $+C_4$-Ben treatment. For the $+C_4$+Ben treatment, *Epulorhiza* colonized 18 ± 3 % of the maize root length.

Carbon isotopic composition of *Z. mays* and

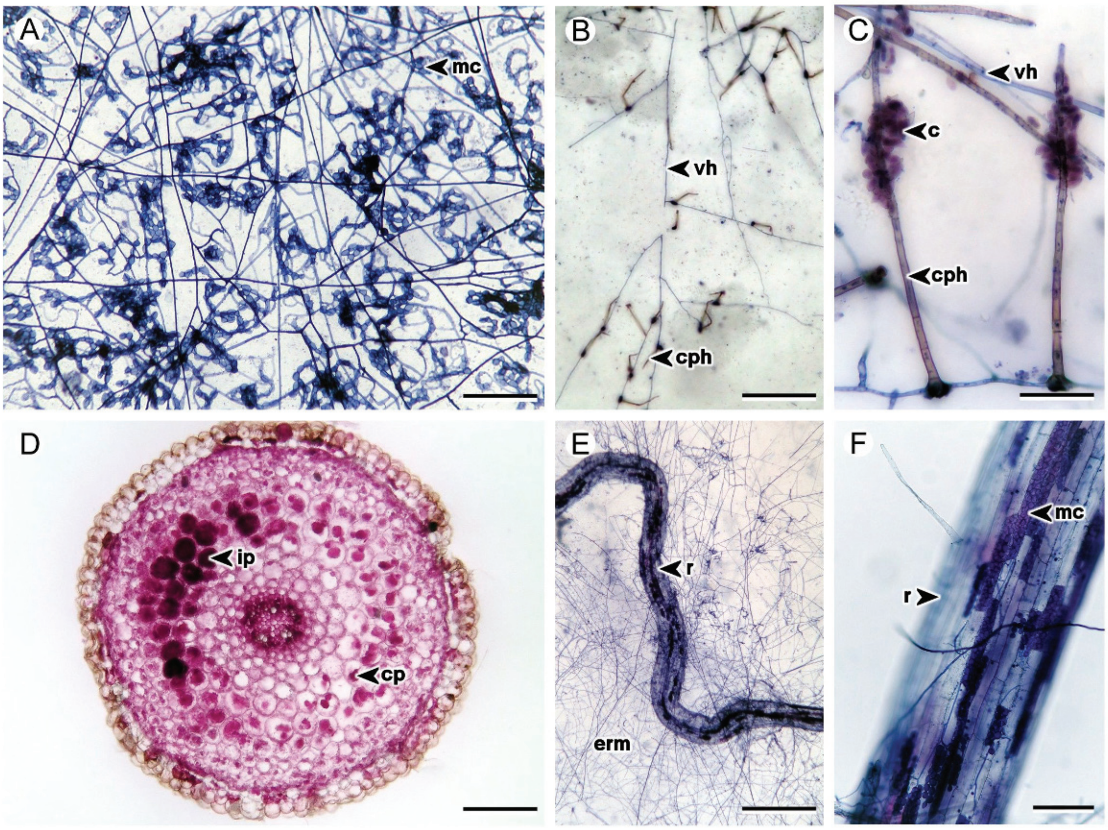

Fig. 1. Structures formed by mycorrhizal *Epulorhiza* sp. associated with *Serapias strictiflora*, and by a dematiaceous hyphomycete, probably *Ramichloridium* sp. *A* - Extraradical mycelia of *Epulorhiza* sp. extracted from the mycorrhizosphere of $-C_4$-Ben treated *S. strictiflora* plant using IMT and stained with trypan blue (*bar* = 100 µm). *B*, *C* - Dematiaceous hyphomycete, probably *Ramichloridium* sp., extracted from the mycorrhizosphere of $+C_4$-Ben treated *S. strictiflora* plant using IMT. Dark pigmented conidiophores and conidia are not stained with trypan blue, while the vegetative hyphae are stained well (*bar* = 250 µm in *B* and 25 µm in *C*). *D* - Mycorrhizal colonization of $+C_4$-Ben treated *S. strictiflora* root. Hand cross section, stained with acid fuchsin (*bar* = 300 µm). *E*, *F* - *Epulorhiza* sp. colonizing decaying maize roots within the rhizosphere of $+C_4$-Ben treated *S. strictiflora* plants (stained with trypan blue, *bar* =500 µm in *E* and 100 µm in *F*). c - conidium, cp - collapsed peloton, cph - conidiophore, erm - extraradical mycelium, ip - intact peloton, mc - monilioid cell, r - root, vh - vegetative hypha.

S. strictiflora tissues (Table 2) shows patterns typical for C_3 and C_4 photosynthetic pathways, as well as for relative enrichment of plant heterotrophic underground organs compared to autotrophic leaves. While δ^{13}C of maize leaves was -15.2 ± 0.1 ‰ and the roots were enriched in ^{13}C by 0.6 ‰, the corresponding values for *S. strictiflora* plants (calculated as overall mean from +C_4-Ben, +C_4+Ben and -C_4-Ben treatments) were -31 ± 0.2 ‰ and 2.5 ‰ enrichment, which almost perfectly fits the mean enrichment ($\varepsilon_{\text{roots-bulk leaves}}$ = 2.4) calculated for C_3 plants from several studies in the review by Bowling *et al.* (2008). These findings are, however, beyond the scope of the present study and were thus not evaluated statistically. The comparison of isotopic composition of roots and new storage tubers of *S. strictiflora* from all three treatments provides evidence of predominant autotrophy in carbon nutrition of *S. strictiflora*, even when the fungus had access to decaying maize roots. Evaluation using one-way *ANOVA* revealed that the δ^{13}C values of roots from different treatments were not significantly different ($F = 2.0$, $df_{error} = 33$, $P = 0.152$), nor were the δ^{13}C values of new tubers from these three treatments ($F = 2.9$, $df_{error} = 33$, $P = 0.072$).

Evaluation of the δ^{13}C values of mycorrhizal ERM using a Welch *t*-test revealed that the ERM enrichment in ^{13}C for the +C_4+Ben treatment compared to -C_4-Ben (ε = 4.7 ‰) was statistically significant ($t = 10.95$, $df = 14.34$, $P < 0.001$). By fitting mean δ^{13}C values for *S. strictiflora* root tissues and mycorrhizal ERM subjected to -C_4-Ben treatment, in which the OM fungus met its C demand *via* biotrophy, it was calculated that the ERM was enriched in ^{13}C by 2.3 ‰ compared to the roots. Assuming the same pattern of enrichment for +C_4+Ben treatment, in which the OM fungus colonized decaying maize roots and thus also obtained nutrients by saprotrophy, the percentage of carbon derived by the ERM from saprotrophy was calculated as 31 ± 3 %.

Discussion

The mystery of whether mycorrhizal fungi gain anything from host orchids in OM associations persisted for over a century. The matter was often debated, but experimental evidence was generally lacking (Smith and Read 1997). Smith (1967) observed spreading of OM fungus from non-photosynthetic *Dactylorhiza purpurella* seedlings onto a sugar-free medium, but this could be explained by reallocation of resources within the living hyphae (Rasmussen 1995). Further attempts to demonstrate a flow of carbon compounds from the orchid to the fungus were also unsuccessful (Hadley and Purves 1974, Purves and Hadley 1975, Alexander and Hadley 1985). Cameron *et al.* (2006) made the first breakthrough. They demonstrated that 2.6 % of $^{14}CO_2$ photoassimilated by *Goodyera repens* was readily (over 72 h) translocated to the ERM of the associated fungus *Ceratobasidium cornigerum*. This finding was recently confirmed in another experiment using the same mycorrhizal association (Cameron *et al.* 2008). Despite the fact that carbon flux from the orchid to the mycorrhizal fungus was quantified in these two studies, some aspects of this phenomenon, such as its occurrence in tuberous orchids, the major group of European orchids, remains unrevealed. Moreover, some authors still call this direction of carbon flow into question (Rasmussen and Rasmussen 2009).

In the present study we addressed this issue using a different approach. We cultivated *S. strictiflora* associated with *Epulorhiza* sp. in a substrate either lacking or containing decaying maize roots and assessed mycorrhiza development and carbon isotopic composition of C_3 (*S. strictiflora*) and C_4 (maize) plant tissues and of the mycorrhizal ERM. The results obtained indicate: *1*) full biotrophy of the mycorrhizal fungus *Epulorhiza* sp. in the absence of organic matter in the cultivation substrate; *2*) mixotrophy of *Epulorhiza* sp. (biotrophy + saprotrophy) when the cultivation substrate contained organic matter; and *3*) autotrophy as the predominant nutritional mode in mature *S. strictiflora* plants even if they were cultivated in substrate containing decomposing maize roots.

The main aim of our experiment was to quantify the carbon nutrition of the mycorrhizal ERM. We utilized the knowledge that OM develops well even if the whole association is cultivated in pure mineral substrate (clinoptilolite) lacking organic matter, thus preventing OM fungal saprotrophy (Baláž and Vosátka 2001). Under these conditions, the mycorrhizal fungus met its carbon demands *via* biotrophy, as confirmed by δ^{13}C values. The ^{13}C enrichment of mycorrhizal ERM compared to *S. strictiflora* root tissues (2.3 ‰) reflects its nutritional dependence on orchid roots. This value is comparable to the 1.5‰ enrichment of ectomycorrhizal fungi relative to the fine roots of *Pinus sylvestris* (Hobbie and Colpaert 2004) and to the mean 2.3 ‰ enrichment calculated from seven different ectomycorrhizal associations (Boström *et al.* 2008). The good ERM development under conditions precluding saprotrophic nutrition (-C_4-Ben) implies sufficient transfer of carbon for the OM fungus nutrition from the orchid and full fungal biotrophy.

Wild terrestrial orchids usually grow in soils containing organic matter, and their mycorrhizal fungi are able to utilize a variety of carbon compounds for saprotrophic nutrition (for a review see Midgley *et al.* 2006). To investigate the nutrition of the OM fungus under more realistic environmental conditions, we co-cultivated *S. strictiflora* plants and *Epulorhiza* sp. in a substrate containing decaying maize roots. Under these conditions, the ERM biotrophy/ saprotrophy ratio (in terms of carbon origin) was approximately 2:1. This result is based on the assumption that the ^{13}C enrichment of mycorrhizal ERM in comparison to the organic material utilized as its carbon source is similar for both biotrophy and saprotrophy. We tried to measure the enrichment of ERM nourished saprotrophically, but our attempt to inoculate maize roots with surface-sterilized mycorrhizal *S. strictiflora* roots failed. However, considering that saprotrophic fungi are approximately 4 ‰ enriched in ^{13}C compared to the organic matter they use as their carbon source (Gleixner *et al.* 1993, Hobbie *et al.* 1999, Bowling *et al.* 2008), we suppose this assumption is fulfilled. To the best of our knowledge, the data presented here, which locate orchid mycorrhizal ERM along the biotrophy-saprotrophy continuum (Koide *et al.* 2008), provide the first evidence published and no direct comparison can be made even with the other mycorrhizal types (*e.g.*, ectomycorrhiza and ericoid mycorrhiza), in which the fungus may feed both biotrophically and saprotrophically (Johnson 2008).

In our experiment we were not able to demonstrate that myco-heterotrophy contributed significantly to the total carbon nutrition of adult *S. strictiflora* plants even if cultivated in substrate containing decaying maize roots. When $\delta^{13}C$ of new storage tubers, which just started to grow during the experiment and thus represented a sink for C compounds, were assessed, no significant differences were found. However, the experimental design and analytical technique we used are not suitable for determination of small amonunts of C, *i.e.* if myco-heterotrophy contributes only marginally to the orchid C nutrition. It is possible that *S. strictiflora* plants in our experiment received low amounts of C compounds from the associated fungus as was demonstrated for *G. repens* (Cameron *et al.* 2006, 2008), but we were not able to detect it. We thus conclude that autotrophy is the predominant nutritional mode of mature tuberous *S. strictiflora* plants.

The autotrophy/myco-heterotrophy ratio of adult green-leaved orchids has already been quantified. *G. repens* was reported to be fully autotrophic on the basis of experiments tracking radioactivity from ^{14}C-labelled insoluble sugars (Alexander and Hadley 1985) and on the basis of ^{13}C natural abundances in plant tissues (Hynson *et al.* 2009). In two studies on *G. repens* by Cameron *et al.* (2006, 2008) transfer of [^{14}C]glycine or [^{14}C]amino acids mixture from the fungus to the plant was detected. These experiments clearly demonstrate that different C compounds can be transferred in opposite directions between mycorrhizal partners at the same time. The proper quantification of net carbon compound transfer between the orchid and fungus thus requires simultaneous application of an organic compound mixture that best reflects the composition available to OM fungi in the field. Moreover, all these compounds must be homogeneously labeled with either ^{13}C or ^{14}C. To address this issue, we used the difference in natural ^{13}C abundance between C_3 and C_4 plants for quantification. A similar approach has already been used many times (for a review see Fry 2006), and its easy implementation makes it promising for future experimentation with OM and other mycorrhizal types.

Benomyl was applied in order to suppress the OM and mainly ERM development. However, this expectation was not fulfilled as the suppression of ERM growth was not complete. The lack of benomyl efficiency, reported elsewhere by Čuříková *et al.* (2009), was ultimately beneficial because it allowed us to collect ERM samples for the assessment of ERM mixotrophy.

We observed that *Epulorhiza* readily colonized the decaying maize roots after removal of the above-ground parts. It is known that OM fungi can use a wide spectrum of organic compounds for their C nutrition, but there is almost no information about the colonization of live or decaying roots of other plants. The presence of orchidaceous rhizoctonias in pot cultures of arbuscular mycorrhizal fungi was reported by Williams (1984), but the actual extent of such interaction in nature remains unexplored. If OM fungi can efficiently colonize decaying roots of accompanying plants, this can have far-reaching consequences for both the orchid, which may benefit from indirect access to the additional source of nutrients, and the species being invaded, for which a negative influence is the most likely scenario. This may strongly influence the competitiveness of orchids in natural habitats and the establishment of orchid seedlings (Rasmussen 1995).

References

Alexander, C., Hadley, G.: Carbon movement between host and mycorrhizal endophyte during the development of the orchid *Goodyera repens* Br. - New Phytol. **101**: 657-665, 1985.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J.: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. - Nucl. Acids Res. **25**: 3389-3402, 1997.

Baláž, M., Vosátka, M.: A novel inserted membrane technique for studies of mycorrhizal extraradical mycelium. - Mycorrhiza **11**: 291-296, 2001.

Bidartondo, M.I., Burghardt, B., Gebauer, G., Bruns, T.D., Read, D.J.: Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaison between forest orchids and trees. - Proc. roy. Soc. London B **271**: 1799-1806, 2004.

Boström, B., Comstedt, D., Ekblad, A.: Can isotopic fractionation during respiration explain the ^{13}C-enriched sporocarps of ectomycorrhizal and saprotrophic fungi? - New Phytol. **177**: 1012-1019, 2008.

Bowling, D.R., Pataki, D.E., Randerson, J.T.: Carbon isotopes in terrestrial ecosystem pools and CO_2 fluxes. - New Phytol. **178**: 24-40, 2008.

Cameron, D.D., Johnson, I., Read, D.J., Leake, J.R.: Giving and receiving: measuring the carbon cost of mycorrhizas in the green orchid, *Goodyera repens*. - New Phytol. **180**: 176-

184, 2008.
Cameron, D.D., Leake, J.R., Read, D.J.: Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. - New Phytol. **171**: 405-416, 2006.
Čuříková, M., Látr, A., Vosátka, M.: Growth and viability of mycorrhizal extraradical mycelia associated with three temperate orchid species. - Biologia (Sect. Bot.) **64**: 63-68, 2009.
Dearnaley, J.D.W.: Further advances in orchid mycorrhizal research. - Mycorrhiza **17**: 475-486, 2007.
Domsch, K.H., Gams, W., Anderson, T.H. (ed.): Compendium of Soil Fungi. - IHW-Verlag, Eching 1993.
Fry, B. (ed.): Stable Isotope Ecology. - Springer, New York 2006.
Gardes, M., Bruns, T.D.: ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. - Mol. Ecol. **2**: 113-118, 1993.
Gebauer, G., Meyer, M.: ^{15}N and ^{13}C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. - New Phytol. **160**: 209-223, 2003.
Gleixner, G., Danier, H.J., Werner, R.A., Schmidt, H.L. Correlations between the ^{13}C content of primary and secondary plant products in different cell compartments and that in decomposing Basidomycetes. - Plant Physiol. **102**: 1287-1290, 1993.
Hadley, G., Purves, S.: Movement of ^{14}carbon from host to fungus in orchid mycorrhiza. - New Phytol. **73**: 475-482, 1974.
Hobbie, E.A., Colpaert, J.V.: Nitrogen availability and mycorrhizal colonization influence water use efficiency and carbon isotope patterns in *Pinus sylvestris*. - New Phytol. **164**: 515-525, 2004.
Hobbie, E.A., Macko, S.A., Shugart, H.H.: Insights into nitrogen and carbon dynamics of ectomycorrhizal and saprotrophic fungi from isotopic evidence. - Oecologia **118**: 353-360, 1999.
Hynson, N.A., Preiss, K., Gebauer, G.: 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*. - New Phytol. **182**: 8-11, 2009.
Johnson, D.: Resolving uncertainty in the carbon economy of mycorrhizal fungi. - New Phytol. **180**: 3-5, 2008.
Julou, T., Burghardt, B., Gebauer, G., Berveiller, D., Damesin, C., Selosse, M.A.: Mixotrophy in orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals of *Cephalanthera damasonium*. - New Phytol. **166**: 639-653, 2005.
Koide, R.T., Sharda, J.N., Herr, J.R., Malcom, G.M.: Ectomycorrhizal fungi and the biotrophy-saprotrophy continuum. - New Phytol. **178**: 230-233, 2008.
Koske, R.E., Gemma, J.N.: A modified procedure for staining roots to detect VA mycorrhizas. - Mycol. Res. **92**: 486-505, 1989.
Leake, J.R.: The biology of myco-heterotrophic (saprophytic) plants. - New Phytol. **127**: 171-216, 1994.
Leake, J.R.: Myco-heterotroph/epiparasitic plant interactions with ectomycorrhizal and asrbuscular mycorrhizal fungi. - Curr. Opin. Plant Biol. **7**: 422-428, 2004.
Ma, M., Tan, T.K., Wong, S.M.: Identification and molecular phylogeny of *Epulorhiza* isolates from tropical orchids. - Mycol. Res. **107**: 1041-1049, 2003.
Midgley, D.J., Jordan, L.A., Saleeba, J.A., McGee, P.A.: Utilisation of carbon substrates by orchid and ericoid mycorrhizal fungi from Australian dry sclerophyll forests. - Mycorrhiza **16**: 175-182, 2006.
Purves, S., Hadley, G.: Movement of carbon compounds between the partners in orchid mycorrhizas. - In: Sanders, F.E., Mosse, B., Tinker, P.B. (ed.): Endomycorrhizas. Pp. 173-194. Academic Press, London 1975.
Rasmussen, H.N. (ed.): Terrestrial Orchids: from Seed to Mycotrophic Plant. - Cambridge University Press, Cambridge 1995.
Rasmussen, H.N., Rasmussen, F.N.: Orchid mycorrhiza: implications of a mycophagous life style. - Oikos **118**: 334-345, 2009.
Rasmussen, H.N., Whigham, D.F.: Phenology of roots and mycorrhiza in orchid species differing in phototrophy strategy. - New Phytol. **154**: 797-807, 2002.
Selosse, M.-A., Roy, M.: Green plants that feed on fungi: facts and questions about mixotrophy. - Trends Plant Sci. **14**: 64-70, 2009.
Shefferson, R.P., Taylor, D.L., Weiss, M., Garnica, S., McCormick, M.K., Adams, S., Gray, H.M., McFarland, J.W., Kull, T., Tali, K., Yukawa, T., Kawahara, T., Miyoshi, K., Lee, Y.I.: The evolutionary history of mycorrhizal specificity among lady's slipper orchids. - Evolution **61**: 1380-1390, 2007.
Smith, S.E.: Carbohydrate translocation in orchid mycorrhizas. - New Phytol. **66**: 371-378, 1967.
Smith, S.E., Read, D.J. (ed.): Mycorrhizal Symbiosis. 2nd Edition. - Academic Press, London 1997.
Smith, S.E., Read, D.J. (ed.): Mycorrhizal Symbiosis. 3rd Edition. - Academic Press, London 2008.
Štorchová, H., Hrdličková, R., Chrtek, J., Tetera, M., Fitze, D., Fehrer, J.: An improved method of DNA isolation from plants collected in the field and conserved in saturated NaCl/CTAB solution. - Taxon **49**: 79-84, 2000.
Williams, P.G.: Orchidaceous rhizoctonias in pot cultures of vesicular-arbuscular mycorrhizal fungi. - Can. J. Bot. **63**: 1329-1333, 1984.
Zimmer, K., Hynson, N.A., Gebauer, G., Allen, E.B., Allen, M.F., Read, D.J.: Wide geographical and ecological distribution of nitrogen and carbon gains from fungi in pyroloids and monotropoids (Ericaceae) and in orchids. - New Phytol. **175**: 166-175, 2007.