# Mycorrhizas of *Cephalanthera longifolia* and *Dactylorhiza majalis*, two terrestrial orchids

Aleš Látr<sup>1,\*</sup>, Martina Čuříková<sup>1</sup>, Milan Baláž<sup>2</sup> & Jaroslav Jurčák<sup>1</sup>

<sup>1)</sup> Department of Botany, Faculty of Science, Palacky University, Slechtitelu 11, CZ-783 71 Olomouc-Holice, Czech Republic (\*corresponding author's e-mail: ales.latr@seznam.cz)

<sup>2)</sup> Department of Experimental Biology, Faculty of Science, Masaryk University, Kotlarska 2, CZ-611 37 Brno, Czech Republic

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Dynamics of mycorrhizal colonization of two orchid species, rhizomatous *Cephalanthera longifolia* and tuberous *Dactylorhiza majalis*, was studied during two growing seasons, with special emphasis on the occurrence of collapsed pelotons. No complete lysis of pelotons in digestion cells was found. Mycorrhizal colonization of subterranean organs of both species was observed to take place even before aerial shoots developed, indicating that colonization is not restricted to leafy season, and was constantly present throughout the growing seasons with no distinct coincidence with flowering or fruiting time. Mycorrhizal colonization of *C. longifolia* was patchy and of low degree (mean = 4.4%, SE = 0.3%). It occurred mostly in the distal parts of main roots, lateral roots and/or in isolated root parts. Intensity of mycorrhizal colonization of *D. majalis* was generally higher (mean = 12.1%, SE = 0.7%). Tubers lacked colonization except in the root-like extensions.

Key words: *Cephalanthera*, colonization, *Dactylorhiza*, peloton lysis, orchid mycorrhiza, symbiosis

## Introduction

Regular occurrence of mycorrhizal fungi within orchid tissues has been well documented for over a century (Bernard 1909, Burgeff 1909). However, until now we have insufficient information about the roles that mycorrhizas play in compound exchange between the host and its symbiotic fungus. It is widely accepted that during early developmental stages of all orchids the fungus supplies carbon compounds. This is a prerequisite for the successful development of an orchid seed into a protocorm and then into a young plantlet, because orchid seeds possess only very limited reserves (Peterson *et al.* 1998). The fungus obtains carbon compounds either saprotrophically (Hadley & Perombelon 1963, Hadley 1969, Midgley *et al.* 2006) or from ectomycorrhizal symbiosis with trees, which in some cases it is also able to form (Zelmer & Currah 1995, McKendrick *et al.* 2000, Gebauer & Meyer 2003). This kind of plant nourishment, known as myco-heterotrophy, persists in some orchid species lacking chlorophyll in the adult stage, e.g. Cephalanthera austinae, which receives nutrients required for growth and development from its mycobionts belonging to Thelephoraceae (Taylor & Bruns 1997). Most adult orchid species, however, are capable of photosynthesis. There is some evidence that upon these conditions mycorrhizal fungi and green orchids are mutually independent as regards their carbon demand (Hadley & Purves 1974, Smith 1967, Cameron et al. 2006) and that the main function of mycorrhizal symbiosis is to enhance the mineral nutrition of the host plants (mainly of nitrogen and phosphorus) (Alexander et al. 1984, Smith & Read 1997, Cameron *et al.* 2007), a situation well known in other mycorrhizal types, e.g. arbuscular, ecto- or ericoid mycorrhizas. Despite this experimental evidence, some authors have speculated that even in these circumstances the fungus might supply the orchid with carbohydrates, which can supplement the photosynthetically fixed carbon. The possibility that the green orchid can supply associated mycorrhizal fungus with its own carbon compounds was often discussed and in some cases also experimentally studied, for a long time without success. Cameron et al. (2006) were the first to show that an orchid could supply the mycorrhizal fungus with a measurable amount of photosynthetically fixed carbon.

Although a considerable amount of information has already been published on orchid mycorrhizas, there is little evidence for a possibility that hyphal pelotons may completely disappear during their disintegration within host cells. Rasmussen and Whigham (2002) first observed this phenomenon in Corallorhiza odontorhiza, a chlorophyll-deficient, presumably fully mycoheterotrophic species, as well as in the summergreen orchid Galearis spectabilis, which at least supplements its myco-heterotrophic nutrition with photosynthetic CO<sub>2</sub> assimilation. If true, a complete lysis of the pelotons is essential for proper understanding of the functioning of orchid mycorrhizal symbiosis, especially regarding the myco-heterotrophic nutrition of orchids. For this reason we studied in detail the dynamics of the mycorrhizal colonization in two summergreen orchid species, Cephalanthera longifolia and Dactylorhiza majalis, during two growing seasons, with special emphasis on the occurrence of collapsed pelotons. We tested the possibility that different patterns of mycorrhizal colonization occur during flowering and/or seed maturation and during the rest of the growing season. Root phenology of the species was also recorded.

## Material and methods

This study was carried out on plants from two localities near Vsetin, in the Zlin region of the Czech Republic, from April 2004 to September 2005. The investigated population of Cephalanthera longifolia, with approximately ten plants per m<sup>-2</sup> occurs in a Fagus sylvatica forest (49°21'N, 18°02'E; elevation 480 m above sea level), where Carex pilosa is abundant and two other orchid species, Neottia nidus-avis and Orchis mascula subsp. signifera are also relatively abundant. The population of Dactylorhiza majalis with a usual abundance of two or three plants per m<sup>-2</sup> grows in a damp meadow (49°23'N, 18°01'E; elevation 530 m above sea level) together with other orchid species such as Listera ovata, Platanthera bifolia and Gymnadenia conopsea subsp. conopsea. The meadow is regularly mowed and/or grazed by cattle in June and July.

Both *C. longifolia* and *D. majalis* are summer-green species that produce green leafy shoots and inflorescences in spring and overwinter underground, either as a tuber (*D. majalis*) or as a rhizome with perennial roots (*C. longifolia*). Both species flower in May and June.

Owing to the protected status of the two species, a limited number of plants were harvested in accordance to a permit issued by the Regional authority of the Zlin region (KUZL 5898/2003 and KUZL 5947/2003). From two to eight mature specimens of *C. longifolia* were collected per sampling (May to September/October). Of *D. majalis*, two specimens were sampled once a week from the beginning of the growing period until the aerial shoots were visible (May to June–July). Due to the inaccessibility of the localities, no specimens were harvested during the winter.

Mycorrhizal colonization was evaluated on sections of all roots (D. majalis, C. longifolia),

tubers (D. majalis) and rhizomes (C. longifolia). Underground organs of each plant were carefully removed from the soil, washed in tap water, fixed in FAA for 48 hours and transferred to a mixture of glycerol and 90% ethanol (1:1, vol/vol). Transverse sections were made either using a manually operated microtome or by free hand. The sections were stained with an aqueous safranin and phluoroglucinol-HCl solution. Part of our study was made on unstained transverse sections. Transverse sections of rhizomes and roots except lateral ones were taken from three zones: apical (5-11 mm behind the root/rhizome tip, zone A), middle (in the middle of the root/ rhizome, zone B) and basal (zone C, 5-11 mm from the rhizome/tuber in the roots and from the rhizome end in the rhizome itself). In tubers proper and lateral roots of C. longifolia, sections were made approximately in the middle of the tuber/root length. One thin and complete section from each zone was then randomly picked for analysis. The sections were mounted in distilled water or glycerol and immediately observed using an Olympus BX-40 compound microscope.

According to Jurčák (2003), degree of mycorrhizal colonization was quantified as the proportion of the colonized cortical cells to all cortical cells assessed by counting cells in the whole section. Three types of colonized cortical cells were distinguished: passage cells with intact unbranched hyphae, host cells with a loose intact hyphal peloton assumed to be living, and digestion cells with an aggregate of poorly distinguishable hyphal structure, which were considered to be dead (Rasmussen & Whigham 2002). Relative proportions of these plant cell types to all colonized cortical cells were also calculated.

Overall degree of mycorrhizal colonization was assessed as a weighted mean of degrees of mycorrhizal colonization of all roots, root-like extensions and tubers proper per plant per sampling. Life span of rhizomes of *C. longifolia* was estimated by counting circles of bud scale scars. Differences in relative proportions of colonized cortical cells in root zones were statistically analyzed with ANOVA followed by a Tukey-Kramer multiple-comparison test ( $P \le 0.05$ ) to assess significance levels. A paired *t*-test was used to compare colonization of lateral and main roots of *C. longifolia*, and colonization between adventitious roots and root-like extensions of *D. majalis*. Analyses were performed using a NCSS software package. Counts of digestion cells in separate root zones were analyzed using hierarchical ANOVA, where the random effect *plant* (assigning roots to individual plants in every harvest) was tested within the fixed factor *harvest*. When the overall ANOVA table showed the effect *harvest* to be significant, then pairwise comparisons between means were made using a Scheffé test. Calculations were done using a Statistica 6 software package. Values are given as means  $\pm 1$  SE.

## Results

### Cephalanthera longifolia

Adventitious roots of C. longifolia showed patchy and very low overall degree of mycorrhizal colonization:  $4.5\% \pm 0.5\%$  (n = 26) in 2004 and  $4.3\% \pm 0.3\%$  (*n* = 26) in 2005 (Table 1), concentrated in the distal parts of the long roots, lateral roots and/or in short isolated root parts. Even partly decayed roots participated in a mycorrhizal symbiosis. Root color was found to be a good indicator of colonization degree. Light-whitish parts lacked or showed only poor colonization with only a few cortical cells containing pelotons, whereas dark root parts were regularly highly colonized. Up to 22-year-old unbranched subterranean rhizomes (Fig. 1) and many roots themselves completely lacked mycorrhizal fungi.

Brown, septate fungal hyphae started to enter new roots by penetrating rhizodermal cells or some of the root hairs when the roots reached a length of about 7–8 cm. The hyphae passed through a rhizodermis and passage cells in the outermost cortical layer and did not branch or form pelotons (Fig. 2a). The hyphae spread from one cell to another through attenuations in the cell walls. In host cells (Fig. 2b), typical for 2–3 outer layers of the cortex, the apparently intact hyphae branched and formed loose pelotons. The hyphae within the root cortex were hyaline to brown, smooth, always septate, with clamp connections, and on average 5  $\mu$ m in diameter.



Fig. 1. Life span of rhizomes of *Cephalanthera longifolia*, estimated by counting circles of bud scale scars. Data based on 48 specimens harvested during two growing seasons.

Larger digestion cells were typical for deeper parts of the cortex. Hyphae from neighboring cells were also seen to recolonize the digestion cells and thus two aggregates were sometimes seen in a cell. In host and digestion cells hypertrophied nuclei were observed (Fig. 2c).

Starch deposits in cortical cells disappeared when fungi colonized the cells. However, in a few cases the cortical cells were seen containing both an aggregate of collapsed fungal tissue typical for digestion cells and starch grains typical for storage cells, indicating that the storage function can be restarted during or immediately after breakdown of hyphae (Fig. 2c).

Distribution of the host and digestion cells in the cortex mostly followed a regular pattern of concentric layers. However, in some older roots the host cells were sometimes seen to be restricted to a few cortical cells, digestion cells filling up the rest of the cortex (Fig. 2c). No colonization was found in the innermost layers of the cortex or in the central cylinder.

In 2004, degree of mycorrhizal colonization was highest in the youngest root parts (zone A:  $16.7\% \pm 1.0\%$ ) and decreased towards the basal parts of the roots (zone C:  $0.6\% \pm 0.3\%$ ), whereas in 2005 the middle root parts (zone B:  $26.1 \pm 1.6\%$ ) showed the highest degree of mycorrhizal colonization, followed by the apical parts (zone A:  $24.2\% \pm 1.2\%$ ).

No statistically significant decrease in the digestion cell numbers was observed for *C*. *longifolia* during the course of both vegetation seasons in sections from all three root zones tested. There was high variability in the digestion cell numbers. The variance components attributable to the factor *plant* were low, the highest value found being only 6.9% of the total variability (Table 2).

In general, colonization of the basal root parts was rare, occurring only in older short roots. Digestion cells were the dominant form of the mycorrhizal cells in colonized roots. The highest relative proportion of digestion cells was observed in the middle parts of the roots. It decreased significantly ( $P \le 0.05$ ) towards root tips and rhizomes. The relative proportion of

**Table 1.** Overall degrees of mycorrhizal colonization in underground organs (adventitious roots, root-like extensionsand tubers proper) of *Cephalanthera longifolia* and *Dactylorhiza majalis* over two growing seasons. The values aremeans of all roots  $\pm$  1 SE from 2–8 plants per sampling time (%). \* data not available.

Sampling time		C. longifolia	D. majalis				
		4.0 ± 1.2	Overall degree	Adventitious roots	Root-like extensions		
2004	May		15.9 ± 2.0	17.5 ± 1.3			
	June	$5.0 \pm 0.5$	12.0 ± 1.4	$14.5 \pm 1.4$	$10.0 \pm 2.7$		
	July	3.6 ± 1.3	9.7 ± 1.9	13.3 ± 2.3	$6.14 \pm 2.3$		
	August	5.1 ± 2.2	*	*	*		
	September	5.2 ± 1.3	*	*	*		
	October	$3.7 \pm 0.3$	*	*	*		
2005	April	$5.2 \pm 0.4$	*	*	*		
	May	$3.9 \pm 0.4$	13.0 ± 1.3	12.0 ± 1.8	2.8 ± 2.1		
	June	$5.7 \pm 0.7$	10.6 ± 0.9	15.6 ± 1.4	$6.1 \pm 0.6$		
	July	3.3 ± 1.2	6.6 ± 1.5	$4.8 \pm 0.0$	$6.4 \pm 1.7$		
	August	$3.6 \pm 0.9$	15.9 ± 0.0	17.5 ± 1.1	13.9 ± 1.3		
	September	$4.5 \pm 0.3$	*	*	*		



**Fig. 2.** Transverse sections of adventitious roots of the studied orchid species. — **a**: Hypha penetrating the rhizodermis and proceeding into cortex of *Cephalanthera longifolia* root; rh = rhizodermis, co = cortex, hy = hypha, pa = passage cell, di = digestion cell. — **b**: Peloton formed of intact brown septate hyphae with clamp connections, filling the host cell in primary cortex of *C. longifolia* root; hc = host cell with peloton, arrows = clamp connections. — **c**: Root cortex of *C. longifolia* with collapsed pelotons and starch grains in digestion cells; rh = rhizodermis, co = cortex, di = digestion cell, sg = starch grains, cc = central cylinder with actinostele, arrows = hypertrophied nuclei. — **d**: Mycorrhizal colonization of *Dactylorhiza majalis* root; rh = rhizodermis, co = cortex, di = digestion cell, sg = starch grains in innermost cortical cells, cc = central cylinder; arrows = hypertrophied nuclei (**d**). Hand-made unstained transverse sections.

host cells decreased from the apical to basal root parts, and the relative proportion of the passage cells decreased in the opposite order (Table 3).

Degree of mycorrhizal colonization of short lateral roots was significantly higher (2004: mean  $\pm$  SE = 37.1  $\pm$  3.0, t = 9.5, df = 10,  $P \le 0.001$ ; 2005: mean  $\pm$  SE = 36.1  $\pm$  2.3, t = 7.5, df = 26,  $P \le 0.001$ ) as compared with the main roots' colonization. Digestion cells (69.1% of all colonized cells) followed by the host ones (23.2%) were most abundant in the cortex of lateral roots.

#### Dactylorhiza majalis

During spring, flattened and palmately divided daughter tubers of D. majalis developed on the stem base. During the first year, the tuber elongated and formed 2–4 root-like extensions, emerging from the distal end of the tuber proper. When the apical bud grew into a short stem, adventitious roots, which were the last to form, developed. They became colonized from the soil soon after their appearance (as soon as they

reached the length of at least 1 cm). The hyphae proceeding into the root cortex were hyaline, septate, unclamped and  $2-3 \mu m$  in diameter. The passage and host cells were typical for the outermost layers of the primary cortex, whereas the rest of the cortical cells were usually digestion cells, except in the innermost layer, which did not contain any fungal hyphae but starch grains (Fig. 2d). The finger-like extensions and the tubers proper were polystelic; each stele had its own endodermis. Parenchyma cells among the steles were often colonized by mycorrhizal fungi and contained collapsed pelotons of fungal tissues. However, newly grown root-like extensions did not form a mycorrhiza until July-August. Adventitious roots and root-like extensions of old tubers maintained the mycorrhizal fungus until decaying at the end of the growing season.

Overall degree of mycorrhizal colonization was approximately the same throughout the

growing seasons:  $12.2\% \pm 1.1\%$  (n = 16) in 2004 and  $11.9\% \pm 0.9\%$  (n = 20) in 2005. Degree of mycorrhizal colonization of root-like extensions was  $9.6\% \pm 1.0\%$ , whereas that of adventitious roots was  $13.7\% \pm 0.9\%$ . In 2004, the adventitious roots showed significantly higher degree of colonization than the root-like extensions (t = 3.34, df = 15, P = 0.004), whereas in 2005, degrees of colonization were approximately the same (t = 1.27, df = 19, P = 0.22) (Table 1). The tubers proper were not colonized at all.

In 2004, degree of mycorrhizal colonization of the roots increased from the basal parts (zone C: 9.9%  $\pm$  1.4%) to the apical ones (zone A: 19.5%  $\pm$  1.6%), whereas in the 2005 growing season, the middle root parts (zone B: 19.7%  $\pm$  1.7%) followed by the apical parts (zone A: 17.7%  $\pm$  2.0%) showed the highest degree of mycorrhizal colonization. The relative proportion of passage cells in the three defined root

Species	Sampling year	Root zone	df	F	Ρ	Coefficient of variance (%)	Variance component (%)
Cephalanthera longifolia	2004	А	12	0.79	0.66	29–149	5.2
		В	12	2.36	0.08	45–284	0
		С	12	0.79	0.65	0-226	6.9
	2005	А	12	1.03	0.48	31–90	2.8
		В	12	2.20	0.08	71-202	0
		С	12	0.48	0.89	0-412	4.7
Dactylorhiza majalis	2004	А	7	0.96	0.51	29-120	8.5
		В	7	0.58	0.76	42-100	21
		С	7	1.74	0.23	65–176	10.4
	2005	А	9	1.69	0.21	71–268	10.6
		В	9	1.82	0.18	45–131	0
		С	9	3.31	0.04	56–190	0

Table 2. Analysis of variance (hierarchical ANOVA) of the effect of counts of digestion cells in separate root zones of *Cephalanthera longifolia* and *Dactylorhiza majalis* in 2004–2005 growing seasons.

**Table 3.** Passage, host and digestion cells in root zones of *Cephalanthera longifolia* and *Dactylorhiza majalis* in 2004–2005 expressed as the relative proportions of colonized cortical cells (in %). Means of colonized roots ± 1 SE from 26 plants per growing seasons in *C. longifolia*; means of all roots and root-like extensions ± 1 SE from 16 plants per 2004 season and 20 plants per 2005 season in *D. majalis*.

Species	Zone	Passage cells	Host cells	Digestion cells
Cephalanthera longifolia	А	8.5 ± 2.9/8.8 ± 0.6	16.5 ± 3.5/13.0 ± 2.5	75.0 ± 4.1/78.2 ± 2.4
,	В	8.3 ± 1.0/9.4 ± 1.2	11.1 ± 1.9/8.0 ± 1.7	80.6 ± 2.2/82.6 ± 1.7
	С	19.8 ± 2.1/12.0 ± 1.7	4.5 ± 1.0/8.0 ± 1.8	75.7 ± 2.4/80.0 ± 2.5
Dactylorhiza majalis	А	4.9 ± 0.3/8.2 ± 1.4	13.1 ± 1.4/9.8 ± 1.7	82.0 ± 1.6/82.0 ± 1.8
, ,	В	5.2 ± 0.8/6.6 ± 0.8	8.4 ± 1.4/8.3 ± 1.8	86.4 ± 1.4/85.1 ± 2.1
	С	6.2 ± 1.0/7.7 ± 1.5	7.1 ± 1.8/5.6 ± 1.6	86.7 ± 2.3/86.7 ± 1.8

zones was almost equal, and the situation was similar for digestion cells. The relative proportion of the host cells significantly decreased ( $P \le 0.05$ ) from the apical to basal parts of the roots (Table 3).

No statistically significant decrease in the digestion cell numbers in the sections from all three root zones tested was observed for *D. majalis* during the growing seasons. In 2005 in root zone C, ANOVA showed a significant effect *harvest* (P = 0.037), but the Scheffé test did not reveal any statistically significant decrease in the digestion cell numbers during the growing season (Table 2). As in *C. longifolia*, there was a high variability in the digestion cell numbers. The variance components attributable to the factor *plant* were low; the highest value found was 21% of the total variability (Table 2).

## Discussion

Despite more than one century of research, remarkable gaps in our knowledge of orchid mycorrhiza persist. For example, the nature of carbon transfer between the fungus and the host remains unclear. Principally, two possibilities exist (Smith & Read 1997). Orchids can gain carbon compounds across the interface between the host cytoplasm and the intact hyphal coil within the host cell, i.e. biotrophically. There is one experimental study supporting this idea: the growth stimulus in Dactylorhiza purpurella protocorm occurred prior to degeneration of hyphal pelotons (Hadley & Williamson 1971). An alternative is a necrotrophical assimilation of fungal carbohydrates after the pelotons collapse, an idea originating from Burgeff (1936) and still often accepted. Some authors studied these processes using electron microscopy (Nieuwdorp 1972, Hadley 1975); however, although the methods seemed promising, they were not able to answer the question clearly.

Determining the ratio between myco-heterotrophy and autotrophy in orchid nourishment is thus a difficult task. Although direct measurement using radioisotopes or stable isotopes is possible, to our knowledge the studies of Smith (1967), Hadley and Purves (1974) and Cameron *et al.* (2006) are the only ones published to date; moreover, all of those studies were carried out *in vitro*. Some authors have attempted to correlate the extent of mycorrhizal colonization with the degree of myco-heterotrophy. However, this concept is apparently false because many orchid species are highly colonized even though they possess the leaf apparatus necessary for successful photosynthesis (*D. majalis*, Jurčák 2003). Furthermore, a flow of carbon from orchid to fungus was recently confirmed (Cameron *et al.* 2006).

Novel information has appeared rather recently (Rasmussen & Whigham 2002), suggesting that a complete lysis (disappearance) of pelotons in cortical cells may occur. That could be considered as indirect evidence that orchids gain fungal carbohydrates after peloton lysis rather than through a living interface. In this case, the mere presence of collapsed pelotons within roots implies that myco-heterotrophy plays a role in orchid carbon nourishment. However, even though the phenomenon described is important, the authors used a very vague technique to evaluate the extent of colonization (a six-step scale). For this reason we evaluated the development of mycorrhizae more precisely, by direct counting of all types of colonized and uncolonized cells within the root cortex. However, we did not confirm the findings of Rasmussen and Whigham (2002) because no statistically significant decline of collapsed pelotons from one sampling time to the next was recorded for C. longifolia or D. majalis. In the light of our finding, we will discuss our results from the point of view that the presence of collapsed pelotons does not imply participation and extent of myco-heterotrophy in orchid carbon nourishment.

In both studied orchid species the mycorrhizal colonization was constantly present with considerable fluctuations, but with no obvious coincidence with the flowering and/or seed maturation time throughout the growing seasons, which is in agreement with Rasmussen and Whigham (2002). Overall degree of mycorrhizal colonization of *C. longifolia* roots was lower as compared with that of *D. majalis* roots. The results thus confirmed that phototrophic rhizomatous orchid species are less colonized than tuberous ones (Tatarenko 2002). New roots of *C. longifolia* 

began to develop accordingly to shoot development suggesting that their main function might be uptake of water and mineral nutrients. Many roots of *C. longifolia*, especially young and short ones, often completely lacked mycorrhiza. The perennial roots serve as storage organs and, if colonized, can participate in myco-heterotrophy (Tatarenko 2003), which is not in agreement with the findings of Filipello Marchisio *et al.* (1985). In comparison with other *Cephalanthera* species, *C. longifolia* as well as *C. longibracteata* belong among the species with the least intense mycorrhizal colonization (Tatarenko 2002).

The tuberous species *D. majalis* formed mycorrhiza in adventitious roots soon after those roots appeared. Overall degree of mycorrhizal colonization was lower than that found by Jurčák (2003) being 28.5%. The palmately divided rootlike extensions of various orchid species are reported to develop mycorrhiza with the same degree as the roots (Fuchs & Ziegenspeck 1927, Cudlín 1974, Marakaev 2002, Tatarenko 2002). Our study showed that in the 2004 season the extensions were significantly less colonized by mycorrhizal fungi in comparison with the roots, whereas in 2005 the differences were not statistically significant.

There was very high variability in the digestion cell numbers in the individual root sections, from which only a small proportion (in *C. longifolia* less than 6.9%, in *D. majalis* less than 21%) was attributable to variations between individual plants. In other words, the colonization of roots in these species is very patchy within a singleroot system. For this reason, we recommend the approach where whole root systems of fewer plants should be assessed in detail instead of assessing more plants and making only few sections from a root system. A side effect of this approach is that fewer, usually rare, plants are needed to attain the same precision in the studies on mycorrhizal colonization.

To properly understand orchid mycorrhiza functioning and plasticity, it would be highly valuable to quantify myco-heterotrophy and autotrophy in a range of orchid species. The use of isotopes, as already mentioned, is possible but technically demanding. Combining the isotope method with mycorrhizal colonization assessment would bring novel insight into this type of symbiosis, because we have no idea about the possible correlation of myco-heterotrophy and autotrophy. So far, the simple assessment of the degree of myco-heterotrophy done by counting the collapsed pelotons, which seemed promising according to Rasmussen and Whigham (2002), is inadequate according to our results. However, further research using a wide spectrum of orchid mycorrhizal associations and highly accurate measurements based on many replicates is needed before the possibility and significance of a complete lysis of pelotons can be definitely rejected.

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