Phenology of roots and mycorrhiza in orchid species differing in phototrophic strategy

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Summary

• The mycorrhiza of orchids represents an energy source that may replace or supplement photosynthesis. Dependency on mycotrophy in adult life stages would thus be expected to be inversely related to the prevalence of phototrophic structures.
• The phenology of underground parts and mycorrhizal infection were monitored in five terrestrial species differing in leaf phenology (and thus in phototrophic strategy): *Goodyera pubescens* (evergreen), *Tipularia discolor* (wintergreen), *Galearis spectabilis* and *Liparis lilifolia* (summergreen) and *Corallorhiza odontorhiza* (chlorophyll deficient), growing sympatrically in a North American deciduous forest.
• Mycorrhizal infection was extensive in *T. discolor* roots and *C. odontorhiza* rhizomes. Only the proximal part of roots was infected in *G. pubescens,* and mycorrhizal colonisation was patchy in roots and tubers of *G. spectabilis* and localized in the rhizome in *L. lilifolia.* Mycotrophic roots were long-lived (1.5–3 yrs) determinate structures and mycorrhizal infection reached maximum intensity 2–6 months after development. Mycotrophy appeared to be active all year round in mature organs.
• The phenology of mycotrophic roots and patterns of mycorrhizal infection were not related to the leafy season. The hypothesis that phototrophic and mycotrophic nutrition alternate through the seasons could not be confirmed.

Key words: *Corallorhiza*, *Galearis*, *Goodyera*, heterotrophy, *Liparis*, terrestrial orchids, mycotrophy, Orchidaceae, *Tipularia*.

Introduction

The nutritional role of orchid mycorrhiza in the early development of seedlings and in the entire life cycle of chlorophyll-deficient species is well established (Burgeff, 1909; Rasmussen, 1995). There is direct evidence that carbon energy is transported from fungus to cultivated seedlings (Smith, 1966, 1967; Lan *et al.*, 1996), but attempts to demonstrate carbon transfer in the opposite direction have failed (Hadley & Purves, 1974; Purves & Hadley, 1975) or shown very low traces (Lan *et al.*, 1994). Furthermore, there is substantial indirect evidence of unidirectional transfer. First, young orchid seedlings need external soluble carbohydrates to grow, light does not increase their growth and seedlings apparently cannot photoassimilate even after visible chlorophyll has developed. Second, seedlings in some cases die, when exposed to light (Thomale, 1957; van Waes, 1984).

Species belonging to *Corallorhiza*, *Epipogium*, *Galeola*, and several other genera have low photosynthetic capacity as adults, as judged by small leaf area, brief aboveground growing season, low chlorophyll content or apparent lack of chlorophyll (Leake, 1994). These plants are assumed to be entirely or predominantly mycotrophic (Rasmussen, 1994). In species that do develop green leaves, the adult stages are often assumed to be entirely phototrophic, but little is known about transition from the mycotrophic seedling stage. The study by Alexander and Hadley (1984, 1985) seems to show a transition in *Goodyera repens* from entirely mycotrophic seedlings to leafy young plants that could grow despite fungicide treatment. However, it seems likely that in some species mycotrophy persists and supplements photosynthesis for the entire life history. This might be the case in species of *Dactylorhiza* (Fuchs & Ziegenspeck, 1924) and *Tipularia discolor* (Whigham, 1984) that increase their dry biomass during the leafless season. Another indication of continued dependence

on fungi is that most adult terrestrial orchids have few and unbranched roots that are thick, owing to a highly developed cortex (Rasmussen, 1995). Such root characteristics provide the root system with a small surface area, not usually considered favourable to water and ion uptake, but a large volume of potentially mycotrophic tissue.

However, the extent to which a combination of phototrophy and mycotrophy is possible or important to the survival and competition of terrestrial orchids is conjectural. Rasmussen (1995) suggested that if mycotrophy does supplement photosynthesis in adult life stages, a range of different strategies would be available to orchids with respect to duration, season and intensity of mycotrophy and photosynthesis, respectively. The anatomy of conducting tissue in orchid roots supports the assumption that phloem is involved in the translocation of mycotrophic products from the roots (see Rasmussen, 1995). It seems conceivable that photosynthesis and mycotrophy should be separated in time, in annual or diurnal cycles, rather than that the two processes should go on simultaneously.

The purpose of this study was to describe the seasonal dynamics of mycorrhizal infection in five orchid species with differing photosynthetic seasons and dependency. Our working hypothesis was that the infection pattern should be controlled by the plant over an annual cycle. We predicted that the five species would differ in seasonal dynamics of root development, infection and digestion of fungi according to their differing phenology of foliage leaves and thus of photosynthesis. We recorded root phenology of all species, leaf phenology of those species for which such information was lacking and sampled underground structures during an annual growth cycle to evaluate the state and stage of mycotrophic infection. Because many of the underground structures are perennial, and the age can be inferred from either position or appearance, the material also allowed us to infer the development over more than 1 yr.

Our study was conducted in upland forests on the Smithsonian Environmental Research Center (SERC, 38°53′ N, 76°33′ W) property, located near Annapolis, Maryland, USA. All of the orchids except *L. lilifolia* occur in a mature forest, approximately 135 yrs old, that is dominated by more than 20 hardwood tree species. The most common tree species are *Quercus alba*, *Quercus falcata*, *Liriodendron tulipifera*, *Liquidambar styraciflua*, *Fagus grandifolia*, *Carya glabra* and *Carya tomentosa*. *Liriodendron tulipifera* and *Liquidambar styraciflua* are the dominant tree species in the mid-successional forests, approximately 60 yrs old, where *T. discolor* and *L. lilifolia* occur.

Tipularia discolor (Pursh.)Nutt. is a wintergreen species, producing a single foliage leaf in September to October and losing it in spring. The base of the leafy shoot develops into a corm that adds a segment annually to the perennial rhizome. An annual cohort of roots is produced from the node below the youngest corm. Usually two to three generations of corms persist on the plant and at least two cohorts of roots are functional (i.e. turgid). A chlorophyll-deficient inflorescence appears above ground in June, approximately 2 months after leaf senescence (Whigham, 1984). *Goodyera pubescens* is phototrophic all year, having an evergreen leafy rosette arising from a creeping, perennial, sparsely-rooted rhizome. It flowers in July and early August at SERC. *Galearis spectabilis* and *Liparis lilifolia* both produce green leaves in the summer season and persist underground during the winter, either as a rooted tuber (*G. spectabilis*) or as a greenish corm with withering roots (*L. lilifolia*). Both species flower in May–June Finally, *Corallorhiza odontorhiza* (Willd.)Nutt. develops no foliage leaves. The below-ground parts consist of a branched, rootless rhizome. The plants only appear above ground with a small brownish inflorescence for about 2 months in late autumn (October–December). The inflorescence of its European and North American relative *C. trifida*, which is similar in habit but summer flowering, has been shown to photoassimilate during flowering, and the energy thus gained is estimated to be sufficient to support the development of fruits and seeds (Montfort & Küsters, 1940).

Materials and Methods

Slightly different sampling and recording strategies were adopted, depending on the information already available on the life-history attributes, and on the availability of the five species in the field.

Generally, plants were sampled once every 6 weeks. Plants disappearing below ground seasonally were flagged to enable retrieval when no above-ground structures were present. We attempted to sample the same number of individuals on each sampling date but mortality in some cases resulted in varying sample sizes and missing data points.

Mycorrhization was evaluated on hand-sections of roots (*T. discolor*, *G. spectabilis*, *G. pubescens*), tubers (*G. spectabilis*), rhizomes (*C. odontorhiza*, *G. pubescens*, *G. spectabilis*) or corms (*L. lilifolia*, *T. discolor*). Roots and other underground structures were fixed in FAA (formaldehyde, glacial acetic acid and 70% ethanol in a 5 : 5 : 90 mixture) and transferred to 70% ethanol. Sections were stained overnight in acid fuchsin (0.01% w : v in lactic acid–glycerol–demineralized water 14 : 1 : 1), and observed in a Leitz Labovert microscope at ×40 magnification. In roots, rhizomes and elongated tubers, the transverse sections were taken at least 2–3 mm from each other, and 8–10 mm apart in the long roots of *T. discolor*; thin and complete transverse sections were then picked randomly for analysis. Loose pelotons with apparently intact hyphae were assumed to be living, whereas strongly staining dense pelotons with poorly distinguishable hyphal structure were considered to be dead. Infection at each sampling date was quantified both by number of sections showing infection, indicative of the longitudinal expansion of the infection ('spread of infection'), and by the proportion of the cortical

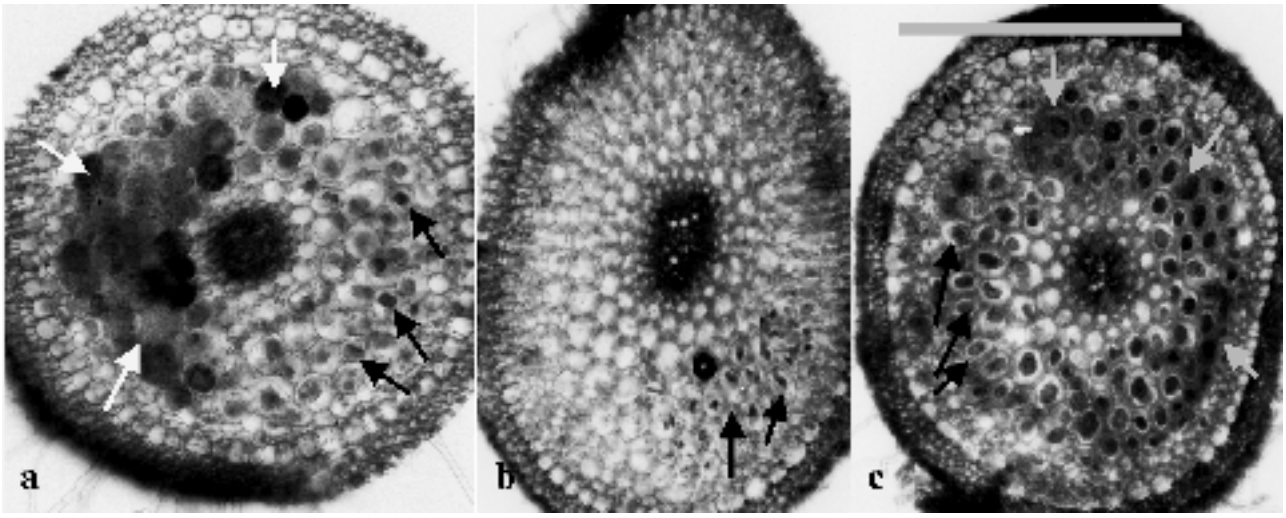

Fig. 1 Transverse sections of roots of *Tipularia discolor* showing various intensities of infection. (a) Young root (12 December) with live infection in about 50% of cortex (white arrows) and dead pelotons in the remainder (black arrows). (b) Root, about 1 yr old (16 July) showing sporadic peloton formation (dead) in less than 25% of cortex (arrows). (c) Old root (23 April) with dead pelotons in almost 100% of cortex (black arrows) and live infection in the same cells in about 75% of cortex (grey arrows). Hand-made transverse sections stained in acid fuchsin. Bar, 0.5 mm.

space occupied by pelotons ('intensity of infection'). Intensity was scored on each section using a six-step scale, representing 0, 12.5%, 25%, 50%, 75% and 100%, respectively, of the cortical area (Fig. 1). Live and dead pelotons were estimated separately and the total amount could thus exceed 100% because cells in some cortical areas could simultaneously contain both. Similarity between subsequent sampling times was tested by χ^2 tests. Two diameters of each cross-section were recorded with a graticule eyepiece. The amount of cortical starch in tubers of *G. spectabilis* and rhizomes of *C. odontorhiza* was estimated in the same manner as infection on the same sections.

For *T. discolor*, 10 nonflowering specimens were harvested each time. Root lengths and numbers of the youngest and second youngest cohort of roots were recorded, before 8–10 randomly chosen roots were examined for mycorrhizal infection. In *G. pubescens*, leaf and root phenology was monitored in marked plants, beginning with 12 plants distributed in four locations. Marked plants that died during the year were replaced by newly marked plants. We thus monitored 20 individuals distributed in five different locations. In each plant the growth of the youngest root was measured by nondestructive excavation every 6 wks, when lengths of all leaves on the shoot were also recorded. New leaves and roots appearing on marked plants were included in the measurements. A regression of leaf length to area based on 20 leaves was used to estimate the total leaf area per plant on each sampling date. Three unmeasured specimens were harvested on each sampling data and fixed for analysis of mycorrhiza. Roots were sorted into young roots, defined by being closest to the tip of the rhizome and having an intact root tip, and older roots, which were further back on the rhizome and obviously no longer growing. As the rhizome is perennial, the roots probably spanned a period of more than 2 yrs.

In *G. spectabilis*, 88 small plants with a single narrow leaf (presumed to be seedlings above ground for the first time) and 88 larger plants with one or two broad leaves were marked during early summer in one population. Ten plants in each group were sampled destructively at each harvest to evaluate organ phenology and biomass allocation. Plants were separated into protocorm (if present), roots, tubers (new and old), rhizome, bud and above-ground shoot, including foliage leaves. All elements (usually 10) in each organ category were pooled and fresh weight determined. After drying at 60°C to constant weight, dry weight (per plant) was determined. Additional plants were marked, sampled and fixed for analysis of mycorrhizal infection. The plants were divided into new roots, older roots, new tubers and older tuber and each category (when present) was sectioned separately.

No growth study of the rhizomes of *C. odontorhiza* was attempted. The extent of individual plants is difficult to evaluate, since side-branches of the mycorrhizome are easily detached when plants are extracted. Inflorescences were marked with flags during autumn, and as long as they were visible, flowering individuals were sampled for mycorrhizal evaluation. A few nonflowering specimens were also sampled when accidentally found. When inflorescences had withered, and flowering specimens presumably were dead (the species is mostly monocarpic) samples of vegetative specimens were collected by gently searching the soil around flags. We were able to collect two or three specimens at 6-wk intervals.

The phenology of *L. lilifolia* has been described previously (Whigham & O'Neill, 1991). Because of small population size only one individual was collected at each harvest date and fixed for mycorrhizal evaluation. Hand-sections of corm and roots were inspected for the presence of live and dead

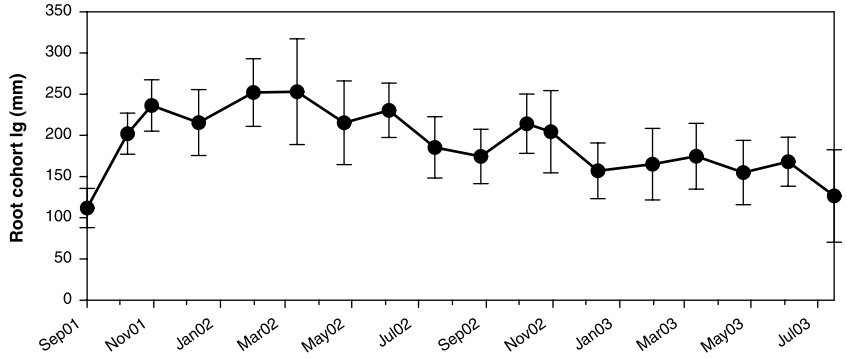

Fig. 2 *Tipularia discolor*, length of root cohort (typically four to five roots per yr per plant) over time. Roots develop in summer and grow in length until winter of year 1. During ageing, length is gradually reduced owing to predation and decay. Means of 20 replicate plants (10 nonflowering and 10 flowering) with 95% significance intervals.

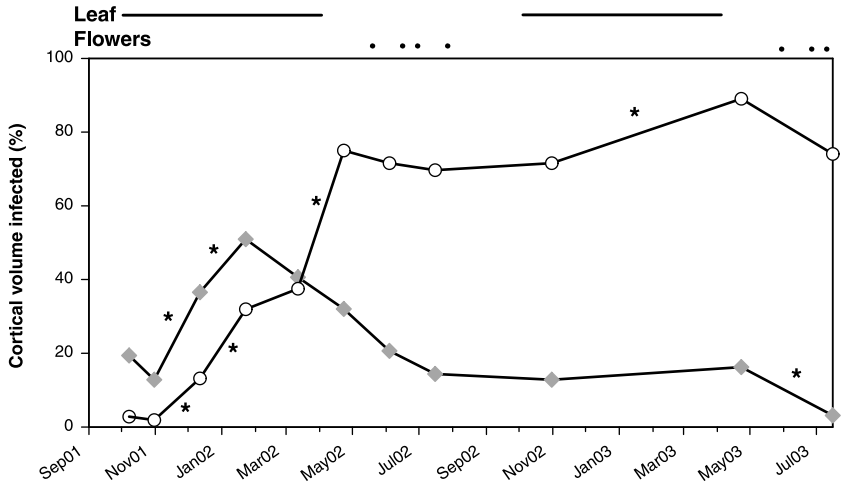

Fig. 3 Peloton formation in roots of *Tipularia discolor,* quantified in relation to available cortical space. The amount of live pelotons (filled diamonds) increases during the root growing stage, reaching maximum in late winter of year 1, followed by a steady decline, stabilizing during year 2, eventually approaching zero. The amount of dead pelotons (empty circles) increases steeply over the first winter and spring, eventually stabilizing at about 75% of cortex. Another increase occurs in the second winter–spring. The duration of leafy season and flowering are indicated above the graph, according to the time-scale of graph. Means of 40 sections. Lines connecting successive points are marked with an asterisk when the increase or decrease is significant ($P \leq 0.05$, χ^2-test).

pelotons. The infected areas were irregular in shape and no quantification of infection was attempted.

Results

Tipularia discolor

Roots developed in late summer. Three to seven unbranched new roots were formed (mean = 4.6). In early winter, roots stopped expanding; the total length of the annual root cohort averaged 250 mm per plant (Fig. 2). Length declined with the progression of the season, probably owing to predation and mechanical damage. Average root diameter was 1.0–1.2 mm and did not change seasonally (data not shown).

In early autumn, when roots were young, live infection was observed in about 40% of the root length and there were very few patches of dead pelotons. Live infection spread to a maximum (about 75% or root length) in winter but declined during the leafless season in summer. The older cohort displayed a similar (though less marked) annual cycle, with the largest expansion of live infection in winter. The patches of dead pelotons expanded until they occurred in 85–100% of the root length in early summer when the roots were about 1 yr old. Intensity of live infection also increased from late October to mid-December (Fig. 3; $P = 0.003$) and further increased until late January, when live pelotons occupied about 50% of the cortical cells. There was a significant decline in live infection intensity from January to early June ($P = 0.007$). The intensity remained low (approx. 15%) during the second winter (i.e. although the infected regions expanded again in the second winter, the concentration of pelotons was much lower than in the first). There was a marked final decline from the spring to summer, when roots were approx. 2 yrs old ($P = 0.001$). Dead pelotons then occupied about 75–90% of the cortical cells (Fig. 3).

The corms did not show any mycorrhizal infection.

Goodyera pubescens

The plants had, on average, seven foliage leaves with new leaves developing from May to September (Fig. 4). Leaf loss was maximum in August and early autumn, but occurred throughout much of the year and was caused by leaf senescence, herbivory and death during periods of drought. Leaf area ranged from 18 to 56 cm^2 in individual plants and was stable through most of the year, except during summer when additions were higher.

New roots were initiated from April to August (data not shown) and increased in length until October. Root diameter tapered from the rhizome towards the root tip and most

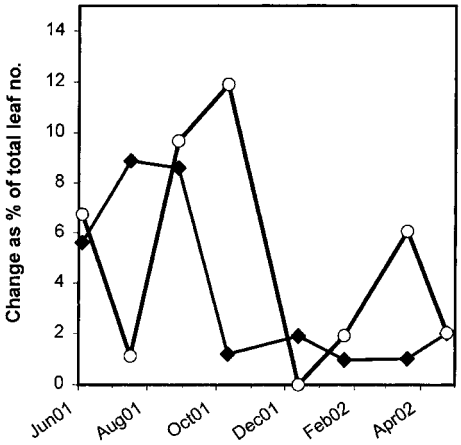

Fig. 4 Appearance of new leaves (additions, filled diamonds) and loss (losses, open circles) of mature leaves over time in *Goodyera pubescens*, in percentage of total leaf numbers. Data based on 85–100 leaves.

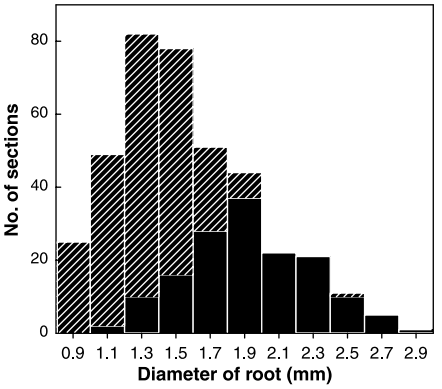

Fig. 5 Diameter of transverse sections of roots of *Goodyera pubescens*, showing infection mainly in the thickest, proximal end of roots (filled bars, infected; hatched bars, not infected). Data from 400 sections collected throughout the year.

infection was found in the thickest, and hence most proximal part of the roots (Fig. 5). Infection never spread to more than 45% of the root length and there was no infection in the cortex of the rhizome (Fig. 6). From January of the second year until spring there was a significant decrease in the expansion of the infected area along the root.

There were numerous live pelotons in young roots in autumn, winter and spring (Fig. 7). From November to January the part of cortex occupied by live infection increased significantly ($P \leq 0.001$) and decreased during early spring ($P = 0.012$). The intensity of live infection tended to decrease further in the older roots but in their second winter, the amount of live infection showed another rise and fall. In spring of the third year, live pelotons were disappearing (Fig. 7). As late as early spring, when the roots were about 1 yr old, numerous living root hairs could still be found, apparently serving as entrances and exits for hyphae. In roots more than 1 yr old the root hairs were no longer intact but apparently

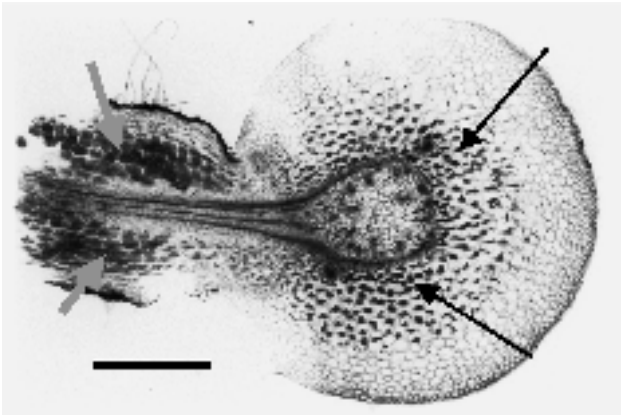

Fig. 6 Transverse section of rhizome (left) and longitudinal section of upper root in *Goodyera pubescens* showing infection (grey arrows) in root cortex, which does not extend into the rhizome. Dark particles in rhizome cortex (black arrows) represent starch. Bar, 1 mm

live hyphae were still passing through their bases. The amount of cortex occupied by dead pelotons fluctuated during the life of the root but generally increased (Fig. 7). It appeared to be correlated with the amount of live pelotons: positively correlated during the first year and negatively correlated during the second year.

Galearis spectabilis

Plants produced one or two foliage leaves in May and leaves persisted until October–November (Fig. 8). Roots developed at the base of the bud for next year's above-ground shoot (Fig. 9a); roots were initiated in summer and expanded during autumn. They remained on the plant for more than a year, eventually to wither in late winter of the second year, at about 1.5 yrs of age. The root–stem tuber, consisting of an elongated tuberous root with a connecting rhizome segment (Fig. 9), began to develop in spring as a side-shoot at the base of a scale leaf, the leaf sheaths are tubes. Hence a sideshoot must emerge either by growing along the sheath of the supporting leaf until the top, or by growing through a basal crack in the leaf sheath. The new tuber does the latter (Fig. 9e). The new tuber continued to grow until autumn (Fig. 8), and persisted throughout the following summer and autumn, eventually to end its biennial cycle towards spring. Hence, two generations of roots and tubers could be seen in autumn but the older roots tended to have disappeared by December. There was no rootless season.

The protocorm of *G. spectabilis* was an elongated, usually branched structure, with short root-like extensions (see also Rasmussen, 1995, Fig. 7.5). In its first year above ground the seedling in spring produced an aerial shoot with basal scale leaves and a single narrow foliage leaf. A tuber developed during the summer at the base of one of the scale leaves. In late October, a bud for next year's leafy shoot had formed, and at the base of this bud the first roots had initiated. These roots

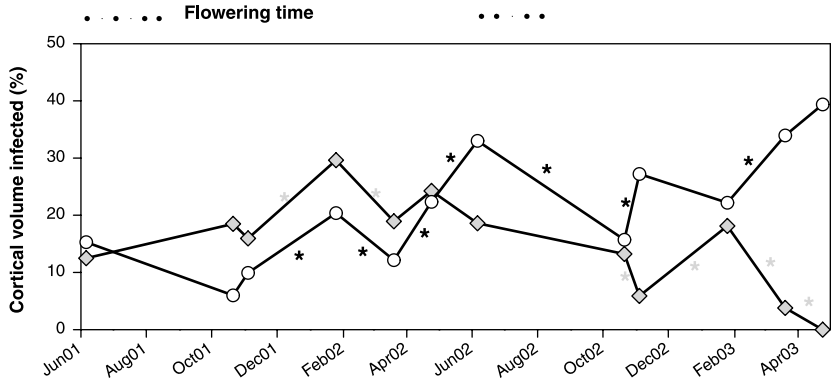

Fig. 7 Infection in roots of *Goodyera pubescens* over time; living (diamonds) and dead (circles) pelotons, respectively, in percentage of total cortical space. Many distal sections were not infected at all, hence overall infection percentage appears low. The duration of flowering season is indicated above the graph, according to the time-scale of graph. Means of 20 (or more) sections. Lines connecting successive points are marked with an asterisk when the increase or decrease is significant ($P \leq 0.05$, χ^2-test).

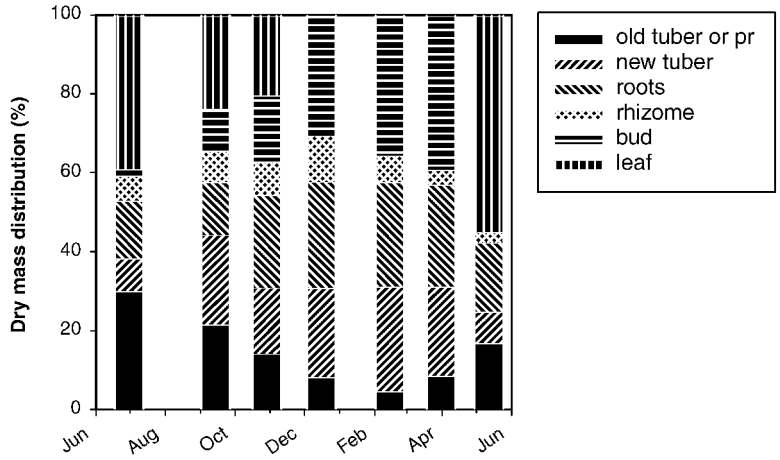

Fig. 8 *Galearis spectabilis* temporal pattern of biomass distribution between organs. Means of 20 plants: 10 young plants with protocorm (pr) and 10 more mature plants.

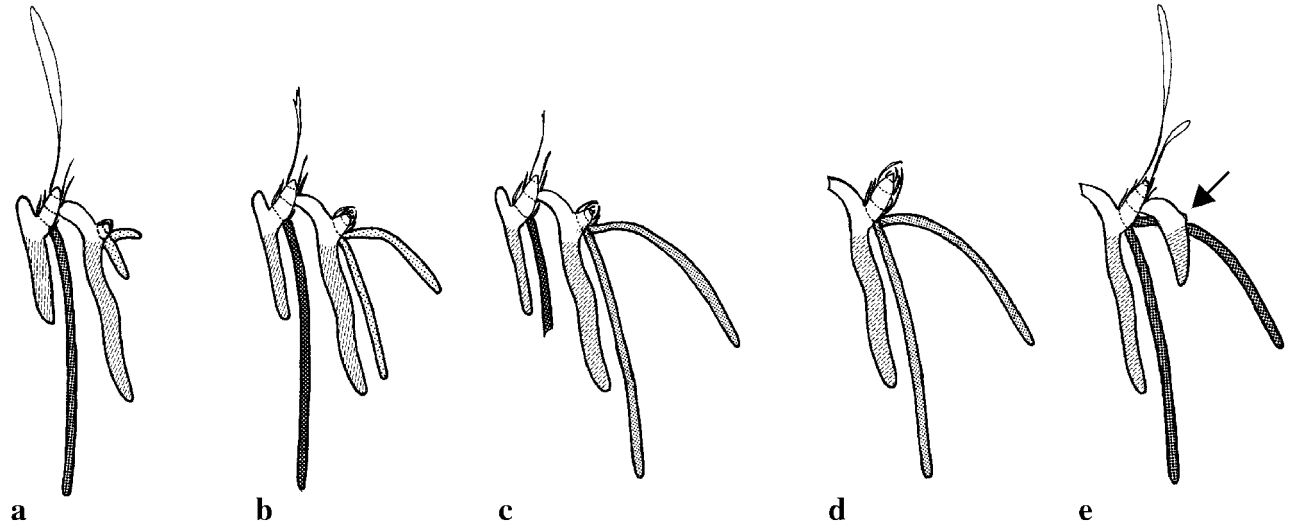

Fig. 9 *Galearis spectabilis* life history stages. (a) Late July: plant in leaf and young roots developing at the base of the renewal bud for next year's above-ground shoot; two generations of tubers (stippled), old roots (shaded) and two segments of rhizome, one from the previous year, and one from the current year (white). (b) Mid-September: leaves withered, young roots and renewal bud growing. (c) Mid-December: new cohort of roots is fully developed. (d) Late March: renewal bud about fully developed; old tuber, old roots and rhizome have disappeared. (e) Mid–late May: a new 'sinker' (i.e. a continuation of the rhizome with a tuber attached) is developing at the base of the leafy shoot; a new renewal bud is barely visible (arrow).

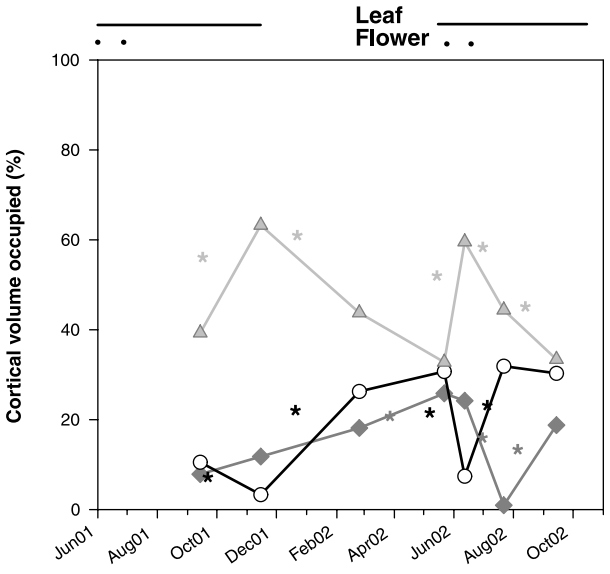

Fig. 10 *Galearis spectabilis.* Live infection (diamonds), dead infection (circles) and starch (triangles) in roots over time (quantified in percentage of cortical volume). The duration of leafy season and flowering are indicated above the graph, according to the time-scale of the graph. Means of 10–40 sections from two to three plants per sampling date. Lines connecting successive points are marked with an asterisk when the increase or decrease is significant ($P \leq 0.05$, χ^2-test).

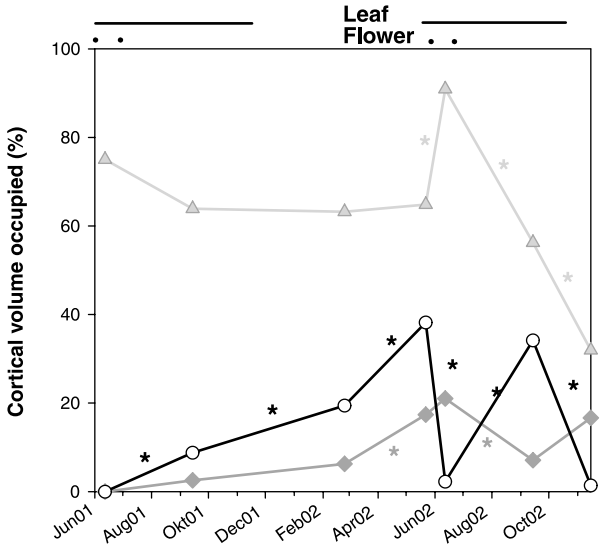

Fig. 11 *Galearis spectabilis.* Live infection (diamonds), dead infection (circles) and starch (triangles) in tubers. Means of 10–27 sections from two to three plants per sampling date. (Material for 15 November missing). Lines connecting successive points are marked with an asterisk when the increase or decrease is significant ($P \leq 0.05$, χ^2-test).

expanded during autumn, while the first leafy shoot, and later the protocorm, disappeared. Development now conformed to that of older plants (Fig. 9, beginning at d).

In autumn, the infection level in young roots was low, occupying less than 10% of the cortical volume (Fig. 10). From February to May live infection increased significantly ($P = 0.04$) and reached a maximum of 25–30% of the cortical volume. There was a decline in live infection from June to July ($P \leq 0.001$) and another increase in early autumn when the roots approached senescence. The amount of cortex occupied by dead pelotons fluctuated greatly and appeared to be inversely related to the amount of starch; hence, maximum starch was present when the occupancy of live infection exceeded that of dead pelotons (Fig. 10).

The main function of the tuber as a storage organ is indicated by the high concentrations of starch in both young and old tubers (Fig. 11), but all parts of the tuber contained some mycorrhizal infection. The starch level was high (approx. 75% of cortex) when the tuber developed but tended to decline (ns) with the increasing mycorrhization during the first year. When live infection peaked in summer and the amount of live pelotons exceeded the amount of dead pelotons, starch levels rose significantly to a maximum. The eventual withering of the tuber in autumn was accompanied by a steep decline in starch levels, and in the amount of dead pelotons. A low incidence of live pelotons was still observed (Fig. 11). Infection was concentrated in the thickest upper part of the tuber. The rhizome was consistently filled with cortical starch but in the September sample, decomposed pelotons were observed in the outer cortex.

Corallorhiza odontorhiza

In sections taken close to the growing tip of the mycorhizome, starch filled most of the cortical cells and there were no pelotons. Sections through young, still uninfected tissues with starch deposits throughout the cortex occurred much more frequently in the summer samples than in winter, suggesting that the mycorhizome grew more rapidly during summer, resulting in a comparatively long, yet uninfected shoot tip. The amount of starch was high from summer to autumn, significantly decreasing in midwinter and subsequently rising during the spring samples (Fig. 12; $P \leq 0.001$). Live pelotons were invariably present in the hypodermis, from which they extended into midcortex. The extent of live infection was roughly stable throughout the year and fairly low, with only an apparently brief high season in April ($P \leq 0.001$). Dead pelotons varied in prominence but the extent was mostly higher than that of live infection, and highest in late winter and spring (Fig. 12).

Liparis lilifolia

The corm is a swollen internode below the inflorescence that is enclosed in leaf sheaths that are succulent at the base. The cormous internode could be seen in early spring before the inflorescence expanded (Fig. 13a). At this time, therefore, there were two generations of corms, (i.e. the youngest corm

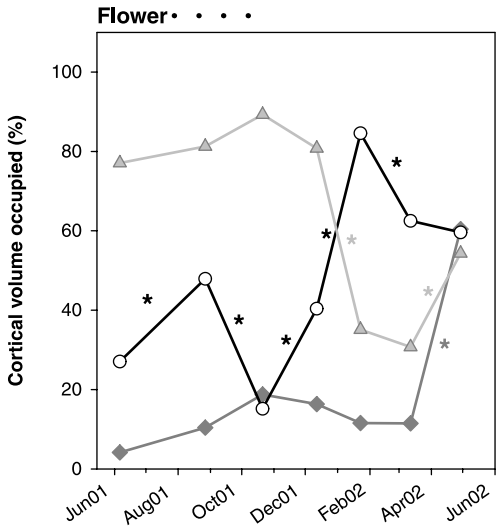

Fig. 12 *Corallorhiza odontorhiza*. Live infection (diamonds), dead infection (circles) and starch (triangles) in rhizome over time. Means of 12–14 sections per sampling date. Starting date arbitrarily chosen, since phenology of rhizome is unknown. The duration of flowering season is indicated above graph, according to time-scale of graph. Lines connecting successive point are marked with an asterisk when the increase or decrease is significant ($P \leq 0.05$, χ^2-test).

inside the bud, and the older corm, wrapped in the bases of least year's foliage leaves, which functions as storage organ). Below the older corm there could be more or less decaying rhizome parts (not seen in Fig. 13), of which the central part with conducting strands was most persistent and which was penetrated by roots from the two younger shoot generations. The older corm was depleted during summer, to become completely replaced by the new corm in autumn (Fig. 13b,c). At this time, a renewal bud was initiated in the axil of the innermost leaf sheath and the axis of this bud developed into a short rhizome. By December, root primordia had formed on the rhizome at the base of the bud. These roots developed slowly during spring, first elongating in May in the direction of the decaying old rhizome tissue below the functioning corm and eventually into the surrounding soil.

The rhizome below the renewal bud was infected in the lowermost part in October. Infection was about the same in May the following year and by then the uppermost cortex of the new roots, and the lowermost periphery of the functioning corm were also infected (Fig. 14a). These parts remained infected during summer, the infected areas enlarging with the growing of the rhizomatous base of the young corm. The occurrence of dead pelotons was greatest in autumn. Little infection was observed in the rather thin roots and only in the most proximal part.

Discussion

The time samples studied here only give a fragmentary picture of the dynamics of mycorrhizal activity. However, when the amount of dead pelotons decreased markedly from one sampling time to the next, this indicates that pelotons eventually become completely lysed. This appears to happen in *G. spectabilis* and *C. odontorhiza*. In *T. discolor* and *G. pubescens*, however, dead pelotons in the oldest samples virtually filled the infected tissue. It is possible that complete breakdown would have been observed if even older roots had been sectioned, but it is also possible that in these species the last pelotons to form are never completely lysed before the tissue loses its mycotrophic function. The data from *G. spectabilis* and *C. odontorhiza* suggest that periodic degradation of pelotons is associated with local accumulation of starch (Figs. 10–12), but in the thick hand-sections masses of starch grains would also tend to mask old pelotons so that their presence could be underestimated. The changing stock of live pelotons at any time reflects the number of new pelotons formed minus the number killed, the latter adding to the stock of dead pelotons. When live infection and amount of dead pelotons increase at the same time, new pelotons are obviously formed at a more rapid pace than they are killed, indicating high activity of invading hyphae. When the

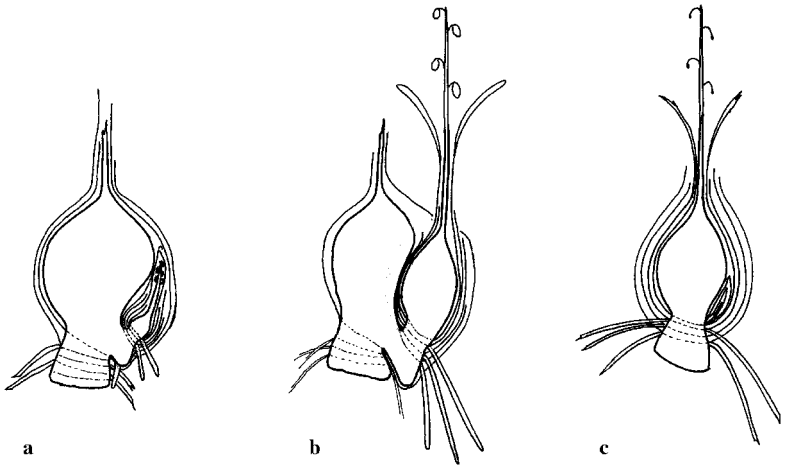

Fig. 13 *Liparis lilifolia*, life history stages. (a) May: renewal bud generated in the axil of one of the foliage leaves, developing a short rooted rhizome and a terminal inflorescence. (b) August: new shoot in fruiting stage, with foliage leaves and the base of the inflorescence swelling into a corm. The old corm, still wrapped in withered leaf sheaths, is decreasing. October: young corm has replaced old corm; renewal bud initiated.

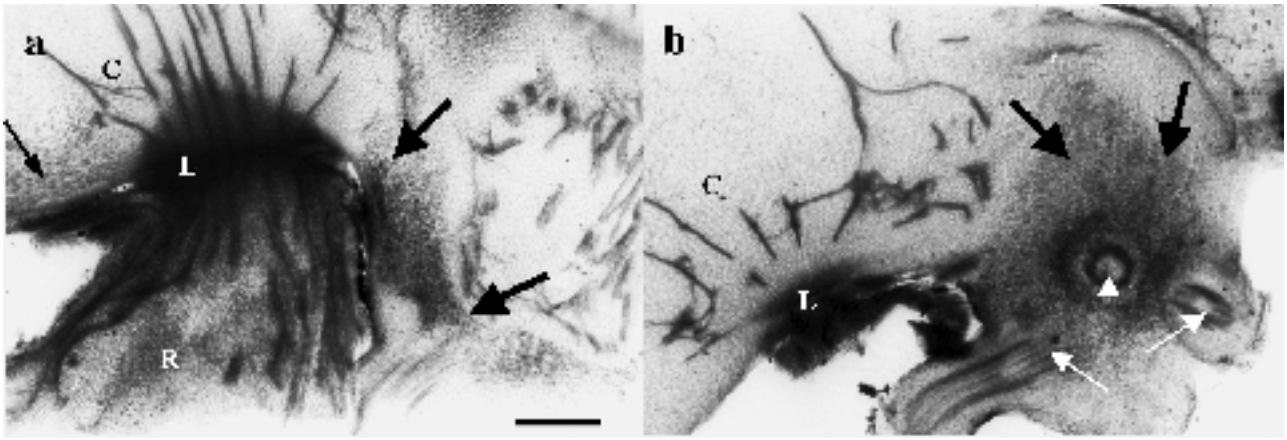

Fig. 14 *Liparis lilifolia.* Longitudinal sections through infected areas. (a) May: lower part of corm (C) and upper part of rhizome (R), separated by a strongly lignified node. To the right is the base of the renewal shoot, in which areas are infected (arrows). Some infection is also seen on left side of corm (small arrow). (b) August: infection at base of fruiting shoot is now more extensive (arrows); base of several roots are sectioned as they traverse the cortex (small arrows). Section roughly corresponds to (a) except that upper part of rhizome is broken off. Bar, 1 mm.

amount of live pelotons decreases while the amount of dead pelotons rises, it can be assumed that there is less new invasion than death of pelotons, but that the final lysis phase is lagging behind.

Seasonal and developmental changes

In plants with arbuscular mycorrhiza, the colonization of individual species seems to be linked to root longevity and shoot phenology rather than a direct seasonal regulation (Brundrett & Kendrick, 1988). This apparently also applies to orchid mycorrhiza in the species studied here, insofar that there was no common seasonal pattern of mycotrophy. Colonization did follow soon after root development, but neither root phenology nor colonization appeared to be connected with leaf phenology.

In *T. discolor*, roots began to develop in the nonphototrophic season in summer and the long infection period began some months later during the leafy season. Maximum colonization of new roots was reached in winter after about 6 months. Mycotrophic activity extended for at least 2 yrs and involved most of the root system.

The leaf area in *G. pubescens* was fairly stable around the year, with losses and additions of leaves taking place mainly during summer. The analysis did not give any indication of a seasonality in phototrophy. In summer, when roots developed, they were soon fully infected. After that, infection was constantly present with considerable fluctuations but no obvious seasonality. Dead pelotons accumulated with the ageing of the root until no more live infection could be observed. A similarly fluctuating and not seasonal infection pattern was observed in roots of *Goodyera repens* (Alexander & Alexander, 1984), which appears to be a species with a much higher dependency on mycotrophy, as the rhizome also contains a considerable amount of infection. A possible explanation for these fluctuations is that infection is governed by climatic events, such as rainy or droughty periods, rather than by an annual cycle. A relationship between humidity and mycotrophic activity was suggested for *Bletilla striata* by Masuhara *et al.* (1988) and for *Spiranthes sinensis* by Masuhara and Katsuya (1992), who found maximum infection for these species during spring and autumn in Japan.

The phototrophic season of *G. spectabilis* extended from May to October. Roots developed towards the end of the leafy season and infection increased from the onset of root development until midwinter. The infection level then appeared to remain at a steady level except for a decline in live infection in midsummer, when leaves were present. Hence activity seemed highest in spring and in the second autumn before the roots eventually died. Alternation between the leafy season and the peaks of mycotrophy could be postulated but climatic factors such as humidity could also be the cause of the apparent decline in mycotrophic infection in summer.

In *L. lilifolia* the roots showed little mycorrhizal infection; perhaps significantly, this is the only species in which root development was related temporally to shoot development, indicating that their primary function is water uptake. The highest mycorrhizal infection within the rhizome appeared to coincide with the leafy season. This is in agreement with the observations of Huber (1921) on *L. loeselii.*

Corallorhiza odontorhiza showed evidence of a quite dynamic annual infection pattern, although this species relies almost entirely on mycotrophy. The main mycotrophic seasons appeared to be spring, when live peloton concentration increased to 60% of the cortical volume, and in autumn and winter (during flowering and fruiting), when dead pelotons accumulated. The rather low concentration of infected cells in summer might indicate that this is the time of most mycorrhizome growth, but it could also reflect a summer decline of infection, as in *T. discolor* and *G. spectabilis.* The short

above-ground season coincided with a decline in starch reserves. Owing to the hapaxanthic life form of *C. odontorhiza*, specimens studied in winter were mainly fruiting and ageing, whereas in spring, only nonflowering specimens were available. However, except for a decrease in the amount of dead pelotons, there was little change from January to March where a shift in the cohort studied might create discontinuity in the results. The increases in live infection and starch content from March to April thus appear to be brought on by season.

Dependency

In this study, no measurements were attempted of the actual carbon gain obtained by the five orchid species by means of mycotrophy. However, the infected organs and the infection patterns do suggest differing mycotrophic dependency among the five species, although all had notable mycorrhizal infections as adults. *Liparis lilifolia* appeared to have only a small amount of mycotrophic tissue within the rhizome and a short mycotrophic season. It is notable for *T. discolor*, G. pubescens and *G. spectabilis* that roots had determinate growth and only grew for a relatively short period of time compared with their longevity. The extended root life and heavy infection in at least two root cohorts in *T. discolor* suggest that this is a highly mycotrophic species. Since live infection continued to appear in the roots, reinfection and active mycotrophy presumably occurred in roots as old as 2 yrs or more. Experimental removal of the foliage leaf in *T. discolor*, however, caused considerable inhibition of growth (Whigham, 1984) for which mycotrophy apparently could not compensate. It has not been tested whether phototrophy could replace mycotrophy, should the latter be impeded, but difficulty in transplanting *T. discolor* into garden situations suggests that mycotrophy is essential. There appears little doubt that in a natural environment both types of nutrition are used, and the infection patterns seen in this study indicate that they are used simultaneously.

Studies of *G. repens* indicated a marginal importance of mycotrophy in that species (Alexander & Hadley, 1984, 1985), although the level of infection was high (up to 40–80%) in both roots and rhizome (Alexander & Alexander, 1984). In contrast to its relative, in *G. pubescens* infection was restricted to the proximal parts of roots. However, the roots are long-lived and seem to be reinfected and continue functioning at least into their second year; the mycotrophic parts are densely infected (Fig. 6). Roughly the same location of infection as in *G. pubescens* was described for *G. oblongifolia* (Zelmer, 1994).

In contrast to many other species in this subfamily (Orchidoideae, e.g. species of *Orchis*), there is no rootless season in *G. spectabilis* and the tubers appear to contain live infection and participate in peloton lysis at almost the same level as the roots.

Maintenance of infection

In none of the species could we detect a season without live infection in some tissues. The transfer of infection from one shoot generation to the next by means of one or more penetrating roots was described by Fuchs and Ziegenspeck (1927) in European species of *Liparis* and by Rasmussen (1995) in the related *Hammarbya paludosa.* Such an arrangement was not observed with certainty in *L. lilifolia* but could have occurred between the sampling dates when the roots penetrated the old corm. An internal transfer of the endophyte from year to year seems likely, since we have consistently found 'the same' fungus in all *L. lilifolia* plants examined (unpubl. data).

The mycotrophic tissues of *C. odontorhiza* are contiguous, so that the fungus originating from the seedling can be preserved throughout. Zelmer *et al.* (1996) observed a permanent layer of living pelotons in the cortex in several other species of *Corallorhiza*, suggesting a possible location for reinfection. Some species of *Corallorhiza* do appear to be consistent in their association with certain fungal genera and species (Taylor & Bruns, 1999; McKendrick *et al.*, 2000). This apparently does not preclude the subsequent invasion by other fungi, indicated by the diversity of simultaneous endophytes found by Zelmer (1994) in *Corallorhiza*.

Separation between roots in *G. pubescens* and between cohorts of roots in *T. discolor* by an uninfected rhizome, however, makes it likely that new fungi could be introduced from the soil to emerging roots. This infection pattern would enable a diversity of endophytes to invade these species. In *G. spectabilis*, however, there seems to be at least a brief infection of the rhizome connecting the tubers, and since roots are contiguous with the tubers, internal transfer into the new shoot generation is possible. A similar phenomenon could have been overlooked in *G. pubescens* and *T. discolor*. However, in most of the orchids of Western Australian studied by Ramsey *et al.* (1986) plants were reduced during the dormant season to uninfected structures. Annual reinfection from the soil thus appears to be the rule in these species.

Conclusions

In the orchids examined in this study, phototrophic strategy apparently does not restrict mycotrophic activity. Infection depended largely on age, with the youngest roots or rhizome parts showing patchy and mainly live infection, and the oldest accumulating dead pelotons. There was continuous mycorrhizal infection in tissues of the orchids examined. The amount of infection may vary temporally, more likely in response to climatic stresses or events than according to phototrophic strategy or other annual patterns. Climate conditions in Maryland, USA, appear to allow mycotrophic activity to continue during the winter. The relationships we have described need to be evaluated further because, except

for *T. discolor*, the data are based on few individuals, varying in size and age, as necessitated by the comparative rarity of these species.

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