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Fungi associated with terrestrial orchid mycorrhizas, seeds and protocorms

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The identity and ecological role of fungi in the mycorrhizal roots of 25 species of mature terrestrial orchids and in 17 species of field incubated orchid seedlings were examined. Isolates of symbiotic fungi from mature orchid mycorrhizas were basidiomycetes primarily in the genera *Ceratorhiza*, *Epulorhiza* and *Moniliopsis*; a few unidentified taxa with clamped hyphae were also recovered. More than one taxon of peloton-forming fungus was often observed in the cleared and stained mycorrhizas. Although *Ceratorhiza* and *Epulorhiza* strains were isolated from the developing protocorms, pelotons of clamped hyphae were often present in the cleared protocorms of several orchid species. These basidiomycetes are difficult to isolate and may be symbionts of ectotrophic plants. The higher proportion of endophytes bearing clamp connections in developing seeds than in the mycorrhizas is attributed to differences in the nutritional requirements of the fully mycotrophic protocorms and partially autotrophic plants. Most isolates of *Ceratorhiza* differed enzymatically from *Epulorhiza* in producing polyphenol oxidases. Dual cultures with thirteen orchid isolates and five non-orchid hosts showed that some taxa can form harmless associations with non-orchid hosts. It is suggested that most terrestrial orchid mycorrhizas are relatively non-specific and that the mycobionts can be saprophytes, parasites or mycorrhizal associates of other plants.

Key Words——*Ceratorhiza*; *Epulorhiza*; *Moniliopsis*; orchid mycorrhizas.

The roots, rhizomes and tissues of terrestrial Orchidaceae are usually inhabited by fungi that form complex intracellular masses of hyphae called pelotons (Arditti, 1992). Experimental evidence indicates that these endophytes are playing an important role in the uptake of nutrients, including carbon, in mature plants (Alexander and Hadley, 1983; Alexander et al., 1984). Orchid seed germination can be initiated and seedling development accelerated in vitro if certain fungi can establish pelotons in the developing protocorms (Masuhara and Katsuya, 1989; Smreciu and Currah, 1989; Zettler and McInnis, 1992; Rasmussen, 1995). Recently, in situ germination studies have shown that mycorrhizal associations develop in very young orchid protocorms in nature (Masuhara, 1991; Rasmussen and Whigham, 1993; Currah et al., 1996) but there is relatively little information concerning the identity of these fungi or their relationship with the mycorrhizal fungi of mature plants.

Orchid mycorrhizal fungi are taxonomically problematic; the majority of the isolates are sterile in culture and represent a broad range of basidiomycetous taxa (Currah and Zelmer, 1992). For example, one of the largest artificial groups of mycorrhizal endophytes from orchids, often referred to as orchidaceous rhizoctonias, comprises the anamorphic genera *Ceratorhiza* Moore, *Epulorhiza* Moore and *Moniliopsis* Ruhland. These genera were established on features of nuclear cytology and septal ultrastructure but can be distinguished in some cases on the basis of cultural and microscopic morphology (Currah and Zelmer, 1992). A simple enzymatic test to assist in separating these groups would be an invaluable adjunct to morphological data.

The ecology of the extramatrical phase of orchid mycorrhizal fungi is also poorly known (Rasmussen, 1995). Mycobionts may be involved with other plants as pathogens (Downie, 1957; Terashita, 1985; Peterson and Currah, 1990), as mycorrhizal fungi (Warcup, 1985; Zelmer and Currah, 1995a), or they may be saprophytes (Masuhara, 1991; Umata, 1995).

In this paper we examine and compare the fungi that form mycorrhizas with mature plants and protocorms of terrestrial orchids native to western Canada. We also report on the use of simple enzymatic tests to distinguish between the genera *Epulorhiza* and *Ceratorhiza*, and, using in vitro inoculated plants, describe the relationship of some orchid mycorrhizal fungi with some non-orchid hosts.

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Materials and Methods

Mycorrhizal endophytes Mature plants of 25 orchid spe-

Table 1. Identity and location of collection sites for orchid species used for the recovery of mycorrhizal (basidiomycetous) endophytes.

Orchid (No. of plants)	Locations	Fungal taxa (No. of isolates)
Amerorchis rotundifolia (19)	b	*Epulorhiza* (7), *Moniliopsis* (3)
Calypso bulbosa (1)	e	None
Coeloglossum viride (12)	c, j	*Ceratorhiza* (17), *Moniliopsis* (5)
Corallorhiza maculata (9)	h, k, l	*Moniliopsis* (7)
Corallorhiza striata (5)	h	None
Corallorhiza trifida (18)	b, e, g	*Ceratorhiza* (1), Yellow basidiomycetes (2)
Cypripedium acaule (8)	i	None
Cypripedium calceolus (19)	a, g, j	*Epulorhiza* (8)
Cypripedium candidum (10)	f	*Epulorhiza* (1)
Cypripedium montanum (2)	l	None
Cypripedium passerinum (6)	g, e, l	*Moniliopsis* (3)
Cypripedium reginae (1)	a	None
Goodyera oblongifolia (8)	h	*Epulorhiza* (1), *Ceratorhiza* (34), *Moniliopsis* (3)
Goodyera repens (3)	m	None
Listera cordata (10)	g, m	None
Malaxis monophyllos (1)	g	None
Piperia unalascensis (3)	h	*Ceratorhiza* (1)
Platanthera dilatata (10)	b, h	*Epulorhiza* (2)
Platanthera hyperborea (13)	c, g	*Epulorhiza* (7), *Ceratorhiza* (7), *Moniliopsis* (5)
Platanthera obtusata (19)	e	*Epulorhiza* (10) *Ceratorhiza* (24), *Moniliopsis* (3)
Platanthera orbiculata (1)	n	None
Platanthera praeclara (11)	d	*Epulorhiza* (1), *Ceratorhiza* (15)
Spiranthes lacera (4)	i	*Epulorhiza* (6), *Ceratorhiza* (4)
Spiranthes magnicamporum (7)	f	*Epulorhiza* (6), *Ceratorhiza* (1)
Spiranthes romanzoffiana (6)	b, g	*Epulorhiza* (2), *Ceratorhiza* (1)

Location codes: a: Anola, MB; b: Calahoo Fen, AB; c: Cardinal River Divide, AB; d: Gardenton/Vita, MB; e: Obed Summit, AB; f: Tolstoi, MB; g: Near or within Wagner Natural Area, AB; h: West Castle River, AB; i: Whiteshell Prov. Forest, MB; j: Argyle, MB; k: Edmonton (River Valley), AB; l: Provided by collectors, AB; m: Swan Hills, AB; n: Edson Forest, AB.

cies were collected between 1991 and 1994 from sites in Alberta and Manitoba, Canada (Table 1). For most species, whole plants were dug and kept cool until examined within 5 d of collecting. Roots were cleaned and one to several undamaged, healthy segments were selected for processing. For the endangered taxa, *Platanthera praeclara* Sheviak & Bowles and *Cypripedium candidum* Muhlenberg, samples were collected by removing only a few of the easily recognized roots from the plants in situ.

Roots were surface sterilized for 1 min in a 20% solution of household bleach (5.25% sodium hypochlorite) to which a drop of detergent had been added. After rinsing twice for 1 min in sterile distilled water, the roots were cut into serial 1 cm segments. Each section was cut longitudinally with a sterile scalpel. One half was used for isolating fungi and the other was preserved in FAA (formalin–50% ethanol–acetic acid, 10 : 45 : 5, v/v). This aided correlation between hyphae within the root segment and isolated fungi.

Segments preserved in FAA were cleared in 10% KOH, stained with either Trypan Blue or Chlorozol Black, squashed and mounted in glycerine jelly or polyvinyl alcohol. Cultures were prepared by plating small pieces of root segments on cornmeal agar (CMA, Difco) with and without 100 mg/L oxytetracycline. Hyphal tip transfers were made to potato-dextrose agar (PDA, Difco).

Enzyme tests The production of cellulase was determined using the cellulose azure method of Smith (1977) modified by the use of Murashige and Skoog basal salts medium (BSM, Sigma) as the nutrient medium. Tubes containing a basal layer of BSM and a layer of cellulose azure medium were inoculated with small plugs of actively growing mycelium and incubated for 5 d in the light at room temperature. A positive reaction was indicated by diffusion of the blue dye from the cellulose azure medium layer so that this layer and the basal layer of BSM were indistinguishable. A weak reaction was scored if the interface between the two layers of media was still visible. No diffusion of the dye denoted a negative reaction.

Polyphenol oxidases were detected using the tannic acid medium (TAM) of Davidsonet al. (1938). Plates of TAM were inoculated with small blocks of mycelium and agar from the margin of an actively growing colony. The results were read after 5 d at room temperature in the dark. In a positive reaction, the medium around the in-

oculum became dark brown from both top and bottom of the plate. A weak reaction was visible only from the top. No change denoted a negative test.

Seed and protocorm endophytes In the fall of 1991 and spring of 1992, seeds of 17 orchid species (Table 2) collected in 1991 were enclosed, in lots of approximately 1000, in 2.5 cm^2 packets made of polyester filter fabric (10 mm Spectrum Filter Fabric, Canadawide Scientific). Packets were buried at the bottom of the live moss layer in peatland sites, or in the humus layer on mineral soils. At each site, packets were planted in groups of three. In most cases seeds were placed on site in the fall of 1991 and retrieved one at a time during the spring, summer and fall of 1992. Packets at the Manitoba sites were set out in the spring of 1992, and recovered during the summer and fall of the same year and finally in the spring of 1993.

Collected packets were rinsed in 20% household bleach for 1 min and then twice in sterile distilled water. Three subsamples of seeds from each packet were removed. Seeds from the first subsample were plated onto CMA in order to culture fungal endophytes using the same protocol as for root segments. The seeds from the second subsample were preserved in FAA and later cleared and stained as above. The third subsample of seeds was mounted in lactofuchsin for immediate observation with a light microscope. Germination was recognized by production of epidermal hairs or by a large increase in size of the proembryo beyond its imbibed size.

Interactions of orchid endophytes with non-orchid hosts Strains of *Ceratorhiza* (4 each), *Epulorhiza* (4 each), and *Moniliopsis* (5 each), isolated from the mature orchid mycorrhizas above were selected for growing along with a range of herbaceous and woody non-orchid hosts. Seeds of *Pinus resinosa*, *Picea glauca*, *Allium sativa*, *Brassica napus* and *Medicago sativa* were surface sterilized for 1 min in a 20% solution of household bleach, rinsed three times with sterile distilled water and plated on water agar. Dual cultures were prepared for plants and fungi as follows. Glass test tubes 15 cm long and 2.5 cm diam filled one quarter full of CMA and slanted, were inoculated with plugs of actively growing mycelium on PDA or CMA. Controls were prepared in the same way, but not inoculated. After 7 d, the tubes were half filled with sterile perlite. Seedlings with green cotyledons and emerged radicle were planted one per tube and the tubes placed in a growth chamber at 18°C with 18 h light at 2400 lx. Four replicate tubes were prepared for each fungal isolate-plant combination. Seedlings were monitored and compared to controls for visible pathogenic effects. At 8 wk, the plants were harvested, the roots excised, cleared and stained as above, and examined with a light microscope.

Results

Root endophytes With the exception of *Cypripedium acaule Ait.* and *Malaxis monophyllos (L.) Swartz*, the mycorrhizas of all the orchid species yielded fungal isolates. Pelotons (apparently intact or degraded) were observed in the cleared roots of all the orchid species, except for those of *M. monophyllos*, for which few root samples were obtained. Most orchid roots were heavily colonized (60% or more of root cortical cells were visually estimated to contain pelotons at some stage of development or breakdown).

In the roots of some orchid species, such as *Corallorhiza trifida* Chatelain, *C. maculata* (Raf.) Raf. and *C. striata* Lindl., pelotons in the outermost cortex were not degraded. In other orchids, especially species of *Platanthera* and *Spiranthes*, groups or patches of pelotons were seen throughout the root cortex. Within each patch, formation and breakdown of the pelotons was synchronous. However, there was seldom synchrony among the patches within a root.

Basidiomycetes were obtained from only 16 species (Table 1). Most isolates of filamentous, non-basidiomycetous fungi were identified at least to genus but are not considered further here.

All but four of the 187 basidiomycetes isolated could be assigned to one of three genera, *Epulorhiza* (Fig. 1), *Ceratorhiza* (Fig. 2) or *Moniliopsis* (Fig. 3) based on morphological criteria. Species identifications were not made except for isolates clearly assignable to *Ceratorhiza pernacatena* Zelmer & Currah, *Epulorhiza calendulina* Zelmer & Currah, *E. anaticula* (Currah) Currah, *E. albertiensis* Currah & Zelmer and *E. repens* (Bernard) Moore. An isolate (Z123-or-2c=UAMH 7572 from *Goodyera oblongifolia* Raf.) representing a species of *Moniliopsis* formed basidia and basidiospores (Fig. 4) and was identified as *Thanatephorus cucumeris* (Frank) Donk. Two isolates that produced slow-growing yellow mycelia composed of clamped hyphae were isolated from *Corallorhiza trifida*. These are described in Zelmer and Currah (1995a). Two isolates of an unidentified species of *Sistotrema* (from *Amerorchis rotunidfolia* (Pursh) Hult., Calahoo fen, AB), were also recovered.

Several root segments yielded two distinct fungi and pelotons of both constituents could be recognized by their hyphal morphology in the cleared and stained roots. One, *Amerorchis rotundifolia* from Calahoo fen, contained pelotons with narrow hyphae, a second type with broad hyphae (and sometimes intermingled in the same peloton), and a third with clamped hyphae in the same root (Figs. 5, 6). Narrow, clamped hyphae were also noted in the pelotons of one root of *Platanthera dilatata* (Pursh) Lindl. but this fungus was not recovered.

Seed and protocorm endophytes Of the 17 orchid species tested, ten had germinated seeds in one or more of the packets (Table 2). In most cases, ungerminated seeds appeared healthy and undamaged by microbes or microfauna even after almost a year in situ. At least a few seeds in most seed packets displayed olive-coloured sclerotia filling the cells of the proembryo (Fig. 7). *Pythium*-like oospores (Fig. 8) were found inside the deteriorating proembryos of *Spiranthes magnicamporum* Sheviak and *Platanthera dilatata*. Some seeds had also been invaded by a variety of other fungi (including basidiomycetes) which produced non-descript hyphae ramifying throughout all areas of the proembryo including the meristematic region.

Table 2. Orchid species, planting locations and basidiomycetes recovered from field incubated orchid seeds.

Orchid (Location, No. of packets)	Fungal taxa (no. of isolates)	Germination/colonization†
Amerorchis rotundifolia	None	
(a,9 pkts)		‡ Germination of some seeds. Epidermal hairs produced by 281 d. No pelotons.
Calypso bulbosa	Unidentified isolate (1)	
(e, 6 pkts)		No germination. *Mycelium radicis atrovirens* (MRA) microsclerotia in seeds.
Ceologlossum viride	None	
(c, 6 pkts)		Many seeds germinated. Epidermal hairs produced. MRA microsclerotia common. Pelotons appear by 303 d; none seen by 337 d.
Corallorhiza maculata	*Ceratorhiza* (1)	
(e, 3 pkts)		‡ Dense peloton-like structures in a few cells by 61 d. Germination of a few seeds by 93 d; no pelotons.
(h, 3 pkts)		No germination.
Corallorhiza striata	Clamped basidiomycetes (3)	
(h, 6 pkts)		‡ No germination. A few seeds with dense pelotons by 61 d.
Corallorhiza trifida	None	
(e, 3 pkts)		No germination. Seeds either free of fungi or overrun.
Cypripedium acaule	*Epulorhiza* (1)	
(b, 2 pkts)		No germination. Seeds free of fungi.
(i, 4 pkts)		No germination. Seeds free of fungi.
Cypripedium calceolus	*Epulorhiza* (3)	
(a, 3 pkts)		‡ Pelotons of clamped hyphae formed by 107 d.
(b, 6 pkts)		No germination
(g, 6 pkts)		Pelotons of clamped hyphae formed by 342 d; no germination indicators‡
Cypripedium candidum	None	
(f, 6 pkts)		No germination. Seeds free of fungi.
Cypripedium passerinum	None	
(b, pkts)		No germination.
(e, pkts)		Seeds heavily parasitized. Clamped pelotons appeared in germinating seeds by 337 d.
(g, pkts)		Pelotons of a broad *Rhizoctonia*-like fungus common by 342 d, but no germination indicators.
Goodyera oblongifolia	None	
(h, 6 pkts)		No germination.
Platanthera dilatata	*Ceratorhiza* (1)	
(b, 6 pkts)		Many seeds germinated and produced epidermal hairs by 262 d; pelotons rare. By 388 d, pelotons (some clamped) common, degraded.
(h, 9 pkts)		‡ Germination without fungal penetration and production of epidermal hairs by 61 d. Proembryos were not invaded by fungi, and did not develop further.
Platanthera hyperborea	*Epulorhiza* (3), *Ceratorhiza*, (1), clamped (4), unidentified (1)	
(b, 9 pkts)		Many seeds germinated and produced epidermal hairs by 262 d. Pelotons, some with clamped hyphae common by 310–388 d.
(e, 6 pkts)		‡ Many seeds germinated by 263 d. Pelotons rare at this time; common by 304 d; rare again by 388 d. Pelotons invaded meristematic region in some seeds.
Platanthera praeclara	*Epulorhiza* (1)	
(d, 6 pkts)		‡ A few seeds germinated by 45 d. Epidermal hairs formed by 108 d. No pelotons. One large protocorm formed-mycorrhizal status unknown.
Spiranthes lacera	*Ceratorhiza* (2)	
(i, 6 pkts)		Many seeds germinated by 49 d; pelotons rare initially; common by 112 d. No epidermal hairs formed.

Spiranthes magnicamporum	*Epulorhiza* (1)	
(f, 6)		‡ Many seeds germinated. Although pelotons present, no further increase in size observed. Oospores common in proembryos.
Spiranthes romanzoffiana	None	
(b, 9 pkts)		Germination and clamped peloton formation in some seeds by 262 d. Subsequent growth uncommon. 1 large colonized protocorm formed epidermal hairs.
(e, 3 pkts)		‡ As for site b, but pelotons of simple septate hyphae.

Location codes: a: Anola, MB; b: Calahoo Fen, AB; c: Cardinal River Divide, AB; d: Gardenton/Vita, MB; e: Obed Summit, AB; f: Tolstoi, MB; g: Near or within Wagner Natural Area, AB; h: West Castle River, AB; i: Whiteshell Prov. Forest, MB; j: Argyle, MB; k: Edmonton (River Valley), AB; l: Provided by collectors, AB; m: Swan Hills, AB; n: Edson Forest, AB.
‡ Results differ between sites. Germination absent at one site. † Germination indicators: production of epidermal hairs, or enlargement of proembryo beyond imbibed state.

In protocorms in which pelotons had formed, hyphae were generally simple septate but in some seeds of *Platanthera dilatata*, *P. hyperborea* (L.) Lindl., *Cypripedium passerinum* Richards., *C. calceolus* L. and *Spiranthes romanzoffiana* Chamisso, pelotons of hyphae with clamp connections were observed (Fig. 9). Pelotons often formed in the proembryos of *C. passerinum* and *C. calceolus* seeds before germination was evident (Fig. 10). The protocorms of *P. hyperborea*, *C. passerinum* and *S. romanzoffiana* contained pelotons with clamped hyphae at one site and unclamped hyphae at another. Isolating the endosymbiotic basidiomycetes from the protocorms was rarely successful and the majority of seed and protocorm isolates were *Mycelium radicis atrovirens* Melin. Genera of basidiomycetes isolated from the seeds and protocorms are also presented in Table 2.

Despite the abundance of germinated seeds in some *Platanthera hyperborea*, *P. dilatata*, *Amerorchis rotundifolia* and *Coeloglossum viride* (L.) Hartman packets, pelotons were rare. Seedlings of these species produced long epidermal hairs early in development before the establishment of endosymbiotic fungi. Further development of the protocorms proceeded only when pelotons were present. In some cases, endosymbiotic fungi appeared to have entered protocorms through the epidermal hairs (Fig. 11). In *Spiranthes* spp. and *Cypripedium* spp., ingress of endosymbiotic fungi occurred via the epidermal cells of the basal portion of the proembryo (Fig. 12). A few seedlings of *Spiranthes magnicamporum*, *S. lacera*, *C. calceolus* and *P. praeclara* had a distinct meristem by the end of the incubation period.

Enzyme tests The colorimetric tests for enzyme production did not clearly differentiate between all genera of *Rhizoctonia*-like endophytes, but were useful in distinguishing between isolates of *Ceratorhiza* and *Epulorhiza*. The majority of *Ceratorhiza* isolates tested produced at least small amounts of cellulase and polyphenol oxidases

Table 3. Effects of orchid mycorrhizal fungi on non-orchid hosts in dual culture.

Taxon	Isolate	Hosts[a]				
		Pinus resinosa	*Picea glauca*	*Allium sativa*	*Brassica napus*	*Medicago sativa*
Ceratorhiza sp.	Z12-or-2b(1)	H 2 m	H 2	U 2 m	H 2 m	H 2 m
Ceratorhiza pernacatena	Z132-or-a	H 1 m	H 2 m	H 2 m	H 2 m	H 2 m
Ceratorhiza sp.	s1.28	H 2 m	H 2 m	D 2 m	H 2 m	H 2 m
Ceratorhiza sp.	Z48-or-1a(2)	H 2 m	H 2 m	D 2	H 2 m	H 2 m
Moniliopsis sp.	Z30-orm-4a(1)	H 2 m	H 2	H 1	H 1	H 2
Moniliopsis sp.	Z3-orf-5a(1)	H 1 m	H 0	H 2	H 2	H 1
Moniliopsis sp.	Z110-rh-c	H 1 m	H 1 m	U 1	H 1 m	H 2
Moniliopsis sp.	Z112-rh-a	H 2 m	H 0	H 1	H 1	H 1
Thanatephorus cucumeris	Z123-or-2c	H 2 m	H 2	H 2 m	H 2 m	H 1
Epulorhiza repens	Z72-r-1a(1)	H 2 m	H 2 m	U 2H 2 m	H 2	H 2
Epulorhiza sp.	Z16-or-4a(1)	H 2 m	H 2 m	H 1, H 2	H 2	H 2
Epulorhiza sp.	s1.10	H 2 m	H 0	D 2	H 2 m	H 2 m
Epulorhiza anaticula	Z50-or-1a	H 2 m	H 2 m	H 1	H 1 m	H 2 m
Control		H 0	H 0	H 0	H 0	H 0

a) H=healthy, U=some necrosis compared to controls, D=dead. Amount of colonization 0-2; 0=no intracellular colonization, 1=slight colonization, 2=heavy colonization. m=monilioid cells formed in cortical cells of root.

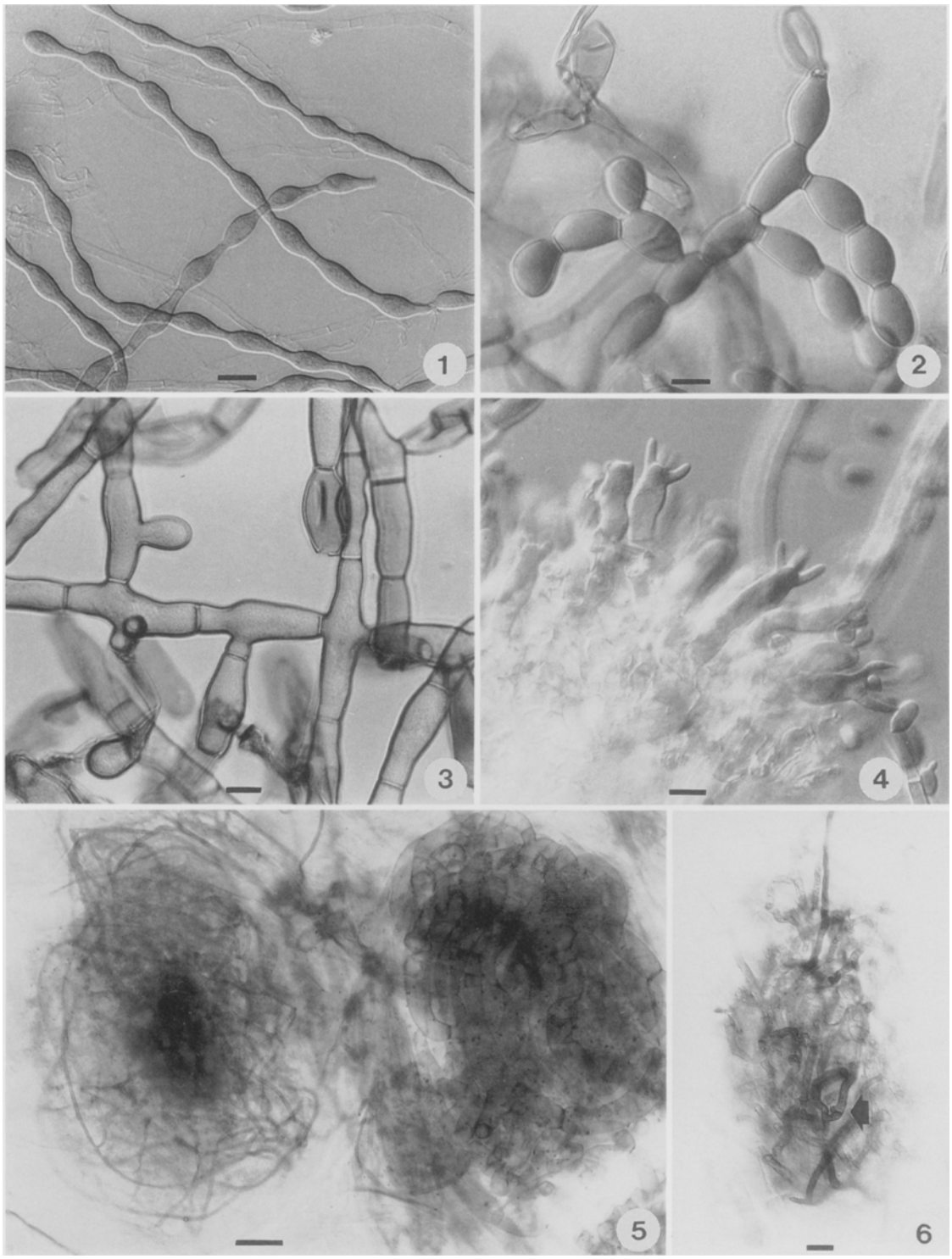

Fig. 1. *Epulorhiza anaticula* (strain Z50-or-1a) monilioid cells on CMA; lactofuchsin stained. Bar = 10 μm. Fig. 2. *Ceratorhiza* sp. (Z48-or-1a(1)) monilioid cells on CMA; lactofuchsin stained. Bar = 10 μm. Fig. 3. *Moniliopsis solani* (Z123-or-2c) broad, widely attached hyphal cells on CMA; lactofuchsin stained. Bar = 10 μm. Fig. 4. *Thanatephorus cucumeris* (Z123-or-2c) basidia and basidiospores; lactofuchsin stained. Bar = 10 μm. Fig. 5. *Amerorchis rotundifolia* root with pelotons of narrow (left) and broad (right) simple septate hyphae. Cleared in KOH; chlorozol black stained. Bar = 20 μm. Fig. 6. Peloton of clamped (arrow) hyphae from same root as Fig. 5. Cleared in KOH; chlorozol black stained. Bar = 10 μm.

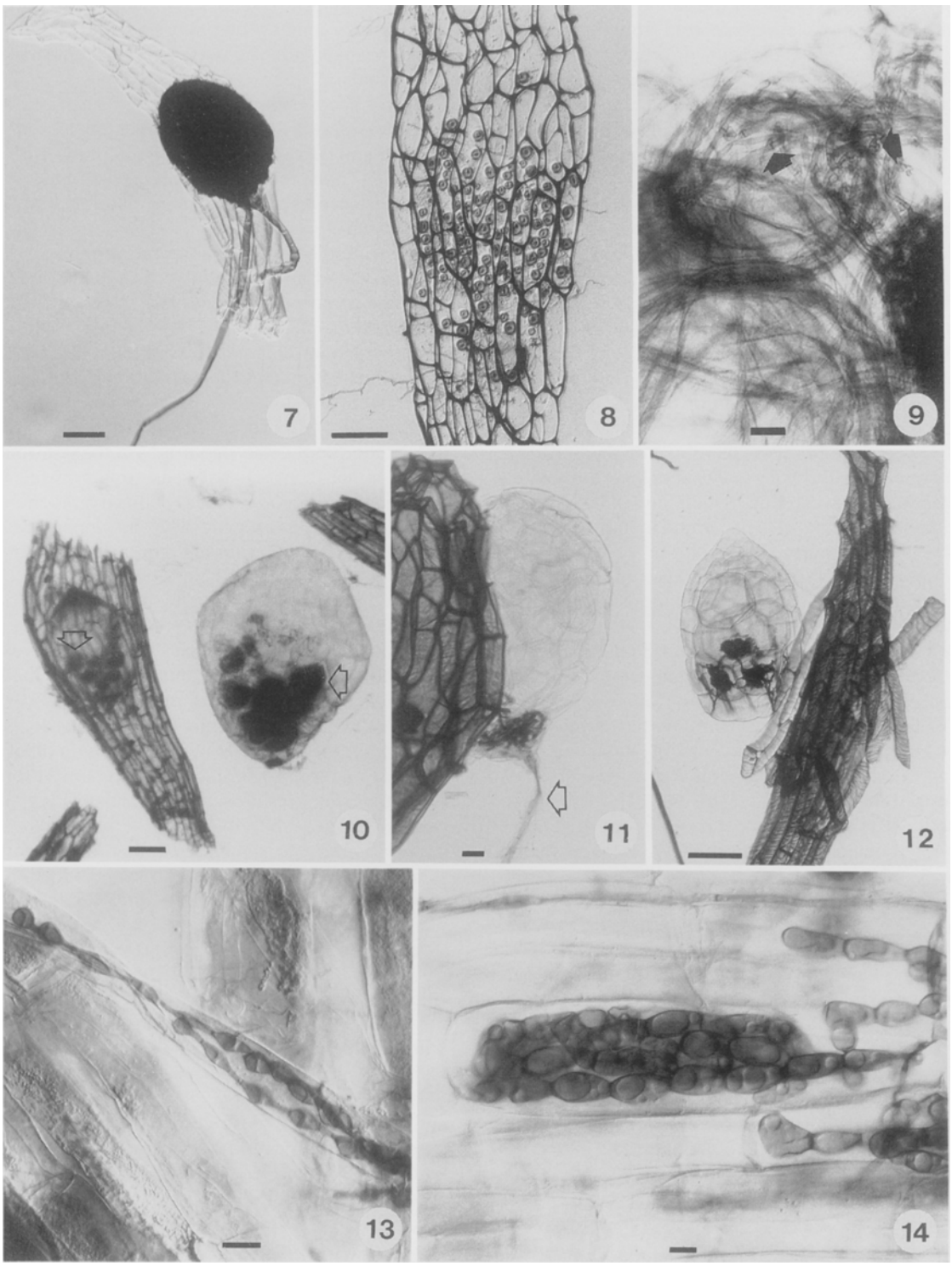

Fig. 7. Sclerotium in *Amerorchis rotundifolia* seed. Lactofuchsin stained. Bar = 50 μm. Fig. 8. *Pythium*-like oospores in proembryo of *Platanthera dilatata*. Cleared in KOH; stained with chlorozol black. Bar = 50 μm. Fig. 9. Peloton of clamped (arrows) hyphae from *Cypripedium calceolus* protocorm. Cleared in KOH; stained with chlorozol black. Bar = 10 μm. Fig. 10. Pelotons (arrows) in proembryo and protocorm of *Cypripedium calceolus*. Cleared in KOH; stained with chlorozol black. Bar = 50 μm. Fig. 11. Colonization of *Amerorchis rotundifolia* via epidermal hair. Cleared in KOH; stained with chlorozol black. Bar = 10 μm. Fig. 12. Colonization of *Spiranthes lacera* via basal cells. Cleared in KOH; stained with chlorozol black. Bar = 50 μm. Fig. 13. *Epulorhiza anaticula* (Z50-or-1a) monilioid cells within root cortical cell of *Pinus contorta*. Cleared in KOH; stained with chlorozol black. Bar = 20 μm. Fig. 14. *Ceratorhiza* sp. (Z48-or-1a(2)) monilioid cells within root cortical cell of *P. contorta*. Cleared in KOH; stained with chlorozol black. Bar = 10 μm.

and many were able to grow on the tannic acid medium. A few morphologically distinct isolates from *Platanthera praeclara* produced cellulase but not polyphenol oxidases, and were identified as *C. pernacatena* (Zelmer and Currah, 1995b). A small number of isolates were either polyphenol oxidases positive and cellulase negative or negative for both attributes. Isolates negative for both enzyme tests were short-lived.

In contrast with the *Ceratorhiza* results, all 32 isolates of *Epulorhiza* spp. were negative for polyphenol oxidases. All but three were positive for cellulase.

Although several isolates of *Moniliopsis* were vigorous in culture, many grew poorly and only a few could be transferred successfully to test media. Results varied among the isolates.

Interactions of orchid endophytes with non-orchid hosts

The effects of the fungal isolates on all host plants are summarized in Table 3. After 8 wk of incubation, most plants in the dual cultures were as robust as the controls. Exceptions occurred in *Allium sativa* seedlings inoculated with *Ceratorhiza* spp. strains S1.28 and Z48-or-1a(2) and *Epulorhiza* sp. strain s1.10. In these combinations, necrosis and death occurred by 8 wk.

The root cortices of the majority of apparently healthy, inoculated plants exhibited considerable development of monilioid cells after 4 wk. Hyphae in the cortex were observed less often in the dual cultures involving *Moniliopsis* strains than in those using *Ceratorhiza* and *Epulorhiza* isolates. Heavy aggregations of monilioid cells occurred intracellularly with all strains (except Z110-rh-c) in combination with at least one host species. The roots of the two conifer species were heavily and consistently colonized (Figs. 13, 14). Intracortical monilioid cell morphology of the inoculants was comparable with monilioid cell morphology on agar media (e.g., Figs. 1, 13).

Discussion

Peloton-forming basidiomycetes were common in all the mature terrestrial orchid species examined with the exception of *Cypripedium acaule* and *Malaxis monophyllos*. Representatives of the fungi were isolated in pure culture with relative ease but in a few instances efforts to establish cultures from segments with pelotons failed. These fungi may have been moribund, or fastidious in their cultural requirements, or sensitive to the antibiotic in the isolation medium. In replicate platings, isolates were sometimes obtained on media without tetracycline but not on plates containing the antibiotic. Alternatively, they may have required a growth factor not present in the isolation medium, or were involved in an obligate symbiosis with other plants in the vicinity, such as the endosymbiont of *Corallorhiza trifida* (Zelmer and Currah, 1995a).

Two patterns of colonization by mycorrhizal fungi were seen in the mycorrhizas of mature orchids. In the first, the pelotons formed in the outermost layer of cortical cells were not degraded. These pelotons may serve as a source of inoculum for the recolonization of cells deeper in the root cortex, where the pelotons are utilized by the orchid. This pattern was seen particularly in *Corallorhiza* species, and may be important to the persistence of the symbiosis between the orchids and their endophytes.

The second pattern, seen especially in species of *Platanthera* and *Spiranthes*, consisted of synchronously formed and degraded patches of pelotons within the root cortex. These patches may represent individual colonization events because adjacent patches may be formed by different fungi.

Not all the orchid seeds that germinated in the field trials required an endosymbiont initially, but further development did not occur in the absence of mycorrhizal colonization. Germination before establishment of the endosymbiotic relationship was seen most often in species which produced long epidermal hairs, such as the *Platanthera* species, *Coeloglossum viride* and *Amerorchis rotundifolia*. Contact between the proembryos and colonizing fungi often occurred via these structures. In vitro, initial contact between mycorrhizal fungi and *Platanthera hyperborea* was reported by Richardson et al. (1992) to be primarily via dead suspensor cells. Other orchids, such as *Spiranthes* spp. and *Cypripedium* spp., did not initially produce epidermal hairs, and colonization was initiated by fungal penetration into basal cells of the proembryo.

Species of *Epulorhiza* and *Ceratorhiza* were isolated from both the protocorm tissues of germinating seeds and mycorrhizas of mature plants. *Moniliopsis* species were only obtained from mature plants. Clamped basidiomycetes were rarely seen forming pelotons in the roots of mature plants, but were present in the pelotons of protocorms from five orchid species (*Cypripedium calceolus*, *C. passerinum*, *Platanthera dilatata*, *P. hyperborea*, *Spiranthes romanzoffiana*). This suggests that these fungi play a more important role in the establishment of orchids than in the maintenance of mature plants. Clamped basidiomycetes have been reported from orchid mycorrhizas from our study area (Currah et al., 1988) as well as from other geographic regions (Richardson et al., 1993; Umata, 1995).

The clamped basidiomycetes were rarely isolated, and their identities are not known. However, it is possible that these fungi are also ectomycorrhizal symbionts of nearby woody plants. If so, the absence of cellulase and polyphenol oxidase enzymes characteristic in most ectomycorrhizal fungi (Hutchison, 1991) might be more conducive to the development of a mycorrhizal protocorm. As well, hyphae connected to ectomycorrhizas may provide a richer source of nutrients to the developing mycotrophic protocorms than the saprophytic fungi typically associated with the mature plants.

In contrast to these clamped isolates, most other endosymbiotic fungi from seeds and adult plants produced either cellulase or polyphenol oxidases or both. Because the vigour of the *Moniliopsis* cultures varied greatly, the results of the enzymatic tests are not reliable. However, the *Epulorhiza* strains tested negative for polyphenol oxidases, while *Ceratorhiza* strains, with the exception of the *Ceratorhiza pernacatena* isolates, produced poly-

phenol oxidases. In combination with morphology, this enzyme test is taxonomically informative. Most *Ceratorhiza* and *Epulorhiza* isolates produced cellulase. Three *Epulorhiza* isolates produced neither enzyme, inviting speculation that in nature they may be ectomycorrhizal symbionts. Ectomycorrhizas formed by fungi with *Epulorhiza*-like parenthesomes have been reported by Haug and Oberwinkler (1987). Ectomycorrhizas were not formed by our orchid endophytes in dual culture, but production of these organs by known mycorrhizal fungi in vitro is often unsuccessful (Fortin et al., 1980).

The broader role of these orchid endophytes needs to be explored in more detail. The production of cellulase and/or polyphenol oxidases would be consistent with a saprophytic or pathogenic mode of nutrition, however, the slow growth of some strains, and lack of mitospore production, suggests a different niche than expected for soil saprophytes. A clear pathogenic relationship was observed with some strains in combination with *Allium sativa*, although extrapolations to nature must be made with caution.

Unidentified species of "*Rhizoctonia*" have been recognized in the roots of non-orchid hosts by Milligan and Williams (1987), Väre et al. (1992) and Dhillion (1994) although the role of these endophytes in nature is unclear. The ecological role of different orchid and non-orchid endophytes is difficult to establish from the literature as many researchers refer to unidentified and uncharacterized fungi only as "*Rhizoctonia* sp."

Although some species of *Ceratorhiza*, *Moniliopsis* and *Epulorhiza* are known to be pathogens (Sneh et al., 1991) it is apparent that some taxa can form relatively harmless or even beneficial associations with non-orchid hosts (Mazzola, 1996). The relationship between these plants and orchidaceous rhizoctonias may not parallel that of the fungi with orchids, though, because monilioid cells are formed within the cells of the non-orchidaceous host.

Monilioid cells are rarely seen in orchid mycorrhizas, but are common in agar cultures of these fungi. Although not considered a reliable diagnostic feature by Andersen (1990), the morphologies of *E. anaticula* and *C. pernacatena* monilioid cells in the pine roots were distinctive for the these species.

The difficulty of distinguishing between endophyte taxa has also impeded our knowledge of the specificity of orchid fungus relationships. The seedlings of *Spiranthes lacera* yielded *Ceratorhiza* isolates morphologically similar to those isolated from the mycorrhizas of mature *S. lacera* plants. However, species of *Ceratorhiza* in this study, with the exception of *C. pernacatena*, could not be distinguished, and so it is not known how many species are represented.

Many stained squash mounts of orchid mycorrhizas contained pelotons of two (or more) fungal genera (often *Epulorhiza* and *Ceratorhiza*), so specificity of these orchids for particular fungi is expected to be low. Seedling orchids may have different endophytes than mature plants of the same species, evidenced by the occurrence of clamped endophytes in pelotons of seedlings without similar pelotons in the mature plants. This contrasts with the observations of Currah et al. (1996), who found that seedlings of *Platanthera integrilabia* (Correll) Luer from outplanted packets in the vicinity of mature plants of the same species were mycorrhizal with a single species, *Epulorhiza inquilina* Currah, Zettler & McInnis.

Germinating seeds may accept different fungi depending upon their availability. For three orchid species, pelotons of a *Rhizoctonia*-like fungus were seen in protocorms at one site and pelotons with clamped hyphae at a different site. As the enzymatic abilities and carbon source of the endosymbiotic fungi are known to differ, it may be advantageous to the orchid seedling to accept several potential symbionts. The ability to accept several potential fungal symbionts could also increase the chances of colonization. Many of the ungerminated seeds we observed may have been unsuccessful in contacting an appropriate fungus to initiate and support their development. By associating with several taxa of fungi, mature plants may have access to a wider range of nutrient sources, tapping into the resources of parasitic, saprophytic and ectomycorrhizal fungi.

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