Cryptosporiopsis species isolated from the roots of aspen in central Alberta: identification, morphology, and interactions with the host, in vitro[1]

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Abstract: *Cryptosporiopsis* Bubák & Kabát isolates were obtained for the first time from roots of apparently healthy aspen seedlings in Alberta. These isolates were similar in all the major morphological features previously used to separate *Cryptosporiopsis* species, but sequencing data of the ITS1-5.8S-ITS2 region indicated that they were separated into two groups, one belonging to *Cryptosporiopsis ericae* Sigler and the other to *Cryptosporiopsis radicicola* Kowalski & Bartnik. Scanning electron microscopy of ex-type cultures and selected isolates from aspen roots revealed that *C. ericae* and *C. radicicola* differed in morphogenesis and structure of conidiomata: those of *C. ericae* were either synnematous or sporodochial, whereas those of *C. radicicola* possessed a peridium-like mycelial envelope bearing amorphous adhesive material. Phialides in the hymenium of *C. radicicola* were also embedded in amorphous matrix material but such material was absent in *C. ericae*. Microscopic examination of artificially inoculated aspen roots indicated that both species are endophytes of the host. Hyphal penetration by *C. ericae* was only occasional and confined to the host epidermis, whereas *C. radicicola* was more aggressive and its hyphal ingress extended to the cortical region.

Key words: conidiomata, DSE, ITS, endophytic fungi, *Populus tremuloides*, SEM.

Résumé : Les auteurs ont obtenu des isolats de *Cryptosporiopsis* Bubák & Kabát pour la première fois, à partir de racines de plantules de peupliers faux-trembles, en Alberta. Ces isolats comportent tous les caractères morphologiques principaux déjà utilisés pour séparer les espèces de *Cryptosporiopsis*, mais les données de séquençage de la région ITS1-5.8S-ITS2 révèlent l'existence de deux groupes, un appartenant au *Cryptosporiopsis ericeae* Sigler et l'autre au *Cryptosporiopsis radicicola* Kowalski & Bartnik. La microscopie électronique par balayage de cultures de l'ex-type et d'isolats sélectionnés montre que le *C. ericeae* et le *C. radicicola* diffèrent par leur morphogenèse et leur conidiomata : ceux du *C. ericeae* sont synnématique ou sporodochiaux, alors que ceux du *C. radicicola* possèdent une enveloppe mycélienne ressemblant à un péridium, portant du matériel adhésif amorphe. Les phialides de l'hyménium se retrouvent également dans un matériel matriciel amorphe, mais on ne retrouve pas ce matériel chez le *C. ericeae*. L'examen microscopique de racines de trembles artificiellement inoculées indique que les deux espèces constituent des endophytes de l'hôte. La pénétration occasionnelle des hyphes du *C. ericeae* se confine à l'épiderme de l'hôte, alors que le *C. radicicola,* plus agressif, s'étend à la région corticale.

Mots-clés : conidiomata, DSE, ITS, champignon endophyte, *Populus tremuloides*, microscopie électronique par balayage.

Introduction

During a recent survey of dark, septate fungal endophytes (DSE, see Addy et al. 2005) in the roots of apparently healthy aspen (*Populus tremuloides* Michx.) saplings growing in different soils and moisture regimes in Alberta, we recovered an unexpectedly large number of isolates belonging to the anamorphic genus *Cryptosporiopsis* Bubák & Kabát, which is characterized by ellipsoid, pluriguttulate, aseptate macroconidia and much smaller microconidia developed from integrated, hyaline, phialidic conidiogenous cells, and acervular or stromatic conidiomata (Sutton 1980; Verkley 1999). Although many species are known, there are few morphological differences among them and the characters used to distinguish taxa are few and unreliable (Sutton 1980). Most species are anamorphs of *Pezicula* Tul. & C. Tul. or *Neofabraea* H.S. Jacks. (Helotiales) (Verkley 1999) and occur mainly on aerial plant parts, especially the branches and trunks of forest and fruit trees (Sutton 1980; Dugan et al. 1993).

Six species of *Cryptosporiopsis* have thus far been reported from the roots of woody plants (Verkley et al. 2003; Sigler et al. 2005): viz., *Cryptosporiopsis grisea* (Pers.) Petr., the anamorph of the wood and bark endophyte *Pezi-*

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Table 1. Isolates of *Cryptosporiopsis* species from roots of aspen (*Populus tremuloides*) collected in Alberta in 2004.

Collection number (accession No.)	Locality	GenBank accession No.
Cryptosporiopsis ericae		
BWC-42-126d[a] (UAMH 10819)	Bilby Lake	EF413591
MDL-10-28a[a]	Lamont	—
MDL-15-43a[a]	Lamont	—
BWC-31-92a[a] (UAMH 10818)	Bilby Lake	—
BWC-43-127c[a]	Bilby Lake	—
BDC-21-61b[a]	Bilby Lake	—
BDC-21-61c[a]	Bilby Lake	—
LDL-58-172b[a]	Lily Lake	—
LDL-58-172d[a]	Lily Lake	—
BWC-31-91b[b]	Bilby Lake	EF413592
MWL-4-1a[b]	Lamont	—
MWL-4-11b[b]	Lamont	—
MDL-7-20e[b]	Lamont	—
MDL-7-20b[b]	Lamont	—
MDL-10-28b[b] (UAMH 10820)	Lamont	—
MDL-13-37b[b]	Lamont	—
MDL-13-38d[b]	Lamont	—
MDL-14-40b[b]	Lamont	—
BWC-43-127a[b]	Bilby Lake	—
BWC-43-127d[b]	Bilby Lake	—
BDC-20-59b[b]	Bilby Lake	—
BDC-21-61h[b]	Bilby Lake	—
BDC-23-69a[b]	Bilby Lake	—
BDC-26-76d[b]	Bilby Lake	—
BDC-33-98e[b]	Bilby Lake	—
BDC-34-101d[b]	Bilby Lake	—
LWL-47-139a[b]	Lily Lake	—
LDL-57-169h[b]	Lily Lake	—
LDL-58-173e[b]	Lily Lake	—
LDL-59-175e[b]	Lily Lake	—
LDL-60-178b[b]	Lily Lake	—
YDL-71-211a[b]	Lloyd Creek	—
MDL-13-38a[c]	Lamont	EF413595
BDC-26-77b[d] (UAMH 10865)	Bilby Lake	EF413593
MDL-17-50c[e] (UAMH 10866)	Lamont	EF413594
C. radicicola		
BWC-39-117d[f] (UAMH 10822)	Bilby Lake	EF413597
BDC-22-65d[f]	Bilby Lake	—
BWC-35-103h[f]	Bilby Lake	—
LWL-54-160b[f]	Lily Lake	—
LDL-56-166b[f]	Lily Lake	—
LDL-59-176e[f]	Lily Lake	—
BWC-30-89a[g] (UAMH 10864)	Bilby Lake	EF413598
BWC-36-107e[h]	Bilby Lake	EF413596
YDL-70-208e[i] (UAMH 10821)	Lloyd Creek	EF413599

Note: Isolates with a common superscript showed 100% similarity based on pairwise comparison in Se-Al.

cula cinnamomea (DC.) Sacc., capable of spreading into roots of dying trees (Kowalski 1983); *Cryptosporiopsis melanigena* Kowalski & Halmschlager and *Cryptosporiopsis radicicola* Kowalski & Bartnik, isolated from the roots of *Quercus*; and *Cryptosporiopsis rhizophila* Verkley & Zijlstra (Verkley et al. 2003), *Cryptosporiopsis ericae* Sigler, and *Cryptosporiopsis brunnea* Sigler (Sigler et al. 2005), all from the roots of ericaceous plants. Verkley et al. (2003) distinguished among the four species, *C. grisea*, *C. radicicola*, *C. melanigena*, and *C. rhizophila*, by means of morphological as well as DNA sequence characters. Sigler et al. (2005) established the other two, i.e., *C. ericae* and *C. brunnea*, based on both ITS region sequences and morphological features. However, the nature of the associations of these *Cryptosporiopsis* species with their host roots is largely unknown. The main objectives of this study were to determine (*i*) the specific taxonomic disposition of the *Cryptosporiopsis* isolates from aspen roots using morpholog-

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Table 2. Sequences included in this study with GenBank accession No., species name, strain, geographic origin, and source publication.

GenBank accession No.	Taxon	Strain	Host	Geographic origin	Source
AY359234	*Cryptosporiopsis actinidiae*	Laundon 20074	*Malus* ×*domestica*	New Zealand	Johnson et al. (2004)
AF149074	*C. brunnea*	UBCtra 288	*Gaultheria shallon*	B.C., Canada	Berch et al. (2002)
AF141165	*C. diversispora*	CBS 185.50 (ex type)	*Picea abies*	Norway	Abeln et al. (2000)
AY540126	*C. ericae*	UAMH 9445	*Erica* sp.	Western North America	Sigler et al. (2005)
AF141196	*C. melanigena*	CBS 898.97; UAMH 10731 (ex type)	*Quercus petraea* (root)	Austria	Abeln et al. (2000)
AF141193	*C. radicicola*	CBS 640.94; UAMH 10729 (ex type)	*Quercus robur* (root)	Poland	Abeln et al. (2000)
AY176753	*C. rhizophila*	CBS 109839; UAMH 10730	*Erica tetralix*	Netherlands	Verkley et al. (2003)
AF141163	*Dermea viburni*	CBS 145.46	*Viburnum* sp.	Ontario, Canada	Abeln et al. (2000)
AF141190	*Neofabraea alba*	CBS 452.64	*Malus sylvestris*	England	Abeln et al. (2000)
AF141189	*N. malicorticis*	CBS 355.72	*Malus sylvestris*	Portugal	Abeln et al. (2000)
AY359236	*N. alba*	MM 159	Unknown	New Zealand	Johnson and Park (2003)*
AF141175	*Pezicula acericola*	CBS 245.97	*Acer spicatum*	Ontario, Canada	Abeln et al. (2000)
AF141170	*P. aurantiaca*	CBS 201.46	*Alnus crispa* var. *mollis*	N.S., Canada	Abeln et al. (2000)
AF141197	*P. carpinea*	CBS 921.96	*Carpinus betulus*	Germany	Abeln et al. (2000)
AF169306	*P. carpinea*	GB4547	*Carpinus caroliniana*	USA	Bills et al. (1999)
AY344803	*P. cinnamomea*	Unknown	Unknown	New Zealand	Johnson and Park (2003)*
AF141182	*P. corni*	CBS 285.39	*Cornus circinata*	Ontario, Canada	Abeln et al. (2000)
AF141176	*P. corylina*	CBS 249.97	*Corylus cornuta*	Ontario, Canada	Abeln et al. (2000)
AF141195	*P. frangulae*	CBS 778.96	*Rhamnus frangula*	Netherlands	Abeln et al. (2000)
AF141167	*P. heterochroma*	CBS 199.46 (ex type)	*Alnus crispa*	Netherlands	Abeln et al. (2000)
AF141181	*P. ocellata*	CBS 949.97	Unknown	Luxemburg	Abeln et al. (2000)
AF141178	*P. rubi*	CBS 253.97	*Rubus* sp.	New York, USA	Abeln et al. (2000)
AF141192	*P. rubi*	CBS 593.96	*Rubus* sp.	Netherlands	Abeln et al. (2000)
AF141177	*P. rubi*	CBS 251.97	*Rubus* sp.	USA	Abeln et al. (2000)
AF141172	*P. sporulosa*	CBS 224.96 (ex type)	*Larix decidua*	Netherlands	Abeln et al. (2000)
AY344802	*Pezicula* sp.	ICMP 14405	Unknown	New Zealand	Johnson and Park (2003)*
AF141173	*Pezicula* sp.	CBS 101.96	*Abies alba*	Netherlands	Bills et al. (1999)
AF141200	*Pezicula* sp.	CBS 100416	*Amelanchier lamarckii*	Netherlands	Abeln et al. (2000)
AJ301960	Ascomycete sp.	BBA 71218	*Erica* sp.	Germany	GenBank

*Data from the journal article are unpublished data.

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Table 3. Isolates representing *Cryptosporiopsis* species from roots of 5- to 7-year-old aspen saplings (n = 90) obtained from five different collection sites in Alberta in 2004.

	Lamont (luvisol)		Bilby Lake (chernozem)		Lily Lake (luvisol)		Lloyd Lake (luvisol)		Red Water (chernozem)
	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Wet
Number of aspen saplings examined	9	9	9	18	9	9	9	9	9
Number yielding *Cryptosporiopsis* spp.	6	1	7	7	5	2	2	0	0
Number of *Cryptosporiopsis* spp. isolates*	19	2	15	15	18	2	2	0	0

*Number of isolates derived from five root tips per sapling.

ical and ITS sequence analyses, and (*ii*) the nature of their associations with host roots using artificially inoculated seedlings.

Materials and methods

Isolation

Ninety apparently healthy, 5- to 7-year-old saplings of aspen were excavated from five different forested sites within a 110 km radius of Edmonton, Alberta. Five collection sites were chosen so that moist (low lying) and (or) dry (upland) habitats were present at each site and both luvisol and chernozem soils were represented. After washing the coarse debris from each root ball in the laboratory, fine roots were excised 1 cm behind the apex and surface sterilized in 70% ethanol for 1 min and then in a 40% solution of household bleach for 5 min, followed by 70% ethanol for 3 s. After rinsing twice in sterile distilled water, five root tips from each sapling were placed on a plate of MEA [20 g Bacto™ malt extract (Becton, Dickinson and Co., Sparks, Md.), 18 g select agar (Invitrogen, Carlsbad, Calif.), 10 mg oxytetracycline dihydride, 1 L distilled water (dH_2O)], CMA [15 g corn meal agar (Acumedia Manufacturers, Inc., Baltimore, Md.), 10 mg oxytetracycline dihydride, 1 L dH_2O] or OA (20 g rolled oats, 20 g select agar, 1 L dH_2O) and incubated in the dark at room temperature. Colonies representing *Cryptosporiopsis*, i.e., having the characteristic macroconidia, sporodochia, and tan to brown colonies (Kowalski and Bartnik 1995; Sigler et al. 2005), were transferred to fresh MEA (without oxytetracycline dihydride) to obtain pure cultures. Only a few representative isolates of each species were deposited in the University of Alberta Microfungus Collection and Herbarium (UAMH).

DNA extraction and sequencing

Broth cultures were prepared by inoculating 50 mL of 2% malt extract in dH_2O with each of 44 isolates (Table 1) and incubating on a rotary shaker (300 rpm) for 1 week at room temperature. Mycelium was removed from broth cultures with a sterile swab, blotted free of excess medium on sterile paper towels, placed in a pre-cooled sterile porcelain mortar and frozen with liquid nitrogen. Frozen mycelium (ca.100 mg) was ground to a powder in sterile sand and genomic DNA was extracted with approximately 1000 μL of extraction buffer (2× CTAB, 1% *w/v* CTAB; 1 M NaCl; 100 mmol/L Tris; 20 mmol/L EDTA; 1% *w/v* polyvinyl polypyrrolidone). The mixture was transferred to a sterile 2 mL screw-cap microcentrifuge tube with 2 μL β-mercaptoethanol and incubated at 65 °C for 2 h. Proteins were removed by adding 750 μL chloroform – isoamyl alcohol (24:1 *v/v*) to the CTAB solution and mixed by inverting the tube about 20 times. After centrifugation at 13 809g for 20 min at room temperature, the supernatant, which contained crude DNA material, was removed and purified using the QIAquick DNA Purification Kit (Qiagen Inc., Mississauga, Ont.). DNA sequences from the ITS ribosomal DNA (rDNA) region, i.e., ITS1, ITS2 and 5.8S, were determined. The ITS regions were amplified using primers BMBC-R (Lane et al. 1985) and ITS4 (White et al. 1990) (synthesized by CyberSyn, Aston, PA). Amplification was done using the Perkin Elmer GeneAmp 9700 Thermal cycler (PE Applied Biosystems, Foster, Calif.) following these parameters: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. Initial denaturation was at 94 °C for 2 min, and the final extension was at 72 °C for 7 min. There were 30 cycles. Cycle sequencing was done with the ABI PRISM® BigDye™ terminator cycle sequencing kit (Applied Biosystems, Foster, Calif.) using forward and reverse primers BMBC-R (Lane et al. 1985), ITS1, ITS2, ITS3, and ITS4 (White et al. 1990). The sequencing products were cleaned with G50 Superfine Sephadex columns (Amersham Pharmacia Biotech, Arlington Heights, Ill.) and separated and run on an ABI 377 Automated Sequencer (Applied Biosystems, Foster, Calif.). Consensus sequences were determined using Sequencher™ for Windows 4.0.5 (Gene Codes Corp. Ann Arbor, Mich.) and alignment was done manually using Se-Al version 2.0a11 Carbon.

Phylogenetic analysis

Phylogenetic analysis was done using PAUP (phylogenetic analysis using parsimony) version 4.0b10 (Swofford 2003) and the robustness of the resulting tree was evaluated by bootstrapping (Felsenstein 1985). Pairwise comparison of sequences was performed manually in Se-Al version 2.0a11 Carbon (Rambaut 1996). Heuristic searches were done in PAUP version 4.0b10 (Swofford 2003) using random stepwise-addition options with maximum parsimony as the optimality criterion and tree bisection–reconnection (TBR) as the branch swapping algorithm. Alignment gaps were treated as missing characters. Bootstrap analyses were performed using 1000 replicates. BLAST (Altschul et al. 1990) searches using BLASTN in GenBank revealed high similarity to species of *Pezicula*, *Dermea*, and *Cryptosporiopsis*, of which a part of the 18S, ITS1, 5.8S rDNA, and ITS2 had been sequenced. In our analyses only the ITS and 5.8S regions of these sequences were included. GenBank accession numbers, taxon names, and other information about these sequences are given in Table 2. The tree was rooted using *Dermea viburni* Groves as outgroup.

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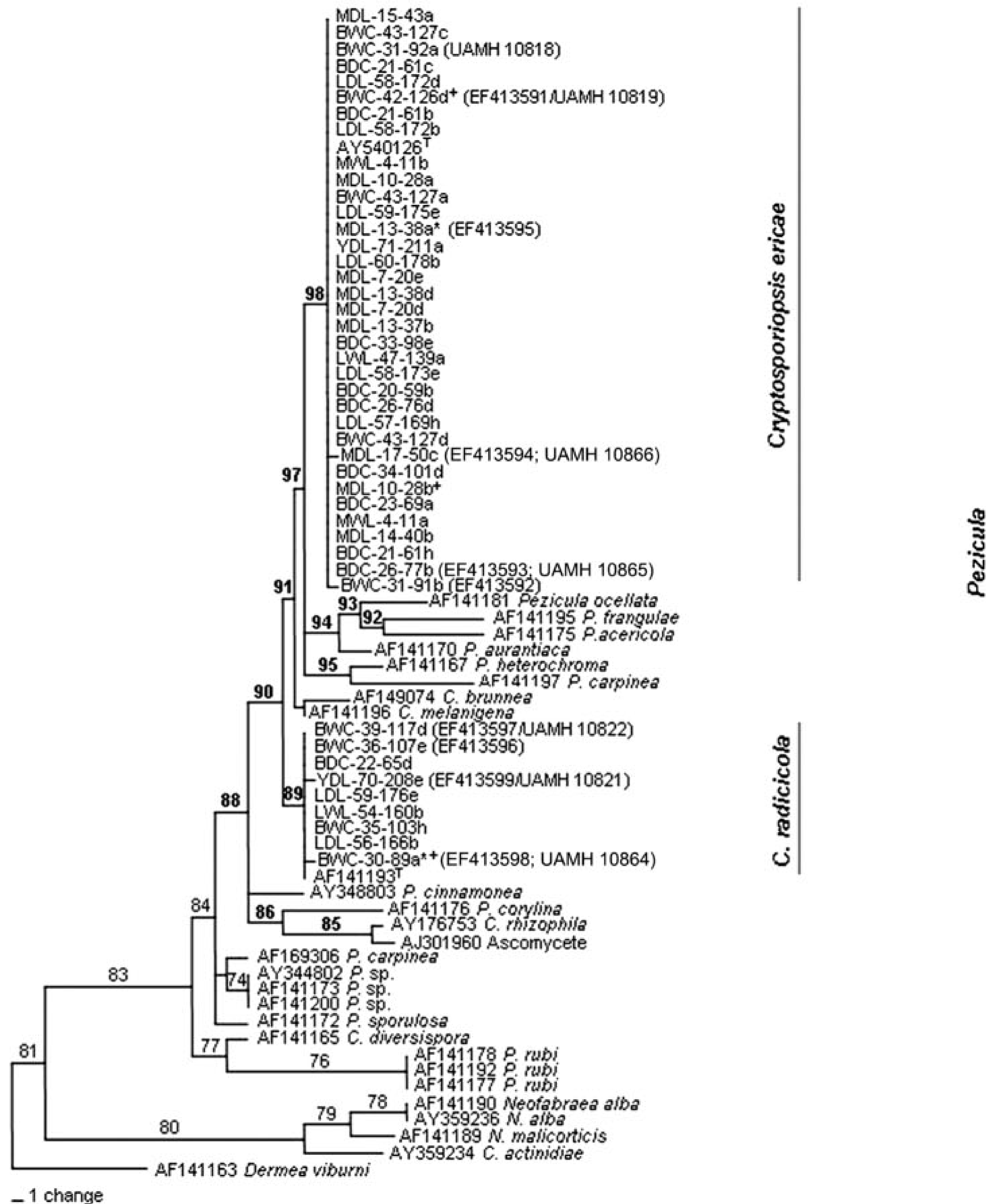
Fig. 1. One of 869 MPTs of 203 steps using 78 parsimony-informative characters of the ITS regions and 5.8SrDNA showing the relationship between isolates identified as *C. ericae* and *C. radicicola* and other anamorphs assignable to *Pezicula* and *Neofabraea*. Bootstrap values from 1000 replications (rounded to the nearest integer) are shown on the branches; those at 85% or higher are in bold. GenBank and UAMH accession numbers are indicated in brackets. Teleomorph names are used where known. *Dermea viburni* was the outgroup. Isolates examined by SEM are flagged with an asterisk and those used in inoculation studies are flagged with a cross.



Preparation of aspen roots inoculated with Cryptosporiopsis *isolates*

Aspen seeds were surface sterilized in a 40% solution of household bleach for 2 min, rinsed twice in sterilized dH_2O, and then plated on TWA (tap water agar, 10 g select agar, 1 L dH_2O) at room temperature. After approximately 2 weeks, seedlings with two foliage leaves were transplanted into peat pellets (Jiffy-7^{TM}, Jiffy Products (NB) Ltd., N.B.) and incubated in a growth chamber (Lab-line Instruments, Inc., Melrose Park, Ill.) for 2 months at 20 °C and a photoperiod of 18 h at an intensity of 200 $\mu E \cdot m^{-2} \cdot s^{-1}$. One month later, seedlings were inoculated with *C. radicicola* (BWC-30-89a, ex-aspen, Bilby Lake; UAMH 10864) or *C. ericae* (BWC-42-126d, ex-aspen, Bilby Lake; UAMH 10819; MDL-10-28b, ex-aspen, Lamont; UAMH 10820) by placing a 4 mm × 4 mm block of mycelium, from a 30-day-old culture on MEA, 2 cm deep into the peat pellet adjacent to the root. Uninoculated plants served as controls. Colonization was detected initially by re-isolation of the inoculant from a 1 cm long root segment that had been surface sterilized (as described above) and plated on MEA.

Microscopy

All isolates were grown on OA or MEA at room temperature for 1–3 months in the dark and examined by light microscopy (LM). For scanning electron microscopy (SEM), *C. ericae* (ex-type, UAMH 9445; isolate from aspen root MDL-13-38a), and *C. radicicola* (ex-type, CBS 640.94; UAMH 10729; isolate from aspen root BWC-30-89a; UAMH 10864) grown and incubated as above were used. To investigate the aspen root – *Cryptosporiopsis* interactions, 2-month-old aspen seedlings, which had been inoculated either with *C. ericae* or *C. radicicola* and prepared as

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Table 4. Measurements of the width and length of macro- and micro-conidia in *Cryptosporiopsis ericae* (CE) and *C. radicicola* (CR).

		Species	Mean	*t*
Macroconidia[†]	Width (μm)	CE	5.76	0.675
		CR	5.88	
	Length (μm)	CE	24.65	0.855
		CR	25.93	
Microconidia[‡]	Width (μm)	CE	1.06	3.576*
		CR	1.29	
	Length (μm)	CE	3.68	3.246*
		CR	2.86	

*Significant at 5% level (*t*-test).
[†]Ten macroconidia were measured in each of 10 isolates.
[‡]Ten microconidia were measured in each of 5 isolates.

described above, were used. Root segments 1 cm in length, 10 per batch of seedlings, were cut in half for SEM and for thin-section observations by LM. Preparation methods for thin sections and SEM samples were the same as described by Tsuneda et al. (2004). SEM samples were examined and photographed at 10 or 15 kV using a Hitachi S-510 microscope.

Results

Fungi belonging to the genus *Cryptosporiopsis* were obtained from the roots of 29 of the 90 aspen saplings examined and across four of the five sites sampled (Table 3). Soil type did not have a marked effect (43 isolates from sites with luvisols; 30 from one site with chernozem), but roots from trees growing in drier soils yielded the majority of isolates (74% from drier soils). Preliminary microscopic examinations of these isolates indicated that more than one species was represented and this was confirmed by molecular analyses.

Molecular analyses

Analysis of the ITS1-5.8S-ITS2 sequences yielded a total length of 525 characters of which 427 were constant, 20 were variable but parsimony uninformative, and 78 were parsimony informative. A heuristic search using maximum parsimony yielded 869 most parsimonious trees (MPT) of 203 steps, with a consistency index (CI) of 0.6158, retention index (RI) of 0.8278, and a homoplasy index (HI) of 0.3842. One of the 869 maximum parsimony trees is shown in Fig. 1.

Of the 44 isolates analyzed, 35 formed a highly supported clade (98%) with the ex-type of *C. ericae* (AY540126), and 9 formed a fairly highly supported clade (89%) with the ex-type of *C. radicicola* (AF141193). Both clades, along with the oak root endophyte *C. melanigena* and a root endophyte from Ericaceae, *C. brunnea*, were included in a clade with 90% bootstrap support. Likewise, *C. ericae*, *C. radicicola*, *C. melanigena*, *C. rhizophila*, and *C. brunnea* were in the clade of *Pezicula* with 83% bootstrap support.

Morphological observations

We first examined 35 and 9 isolates from aspen roots in the *C. ericae* and the *C. radicicola* clade, respectively, for cultural characteristics and for morphological features using LM. Colonies of *C. ericae* were initially smooth and pale in colour, but by 28 d they became dark brown to black, and had cream-colored conidiomata scattered over the surface. Macroconidia were aseptate, straight to curved, 20–30 μm × 5.0–6.2 μm, and arose from phialides occurring singly on vegetative hyphae or in conidiomata. Microconidia were mostly oblong, aseptate, and 3–4.2 μm × 1–1.2 μm. Seta-like hyphae were darkly pigmented, 5.2–7.8 μm wide, up to 100 μm long, and had 4 to 6 septa. Chlamydospores were hyaline, brown, or black, globose and up to 13 μm in diameter, to ellipsoidal, 10–15 μm × 5.2–7.8 μm.

Colonies of *C. radicicola* were initially smooth and pale in colour, becoming beige to brown or black by 28 d, by which time beige to brown conidiomata were numerous over the surface. Macroconidia were straight to curved, aseptate, occasionally appearing 1–3 septate in old cultures, 17–35 μm × 5–7 μm, and arose from single phialides on vegetative hyphae or in conidiomata. Microconidia were mostly ellipsoidal, 2–4 μm × 1–1.5 μm. Seta-like hyphae occurred in some isolates and were smooth, with 4–6 septa, darkly pigmented at the base, upper part hyaline, blunt-ended, up to 94 μm long and 2.6 μm wide at the base. Using these morphological data, the only reliable criteria for discriminating between the two species appeared to be the somewhat darker conidiomata in *C. radicicola* and the longer, more slender microconidia in *C. ericae* (Table 4). Consequently we made comparative examinations of ex-type cultures of both species, primarily by SEM, in a search for characters related to morphogenesis and structure of conidiomata.

Conidiomata of the ex-type culture of *C. ericae* (UAMH 9445) were either synnematous (Figs. 2–4) or resembling sporodochia (hereinafter called sporodochial conidiomata) (Figs. 6–8). The synnema was composed of two to many erect conidiophores (Figs. 2–4, arrows), which were cemented together, and a spore-bearing head. In some synnemata, microconidia were predominant and macroconidia were scarce (Figs. 4 and 5). Sporodochial conidiomata were spherical to hemispherical, and formed on a basal mycelial mat that was either cushion-like (Fig. 6) or composed of horizontally arranged sparse hyphae from which bundles of conidiophores arose (Fig. 8, arrow). Phialides of both macro- and micro-conidia were densely arranged and formed the spore-bearing layer of the conidioma (hereinafter called hymenium) (Figs. 6–9). Sometimes, the periphery of the hymenium consisted mostly of phialides bearing microconidia (Figs. 6 and 7), but usually both forms of phialides were intermingled in the hymenium (Figs. 9 and 10). Neither mycelial covering over the sporodochial conidioma nor matrix material in the hymenium was present, thus, the entire length of phialides was clearly visible under SEM (Figs. 7 and 9). Besides typical macro- and micro-conidia, those that were intermediate in size and shape often occurred in sporodochial conidiomata (Fig. 10, asterisks). Septa in conidia were difficult to locate by SEM. Under LM, macroconidia were predominantly aseptate, but occasionally septate in old cultures (Fig. 11). Seta-like hyphae were sparse or absent in both types of conidiomata. Chlamydospores occurred in old cultures (Fig. 12, arrowheads). They were initially hyaline, becoming darkly pigmented

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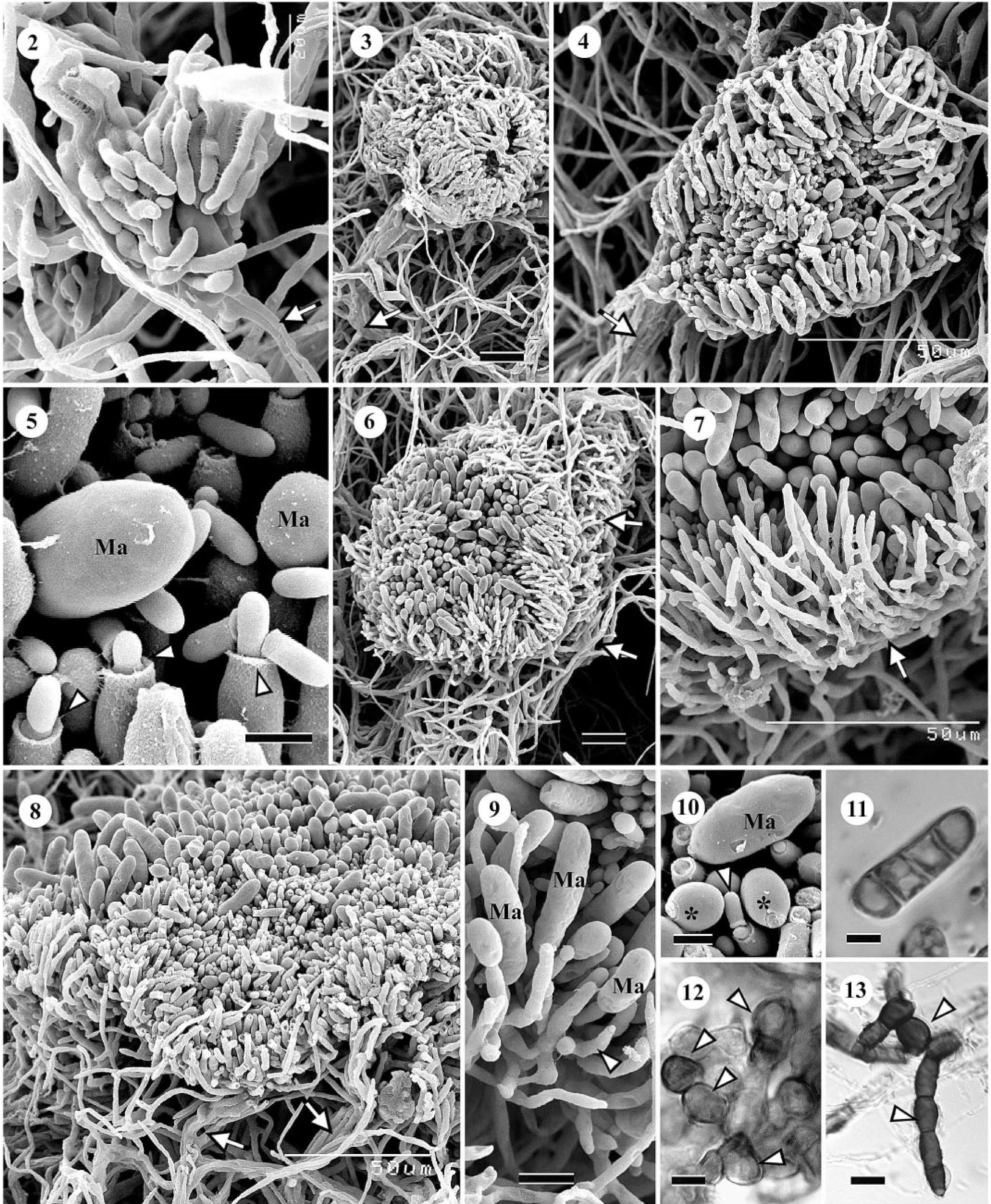

with age, terminal or intercalary, and usually in chains or in clusters. We also confirmed the occurrence of chlamydospores in some of our isolates from aspen roots (Fig. 13, arrowheads).

Unlike *C. ericae*, neither typical synnemata nor sporodochial conidiomata were found in the ex-type culture of *C. radicicola* (CBS 640.94; UAMH 10729). In this strain, conidiomata were initially closed and covered with a large

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Figs. 2–13. SEM (Figs. 2–10) and LM (Figs. 11–13) micrographs of *Cryptosporiopsis ericae* (ex-type culture except Fig. 13, isolate from aspen MDL-13-38a). Figs. 2 and 3. Developing synnemata; arrows indicate erect stalks. Figs. 4 and 5. Sporulating synnema and enlarged view of its sporulating area, respectively; arrow and arrowheads indicate a stalk and phialides of microconidia, respectively. Ma, macroconidium. Fig. 6. Mature sporodochium with basal cushion-like mycelial mat (arrows). Fig. 7. Phialides of microconidia at periphery of conidioma (arrow). Fig. 8. Mature conidioma with bundles of stalks at base (arrows). Fig. 9. Part of hymenium consisting of phialides of both macroconidia (Ma) and microconidia (arrowhead); note the absence of matrix material. Fig. 10. Macro-(Ma), micro-(arrowhead) and intermediate-forms of conidia (asterisks) occurring side by side. Fig. 11. Septate macroconidium. Figs. 12 and 13. Chlamydospores (arrowheads). Scale bars = 20 μm (Fig. 3), 2 μm (Fig. 5), 30 μm (Fig. 6), 8 μm (Fig. 9), 3 μm (Fig. 10), 5 μm (Figs. 11 and 12), 10 μm (Fig. 13).

amount of amorphous material. In the early stages of conidioma development, a group of conidiophores arose from vegetative hyphae and gave rise to closely packed phialides that often formed conidia soon after their development (Figs. 14 and 15). The conidioma initials were soon covered by a mycelial envelope that exuded a large amount of amorphous adhesive material (Fig. 16, arrow) and thus, further examination of their developmental process by SEM was difficult. As conidiomata matured, however, they opened up to expose the hymenium (Fig. 17). In the hymenium, phialides of both macro- and micro-conidia were embedded in the matrix material and only their tips were usually visible by SEM (Figs. 17–21). Ruptured phialide apices after conidial secession were abundant (Fig. 18, arrowheads) and seta-like hyphae, some of which showed percurrent proliferation (Fig. 20, arrow), occurred in most conidiomata (Figs. 18–20). To confirm these features of the ex-type strain, we examined one of the aspen isolates (BWC-30-89a; UAMH 10864) by SEM and found that its conidiomata were also provided with a mycelial envelope, enclosing the hymenium until later stages of development. However, conidiomata varied in the density of enveloping mycelium, ranging from sparse to dense, and in the amount of adhesive material. Some conidiomata resembled pycnidia, having an ostiole-like opening (Fig. 22, arrow) through which both macro- and micro-conidia could be seen inside (Figs. 23 and 24). In many conidiomata, the enveloping mycelium resembled a peridium (Figs. 25 and 26, arrows) that ruptured during the maturation of the hymenium (Fig. 26) leaving broken pieces of the peridium-like envelope to the side (Figs. 25 and 27, arrowheads; some broken pieces appeared to have been removed during the dehydration procedure in the SEM sample preparation). Both macro- and micro-conidia varied in size and shape (Figs. 24 and 28) and some were intermediate between the two forms (Fig. 29). Septate macroconidia occurred only in old cultures (Fig. 28), whereas solitary phialides borne on vegetative hyphae (Fig. 30) were found only in young cultures.

Cryptosporiopsis – aspen root associations

On artificially inoculated roots, hyphae of *C. ericae* were sparse and grew in random directions (Fig. 31), while those of *C. radicicola* were abundant and often grew along the host epidermal cell junctions (Figs. 32 and 36, arrowheads). Hyphae of *C. radicicola* were usually covered with a thick sheath of adhesive material (Fig. 33), but such a sheath was absent in *C. ericae* (Fig. 31). Hyphal penetration into the host epidermis was occasional in *C. ericae* (Figs. 31, arrow; 34, and 35), but frequent in *C. radicicola* (Figs. 36 and 37). Sites of hyphal penetration by *C. radicicola* were difficult to locate by SEM because of the sheath. Appressoria did not occur in either species. Thin sections of infected roots revealed that internal hyphae of *C. ericae* were confined to the host epidermis (Figs. 34 and 35), whereas hyphal cells of *C. radicicola* occurred both in epidermal (Fig. 36, arrows) and cortical cells (Fig. 37, arrows), although the frequency of occurrence was much less in the cortical region and no hyphal ingress was evident in the vascular region.

Discussion

Identifying *Cryptosporiopsis* species is difficult because there are few morphological differences between species and the characters used to distinguish taxa are few and unreliable (Sutton 1980). Furthermore, all species that have been isolated exclusively from roots of woody plants are anamorphic and thus lack the teleomorph–anamorph connection data that could be useful for identification (Verkley 1999; Sigler et al. 2005). Indeed, we encountered great difficulties in placing our isolates from aspen roots into previously described species based on cultural and routine morphological data alone. Phylogenetic analyses using DNA sequence data, however, separated the isolates into two groups (Fig. 1). Furthermore, pairwise comparisons using Se-Al showed that *C. ericae* isolates had 99%–100% similarity to the ex-type strain and those identified as *C. radicicola* had 98%–100% identity to the ex-type of this species (Table 1). According to Sigler et al. (2005), *C. ericae* is most similar both phylogenetically and morphologically to *C. radicicola*, a species presumed to belong to the monophyletic genus *Pezicula* sensu Verkley (1999) on the basis of partial 18SrDNA and ITS sequence analyses (Abeln et al. 2000). Thus far, however, ITS sequences show little variation within species of *Pezicula* (Verkley et al. 2003) and have limited usefulness in reconstructing relationships among species. Additional characters such as sequencing of mitochondrial rDNA and β-tubulin gene, or utilization of a diagnostic PCR assay (de Jong et al. 2001) may be necessary to clarify whether the root-inhabiting species of *Cryptosporiopsis* represent one or more lineages within *Pezicula*.

Verkley et al. (2003) presented a key to the four root-associated species of *Cryptosporiopsis*, including *C. radicicola*. The major key characters were colony morphology, type of conidioma (sporodochial or initially closed and eustromatic), presence or absence of chlamydospores as well as seta-like hyphae, and size of macroconidia. Later, Sigler et al. (2005) added two more root-associated species, *C. ericae* and *C. brunnea*, and stated that *C. ericae* was similar to *C. radicicola* but could be distinguished by such characteristics as the absence of chlamydospores and occurrence of fewer seta-like hyphae in *C. ericae*. However, we could not find chlamydospores in either the ex-type culture of *C. radicicola* or our isolates from aspen roots, but found darkly pigmented ones in cultures of both ex-type and isolates from aspen roots of *C. ericae* (Figs. 12 and

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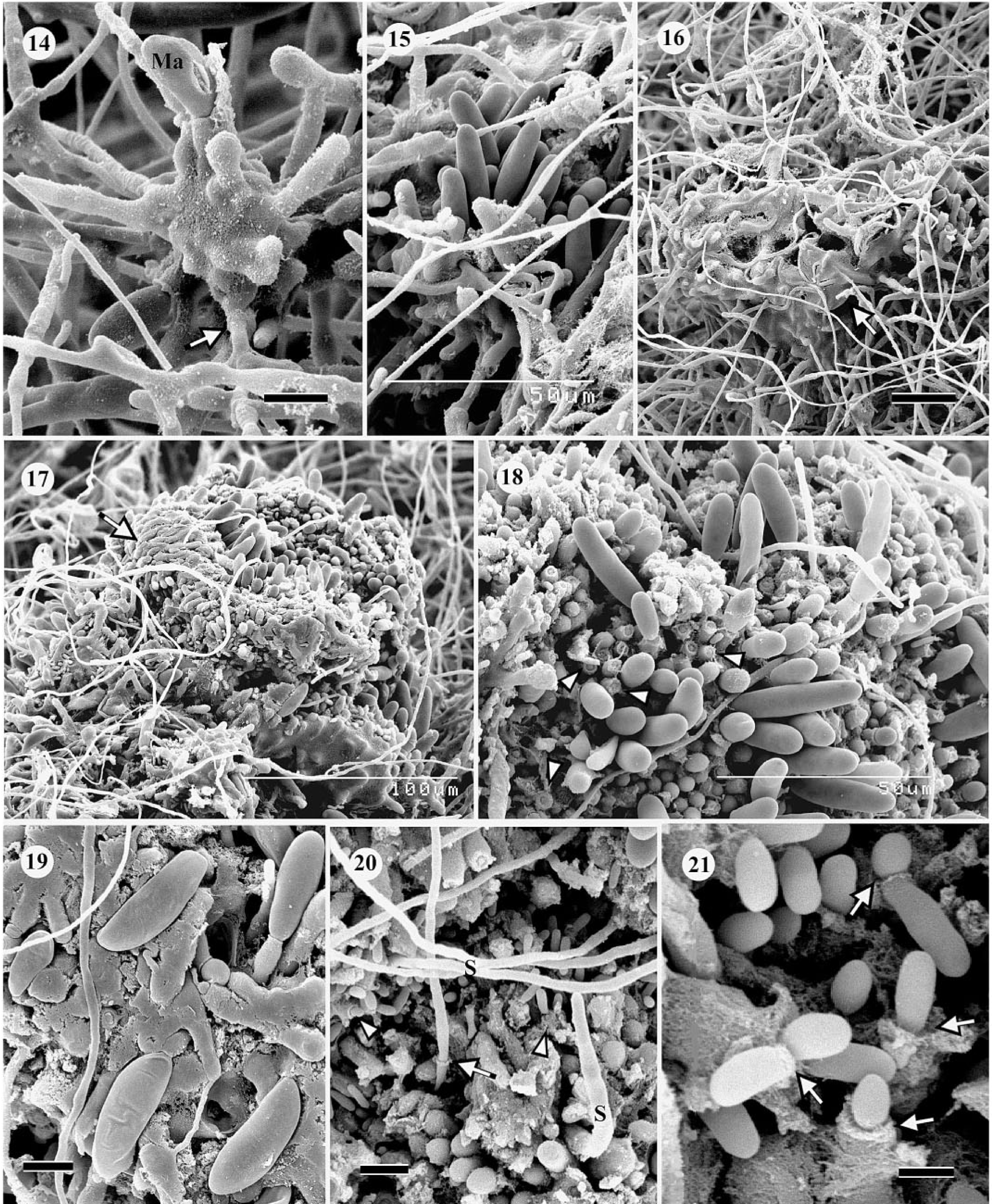

13). In fact, chlamydospores were not mentioned in the original description of *C. radicicola* (Kowalski and Bartnik 1995), although Verkley et al. (2003) treated the structure as an important feature of the species. With regard to seta-like hyphae, we found that they were much fewer in the ex-type strain of *C. ericae* than in the ex-type of *C. radicicola*, as Sigler et al. (2005) observed. However, their frequency of occurrence was variable among isolates from aspen roots of both species and therefore the abundance of seta-like hyphae may not be a reliable distinguishing

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Figs. 14–21. SEM micrographs of *Cryptosporiopsis radicicola* (ex-type culture). Fig. 14. Conidiophore (arrow) giving rise to phialides (see also in Fig. 15). Ma, macroconidium. Figs. 15 and 16. Conidioma initials; arrow indicates developing mycelial envelope with amorphous adhesive material. Fig. 17. Opening conidioma exposing hymenium; arrow indicates peridium-like envelope. Fig. 18. Mature hymenium; arrowheads indicate ruptured apexes of phialides embedded in matrix material. Fig. 19. Macroconidia showing variation in size and shape. Fig. 20. Seta-like hyphae (S) and microconidia (arrowheads); arrow indicates percurrently proliferated seta-like hypha. Fig. 21. Phialides of microconidia (arrows) bearing matrix material. Scale bars = 10 µm (Fig. 14), 50 µm (Fig. 16), 7 µm (Figs. 19 and 20), 2 µm (Fig. 21).

characteristic, either. The two species were also similar in size and shape of macro- as well as micro-conidia, although a slight but significant difference in microconidial size was detected. Unfortunately, microconidia are so minute that precise measurements can be difficult to make. Moreover, forms intermediate between macro- and micro-conidia were common in both species. Therefore, we consider all these morphological features to be unreliable in separating the two species.

Conidiomata of *Cryptosporiopsis* species on natural substrates are acervular to stromatic, peridermal to subperidermal, separate or confluent, with outer walls formed of pale brown to hyaline, thin-walled textura angularis (Sutton 1980). In neither *C. ericae* nor *C. radicicola* have conidiomata been recorded from host roots and we also failed to induce their production on aspen roots: therefore, an in vitro comparison was made in this study. SEM, which was essential to ascertain the three-dimensional architecture of conidiomata, revealed that *C. ericae* differed from *C. radicicola* in the structure and morphogenesis of conidiomata. In *C. ericae*, conidiomata were either synnematous or sporodochial, whereas those of *C. radicicola* possessed a mycelial envelope that was embedded in amorphous adhesive material, although details of conidiomal structure varied in both species. In *C. ericae*, a clear distinction between the two forms of conidiomata was often difficult to make. Some conidiomata occurred on a basal cushion-like mycelial mat (Fig. 6, arrows) and thus resembled sporodochia, but most of the seemingly sporodochial conidiomata lacked such a cushion and could be regarded as aggregated synnemata (see Fig. 8). In *C. radicicola*, the density of mycelium as well as the adhesive material in the envelope varied with the conidioma. Some conidiomata resembled pycnidia, possessing an ostiole-like opening at the apex. According to Sutton (1980), dehiscence in some *Cryptosporiopsis* species is by a large ostiole. In most cases, however, mycelial envelopes were entirely closed until ruptured by the maturing hymenium: these conidiomata may be best described as acervuloid. Conidiogenous cells of both macro- and microconidia were phialides in both species and annelides, as reported in *Cryptosporiopsis* sp. on *Acer negundo* L. (Sutton and Sandhu 1969) and in *Cryptosporiopsis phaeosora* (Sacc.) Arx [teleomorph *Pezicula rubi* (Lib.) Niessl] (Verkley 1999), were not observed under SEM. For *C. radicicola*, however, transmission electron microscopy may be necessary to determine the precise nature of conidiogenesis because the amorphous matrix material hindered the examination of conidiogenous process. Such matrix material was absent in the hymenium of *C. ericae*. With regard to chemical nature of the matrix material in *C. radicicola*, we assume that it is probably composed of fatty acids, waxes, or oils because the matrix was readily fixed with OsO_4. In fact, Kowalski and Bartnik (1995) described that hyphae of *C. radicicola* bore numerous oil droplets and we often observed a large amount of oily material in squash mounts of conidiomata. This material may contribute to the darker colour associated with the conidiomata of *C. radicicola*.

The genus *Cryptosporiopsis* includes several important plant pathogens (Butin 1983; Kowalski 1983; Taylor 1983; Kehr 1991) as well as endophytes (Kowalski and Kehr 1996; Schulz et al. 1999). *Cryptosporiopsis ericae* and *C. radicicola* were described from roots of ericaceous plants and *Quercus*, respectively (Kowalski and Bartnik 1995; Sigler et al. 2005) but their ecology, particularly the nature of associations with host roots were largely unknown. Ericoid mycorrhizal fungi produce hyphal coils in host epidermal cells (e.g., Currah et al. 1993; Smith and Read 1997), but Berch et al. (2002) found no formation of mycorrhizal structures in their resynthesis experiments done with salal and *C. ericae* (UBCtra-29 isolate). *Cryptosporiopsis radicicola* was suggested to have an important role in soil and in the health of the host plant because of its high frequency of association with oak roots (Kowalski and Bartnik 1995), and to be host specific (Verkley 1999). We found that both *C. ericae* and *C. radicicola* were commonly associated with apparently healthy aspen roots, whether the habitat was dry or wet and the soil type was luvisol or chernozem. Therefore, these species are not host specific and may be adaptable to various soil conditions.

Both *C. ericae* and *C. radicicola* were capable of penetrating epidermal cells of artificially inoculated aspen roots, but they differed in the growth habit and morphology of hyphae on the root surface. Hyphae of *C. ericae* were sparse and grew in random directions and penetrated only epidermal cells, which probably were mostly moribund, and formed no hyphal coils in the host cells, suggesting that this species is an endophyte of aspen roots but causes neither markedly favorable nor adverse effects in the host. Such an association, however, is certainly advantageous for *C. ericae* because the internal hyphae are more or less protected from adverse environmental conditions, including the attack by antagonistic microorganisms. *Cryptosporiopsis radicicola* was more aggressive and differed from *C. ericae* in hyphal morphology on the root surface, i.e., hyphae had a thick sheath around them. Hyphae grew preferentially along the host epidermal cell junctions and their penetration extended to the host cortical region. Our preliminary investigation by transmission electron microscopy indicated that (*i*) the hyphal sheath of *C. radicicola* was highly osmiophilic and thus similar to the matrix material of the conidiomata, and (*ii*) its hyphal ingress incited a series of host defense reactions, including papilla formation (A. Tsuneda et al., unpublished data, 2006). However, *C. radicicola* is apparently nonpathogenic because it does not infect host vascular tissue and thus does not impair the water-conducting function of infected roots. Moreover, all of our isolates from aspen roots were obtained from apparently healthy saplings and recovered inoculants all came from seedlings that appeared healthy.

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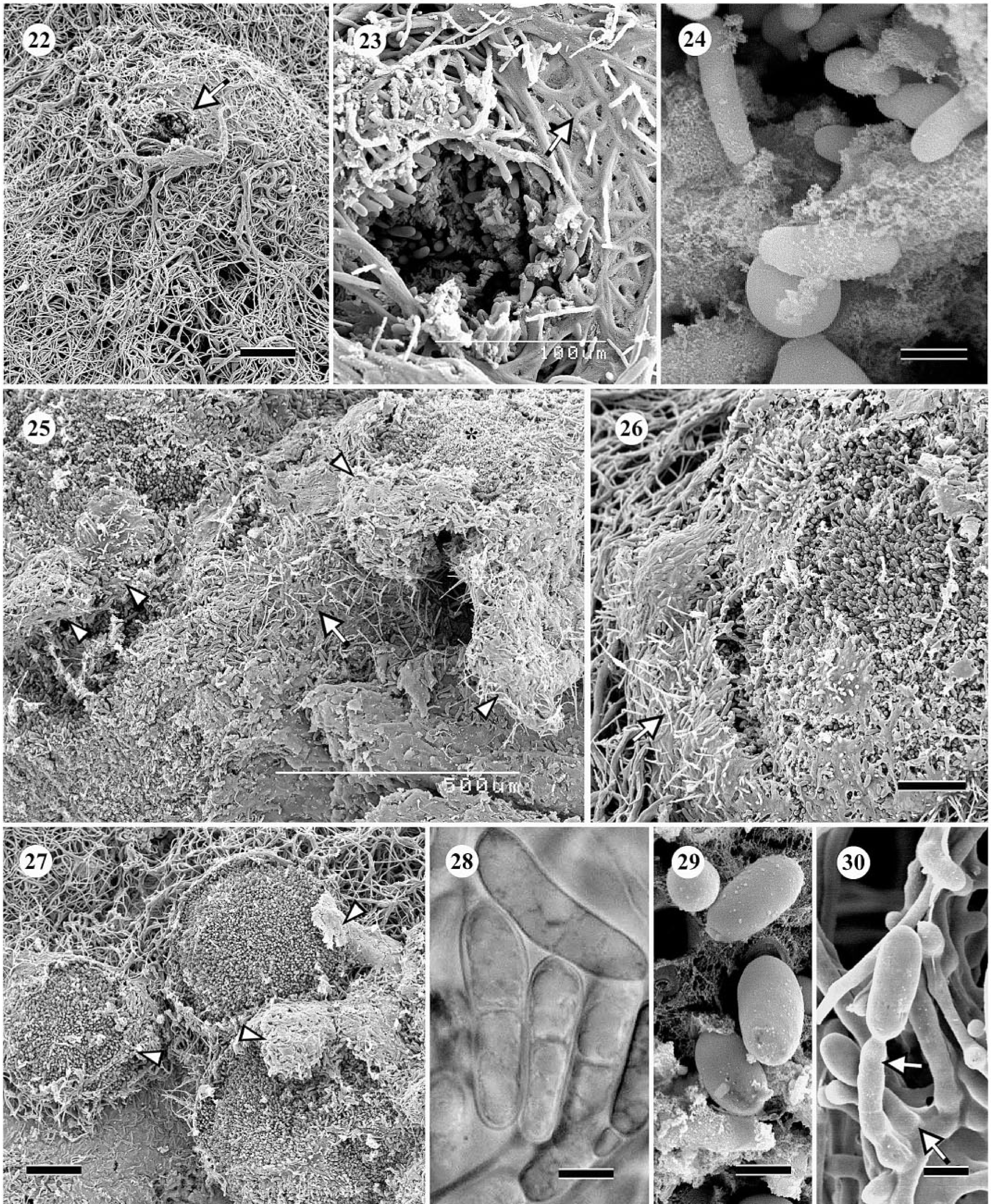

Studies examining the effects of other fungal root endophytes have shown that these symbionts can increase host resistance against more virulent root-pathogenic fungi (e.g., Benhamou and Garand 2001; Sylvia and Chemelli 2001; Narisawa et al. 2002). In this regard, species of *Cryptosporiopsis* may have a similar effect in protecting aspen roots from certain pathogens. Given the potential for these species to play some role in the health of their hosts, further studies of their interaction with aspen, and with known root pathogens of this species are warranted.

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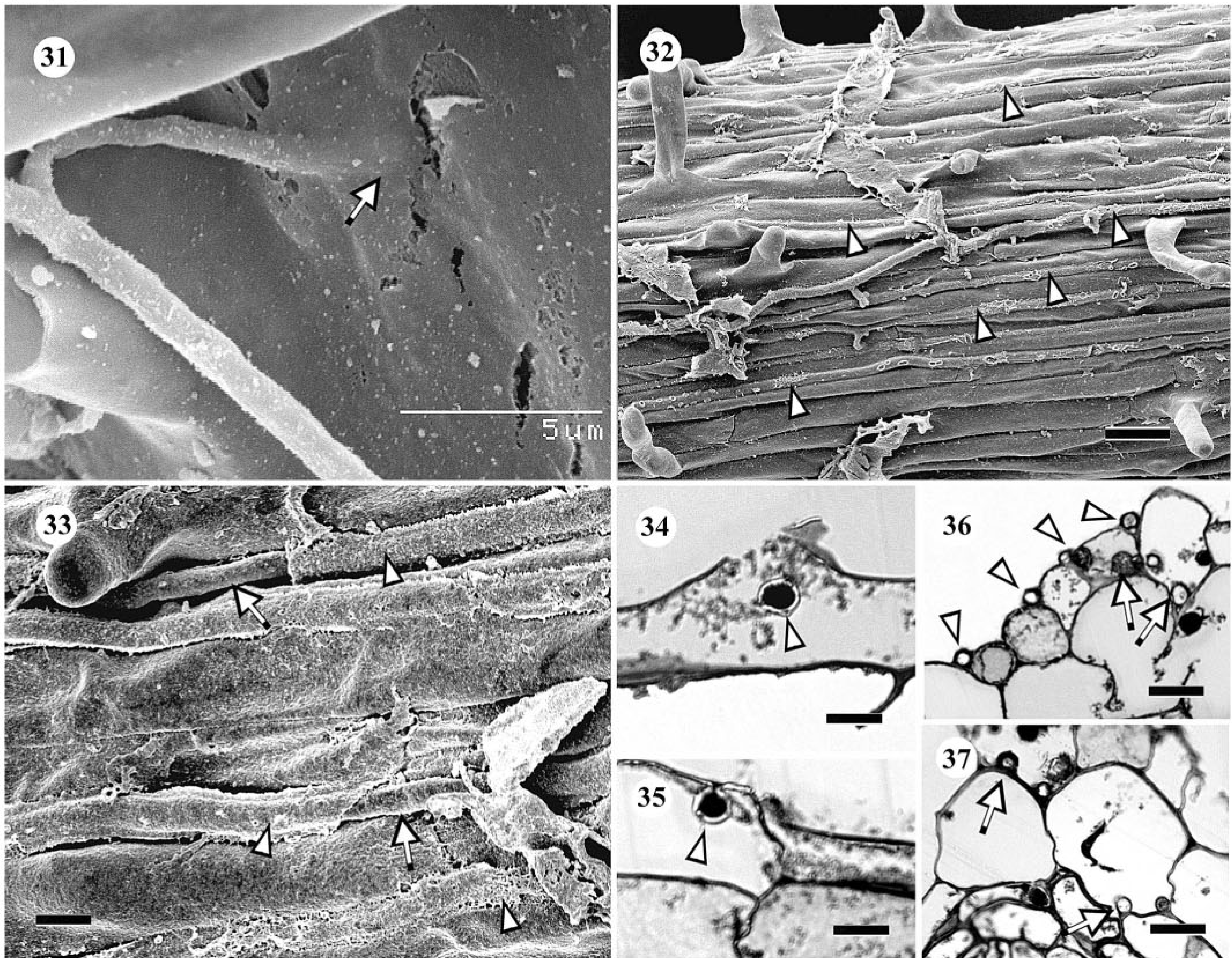
Figs. 22–30. SEM micrographs (except Fig. 28, LM) of *Cryptosporiopsis radicicola* (isolate from aspen BWC-30-89a; UAMH 10864). Fig. 22. Pycnidium-like conidioma with ostiole (arrow); note the presence of mycelial envelope. Fig. 23. Enlarged view of ostiole; arrow indicates meshwork of hyphae (hyphal envelope) bearing relatively small amount of adhesive material. Fig. 24. Conidia viewed through ostiole. Fig. 25. Conidiomata with peridium-like hyphal envelope; arrow and arrowheads indicate relatively intact and broken pieces of peridium-like envelopes, respectively. Broken pieces are seen beside fully-exposed conidioma (asterisk). Fig. 26. Enlarged view of partially opened conidioma with peridium-like hyphal envelope (arrow). Fig. 27. Mature conidiomata; arrowheads indicate remnants of hyphal envelopes. Fig. 28. Aseptate and septate macroconidia. Fig. 29. Conidia intermediate between macro- and micro-conidia in shape and size. Fig. 30. Solitary phialides (arrows) of macroconidia arising from vegetative hypha. Scale bars = 100 μm (Fig. 26), 150 μm (Fig. 27), 10 μm (Fig. 28), 3 μm (Fig. 29), 4 μm (Fig. 30).

Figs. 31–37. Aspen root – *Cryptosporiopsis* associations (Figs. 31–33, SEM; Figs. 34–37, LM, thin sections). Fig. 31. Hyphal penetration (arrow) of host epidermal cell by *C. ericae*. Figs. 32 and 33. Hyphae of *C. radicicola* growing along junctions of host epidermal cells (arrowheads); note that each hypha is surrounded by a thick sheath (arrowheads in Fig. 33). Arrows in Fig. 33 indicate hyphal surfaces where the sheath material was removed, probably during preparation for SEM. Figs. 34 and 35. Hyphal cells of *C. ericae* (arrowheads) within host epidermal cells. Figs. 36 and 37. Hyphal cells of *C. radicicola* in host epidermal (Fig. 36, arrows) and cortical cells (Fig. 37, arrows). Arrowheads indicate hyphal cells at epidermal cell junctions. Scale bars = 20 μm (Fig. 32), 5 μm (Fig. 33), 2 μm (Figs. 34 and 35); 8 μm (Figs. 36 and 37).



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