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Endomembrane system of aspen root cells plays a key role in defense against a common fungal root endophyte, *Cryptosporiopsis radicicola*

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Abstract: The host-endophyte interaction between roots of aspen (*Populus tremuloides*) and *Cryptosporiopsis radicicola* was examined primarily by transmission electron microscopy. Hyphae growing on the exterior of the inoculated roots had a thick, electron-dense, adhesive sheath. At hyphal contact and penetration, host epidermal cells exhibited a series of defense responses (viz. formation of papillae and partition walls, general wall thickening and walling-off of internal hyphae). In papilla formation, loop-shaped, rough endoplasmic reticula (rER) gave rise to globose secretory vesicles that accumulated around and then fused to the developing papilla. Unlike papillae, general wall thickening was associated with the Golgi apparatus (GA) that produced cell wall materials; 1–3 layers of Golgi cisternae were in contact with or in the immediate proximity (mostly within 0–0.5 μm) of and lying parallel to the host cell wall, where they budded out numerous subglobose vesicles that fused directly to the host cell wall and made it thicker. Partition wall formation and walling-off of internal hyphae also were common; the former was associated with an extended single cisterna, which was indistinguishable from rER or individual cisternae of GA, and in the latter phenomenon internal hyphae were encased by electron-dense material containing numerous ribosomes and membranous elements that were derived apparently from proliferated rER. These pronounced defense responses protected the stele and contributed to making *C. radicicola* endophytic rather than pathogenic.

Key words: cell wall apposition, ER, Golgi apparatus, plant defense response, transmission electron microscopy

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INTRODUCTION

Endophytic fungi are extremely common (Petrini 1991) and unlike plant pathogens they do not incite disease in the host (Hirsch and Braun 1992). *Cryptosporiopsis* Bubák & Kabát is a dematiaceous hyphomycete genus that includes anamorphs of the ascomycete genera *Pezicula* Tul. & C. Tul. and *Neofabraea* H.S. Jacks. (Helotiales) (Verkley 1999). Some species have been reported as dark septate endophytes (DSE) (Jumpponen and Trappe 1998, Addy et al 2005) of roots, and others are important plant pathogens (Butin 1983, Kowalski 1983, Taylor 1983, Kehr 1991), occurring mainly on the branches and trunks of forest and fruit trees (Sutton 1980, Dugan et al 1993). DSE might be as abundant as mycorrhizal fungi in plant roots, but our knowledge of their functional role is in its infancy (Mandyam and Jumpponen 2005). In general detailed ultrastructural studies on plant host-fungal endophyte interactions are rare (Peterson et al 2008).

During our recent survey of DSE in the roots of apparently healthy saplings of trembling aspen (*Populus tremuloides* Michx.) in Alberta we recovered a large number of isolates of *Cryptosporiopsis radicicola* Kowalski & Bartnik, a species that originally was described from roots of oaks in Europe. Kowalski and Bartnik (1995) suggested that *C. radicicola* has an important role in soil and in the health of the host plant because of its high frequency of association with oak roots, but its ecology, particularly the nature of its association with its host, was unknown. Our light microscopic observations of artificially inoculated aspen roots indicated that *C. radicicola* is endophytic, causing no disease symptoms, despite frequent penetrations of epidermal cells (Wang et al 2007). These observations prompted us to conduct a cytological study of the host-endophyte interaction to investigate (i) ultrastructural details of host responses to fungal ingress and (ii) how hyphae of *C. radicicola* are able to grow within root tissue without causing overt signs of disease.

MATERIALS AND METHODS

Aspen seeds were surface sterilized in a 40% solution of household bleach (concentration of active Cl = ca. 10%) for 2 min, rinsed twice in sterilized distilled water (d-H_2O), and plated on tap water agar (10 g select agar [Invitrogen, Carlsbad, California], 1 L d-H_2O) at room temperature.

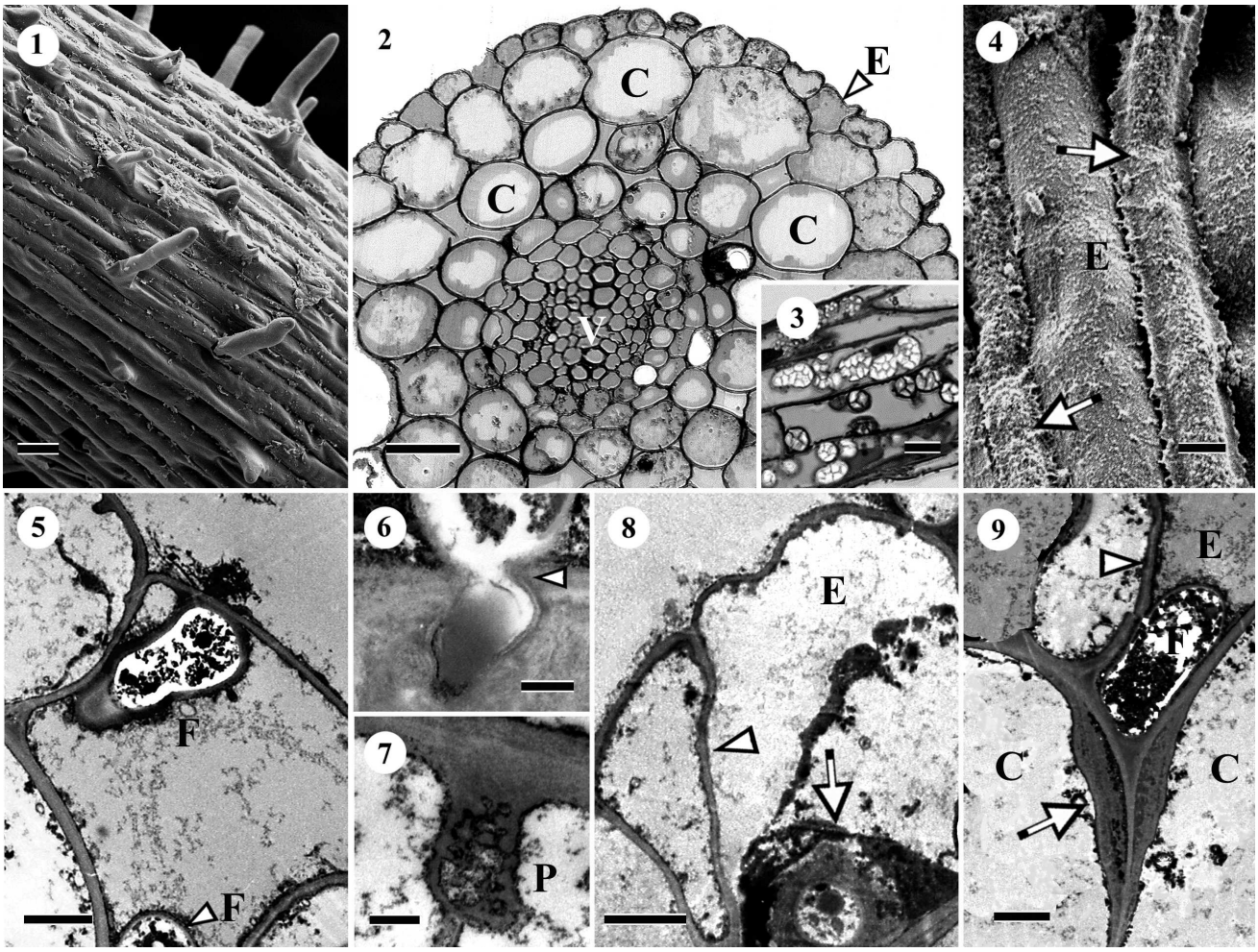
After approximately 2 wk seedlings with two foliage leaves were transplanted into peat pellets (Jiffy-7™, Jiffy Products Ltd., New Brunswick) and incubated in a growth chamber (Lab-line Instruments Inc., Melrose Park, Illinois) 2 mo at 20 C and a photoperiod of 18 h at an intensity of 200 μE m^{-2} s^{-1}. After 1 mo incubation seedlings were inoculated with *C. radicicola* (UAMH 10869, see Wang et al 2007) by placing a 4 × 4 mm block of mycelium from a 30 d old culture on malt extract agar (20 g Bacto™ malt extract [Becton, Dickinson & Co., Sparks, Maryland], 18 g select agar [Invitrogen], 10 mg oxytetracycline dihydride [Sigma, St Louis, Missouri], 1 L d-H_2O), 2 cm deep into the peat pellet adjacent to the root. Uninoculated plants served as controls. The inoculated aspen seedlings were incubated further in the growth chamber 1 mo and were used for microscopic observations. This incubation period was chosen based on observations from our previous LM study (Wang et al 2007) that showed that root cells at this point were in various stages of fungal colonization (viz. some were free of colonization, some were in the early stages of fungal penetration and some had internal fungal cells) and thus were suitable for TEM observations. Five seedlings were selected for microscopy after confirming both the colonization by *C. radicicola* and absence of contaminants with the method described in Wang et al (2007). Root segments (1 cm long) excised from these seedlings, 10 per seedling, were cut into halves: one-half for thin-section observations by light microscopy (LM) and the other for scanning and transmission electron microscopy (SEM and TEM respectively). Root segments were washed in phosphate buffer (pH 7.0) and fixed in 2% glutaraldehyde (Sigma) in buffer 2 h at room temperature. After rinsing with buffer, root segments were postfixed overnight in 2% OsO_4 (Sigma) at 5 C. For SEM the fixed samples were dehydrated in an ethanol series, taken to amyl acetate (Fisher Scientific, Fair Lawn, New Jersey) and critical-point dried in a Polaron E-3000 dryer using carbon dioxide. The dried samples were coated with gold and examined with a Hitachi S-510 electron microscope at 10 kV. For LM and TEM the fixed samples were dehydrated in an ethanol series and embedded in Spurr's resin (Canemco-Marivac, St Laurent, Quebec). Sections (about 1 μm) of the embedded material for LM were prepared with the method of Meek (1976). Ultrathin sections for TEM were stained with uranyl acetate (Fisher Scientific) and lead citrate (Tabb Laboratories Equipment Ltd., Reading, Berkshire, UK), and examined at 75 kV with a Hitachi H-7000 electron microscope.

RESULTS

SEM of control roots revealed scattered root hairs and narrow, longitudinally oriented rows of epidermal cells whose junctions were noticeably indented (FIG. 1). In thin (FIG. 2) and ultrathin sections the epidermal and cortical cells were occupied mostly by large vacuoles whereas root hairs and parenchyma cells in the vascular cylinder (stele) were filled with cytoplasm. Some parenchyma cells contained abundant starch granules (FIG. 3). On the exterior of the inoculated roots hyphae of *C. radicicola* grew preferentially along epidermal cell junctions within a thick adhesive sheath, consisting of highly electron-dense granular as well as less electron-dense fibrillar elements (FIGS. 4, 10, 12). LM and TEM observations of many serial sections showed that hyphae were frequent in epidermal cells (FIGS. 5, 8, 9), sparse in cortical cells and absent from the tissues of the stele (i.e. xylem, phloem and parenchyma) (also see FIGS. 36 and 37 in Wang et al 2007). Hyphal penetration of epidermal cells was either direct, without appressoria (FIG. 6) or more commonly intercellular where hyphae pushed adjoining epidermal cells apart and grew through anticlinal walls (FIG. 12, single arrowheads). Hyphae constricted markedly at cell wall penetration (FIG. 6). Cytoplasm of internal hyphae often appeared necrotic.

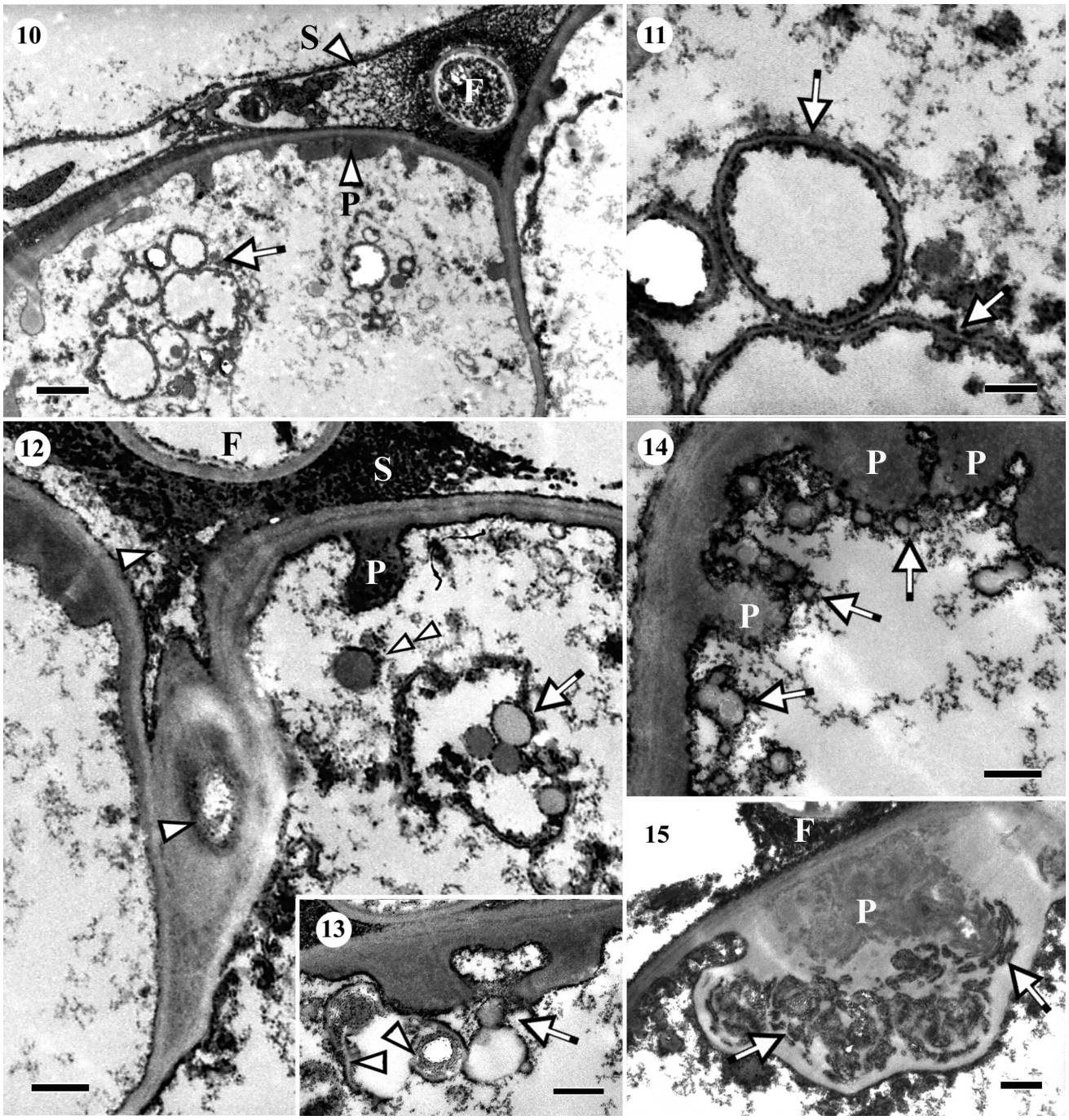
At hyphal contact and penetration host epidermal cells sometimes showed no visible sign of resistance responses (FIG. 5) but more often exhibited a series of defense reactions, viz. formation of papillae (FIG. 7), and partition walls (FIGS. 8, 9; arrowheads), general wall thickening, and walling-off (i.e. accumulation of encasing materials around internal hyphae) (FIG. 8, arrow). Thickening of anticlinal walls in response to attempted hyphal ingress was more pronounced in cortical cells (FIG. 9), and hyphal penetration of such areas was not observed. Loop-shaped, rough endoplasmic reticula (rER), about 30 nm thick, were evident in host cells near developing papillae (FIGS. 10–12) and gave rise to secretory (transport) vesicles that were typically globose in cross section and possessed translucent contents (FIG. 12, arrow). Vesicles subsequently moved toward the developing papilla where they surrounded and eventually fused to it (FIGS. 12–14). Outlines of vesicles often were identifiable in papillae during early stages of development (FIG. 7). In some cases secretory vesicles released translucent contents (exocytosis) that fused to the papilla (FIG. 13, arrow), leaving vacated but persistent membranes behind. Direct fusion of rER loops to the papilla also was frequent (FIG. 13, arrowheads), and as a result fully developed papillae were heterogeneous, having remnants of rER embedded in wall material (FIG. 15, arrows). Mature papillae were resistant to hyphal penetration, but immature ones occasionally were found penetrated (FIG. 6).

General thickening of host cell walls also was elicited by hyphae, but unlike papillae this form of wall thickening was associated with 1–3 layers of membrane cisternae (FIGS. 16–18). These were mostly 30–50 nm thick (except in dilated areas; FIG. 16, arrow), distended at the ends (FIG. 18, arrowhead), frequently interconnected (FIG. 17) and in contact



FIGS. 1–3. Uninoculated roots of aspen (*Populus tremuloides*). 1. Scanning electron micrograph of a portion of a mature root showing longitudinally oriented rows of epidermal cells with scattered root hairs. 2. Cross sectional view of root consisting of epidermal (E) and cortical (C) cells with central vascular cylinder (V). 3. Longitudinally sectioned parenchyma cells in vascular cylinder containing abundant starch granules. FIGS. 4–9. Scanning (FIG. 4) and transmission electron micrographs showing aspen root-*Cryptosporiopsis radicicola* host-endophyte interface. 4. Hyphae growing along junctions of host epidermal cells (E). Note that the hyphae are covered with a thick sheath of adhesive material (arrows). 5. Hyphal cells (F) in host epidermal cell. In this case no visible sign of defense responses is seen and cytoplasm of the hyphal cells appears to have degenerated. 6. Hyphal penetration of host epidermal cell wall with marked constriction of the hypha at the point of penetration (arrowhead). The penetration peg further is growing into an immature papilla. 7. Early stage of papilla (P) formation. 8. Partition wall (arrowhead) and electron-dense material encasing hyphal cell (arrow) in host epidermal cell (E). 9. Markedly thickened anticlinal wall (arrow) between adjacent cortical cells (C), and partition wall (arrowhead) in epidermal cell (E) formed in response to internal hypha (F). Bars: 1, 2 = 20 μm; 3, 4 = 5 μm; 5, 8, 9 = 2 μm; 6 = 0.5 μm; 7 = 0.2 μm.

with or in the immediate proximity of (mostly within 0–0.5 μm) and lying parallel to the host cell wall. These characteristics are compatible with Golgi apparatus, and the organelle is called GA hereafter. The GA was up to 13 μm long and the point of fungal contact usually was located near the middle of the extended GA. Numerous subglobose vesicles of varying size budded from the cisternae and directly fused to the host cell wall (FIG. 16, arrowheads), making it two to three times thicker than average epidermal and cortical cell walls. A marked thickening of anticlinal walls in response to attempted hyphal ingress was particularly common in cortical cells and occurred in a similar way and simultaneously on the inner face of cell walls on both sides of the invading hypha (FIGS. 9, 18, 19). Partition wall formation (FIGS. 8, 9) and what appeared to be analogous to ''walling-off'' of internal hyphae (FIGS. 8, 22) also were common. Partition wall formation was associated with a single cisterna that was straight or sometimes zigzag in profile, resembled rER (both were ca. 30–40 nm thick) and budded out numerous vesicles on



FIGS. 10–15. Development of papillae in epidermal cells of *Populus tremuloides* root in response to *Cryptosporiopsis radicicola*. 10. Overall view showing hyphal cell (F) covered with slime sheath (S), early developmental stage of papilla (P) and loop-shaped rER (arrow). 11. Enlarged view (FIG. 10, the portion pointed by arrow), showing loop-shaped rER (arrows). 12. Loop-shaped rER with globose secretory vesicles (arrow). The hypha (F) with sheath (S) is pushing adjoining epidermal cells apart and growing through the anticlinal wall (arrowheads). Double arrowheads indicate a released vesicle that probably is moving toward the developing papilla (P). 13. Excreted content (arrow) of secretory vesicle and rER (arrowheads) fusing to developing papilla. 14. Many secretory vesicles accumulating to form papillae (P). 15. Heterogeneous, fully mature papilla (P) containing remnants of rER (arrows). F = fungal hypha. Bars: 10 = 1.5 μm; 11, 13, 14 = 0.3 μm; 12, 15 = 0.5 μm.

both sides (FIG. 20). Therefore developed walls were sometimes zigzag in profile (FIG. 21). During walling-off internal hyphae were encased by electron-dense material containing numerous ribosomes and membranous elements (FIG. 22, arrows) that appeared to be derived from proliferated rER, as seen inside papillae (FIG. 15, arrows). Hyphae were rare in the cortex and could not be found in the stele, despite extensive microscopic examination of both thin and ultrathin sections.

DISCUSSION

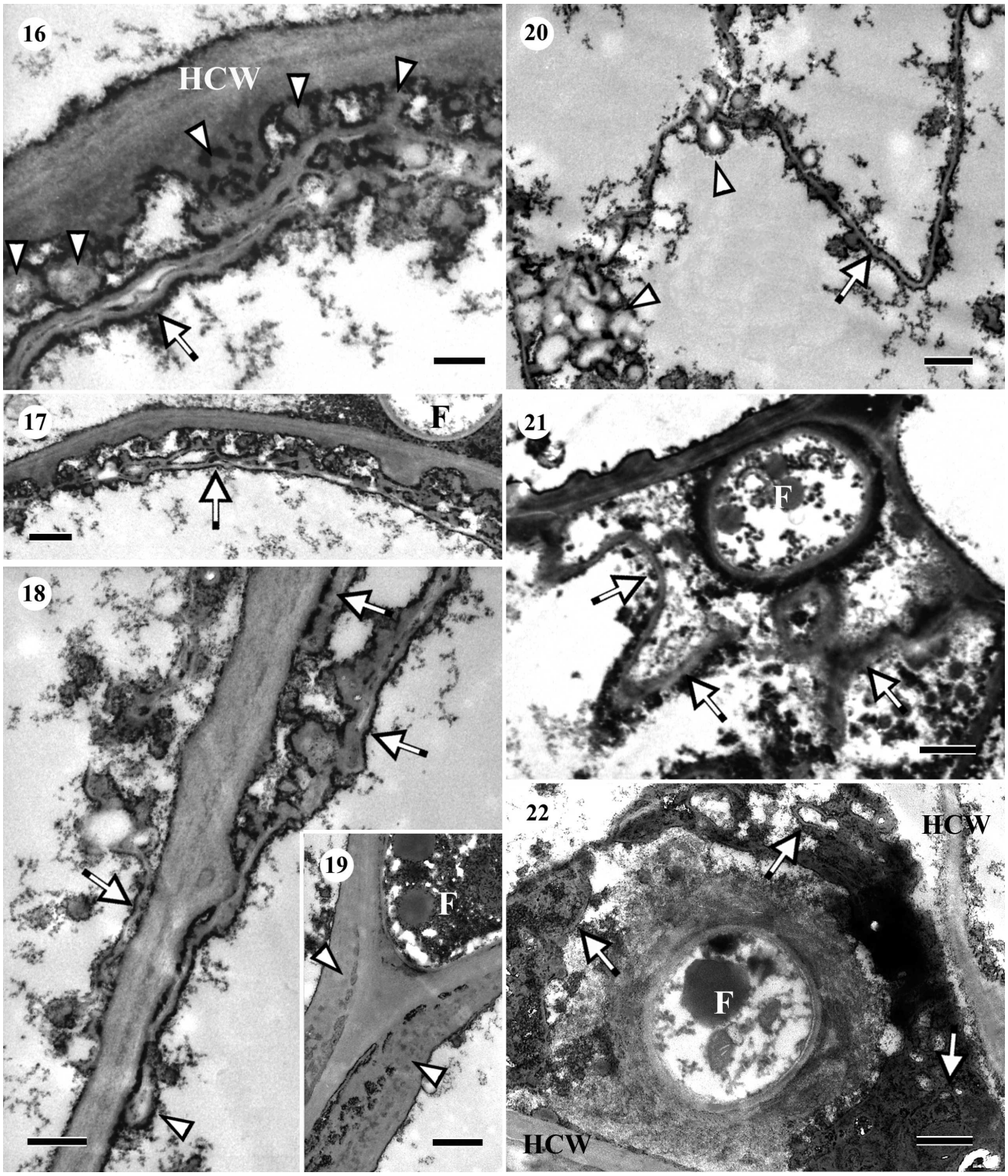
In plant-fungus interactions hyphae often are surrounded by a matrix or sheath that is distinct from wall-like appositions and not restricted to any particular biochemical characterization or to a single or specific origin (i.e. not necessarily host-derived or symbiont-derived) (Bracker and Littlefield 1973). In *C. radicicola* hyphae growing on the root surface possessed a thick, extracellular, mucilaginous sheath. Such an extensive sheath would prevent direct contact between the walls of the two organisms and also might slow down the exchange of chemicals, including elicitors of plant defense reactions.

Hyphal behavior on the surface of epidermal cells and mode of penetration differ according to the type of host plant and the fungal endophyte. For example penetration by a sterile, white endophytic fungus in eggplant was via appressoria in early stages of infection but later through voids created in host cell walls by accumulated hyphae (Narisawa et al 2003). In the aspen-*C. radicicola* system hyphae grew preferentially along epidermal cell junctions, remained sparse on the host surface and penetrated the root either directly into individual cells or by pushing adjoining epidermal cells apart and growing intercellularly. A markedly similar mode of intercellular penetration was reported in the *Phragmites australis-Stagonospora* host-endophyte system (Gao and Mendgen 2006).

Unlike the roots of crop plants, epidermal and cortical cells in mature roots of trees are highly vacuolated and have little cytoplasm, unless occupied by a mycorrizal fungus (Brundrett 2008), and even cells of the apical meristem have some vacuoles (Cutter 1971). This fact is relevant here because the highly vacuolated cells of aspen roots were able to mount a strong defense response against *C. radicicola*. In pathogenic interactions elicitors of plant defense reactions are targeted to specific, high-affinity binding sites of host membranes (Haln 1996, Tenberge et al 1999, Garcia-Brugger et al 2006). Elicitor(s) appear to be involved in the aspen-*C. radicicola* host-endophyte interaction because a cascade of defense responses occurred in host epidermal cells in response to physical contact of hyphae. rER was the first endomembrane system that visibly responded to *C. radicicola* and participated in the formation of papillae through the production of secretory vesicles and their later fusion to the developing papilla. Involvement of membrane fragments (ER- or plasma membrane-derived or in the form of multivesicular bodies) in papilla formation has been reported in various host-parasite systems (e.g. Bracker and Littlefield 1973; Tsuneda et al 1977; Narisawa et al 2003; Shimizu et al 2005, 2006). Although such membrane fragments once were suspected to be artifacts of chemical fixation, the rapid freezing method later confirmed their constitutive occurrence (Shimizu et al 2005, 2006; Park and Ikeda 2008). As in aspen roots, secretory vesicles participated in the formation of wall appositions in roots of *Gossypium hirsutum* (cotton) and *P. australis* (reed) after infection with *Fusarium oxysporum* f. sp. *vasinfectum* and *Stagonospora* respectively (Rodriguez-Gálvez and Mendgen 1995, Gao and Mendgen 2006). In the cortex of reed, wall appositions were enriched by callose but also included many plant components produced during normal cell wall synthesis and a walling-off phenomenon similar to that in aspen occurred (Gao and Mendgen 2006).

The general thickening of aspen-root cell walls in response to *C. radicicola* was associated with Golgi apparatus (GA), consisting of 1–3 cisternae that gave rise to numerous subglobose vesicles. These vesicles fused to the host cell wall and made it thicker. Similarly fusion of Golgi vesicles with host plasma membrane was observed in Japanese pear treated with a host specific toxin (Park et al 1988). However, as far as we are aware, our report is the first demonstration of a plant defense response in which Golgi cisternae are aligned parallel to the host cell wall during its fortification. Cisternal structure is simpler than in animal cells, but GA in general is well known for its variability in form (Mollenhauer and Morré 1980, 1994; Park and Ikeda 2008). Sometimes GA appear in simple configurations, making them difficult to distinguish from specialized regions of ER, and in fact with regard to the single cisternae that were associated with partition-wall formation we are uncertain whether they represent a simple form of GA (similar to the one reported in *Saccharomyces cerevisiae* [Rossanese et al 1999]) or ER. Furthermore the GA in *Aspergillus nidulans* sometimes takes a loop-like configuration (Mims et al 1988), resembling rER reported here.

We presumed that because of the profound defense reactions in aspen roots *C. radicicola* hyphae were unable to reach the stele and affected neither



FIGS. 16–22. High-magnification transmission-electron micrographs showing defense reactions of *Populus tremuloides* against *Cryptosporiopsis radicicola*. 16. Vesicles (arrowheads) budded out of Golgi cisternae, directly fusing to host epidermal cell wall. The arrow indicates a dilated portion of a cisterna. 17. Frequently interconnected Golgi cisternae giving rise to numerous vesicles. 18. Anticlinal wall of cortical cells and associated Golgi cisternae (arrows) on both sides. The arrowhead indicates the distended end of a cisterna. 19. Markedly thickened anticlinal wall (arrowheads). 20. Single zigzag-shaped cisterna giving rise to numerous vesicles (arrowheads) to form partition wall. 21. Zigzag partition wall (arrows). 22. Internal hypha encased by electron dense material containing numerous membranous elements (arrows) and ribosomes. F = fungal hypha, HCW = host epidermal cell wall. Bars: 17, 22 = 0.6 μm; 16 = 0.2 μm; 18, 20 = 0.3 μm; 19, 21 =1 μm.

the water-conducting function nor the associated nutrient reserves in the parenchyma, and consequently the colonized aspen seedlings remained symptom free and the fungus functioned as an endophyte instead of a pathogen. Our conclusion is supported by comparisons of invasive abilities of virulent and avirulant strains of *F. oxysporum* in pea roots. The pathogenic strain could invade the stele and cause wilting whereas its nonpathogenic counterpart (Fo 47) was unable to reach the vascular tissue (Benhamou and Garand 2001).

Host-endophyte associations initially were interpreted as variants of mutualistic symbioses, but recent perception is more complicated. For example Peterson et al (2008) considered that there may be a continuum along a gradient from mutualism to commensalisms to parasitism, depending on fungal species, host species and the environment in which colonization occurs. Variation in the nature of interactions depending on the host species has been well documented in host plant-*Phialocephala fortinii* associations (Currah et al 1993, Yu et al 2001, Peterson et al 2008). Saikkonen et al (2004) interpreted host-endophyte associations as mutual exploitation, while Schulz and Boyle (2005) interpreted them as balanced antagonism in which there is some degree of virulence on the part of the fungus and an active defense response. Ortiz-Garcia et al (2003) suggested that pathogenic species in *Lophodermium* were derived from endophytic predecessors, and Redman et al (1999) demonstrated that a pathogenic fungus, *Colletotrichum magna*, was rendered endophytic through gene disruption. Like *Lophodermium*, *Cryptosporiopsis* includes both pathogenic and endophytic species with differing invasive abilities. Wang et al (2007) showed by LM that *C. radicicola* was able to penetrate deeper into root tissues than *C. ericae*, another endophytic *Cryptosporiopsis* of aspen, whose hyphae were strictly confined to the root epidermis. Defense reactions of aspen roots in response to *C. radicicola* revealed by TEM here markedly resembled those induced by necrotrophic or hemibiotrophic pathogenic fungi (Hardham and Mitchell 1998, Harrison 1999). Therefore the aspen-*C. radicicola* association is probably best considered an antagonism in which host defense and fungal invasive ability balance to produce asymptomatic symbioses (Schulz and Boyle 2005) instead of mutual exploitation (Saikkonen 2004).

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