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FULL PAPER

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Anatomical study on the interaction between the root endophytic fungus *Heteroconium chaetospira* and Chinese cabbage

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Abstract Chinese cabbage roots colonized by the dematiaceous fungal taxon *Heteroconium chaetospira* were previously found to become highly resistant to clubroot and *Verticillium* yellows. The dematiaceous fungus possesses an endophytic nature, but no detailed anatomical studies on endophyte–host plant interactions have so far been provided. Light and electron microscopy revealed that hyphae of *H. chaetospira* were abundant on and inside the root epidermal cells by 3 weeks following inoculation. The penetration pegs easily breached into epidermal cells, and the infection hyphae penetrated into cortical cells. Some appressorium-like swollen structures formed from intracellular hyphae, but no visible degradation of the host cell walls was evident where the hyphae contacted. No visible signs of host reactions and no invagination of the host plasma membrane around the hyphae were seen in the host cells. By 8 weeks following inoculation, masses of closely packed fungal cells had been formed in some cells of the epidermis and cortical layers, but further hyphal ingress was halted, mostly in the inner cortical cell layer. Thus, root vascular cylinders remained intact.

Key words Anatomy · Chinese cabbage · Dark septate fungal endophyte · *Heteroconium chaetospira* · Host cell response

Introduction

Dark septate root endophytes (DSE) are dematiaceous fungi that occur with some regularity in the roots of apparently healthy plants, where they usually form distinctive intracellular structures or colonization patterns (Jumpponen and Trappe 1998). In spite of their widespread occurrence, relatively little is known about the taxonomic diversity of DSE fungi. Among identified taxa that have sporulated in culture are *Phialocephala fortinii* Wang & Wilcox, *Phialophora finlandia* Wang & Wilcox, *Chloridium paucisporum* Wang & Wilcox, and *Leptodontidium orchidicola* Sigler and Currah (Jumpponen and Trappe 1998).

Recently, *Heteroconium chaetospira* (Grove) M.B. Ellis was isolated from roots of Chinese cabbage and eggplant grown in field soils in Japan (Narisawa et al. 1998, 2002). When used as a preinoculum, *H. chaetospira* suppressed the incidence of clubroot, *Verticillium* yellows, and *Verticillium* wilt when test plants were postinoculated with the causal agents of these diseases. In vitro resyntheses confirmed that these isolates could be reestablished as root endophytes in axenically reared plants without causing necrosis or disruption of growth in the host. This dematiaceous fungal taxon should be considered among the named fungi in the DSE group.

The early infection events of *H. chaetospira*, involving the formation of appressoria on cell surfaces and the subsequent growth of hyphae within cells of host plant roots, including microsclerotia formation, have been explained (Narisawa et al. 1998, 2000; Ohki et al. 2002), but no detailed anatomical studies on endophyte–plant interactions are available. Herein we report anatomical studies on *H. chaetospira*–Chinese cabbage interactions to clarify the details of the infection process and subsequent development of the fungus on the host plant.

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Materials and methods

Host plant and fungal isolate

Chinese cabbage (*Brassica campestris* L.) line "W4107" (Watanabe Seeds, Miyagi, Japan), and *H. chaetospira* (MAFF238955), obtained from the roots of Chinese

cabbage grown in a sample of soil from a wheat field at Shimodate, Ibaraki, Japan (Narisawa et al. 1998), were used for the experiments.

Inoculation of Chinese cabbage seedlings with *H. chaetospira*

Heteroconium chaetospira was grown on oatmeal agar (OMA) medium [10 g oatmeal, 18 g Bacto agar (Difco), 1 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, and 1 g $NaNO_3$ per liter] in 5-cm Petri dishes at approximately 23°C. Three axenically grown, 2-day-old Chinese cabbage seedlings were transferred onto the surface of a 3-week-old fungal colony. Seedlings placed on uninoculated OMA served as controls. Seedlings were then kept in plastic trays (Sigma, St. Louis, MO, USA) and incubated in a growth chamber at 20°–25°C under a 16-h light:8-h dark photoperiod. Lighting was approximately 180 $\mu mol\, m^{-2} s^{-2}$. There were three seedlings in each tray and three replicate trays for each incubation period, 3 or 8 weeks after inoculation.

Periodic observation of endophyte growth within host roots

After each incubation period, root segments were recovered from the trays, washed with 0.01 M phosphate buffer (pH 7), and cut into lengths of approximately 3 cm for light microscopy and scanning electron microscopy (SEM) and 5 mm for transmission electron microscopy (TEM).

Light microscopy

Root segments recovered were hand-sectioned with a razor blade and stained with 0.05% cotton blue in 50% acetic acid. Fifty root segments were randomly selected from each plant in each incubation period and mounted on glass slides. Colonization patterns of the fungal endophytes in Chinese cabbage roots were observed under an Olympus BX50 microscope with UplanFI20 and 40/0.30 objectives.

Electron microscopy

Root segments for SEM were washed in 0.01 M phosphate buffer (pH 7), fixed in 2% glutaraldehyde for 3 h at 4°C, and washed again in the buffer. The samples were soaked in 2% tannic acid, 2% guanidine hydrochloride solution for 4–6 h at 5°C, washed 1 h in distilled H_2O, and postfixed overnight in 2% OsO_4 at 4°C. After washing in distilled H_2O, the postfixed material was dehydrated in an ethanol series, taken to amyl acetate, and dried on a Hitachi HCP-1 critical point dryer using CO_2. These dried samples were sectioned by using a blade in case of the transectional observations. These samples then were mounted on stubs using double-sided carbon tape, coated with gold-palladium in a Hitachi E1030 ion sputter coater, and observed with a Hitachi S4200 scanning electron microscope at 60 kV.

Root segments for TEM were fixed in 2.5% glutaraldehyde in 0.05 M cacodilate buffer (pH 7.2) for 2–4 h at 4°C. After rinsing in phosphate buffer under light vacuum, the segments were postfixed in buffered 2% osmium tetroxide for 2 h, and dehydrated in a graded ethanol series before being transferred to propylene oxide and embedded in spur epoxy resin. Ulrathin transverse sections were cut on an LKB ultramicrotome with a glass knife. Sections were stained with 2% uranyl acetate for 20–30 min, followed by lead citrate for 20–30 min. Electron micrographs were taken with a Hitachi H-7000 microscope at 100 kV.

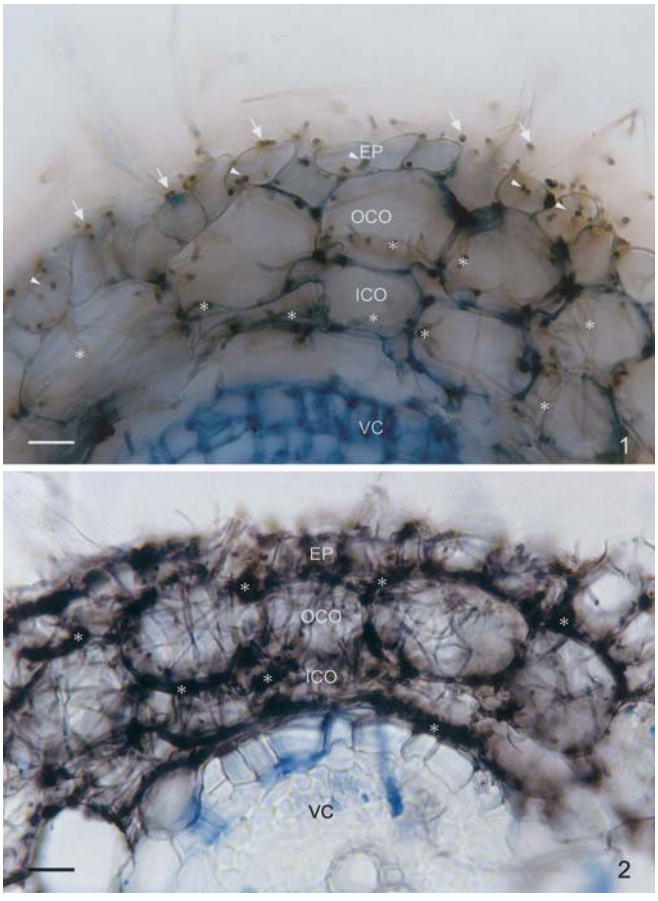
Results

Light microscopy and SEM of cross sections of Chinese cabbage roots showed that they were composed of an epidermis, a cortex consisting of two or three cell layers, endodermis, and a vascular cylinder (Figs. 1–3). At 3 weeks after inoculation with *H. chaetospira*, hyphae were abundant in the epidermis and outer cortical layer (Figs. 1, 3) but were fewer in the inner cortical layer and completely absent in the vascular cylinder. At 8 weeks of incubation, hyphae were numerous in the epidermis and cortical layers (Fig. 2) and some had become irregularly lobed hyphae or microsclerotia on and in the host cells (Fig. 2, asterisks), but no structures indicative of a typical mycorrhizal symbiosis were developed. Masses of closely packed fungal cells had been formed in most cells of the epidermis and cortical layers, but hyphal penetration into the vascular cylinder was never observed (Fig. 2).

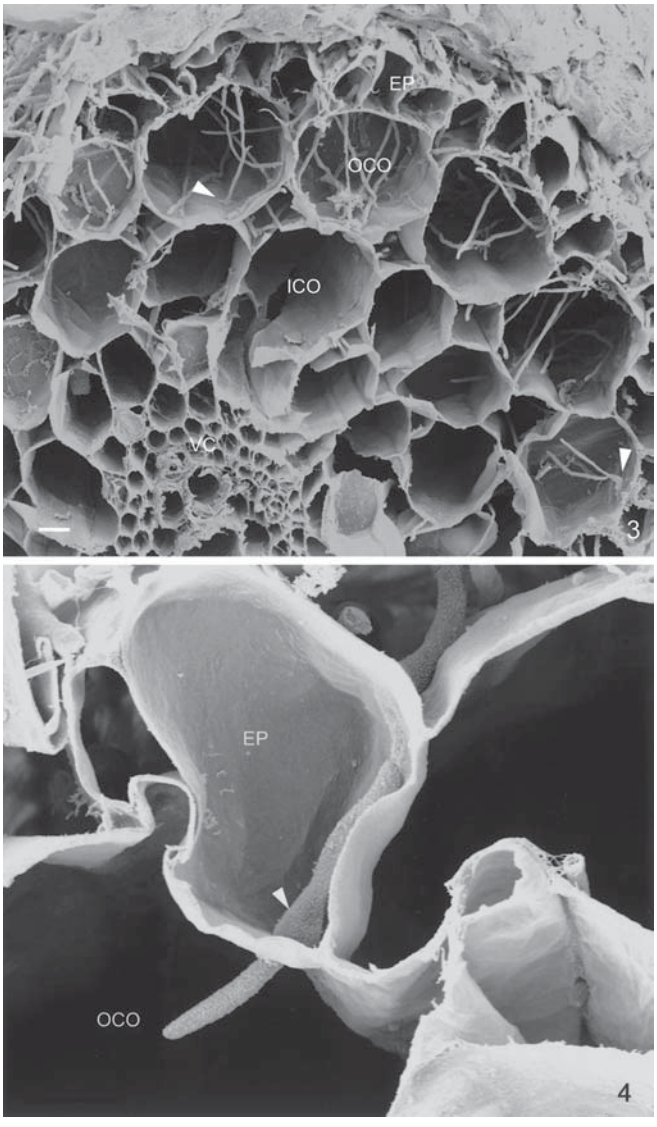
SEM revealed that hyphae of *H. chaetospira* were abundant inside the root epidermal and cortical cells 3 weeks after inoculation (Fig. 3). Some appressorium-like swollen structures occurred in epidermal and cortical cells (Fig. 4, arrowhead). No visible degradation of the host cell walls was evident where hyphae contacted. Under TEM, narrow infection hyphae penetrated into the adjacent cortical cell wall (Fig. 5, arrowhead). In response to hyphal ingress, no ultrastructural signs of host resistance responses were evident, and the penetrated host cells appeared almost empty (Figs. 5, 6). Similarly, the cytoplasm of control plant within most of the epidermal and cortical cells might not have remained intact (Fig. 7). There was no invagination of the host plasma membrane around penetrating and intracellular hyphae (Figs. 5, 6), but fibrillar materials were distributed around intracellular hyphae (Fig. 6).

Discussion

Seedlings of Chinese cabbage inoculated with *H. chaetospira* were known to be morphologically healthy and had no external symptoms of disease (Narisawa et al. 1998, 2000). In addition, our previous work (Narisawa et al. 1998) showed that seedlings of Chinese cabbage inoculated with *H. chaetospira* in a nursery setting had dry weights approxi-



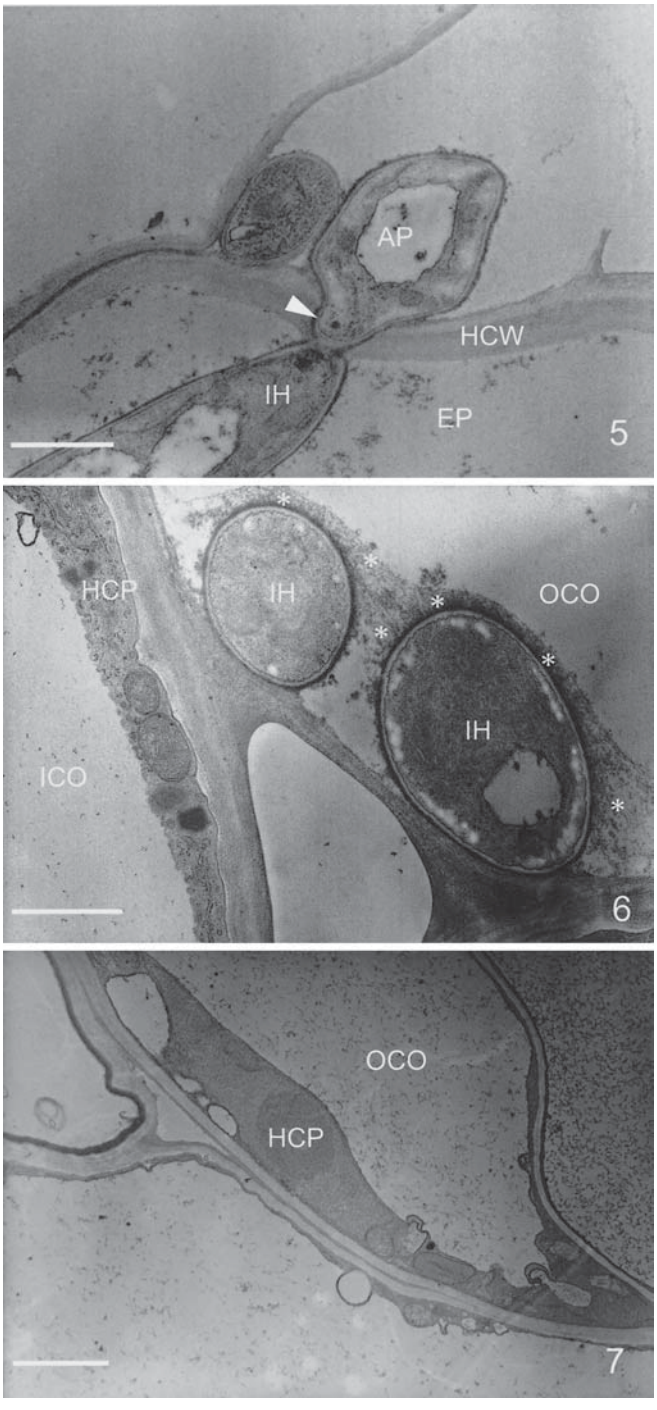
Figs. 1,2. Interactions between Chinese cabbage root and *Heteroconium chaetospira*. Light micrographs of cross section of Chinese cabbage root stained with 0.005% cotton blue. Most of the cortex consists of outer (*OCO*) and inner cortical cell (*ICO*) layers. Fungal hyphae were seen on root surfaces (*arrows*), within epidermal cells (*EP*, *arrowheads*), and within cortical cells (*asterisks*). Fungal colonization was restricted to epidermal and cortical cells. *VC*, vascular cylinder. **1** Four weeks after inoculation. **2** Eight weeks after inoculation. Masses of closely packed, irregularly lobed hyphae or microsclerotia had formed in most cells of the epidermis and cortical layers (*asterisks*). *Bars* 10 μm



Figs. 3,4. Scanning electron micrographs of cross sections of Chinese cabbage root infected by *H. chaetospira*. Appressorium-like swollen structures (*arrowheads*) of the fungus created in host epidermal (*EP*) and outer cortical cell walls (*OCO*). **3** Abundant fungal hyphae developed mostly within epidermal cells (*EP*) and within outer cortical cells (*OCO*). **4** Hyphae of *H. chaetospira* penetrated into epidermal and cortical cells. *Arrows* indicate appressorium-like swollen structures. **3,4** Four weeks after inoculation. *Bars* 5 μm

mately four times those of uninoculated controls. Also, experiments with some morphologically similar DSE fungi in the roots of plants have shown positive effects on the growth of host plants (Jumpponen and Trappe 1998). The current anatomical observations indicated that no intracellular hyphae of *H. chaetospira* reached the vascular cylinder; thus, the root vascular cylinders remained intact. This result might explain why *H. chaetospira* did not cause wilting or disease of inoculated host plants. In contrast, in other host plant–fungal endophyte interactions, such as *Phialocephala fortinii*, the hyphae occasionally penetrated vascular tissue of host roots (Yu et al. 2001; Narisawa et al. 2004) and can have a detrimental effect on the host plant, depending on growth conditions (Jumpponen and Trappe 1998). Consequently, *H. chaetospira* is assumed to be symbiotic with Chinese cabbage, i.e., at least the host derives benefit from the association, although *P. fortinii* functioned as a weak pathogen or as an opportunistic saprophyte under the conditions of the growth pouch method.

We observed fibrillar materials morphologically resembling those that commonly occur in mycorrhizal fungi and host plant associations distributed around intracellular hyphae of *H. chaetospira*. However, no invagination of the host plasma membrane around penetrating or intracellular hyphae was recognized in the current study. This feature is not unique to *H. chaetospira.* In other DSE fungus such as *P. fortinii*, hyphae also were not separated from host cell plasmalemma by space that contains sparsely distributed fibrillar material (Yu et al. 2001). The origin of the fibrillar material that coats the intracellular hyphae of mycorrhizas is a subject of controversy (Duddridge and Read 1981), and thus the meaning of the fibrillar material observed in the current study is not clear. These materials are morphologi-



Figs. 5–7. Transmission electron micrographs of cross sections of *H. chaetospira*–treated Chinese cabbage root. **5** Narrow infection hyphae (*arrowhead*) of *H. chaetospira* penetrate the epidermal cell wall (*HCW*). No ultrastructural signs of host resistance responses were seen in response to hyphal ingress. **6** Outer (*OCO*) and inner cortical cells (*ICO*) of *H. chaetospira*–treated Chinese cabbage root. Fibrillar materials (*asterisks*) were seen around intracellular hyphae (IH). **7** Outer cortical cell (*OCO*) of Chinese cabbage root in control treatment. **6,7** The host cytoplasm (*HCP*) within the cortical cells does not remain active. **5–7** Four weeks after inoculation. *AP*, appressorium-like swollen structure; *IH*, intercellular hyphae. *Bars* 3 μm

cally similar to those observed with mycorrhizal symbiosis, but probably have a different role because no invagination of the host plasma membrane around *H. chaetospira* hyphae was recognized.

In associations between mycorrhizal fungi and host plants, it is common to observe a perifungal membrane across which nutrient transfer occurs (Smith and Read 1997). In the case of DSE fungi, only a *P. fortinii* – host plant association was reported, which was not biotrophic because of the lack of a perifungal membrane (Yu et al. 2001). In the current study, in spite of the obvious beneficial effects on the host and the endophytic nature of the fungus in its roots, no structures indicative of a conventional mycorrhizal symbiosis were observed. The Brassicaceae is an exceptional family of angiosperms because most species are nonmycorrhizal (Ocampo et al. 1980) as well, and *H. chaetospira* is assumed not to be a typical mycorrhizal fungus from the morphological characteristics in host cells. We must confirm whether the bidirectional nutrient transfer occurs between *H. chaetospira* and Chinese cabbage to clarify the role of the membrane and the nature of the relationship between them.

We did not find ultrastructural signs of host resistance responses in host epidermal and cortical cells against hyphal ingress of *H. chaetospira*, and the anatomical structure of the roots infected by the fungus is basically the same as that of the uninfected (control). This result suggests that the host cells do not activate their resistance reactions for the hyphal ingress of *H. chaetospira*. In contrast, wall appositions that are assumed to be a host response against hyphal ingress were sometimes recognized in a morphologically similar DSE fungus, *P. fortinii* (Yu et al. 2001). This function is not included in typical responses to pathogen ingress at the penetration sites, e.g., formation of papillae, but may be categorized as a defense reaction of host plant (Yu et al., 2001). Because the cytoplasm of control plants within most of the epidermal and cortical cells appeared to be nearly empty in the current study, we should examine whether root cells of Chinese cabbage can react against other DSE fungus, such as *P. fortinii*, or whether pathogens ingress on the same experimental conditions to clarify the host resistance responses against *H. chaetospira*.

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