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SHORT COMMUNICATION

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Colonization process of the root endophytic fungus *Heteroconium chaetospira* in roots of Chinese cabbage

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Abstract Dark septate endophytic fungi (DSE) may have an important functional relationship with host plants, but these functions and the colonization process remain unknown. We made microscopic observations of the growth of an endophytic hyphomycete in Chinese cabbage roots to understand its colonization process. This hyphomycete was *Heteroconium chaetospira*, a suspected DSE. Three weeks post inoculation, some hyphae became irregularly lobed and formed microsclerotia within host epidermal cells of healthy plants. In stunted plants, hyphae formed closely packed masses of fungal cells within host epidermal cells, but conidiophores rarely broke through the cell walls to produce conidia.

Key words Colonization process · *Heteroconium chaetospira* · Microsclerotium · Root endophytic fungus · Dematiaceous hyphomycete

Roots of most plants are infected by fungi, and their relationships range from antagonism to mutualism (Read et al. 1992). For example, mycorrhizal fungi associate with many plant species and benefit their host plants. Besides mycorrhizal fungi, root-associated dark septate endophytic fungi (DSE), including *Mycelium radicis atrovirens* (MRA) (Melin 1922, 1923), have been reported from approximately 600 plant species of 320 genera in 114 families (see review in Jumpponen and Trappe 1998). They may have a potentially important functional relationship with host plants; however, little is known about these relationships and functions. Microscopic observations are crucial to show how DSE interact with their host plants, but only a few observations have been made so far.

Heteroconium chaetospira (Grove) M.B. Ellis has been isolated from the wood of deciduous trees, millipede droppings, arable soils, and alpine habitats (Matsushima 1975; Ellis 1976; Petrini et al. 1992; Domsch et al. 1993). Recently, *H. chaetospira* was isolated from roots of Chinese cabbage grown in wheat field soil. This fungus suppressed incidents of clubroot and *Verticillium* yellows in pot and field trials (Narisawa et al. 1998, 2000). The endophytic, dematiaceous hyphomycete *H. chaetospira* colonized the root tissues of 19 plant species, including Chinese cabbage, without visible symptoms such as wilt or necrosis (Narisawa et al. 1998, 2000), suggesting its affinity with DSE.

Potential agents of biocontrol for soil-borne diseases require an organism that not only is persistent in the field but which also can be recovered readily (Narisawa et al. 2000). *Heteroconium chaetospira* can be recovered with relative ease during the course of an experiment in Chinese cabbage fields, but formation of any dispersal or resistant structures, such as conidia or microsclerotia, has not been found in the root tissue of the Chinese cabbage (Narisawa et al. 2000). The initial infection process of *H. chaetospira* involved the formation of appressoria on root epidermal cells and the subsequent growth of hyphae within root cortical cells of the host plant (Narisawa et al. 1998, 2000); however, the morphological development of the fungus in host plant roots following the initial infection process remains unclear.

We proposed the hypothesis that *H. chaetospira*, which can be recovered with ease, may produce dispersal or resistant structures in the root tissues of host plants in the life cycle. To test this hypothesis, we observed the hyphal development of the fungus in the root cortex of Chinese cabbage mainly after the initial infection process in pot cultures.

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Materials and methods

Heteroconium chaetospira (isolate number H4007, available from KN) was isolated from the roots of Chinese cabbage grown in natural soil. The soil originated from the root zones (5–20 cm) in wheat fields (Shimodate, Ibaraki, Japan), and the isolation method followed the procedure reported by Tokumasu (1978) with slight modification.

Heteroconium chaetospira was grown on malt extract agar [25 g malt extract agar, 15 g bacto agar (Difco, Detroit, MI, USA), 1 l H_2O] in petri dishes for 1 month at 20°C. Pieces of colonies (5 × 5 mm) were excised and transferred onto autoclaved (121°C, 15 kg cm^{-2} for 30 min), 40-mm-diameter peat pellets (Jiffy 7, Sakata seed, Yokohama, Japan) containing 50 ml malt yeast medium (10 g Difco malt extract, 2 g Difco yeast extract, 1 l H_2O) per pellet. These peat pellets were incubated in sealed plastic bags at 20°C for 1 month in the dark.

We used Chinese cabbage (*Brassica campestris* L.) cv. Shin-Riso (Nihon-norin, Tokyo, Japan) susceptible to both clubroot disease and *Verticillium* yellows. Surface-disinfected Chinese cabbage seeds were placed on corn meal agar (25 g Difco corn meal, 15 g Difco bacto agar, 1 l H_2O) in petri dishes. After 2 days, the seedlings were transferred onto the peat pellets, which were visibly colonized by *H. chaetospira*. Seedlings were incubated in a growth chamber at 20°C under a light : dark cycle (16 : 8, approximately 180 μmol m^{-2} s^{-2}) for 1 month. A total of 30 inoculated seedlings were prepared for observation. We randomly sampled 10 seedlings per week, all of which were infected with *H. chaetospira*, for 3 weeks. At the same time, these plants were recovered from the pots to determine the dry weight of the plants. The mean dry weight of the plants was calculated and analyzed by analysis of variance followed by Student's least significant difference test.

Roots of sampled seedlings were cut at 2-cm intervals, cleared for 30 min in 10% KOH at 95°C, and stained with 0.005% Trypan blue. Roots were observed under an Olympus (Tokyo, Japan) BX50 microscope with UPlan FI40/0.75 and UPlan FI100/1.30 objectives.

Results and discussion

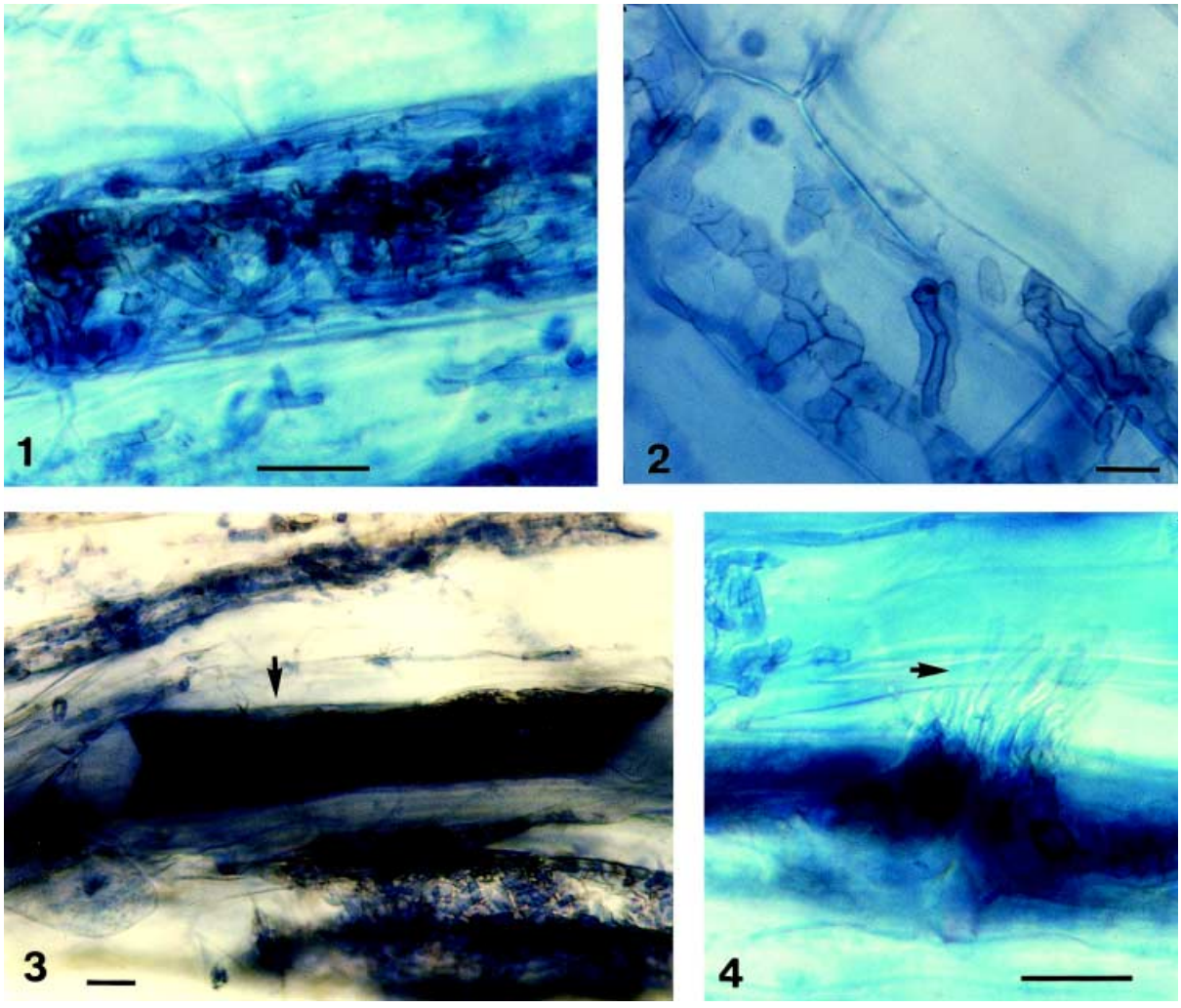
One week post inoculation, conidia or mycelial fragmentation of *H. chaetospira* had germinated, produced small amounts of hyphae on the root epidermis, and formed appressoria. Hyphae of the fungus penetrated the host epidermal cell wall via narrow infection hyphae. Two weeks post inoculation, fungal hyphae grew over the root surfaces and extensively colonized the inner root cortical tissues. Hyphae of the fungus elongated, branched, and anastomosed in the root cortical cells, similar to previous reports (Narisawa et al. 1998, 2000).

Three weeks post inoculation, hyphae formed masses of closely packed, pigmented fungal cells in the root cortical cells. These hyphae developed irregularly lobed, rounded, thick-walled cells referred to as microsclerotia in 50% of the seedlings (Figs. 1, 2). Microsclerotia formation has been reported previously by some identified DSE, e.g., *Phialocephala fortinii* Wang & Wilcox and *Leptodontidium orchidicola* Singer & Currah (Stoyke and Currah 1991; Currah et al. 1993; Fernando and Currah 1995, 1996), and many unidentified DSE (see review in Jumpponen and Trappe 1998). This is the first report of microsclerotia formation in host root cells by species of *Heteroconium*. The ecological significance of the formation of microsclerotia remains unclear; however, Currah et al. (1993) hypothesized that the intracellular microsclerotia of *P. fortinii* can be effective dispersal propagules. As the colonized roots mature, the epidermal cells frequently loosen and slough off the root. The sloughed-off cells can then disperse with soil movement.

At the same time, 20% of seedlings were stunted, but any typical pathogenic symptoms, such as wilting or necrosis, were not observed on the plant shoots and leaves. The dry weights (aerial parts) of these stunted seedlings were significantly decreased compared to other plants ($P = 0.05$). Invasive hyphae filled the cortical cells of these plant roots (Fig. 3), but the hyphae were restricted to the cortex and were unable to penetrate into the cells of the vascular tissues. Occasionally, conidiophores broke through the epidermal cell walls of these seedling roots to produce conidia (Fig. 4). Conidia were formed when host roots became stunted, which is similar to the foliar fungal endophyte *Rhabdocline parkeri* Sherw. (Sherwood-Pike et al. 1986; Stone 1987). *Rhabdocline parkeri* was latent when Douglas-fir needles were healthy, but it produced conidia when the needles were dead. Fifteen species of DSE have been reported from the family Brassicaceae (Jumpponen and Trappe 1998; Narisawa et al. 1998), but the effects of DSE on the *Brassica* host were not clearly understood. We first recorded the stunted growth of *Brassica* plants inoculated with *H. chaetospira* and the conidial formation of the fungus on stunted plants. However, typical pathogenic symptoms were not found on the plants. Consequently, the association between species of *Heteroconium* and its *Brassica* host is not easily classified into any category of mutualistic, parasitic, or pathogenic associations.

Melin (1922, 1923) first described that MRA overgrew and killed *Pinus sylvestris* L. and *Picea abies* (L.) Karst., appearing as a parasite. Furthermore, some DSE, such as *P. fortinii*, showed weak pathogenicity on *Pinus resinosa* Ait. and *Picea rubens* Sarg., depending on environmental conditions (Wang and Wilcox 1985; Wilcox and Wang 1987). However, Currah et al. (1993) found that one of three strains of *P. fortinii* caused no pathological effects on *Rhododendron brachycarpum*. G. Don. Thus, effects of DSE on their host were variable and the host response depended on the species or even strains of the fungus, as well as the environmental conditions (Jumpponen and Trappe 1998).

The reproduction and dispersal mechanisms of DSE are almost completely unknown (Jumpponen and Trappe 1998). As already mentioned, mycelial fragmentation, such as intracellular microsclerotia and conidia, of *H. chaetospira*



Figs. 1–4. Colonization process of *Heteroconium chaetospira* in roots of Chinese cabbage. **1** Irregularly lobed hyphae colonizing cortical cells. **2** Microsclerotia of *H. chaetospira* in cortical cells. **3** Invasive hyphae filling the cortical cells of stunted seedlings (*arrow*, hyphae filling host cell). **4** Conidia of *H. chaetospira* growing through epidermal cell walls (*arrow*, conidia). *Bars* 10 μm

is among the suggested means of dispersal. How the fungal endophyte *H. chaetospira* behaves in host roots in natural soils has yet to be determined, and more observations are needed to understand how populations of *H. chaetospira* disperse and maintain themselves in natural soils.

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