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A Fluorescence Microscopic Study of the Infection Process of *Discula theae-sinensis* in Tea

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Abstract

The infection process of the anthracnose fungus *Discula theae-sinensis* in tea was investigated in detail by fluorescence microscopy. Leaves of the susceptible tea variety 'Yabukita' spray inoculated with the conidial suspension were collected sequentially and examined after fluorescence staining with fluorescein-conjugated wheat germ agglutinin. Conidia adhering to the trichomes germinated and formed very short germ tubes. Penetration hyphae grew from the germ tube tips into the cell walls of the trichomes, and formed infection hyphae by 12 h after inoculation. Hyphae that had invaded the mesophyll were confined to the small round spots that formed surrounding the infected trichomes. However, hyphae that had reached the veins extended through the phloem, causing necrosis of the neighboring mesophylls and eventually the formation of large necrotic lesions.

Discipline: Plant disease
Additional key words: anthracnose, *Camellia sinensis*, fluorescein-conjugated wheat germ agglutinin

Introduction

Discula theae-sinensis (I. Miyake) Moriwaki & Toy. Sato [syn. *Gloeosporium theae-sinensis* I. Miyake, *Colletotrichum theae-sinensis* (I. Miyake) Yamamoto] causes anthracnose on tea [*Camellia sinensis* (L.) Kuntze], which is the most common disease in Japanese tea cultivation (Moriwaki & Sato 2009). Previous studies (Ando & Hamaya 1986, Hamaya 1982) have shown that *D. theae-sinensis* have a unique infection process. *D. theae-sinensis* enters young tea leaves only through the trichomes, small outgrowths on the plants' surface. The trichomes of tea are simple unicellular hairs, and those of most Japanese tea varieties are over 800 μm in length and 20-40 μm in diameter, with more than 10 trichomes/cm^2 on the entire lower surface of the leaves (Takeda et al. 1993). Some plant pathogenic fungi enter the trichomes, such as *Colletotrichum acutatum* (syn. *Gloeosporium laeticolor*) (Kitajima 1951, 1952; Moriwaki et al. 2002) and *Phoma clematidina* (van de Graaf et al. 2002). However, as far as we know, *D. theae-sinensis* is the sole fungus that enters the host plant only through the trichomes (Hamaya 1982). Conidia germinate on the trichomes and penetration hyphae grow into the cell walls of the trichomes. Hyphae then elongate into the lumen and enter the mesophyll through small pores at the base of the trichomes. Small round spots 0.2-0.6 mm in diameter and centered on the infected trichomes, develop after 10 to 30 days. Veins around the spots become brown, neighboring mesophyll necrotize, and finally typical anthracnose lesions form after 15 days to 1 month. However, the behavior of the causal fungus during this process, particularly before penetrating the trichome and after entering the mesophyll, remains unclear. In this study, we investigated the detailed infection process of *D. theae-sinensis* by fluorescence microscopy.

Materials and methods

1. Inoculation and sampling

The susceptible tea variety 'Yabukita' was inoculated with *D. theae-sinensis* isolate CT001, which had been maintained at the NARO Institute of Vegetable and Tea Science. Conidia were obtained by incubation on autoclaved tea leaves at 26°C for 3-4 weeks, whereupon the conidial suspension (10^6–10^7 conidia/mL) was sprayed onto new shoots on detached tea stems collected from the field, or intact shoots on potted or field-grown tea plants. The detached

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stems and potted plants were placed in a dew chamber at 26°C for 48 h, and grown at 26°C with a 12-h light/dark cycle. The upper 2-3 leaves of the new shoots on detached stems were sampled at 8, 10, 12, 16, 20, 24 h and 2, 3, 4, 5, 6, 7, 8, 10 days after inoculation and at least 8 leaves on 4 shoots were examined for each sampling period. The experiment was repeated twice. Lesion development after 10 days was observed on intact shoots on potted and field-grown plants, and leaves were sampled depending on the lesion development.

2. Fluorescence microscopy

Fungi in the sampled leaves were stained with fluorescein-conjugated wheat germ agglutinin (F-WGA; Molecular Probes, Eugene, OR, USA) as previously described (Yamada et al. 2009). Briefly, leaves were fixed and decolorized in a mixture of ethanol and acetic acid (96:4, v/v) at 40°C for a few days with one or two exchanges of fixing solution, and stored in 70% (v/v) ethanol at 4°C. Fixed leaves were sectioned at 10 to 20 μm with the MicroSlicer Zero 1 (Dosaka EM, Osaka, Japan), or cleared for the whole mount in 10% (w/v) potassium hydroxide solution at 40°C overnight. After washing with distilled water (DW) twice, the samples were immersed in F-WGA solution (10 μg/mL in phosphate-buffered saline) for 10 min. After washing with DW, the samples were mounted in DW and examined under blue excitation [excitation filter (EX) 450-490 nm, dichroic mirror (DM) 505 nm, and barrier filter (BA) 520 nm] using an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan) and a DXM1200F digital camera (Nikon). Callose was visualized by mounting the samples in aniline blue (Wako Pure Chemical Industries, Osaka, Japan) solution [0.05% (w/v) in 0.067 M phosphate buffer, pH 8.5] and examined under ultraviolet excitation (EX 365/10 nm, DM 400 nm, BA 400 nm) (O'Brien & McCully 1981).

Results

Some of the conidia adhering to the trichomes had already begun germinating, and had produced germ tubes and penetration hyphae 8 h after inoculation. The germ tubes were usually very slight protrusions, less than 1 μm in length (Fig. 1A), but occasionally grew longer and formed a distinct germ tube or an appressorium-like shape (Fig. 1B). Thin penetration hyphae grew from the germ tube tips into the cell walls of the trichomes (Figs. 1A-C). Penetration hyphae changed their direction of growth to longitudinal and thickened to form infection hyphae in the cell wall or the lumen of the trichomes 12 h after inoculation (Fig. 1C). Infection hyphae grew longitudinally in the trichomes and reached the basal part of the latter at the earliest 2 days after inoculation. Hyphae entered the mesophyll through the small pores at the base of the trichomes by 4 days, in the most rapid case, after inoculation, but usually entered on and after the 7th day (Fig. 1D). Hyphae grew irregularly through the mesophyll (Fig. 1E), and a small round spot formed surrounding each infected trichome (Fig. 2A). These could occur by 7 days after inoculation, but they usually formed at 10 to 14 days after inoculation. Hyphae radiated in all directions from the trichomes at the centers of these spots, and were confined to the central necrotic areas of the spots surrounded by callose (Figs. 1F-I). However, once the hyphae reached the veins, they spread rapidly; mainly through the phloem (Figs. 1G, I-K). The infected phloem turned brown and collapsed, followed by the discoloration and necrosis of neighboring mesophyll tissues (Figs. 1J, K, 2B). The hyphae subsequently entered the necrotic mesophyll from the veins (Fig. 1L). In the large necrotic lesions, which formed 2 to 4 weeks after inoculation, the mesophyll and veins were filled with hyphae (Figs. 1M, 2C).

Discussion

The behavior of *D. theae-sinensis* on the trichomes elucidated in this study is generally in agreement with earlier studies. However, there are some differences that we attribute to the detailed examination by fluorescence microscopy. Previously, it was shown that conidia transform into dome-shaped appressorium-like bodies on trichomes before penetration (Ando & Hamaya 1986). However, we consider that conidia merely swell slightly before germination. When the lower part of a swollen conidium, including the short germ tube, is hidden by the trichome it resembles an appressorium. Other appressoria, which are produced from conidia on cellophane film, have also been reported (Moriwaki & Sato 2009). However, such appressoria have never been observed, in earlier (Ando & Hamaya 1986) or present studies on trichomes. These results indicate that appressoria formation is not necessary for penetration. Trichome penetration occurred within 12 h after inoculation in this study, while a previous study showed that it took 2 to 3 days (Ando & Hamaya 1986). Our result supports an earlier finding, obtained by inoculation experiments, that a dew period of at least 12 h is required to establish infection (Hamaya 1982).

This study confirmed the existence of the causal fungus in the mesophyll and veins of diseased tea leaves, which was previously unproven. We found that the hyphal extensions in the mesophyll were confined to small round spots during the initial stage of lesion development, which may be attributable to the defense reactions of the host plants, since callose deposition was observed around all the small spots. These spots formed on both susceptible and resistant varieties; but subsequent larger necrotic lesion development did not occur on resistant varieties (Ando & Hamaya 1986). These findings indicate that both susceptible and resistant varieties can inhibit the causal fungus in the mesophyll by

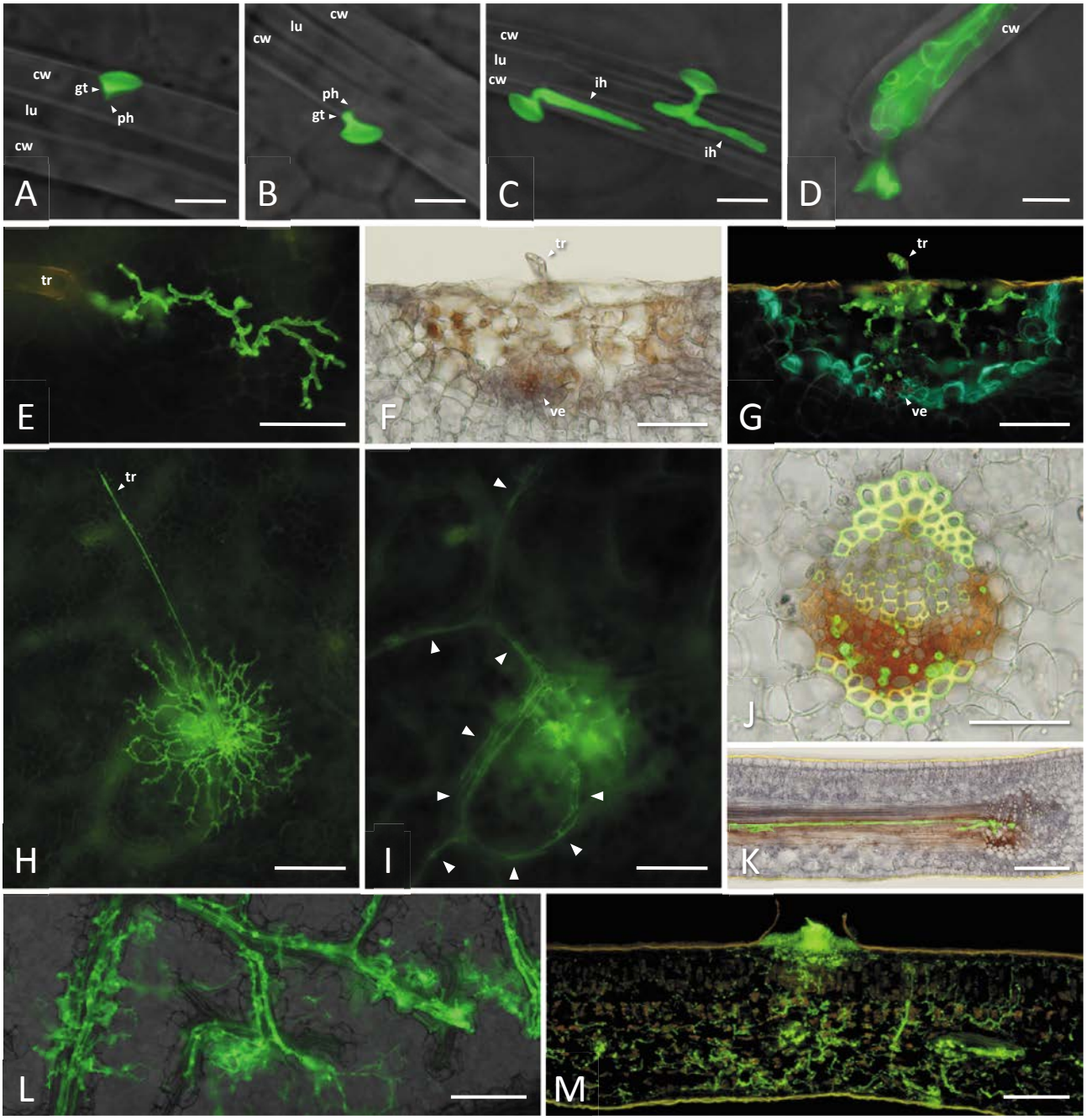

Fig. 1. ***Discula theae-sinensis*** **in tea leaves**

(A-C) Conidia and germ tubes (gt) on trichomes, and penetration hyphae (ph) and infection hyphae (ih) in cell walls (cw) and lumens (lu) of trichomes. (A) Conidium with a short germ tube and a thin penetration hypha. (B) Conidium with an appressorium-like germ tube. (C) Infection hyphae in the lumen and cell wall of a trichome. (D) Hypha entering the mesophyll from the trichome. (E) Elongation of hyphae from a trichome (tr) through the mesophyll. (F, G) Bright field (F) and fluorescence (G) images of the cross-section of a small round spot surrounding an infected trichome. Hyphae grow from a trichome and enter a vein (ve). Callose deposit around a spot (bluish white fluorescence). (H, I) Hyphae in the trichome and small round spot (H), and veins under it (I, arrowheads). (J, K) Transverse (J, K) and longitudinal (K) sections of infected veins. The infected phloem becomes brown and collapses. (L) Hyphae entering the mesophyll from the veins. Thin hyphae irregularly elongate through the mesophyll from thick hyphae in the veins. (M) Cross-section of a mature large necrotic lesion. The acervulus and hyphae fluoresce yellow-green. Cuticular layers and necrotic mesophyll cells show yellow to brown intrinsic fluorescence. (A-D, J-L) Composites of fluorescence and bright field images. Scale bars A-C 5 μm; D 10 μm; E, J 50 μm; F-I, K-M 100 μm.

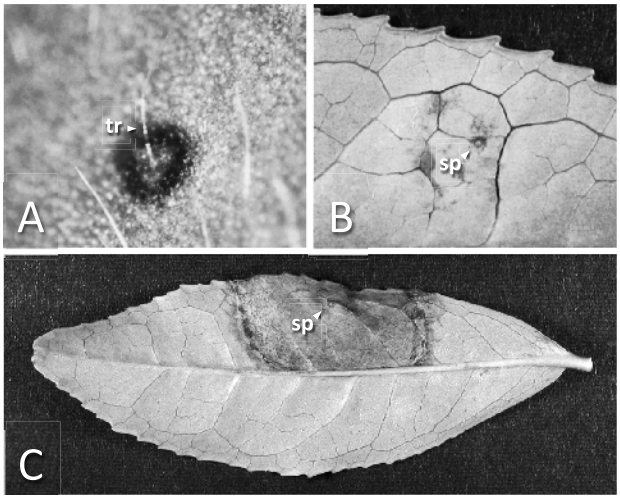

Fig. 2. Lesion development of anthracnose on tea leaves

(A) Small round spot surrounding the infected trichome (tr). (B) Browning of veins and neighboring mesophylls near the small round spot (sp). (C) Mature large necrotic lesion.

defense reactions, but susceptible varieties cannot defend against the fungus in the veins. Therefore, the collapse of the phloem and formation of large lesions can ensue in susceptible varieties. Further investigations into the mechanisms of varietal resistance to tea anthracnose are necessary to facilitate breeding of resistant varieties.

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