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Studies on turfgrass snow mold caused by *Typhula ishikariensis*. II. Microscopical observation of infected bentgrass leaves

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Light and transmission electron microscopy revealed that *Typhula ishikariensis* penetrated into bentgrass leaves either through cuticles or stomata either by single hyphae or infection cushions formed on host surfaces. Time course study on infected leaves showed that penetration through stomatal subsidiary cells and their adjacent cells seemed to occur earlier than that through epidermal cells located farther from stomata. More than 30% of epidermal cells were infected by 10 days after inoculation. When hyphae penetrated through an intact cuticle of epidermal cells, they seemed to dissolve host cell walls enzymatically at penetration sites. Physical pressure also seemed to be involved in penetration.

Key Words——Turfgrass snow mold; *Typhula ishikariensis*; Ultrastructure.

Typhula ishikariensis S. Imai infects turfgrass, pasture and wheat under drifted snow, kills these plants by the time of thawing of snow, and thus causes snow mold disease of these plants. Although several researchers worked on resistance of host plants to *Typhula* species (Araki, 1985; Iriki and Kuwabara, 1993; Matsumoto, 1989; Matsumoto and Sato, 1983; Takenaka and Yoshino, 1988), few reports are available on microscopical observation of the infection process of this species. A major reason is apparently the difficulty of specimen preparation for observation, because infection occurs under drifted snow. Takenaka and Yoshino (1987) prepared specimens for light microscopy from wheat leaves in pots inoculated with *T. incarnata* Lasch ex Fr., covered with a wet cotton sheet, and incubated in a cold room. Their observation revealed that hyphae of this fungus invaded wheat leaves either through cuticles or stomata by hyphal masses or single hyphae. However, no reports are available on electron microscopical observation of the infection process of this species.

As a part of our studies to develop biological control of turfgrass snow mold caused by *T. ishikariensis*, we have examined the infection process of this fungus in bentgrass by light and electron microscopy. Understanding the infection process of the pathogen will help us to screen effective antagonistic microorganisms which might suppress infection of the pathogen and to determine the proper time for treatment of turfgrasses by the selected microorganisms to prevent turfgrass snow mold.

Materials and Methods

Inoculation of bentgrass leaves with *T. ishikariensis*

Bentgrass sods (*Agrostis palustris* cv. Penncross) purchased from Nasu Nursery Inc. (Tochigi Prefecture) were inoculated with *T. ishikariensis* by the sod cuptest method (Oshiman et al., 1993). Briefly, four or five mycelial discs of this fungus obtained from a stock culture were inoculated into flasks containing 500 ml of autoclaved wheat-vermiculite medium (wheatbran, 1000 ml; vermiculite, 1000 cm^3; glucose, 14 g; distilled water, 700 ml) and incubated at 9°C for 30 days. Grown mycelia were stirred and used as an inoculum source. One gram of the inoculum was stuffed into a cylindrical plastic case (inside diameter, 22 mm; depth, 9 mm), which was placed upside down at the center of a bentgrass sod grown in a box ($108 \times 259 \times 85$ mm^3). The surface of the sod was covered with a fine-mesh synthetic fiber sheet and a wet cotton sheet. The fiber sheet prevents hyphae from entering into the wet cotton sheet. The box was covered with a lid and incubated at 0-1°C, which corresponds to the temperature under the drifted snow. Hyphae extended from the edge of the inoculum case, attached to grass stems and leaves outside the inoculum case by 14 days after the start of incubation, and extended further thereafter. Thus, day 15 of incubation was defined as the first day of inoculation in this paper. Leaves and stems outside the edge of inoculum case were harvested for specimen preparation as described below.

Light microscopy

1. Whole-leaf clearing and staining technique

Penetration of *T. ishikariensis* in epidermal cells of bentgrass leaves was observed as follows. Fifty leaf segments (2 mm length × 1-2 mm width) were harvested from the edge of mycelial colony on leaves daily on days

1 to 10 of inoculation. These segments were cleared and stained by the whole-leaf clearing and staining technique (Shipton and Brown, 1962) and observed with a Nomarski differential interference contrast microscope (NIKON Optiphoto-2). Briefly, the leaf segments were boiled in alcoholic lactophenol for about 10 min until chlorophyll was removed completely. Decolorized segments were boiled again in alcoholic lactophenol containing 0.05% aniline blue for 10 min to stain superficial hyphae.

As described below, hyphae invaded leaves through stomata, subsidiary cells, or epidermal cells other than stomata and their related cells. To avoid confusion, epidermal cells other than stomatal guard and subsidiary cells and their adjacent cells are defined as "regular" epidermal cells, and subsidiary and their adjacent cells are collectively defined as subsidiary cells in this paper. Because hyphal masses frequently covered the entire stomatal openings, it was difficult to judge penetration through stomata by those masses even though leaf color had been cleared. Thus, the incidences of hyphae observed inside regular epidermal cells and in subsidiary cells were counted to evaluate penetration efficiency (percentage of successful infection). This efficiency was expressed as a percentage of the number of cells in which hyphae were observed out of the total number of observed cells.

2. Observation of thick sections

To observe penetration sites in more detail, thick sections were prepared from specimens embedded in electron microscopical resin (Shigemitsu et al., 1981). Inoculated leaf segments (5 mm length × 1-2 mm width) were cut on days 5 and 25 of inoculation, prefixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature for 4 h, then rinsed in the same buffer. Subsequently, specimens were postfixed with 2% osmium tetraoxide in the same buffer at room temperature for 4 h and washed in distilled water. They were dehydrated through an ethanol series from 30 to 100% and embedded in Spurr's low viscosity resin (Spurr, 1969). Sections of ca. 5 μm thickness were cut with a ultramicrotome (Ultracut N Reichert Nissei) and attached to glass slides one by one with forceps. After sections had been dried to affix glass slides tightly, they were stained with 1% toluidine blue at 70°C for 8 h and observed with a light microscope.

Transmission electron microscopy

Specimen fixation and embedding methods for transmission electron microscopy were exactly the same as above except for block staining in 1% uranyl acetate in 70% ethanol for 1 h during the dehydration process. Ultrathin sections were prepared using a ultramicrotome and stained with uranyl acetate and lead citrate by the method of Reynolds (1963). The sections were observed with a Hitachi H700 transmission electron microscope at 100 kV accelerating voltage.

Results and Discussion

Estimated time of penetration by *T. ishikariensis*

Sparse mycelia extending from the edge of inoculum case began to cover the surface of leaves by the 5th day after the start of incubation and formed a thick mycelial mat surrounding the case by the 25th day. To estimate the time of penetration, the penetration efficiency in "regular epidermal cells" and "subsidiary cells" was determined by observing cleared and stained leaf segments (Table 1).

On surfaces of uninoculated control leaves, no fungal hyphae were observed. Hyphae were first observed in subsidiary cells of both adaxial and abaxial leaf surfaces on day 6 of inoculation. Penetration efficiencies in these cells were respectively 10 and 12% on these surfaces, and increased 32 and 28% by the 7th day. Thereafter, we could not observe the cells under the mycelial mat, because the mat became too thick. Hyphae which had invaded regular epidermal cells were first observed on the 8th day on both adaxial and abaxial surfaces. Penetration efficiencies in these cells were respectively 8-10% and 23-24% on these leaf surfaces on the 8th and 9th days. These results suggest that hyphae probably begin to penetrate into leaves within 6-8 days after making contact with leaf surfaces, preferentially through subsidiary cells and their adjacent cells. However, the possibility remains that they may invade leaves through stomatal openings much earlier than through these intact epidermal cells.

Hyphae which had invaded both regular epidermal and subsidiary cells remained in these cells until the 8th and 10th day, respectively. Thereafter, hyphae could be seen in cells surrounding these original cells, which made it impossible thereafter to determine the original penetration site. These observations suggest that hyphae might require approximately 2 days to expand from the original penetration cells to their surrounding cells.

Mode of penetration

Thick sections prepared from inoculated leaves were observed by light microscopy. No hyphae were observed

Table 1. Penetration efficiency of *Typhula ishikariensis* in adaxial and abaxial surfaces of bentgrass leaves.

Day of inoculation	Subsidiary cells		Epidermal cells	
	adaxial	abaxial	adaxial	abaxial
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	10	12	0	0
7	32	28	0	0
8	ND	ND	8	10
9	ND	ND	24	23
10	ND	ND	ND	ND

ND: not determined.

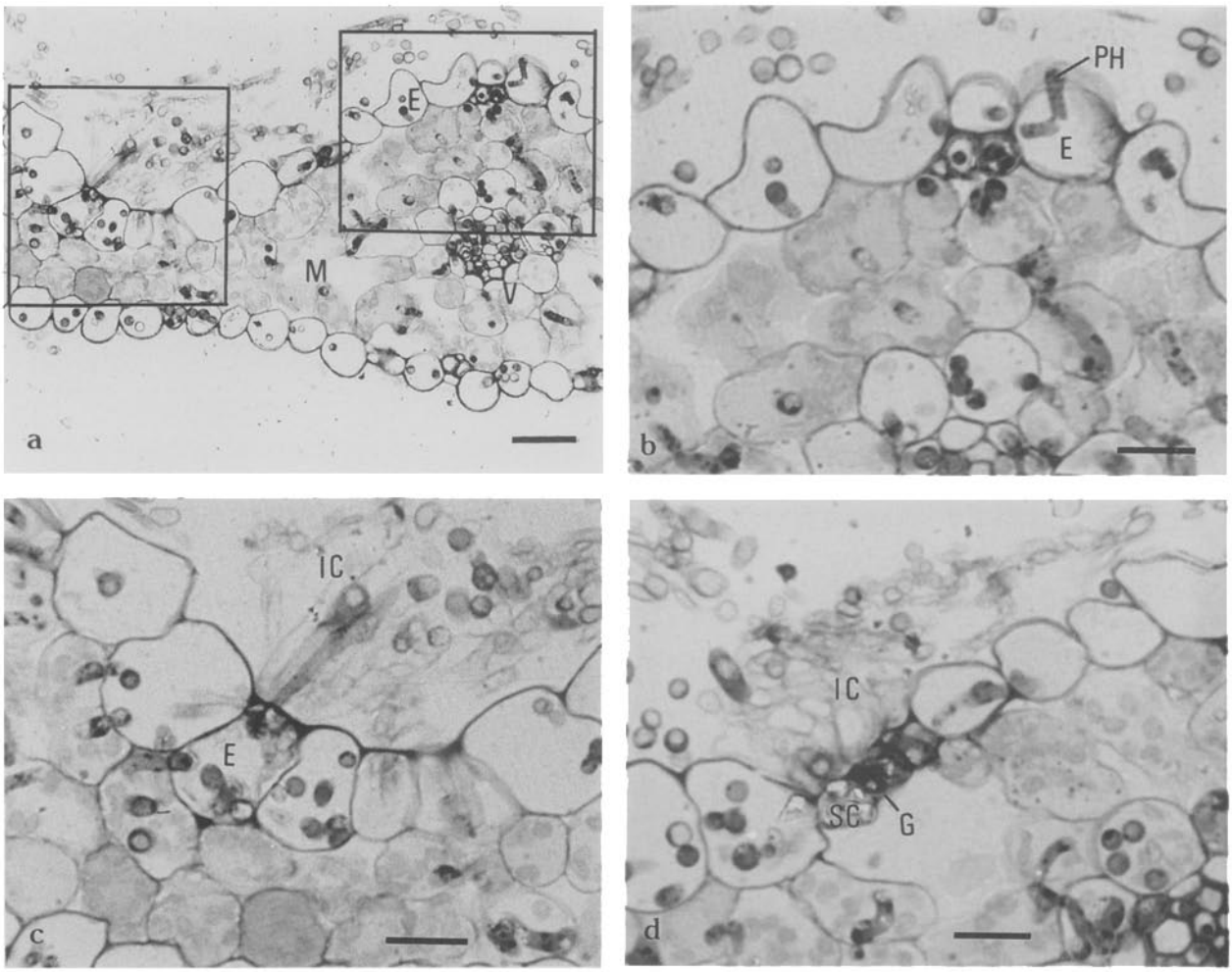

Fig. 1. Light micrographs showing bentgrass leaves infected with *Typhula ishikariensis*, day 25 of inoculation.
a. A transverse section of a leaf. Many hyphae grow in epidermal and mesophyll tissues and vascular bundles. Scale=30 μm. b. An enlarged portion of the right rectangle in panel a. A single hypha penetrates into a regular epidermal cell directly through its cell wall. Scale=20 μm. c. An enlarged portion of the left rectangle in panel a. Multiple hyphae extending from an infection cushion formed on the epidermal surface invade regular epidermal cells through their cell walls. Scale=20 μm. d. An infection cushion formed on a stoma. Several hyphae can be seen in the stoma and its surrounding cells. Hyphae which grew in mesophyll cells surrounding a substomatal cavity probably originated from hyphae which had invaded the leaf through the stomatal opening. Scale=20 μm.
Abbreviations: C=chloroplast, CU=cuticle, E=epidermal cell, G=guard cell, HCW=host cell wall, IC=infection cushion, IH=infection hypha, M=mesophyll cell, PH=penetration hypha, SC=subsidiary cell, SH=superficial hypha, V=vascular bundle.

in leaf tissues of 20 specimens prepared on day 5 of inoculation. This observation was consistent with the results mentioned above.

By contrast, a large number of hyphae were observed in epidermal and mesophyll tissues and also in vascular bundles of leaves which had been fixed on day 25 of inoculation (Fig. 1a). Infection was confirmed in 25 out of 30 sections of these leaves. In some cases single hypha penetrated independently through a regular epidermal cell by breaching its cell wall (Fig. 1b). In other cases multiple hyphae emerging from the same hyphal mass invaded an epidermal cell through its cell wall (Fig. 1c). Hyphal masses were also produced frequently on stomata and a few hyphae extended from these masses into substomatal cavities through stomatal openings (Fig. 1d). These hyphal masses probably correspond to infection cushions, which are frequently produced as an infection structure by *Rhizoctonia* (Smiley et al., 1992; Kousik et al., 1994), *Pellicularia* (Ishizaki et al., 1979), *Sclerotinia* (Lumsden and Dow, 1973) and other fungi such as *Diplodia* (Chou, 1978). The mode of penetration by the present fungus resembles that of *T. incarnata* in wheat leaves (Takenaka and Yoshino, 1987).

In the following electron micrographs, hyphae which are passing in host epidermal cell walls and those which have developed in host mesophyll cells are distinguished, being labeled as penetration hyphae (PH) and infection hyphae (IH), respectively. Figs. 2a and 2b illustrate a penetration pattern through a cuticle and cell wall of a regular epidermal cell by a single hypha. As shown in Fig. 2b, three layers were distinguished in host cell walls by electron density: 1) a very thin, electron-lucent, outer-

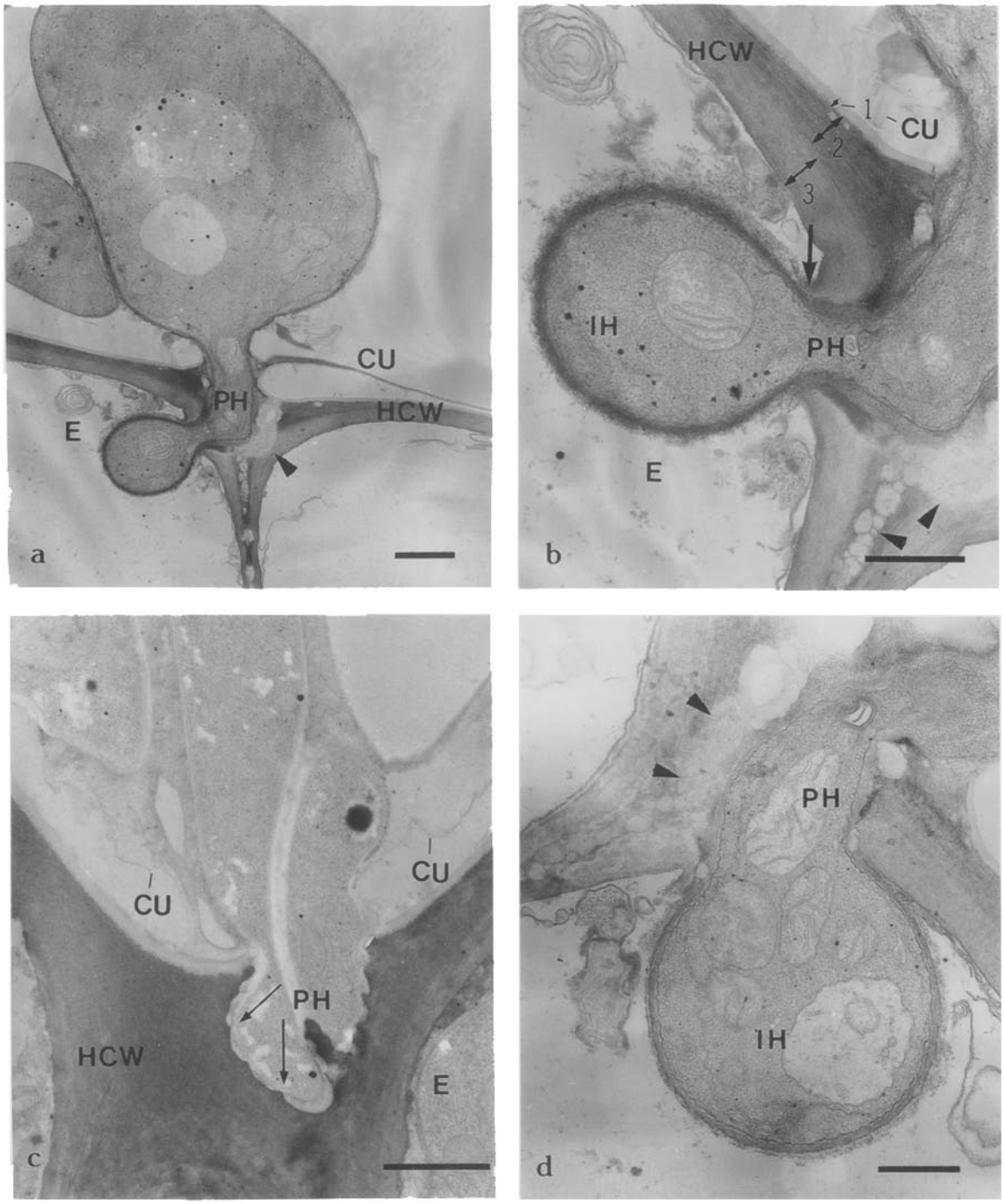

Fig. 2. Transmission electron micrographs showing penetration process of bentgrass epidermal tissues by *Typhula ishikariensis*. a. A single hypha penetrates into a leaf epidermal cell through a junction of anticlinal cell wall. Note that a cuticle layer is detached from the epidermal cell wall and the host cell wall is bent inwards around the penetration hypha. The arrowhead shows the cell wall portion of low electron density, probably caused by the fungal enzyme. Scale = 1 μm. b. A higher magnification of Fig. 2a. Three layers numbered 1-3 are distinguished in the host cell wall. The penetration hypha reduced its thickness within the cell wall and recovered it after passing the cell wall. The arrow shows a cell wall bending inwards. The arrowheads show cell wall portions of low electron density, which might have been caused by the fungal enzyme. Scale = 0.5 μm. c. An apical portion of a penetration hypha within a host cell wall. Note that the edge of the apical portion is irregularly shaped (arrows), suggesting that this portion of host cell wall has been softened at least partially by wall-degrading enzymes of the fungus. Scale = 1 μm. d. Electron density of the host cell wall portion (arrowheads) which contacts a penetration hypha, has been prominently reduced leaving an amorphous appearance. Scale = 0.5 μm.
Abbreviations are shown in Fig. 1.

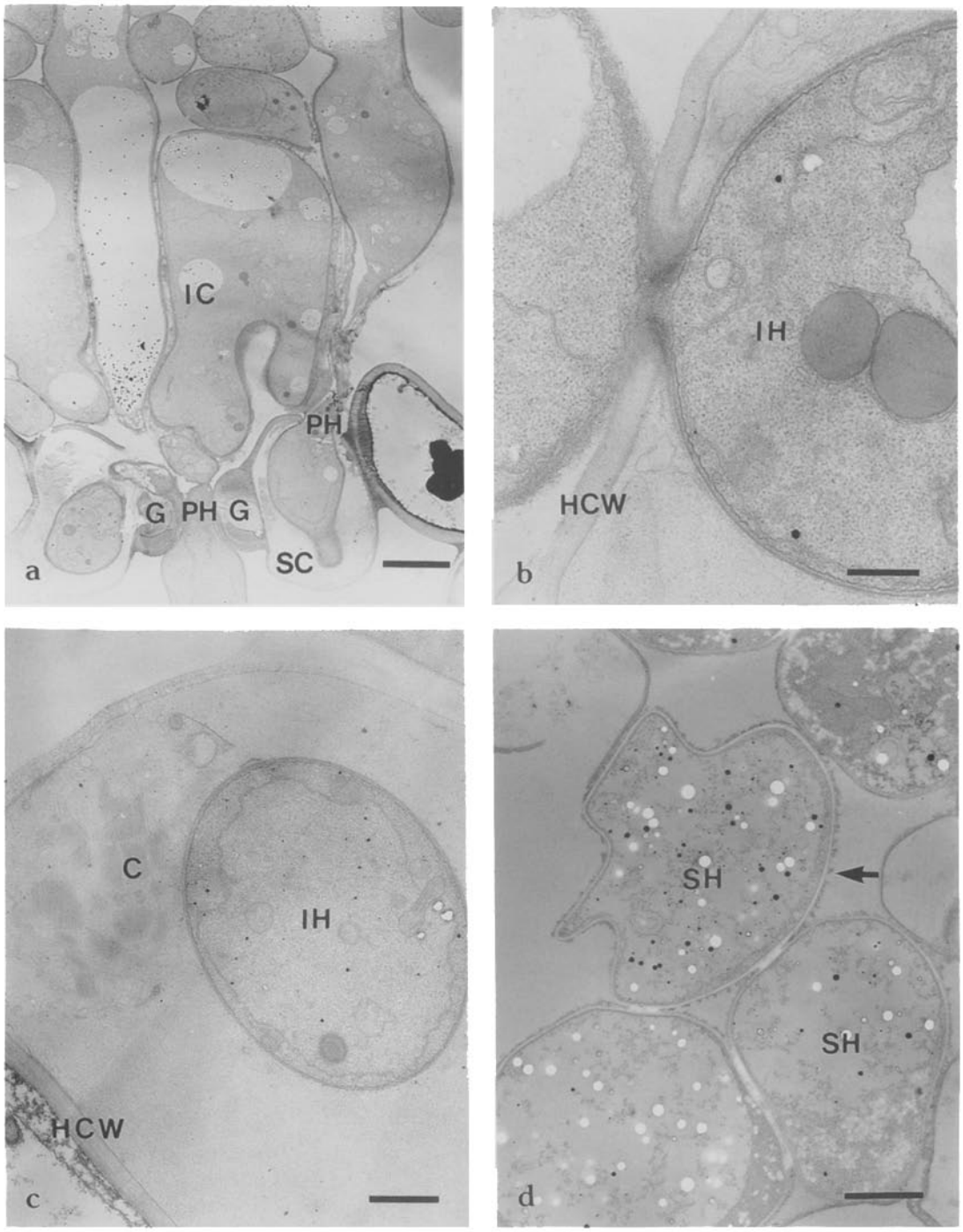

Fig. 3. Transmission electron micrographs of hyphae in subsidiary cells (a), mesophyll cells (b and c) and an infection cushion (d). a. Single hyphae emerging from an infection cushion have invaded a substomatal cavity through a stomatal opening between guard cells and directly a subsidiary cell through the outer epidermal cell wall. Scale = 2 μm. b. A single hypha extending into a mesophyll cell from an epidermal cell by breaching a bordering cell wall. Scale = 0.5 μm. c. All organelles in a mesophyll cell in which hyphae grew have degenerated leaving only a cell wall. Scale = 1 μm. d. A layer composed of electron-dense granules (arrow) on the outer surface of hyphae within an infection cushion. Scale = 1 μm. Abbreviations are shown in Fig. 1.

most layer (cuticle layer); 2) an electron-dense, middle layer; 3) an innermost layer of lower electron density than the middle layer. Around the penetration site, the outermost cuticle layer was often detached from the cell wall (Figs. 2a-d). Both middle and innermost layers bent inwards along the penetration hypha (Figs. 2a, 2b). These phenomena suggest that physical pressure might be involved in the penetration. Fig. 2c demonstrates the apical region of a penetration hypha within the host cell wall. The edge of this hypha is irregularly shaped. The

electron density of the host cell wall around this hyphal tip has become higher than elsewhere. By contrast, the electron density of the host cell wall which contacts a penetration hypha is noticeably reduced in Fig. 2d. Similar areas of low electron density are also seen in Figs. 2a and 2b, as indicated by arrowheads. As reviewed by Cooper (1983), such alterations of electron density probably reflects the secretion of wall-degrading enzymes by the fungus when it breaches host cell walls. The cell wall retained its fibrous appearance but became in the area of high electron density rather amorphous in appearance in the area of low electron density. Thus, it is plausible that low electron density might represent an advanced stage of wall degradation. Matsumoto and Sato (1982) reported that *T. ishikariensis* and *T. incarnata* produced polygalacturonase (PG), which might be associated with softening of tissues in infected grasses, and that productivity of this enzyme was positively correlated with the degree of pathogenicity of each species. This study has focussed on wall alterations only at the fungal penetration sites, and further observation of cell wall degradation at later infection stages will be required to discuss the significance of wall degradation enzymes in symptom development.

The present observations show that when hyphae penetrate host cell walls, chemical and physical stimuli might be given to host walls. Similar observations were reported in the cases of infection of apple leaves by *Venturia inaequalis* (Cooke) Aderhold (Valsangiacomo and Gessler, 1988), barley leaves by *Erysiphe graminis* de Candolle (McKeen and Rimmer, 1973), and rice leaves by *Pyricularia oryzae* Cav. (Hashioka et al., 1968; Koga and Yoshino, 1991a, b). Fig. 3a illustrates penetration from an infection cushion produced on a stoma. A single hypha emerging from the infection cushion penetrates into a substomatal cavity through a stomatal opening. This hypha reduced its thickness when passing the stomatal opening and recovered it in the substomatal cavity. Another hypha emerging from the same infection cushion penetrates into a subsidiary cell of the stoma. This micrograph demonstrates that hyphae originating from the same infection cushion can invade host cells by either stomatal or cuticle penetration. Such a double penetration mode by a single infection cushion is very similar to that by *Pellicularia sasakii* (Shirai) Ito (Ishizaki et al., 1979) and *Rhizoctonia solani* Kühn (Smiley et al., 1992).

Hyphae which had invaded epidermal tissues seemed to continue growing and then to breach the lower epidermal cell walls towards adjacent mesophyll cells (Fig. 3b). They seemed to produce a thin penetration hypha in host cell walls and recover their thickness in adjacent cells. All organelles including chloroplasts often degenerated leaving only a cell wall of mesophyll cells in which infection hyphae had grown (Fig. 3c). Almost complete degeneration of chloroplasts in infected cells was probably associated with the chlorotic symptoms seen in the following spring.

Fig. 3d demonstrates hyphae composing an infection cushion formed on the surface of a leaf. The hyphal surface was covered with a thin layer composed of electron-dense granules, as indicated by the arrow. Armentrout and Downer (1987) showed a similar layer surrounding hyphae in an infection cushion of *Rhizoctonia solani*, and suggested that this layer might be composed of adhesive materials which are secreted by the hyphae and contribute to forming an infection cushion by holding hyphae together. Electron-dense granules similar to those in Fig. 3d were observed more prominently in hyphae constituting infection cushions than single hyphae. Thus, this granulous material probably has an identical, adhesive role to that of *R. solani*.

The present study revealed that *T. ishikariensis* penetrated into bentgrass leaves similarly as *T. incarnata* did into wheat leaves (Takenaka and Yoshino, 1987). Ultrastructure of the present host-fungus interface showed that the fungus might secrete wall-degrading enzymes when it penetrates host cell walls. Thus, one possible approach for developing biocontrol of snow mold disease caused by *T. ishikariensis* might be to search for antagonistic microorganisms which could effectively suppress secretion and/or activity of wall-degrading enzymes from the fungus.

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