

Fungal Penetration of the Cyst Wall of *Heterodera glycines*

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ABSTRACT

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Twelve fungal species were examined with the aid of light, scanning electron, and transmission electron microscopy to determine their capability to penetrate the cyst wall of *Heterodera glycines*. *Arthrobotrys dactyloides*, *Dictyochaeta heteroderae*, *Exophiala pisciphila*, *Fusarium oxysporum*, *Neocosmospora vasinfecta*, *Paecilomyces lilacinus*, *Pyrenochaeta terrestris*, *Stagonospora heteroderae*, *Verticillium chlamydosporium*, and a sterile fungus (black, yeastlike fungus) were observed to penetrate the cyst wall of *Heterodera glycines*. The light microscopy procedure reported herein was useful for determining the direction of

fungal penetration of the cyst wall. Also, the procedure allowed for the examination of many specimens. Nine of these fungi penetrated the cyst wall from inside the cyst. At least three species, *Exophiala pisciphila*, *Fusarium oxysporum*, and *Pyrenochaeta terrestris*, also penetrated the cyst wall from the outside. Penetration by the black, yeastlike fungus was limited to the outer layers of the cyst wall. No fungal penetration was observed on cysts exposed to *Gliocladium catenulatum* or *Hirsutella rhossiliensis* for up to 1 month.

Additional keywords: biological control, nematode fungal antagonist, soybean cyst nematode.

Many fungi have been isolated from cyst nematodes and some are parasites of females and eggs (4,16). The modes of infection of females and colonization of cysts by fungi are poorly understood. Tribe (20) speculated that routes of fungal invasion into cysts are generally through natural body openings. He stated that penetration through the hardened cuticle did not seem to occur. Kerry (10), however, suggested that fungi may invade cysts directly through the cyst wall, and a few reports show that fungi can penetrate the cyst wall (7,11,12). The cyst wall of *Heterodera schachtii* Schmidt was penetrated by *Verticillium lecanii* (Zimm.) Viégas 60 h after inoculation on glass slides (7). Kim et al. (12) reported that a sterile fungus, designated "ARF 18", could penetrate the cyst wall of *Heterodera glycines* Ichinohe. However, little evidence is available to determine whether these fungi penetrate the cyst wall from outside or inside the body cavity.

More than 40 fungal species have been isolated from females and cysts of *H. glycines* collected from a Florida soybean field (1,2). Some isolates have been tested for their pathogenicity to nematode eggs (3). The objective of this study was to examine fungi encountered at high frequencies for their capability of penetrating the cyst wall of *H. glycines*.

MATERIALS AND METHODS

Fungi. The following 12 species of fungi were examined: *Arthrobotrys dactyloides* Drechsler, *Dictyochaeta heteroderae* (Morgan-Jones) Carris & Glawe, *Exophiala pisciphila* McGinnis

& Ajello, *Fusarium oxysporum* Schlecht., *Gliocladium catenulatum* Gilman & Abbott, *Hirsutella rhossiliensis* Minter & Brady, *Neocosmospora vasinfecta* E. F. Smith, *Paecilomyces lilacinus* (Thom) Samson, *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker, & Larson, *Stagonospora heteroderae* Carris, Glawe, & Morgan-Jones, *Verticillium chlamydosporium* Goddard, and a sterile fungus (black, yeastlike fungus). *Verticillium chlamydosporium* was isolated from an egg mass of *Meloidogyne* sp. in a tobacco field near Gainesville, Florida. *Paecilomyces lilacinus* was obtained from P. Jatala at the International Potato Research Center, Peru. *Hirsutella rhossiliensis*, an endoparasite of vermiform nematodes, was obtained from B. A. Jaffee at the University of California, Davis. The remaining species were isolated from females or cysts of *H. glycines* collected from a Florida soybean field (1). The fungi were cultured on cornmeal agar (Difco Laboratories, Detroit) for 1 to 3 weeks before being inoculated to cysts contained on a glass microscope slide.

Nematodes. *H. glycines* were grown on a modified tissue culture (13). Second-stage juveniles (J2) that showed no signs of fungal growth after plating on potato-dextrose agar (PDA) (Difco Laboratories) were obtained by following the procedures described previously (2) and used as inoculum for the tissue culture. Soybean seeds were washed with water, treated with 70% ethanol for 1 min, washed with sterile water at least three times, treated with 0.001% HgCl₂ for 5 min, washed with sterile water three more times, and rinsed with sterile water for at least 10 min. The treated seeds were placed on PDA in petri dishes and incubated at room temperature (23 to 25°C) for 5 days. Root radices were aseptically excised about 3 cm from the root tip, and three root tips were transferred to each petri dish containing Gamborg's B-5 medium (GIBCO Laboratories, Grand Island, NY) at pH 5.7 to 5.8 (6). After 1 to 2 weeks, each dish received about 1-ml suspension of J2 (about 300 J2). The cultures were maintained in the dark at 28°C. Light brown cysts that formed on roots were inoculated with fungi.

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Inoculation of cysts with fungi. Thirty cysts were placed on a glass microscope slide. Spore and mycelium suspensions were made for each fungus that sporulated. About 1 ml of sterile water was added to each petri dish containing a fungal colony. Spores and mycelium were scraped and mixed with a knife to make a fungal suspension. The suspension was removed with a sterile Pasteur pipet and placed on the cysts. The slides with treated

TABLE 1. Summary of penetration of *Heterodera glycines* cyst wall by fungi

Fungal species	Penetration from inside to outside	Penetration from outside to inside	Time for penetration (day) ^a
<i>Arthrobotrys dactyloides</i>	Yes	ND ^b	4
<i>Dictyochoaeta heteroderae</i>	Yes	ND	7
<i>Exophiala pisciphila</i>	Yes	Yes	2
<i>Fusarium oxysporum</i>	Yes	Yes	1
<i>Gliocladium catenulatum</i>	No	No	
<i>Hirsutella rhossiliensis</i>	No	No	
<i>Neocosmospora vasinfecta</i>	Yes	ND	3
<i>Paecilomyces lilacinus</i>	Yes	ND	2
<i>Pyrenochaeta terrestris</i>	Yes	Yes	1
<i>Stagonospora heteroderae</i>	Yes	ND	2
<i>Verticillium chlamydosporium</i>	Yes	ND	3
Sterile fungus I (a black, yeastlike fungus)	ND	Limited in outer layers	4

^a The earliest time when penetration was observed after exposure of cysts to fungi.

^b ND = not detected.

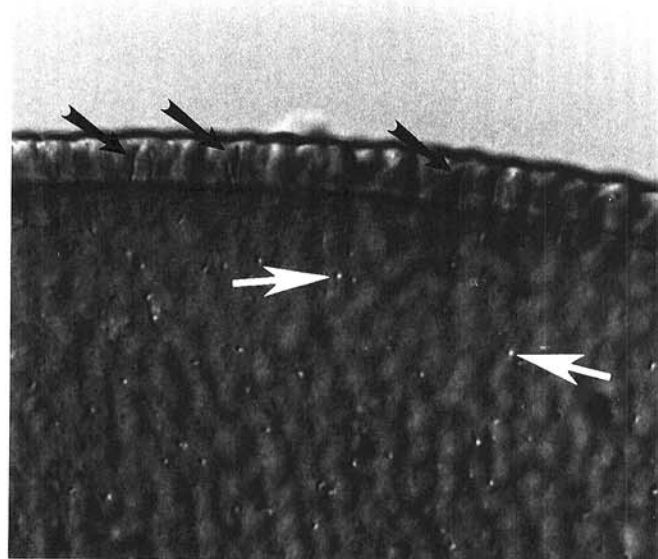


Fig. 2. Photomicrograph of the penetration of *Heterodera glycines* cyst wall by *Dictyochoaeta heteroderae* 7 days after inoculation. Black arrows indicate hyphae penetrating cyst wall from the cyst cavity toward the outside. White arrows indicate hyphal pegs or holes on the cyst wall resulting from fungal penetration ($\times 650$).

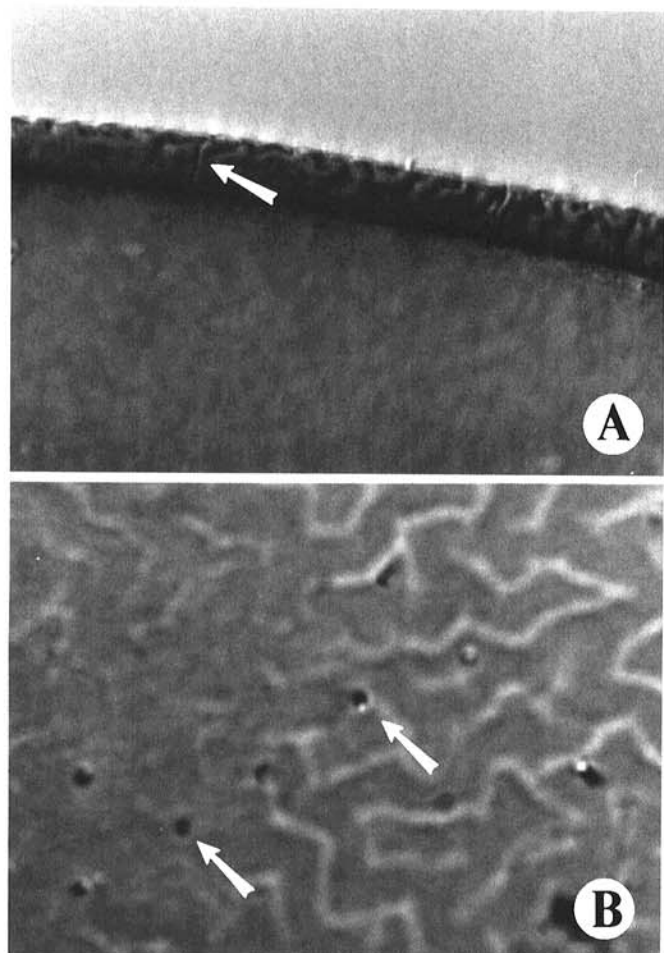


Fig. 1. Photomicrographs of the penetration of *Heterodera glycines* cyst wall by *Arthrobotrys dactyloides* 7 days after inoculation. A, Hyphae (arrow) penetrating the cyst wall ($\times 650$). B, Top view of hyphal pegs or holes (arrows) in the cyst wall resulting from fungal penetration ($\times 1,610$).

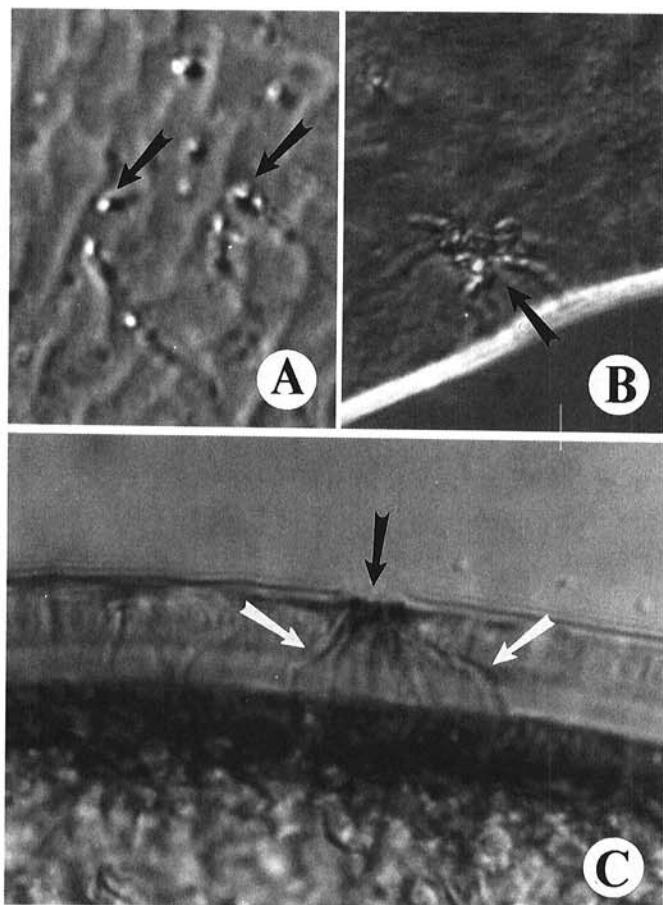


Fig. 3. Photomicrographs of the penetration of *Heterodera glycines* cyst wall by *Exophiala pisciphila*. A, Top view of hyphal pegs or holes (arrows) in the cyst wall resulting from fungal penetration, 3 days after inoculation ($\times 1,610$). B, A cluster of hyphae penetrating cyst wall 7 days after inoculation ($\times 650$). C, The fungus penetrated the cyst wall from the outside to enter the cyst cavity 7 days after inoculation. Fungal penetration started at a point (black arrow) on the surface of cyst wall, and formed several penetrating hyphae (white arrows) from this point ($\times 1,610$).

cysts were incubated in petri dishes containing a small amount of sterile water. Cysts were added directly to fungal colonies of the sterile species and *Arthrobotrys dactyloides*, which had few spores. The cysts were incubated at 24°C for 12 h to 1 month. A group of cysts without fungal treatment was included as a control.

Light microscopy observations. The cuticles of cysts were examined with a light microscope at 12 h, 1, 2, 3, 4, 7, 14, 21 days, and 1 month after inoculation. They were prepared for examination by modifying a technique previously used to prepare perineal patterns of root-knot nematodes (19). The cysts were transversely cut in half with a scalpel in water on a slide and the body contents pulled out. The cuticles were placed in a drop of 45% lactic acid on a slide and the body tissues that adhered to the

cuticle were removed by brushing the inner surface of the cuticle with a bristle made from bamboo. The cleaned cuticle was transferred to a drop of glycerin or lactophenol, folded inward, and covered with a coverslip. Fungal pegs or holes in the cuticle resulting from fungal penetration appeared as bright spots when observed at $\times 200$ or higher magnification with an Olympus light microscope (model Vanox; Olympus America Inc., Melville, NY). The direction of fungal penetration could be determined by observing the folded edge of the cuticle at $\times 400$ or higher magnification with the light microscope. By rolling the coverslip, the folded cuticle edge could be changed from one position to another. Most penetration sites could be brought into view at the folded edge, thus allowing determination of the direction of

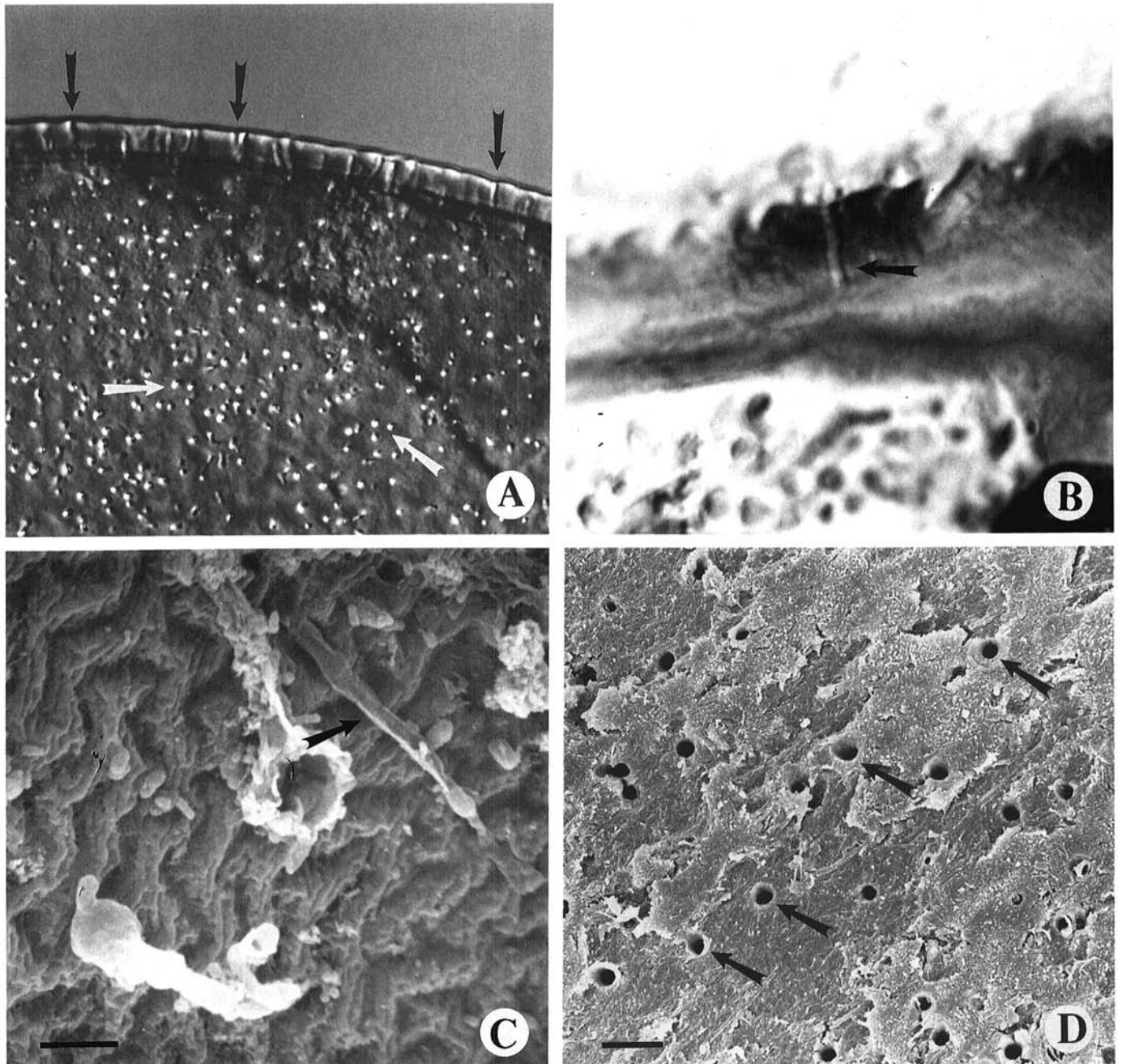


Fig. 4. Photomicrographs (A and B) and scanning electron micrographs (C and D) of the penetration of *Heterodera glycines* cyst wall by *Fusarium oxysporum* 7 days after inoculation. **A**, Black arrows show hyphae penetrating from cyst cavity toward the outside and white arrows indicate hyphal pegs or holes resulting from fungal penetration ($\times 650$). **B**, A hypha (arrow) penetrated the cyst wall from outside toward the cyst cavity ($\times 1,880$). **C**, Hyphae (arrow) growing from cyst cavity. Scale bar = 3 μm . **D**, Holes (arrows) in cyst wall resulting from fungal penetration. Scale bar = 3 μm .

penetration. Semithin sections (3 to 5 μm thick) obtained from samples prepared for transmission electron microscopy were also viewed. Sections were stained with 0.1% toluidine blue O in 1% sodium borate or with Nile blue A and mounted in immersion oil. Photographs were taken using Normarsky optics with the Olympus light microscope (Olympus America Inc.).

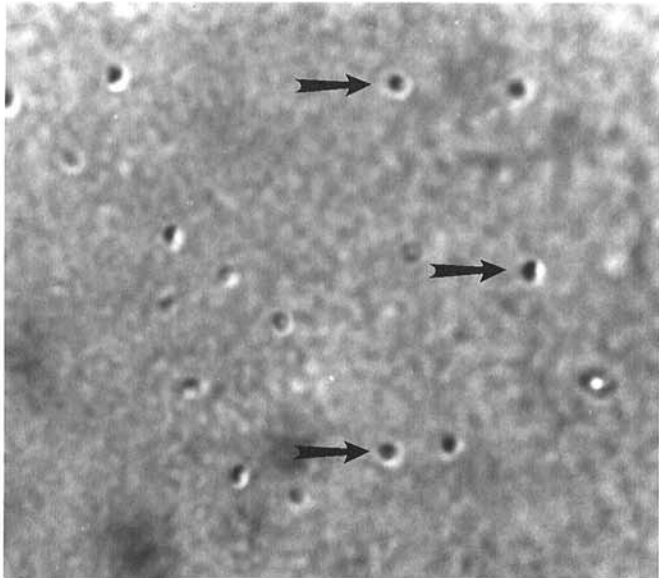


Fig. 5. Photomicrograph of the penetration of *Heterodera glycines* cyst wall by *Neocosmospora vasinfecta* 4 days after inoculation ($\times 1,880$). Arrows indicate hyphal pegs or holes resulting from the penetration by the fungus.

Scanning electron microscopy observations. The mycelium on the surface of the cyst cuticle was removed by carefully brushing with a bamboo bristle. The cysts were cleaned with ultrasound treatment in deionized water. Following the treatment, the cysts were fixed for 24 h in a modified Karnovsky's fixative (2% formaldehyde + 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2) (5). The fixed cysts were rinsed twice in phosphate buffer (pH 7.2) for 20 min each time and postfixed with 1% osmium tetroxide in the same buffer for 2 h. After two rinses of 10 min each in deionized water, the specimens were dehydrated through an ethanol series of 30, 50, 70, 80, 90, 100, 100, and 100%, 20 min each. The ethanol was replaced by one change of 1:1 of ethanol/amylic acetate, and two changes of 100% amylic acetate, 20 min each. The specimens were critical point dried, mounted on aluminum stubs, coated with gold and palladium, and were viewed with a Hitachi S-517 scanning electron microscope at 20 kV or Hitachi S-4000 scanning electron microscope at 10 kV (Hitachi Scientific Instruments, Mountain View, CA).

Transmission electron microscopy observations. Specimens examined with transmission electron microscopy included cysts inoculated with *Exophiala pisciphila*, *Fusarium oxysporum*, *Neocosmospora vasinfecta*, *Paecilomyces lilacinus*, *Pyrenochaeta terrestris*, *Stagonospora heteroderae*, *Verticillium chlamydosporium*, and the sterile fungus. The specimens were fixed in the modified Karnovsky's fixative for 1 h at room temperature, 2 h at 67°C, and cut in half with a small knife in the same fixative in a hood. The fixative was replaced with fresh fixative and incubated at 4°C for 22 h. The specimens were washed twice with the phosphate buffer for 20 min each, and postfixed with 1% osmium tetroxide for 3 h at room temperature in the same buffer. After rinsing once in buffer and once in deionized water for 10 min each, the specimens were dehydrated in a series of ethanol: 10, 25, 50, 75, 85, and 95% ethanol, 20 min each, and three changes

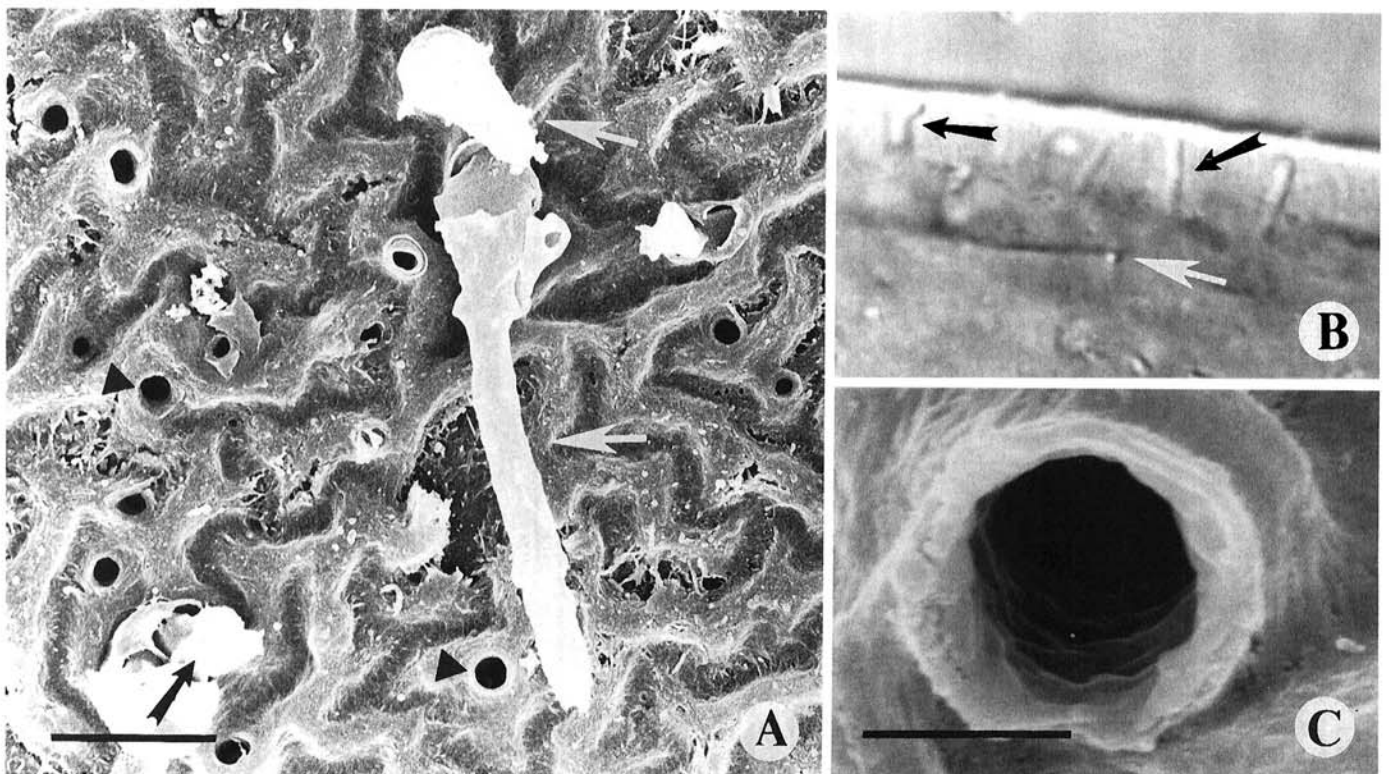


Fig. 6. Micrographs of the penetration of *Heterodera glycines* cyst wall by *Paecilomyces lilacinus* 7 days after inoculation. A, Scanning electron micrograph shows hyphae growing from the cyst wall (white arrows) or hyphal pegs protruding from the cyst wall (black arrow), or holes (arrow tips) resulting from fungal penetration. Scale bar = 5 μm . B, Photomicrograph shows hyphae (black arrows) penetrating the cyst wall from the cavity toward the outside and hyphal pegs or holes (white arrow) resulting from fungal penetration ($\times 1,610$). C, Hole caused by fungal penetration. Scale bar = 1 μm .

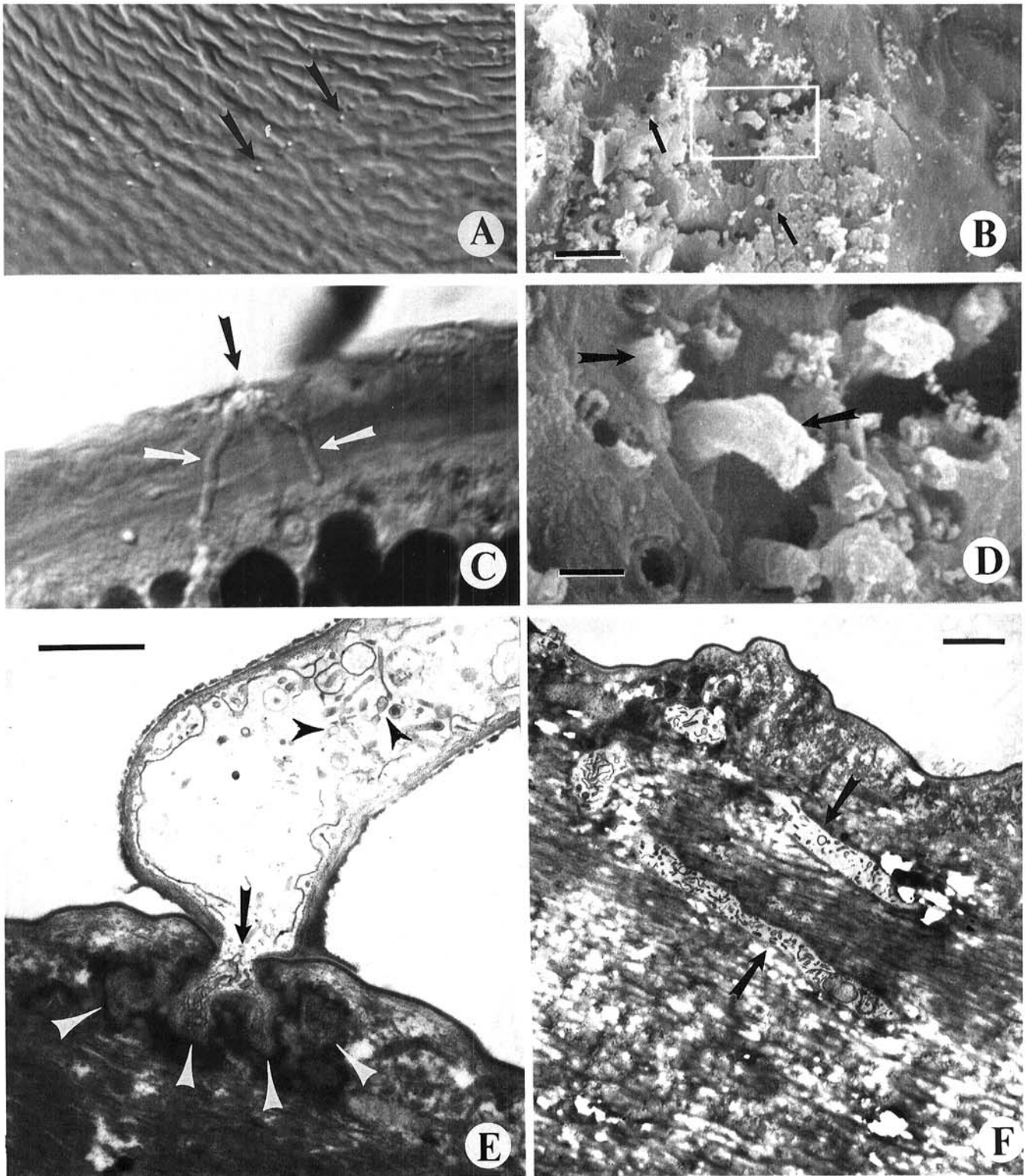
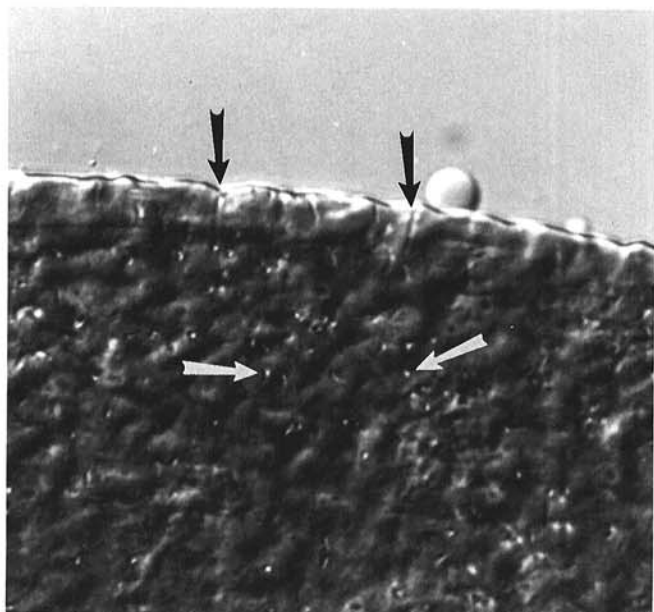


Fig. 7. Photo- (A and C), scanning electron (B and D), and transmission electron (E and F) micrographs of the penetration of *Heterodera glycines* cyst wall by *Pyrenochaeta terrestris* 7 days after inoculation. **A**, Top view of hyphal pegs or holes (arrows) resulting from fungal penetration ($\times 650$). **B**, Holes (arrows) caused by fungal penetration. Scale bar = 5 μm . **C**, Semithin section of a cyst exposed to the fungus. The fungus initiated penetration at a point (black arrow) from which penetration hyphae (white arrows) developed ($\times 1,880$). **D**, Higher magnification of the boxed area of Figure 7B showing hyphal pegs (arrows) protruding from the cyst wall. Scale bar = 1 μm . **E**, Hypha initiated penetration on surface of cyst wall at a point (black arrow) from which several branches of hyphae (white arrow tips) developed and moved toward the inside of the cyst wall. Organelles (black arrow tips) are distinguishable in the fungal cell. Scale bars = 1 μm . **F**, Arrows indicate two penetration hyphae with condensed organelles within the cyst wall. Scale bars = 1 μm .



of 100% ethanol, 30 min each. The ethanol was replaced by acetone with two changes for 20 to 30 min each. Spurr's standard resin (5) was prepared with the following modification, dimethylaminoethanol (DMAE) was replaced with 1:1 of DMAE/lecithin. The specimens were infiltrated through a series of the resin: 30% (in acetone) for 12 h, 70% for 12 h, 100% for 12 h, and 100% for 24 h. Following the infiltration, the resin was replaced with fresh 100% resin. The resin-infiltrated specimens and support resin medium were polymerized in an oven at 60°C for 48 h. Ultrathin sections (60 to 100 nm) were cut with a glass knife on a LKB 8000 Ultratome III ultramicrotome (LKB, Bromma, Sweden) or with a diamond knife on a Sorvall Porter-Blum MT2-B ultramicrotome (Ivan Sorvall Inc., Norwalk, CT). The sections were mounted on formvar film on 100-mesh copper grids and stained in 1% uranyl acetate for 15 min and in lead citrate for 5 min (5). The specimens were examined with a JEOL 100 CX transmission electron microscope (JEOL, Ltd., Tokyo) at 60 kV.

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Fig. 8. Photomicrograph of the penetration of *Heterodera glycines* cyst wall by *Stagonospora heteroderae* 3 weeks after inoculation. Black arrows show hyphae growing from the cyst cavity toward the outside. White arrows show top view of hyphal pegs or holes formed during fungal penetration (×1,880).

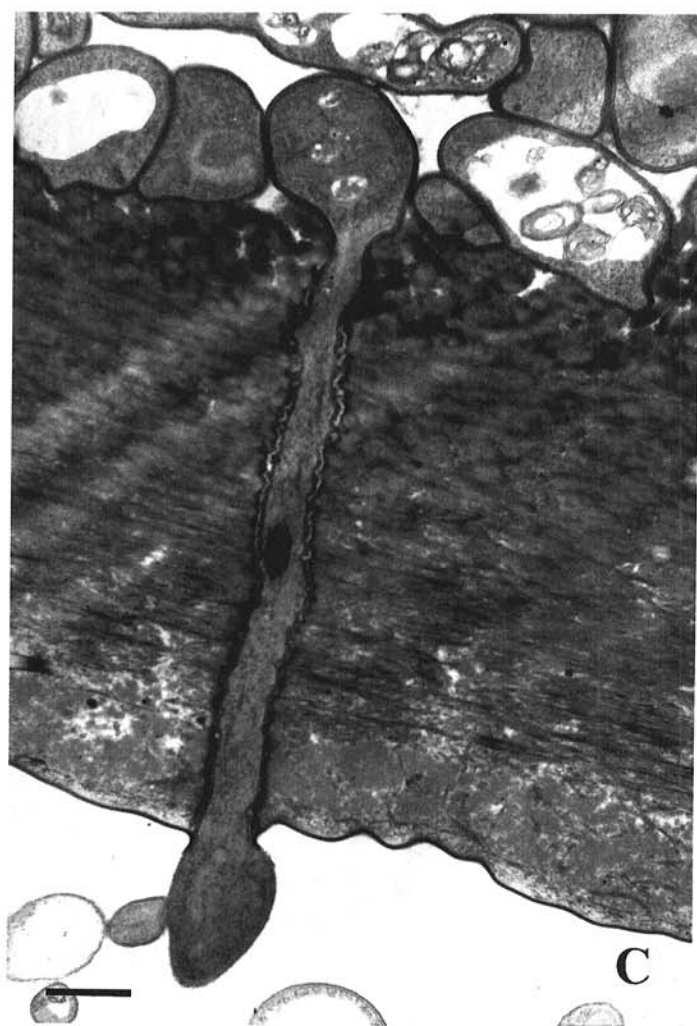
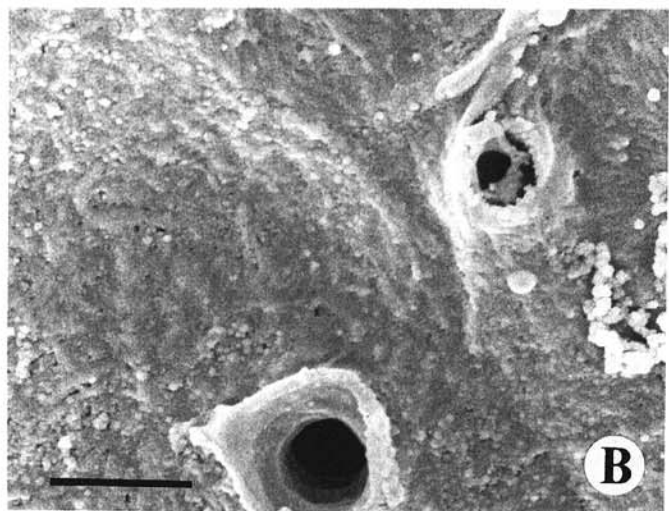
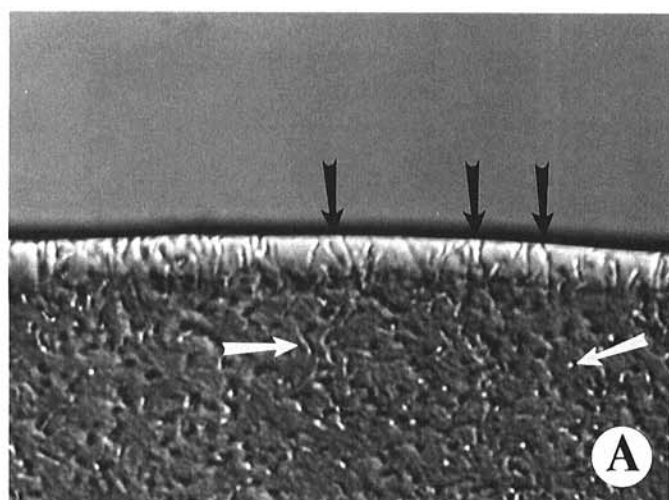


Fig. 9. Micrographs of the penetration of *Heterodera glycines* cyst wall by *Verticillium chlamyosporium*. **A**, Photomicrograph of the fungal penetration 3 weeks after inoculation. Black arrows indicate hyphae growing from the cyst cavity toward the outside. White arrows show top view of hyphal pegs or holes resulting from fungal penetration (×760). **B**, Scanning electron micrograph shows two holes resulting from fungal penetration 7 days after inoculation. Scale bar = 1 μm. **C**, Transmission electron micrograph shows the fungal penetration from the cyst cavity through the cyst wall 7 days after inoculation. Scale bar = 1 μm.

RESULTS

The fungal penetration of the *H. glycines* cyst wall is summarized in Table 1. Ten of 12 fungal species were found to penetrate the cyst wall (Table 1; Figs. 1 to 11). Penetration of the cyst wall from outside to inside, however, was observed only in four species, namely *Exophiala pisciphila* (Fig. 3C), *Fusarium oxysporum* (Fig. 4B), *Pyrenochaeta terrestris* (Fig. 7C and E), and the black, yeastlike fungus (Fig. 11A and B). After exposure of the cysts to fungi, the earliest time penetration was observed at 1 day for *Fusarium oxysporum* and *Pyrenochaeta terrestris*; 2 days for *Exophiala pisciphila*, *Paecilomyces lilacinus*, and *Stagonospora heteroderae*; 3 days for *Verticillium chlamydosporium*; 4 days for *Arthrobotrys dactyloides*; and 7 days for *Dictyoachaeta heteroderae*. No fungal penetration was observed on cysts exposed to *Gliocladium catenulatum* or *Hirsutiella rhossiliensis* for up to 1 month. The cyst wall without fungal penetration is illustrated in Figure 12.

Generally, penetration could be completed by a single hypha. After mycelium on the surface of the cyst wall was removed, some penetration hyphal pegs either remained on the cuticle or were removed along with the mycelium, leaving circular holes in the wall (Figs. 4C and D, 6A and C, 7B and D, and 9B). The penetration hyphal pegs within the cyst wall were generally less than 1 μm (usually about 0.5 μm) in diameter, which is much less than the diameter of a regular hypha. Numerous organelles were observed in penetration hyphae of *Pyrenochaeta terrestris* (Fig. 7E and F). When penetration of the cyst wall was initiated from outside to inside, hypha was observed attached to the cyst wall and appeared to dissolve a hole through the wall. The contents of the fungal cell with condensed organelles extended into the cyst wall, from which several branches of hyphae may have developed. At this stage, the wall of the penetrating hypha was not obvious. The penetrating hyphae of *Verticillium chlamydosporium* in the cyst wall, however, appeared to have cell walls (Fig. 9C).

The black, yeastlike fungus exhibited a distinctly different mechanism of interacting with the cyst wall than other fungi. When the fungus contacted the surface of the cyst wall, hyphal cells enlarged (Fig. 10A and B) and were tightly attached to the

cyst wall (Figs. 10B and 11A). The fungus penetrated through cuticle layers A1, A2, and A3 (18) and proliferated between layers A3 and C (Fig. 11B, C, and D). The fungal cells enlarged and were tightly compacted in the cuticle so that they appeared to form a pseudoparenchymatous structure. The fungus, however, was not observed to penetrate the cyst wall.

DISCUSSION

Most fungi penetrated the cyst wall, and at least three species, *Exophiala pisciphila*, *Fusarium oxysporum*, and *Pyrenochaeta terrestris*, penetrated the cyst wall from the outside. This suggested that direct fungal penetration of cyst walls may be common among nematophagous fungi. Although penetration of the cyst wall from outside to inside was not observed by most of the fungi, additional studies are needed to verify different modes of penetration.

The ability to penetrate the cyst wall from the outside may not be important for a fungus to colonize a cyst, because fungi can probably enter cysts readily through natural body openings (10,14). Fungi were usually found inside cyst cavities within 1 day after inoculation. This occurred before there was any evidence that the cyst wall had been penetrated. Whether these fungi have the same ability to enter young females or cysts through natural body openings is yet to be determined. If the natural body openings of the young females and cysts repel penetration, the ability of cuticle penetration may be an important factor in the infection process. If a fungus can invade young females and cysts, the fungus may have a greater opportunity to destroy eggs within the cysts, because eggs in the early developmental stage are more vulnerable to fungal infection (8).

Hyphae were often found to penetrate the cyst wall from within the cyst cavity to the outside. In contrast, fewer hyphae were found to penetrate the cyst wall from the outside to inside the cyst cavity. The reason for this is not clear. It may be that the nutrient contents within the cysts provide for a rapid increase of fungal biomass, which facilitates fungal penetration of the cyst wall from inside the cyst cavity. An alternative possibility is that the chemical interaction between fungi and inner layers of the cyst wall

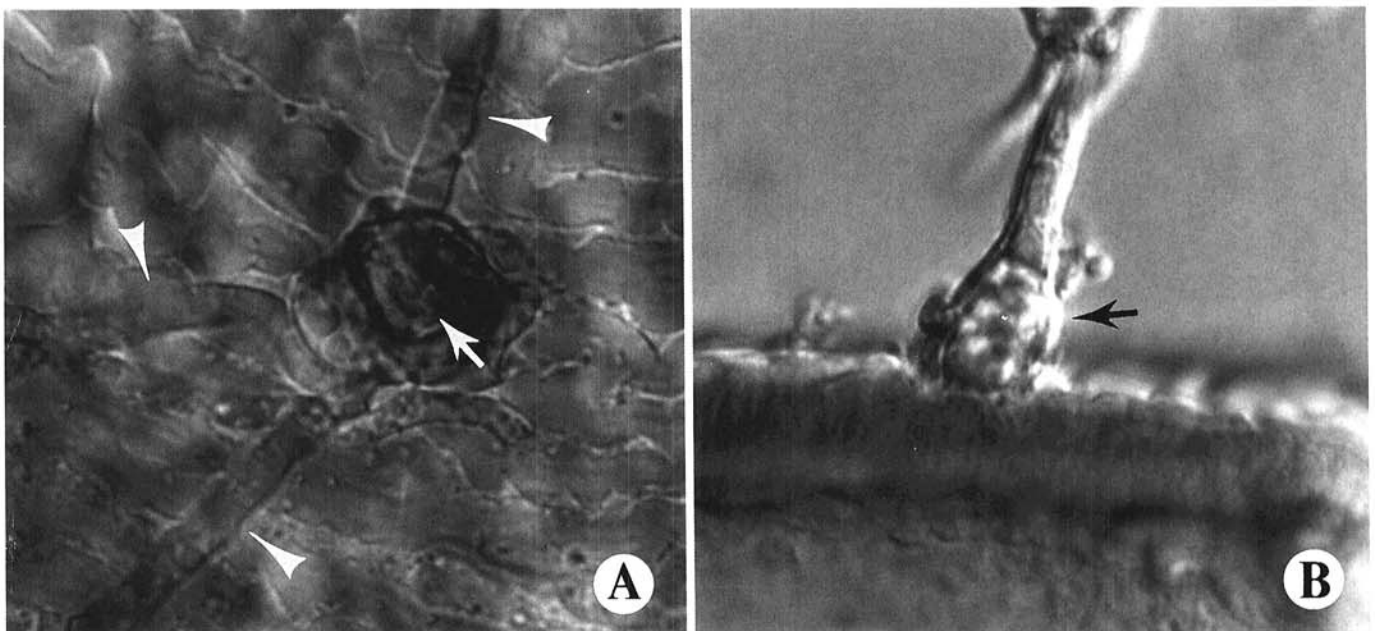


Fig. 10. Photomicrographs of the penetration of *Heterodera glycines* cyst wall by a sterile fungus (black, yeastlike fungus) 4 days (A) or 7 days (B) after inoculation. A, Top view of hyphae (white arrow tips) with swollen cells (white arrow) that may have attached to the cyst wall ($\times 1,880$). B, Lateral view of a swollen fungal cell (arrow) attached to the cyst wall ($\times 1,880$).

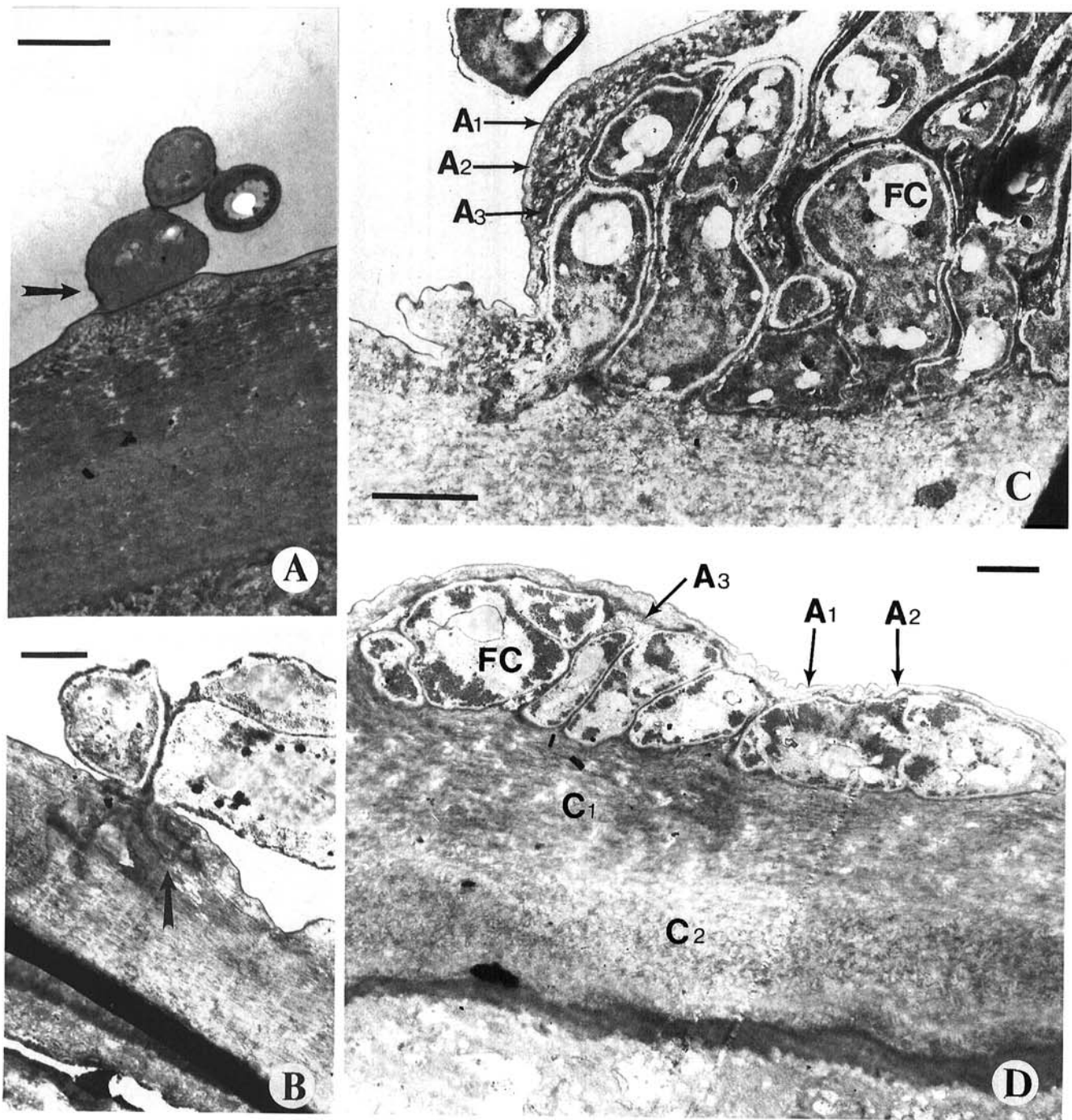


Fig. 11. Transmission electron micrographs of the penetration of *Heterodera glycines* cyst wall by a sterile fungus (black, yeastlike fungus) 7 days after inoculation. Scale bars = 2 μ m. A, A hypha (arrow) attached to the cyst wall. B, Hyphae initiated penetration (arrow) of cyst wall. C and D, Compacted pseudoparenchyma-like cells within the cyst wall. A₁, A₂, A₃, C₁, and C₂ represent layers of the cuticle (18). FC = fungal cells.

differs from the interaction between fungi and outer layers of the cyst wall.

The mechanisms involved in penetration of the nematode cuticle are not yet clear (9), although both enzymatic (17) and mechanical (9,15,21,22) mechanisms are thought to be involved. In the present study, penetration hyphae of *Pyrenochaeta terrestris* observed within the cyst wall were rich in organelles (Fig. 7F). This may suggest involvement of enzymatic activity. The penetration of the cyst wall by the black, yeastlike fungus was confined between layers A and C (Fig. 11C and D). It is possible that this fungus has some enzymes that can dissolve layer A, but not layer C, of the nematode cuticle. Since the collagen-like protein is a main component in layer C, but not in layer A (18), the fungus

may lack collagenase. Further study is needed, however, to confirm this possibility. Diameters of penetration pegs were usually less than those of regular hyphae. This may be a mechanism that evolved for saving energy during penetration. The small size of penetration pegs may facilitate mechanical penetration if this mechanism is involved.

Electron microscopy revealed some details of the fungal penetration process into cyst walls. Scanning electron microscopy, however, provided little information for detecting fungal penetration from the outside to inside. Although transmission electron microscopy provided clearer details of the fungal penetration process than either light microscopy or scanning electron microscopy, it required a great deal of time for sample preparations. The

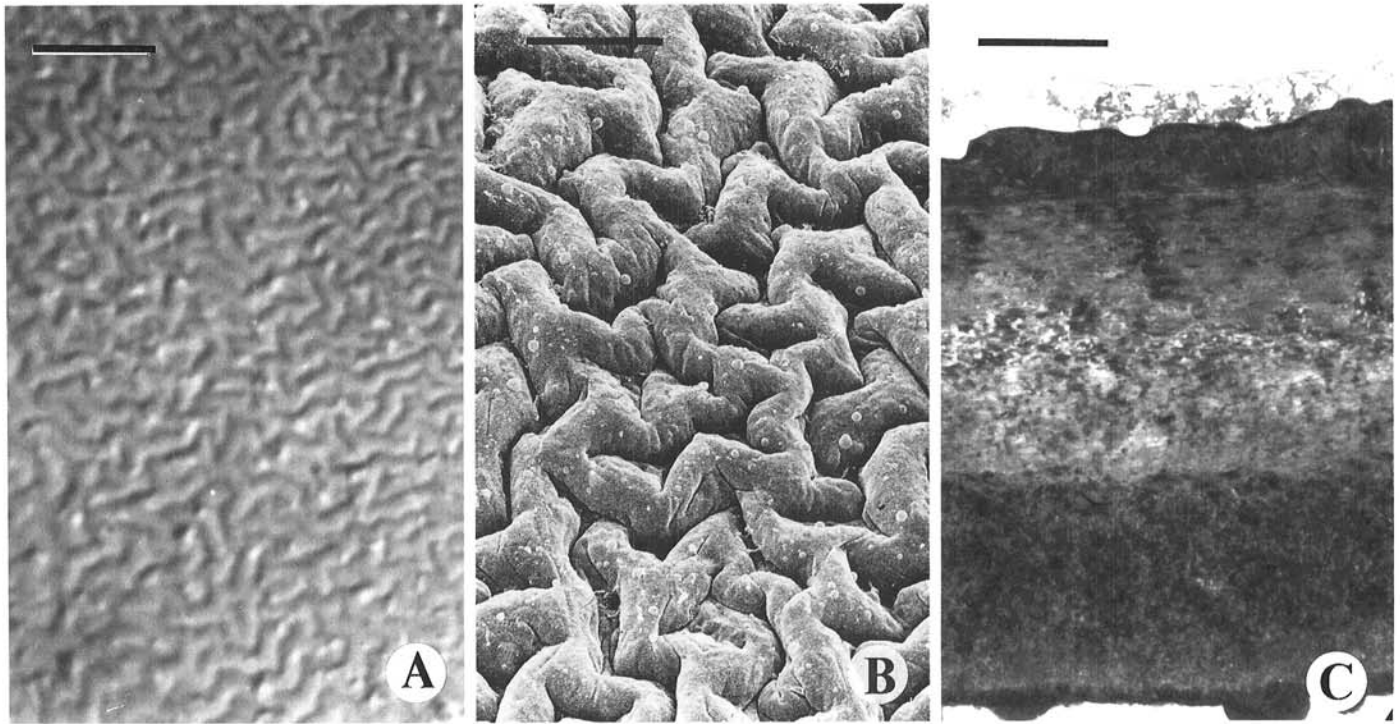


Fig. 12. Micrographs of *Heterodera glycines* cyst cuticle without mycelium on surface or fungal penetration. A, Photomicrograph of the surface of cuticle ($\times 760$). B, Scanning electron micrograph shows surface of cuticle. Scale bar = 5 μm . C, Transmission electron micrograph shows section of cuticle. Scale bar = 2 μm .

penetration of the cyst wall by fungi could be examined most easily by using the light microscopy procedure developed in this study. This procedure is simple and can be used to examine many specimens. Care must be taken, however, to distinguish granules or bubbles on the surface of the cuticle from fungal hyphae or penetrating holes.

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