Ultrastructure of *Heterodera glycines* Parasitized by Arkansas Fungus 18

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ABSTRACT

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Ultrastructure of *Heterodera glycines* cysts, eggs, and juveniles parasitized by Arkansas fungus 18 (ARF18) was studied with transmission and scanning electron microscopy. Transmission electron microscopy revealed that parasitized cysts, eggs, and first-stage juveniles in eggs were filled with ARF18 fungal hyphae. Numerous pores, apparently caused by fungal penetration, were found below the sclerotiumlike structures on the cyst cuticles. The cyst cuticles at the site of the sclerotiumlike structures appeared to have been dissolved. These results suggest that ARF18 penetrates the cyst cuticle enzymatically. The surface of diseased eggs was heavily furrowed. Hyphae of ARF18 have electron-transparent septa that contain simple pores associated with Woronin bodies. These characteristics suggest that ARF18 is an ascomycete fungus. This is the first descriptive report of direct penetration of a nematode cyst wall by a fungal biological control agent.

Additional keywords: biological control, SEM, soybean-cyst nematode, TEM.

Plant pathogenic fungi penetrate hosts directly (enzymatically or physically) or through natural openings (1,7). Most fungi that parasitize eggs of cyst nematodes enter the cysts through natural openings such as the oral aperture or the vulva (13). Nematode egg shells, however, do not have natural openings, and therefore, parasitic fungi must penetrate directly, either by enzymatic digestion, physical penetration, or both.

Arkansas fungus 18 (ARF18) was first isolated from eggs of the soybean-cyst nematode (SCN), *Heterodera glycines* Ichinohe, collected from field soil in Arkansas in 1986. Subsequent studies showed that this fungus can be a potential biological control agent for several important nematode pests including *H. glycines*, *H. schachtii* Schmidt, and *Meloidogyne incognita* (Kofoid &

White) Chitwood (4,6). The fungus can parasitize eggs in cysts and immature females of SCN (4,5,6). However, the fungus has not been identified taxonomically, because it does not sporulate. The mode of parasitism of SCN eggs and cysts has not been determined.

This paper presents ultrastructural evidence of parasitism of eggs and juveniles in cysts and females by ARF18 and clues to the possible identity of the fungus.

MATERIALS AND METHODS

Healthy and parasitized cysts of SCN were obtained from an artificially inoculated greenhouse culture. Soybean (*Glycine max* [L.] Merr. 'Lee') was planted in soil inoculated with both SCN eggs (15 eggs per gram of soil) and ARF18 or SCN alone. An isolate of ARF18 isolated from eggs of SCN at Pine Tree Station, Colt, AR, and maintained in half-strength cornmeal agar (4,6) was used in these studies. After 30 days, females were extracted by sieving and sugar flotation (11). For transmission electron microscopy (TEM), both healthy and parasitized cysts were fixed in a modified Karnovsky fixative consisting of 2% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.2. Fixed cysts were stored in a refrigerator at 5 C until processed further. Cysts that had been in the fixative for at least 24 h were rinsed twice (20 min each) in cacodylate buffer, and postfixed in 1% osmium tetroxide in the same buffer for 2 h. After two rinses of 1–2 min each in deionized water, the nematodes were prestained overnight in aqueous 0.5% uranyl acetate in a refrigerator and dehydrated through an ethanol series of 30, 50, 70, 80, 90, and 100% (three changes of 20 min each) and propylene oxide (two changes of 20 min each). The cysts were infiltrated under vacuum through a series consisting of 50% propylene oxide, 50% Spurr's medium for 24 h; 25:75 for 24 h; and three changes of 100% Spurr's medium each for a period of 24 h (12). Cyst samples were polymerized by incubation for 24 h in an oven at 70 C. Sections were cut 60–100 nm thick with a Sorvall MT-2 ultra microtome with a glass knife, mounted on uncoated 300-mesh copper grids, and double-stained in 2% uranyl acetate and lead citrate. The sections were examined with a JEOL-100 CX transmission electron microscope at 80 KV.

For scanning electron microscopy (SEM), nematode samples were processed as for TEM until dehydration. After dehydration, the nematode samples were critical point dried, mounted on aluminum stubs, coated with gold and palladium, and viewed with an ISI-60 scanning electron microscope at 20 KV. For the SEM pictures of healthy and diseased eggs, the cysts were broken with forceps after critical point drying, and separated eggs on stubs were coated with gold and palladium.

To elucidate the possible roles of the sclerotiumlike structures, the structures were separated from the cyst surface, and the area under them was observed with SEM. Cysts with the sclerotiumlike structures were placed in a 0.5% sodium hypochlorite solution for 1–3 min, and the sclerotiumlike structures were carefully separated from them without breaking the cyst wall.

RESULTS

SEM of whole mounts of *H. glycines* cysts revealed the occurrence of the sclerotiumlike structures on several areas of the cyst surface (Fig. 1A) that appeared to be attached tightly to the cyst wall. In some cases, the sclerotiumlike structures were so tightly attached that treatment with sodium hypochlorite was necessary to separate them from the cyst without tearing or breaking the cyst wall. Hyphae and the sclerotiumlike structures always occurred together. Scattered hyphae around the sclerotiumlike structures appeared to have originated from the sclerotiumlike structures (Fig. 1B).

After the removal of a sclerotiumlike structure from a cyst, the surface of the cyst wall revealed two types of topographic morphology; the surface was somewhat sunken (Fig. 2A,B) and perforated with numerous circular pores approximately the same diameter as the fungal hyphae. Many pores were plugged with a single hypha (Fig. 2B). Alternatively, the cyst surface beneath the sclerotiumlike structure was opened into a large single hole through the cyst wall (Fig. 2C,D). Tightly woven masses of fungal hyphae were inside the cyst (Fig. 2C,D).

TEM revealed that fungal hyphae were also found inside cysts (Fig. 3). In some infected cysts, the entire cavity of the cyst was filled with tightly packed hyphae. Elongated hyphae embedded in the thick cyst wall were oriented in a way that suggested that they were in the process of direct penetration. The structure of the cyst wall surrounding the embedded hyphae appeared intact. The diameter of the hyphae embedded in the wall was much smaller than that of hyphae inside the cyst cavity, which suggested that embedded hyphae may have been penetrating pegs.

Eggs parasitized by ARF18 exhibited a distinct change in surface appearance. The surface of healthy eggs was taut and smooth, whereas the surface of diseased eggs was severely shrunken, unevenly sunken, and/or furrowed (6). Some sunken areas had pores apparently caused by fungal penetration. At the site of fungal penetration, the fungal wall and egg shell were attached and not easily distinguished under TEM (Fig. 4A,B). Diseased eggs often were filled with tightly packed hyphae. Hyphae have a simple septum with a central pore, and the walls taper gradually and evenly toward the pore, forming a wedge in cross section; Woronin bodies occur adjacent to it (Fig. 4A,B). Woronin bodies

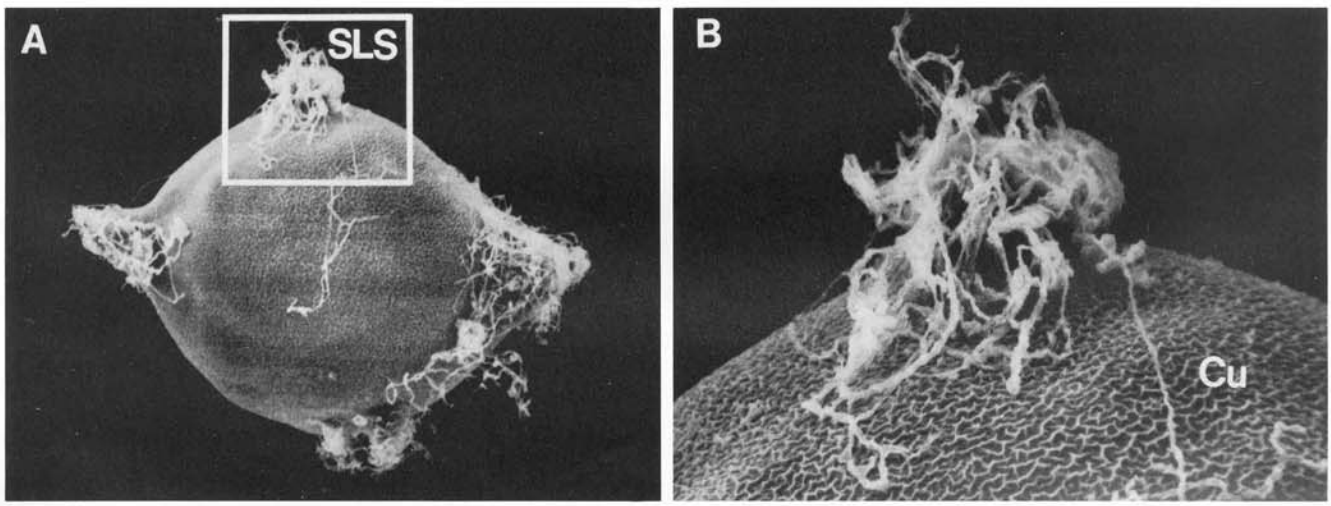

Fig. 1. Scanning electron micrographs of the cyst of *Heterodera glycines* parasitized by Arkansas fungus 18. **A,** a whole cyst with several sclerotiumlike structures (SLS) attached (×120). **B,** higher magnification of the boxed area of Figure 1A shows details of the cyst cuticle (Cu) and the sclerotiumlike structure (×400).

are surrounded by double-unit membrane.

First-stage juveniles in eggs also were infected by ARF18 (Fig. 5). The cuticles of infected juveniles were wrinkled, and individual cuticular layers and the hypodermis were hardly discernible. Contents of some juveniles were largely replaced by tightly packed fungal hyphae, therefore, internal structures of the nematode were often not discernible (Fig. 5).

DISCUSSION

These observations clearly demonstrated that hyphae of ARF18 penetrate directly through the cyst wall. Whether penetration was accomplished by physical force or enzymatic degradation is unclear. However, the cyst wall at the site of penetration appeared to have been enzymatically dissolved rather than mechanically

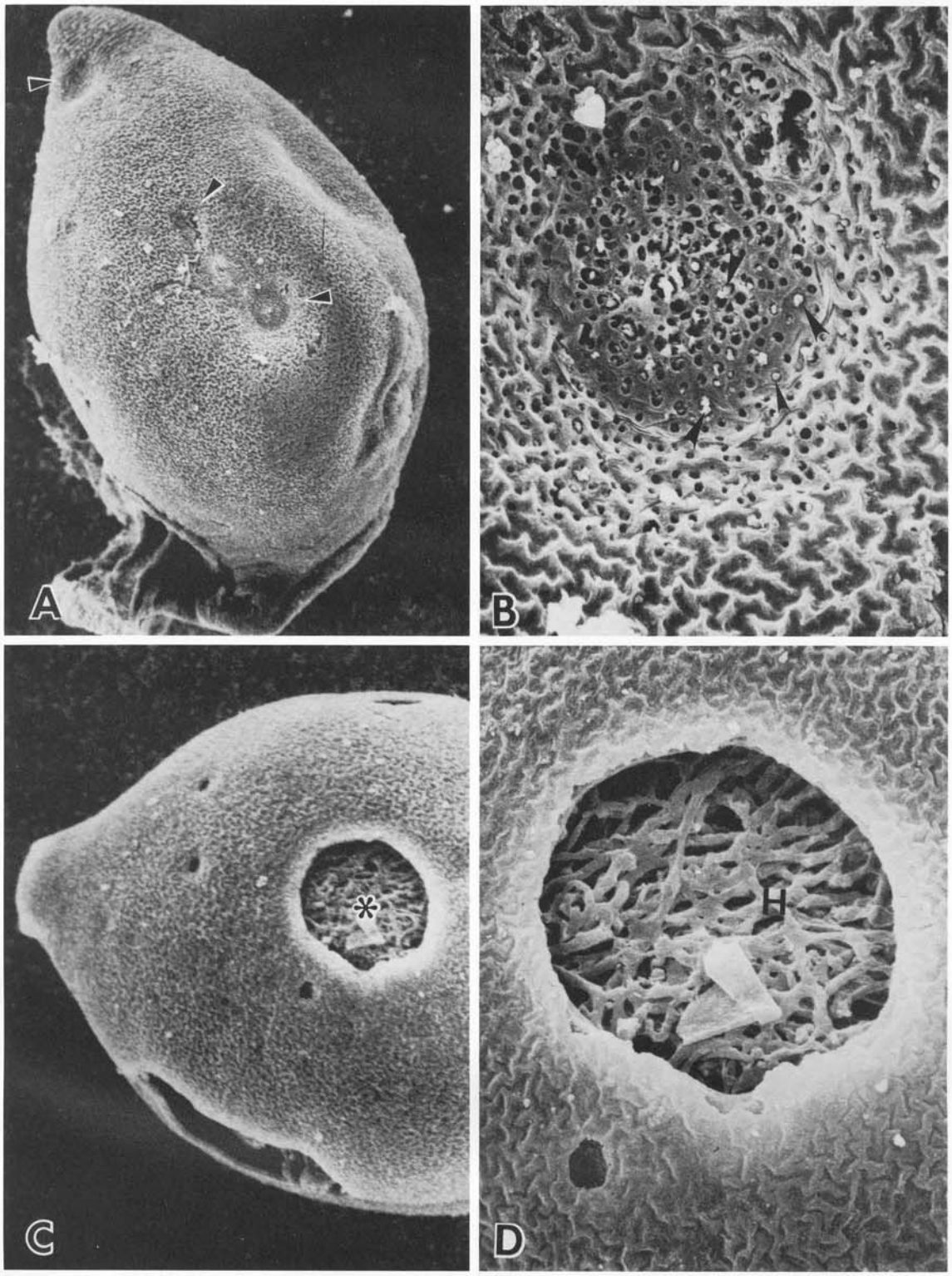

Fig. 2. Scanning electron micrographs of the cyst cuticle beneath the sclerotiumlike structure after removal of the sclerotiumlike structure. **A,** sunken areas (arrow) on cyst cuticle after the removal of the sclerotiumlike structure (×230). **B,** higher magnification of a sunken area of the cyst wall and the sclerotiumlike structure interface. The entire sunken area is perforated with a great number of pores, some of which are plugged with fungal hyphae (arrows) (×1,700). **C,** a cyst wall with a large hole (*) after the removal of the sclerotiumlike structure (×340). **D,** higher magnification of the hole in Figure 2C, exhibiting tightly packed fungal hyphae (H) inside the hole (×950).

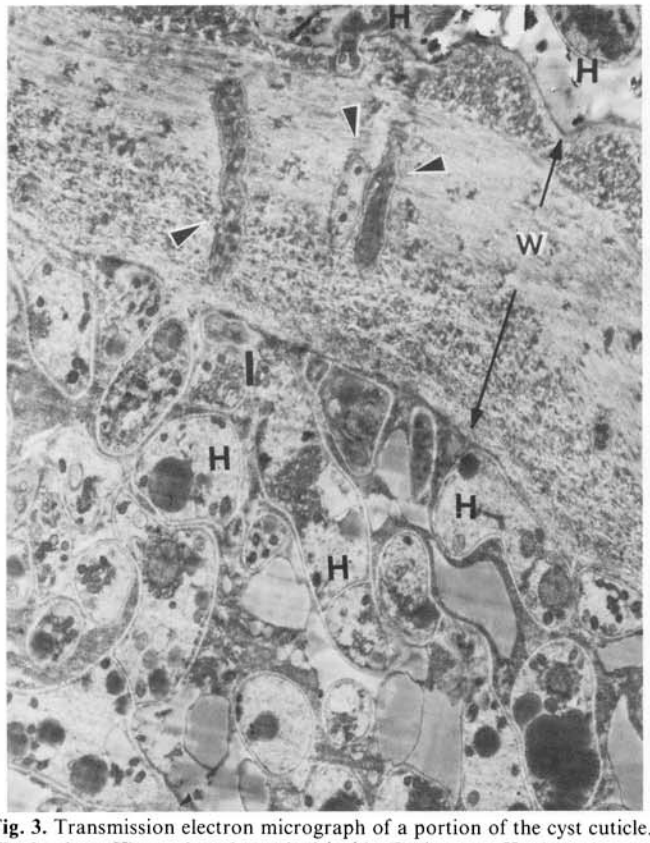

Fig. 3. Transmission electron micrograph of a portion of the cyst cuticle. The hyphae (H) are densely packed inside (I) the cyst. Hyphae also are shown on the surface of the cyst. Three elongated hyphae (arrows) are embedded in the thick cyst wall (W) (×4,500).

pierced. If physical force was the main means of penetration, structural artifacts, such as minor cracks between the pores, indentation, and/or uneven orifices of the pores, would be expected. No such artifacts were observed. Furthermore, the interface between the fungal wall and the egg shell at the penetration site was so tightly and smoothly adhered that distinguishing between the two required close scrutiny. These observations suggest enzymatic penetration.

Whether the fungus penetrated living female cuticle or dead cyst wall seems not important because both have the same structure and composition (10). The cyst wall may be harder than the cuticle because the cyst wall contains polyphenolic materials that stabilize the collagen so that cysts persist for many years in soil (10). However, observations of parasitized nematodes in different stages, eggs, second-stage juveniles, young females, mature females, and brown cysts, containing fully developed eggs suggested that the fungus is able to penetrate both living female cuticle and dead cyst wall (D. G. Kim, *unpublished*).

In plant-pathogenic fungi, the nature of fungal penetration of the plant cuticle (enzymatic versus physical) has been a subject of controversy for many years. Ultrastructural studies and demonstration of the presence of esterase and cutinase at the site of penetration have led to the conclusion that both mechanisms may be involved (7). Weakening of the cuticle by hydrolases in an early phase of penetration would facilitate growth of the infection peg through the cuticle. Thus, neither mechanism of penetrating the cyst wall and egg shell by ARF18 can be ruled out.

The two types of damage to the cyst wall beneath the sclerotium-like structures, one with many small pores in a sunken area and one with a single large hole, probably represent different stages of infection, an early and an advanced stage, respectively. The small pores in the early stage of infection may gradually coalesce (possibly by an enzymatic action) into a large hole as infection progresses.

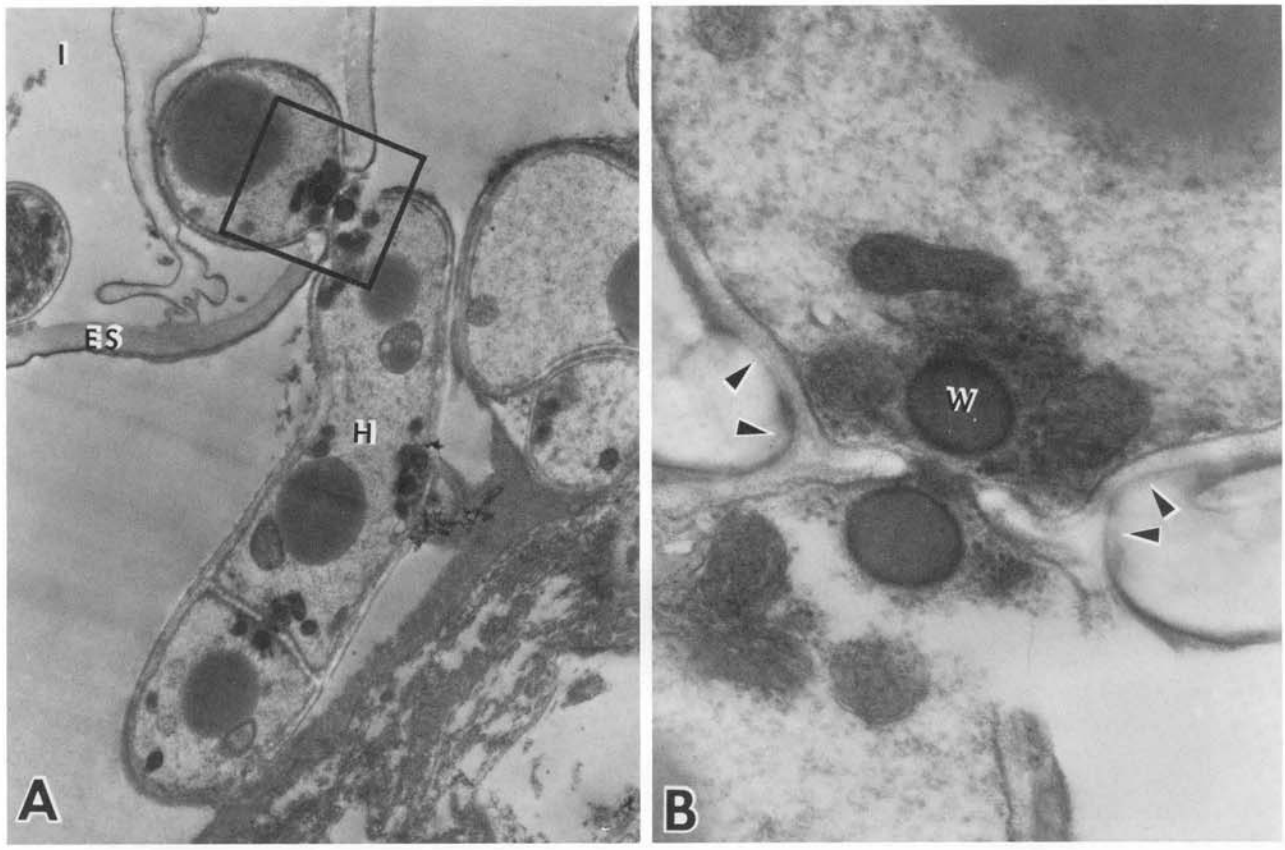

Fig. 4. Transmission electron micrograph of a portion of an egg shell penetrated by Arkansas fungus 18 (ARF18). **A,** ARF18 fungal hypha (H) has penetrated inside (I) the egg shell (ES) through a pore (×9,000). **B,** higher magnification of the boxed area in Figure 4A shows the details of the penetration site. The electron-transparent septum has a simple pore associated with Woronin bodies (W). The fungal wall and egg shell adhere tightly (arrows) (×46,000).

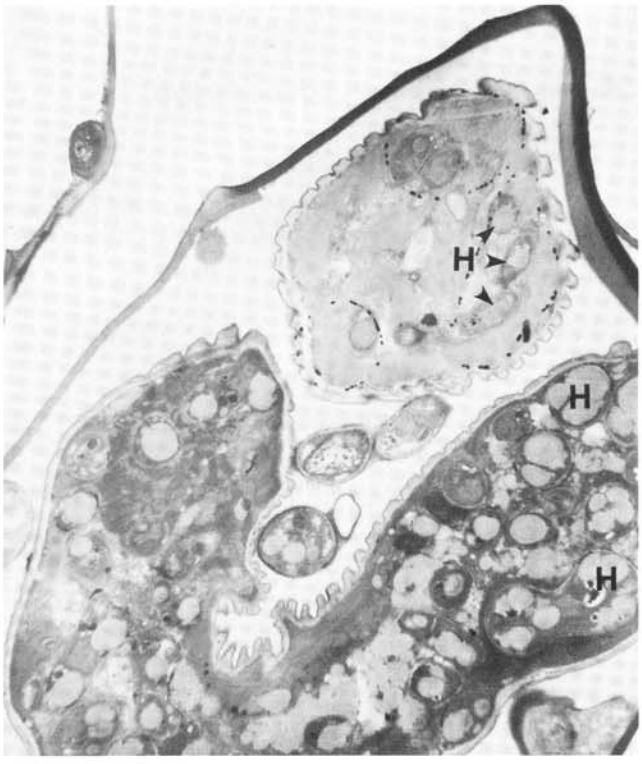

Fig. 5. Transmission electron micrograph of parasitized first-stage juvenile in egg showing numerous hyphae (H) inside (×2,000).

Most egg pathogens appear to enter the cyst through one of the natural openings, but spores of *Verticillium chlamydosporium* penetrate the cyst wall of *H. avenae* (13). Furthermore, zoospores of *Catenaria auxiliaris* and *Nematophthora gynophila* may invade the cysts directly through the cyst wall, even though the mycelium of neither fungus can penetrate the egg shell within the cysts (13). The cyst wall is the primary protective barrier for the eggs and juveniles. The ability of ARF18 to penetrate directly the cyst wall and egg shell add to the effectiveness of this fungus as a parasite of cyst nematode species.

Appressoria or similar structures are reported for many fungal pathogens of plants (1) and insects (14) but were first observed on the cyst nematode parasitized by ARF18 in these studies. The large number of penetration holes that were occupied by hyphae strongly suggests that the sclerotiumlike structures may function as an appressorium.

Cysts and eggs parasitized with ARF18 produce better structural detail for TEM studies than nonparasitized cysts and eggs. In fact, no clear TEM micrographs of intact cysts or eggs were obtained. This apparently is due to the poor penetration of the fixative, which causes inadequate tissue fixation for TEM. A poorly fixed biological specimen is not readily infiltrated and polymerized with the epoxy embedding medium, and sectioning and staining are very difficult (3). The cysts and eggs parasitized by ARF18, however, have pores made by the fungal penetration that provide an excellent pathway for fixatives and result in adequate fixation. Similar observations were made in ultrastructural studies on parasitism of *Meloidogyne arenaria* eggs by parasitic fungi (8,9).

The wedge-shaped electron-transparent septa that have simple pores associated with Woronin bodies have been reported only from the Ascomycotina (2,15). This indicates that ARF18 is an ascomycetous fungus. Pores unplugged by Woronin bodies at the site of penetration indicate that this is an active hypha.

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