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Colonization of Soybean Cyst Nematode Females, Cysts, and Gelatinous Matrices by the Fungus *Verticillium lecanii*

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Abstract: *Heterodera glycines* was grown in monoxenic culture on soybean roots and then inoculated with the antagonistic fungus *Verticillium lecanii*. Use of root explant cultures allowed evaluation of the fungus-nematode interaction with the nematode attached to roots or removed from the host, and avoided contamination with other fungi. From 16 hours to 14 days following inoculation, female and cyst samples were examined with the light microscope, or prepared for either conventional or low-temperature scanning electron microscopy. Within 16 hours, hyphae had begun colonizing the gelatinous matrices (GM). The fungus proliferated in the GM of some specimens within a week, but was rarely seen in unhatched eggs. Fungus penetration holes in female and cyst walls were observed 3 days after inoculation; penetration through nematode orifices was not seen at that time. More cysts than females were colonized at the earliest sampling dates. Specimens associated with external hyphae exhibited variable internal colonization, ranging from no fungal penetration to extensive mycelial growth.

Key words: biocontrol, colonization, fungus, *Heterodera glycines*, light microscopy, nematode, scanning electron microscopy, soybean, *Verticillium lecanii*.

The fungus *Verticillium lecanii* (A. Zimmermann) Viégas is known to have a broad host range, with strains that colonize insects and fungi (Harper and Huang, 1986; Heintz and Blaich, 1990; Hussey, 1984; Uma and Taylor, 1987). This species also has been isolated from *Heterodera glycines* Ichinohe (Gintis et al., 1983) and colonizes eggs, cysts, and(or) females of *H. glycines*, *Heterodera schachtii* Schmidt, and *Globodera pallida* (Stone) Behrens (Hänssler, 1990; Hänssler and Hermanns, 1981; Meyer et al., 1990; Uziel and Sikora, 1992). Consequently, *V. lecanii* has been studied as a potential management agent for *H. glycines* (soybean cyst nematode), which is a leading cause of soybean yield losses (Wrather et al., 1997). Strains of *V. lecanii* reduced *H. glycines* population densities in greenhouse studies (Meyer and Huettel, 1996; Meyer and Meyer, 1995, 1996). In those studies, mutant strains that had been selected for increased benomyl tolerance were more effective against *H. glycines* than the wild type strain. However, microplot and small-scale field tests with one mutant strain produced variable results: cyst counts were reduced 1 out of 2 years in microplots and were not significantly decreased in a single-year field test (Meyer et al., 1997).

As a consequence of the greenhouse and field test results, further studies on soybean cyst nematode and *V. lecanii* were conducted to better understand and possibly improve the activity of the fungus by determining details of the spatial interactions between the two organisms. Light microscopy (LM) and scanning electron microscopy (SEM) were employed to investigate *V. lecanii* colonization of *H. glycines* females and cysts. Female and cyst stages both were examined because a prior study demonstrated that the number of viable eggs from yellow females decreased 1 week after inoculation with *V. lecanii*, but the viability of eggs from cysts was not affected (Meyer et al., 1990). Goals of the current research included determining: time from inoculation to colonization of females and cysts; whether penetration occurred through nematode orifices or body walls; extent of colonization of eggs, females, and cysts; and whether the infection process on yellow females was similar to that on brown cysts. Because some fungi are unable to grow within the *H. glycines* gelatinous matrix (GM) (T. L. Niblack, personal communication), the study also was conducted to find out whether the fungus proliferated within

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the GM, how soon proliferation occurred after inoculation, and whether eggs in the GM were colonized.

Materials and Methods

Heterodera glycines was maintained in monoxenic culture on soybean (*Glycine max* (L.) Merr. cv. Kent) root tips grown on Gamborg's B-5 medium. The nematode isolate was originally obtained from a Maryland soybean field and has been maintained in culture for more than 5 years at the Beltsville Nematology Laboratory.

Initial LM and SEM observations were made with two strains of *V. lecanii:* wild type strain ATCC 58909 and strain M2S1 (Agricultural Research Service Culture Collection, NRRL 18726). The latter was induced with ultraviolet light from strain ATCC 58909 and selected for increased tolerance to the fungicide benomyl (Meyer, 1992). Females and cysts of *H. glycines* were removed from monoxenic culture, placed on 1.5% water agar near a disc of *V. lecanii,* and chemically fixed for SEM at either 7 or 14 days after inoculation (see chemical fixation procedure described below).

The initial study did not demonstrate any differences between the two fungus strains in the way they interacted spatially with the nematode 1 and 2 weeks after fungus inoculation. Further details of the colonization process were studied solely with strain M2S1 because this strain has shown greater efficacy than the wild type strain for suppression of *H. glycines* population levels. For those studies, strain M2S1 was grown on potato dextrose agar for 1 week. An aqueous suspension of conidia and hyphae was prepared in sterile distilled water, and the suspension was pipetted onto petri dishes containing *H. glycines* in monoxenic culture (1 ml of suspension/petri dish). One-month-old nematode cultures were used for female samples, and 2- to 4-month-old cultures were used for cyst samples. The upper lid of each petri dish was marked to identify the locations of females and cysts with attached egg masses so that specimens could be found even after mycelial growth. The selected specimens were simultaneously attached to roots and touching the agar surface so that they would be exposed to the fungus from the time of inoculation. The cultures were incubated at 28 °C in the dark. One, two, three, four, seven, and fourteen days following inoculation, marked females and cysts with *V. lecanii* strain M2S1 growing around them were observed fresh with LM (ca. 5 cysts and 5 females/sample-day) or were immediately chemically fixed for SEM. Cultures used for SEM were prepared on different dates than cultures used for LM.

Samples for chemical fixation were removed from petri dishes and placed into 3% glutaraldehyde (in 0.05 M PO_4 buffer) for a minimum of 2 hours at room temperature (ca. 25 °C), then cooled to and held at 4°C. Samples were then given two rapid rinses in the same buffer, followed by three slower buffer rinses of 20 minutes each. Samples were sequentially dehydrated in EtOH (20%, 40%, 60%, 80%, and 100%, three times) for 30-minute increments, mounted onto aluminum stubs, coated with Au/Pd in a Hummer sputter coater, and viewed in a Hitachi SEM model 570 at 10 kV accelerating voltage. Some of the samples were fixed in glutaraldehyde as whole cysts or females with attached egg masses, and were fractured prior to viewing. To better observe penetration holes in female and cyst walls, other specimens were prepared according to the modified procedure adapted from root-knot nematode perineal preparations (Chen and Dickson, 1996).

Some specimens were collected from monoxenic cultures at either 16 hours or 8 days after inoculation, placed in water, and within an hour prepared for observation by means of low-temperature scanning electron microscopy (Orion et al., 1994; Wergin et al., 1993). For this procedure, cysts and females with attached egg masses were placed in gold-hinged holders mounted on a Denton complementary freeze-etch specimen cap. The cap was placed in an Oxford nitrogen slush chamber, evacuated, and then withdrawn into a cryo-transfer device and moved to an Oxford prechamber. The fracture arm of the complementary cap was

lifted and rotated 180 degrees with a precooled pick, thereby fracturing the samples. The samples were etched for 5 minutes at –90 °C to increase surface relief by removing water, magnetron sputter-coated with platinum metal in the prechamber, and moved to the cold stage in the Hitachi S-4100 field emission scanning electron microscope. Observations were made at accelerating voltages of 10 kV. Specimens that did not fracture initially were moved back to the prechamber, fractured with a precooled knife, and observed again.

Stereo-scanning micrographs were taken of selected specimens to provide information about the three-dimensional images of the fungus-nematode interactions.

Results

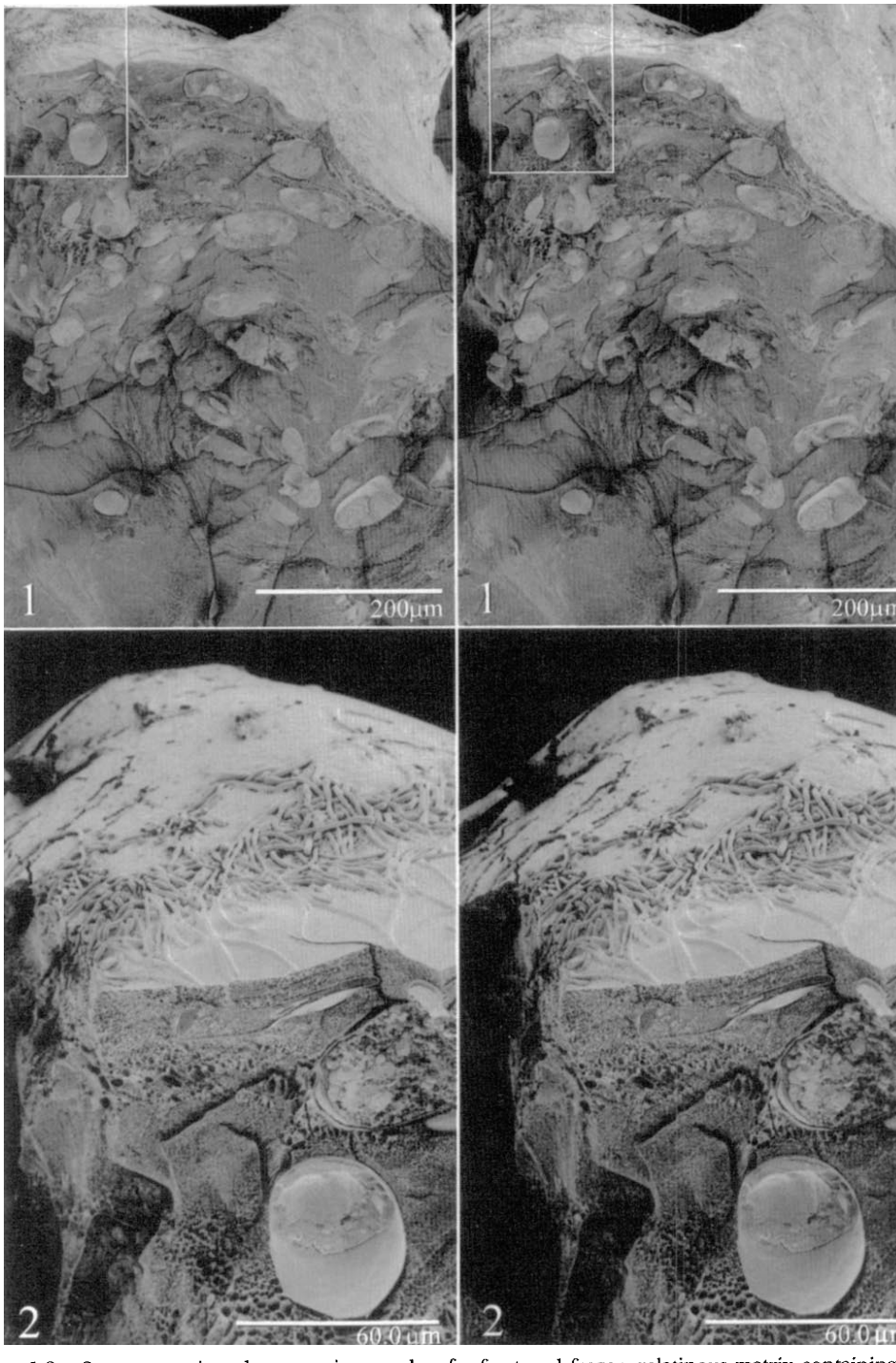
Hyphae of *V. lecanii* grew externally on GM of *H. glycines* females within 16 hours after inoculation onto the nematode (Figs. 1,2) and also began penetration and shallow growth within some GM (Fig. 3). The same growth patterns were recorded from GM of cysts (Figs. 4,5). Observations with LM revealed that some GM were surrounded by "clear zones," with mycelium growing close to, but failing to colonize, the GM. However, the majority of the inoculated GM did not exhibit this phenomenon, and these zones were no longer observed as the fungus continued growing throughout the week.

By day 2, *V. lecanii* had not penetrated the GM as far as the egg mass, even though nine of ten specimens observed with LM had hyphae growing in the GM. The following day, mycelium was in the GM of all observed specimens and was proliferating as far as the outermost eggs in some egg masses. Starting on day 4, the fungus colonized some unhatched eggs in about half of the females and cysts. Colonized eggs contained embryos that had not developed into second-stage juveniles. When the 4, 7, and 14-day samples were combined, the number of infected, unhatched eggs per specimen ranged from 1 to 5 (mean = 2 eggs). Total numbers of unhatched eggs in those GM ranged from 2 to 67 (mean = 29 eggs), so the proportion of colonized eggs was very small. Other colonized GM contained 0 to 169 unhatched eggs (mean = 32) but exhibited no egg penetration. It is not known whether colonized eggs were live or dead prior to fungal entry.

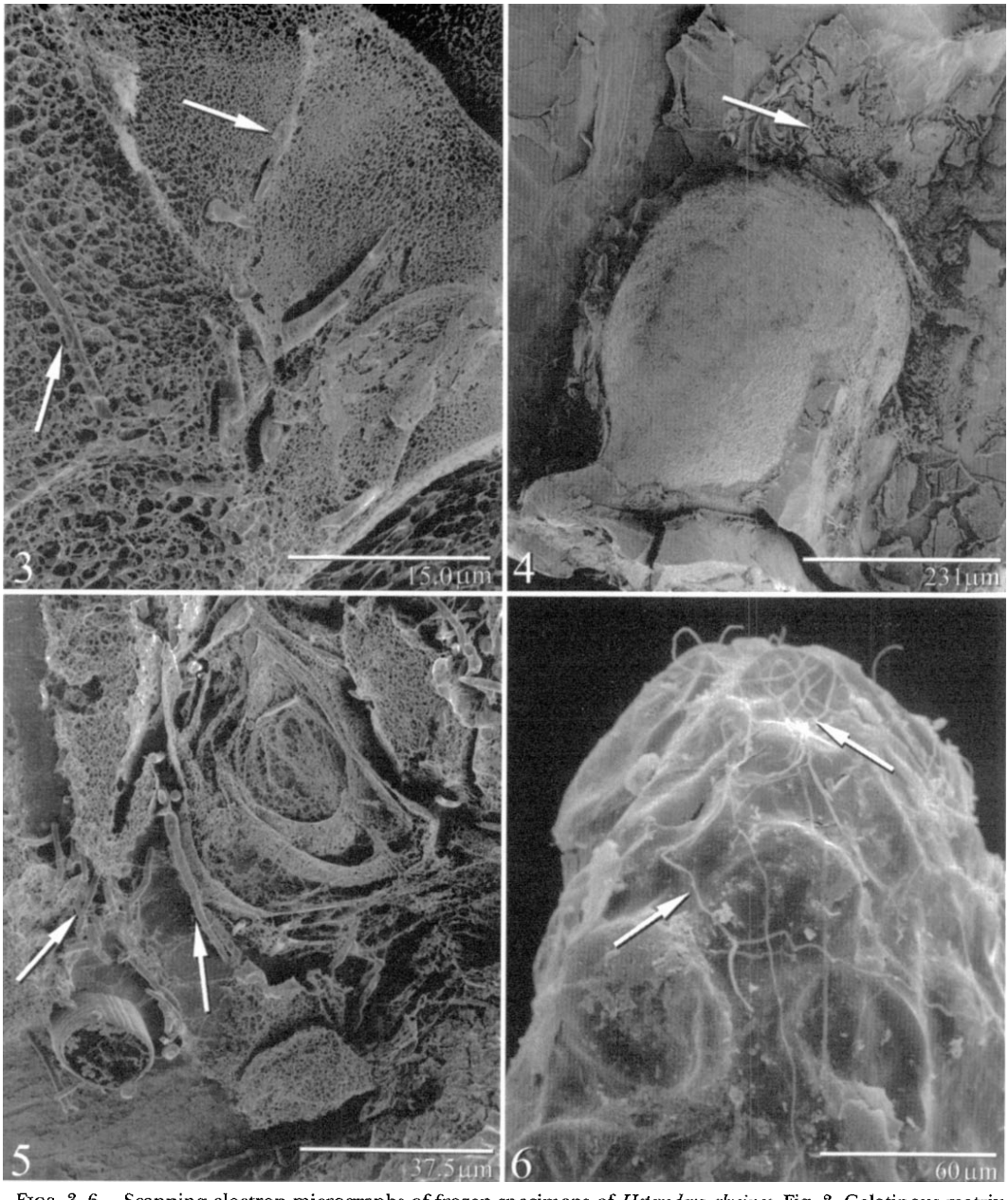
After 1 week, several GM were so heavily colonized that the eggs were difficult to see with LM. Some specimens appeared to have the GM almost replaced by mycelium. Low-temperature SEM proved superior to conventional SEM for clearly discerning the extent of GM colonization, and specimens were not obscured by hyphae as occurred with LM. Conventional SEM revealed hyphae closely associated with the exterior of the GM (Fig. 6), but the egg masses were difficult to fracture with this procedure. Low-temperature SEM on specimens collected 8 days after inoculation clearly demonstrated that the GM supported fungal growth (Figs. 7,8) that penetrated into the eggs mass area (Fig. 8) and proliferated in hatched eggs (Fig. 9).

Cysts and females were also subject to external fungal growth within 16 hours. Hyphae were observed within only one specimen by day 1: a female that had not produced eggs. On day 2, hyphae were not observed within any of the sampled females, but four of the five cysts observed with LM were internally colonized. On day 3, none of the five females observed with LM were colonized internally, but four of five cysts contained internal hyphae. The following day, one of four female specimens was colonized, and three of four cysts.

With SEM, penetration sites were not observed in the female or cyst walls until the third day after inoculation, possibly because penetration pegs were present in small numbers before that time. The cuticles of untreated specimens (Fig. 10) appeared quite different from colonized specimens. In the latter, penetration holes on exterior (Fig. 11) and interior (Fig. 12) body wall surfaces were observed in some samples on days 3 and 4. The penetration sites were more easily located on specimens prepared with the modified perineal preparation technique. Holes made by penetration pegs were gen-

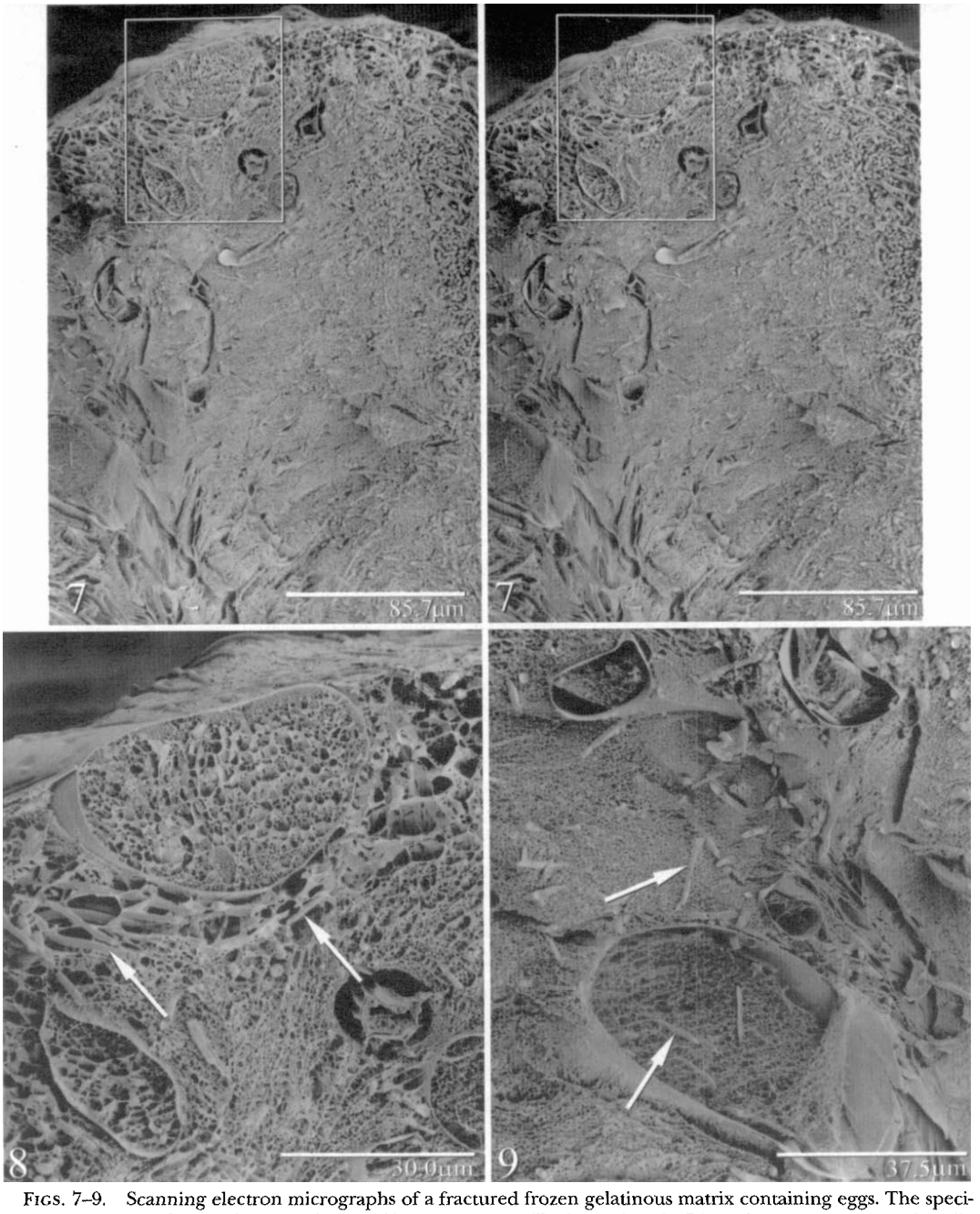


FIGS. 1,2. Stereo scanning electron micrographs of a fractured frozen gelatinous matrix containing eggs. A *Heterodera glycines* female with attached gelatinous matrix was inoculated with *Verticillium lecanii* and observed 16 hours later. The smooth outer surface of the gelatinous matrix contrasted with the spongy internal area. Hyphae did not proliferate extensively within the gelatinous matrix or grow near eggs (Fig. 1) but were closely associated with the external surface (Fig. 2; from the area delineated by the box in Fig. 1). To attain the three-dimensional view, a stereo viewer can be placed on the complementary stereo images so that the left and right lenses are aligned above the left and right images, respectively. The same effect can also be achieved by entraining the left and right eyes on the left and right figures, respectively.



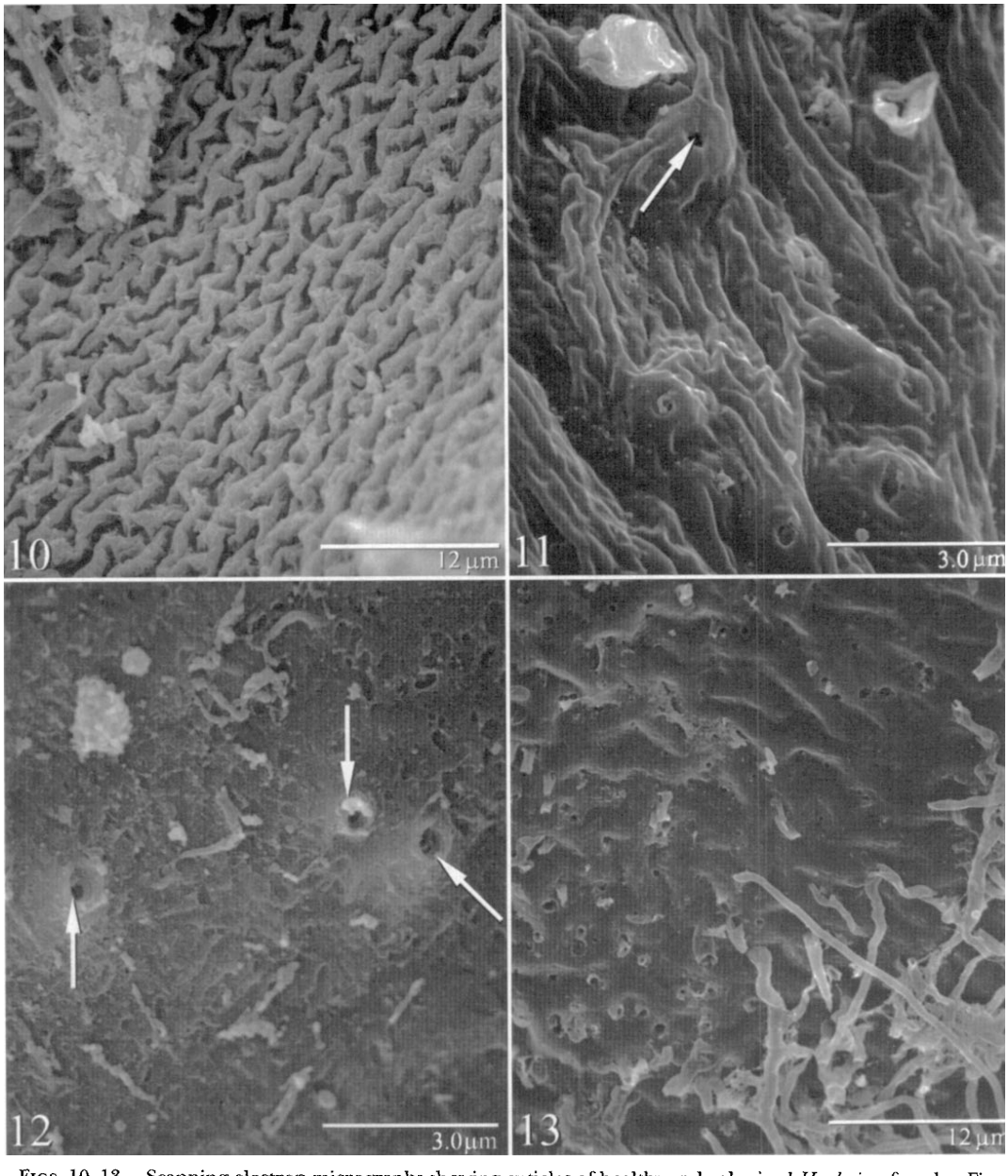
FIGS. 3–6. Scanning electron micrographs of frozen specimens of *Heterodera glycines*. Fig. 3. Gelatinous matrix from Figs. 1 and 2. Hyphae (arrows) also penetrated just under the surface of the gelatinous matrix. Figs. 4, 5. Scanning electron micrographs of a frozen *Heterodera glycines* cyst (Fig. 4) with a fractured egg mass (arrow). Observations were made 16 hours after inoculation with *Verticillium lecanii*. Fig. 5 is a higher magnification of the area around the arrow in Fig. 4. Hyphae grew on the exterior of the cyst and the gelatinous matrix, and inside the gelatinous matrix (arrows, Fig. 5). Fig. 6. Scanning electron micrograph of an egg mass from a *H. glycines* cyst. Observations were made 7 days after inoculation with *V. lecanii*. External hyphae (arrows) grew on the gelatinous matrix, but internal colonization was not observed with conventional SEM techniques. Low-temperature SEM proved superior for such observations.

erally smaller in diameter than nonpenetrating portions of hyphae (Fig. 13). One female specimen was heavily colonized with *V. lecanii* by day 4 (Fig. 14); the body wall contained many hyphae, and the wall structure was degraded (Fig. 15). More typically, fe-



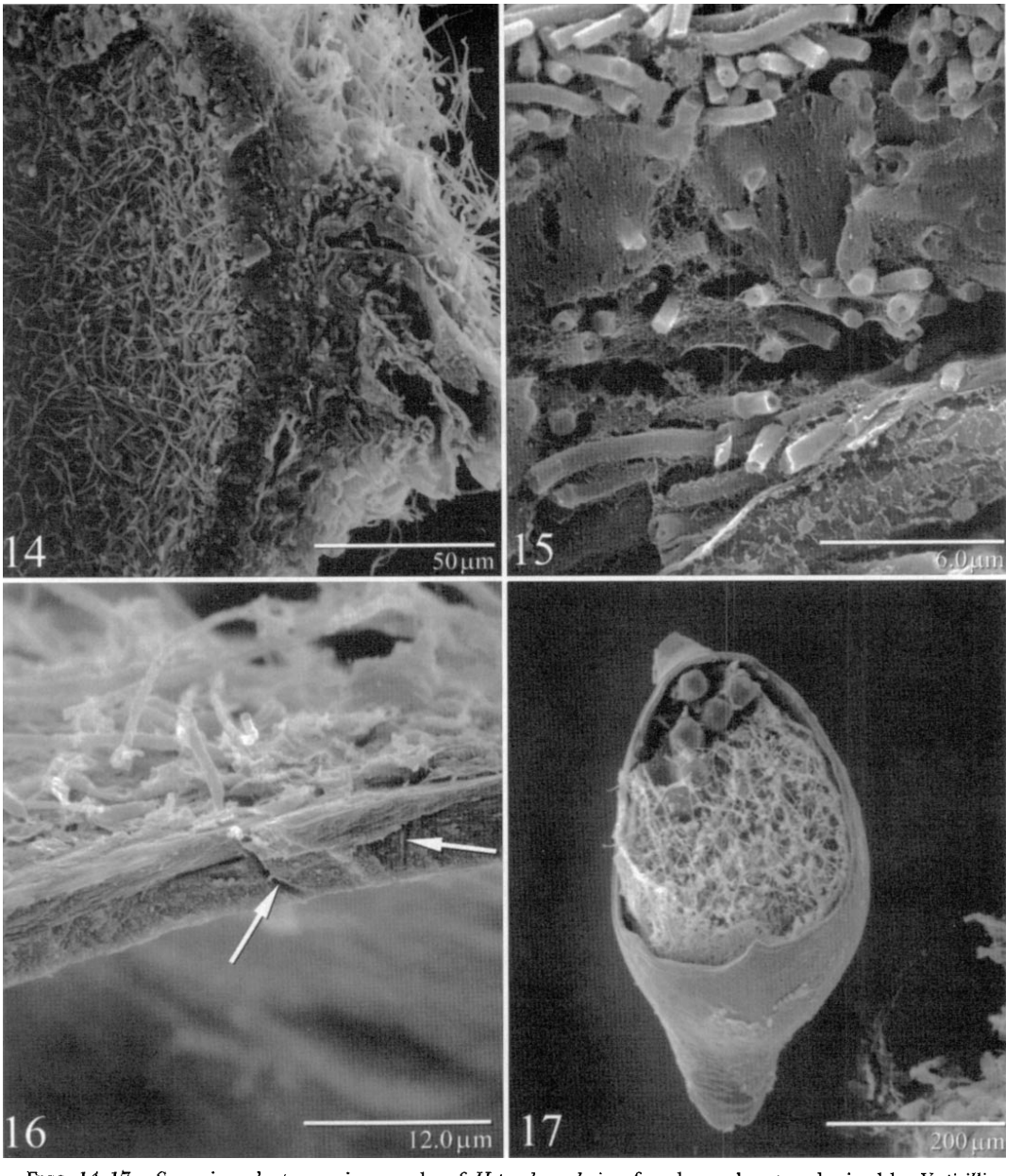
FIGS. 7–9. Scanning electron micrographs of a fractured frozen gelatinous matrix containing eggs. The specimen was prepared 8 days after inoculation of *V. lecanii* onto a *H. glycines* female. The gelatinous matrix was heavily colonized by the fungus (Fig. 7). Hyphae (arrows) proliferated near some eggs (Figs. 8,9, arrows) and grew in hatched eggs (Fig. 9, arrow). Fig. 7 is a stereo pair; Fig. 8 is from the area delineated by the box in Fig. 7.

male and cyst walls were intact but contained "tunnels" where the fungus had penetrated. Presumably, penetration pegs had fallen out of some of the fractured females and cysts during specimen preparation, leaving the sites empty (Fig. 16). The wall layers were not disturbed as they would be by mechanical pressure, and no pushed-out ring of material was observed where penetration pegs had emerged from walls (Fig. 16).



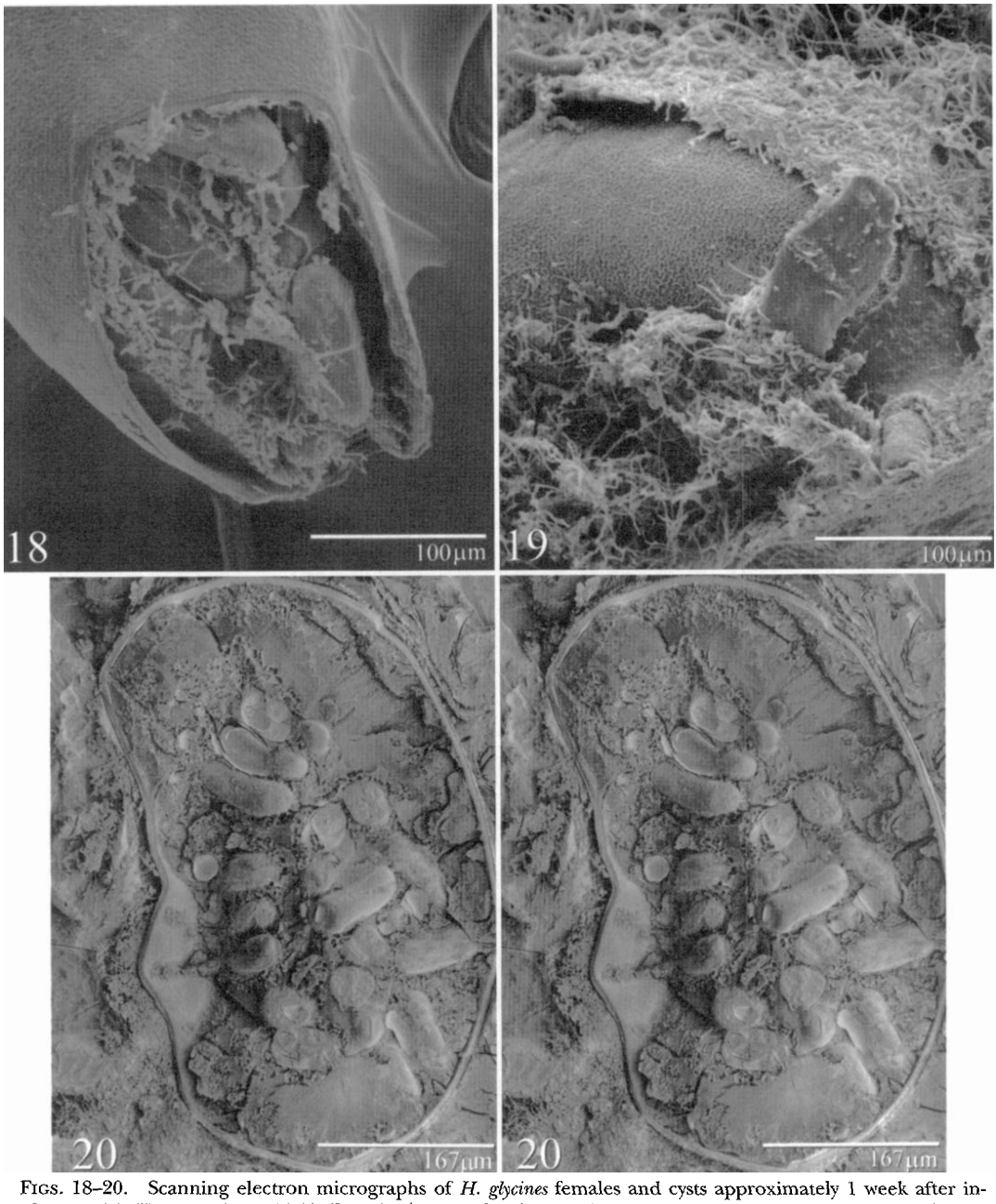
FIGS. 10–13. Scanning electron micrographs showing cuticles of healthy and colonized *H. glycines* females. Fig. 10. Cuticle of a female that was not inoculated with fungus. Figs. 11,12. Penetration holes (arrows) on the external (Fig. 11) and internal (Fig. 12) surfaces of *H. glycines* females. Observations were made 3 days after inoculation with *V. lecanii*. Fig. 13. Penetration holes on the external surface of a *H. glycines* female. Observations were made 4 days after inoculation with *V. lecanii*. Penetration holes were generally smaller in diameter than fungal hyphae on the surface of the female.

Approximately 1 week (days 7 and 8) after inoculation, three of four females observed with LM, and two of four cysts, were colonized. The remaining two cysts were empty, containing no eggs, hyphae, or recognizable internal material. Nematodes removed from root explant culture prior to fungus inoculation (and subsequently observed at the 1- and 2-week sampling dates) provided the same results as nematodes inoculated while still attached to host roots. One week after inoculation, extent of colonization within fe-



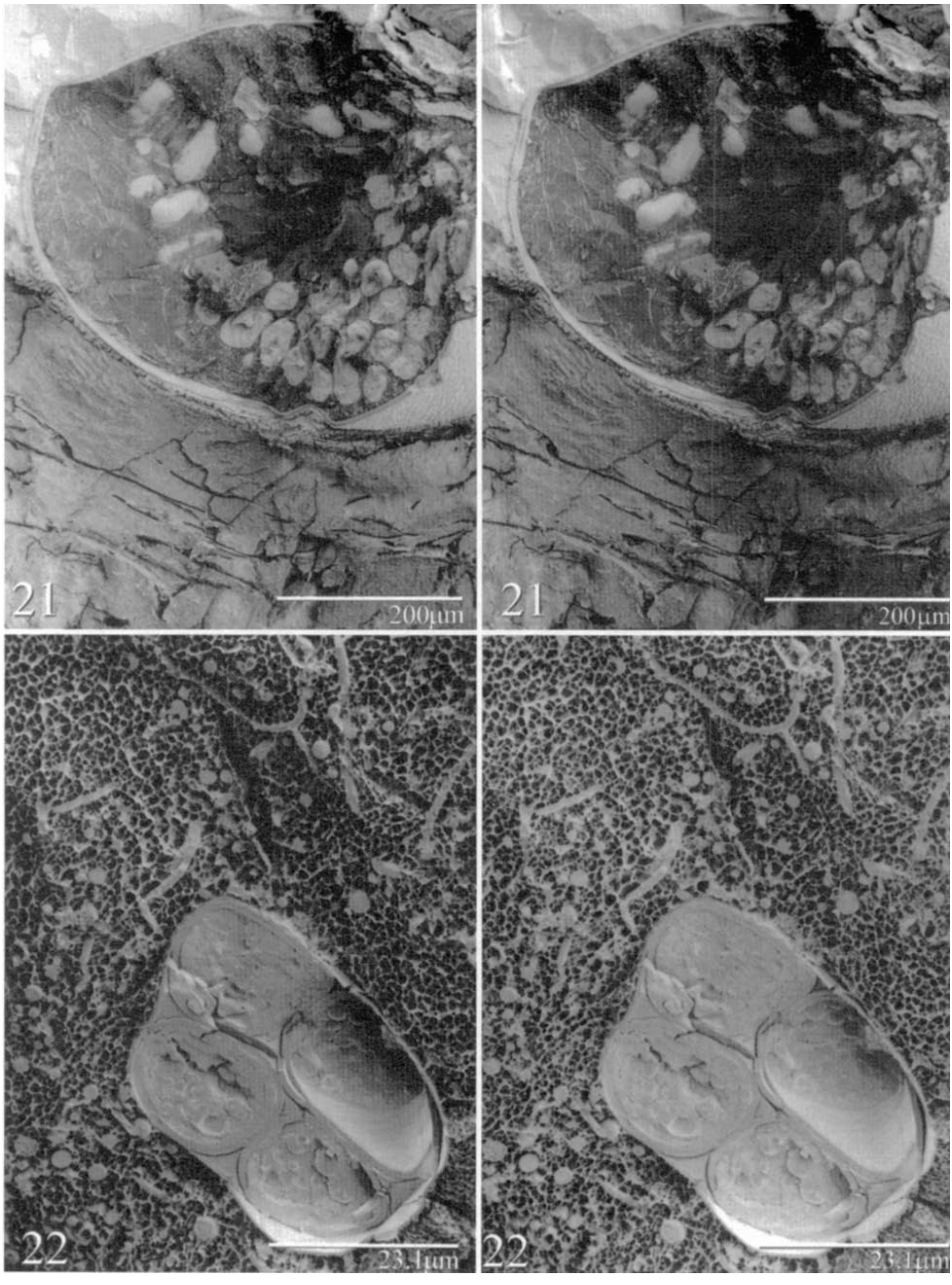
FIGS. 14–17. Scanning electron micrographs of *Heterodera glycines* females and cysts colonized by *Verticillium lecanii*. Figs. 14,15. *Heterodera glycines* female heavily infected with *V. lecanii* 4 days after inoculation. Dense mycelium was present inside and outside the fractured specimen (Fig. 14); numerous hyphae penetrated the degraded body wall (Fig. 15). Fig. 16. Scanning electron micrograph of a *H. glycines* cyst wall 4 days after inoculation with *V. lecanii*. Tunnels (arrows) in the wall were formed by penetration pegs, which probably fell out at the time of fracture. Fig. 17. Scanning electron micrograph of a *H. glycines* cyst, demonstrating extensive fungus colonization 7 days after inoculation with *V. lecanii*.

males and cysts was variable. Some colonized specimens were almost filled with mycelium (Fig. 17), while others contained fewer hyphae (Fig. 18). Although females and cysts observed at this and other times often were covered with a mat of hyphae, the fungus grew on the subcrystalline layer of some specimens and could be peeled off, leaving uninfected cuticle behind (Fig. 19). A number of specimens with external fungus



FIGS. 18–20. Scanning electron micrographs of *H. glycines* females and cysts approximately 1 week after inoculation with *V. lecanii*. Figs. 18,19. Females 7 days after inoculation with *V. lecanii*. Eggs were surrounded by hyphae but did not exhibit signs of being parasitized (Fig. 18). A mat of mycelium surrounding a female illustrates the fungus "peeling" off the nematode, as seen on some specimens (Fig. 19). The cuticle appeared undisturbed. Fig. 20. Stereo scanning electron micrographs of a fractured, frozen, hydrated *H. glycines* cyst, 8 days after inoculation with *V. lecanii*. The hyphae grew external to the cyst but did not penetrate or colonize the body cavity.

growth were not colonized internally (Fig. 20). In specimens that did contain mycelium (Figs. 17, 18, and 21), hyphal degradation of eggs was not observed with SEM (Figs. 17,18,21,22). Two of the eight specimens studied with LM contained colonized eggs: one female (2 of 106 unhatched eggs) and one cyst (4 of 51 unhatched eggs). As observed in the GM, none of the colonized eggs had matured to second-stage juveniles.

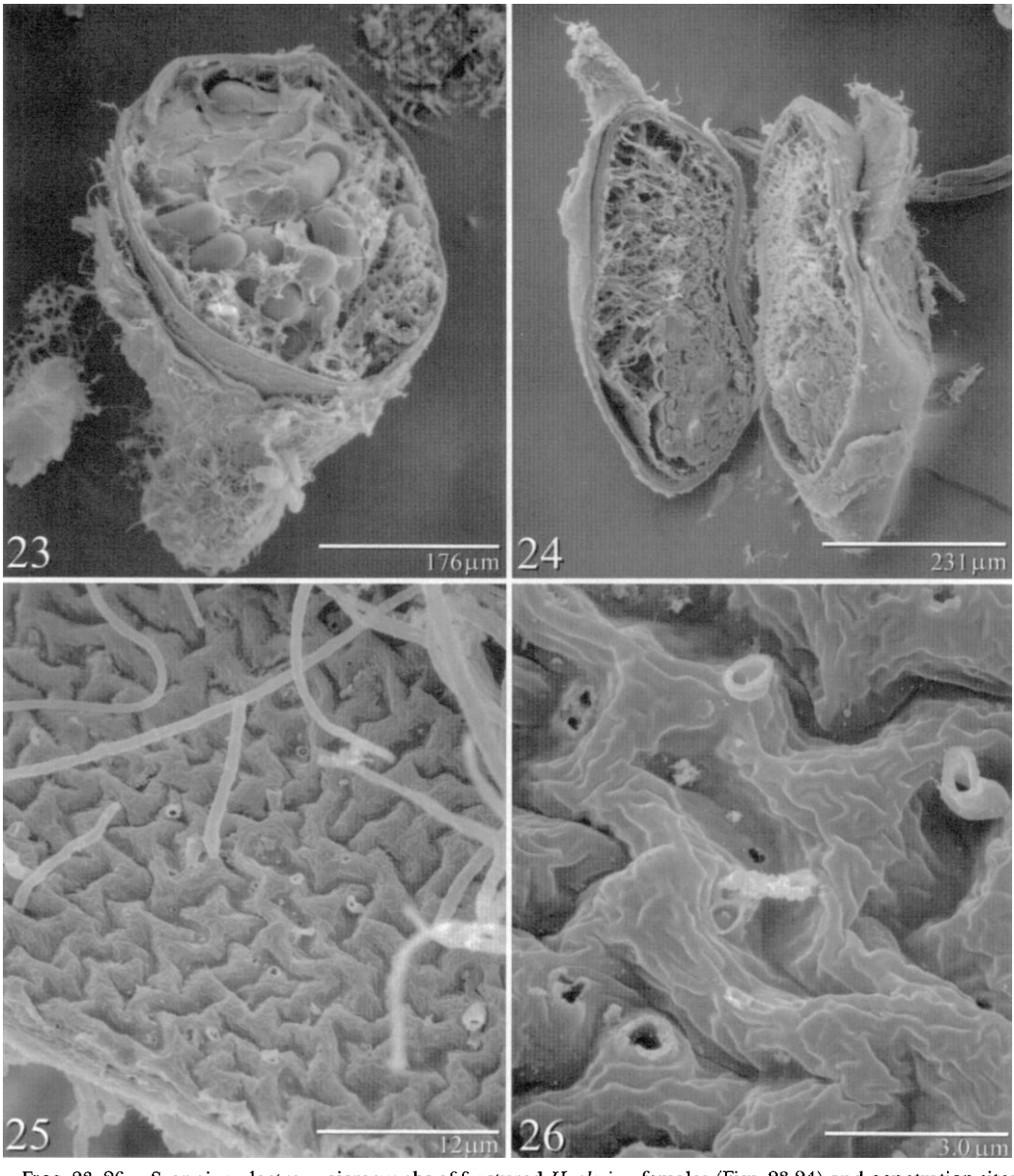


FIGS. 21,22. Stereo scanning electron micrographs of a fractured, frozen, hydrated *H. glycines* cyst, 8 days after inoculation with *V. lecanii*. Hyphae colonized the internal body cavity of the cyst (Figs. 21,22) but did not penetrate eggs (Fig. 22). In those specimens where egg penetration occurred, numbers of colonized eggs were low.

Two weeks (day 14) after inoculation, all four females examined with LM, and two of three cysts, contained internal hyphae. The fungus still was not colonizing large numbers of eggs, even when the eggs were surrounded by mycelium (Fig. 23). Hyphae were in unhatched eggs from three females and one cyst, colonizing 1 to 3 eggs (mean = 2 eggs) in each of those specimens. Total numbers of unhatched eggs in those four samples ranged from 27 to 138 (mean = 50 eggs). However, specimens were observed

with SEM where much of the internal body area was occupied by mycelium, with few eggs present (Fig. 24).

Penetration sites were numerous in females (Fig. 25) and variable in diameter (Fig. 26) 2 weeks after inoculation. One fractured specimen contained a penetrating hypha in the body wall (Fig. 27), clearly demonstrating the origin of the "tunnels." At this time, there were large numbers of tunnels in the body walls of colonized specimens (Figs. 28,29), but the body walls often

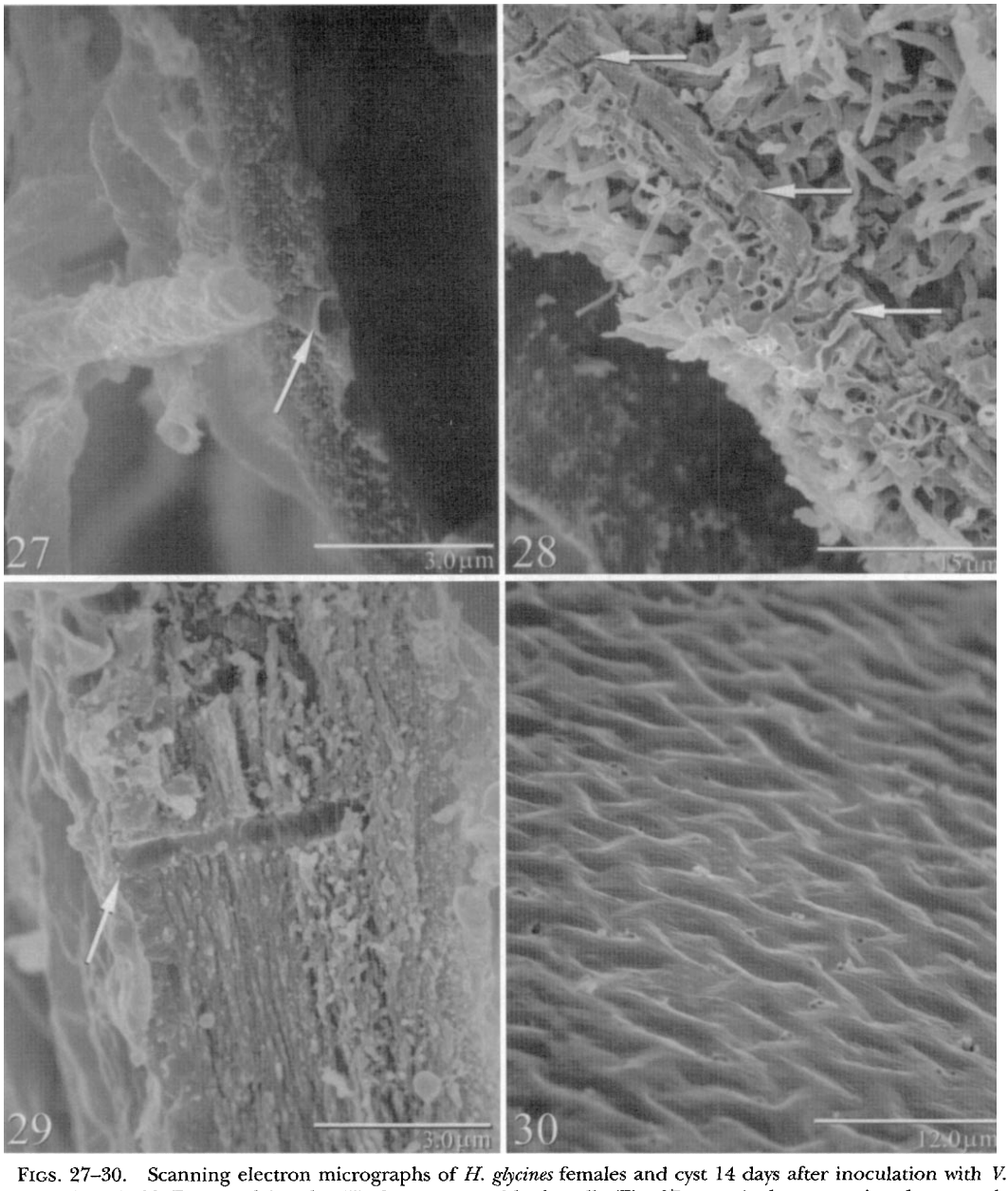


FIGS. 23–26. Scanning electron micrographs of fractured *H. glycines* females (Figs. 23,24) and penetration sites (Figs. 25,26) 14 days after inoculation with *V. lecanii*. Colonization of infected females varied from smaller amounts of mycelium surrounding eggs (Fig. 23) to extensive growth that nearly filled the body cavity (Fig. 24). Numerous penetration sites were often present on internally colonized specimens (Fig. 25); penetration hole sizes were variable (Fig. 26).

were intact despite the high degree of colonization. No evidence of significant mechanical penetration was observed (Fig. 29). Similar numbers and types of penetration sites were found in cysts (Fig. 30). With LM, hyphae were seen growing through natural openings of some specimens by day 14.

Discussion

Light microscopic and scanning electron microscopic observations demonstrated that *V. lecanii* colonized females, cysts, and GM of *H. glycines*. Use of monoxenic cultures for nematode specimens ensured that the ob-



Figs. 27–30. Scanning electron micrographs of *H. glycines* females and cyst 14 days after inoculation with *V. lecanii*. Figs. 27–29. Fractured females. Hypha penetrated body walls (Fig. 27, arrow), demonstrating that tunnels were formed by the fungus. Penetration sites (indicated by arrows) were often numerous by this time (Fig. 28). Tunnels did not exhibit mechanical disruption of wall layers (Fig. 29, arrow). Fig. 30. *Heterodera glycines* cyst. Numerous penetration sites were observed in colonized cysts as well as females at this time.

served fungus was *V. lecanii* and not a contaminant. The procedure also demonstrated that *V. lecanii* colonized *H. glycines* while the nematode was still attached to the roots; earlier research on cyst nematode-fungus interactions often involved females or cysts separated from their hosts.

Colonization of *H. glycines* females was similar to that of cysts, but cysts tended to be colonized more quickly. Penetration holes were seen in the female walls and the cyst walls by the third day following inoculation with the fungus, but hyphae were not observed growing through natural openings until more than a week after inoculation. These observations indicate that the primary method of infection was direct penetration through body walls. This is similar to reports that *V. lecanii* entered *H. schachtii* cysts through narrow penetration holes in the body wall (Hänssler, 1990; Hänssler and Hermanns, 1981), penetrated through the cuticle of thrips (Schreiter et al., 1994; Vestergaard et al., 1995), and did not infect aphids through natural orifices (Grünberg et al., 1990). Penetration holes were observed 48 hours after *H. schachtii* cysts were placed next to *V. lecanii* colonies (Hänssler and Hermanns, 1981); in the current study, such early penetration may have occurred in some specimens but was not observed due to the rarity of penetration sites.

Similar means of penetration have been observed with other fungus-nematode combinations. For example, 10 species of fungi penetrated cyst walls of *H. glycines* (Chen and Dickson, 1996), the fungus ARF18 entered cysts through the cuticle (Kim et al., 1992), and *Trichoderma harzianum* colonized cysts of *Globodera rostochiensis* through the cyst wall (Saifullah and Thomas, 1996). Infection time for *V. lecanii* on *H. glycines* was also comparable to times reported for the 10 fungi on *H. glycines* cysts (1 to 7 days) and for *V. lecanii* on *H. schachtii* (48 to 60 hours; Hänssler, 1990; Hänssler and Hermanns, 1981).

Although mechanical penetration cannot be entirely ruled out, the primary mechanism for infection appeared to be digestion, as has been found in other interactions (Chen and Dickson, 1996; Hänssler, 1990; Saifullah and Thomas, 1996; Segers et al., 1996). *Verticillium lecanii* did not disturb wall layers of *H. glycines* nor push body wall material out at either end of the penetration holes, indicating that this fungus probably entered females and cysts through enzymatic activity. A similar mechanism appeared to be involved in *V. lecanii* penetration of *H. schachtii* cysts (Hänssler and Hermanns, 1981).

Strains of *V. lecanii* colonized eggs inside the cyst cavity of *H. schachtii* (Hänssler, 1990) and attacked eggs of *G. pallida* (Uziel and Sikora, 1992). However, in a previous in vitro assay, only one of four *V. lecanii* strains inoculated onto *H. glycines* was found infecting viable eggs (Meyer et al., 1990). In the current study, few eggs were colonized, and it was not determined whether they were penetrated before or after egg death. While *V. lecanii* has the ability to act as an egg parasite, the strains used for this research apparently do not utilize this mode of nutrition, are weak egg parasites, or consume eggs so quickly after initial penetration that it is difficult to find colonized eggs. The latter hypothesis was not supported by the current study, in which observations were made frequently enough after fungus inoculation that partially digested eggs should have been observed, had they been present in large numbers. Possible modes of action against the nematode include production of a natural substance that kills eggs without prior egg penetration, and infection and death of some females before a full complement of eggs is produced. This microscopy study therefore corroborates the findings from the earlier in vitro assay (Meyer et al., 1990), which indicated that *V. lecanii* did not act against *H. glycines* primarily through direct egg parasitism. Application of the fungus for suppression of nematode populations should consequently include use of growth media and formulations that maximize production of an antagonistic substance or substances.

In the same in vitro bioassay (Meyer et al., 1990), treatment with the wild type strain of this fungus reduced the number of viable

eggs from yellow females, but the number of viable eggs from cysts was not significantly affected. The cyst wall apparently was not a barrier to *V. lecanii;* in fact, the current research indicated that cysts tended to be more rapidly colonized than females, at least under the conditions found in root explant cultures. Eggs in older cysts may be less susceptible to harmful effects of the fungus; eggs in early developmental stages have been found to be more susceptible to fungal infection (Irving and Kerry, 1986; Kerry, 1988; Lopez-Llorca and Duncan, 1991).

Despite the ability of *V. lecanii* to colonize females and cysts of *H. glycines,* specimens were found that were enclosed by hyphae but not penetrated. On some of these samples, the fungus grew extensively on the subcrystalline layer, a structure composed of a fatty acid and a calcium salt in some *Heterodera* species (Brown et al., 1971). This layer is colonized by other fungi (Brown et al., 1971; Lopez-Llorca and Duncan, 1991), which are presumed to be feeding on exudates from the female. It is not known why *V. lecanii* failed to penetrate further than the subcrystalline layer in some cases.

Chemically fixed specimens viewed with SEM were the most efficacious for studying fungus penetration of the body walls, and use of fractured specimens for SEM allowed observation of wall cross-sections. Information about penetration could be obtained in less time than that required to prepare thin sections for transmission electron microscopy. Penetration holes were not seen with low-temperature SEM techniques, possibly because specimens fixed in this fashion still had hyphae filling the penetration sites or because such sites were rare in those particular specimens. However, low-temperature SEM proved invaluable for examination of fungus in the GM. It was often difficult to observe hyphal penetration of egg masses with the light microscope, and the GM did not fracture well with conventional SEM techniques. Low-temperature SEM was also superior for preserving internal structures.

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