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Parasitism of *Xiphinema rivesi* and *X. americanum* by Zoosporic Fungi[1]

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Abstract: Living *Xiphinema americanum* (Xa) and *X. rivesi* (Xr) extracted from soil samples and stored for 1–5 days at 4 or 20 C contained aseptate fungal hyphae. The fungi directly penetrated the nematode's cuticle from spores encysted near the head. Penetration through the stoma, vulva, or anus was rare. *Catenaria anguillulae* (Cat), *Lagenidium caudatum* (Lag), *Aphanomyces* sp. (Aph), and *Leptolegnia* sp. (Lep) were isolated into pure culture from infected nematodes. The pathogenicity of these zoosporic fungi was determined by incubating mixed freshly extracted Xa and Xr in 2% soil extract (pH = 6.7, conductivity = 48 μmhos, 20 ± 2 C) containing zoospores obtained from single-spore isolates. After 4 days, Cat, Lag, Aph, and Lep had infected 78, 18, 13, and 22%, respectively, of the nematodes. Both Xa and Xr were infected by every fungus; however, the relative susceptibility of Xa and Xr to these fungi was not determined. All noninoculated control nematodes remained uninfected and alive. In a second experiment, parasitism of Xa and Xr by Aph and Lep was increased when nematodes were incubated in 2% soil extract for 4 days before exposure to zoospores. In a third experiment, parasitism of Xa and Xr by Cat was greater in diluted saturation soil extract (conductivity = 100–400 μmhos) than in undiluted saturation extract (conductivity = 780 μmhos). Cat produced small zoospores (4-μm-d), bulbous infection hyphae, and assimilative hyphae of varying diameters in nematodes, whereas Lag, Aph, and Lep produced large zoospores (8-μm-d) and tubular, uniform infection and assimilative hyphae in nematodes.

Key words: biological control, *Catenaria, Lagenidium, Aphanomyces, Leptolegnia,* endoparasites, dagger nematodes.

Xiphinema rivesi Dalmasso and *X. americanum* Cobb occur commonly in Pennsylvania fruit plantings (12). Both species are important pests, because they transmit tomato ringspot virus and because nonviruliferous specimens may directly damage peach and apple trees (5,6,12). In routine examinations of nematodes extracted from orchard soils, I regularly observed fungal hyphae within living *X. rivesi* (Xr) and *X. americanum* (Xa). Isolation of these fungi and their parasitism of nematodes in vitro are described here.

Materials and Methods

Fungi isolated from parasitized nematodes: Parasitized nematodes were obtained from peach and apple orchard soil samples. Soil samples were collected from March to September 1984 and processed within 30 days (usually 10) of collection. *Xiphinema* spp. were extracted by soaking 100 cm^3 soil for 1 hour in 500 ml tap water, followed by elutriation (8). Nematodes and debris collected on a 38-μm-pore sieve were placed in a Baermann funnel containing tap water. Samples were collected from the funnels after 48 hours and stored for 1–5 days at 4 or 20 C.

Living nematodes that appeared parasitized were examined by light microscopy at ×40–1,000. Some of these specimens were placed in a vial (one to five nematodes per vial) with 10 ml sterile distilled water and shaken vigorously for 30 seconds. The suspension was then poured into a sterile petri dish, and the nematodes were transferred aseptically with a flame-sterilized needle to 2% water agar. Portions of advancing edges of fungal colonies growing from the nematodes incubated on water agar were transferred to petri dishes containing corn meal agar. Fungus cultures were maintained on corn meal agar at 20 ± 2 C and subcultured monthly.

Poppy (*Papaver* sp.) seeds were used to induce fungus sporulation. Seeds autoclaved in 5 ml distilled water were placed on the surface of fungal colonies. After 4–7 days, the seeds were placed in 6 ml of 2% soil extract (see below). The fungi usually produced spores within 24 hours. Single-spore isolates were obtained by transferring 0.5 ml of a spore suspension to the

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TABLE 1. Electrical conductivity and pH of incubation solutions.

Solution	Electrical conductivity*	pH
2% soil extract	48	6.6
Saturation extract†	780	6.0
Saturation extract (1:1)	400	6.2
Saturation extract (1:3)	190	6.2
Saturation extract (1:7)	100	6.2
Baermann funnel	270	6.9

* μmhos/cm.
† Saturation extract was undiluted or diluted 1:1, 1:3, or 1:7 with distilled water.

surface of 2% water agar. After 6–48 hours, individual germinating spores and surrounding agar were transferred to corn meal agar.

In vitro pathogenicity: Incubation solutions included 2% soil extract and a saturation soil extract that was undiluted or diluted 1:1, 1:3, or 1:7 with distilled water. The 2% soil extract was prepared by mixing 20 g air-dried soil from a fallow orchard site in 1 liter distilled water in a 1-liter flask, allowing the suspension to settle for 4 days and removing 150 ml supernatant fluid (17). A saturation extract was obtained by wetting soil from the same source to a pastelike consistency and extracting the soil solution with a Buchner funnel (20). All extracts were filtered through two layers of Whatman No. 2 filter paper and autoclaved. The conductivity and pH of the extracts were measured (Table 1).

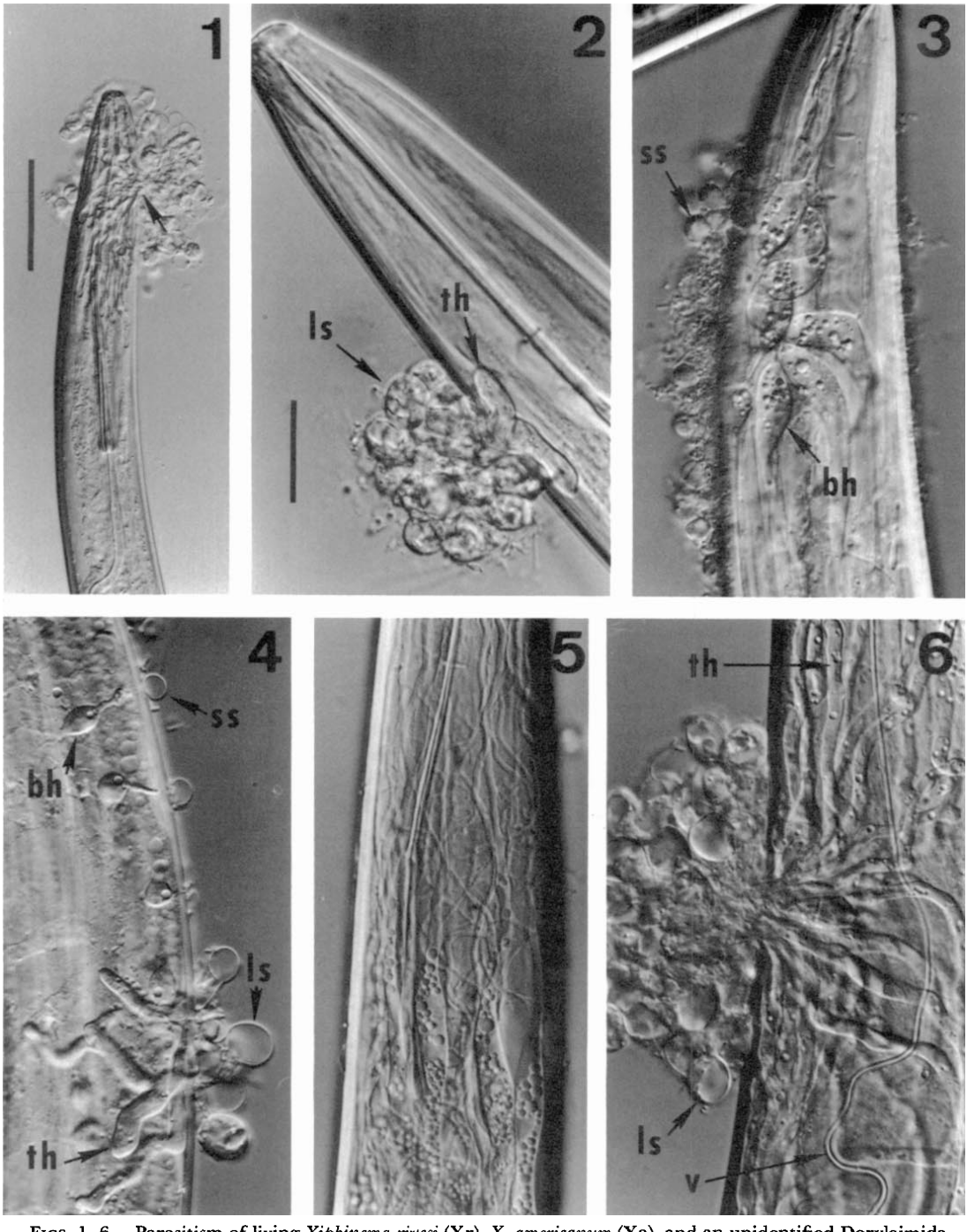
A mixture of hand-picked, healthy adult female Xa and Xr was incubated in solutions in 6-cm-d petri dishes containing poppy seeds infested with fungi derived from single-spore isolates. The nematodes were obtained from a 1-m^2 plot of perennial rye grass (*Lolium perenne*) containing 300–900 *Xiphinema* spp. per 100 cm^3 soil. At the start of these experiments, the species of 100 randomly selected adult females obtained from the plot were determined; 80% were Xa and 20% were Xr. Each petri dish contained 6 ml of soil solution, 15–20 nematodes, and one seed; each was incubated at 20 ± 2 C for 4 days after combining nematodes and seeds. Control dishes contained soil solution, nematodes, and autoclaved, noninfested seeds. The condition of each nematode specimen was determined daily by examination at ×30–70. Active nematodes or those that moved when gently touched were considered alive; only nematodes in control plates were touched. Zoospore concentration was determined by examining (at ×60) three 1–5-μl drops of spore suspension per petri dish on a glass microscope slide; only motile spores were counted.

Each experiment was performed once with three replicate petri dishes per treatment. Data were subjected to analysis of variance, Duncan's multiple-range test, and linear contrasts, when appropriate. In one experiment, the nematodes were extracted (elutriation plus Baermann funnel) and used within 20 hours of sampling. Selected females were placed in petri dishes containing 2% soil extract to which fungus-infested poppy seeds had been added 18 hours earlier. In a second experiment, nematodes were incubated in 2% soil extract for 4 days at 20 ± 2 C before infested seeds were introduced. In a third experiment, nematodes used within 20 hours of soil sampling were incubated in different solutions containing *Catenaria anguillulae*-infested poppy seeds added 18 hours earlier.

RESULTS

Fungi isolated from parasitized nematodes: Living, fungal-infected Xa and Xr were readily identified when examined with a dissecting microscope. The anteriors of healthy nematodes were transparent, whereas the anteriors of infected nematodes had grey, granular, opaque zones. Usually there was one opaque zone per infected nematode, and the zone appeared to originate anteriorly and spread posteriorly. Spores often adhered to the nematode cuticle near the opaque areas.

Examination of infected nematodes at ×200–1,000 by interference contrast light microscopy revealed that, although fungus infections occurred in all regions of the nematode, most infections originated near the stylet and esophagus (Figs. 1–3). The cuticle was almost always penetrated directly by the fungi; penetration through the stoma, vulva, or anus was rare. Penetration was associated with single or few spores or clusters of spores (Figs. 1–4). Groups of spores often encysted prior to penetration. Spores were usually 8 μm (Fig. 2) or 4 μm (Fig. 3) in diameter. Small and



FIGS. 1–6. Parasitism of living *Xiphinema rivesi* (Xr), *X. americanum* (Xa), and an unidentified Dorylaimida by zoosporic fungi. Parasitized nematodes were obtained from peach or apple orchard soil samples. Bar in Figure 1 = 50 μm. Bar in Figure 2 = 20 μm. Figures 3–6 same scale as Figure 2. 1) Zoospores encysted near anterior of Xr. Penetration (arrow) of cuticle has occurred about 40 μm posterior of stoma on right side of nematode. 2) Penetration of Xr near guiding ring by fungus with large (8-μm-d) zoospores (ls). Note presence of wide tubular hyphae (th) and absence of bulbous infection hyphae. 3) Penetration of Xa (about 70 μm posterior of stoma) by fungus with small (4-μm-d) zoospores (ss). Note presence of six bulbous infection hyphae (bh) with attenuated ends. 4) Penetration of Xa by small spores (ss) and large spores (ls). Note bulbous infection hyphae (bh) beneath small spores and tubular hyphae (th) beneath large spores. 5) Infected Xa containing hyphae with variable diameters typical of *Catenaria anguillulae*. Odontophore, portion of odontostyle, and guiding ring are visible. 6) Nonparasitic Dorylaimida containing tubular hyphae (th) typical of *Lagenidium caudatum*, *Aphanomyces* sp., and *Leptolegnia* sp. Note large spores (ls) and esophagus (v) of nematode.

large spores frequently occurred on the same nematode (Fig. 4) and even at the same penetration site. Hyphae within nematodes early during colonization were always aseptate. Two general kinds of infection hyphae were observed. When fungi with large spores (ls) penetrated the cuticle, the hyphae appeared tubular (th) and relatively uniform in diameter (Figs. 2, 4, 6). When fungi with small spores (ss) penetrated the cuticle, a bulbous infection hypha (bh) and variable secondary hyphae developed (Figs. 3–5).

Of the fungi isolated from nematodes, only *Catenaria anguillulae* Sorokin produced sporangia on water agar and corn meal agar. The other fungi grew vegetatively on these media but produced sporangia and zoospores if infested seeds were placed in dilute soil extract. *Catenaria anguillulae* (ATCC 58382) = Cat and *Lagenidium caudatum* Barron (ATCC 58383) = Lag were identified by the author with the aid of published descriptions (1,24). *Aphanomyces* sp. (ATCC 58381) = Aph and *Leptolegnia* sp. (ATCC 58384) = Lep were identified by T. W. Johnson, Jr., Department of Botany, Duke University. Neither Aph nor Lep were identified to species because sexual structures could not be induced (T. W. Johnson, Jr., pers. comm.).

The number of infected nematodes per soil sample and the number of isolations of each fungus were not recorded. However, Cat and Lag occurred more frequently than Aph or Lep. The species of infected *Xiphinema* was determined whenever possible. Infections of Xa and Xr appeared similar and occurred with equal frequency in either species. Similar infections were also observed in specimens of *X. californicum* and *Longidorus elongatus* that were extracted from two soil samples. Tylenchid nematodes were unaffected, whereas nonparasitic dorylaimid nematodes were parasitized by the zoosporic fungi (Fig. 6).

In vitro pathogenicity tests: All of the isolated fungi attacked some of the apparently healthy Xa and Xr when freshly extracted nematodes were added to zoospore suspensions in 2% soil extract (Fig. 7A). Cat infected most nematodes in the test, but Lag, Aph, and Lep infected only 13–29%. All infected nematodes died within 24 hours. Mortality of noninoculated control nematodes was 0% at 4 days and 5% at 18 days. After 2 days, zoospore concentration in the incubation solution was higher ($P = 0.05$) for Cat than for the other fungi (Fig. 7C). Counting the motile spores when spore concentration exceeded 15/µl was difficult, and the accuracy of counts was reduced. Usually, zoospores were not immediately attracted to nematodes when nematodes were introduced. As observed in natural infections, fungal penetration was direct and occurred most frequently near the nematode stylet and esophagus. Cat produced small spores that formed swollen infection hyphae with attenuated ends (as in Figs. 3, 4) whereas Lag, Aph, and Lep produced larger spores that formed tubular hyphae within the nematodes (as in Figs. 2, 4, 6). Characteristic sporangia were produced in these pathogenicity tests. In many cases, however, no fungal reproductive structures were observed, because the entire nematode was attacked by zoospores soon after the initial infection.

Parasitism of nematodes by Aph and Lep was higher ($P = 0.05$) with aged nematodes (incubated for 4 days in 2% soil extract before addition of fungi) than with freshly extracted nematodes (Fig. 7B vs. 7A). Cat produced many spores and a high percentage of infection, whereas infection by Lag remained low (Fig. 7B, D). No infection or mortality occurred among the control nematodes.

Parasitism of freshly extracted nematodes by Cat was significantly ($P = 0.05$) delayed in undiluted saturation extract versus diluted saturation extracts or 2% soil extract (Fig. 8). Although zoospore concentration was variable, it was as high in undiluted extract as in 1:7 extract. After 4 days, parasitism was higher ($P = 0.05$) in solutions of intermediate electrical conductivity (diluted saturation extracts) than in solutions of high (undiluted saturation extract) or low (2% soil extract) electrical conductivity. Mortality of noninoculated control nematodes was less than 3% in all solutions.

Discussion

The four genera of zoosporic fungi isolated in this study include species parasitic and saprophytic on small, aquatic organisms (3,9,14,15,24). *Catenaria* spp. are frequently isolated from, and are recognized

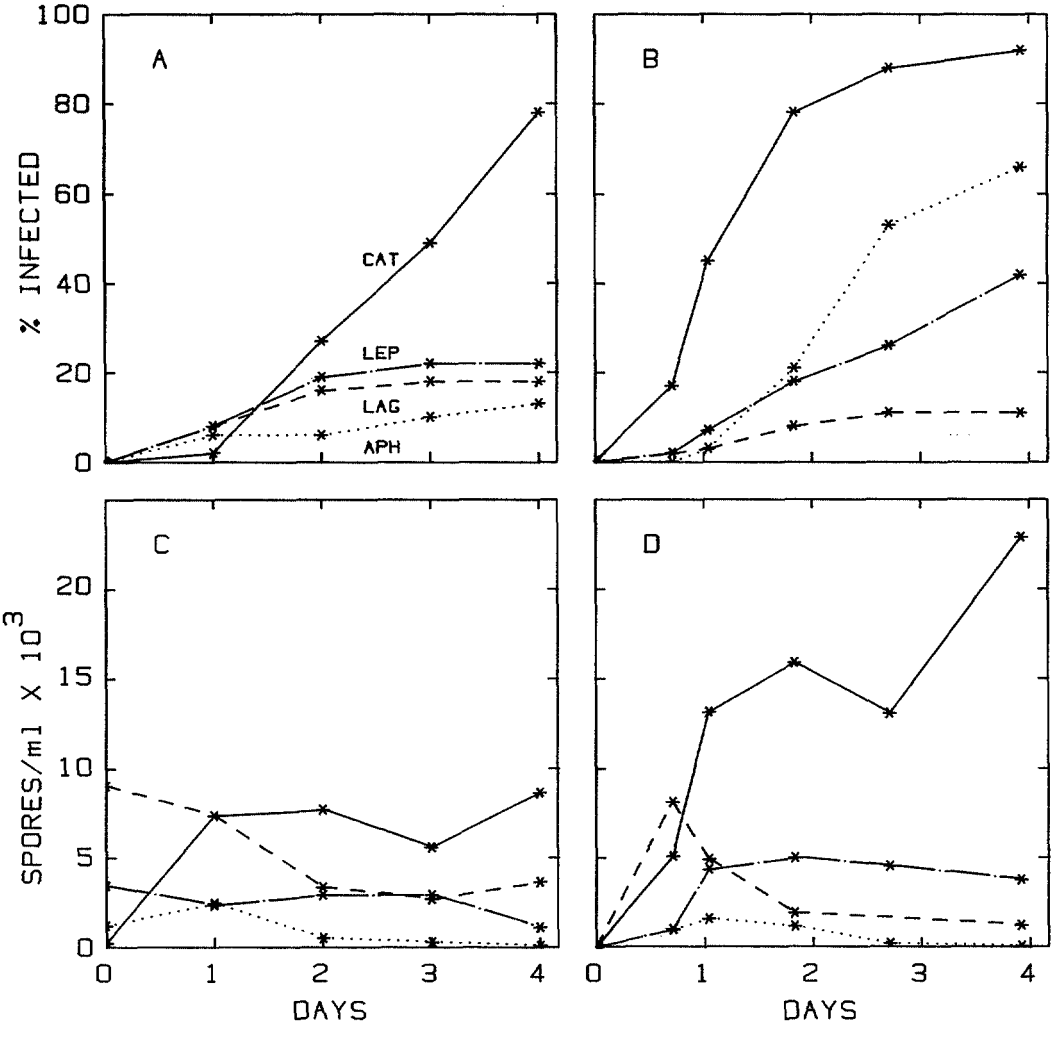


FIG. 7. Infection of *Xiphinema* spp. (mixture of *X. americanum* and *X. rivesi*) by zoosporic fungi in 2% soil extract. CAT = *Catenaria anguillulae*, LEP = *Leptolegnia* sp., LAG = *Lagenidium caudatum*, APH = *Aphanomyces* sp. Extracted nematodes were incubated for 0 hours (A) or 96 hours (B) before exposure to fungi. Day 0 refers to time when nematodes and fungi were brought together in same petri dish. Each value is the mean of three replications, 15–20 nematodes per replication. At day 4, the standard error of the mean was 4.7% for A and 7.5% for B. No infection or mortality of control nematodes occurred. C and D show concentrations of motile spores for A and B, respectively. The average standard error of the mean was 0.8 × 10^3 spores for C and 2.4 × 10^3 spores for D.

parasites of, nematodes (2,4,10,13,22,26). There are fewer reports on parasitism of nematodes by genera of the three other fungi. *Lagenidium* spp. were observed on *Heterodera* spp., *Hoplolaimus* sp., and *Rhabditis* sp. (1,11,14–16), and *Leptolegnia* sp. was isolated recently from a fresh water nematode (19). Other species of *Leptolegnia* attack crustaceans and mosquito larvae (9,18,23). The genus *Aphanomyces* includes species that parasitize roots of higher plants, algae, protozoans, and crustaceans (3); I am unaware of previous reports implicating *Aphanomyces* spp. as parasites of nematodes.

Most researchers have considered *Catenaria anguillulae* a poor biocontrol agent for plant parasitic nematodes. *Ditylenchus dipsaci* and *Panagrellus redivivus* were attacked only in the presence of high concentrations of spores, and the level of suppression of *D. dipsaci* in greenhouse tests was low (22). *Catenaria anguillulae* readily attacked dead or injured nematodes but was only weakly

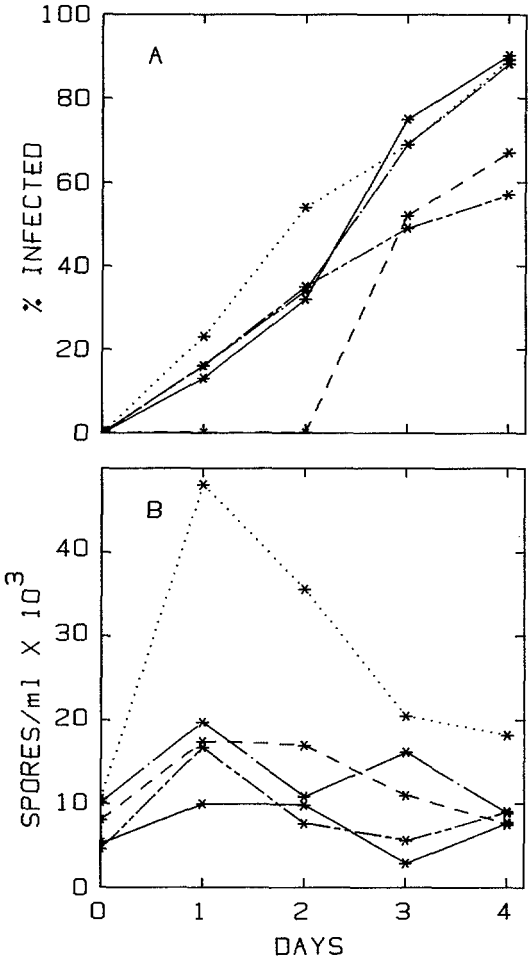


FIG. 8. Infection of *Xiphinema* spp. (mixture of *X. americanum* and *X. rivesi*) by *Catenaria anguillulae*. Extracted nematodes were incubated for 0 hours before exposure to fungi in 2% soil extract (*— — — —*), undiluted saturation extract of soil (*– – –*), or saturation extract diluted 1:1 (*·····*), 1:3 (*—·—*), or 1:7 (*——*) with distilled water. A) Percentage of infected nematodes. Each value is the mean of three replications, 15–20 nematodes per replication. At day 4, the standard error of the mean was 7.9%; infection of control nematodes was zero and mortality of control nematodes was < 3% in all solutions. B) Concentrations of motile zoospores. The average standard error of the mean was 4.5 × 10^3 spores.

parasitic on unstressed nematodes of the order Tylenchida (7). Similarly, only a low proportion of *Rhabditis* spp. was attacked by *Lagenidium caudatum* (1). Although these studies indicated that the zoosporic fungi in question were relatively nonaggressive parasites of vermiform nematodes, most nematodes tested were members of the Tylenchida. Researchers previously observed that dorylaimid nematodes are more susceptible than tylenchid nematodes to zoosporic fungi (7). The mode of penetration of nematodes supports this observation. In previous studies with tylenchid nematodes, penetration usually occurred through natural openings (1,4,10,22); in this study with *Xiphinema* spp., penetration through natural openings was rare but direct penetration of the cuticle was common. Furthermore, encystment on and penetration of *Xiphinema* spp. was not random but was most frequent near the stylet and esophagus. Encystment on particular areas of the cuticle might result from morphological features (e.g., pores) not easily observed with the light microscope (25) or from cuticular chemistry (28).

In general, Cat produced more spores and infected more nematodes than did the other fungi in this test. Infection of nematodes by *Catenaria anguillulae* was previously found to be correlated with zoospore concentration; 10^3–10^4 spores/ml were required for 50% infection (22). It is unclear why infection should be dependent on high spore concentrations. Perhaps single or few spores produce insufficient enzymes and mechanical pressure to penetrate the cuticle. Perhaps zoospores are variable and only a low percentage may initiate infection of nematodes. Finally, zoospore encystment might result from random contact between specific sites on the spore and cuticle; the probability of contact would increase with spore concentration.

The 2% soil extract used in this study was more representative of soil solution than the diluted pond water and salt solutions used by others (10,22). Nevertheless, zoospores failed to encyst immediately upon nematodes when they were combined. It was hypothesized that a more concentrated soil solution, such as a saturation extract, would provide a more favorable environment for rapid spore encystment and parasitism of nematodes. However, encystment and parasitism by Cat were delayed in saturation extract, suggesting that Cat may be more active in dilute solution, such as in the Baermann funnel, than in soil. On the other hand, nematodes in saturation extracts appeared less active than those in more dilute solutions, and delay in parasitism may have reflected the condition of the nematodes. Although several authors reported that Cat was more aggressive on stressed than nonstressed

nematodes (7,21,22), Esser and Ridings (10) reported that apparently healthy active nematodes were the best hosts for this fungus.

The conditions in the in vitro tests favored parasitism in several ways. First, many active spores were present throughout the experiment. Second, these spores were not transferred and thus were not subject to damage. Third, water potential was high and constant. Fourth, the nematodes were very accessible; i.e., the spores did not have to swim around soil particles to locate nematodes. Fifth, although the control nematodes remained alive and appeared healthy, they were probably stressed (27), and stress may predispose nematodes to attack by zoosporic fungi (21). Sixth, the zoosporic fungi were not subject to antagonism by other fungi and bacteria as may occur in soil. In light of what are apparently favorable environmental conditions for nematode parasitism, the failure of the fungi to encyst rapidly and infect 100% of the exposed nematodes suggests that these fungi are not aggressive pathogens of healthy nematodes.

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